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High rate of *Clostridium difficile* among young adults presenting with diarrhea at two hospitals in Kenya

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Abstract

Background: *Clostridium difficile* infection (CDI) is the leading cause of antibiotic-associated diarrhea worldwide. As a result, the US Centers for Disease Control and Prevention have designated *C. difficile* as an urgent threat. Despite the global public health risk posed by CDI, little is known about its epidemiology on the African continent. This article describes the common occurrence of CDI from a cross-section of consecutively seen, randomly enrolled patients presenting with diarrhea at two major hospitals in Kenya.

Methods: Patients presenting with diarrhea at two major hospitals in Kenya from May to July 2017 were enrolled. After signing the informed consent, stool samples, demographic data, medical history, prior antibiotic use, and HIV status were obtained from the patients. *C. difficile* was detected and validated by toxigenic culture and PCR.

Results: The average age of the patients was 35.5 years (range 3–86 years); 59% were male and 41% were female. Out of 105 patient stools tested, 98 (93.3%) were positive for *C. difficile* by culture. PCR analysis confirmed *C. Difficile*-specific genes, *tcdA*, *tcdB*, and *tcdC*, in the strains isolated from the stools. Further, 82.5% of the stools had *C. difficile* isolates bearing the frame-shift deletion associated with hypervirulent strains. Remarkably, 91.9% of the stools that tested positive for *C. difficile* came from patients under 60 years old, with 64.3% being less than 40 years

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Ethical approval

This study was approved by the ethics review boards of Kenyatta National Hospital/University of Nairobi, Kisii Teaching and Referral Hospital, and the Kenyan Ministry of Health.

None of the authors have any conflict to report.

of age. The majority of the patients (85%) reported over-the-counter antibiotic use in the last 30 days before the hospital visit.

Conclusions: Together, the results revealed an unusually high incidence of *C. difficile* in the stools analyzed, especially among young adults who are thought to be less vulnerable. Comprehensive research is urgently needed to examine the epidemiology, risk factors, pathogenesis, comorbidities, clinical outcomes, antibiotic susceptibility, and genetic makeup of *C. difficile* strains circulating on the African continent.

Keywords

Clostridium difficile infections in Africa; *C. difficile* pathogenesis; *C. difficile* epidemiology; CDI in young adults; Antibiotics and CDI

Introduction

Clostridium (*Clostridioides*) *difficile* is a leading cause of antibiotic-associated diarrhea worldwide. Morbidity and mortality associated with *C. difficile* infections (CDI) have increased significantly over the last decade. The annual cost of treatment in the USA ranges between \$1.9 and \$7.0 billion (Zhang et al., 2016). The US Centers for Disease Control and Prevention has designated *C. difficile* as an urgent threat. The risk of CDI increases with broad-spectrum antibiotic use (Owens et al., 2008). Other CDI-associated risk factors include old age, the use of gastric acid-suppressing drugs, comorbidities, immunodeficiency, and inflammatory bowel disease (Khanna and Pardi, 2012).

Toxigenic *C. difficile* strains produce and release toxins A and B, which cause disease (Geric et al., 2004; Kuehne et al., 2010; Lyerly et al., 1985; Rupnik et al., 2001; Voth and Ballard, 2005). During infection, the toxins are internalized by the host cells, leading to monoglucosylation of GTPases of the Rho family in the cytosol (Just et al., 1995). The infiltrated toxins cause the release of various immunomodulatory mediators, resulting in massive inflammation and the accumulation of neutrophils in the colon. The downstream effect of the toxins is mild to severe diarrhea and in more complicated cases, pseudomembranous colitis. Other CDI-associated symptoms include abdominal cramping, profuse diarrhea, and abdominal pain (Borriello, 1998).

Despite the public health risks posed by CDI, little is known about the epidemiology of this life-threatening pathogen in Africa. Such knowledge is critical, because most African countries are popular tourist destinations and may serve as direct routes for disseminating CDI. Given the practice of unregulated antibiotic use on the African continent, it was hypothesized that the incidence of CDI would be high among diarrhea patients. To investigate this hypothesis, the presence of *C. difficile* was determined in the stools of patients reporting diarrhea at two regional hospitals in Kenya, a popular tourist destination. The results revealed an alarming frequency of *C. difficile* in the patients with diarrhea, especially in young adults originally thought to be less vulnerable.

