International Journal of Infectious Diseases 81 (2019) 66–72

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Prevalence of *Clostridium difficile* infections among Kenyan children with diarrhea

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ARTICLE INFO

Article history:
Received 16 October 2018
Received in revised form 9 December 2018
Accepted 10 January 2019
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Clostridium difficile infections in Africa
C. difficile pathogenesis
C. difficile epidemiology
CDI in young children
CDI in infants
C. difficile co-infection

ABSTRACT

Background: Diarrhea causes significant morbidity and mortality among children worldwide. Regions most affected by diarrhea include Sub-Saharan Africa and Southeast Asia, where antibiotics are in common use and can make children more vulnerable to *Clostridium difficile* and pathogens that are not affected by these drugs. Indeed, *C. difficile* is a major diarrhea-associated pathogen and poses a significant threat to vulnerable and immunocompromised populations. Yet, little is known about the role and epidemiology of *C. difficile* in diarrhea-associated illness among young children. As a result, *C. difficile* is often neglected in regions such as Sub-Saharan Africa that are most impacted by childhood diarrhea. The purpose of this study was to establish the frequency of *C. difficile* in young children (<5 years) with diarrhea.

Methods: Children presenting with diarrhea at a national hospital in Kenya from 2015 to 2018 were enrolled consecutively. Following informed consent by a parent or legal guardian, stool samples were obtained from the children and demographic data were collected. The stools were examined for the presence of four common pathogens known to cause diarrhea: *C. difficile*, rotavirus, *Cryptosporidium parvum*, and *Giardia lamblia*. *C. difficile* was verified by toxigenic culture and PCR. The presence of *C. parvum* and/or *G. lamblia* was determined using the ImmunoCard STAT! Crypto/Giardia Rapid assay. Rotavirus was detected by ELISA.

Results: The study population comprised 157 children; 62.4% were male and 37.6% were female and their average age was 12.4 months. Of the 157 stool specimens investigated, 37.6% were positive for *C. difficile*, 33.8% for rotavirus, 5.1% for *Cryptosporidium*, and 5.1% for *Giardia*. PCR analysis identified at least one of the *C. difficile*-specific - genes (*tcdA*, *tcdB*, or *tcdC*). Further, 57.6% of the stools had *C. difficile* colonies bearing a frame-shift deletion in the *tcdC* gene, a mutation associated with increased toxin production. The frequency of *C. difficile* was 32.6% in children ≤12 months old and increased to 46.6% in children 12–24 months old.

Conclusions: In Kenyan children presenting with diarrhea, *C. difficile* is more prevalent than rotavirus or *Cryptosporidium*, two leading causes of childhood diarrhea. These findings underscore the need to better understand the role of *C. difficile* in children with diarrhea, especially in areas with antibiotic overuse. Understanding *C. difficile* epidemiology and its relationship to co-infecting pathogens among African children with diarrhea will help in devising ways of reducing diarrhea-associated illness.

Introduction

Diarrhea accounts for 9.9% of the global mortality among children in the first 5 years of life, and the majority of deaths occur in Sub-Saharan Africa and Southeast Asia (Liu et al., 2012). In 2015,

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https://doi.org/10.1016/j.ijid.2019.01.024

there were nearly one billion cases of diarrhea in children under 5 years of age worldwide (Troeger et al., 2017). In addition to acute morbidity and mortality, frequent diarrheal episodes in early childhood can also have a negative impact on nutritional status, as well as physical and cognitive development later in life (Checkley et al., 2008). Rotavirus and *Cryptosporidium* have been reported as the leading causes of diarrhea-associated morbidity and mortality among children in developing countries, including Kenya (Kotloff et al., 2013). In addition, *Clostridium difficile* is also a major diarrhea-associated pathogen at all ages and poses a significant threat to vulnerable and immunocompromised populations. However, little is known about the burden and role of *C. difficile* diarrhea in children under 5 years of age, despite its increasing incidence and mortality in the adult population (Alcides et al., 2007; Elewa and Sayed-Zaki, 2017; Seugendo et al., 2015; Wang et al., 2013). Such knowledge is important because young children are among the most vulnerable to the negative effects of diarrhea.

C. difficile infection (CDI) is initiated by the disruption of the colonic microbiota following broad-spectrum antibiotic therapy. This allows *C. difficile* overgrowth and subsequent production of its major virulence factors, toxins A and B. These two major virulence factors are encoded by the *tcdA* and *tcdB* genes, respectively, and are located within 19.6 kb of the pathogenicity locus in the genome. Two regulators of transcription encoded by the pathogenicity locus, TcdR and TcdC, have been proposed to control toxin expression at the *tcdA* and *tcdB* promoters. The *tcdR* gene encodes a positively-acting sigma factor that controls transcription of the toxin promoters and its own promoter (Mani and Dupuy, 2001; Mani et al., 2002). In contrast, the *tcdC* gene is proposed to encode a negative regulator of toxin synthesis (Carter et al., 2011; Hundsberger et al., 1997; Matamouros et al., 2007). Epidemic *C. difficile* strains (NAP1/027 strains) have emerged with deletions or frame-shift mutations in the *tcdC* gene, a phenotype that produces high toxin levels (Carter et al., 2011; Curry et al., 2007; Hundsberger et al., 1997; Matamouros et al., 2007; Warny et al., 2005).

