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***Clostridioides difficile* ribotypes isolated from domestic environment and from patients in Bangladesh**

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Abstract

Clostridioides difficile infection (CDI) is an emerging but often understudied infectious disease in developing countries. This study was aimed to isolate and characterize *C. difficile* from shoe sole swabs and diarrheal patient's stool samples in Bangladesh. We collected 94 shoe sole swabs samples from urban communities in Dhaka and 208 diarrheal stool samples from hospitalized patients over a period of 4 months. Samples were incubated anaerobically for *C. difficile* growth, confirmed toxigenic, and PCR-ribotyped. Eleven of 94 (11.7%) shoe sole swabs and 4 of 208 (1.9%) stool samples were culture positive of which 9 shoe sole isolates were toxigenic. Six PCR ribotypes from the 9 toxigenic isolates were identified with ribotype F014-020 being the most common (n = 4; 44%). The recently identified ribotype 106 strain was also identified. To the best of our knowledge, this is the first report of *C. difficile* culture, isolation and characterization from environmental sources in Bangladesh.

Keywords

Clostridioides difficile; Toxins; Fluorescent-ribotyping; Diarrhea; Shoe sole; Dhaka

1. Note

Clostridioides difficile is a Gram-positive, anaerobic, sporeforming bacillus, the most common cause of antimicrobial-associated infectious diarrhea in hospitalized patients in the developed world [1,2]. Pathogenicity of the organism is associated within a 16-kb pathogenicity locus (PaLoc) carrying genes encoding for enterotoxin A (*tcdA*) and cytotoxin

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Conflicts of interest

The authors declare no potential conflict of interest.

B (*tcdB*) [3]. Toxin A is a potent enterotoxin that causes accumulation of fluid in the gut. Toxin B is 1000-fold more potent as a cytotoxin [4]. Epidemic strains of *C. difficile* (ribotype 027/NAP1) have been shown to circulate throughout the globe and *C. difficile* infection (CDI) can occur in travelers to destinations in Asia, Central/South America, the Caribbean, and Africa [5]. The spores of *C. difficile* have been shown to be ubiquitous in the environment including the household environment [6], animal feces [7,8], and the external community environment [9].

The first report of clinical *C. difficile* isolation from diarrheal patients in Bangladesh was published in 1987 [10]. Later in a case-control study, *C. difficile* was found to be a significant enteropathogen causing childhood diarrhea in Dhaka, Bangladesh [11]. The objectives of our study were to isolate and characterize *C. difficile* from domestic environment using shoe sole swabs and compare the isolates with clinically relevant strains via testing fecal samples from patients with diarrhea. This study was approved by the Institutional Review Board at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b). This pilot study was conducted in an urban area in Dhaka, Bangladesh between October 2015 to January 2016. Ninety four shoe sole swab samples were collected from community dwelling persons in Dhaka city, Bangladesh. Researchers approached households of their family, friends and relatives located in 10 different areas of Dhaka city to collect shoe sole swab samples. All members present in the house were requested to provide a shoe for sampling. Shoe soles were swabbed using pre-sterilized cotton gauge lightly soaked with water by trained research staff using sterile gloves. In the same time period, October 2015 to January 2016, altogether 51,880 diarrheic patients have visited the Dhaka hospital of icddr,b of which 1037 patients were enrolled in an ongoing 2% surveillance and from them 208 were enrolled in *C. difficile* testing irrespective of other possible known intestinal pathogen detected. In 2% surveillance, every 50th patient with diarrhea attending the icddr,b Dhaka hospital are enrolled. In addition to providing the regular management, these patients are interviewed by trained personnel to collect information on socioeconomic and demographic characteristics, housing and environmental conditions, feeding practices, particularly of infants and young children, and use of medicines and fluid therapy at home. Information on clinical characteristics, nutritional status measurements, and treatments received at the facilities and outcomes of patients are also recorded. Extensive microbiological assessments of faecal samples (culture, and ELISA) are performed to identify diarrheal pathogens and to determine antimicrobial susceptibility of bacterial pathogens. All samples (shoe swabs and stool) were enriched in brain heart infusion (BHI) broth with 0.1% sodium taurocholate hydrate (Sigma Chemicals) and incubated anaerobically at 37 °C for 2-days. One ml of broth culture from each sample was centrifuged and the pellet was suspended in 100 µl of 70% ethanol, and plated onto cycloserine cefoxitin fructose agar (CCFA) and blood agar plates, and incubated anaerobically at 37 °C for 40–48 h in anaerobic jars with an AnaeroGen™ sachet (Oxoid, Hampshire, England). Suspected *C. difficile* colonies based on colony morphology and smell were tested using latex agglutination reagent (*C. difficile* test kit, Oxoid Microbiology Products, Hampshire, England) and Enzyme Immuno Assay (EIA) for glutamate dehydrogenase and toxins A and B using C. DIFF QUIK-CHEK COMPLETE (Techlab, Blacksburg, VA) according to manufacturer's instruction. Presence of *C. difficile* specific genes in isolated strains were

assessed using multiplex polymerase chain reaction to detect the toxin A (*tcdA*), toxin B (*tcdB*) and *tpi* genes as previously described [12]. A second set of PCR protocol was executed for the detection of binary toxins *cdtA* and *cdtB* [13]. Fluorescent ribotyping was conducted on all *tpi* positive strains and PCR condition and protocol was as previously reported [14]. An online analysis tool is available at <http://walklab.rcg.montana.edu> where a curated and compiled data of *C. difficile* F-ribotypes is available for free.