Materials and methods

Stool sample collection

This study was approved by the ethics review boards of Kenyatta National Hospital/ University of Nairobi, Kisii Teaching and Referral Hospital, and the Kenyan Ministry of Health. The Kenyatta National Hospital is located in an urban area of Nairobi and serves patients from Nairobi and surrounding rural towns, as well as patients needing specialized care from other parts of the country. The Kisii Teaching and Referral Hospital is located in a rural area and serves people living in the surrounding rural and peri-urban areas. Patients who visit this hospital either are referred by doctors or are self-referred.

All patients that reported to these hospitals with diarrhea from May to July 2017 were sequentially enrolled in the study. To limit bias, no diarrhea patient was excluded from the study, except a few patients who refused to participate. After signing a consent form, the patients were administered questionnaires to capture data on demographics, previous antibiotic use, and medical history including HIV status (if known). Each patient was asked to collect a stool sample for the study. The samples were carefully aliquoted into sterile 1-ml tubes with screw caps using a sterile spatula and stored at -80°C until shipped on cold packs to the University of Texas Health Science Center, Houston, Texas, USA for analysis.

Detection of *C. difficile* in stool by culture

The presence of *C. difficile* in the diarrheal stools was determined and validated using toxigenic culture (Darkoh et al., 2011a) and PCR. An anaerobic condition was maintained in a Bactron 600 anaerobic chamber (Sheldon Manufacturing, Cornelius, OR, USA) using 5% CO_2 , 10% H_2 , and 85% N_2 . A loopful of the frozen stools was carefully spread on the *C. difficile*-specific culture plates (Darkoh et al., 2011a) using sterile single-use loops; the plates were then incubated anaerobically at 37°C for 48 h. This medium contains 250 $\mu\text{g}/\text{ml}$ D-cycloserine, 8 $\mu\text{g}/\text{ml}$ cefoxitin, and 0.025% *p*-cresol, which specifically select for *C. difficile* and eliminate other non-*C. difficile* anaerobes on the plate. The numbers of stools that grew colonies were enumerated. To obtain *C. difficile* isolates for further analysis, all of the colonies on each plate were pooled and enriched by culturing for 24 h anaerobically at 37°C in brain heart infusion (BHI) broth containing 300 mg/ml D-cycloserine and 8 $\mu\text{g}/\text{ml}$ cefoxitin. Freezer stocks (1 ml) of each culture were made in 10% dimethylsulfoxide (DMSO) and stored at -80°C . Cell pellets from the remaining culture were stored at -20°C for PCR analysis.

PCR analysis

For PCR analysis, DNA was isolated from each of the bacterial pellets using the Gene Reagent Pack on the Corbett Life Science X-tractor platform (Qiagen, Valencia, CA, USA). The concentration of the extracted DNA was determined using NanoDrop (ThermoScientific, Wilmington, DE, USA). PCR was performed using primers specific for toxins A and B (TcdA2), TcdC (TNC), and the 16S ribosomal RNA gene (16S rRNA) as control (Fiedoruk et al., 2015; Fry et al., 2012; Griffiths et al., 2010; Lemee et al., 2004; Li et al., 2017; Liu et al., 2018; Murray et al., 2009). The sequences of the primers used are: TcdA2 F-50AGATTCCTATATTTACATGACAATAT30, R- 5'GTATCAGGCATAAAGT

AATATACTTT3'; TNC F- 5'GAGCACAAAGGGTATTGCTCTACTGGC3', R- 5'CCAGACACAGCTAATCTTATTTGCACCT3'; 16S rRNA F- 5'ACACG GTCCAAACTCCTACG3', R-5'AGGCGAGTTTCAGCCTACAA3'. The PCR amplification was done using OneTaq Quick-Load 2 Master Mix (New England Biolabs, Ipswich, MA, USA) with an initial denaturation temperature of 94 C for 30 s and 36 cycles of 94 C for 30 s, 55 C for 30 s, and 68 C for 30 s, with a final extension of 68 C for 5 min. The PCR products were analyzed using 1% agarose gel electrophoresis, stained with ethidium bromide, and imaged using BioDoc-It Imaging System (UVP, Upland, CA, USA).