Toxins A and B bind to and enter the host's colonic mucosal cells, where they inactivate Rho family GTPases in the cytosol and induce a variety of other cytotoxic effects (Chen et al., 2015). The downstream effects of the toxins include disruption of the intestinal barrier and neutrophil aggregation in colonic tissues, which together lead to diarrhea and in severe cases, to pseudomembranous colitis (Leffler and Lamont, 2015). While fulminant disease is most commonly observed in adults, infection also occurs in children and is increasing in frequency (Alcides et al., 2007; Elewa and Sayed-Zaki, 2017; Seugendo et al., 2015; Wang et al., 2013). The association between toxigenic *C. difficile* in the stool and diarrheal illness in children has been difficult to establish due to the frequency of asymptomatic carriage in this population. The prevalence of asymptomatic *C. difficile* carriage in young children under 12 months of age has been reported to be as high as 70% (Jangi and Lamont, 2010), but decreases after the first year of life. Subsequently, colonized children then become susceptible to the associated diarrhea when dysbiosis develops, presumably when they begin taking antimicrobial drugs (Wendt et al., 2014), which are often commonly available and used indiscriminately in many countries.

Other enteric pathogens including viruses, bacteria, and parasites have been detected concurrently with *C. difficile*, and the frequency of co-infections is estimated to be 20.7% (de Graaf et al., 2015; Nicholson et al., 2016). In addition, *C. difficile* is frequently detected in co-infection with diarrhea-associated viruses such as rotavirus and norovirus, suggesting that screening exclusively for viruses in children may neglect the role of *C. difficile* in pediatric diarrhea (Fiedoruk et al., 2015). The dynamics of *C. difficile* co-infection in young children are still poorly understood, and available data are primarily from North America and Europe, with limited studies from other regions of the world (Albert et al., 1999; Pinto et al., 2003; Wang et al., 2013). Moreover, rotaviruses account for an estimated 45–50% of diarrhea cases in children globally and up to 56% in Kenyan children (Kiulia et al., 2008), suggesting that other pathogens may be involved.

The standard of care for Kenyan children with diarrhea is to provide antibiotic treatment and supportive measures without an initial microbiological evaluation of the stool. Thus, data on the prevalence of enteric pathogens have relied on research studies. Due to the indiscriminate use of antimicrobials by both adults and children in Kenya, we postulate that *C. difficile* may be an important cause of diarrhea in children. To investigate this hypothesis, screening for the presence of *C. difficile* was performed in the stools of children presenting with diarrhea at a national hospital in Kenya. Stool samples had previously been screened for rotavirus (Nyaga et al., 2014; Sigei et al., 2015) as part of the World Health Organization (WHO) African Rotavirus Surveillance Network. In addition to *C. difficile*, the stool samples were also tested for two enteric parasites, *Cryptosporidium* and *Giardia*, since they have been reported in up to 9.8% and 11.1% of Kenyan children with diarrhea (Squire and Ryan, 2017), respectively, and are common causes of diarrhea in African children (Khalil et al., 2018; Rogawski et al., 2017). Unlike the common enteric bacterial pathogens, none of the potential pathogens tested in the present study would be affected by antibiotic treatment received prior to presentation.

Materials and methods

Study population, sample collection and transport

The study was approved by the ethics review boards of the Kenyatta National Hospital and Kenyan Ministry of Health. Recruitment of the patients was integrated with the services of the clinic and hospital wards under the supervision of the attending physicians. Stool samples were obtained from 157 children aged 5 years or younger, presenting with diarrhea and gastroenteritis at Kenyatta National Hospital (Nairobi County, Kenya) from 2015 to 2018. All children presenting with diarrhea during the study period were sequentially enrolled in the study. Before recruitment, the physicians explained all aspects of the study to the parents or legal guardians in either English or Kiswahili, and a signed informed consent agreement was obtained. To limit bias, no child with diarrhea was excluded from the study, except the few whose parents or legal guardians refused to participate. Following consent, the children were enrolled and stool samples (one from each child) were collected immediately. Stool specimens were collected in sterile containers, kept at 4 °C on ice packs, and transported to the Immunology Laboratory of Kenyatta National Hospital, where they were logged in, aliquoted, and immediately stored at −20 °C until analyzed. Aliquots of the frozen stool samples were transported on ice packs to the Kenya Medical Research Institute (Nairobi, Kenya) for rotavirus testing as part of the WHO African Rotavirus Surveillance Network (Nyaga et al., 2014; Sigei et al., 2015). Another aliquot of the stool samples was sent on dry ice to The University of Texas Health Science Center, School of Public Health, Center for Infectious Diseases (Houston, Texas, USA) for additional analysis.

During the hospital visit, children were evaluated by attending physicians for severity of symptoms and either treated in the clinic as outpatients or hospitalized. The severity of illness was indicated by whether a child was admitted to the hospital (inpatient) or treated as an outpatient.