From 94 shoe sole swab samples 11 samples were culture positive for *C. difficile* (11.7%). Nine of 11 (82%) isolates were toxigenic identified by the presence of toxin genes *tcdA* and *tcdB*. No isolates with the binary toxin genes (*cdtA/cdtB*) were identified. The positivity rates for shoes were lower than that described elsewhere and this could be either due to the true low prevalence of *C. difficile* spores in the environment or due to the methods used. Enrichment for 2 days is probably not enough and a prolonged enrichment of 5 days might have increased the rate of positivity [15]. Contamination of shoe soles with *C. difficile* represents an infection control problem in healthcare settings but also represents a novel approach to better understand the ecology of certain pathogens in the healthcare and community environment. Our research group had previously demonstrated that shoe sole swabs collected in the healthcare and community setting in Houston, Texas were frequently contaminated with *C. difficile* [6,9]. Procedure for shoe sampling are non-technical, quick, and non-obtrusive and therefore shoe sole sampling could provide a method to perform worldwide surveillance of targeted pathogens [15,16]. However, the applicability of this method is still at the evaluation stage and large scale studies with higher sample size need to be carried out in different parts of the world.

Among the 208 stool samples, 4 (1.9%) were positive for *C. difficile* (*tpi* gene positive) but all the isolates were non-toxigenic. All four patients were positive for other known pathogens and thus the detected *C. difficile* was not causing diarrheal symptoms but was colonizing in the gut due to disturbed gut microbiota. A description of the four cases is shown in Table 1. Overall the positivity rate of *C. difficile* in patients is low compared to similar reports from other developing countries [17] and this might be because all patients had acute diarrhea with other known enteric pathogens. However, both typical and atypical risk factors for colonization with *C. difficile* were present in these four patients including predominantly young age (3 of 4 patients were children between 6 and 18 months) and all four patients co-habited with domestic animals (all persons had livestock and live poultry in their kitchens and bedroom). Younger age and frequent exposure to domestic animals have both been previously shown to increase the likelihood of *C. difficile* colonization [7].

PCR ribotyping was done only for the toxigenic strains. Nine toxigenic isolates from shoe swab samples were grouped into six ribotypes. Ribotype F014-020 being the most common (n = 4; 44%), other ribotypes identified were F053-163, F106, F107, FP407, and FP501 (1, each). All these ribotypes had been previously identified in our library allowing for a worldwide comparison of these ribotypes. Ribotype F014-020 has been most frequently isolated from the environments in our previous study [9] and also from other investigators in the USA [18], hospitalized patients in Europe [19] and animals and the environment including water in Slovenia [20]. Ribotype 106 has become one of the more common ribotypes in the USA and Europe, this is the first reported case of ribotype 106 in

Bangladesh [21,22]. The comparison with clinically relevant strains was not possible due to the absence of toxigenic strains in the clinical samples.

This is not the first report of *C. difficile* in Bangladesh. In the 1990's, 13 of 814 children admitted to hospital with diarrhea were infected with *C. difficile* [11]. However, stool samples are not routinely tested for *C. difficile* in Bangladesh as it is not included in the regular diagnostic panel of organisms considered for potential cause of acute diarrhea. Future studies directed at hospitalized patients that received broad-spectrum antibiotics will be required to assess the incidence of *C. difficile* infection in Bangladesh. Nevertheless, this study demonstrates that toxigenic strains are present in the Bangladesh environment and could be a source of infection in high-risk patients.

In conclusion, using the simple shoe swab method we were able to isolate and characterize *C. difficile* from environmental sources in Bangladesh. Various ribotypes were found including the emerging ribotype 106.

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Dr. Islam was a Scientist and head of the Food Microbiology Laboratory at icddr,b in Dhaka, Bangladesh. Recently he moved to USA and joined Washington State University as an Assistant Professor. His research interests include molecular epidemiology and transmission dynamics of enteric and foodborne bacterial pathogens and antimicrobial resistance.

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Table 1

Characteristics of patients with diarrhea positive for non-toxicogenic *C. difficile* colonization.

Case	Age	Sex	Location	Other pathogen	Duration of diarrhea (days)	Treatment given	Stool type	Domestic animal in house
1	6 m	M	Rural	Campy + Rotavirus	1–3	ORS	Watery + mucus (uncomplicated)	Goat + chicken
2	20y	F	Rural	Rotavirus	<1	ORS + Azith	Watery (uncomplicated)	Goat
3	18m	F	Rural	Rotavirus	1–3	ORS	Watery (uncomplicated)	Goat
4	6m	M	Rural	Rotavirus	<1	ORS	Watery (uncomplicated)	Goat + chicken