Toxin assays

Toxins A and B in the culture supernatants of the isolates were detected using the Cdifftox activity assay for toxin activity (Darkoh et al., 2011b) and *C. difficile* TOX A/B II ELISA test (TechLab, Blacksburg, VA, USA) for toxin production. For the Cdifftox activity assay, the culture was centrifuged for 10 min at 10 000 xg and the supernatant (250 µl) was added to 30 µl of 0.2-µ filtered 30 mM p-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich, St. Louis, MO, USA) in a sterile 96-well plate. The sample was incubated aerobically at 37 C for 4–24 h and absorbance at 410 nm was measured using a SpectraMax I3 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

For the ELISA test, 200 µl of the supernatant was used and the manufacturer's instructions were followed.

Statistical analysis

The data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). The non-parametric one-way Kruskal–Wallis test followed by Mann–Whitney *U*-test was used to determine differences in toxin production and toxin activity. Pearson's Chi-square test was used to determine the relationship between history of antibiotic use and CDI positivity. Statistical significance was defined as $p < 0.05$.

Results

A total of 105 stools collected from non-biased, sequentially enrolled diarrhea patients were examined for the presence of *C. difficile*; 92 were collected from Kisii Teaching and Referral Hospital and 13 from Kenyatta National Hospital. Out of the 105 stools, 98 (93.3%) grew *C. difficile* colonies on the culture plates, whereas seven (6.7%) showed no growth (Table 1). To validate the culture results, PCR was performed to detect *C. difficile*-specific genes (*tcdA*, *tcdB*, and *tcdC*). Of the 98 samples analyzed by PCR, 97 (98.9%) were positive for *tcdA*, *tcdB*, or *tcdC* and only one (1.1%) was negative. The *tcdA* and *tcdB* genes were amplified in 95 (97.9%) of the 97 samples. Further, the *tcdC* gene was amplified in all of the 97 samples, of which 80 (82.5%) had isolates bearing the frame-shift deletion associated with hypervirulent strains (Carter et al., 2011; Curry et al., 2007; Hundsberger et al., 1997; Matamouros et al., 2007; Warny et al., 2005). *C. difficile* isolates from all of the 95 stools that had colonies bearing the *tcdA* and *tcdB* genes produced active toxins. However, neither colonies from the single stool sample that tested negative for the *tcdA*, *tcdB*, and *tcdC* genes nor the two samples that tested negative for the *tcdA* and *tcdB* genes produced toxins.

Of the patients enrolled, 59% were male (average age 34 years) and 41% were female (average age 37 years). Male and female patients had a similar prevalence of *C. difficile* and there was no statistically significant correlation ($p = 0.456$) between the age groups (Figure 1). The average age of all of the patients was 35.5 years (range 3–86 years): 13.3% were <20 years of age, 50.5% were 20–39 years old, 28.6% were 40–59 years old, and 7.6% were 60–86 years old. Strikingly, 91.9% of the stools that tested positive for *C. difficile* came from patients under 60 years of age, with 64.3% being less than 40 years old (Figure 1B). About 90.5% of the patients reported having diarrhea for less than 1 month, whereas 9.5% indicated prolonged diarrhea lasting 1–3 months prior to the hospital visit. Moreover, 4.8% of the patients reported being HIV-positive, 28.6% reported being HIV-negative, and 66.7% did not know their HIV status. All of the HIV-positive patients were *C. difficile*-positive.

About 85% of the patients reported over-the-counter antibiotic use 30 days before the hospital visit, and no significant difference was found between the age groups ($p = 0.485$) or sexes ($p = 0.760$) (Table 2). Pearson's Chi-square test demonstrated a significant relationship between history of antibiotics use and CDI positivity ($p = 0.035$). The most widely used over-the-counter antibiotics reported by the patients were amoxicillin, metronidazole, cephalosporins, ciprofloxacin, and azithromycin.