C. difficile *detection by culture*

Detection of *C. difficile* was performed and validated using toxigenic culture (Darkoh et al., 2011a) and PCR, as described

elsewhere (Oyaro et al., 2018). Anaerobic conditions were 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 stool was carefully spread on *C. difficile*-specific culture plates (Darkoh et al., 2011a) with a sterile single-use loop and incubated anaerobically at 37 °C for 48 h. The agar-based culture medium comprised (per liter) 37 g brain heart infusion (BHI) medium (Becton Dickinson, Franklin Lakes, NJ, USA), 14 g agar, 7% defibrinated horse blood, 150 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Zymo Research, Tustin, CA, USA), 0.1% sodium taurocholate (Sigma-Aldrich, St. Louis, MO, USA), 250 μg/ml D-cycloserine, 16 μg/ml cefoxitin, and 0.025% *p*-cresol. Following incubation, the number of stools that grew colonies was enumerated. For further analysis, the *C. difficile* colonies on each plate were pooled and enriched by culturing anaerobically for 24 h at 37 °C in BHI broth containing 300 μg/ml D-cycloserine and 8 μg/ml cefoxitin. Freezer stocks (1 ml) of each culture were made in 10% dimethyl sulfoxide (DMSO) and stored at −80 °C. The remaining culture (3 ml) was centrifuged for 10 min at 10 000 × *g*, and the pellets were stored at −20 °C for DNA isolation and PCR analysis.

PCR analysis

DNA was isolated from each of the bacterial pellets using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the protocol provided by the manufacturer. A NanoDrop (ThermoScientific, Wilmington, DE, USA) was used to determine the concentration of the extracted DNA. 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 were: TcdA2 (F-5′AGATTCCTATATTTACATGACAATAT3′, R-5′GTATCAGGCATAAAGTAATATACTTT3′); TNC (F-5′GAGCACAAAGGGTATTGCTCTACTGGC3′, R-5′CCAGACACAGCTAATCTTATTTGCACCT3′); 16S rRNA (F-ACACGGTCCAAACTCCTACG, R-5′AGGCGAGTTTCAGCCTACAA3′). 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, followed by 36 cycles of 94 °C for 30 s; 55 °C for 30 s; 68 °C for 30 s; and a final extension of 68 °C for 5 min. The PCR products were separated by gel electrophoresis using a 1.0% agarose gel. The DNA in the agarose gel was stained with ethidium bromide and visualized using BioDoc-It Imaging System (UVP, Upland, CA, USA).

Toxin assays

C. difficile toxins A and B present in 48-h 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 *C. difficile* culture was centrifuged for 10 min at 15 000 × *g*, and 250 μl of the supernatant fluid was added to 30 μl of 0.2-micron 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 up to 24 h (depending on the amount of the toxins present), 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 culture supernatant was tested according to the manufacturer's instructions.

Detection of Cryptosporidium, Giardia, *and rotavirus*

The presence of *Cryptosporidium parvum* and/or *Giardia lamblia* in the diarrheal stools was determined simultaneously using the ImmunoCard STAT! Crypto/Giardia Rapid assay according to the manufacturer's instructions (Meridian Bioscience, Inc., Cincinnati, OH, USA). Briefly, 250 mg of the stool was diluted 1:4 with deionized water. Diluted sample (60 μl) was placed in the card well and allowed to react at room temperature for 10 min before visual assessment. Positive reactions for one or both parasites were determined by the development of bands in indicated areas of the card. An internal positive control band indicated a valid test. Each test was done as a single assay; tests with indeterminate or faint bands were repeated to confirm the results.

Stools were screened for the presence of rotavirus by ELISA with confirmatory polyacrylamide gel electrophoresis (PAGE) assays at the Kenya Medical Research Institute, as described previously (Nyaga et al., 2014; Sigei et al., 2015).

Statistical analysis

Data were analyzed using Stata 15 for Windows (StataCorp LLC, College Station, TX, USA). Pearson's Chi-square test and Fisher's exact test were used to assess correlations between the demographic variables and the type of pathogen detected in the stool. Statistical significance was defined as a *p*-value of ≤0.05 for all tests.

Results

To determine the frequency of *C. difficile* in children <5 years old, 157 stool samples from children presenting with diarrhea at a national hospital in Kenya were examined by culture and PCR for the presence of *C. difficile*. Of the 157 stool samples tested, 59 (37.6%) contained viable *C. difficile* colonies, while 98 (62.4%) had no *C. difficile* growth on the culture medium (Table 1). To confirm the identity of the colonies that grew on the culture plates, DNA was extracted from a pool of colonies isolated from each stool sample and analyzed by PCR for the presence of genes specific to *C. difficile*: *tcdA*, *tcdB*, and *tcdC*. Of the 59 samples that underwent PCR analysis, all were positive for at least one of the genes examined. The *tcdA* and *tcdB* genes were identified in 53 (89.8%) of the pooled isolates. The *tcdC* gene was amplified in all 59 samples, and 34 (57.6%) had the frame-shift deletion associated with *C. difficile* hypervirulence. Toxin activity was detected in pooled colonies from 40 (67.8%) of the samples, whereas 19 (32.2%) demonstrated no detectable toxin activity *in vitro*. Of these 19 samples, 13 had the *tcdA* and *tcdB* genes.