Discussion

Most CDI studies have been reported from industrialized regions and little is known about the infection on the African continent. Moreover, *C. difficile* has traditionally been neglected as an important diarrhea-causing pathogen in Africa. Since Kenya is a popular destination for tourists around the world, it was sought to establish the presence of *C. difficile* by examining a cross-section of unbiased, sequentially enrolled diarrhea patients in two hospitals. Astoundingly, the study results demonstrated that a large number of stools from young adults presenting with diarrhea at the two hospitals had *C. difficile*. This rate is unusually higher than that reported in industrialized countries and the few studies conducted on the African continent (Onwueme et al., 2011; Seugendo et al., 2015), but the patient population and demographics are different. Moreover, the methods used in the current study (culture with PCR validation) are more sensitive than those used in the previous studies conducted in Africa.

The higher CDI frequency in Kenya undoubtedly relates to the overuse of antibiotics, which are widely available over-the-counter with no strict regulation governing access to the drugs. The patients in this study were unique because they went to the hospital following unsuccessful treatment of their diarrhea by self-medication. This practice is common in African countries and may potentially exacerbate CDI rates and antibiotic resistance more than previously thought. While beta-lactam antibiotics are well known to predispose people to CDI, 19% of the CDI patients in this study had taken metronidazole before presenting to the hospital. Of importance, metronidazole was not associated with cure when used as self-treatment. Thus, the current study adds to the growing evidence that metronidazole is less effective in treating CDI (Johnson et al., 2014).

Comorbidities such as HIV infection may have contributed to the high prevalence of CDI in this population, a known association (Keeley et al., 2016). HIV infection in the study population was invariably associated with CDI, although only a small number of HIV-positive patients were enrolled. However, other diarrhea-causing pathogens such as norovirus and *Escherichia coli* were not tested for in this study. The finding that 64.3% of the CDI patients were less than 40 years of age and 27.6% were 40–59 years old was unexpected. Such a high frequency of CDI in young adults with diarrhea has not been observed elsewhere, and this may highlight the changing epidemiology and virulence of *C. difficile* strains.

A limitation of this study was the lack of data on the symptoms exhibited by the patients and clinical outcomes, as this was an initial study to establish the presence of *C. difficile* among diarrheal patients in Kenya. Another limitation is that other diarrhea-causing pathogens could not be excluded in this study and therefore *C. difficile* may not have been the sole pathogen associated with diarrhea in these patients. Nonetheless, with such an unusually high level of CDI among the diarrhea patients, a larger study has been planned to comprehensively examine the epidemiology, risk factors, co-infections, pathogenesis, comorbidities, clinical outcomes, antibiotic resistance, and the genetic makeup of *C. difficile* strains circulating in this population.

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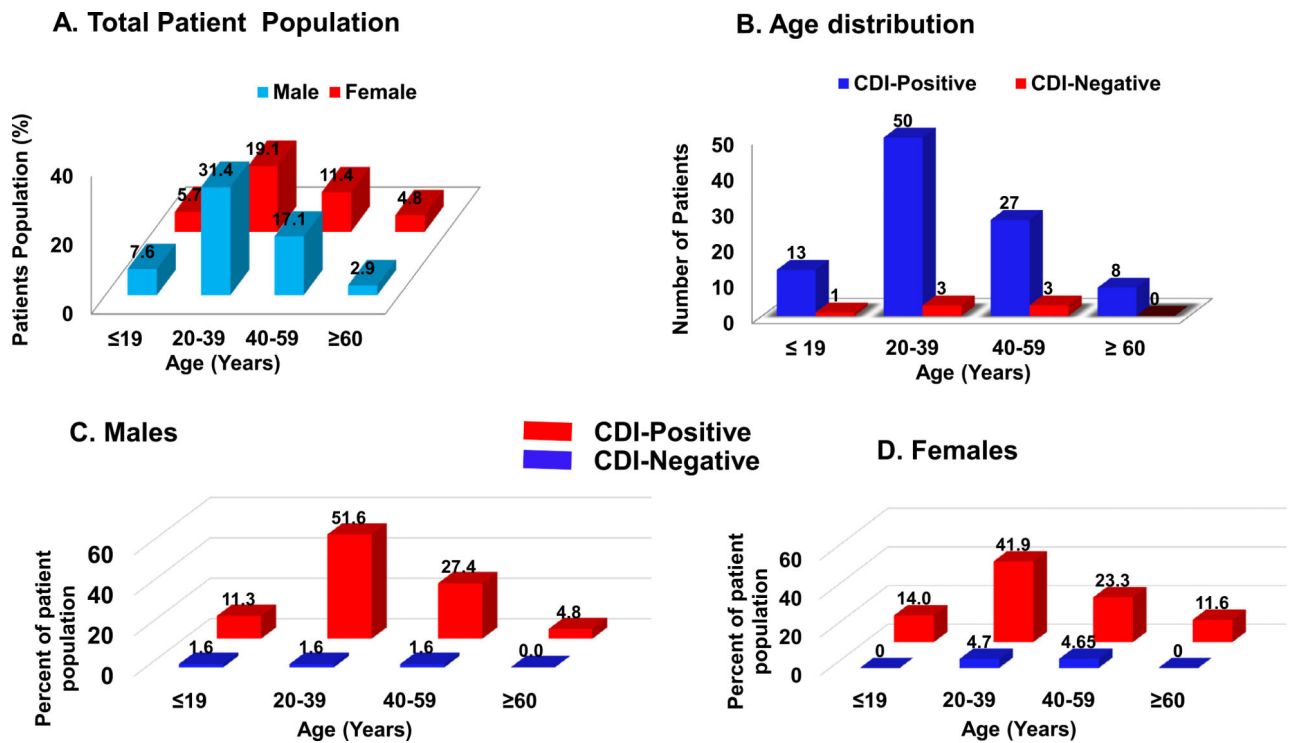


Figure 1. Distribution of the total patient population based on sex and age (A) and by the presence of *Clostridium difficile* in stool based on age (B) and sex (C and D). All patients presenting diarrhea at Kenyatta National Hospital and Kisii Teaching and Referral Hospital in Kenya from May to July 2017 were sequentially enrolled in the study. To limit bias, no diarrhea patient was excluded, except a few patients who refused to participate. The population was 59% male and 41% female.

Table 1Detection and confirmation of *Clostridium difficile* in 105 diarrheal stool samples by culture and PCR.^a

	Number of patients	Percentage (%)
Total number of patients	105	
<i>C. difficile</i> detection by culture		
Positive	98	93.3
Negative	7	6.7
PCR analysis of pooled <i>C. difficile</i> colonies		
Presence of <i>tcdA/tcdB</i> or <i>tcdC</i> genes		
Positive	97	98.9
Negative	1	1.1
<i>tcdA+/tcdB+</i>		
Positive	95	97.9
Negative	2	2.1
<i>tcdA+/tcdB-</i>		
Positive	92	96.8
Negative	3	3.2
<i>tcdA+/tcdB-</i>		
Positive	2	2.1
Negative	95	97.9
<i>tcdC+</i>		
Positive	97	100
Negative	0	0
<i>tcdC</i> deletion		
Wild-type	17	17.5
Deletion	80	82.5

^aStools were streaked on *C. difficile* plates containing 250 g/ml D-cycloserine and 8 µg/ml cefoxitin and incubated anaerobically at 37° C for 48 h. To validate the culture results, PCR was performed using primers specific for *C. difficile* genes (*tcdA*, *tcdB*, or *tcdC*) on the colonies isolated from the plates.

Table 2

Distribution of antibiotics taken by the patients in the last 30 days before the hospital visit.

		Number	%	Age (years)	
				Mean	Range
Antibiotic use in last 30 days					
Yes		89	84.8	35.1	(3–86)
No		16	15.2	37.9	(13–61)
Antibiotic class	Generic name				
Unreported antibiotic		16	15.2	37.9	(13–61)
Penicillins					
	Amoxicillin	31	29.5	36.8	(5–86)
Nitroimidazoles	Ampicillin	2	1.9	34.5	(29–40)
Cephalosporins	Metronidazole	20	19.1	31.4	(3–58)
	Cephalosporin	10	9.5	31.5	(19–60)
	Cephalexin	1	1	35	–
Fluoroquinolones	Ceftriaxone	9	8.6	34.3	(17–61)
	Ciprofloxacin	6	5.7	31.8	(18–42)
Macrolides	Levofloxacin	2	1.9	56.5	(56–57)
Sulfonamides	Azithromycin	4	3.8	31	(22–39)
Glycopeptides	TMP–SMX	2	1.9	45.5	(43–48)
	Vancomycin	1	1	71	–

TMP–SMX, trimethoprim–sulfamethoxazole.