The study population comprised 98 male (62.4%) and 59 female (37.6%) children with an overall average age of 12.4 months and median age of 12.0 months (Figure 1). Age ranged from 1 to 36

Table 1
Detection and confirmation of *Clostridium difficile* by culture and PCR in 157 stool samples from patients with diarrhea. Stools were streaked on *C. difficile*-specific agar-based medium and incubated anaerobically for 48 h. Colonies were pooled and further analyzed by PCR using primers specific for toxin A (*tcdA*), toxin B (*tcdB*), or the TcdC gene (*tcdC*).

	N	%
Detection of *C. difficile* by Culture	157	
Positive	59	37.6
Negative	98	62.4
PCR Analysis of *C. difficile* Colonies		
Presence of *tcdA*/*tcdB* or *tcdC* genes	59	
TcdA/*tcdB* genes		
$tcdA^+/tcdB^+$	40	67.8
$tcdA^-/tcdB^+$	13	22.0
$tcdA^-/tcdB^-$	6	10.2
$tcdC^+$	59	98.3
TcdC deletion		
Wild type	25	42.4
Frame-shift deletion	34	57.6

months, with 25 (15.9%) children being under 6 months old and 27 (17.2%) being over 19 months old; age was not reported for 10 (6.4%). The average age was comparable between the sexes (male, 12.3 months; female, 12.8 months). Of the 59 children positive for *C. difficile*, 57.6% were male and 42.4% were female. *C. difficile* was detected in all age categories, except in children >24 months old ($n = 4$). Children 13–18 months of age had the highest incidence of *C. difficile* (54.8%), followed by those 19–24 months of age (43.5%). The frequency of *C. difficile* was 32.6% in children 12 months of age or younger and increased to 46.6% in children 12–24 months of age. The distribution of *C. difficile* positivity among the age categories was similar between males and females.

Etiology of diarrhea

To investigate the presence of common diarrhea-causing parasites, the stool samples were tested for *C. parvum* and *G. lamblia*. Rotavirus testing was done as part of a previous study and reported elsewhere (Nyaga et al., 2014; Sigei et al., 2015). The combined results from the present and previous studies showed that 66.8% ($n = 105$) of the stools were positive for one or more of the four enteric pathogens (*C. difficile*, *C. parvum*, *G. lamblia*, and rotavirus) (Table 2). None of the four enteric pathogens was detected in the stools of 34.7% of the male children ($n = 34$) and 30.5% of the female children ($n = 18$). Surprisingly, the most prevalent pathogen identified in both sexes was *C. difficile*, followed by rotavirus. *C. difficile* was detected in the stools of 56.5% ($n = 105$) of children identified with one or more of the tested pathogens. Rotavirus was the second most common pathogen with 50.5%, while *Cryptosporidium* and *Giardia* were each detected in 5.1%, respectively, of the children. With regard to the tested pathogens, 37.1% of children had a *C. difficile*-only infection, whereas 31.4% had rotavirus only. Rotavirus with *C. difficile* was the most prevalent type of co-infection and accounted for 16.2% of the diarrhea stools with the pathogen identified. No child in the study was infected with more than two of the four tested pathogens, and 64.8% of children 7–18 months of age had two identified pathogens, a percentage higher than for the other age groups. Further, 75.8% of the rotavirus-only, 56.4% of the *C. difficile*-only, and 73.9% of mixed infections also occurred in children 7–18 months of age.

Severity of disease

Since no data on diarrhea frequency or gravity of the symptoms were available, disease severity was determined by the treatment setting of the patients (Kotloff et al., 2013). Diarrhea that required hospitalization was classified as severe. Of the 157 children examined, 90 (57.3%) were hospitalized and 67 (42.7%) were treated and released as outpatients (Table 3A). No correlation was observed between disease severity and the child's sex or age. There were more inpatients than outpatients in all age categories, except in children 19–24 months of age. Of those identified with any of the tested enteropathogens, 56 (53.3%) were inpatients, whereas 49 (46.7%) were outpatients. Among the inpatients, children 7–9 months old were predominant (21.4%), while 26.5% of the outpatients were 13–18 months of age (Table 3B). Of the 90 inpatients, 56 (62.2%) had one or more of the four pathogens in their stools compared to 73.1% (49/67) of the outpatients. The stools of 24.4% of the inpatients had only *C. difficile*, whereas 17.8% had rotavirus only. When co-infections were considered, the prevalence of *C. difficile* and rotavirus among the inpatients was 33.3% and 30.0%, respectively. Among the outpatients, 25.4% of their stools had *C. difficile* only, while 25.4% had rotavirus only. With regard to co-infections, the total prevalence of *C. difficile* and rotavirus among outpatients was 43.3% and 38.8%, respectively. Overall, the incidence of *C. difficile* and rotavirus was higher among outpatients than inpatients (Table 4).

Table 2
Distribution of the patients based on known diarrhea-associated pathogens detected in their stool samples.

	N	%	Male	%	Female	%
Only Detected Pathogen						
C. difficile	39	24.8	24	24.5	15	25.4
Rotavirus	33	21.0	22	22.5	11	18.7
Giardia	6	3.8	4	4.1	2	3.4
Cryptosporidium	4	2.6	1	1.0	3	5.1
Mixed Infections						
Rotavirus + C. difficile	17	10.8	7	7.1	10	16.9
Rotavirus + Cryptosporidium	3	1.9	3	3.1	–	–
C. difficile + Giardia	2	1.3	2	2.0	–	–
C. difficile + Cryptosporidium	1	0.6	1	1.0	–	–
Unknown	52	33.2	34	34.7	18	30.5
Total	157	100.0	98	62.4	59	37.6

Only 33.8% of children could be diagnosed with a pathogen potentially responsible for their diarrhea when stool samples were tested for rotavirus. The proportion increased to 60.5% when the results from both rotavirus and *C. difficile* tests were considered and 66.8% if all four pathogens were considered. These results highlight the need to test for more than one of these diarrhea-associated enteropathogens during diarrhea diagnosis (Figure 2).

Discussion

Little is known about the role of *C. difficile* in young children in Sub-Saharan Africa, as well as *C. difficile* in co-infection with other enteric pathogens (Emeruwa and Oguike, 1990; Janssen et al., 2016; Seugendo et al., 2015). Due to the increasing incidence of *C.*

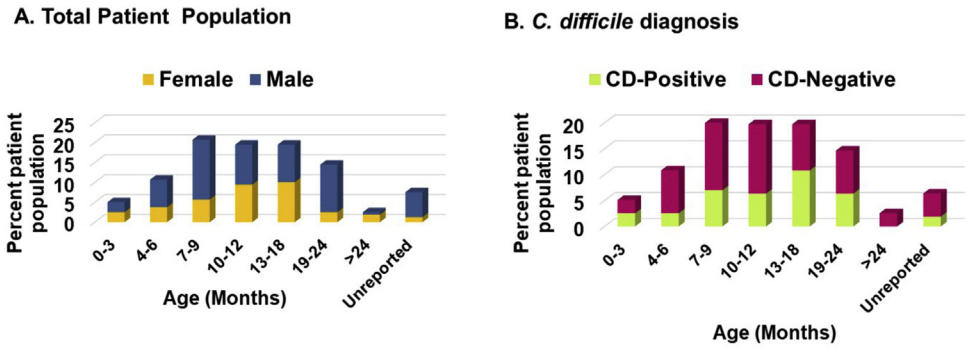

Figure 1. Distribution of the total patient population by sex and age (A), and by the presence of *Clostridium difficile* in stool (B). The population was 62.4% male and 37.6% female.

Table 3
Disease severity by age (A), and by age of patients whose stools contained one or more of the four diarrhea-associated pathogens tested (B). Disease severity was determined on the basis of whether a patient was admitted (inpatient) or not hospitalized (outpatient).

A. Disease severity by age

		Inpatient		Outpatient	
Age group	N	N	%	N	%
0–3	8	6	75.0	2	25.0
4–6	17	10	58.8	7	41.2
7–9	33	18	54.6	15	45.4
10–12	31	19	61.3	12	38.7
13–18	31	17	54.8	14	45.2
19–24	23	10	43.5	13	56.5
>24	4	3	75.0	1	25.0
Not reported	10	7	70.0	3	30.0
Total	157	90	57.3	67	42.7

B. Disease severity by age in patients with identified pathogens

		Inpatient		Outpatient	
Age group	N	N	%	N	%
0–3	6	5	8.9	1	2.0
4–6	10	4	7.2	6	12.3
7–9	23	13	23.2	10	20.4
10–12	20	11	19.6	9	18.4
13–18	25	12	21.4	13	26.5
19–24	13	5	8.9	8	16.3
>24	2	2	3.6	0	0.0
Not reported	6	4	7.2	2	4.1
Total	105	56	53.3	49	46.7

difficile infections globally and the high rate of diarrhea-associated deaths among children in this region, it was sought to establish the frequency of *C. difficile* in a cross-section of young children less than 5 years of age in Kenya. Stools from children presenting with diarrhea at a major hospital in Kenya were examined for the presence of four important pathogens associated with diarrhea: *C. difficile*, rotavirus, *C. parvum*, and *G. lamblia*. Surprisingly, *C. difficile* was the most prevalent pathogen in the diarrhea stools examined, followed by rotavirus, which is thought to be the leading cause of diarrhea globally in children under 5 years old (Kotloff et al., 2013).

Viable *C. difficile* colonies were isolated from 59 (37.6%) out of 157 diarrhea stools collected from children ranging in age from 1 to 36 months, but none over the age of 24 months tested positive. The age group that had the highest incidence of *C. difficile* was 13–18 months (54.8%), followed by 1–3 months (50%), 19–24 months (43.5%), 7–9 months (33.3%), 10–12 month (32.3%), and 4–6 months (23.5%). Most of the *C. difficile* isolates contained the genes encoding toxins A and B (89.8%); furthermore, isolates from 67.8% of the stool samples produced active toxins *in vitro*. Moreover, 57.6% of the stools contained *C. difficile* strains bearing the frame-shift deletion in the *tcdC* gene that is associated with hyper-virulence. These results demonstrate that the *C. difficile* strains circulating in these children exhibit some of the hallmarks of *C. difficile* strains circulating in other parts of the world. However, this study did not investigate the genetic makeup of the strains, differences in toxin production, toxin activity, or sporulation, which are the subjects of an ongoing study.

Table 4
Distribution of the inpatients and outpatients based on the presence of the four diarrhea-associated pathogens in their stools. *Cryptosporidium* and *Giardia* were denoted as parasites.

	All Cases	Inpatient		Outpatient	
		No. cases	%	No. cases	%
Total number of patients	157	90	100.0	67	100.0
One or more pathogens identified	105	56	62.2	49	73.1
Rotavirus only	33	16	17.8	17	25.4
C. difficile only	39	22	24.4	17	25.4
Rotavirus + *C. difficile*	17	8	8.9	9	13.4
Rotavirus + parasites	3	3	3.3	0	0.0
C. difficile + parasites	3	0	0.0	3	4.5
All *Rotavirus*	53	27	30.0	26	38.8
All *C. difficile*	59	30	33.3	29	43.3

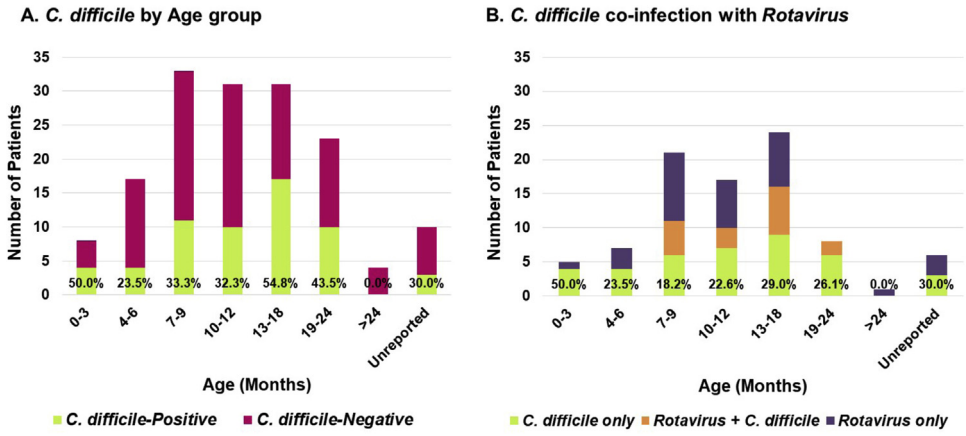

Figure 2. *Clostridium difficile* prevalence by age (A), and *C. difficile* in co-infection with rotavirus (B). The percentages reported in panel A are the prevalence of *C. difficile* only within each age category, whereas the percentages reported in panel B are the prevalence of *C. difficile* only and *C. difficile* in co-infection with any of the four enteric pathogens tested within each age category.

Of the four diarrhea-associated enteric pathogens tested, all were identified in 105 of the 157 stool samples. Of note, 52 (33.2%) of the samples did not contain any of these pathogens, indicating that diarrhea in those children may have been caused by other pathogens such as enterotoxigenic *Escherichia coli* or *Shigella spp*, or conditions not tested. Due to restrictions placed by the ethics review boards, data on possible contributing factors such as antibiotic use, comorbidities, nutritional status, HIV status, and chronic infections (Seugendo et al., 2015) were not available. Among the stools that were positive in the present study, *C. difficile* was the most prevalent pathogen (56.5%), followed by rotavirus (50.5%), *Cryptosporidium* (7.6%), and *Giardia* (7.6%). These prevalence rates in Kenyan children (≤5 years) are consistent with those reported in other studies on rotavirus (Kiulia et al., 2008), *Cryptosporidium* (Squire and Ryan, 2017), and *Giardia* (Squire and Ryan, 2017). Moreover, the percentage of stools in which only *C. difficile* was identified was higher (37.1%) than that for rotavirus (31.4%). Together, the results indicate that *C. difficile* may play a more important role in diarrhea-associated disease in children than previously thought. The rate of *C. difficile* observed may also suggest that infants and young children may serve as reservoirs of infection to susceptible populations (Chitnis et al., 2013) and highlights the need for further research to examine the role of *C. difficile* carriage in young children in diarrheal disease transmission.

The frequency of co-infections among other enteric pathogens makes it difficult to identify the offending pathogen responsible for diarrhea. Of note, the frequency of co-infections among children who tested positive for any of the four pathogens was 21.9%, and the majority of the co-infections involved *C. difficile* and rotavirus. This observation adds to the growing evidence that enteric co-infections are not uncommon among young children (Nicholson et al., 2016). Interestingly, *C. difficile* did not correlate with milder disease as previously reported in young children (de Graaf et al., 2015; Wendt et al., 2014), and comparable levels of *C. difficile* and rotavirus were observed in both inpatient and outpatient children.

The etiology of diarrhea in Kenyan children is also complicated by the widespread availability and use of antibiotics by the general population. As this phenomenon is so common, the microbiological workup for bacterial pathogens is generally unhelpful and not done in Kenyan children presenting to the clinic with diarrhea. Thus, no data on potential bacterial pathogens exist in the medical records for the children studied here. It was decided to test for common enteric pathogens that are not affected by antibiotics and thus may be of increased importance in childhood diarrhea in such populations. However, since no data on prior antibiotic administration were available, the possibility of bacterial pathogens in those receiving no antibiotics or the presence of antibiotic-resistant bacteria in those who did cannot be ruled out.

The presence of different pathogens co-infecting the same child adds to the complexity in deciphering the main cause of diarrhea and underscores the need to test for the presence of other diarrhea-associated pathogens, including *C. difficile* in the diagnostic workup of children with diarrhea. Moreover, research on *C. difficile* epidemiology alone or in relation to co-infecting pathogens among African children with diarrhea will provide new approaches that can help improve infection control measures.

Acknowledgements

This work was supported by NIH R01 grant number R01AI116914, a Molecular Basis of Infectious Diseases Training Grant from the NIH Institute of Allergy and Infectious Diseases (T32AI055449), and the Gillson-Longenbaugh Foundation.

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.

Conflict of interest

None of the authors has a conflict to disclose.

References

Albert MJ, Faruque AS, Faruque SM, Sack RB, Mahalanabis D. Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. J Clin Microbiol 1999;37(11):3458–64.
Alcides AP, Brazier JS, Pinto LJ, Balassiano IT, Boente RF, de Paula GR, et al. New PCR ribotypes of *Clostridium difficile* detected in children in Brazil: prevalent types of *Clostridium difficile* in Brazil. Antonie van Leeuwenhoek 2007;92(1):53–9.
Carter GP, Douce GR, Govind R, Howarth PM, Mackin KE, Spencer J, et al. The anti-sigma factor TcdC modulates hypervirulence in an epidemic BI/NAP1/027 clinical isolate of *Clostridium difficile*. PLoS Pathog 2011;7(10)e1002317.
Checkley W, Buckley G, Gilman RH, Assis AM, Guerrant RL, Morris SS, et al. Multi-country analysis of the effects of diarrhoea on childhood stunting. Int J Epidemiol 2008;37(4):816–30.
Chen S, Sun C, Wang H, Wang J. The role of rho GTPases in toxicity of *Clostridium difficile* toxins. Toxins 2015;7(12):5254–67.
Chitnis AS, Holzbauer SM, Belflower RM, Winston LG, Bamberg WM, Lyons C, et al. Epidemiology of community-associated *Clostridium difficile* infection, 2009 through 2011. JAMA Intern Med 2013;173(14):1359–67.
Curry SR, Marsh JW, Muto CA, O'Leary MM, Pasculle AW, Harrison LH. tcdC genotypes associated with severe TcdC truncation in an epidemic clone and other strains of *Clostridium difficile*. J Clin Microbiol 2007;45(1):215–21.
Darkoh C, DuPont HL, Kaplan HB. Novel one-step method for detection and isolation of active-toxin-producing *Clostridium difficile* strains directly from stool samples. J Clin Microbiol 2011a;49(12):4219–24.
Darkoh C, Kaplan HB, Dupont HL. Harnessing the glucosyltransferase activities of *Clostridium difficile* for functional studies of toxins A and B. J Clin Microbiol 2011b;49(8):2933–41.
de Graaf H, Pai S, Burns DA, Karas JA, Enoch DA, Faust SN. Co-infection as a confounder for the role of *Clostridium difficile* infection in children with diarrhoea: a summary of the literature. Eur J Clin Microbiol Infect Dis 2015;34(7):1281–7.
Elewa A, Sayed-Zaki ME. Evaluation of nested polymerase chain reaction and immunoassay for rapid diagnosis of *Clostridium difficile* in children with community acquired diarrhea. Clin Lab 2017;63(2):321–6.
Emeruwa AC, Oguike JU. Incidence of cytotoxin producing isolates of *Clostridium difficile* in faeces of neonates and children in Nigeria. Microbiologica 1990;13(4):323–8.
Fiedoruk K, Daniluk T, Rozkiewicz D, Zaremba ML, Oldak E, Sciepuk M, et al. Conventional and molecular methods in the diagnosis of community-acquired diarrhoea in children under 5 years of age from the north-eastern region of Poland. Int J Infect Dis 2015;37:145–51.
Fry PR, Thakur S, Abley M, Gebreyes WA. Antimicrobial resistance, toxinotype, and genotypic profiling of *Clostridium difficile* isolates of swine origin. J Clin Microbiol 2012;50(7):2366–72.
Griffiths D, Fawley W, Kachrimanidou M, Bowden R, Crook DW, Fung R, et al. Multilocus sequence typing of *Clostridium difficile*. J Clin Microbiol 2010;48(3):770–8.
Hundsberger T, Braun V, Weidmann M, Leukel P, Sauerborn M, von Eichel-Streiber C. Transcription analysis of the genes tcdA-E of the pathogenicity locus of *Clostridium difficile*. Eur J Biochem 1997;244(3):735–42.
Jangi S, Lamont JT. Asymptomatic colonization by *Clostridium difficile* in infants: implications for disease in later life. J Pediatr Gastroenterol Nutr 2010;51(1):2–7.
Janssen I, Cooper P, Gunka K, Rupnik M, Wetzel D, Zimmermann O, et al. High prevalence of nontoxigenic *Clostridium difficile* isolated from hospitalized and non-hospitalized individuals in rural Ghana. Int J Med Microbiol 2016;306(8):652–6.
Khalil IA, Troeger C, Rao PC, Blacker BF, Brown A, Brewer TG, et al. Morbidity, mortality, and long-term consequences associated with diarrhoea from *Cryptosporidium* infection in children younger than 5 years: a meta-analyses study. Lancet Glob Health 2018;6(7):e758–68.
Kiulia NM, Kamenwa R, Irimu G, Nyangao JO, Gatheru Z, Nyachieo A, et al. The epidemiology of human rotavirus associated with diarrhoea in Kenyan children: a review. J Trop Pediatr 2008;54(6):401–5.
Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 2013;382(9888):209–22.
Leffler DA, Lamont JT. *Clostridium difficile* infection. New Engl J Med 2015;372(16):1539–48.
Lemee L, Dhalluin A, Testelin S, Mattrat MA, Maillard K, Lemeland JF, 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(12):5710–4.
Li N, Jia H, Yang H, Ji B, Liu Y, Peng X, et al. Preliminary screening of type IV secretion system in divergent geographic sources of *Clostridium difficile*. Exp Ther Med 2017;14(5):4405–10.
Liu L, Johnson HL, Cousens S, Perin J, Scott S, Lawn JE, et al. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. Lancet 2012;379(9832):2151–61.
Liu XS, Li WG, Zhang WZ, Wu Y, Lu JX. Molecular characterization of *Clostridium difficile* isolates in China from 2010 to 2015. Front Microbiol 2018;9:845.
Mani N, Dupuy B. Regulation of toxin synthesis in *Clostridium difficile* by an alternative RNA polymerase sigma factor. Proc Natl Acad Sci U S A 2001;98(10):5844–9.
Mani N, Lyras D, Barroso L, Howarth P, Wilkins T, Rood JI, et al. Environmental response and autoregulation of *Clostridium difficile* TxeR, a sigma factor for toxin gene expression. J Bacteriol 2002;184(21):5971–8.
Matamouros S, England P, Dupuy B. *Clostridium difficile* toxin expression is inhibited by the novel regulator TcdC. Mol Microbiol 2007;64(5):1274–88.
Murray R, Boyd D, Levett PN, Mulvey MR, Alfa MJ. Truncation in the *tcdC* region of the *Clostridium difficile* PathLoc of clinical isolates does not predict increased biological activity of Toxin B or Toxin A. BMC Infect Dis 2009;9:103.
Nicholson MR, Van Horn GT, Tang YW, Vinje J, Payne DC, Edwards KM, et al. Using multiplex molecular testing to determine the etiology of acute gastroenteritis in children. J Pediatr 2016;176:50–6 e2.
Nyaga MM, Stucker KM, Esona MD, Jere KC, Mwinyi B, Shonhai A, et al. Whole-genome analyses of DS-1-like human G2P[4] and G8P[4] rotavirus strains from Eastern, Western and Southern Africa. Virus Genes 2014;49(2):196–207.
Oyaro MO, Plants-Paris K, Bishoff D, Malonza P, Gontier CS, DuPont HL, et al. High rate of *Clostridium difficile* among young adults presenting with diarrhea at two hospitals in Kenya. Int J Infect Dis 2018;74:24–8.
Pinto LJ, Alcides AP, Ferreira EO, Avelar KE, Sabra A, Domingues RM, et al. Incidence and importance of *Clostridium difficile* in paediatric diarrhoea in Brazil. J Med Microbiol 2003;52(Pt 12):1095–9.
Rogawski ET, Bartelt LA, Platts-Mills JA, Seidman JC, Samie A, Havt A, et al. Determinants and impact of *Giardia* infection in the first 2 years of life in the MAL-ED birth cohort. J Pediatric Infect Dis Soc 2017;6(2):153–60.
Seugendo M, Mshana SE, Hokororo A, Okamo B, Mirambo MM, von Muller L, et al. *Clostridium difficile* infections among adults and children in Mwanza/Tanzania: is it an underappreciated pathogen among immunocompromised patients in sub-Saharan Africa?. New Microbes New Infect 2015;8:99–102.
Sigei C, Odaga J, Mvundura M, Madrid Y, Clark AD. Cost-effectiveness of rotavirus vaccination in Kenya and Uganda. Vaccine 2015;33(Suppl. 1):A109–18.
Squire SA, Ryan U. *Cryptosporidium* and *Giardia* in Africa: current and future challenges. Parasit Vectors 2017;10(1):195.
Troeger C, Forouzanfar M, Rao PC, Khalil I, Brown A, Reiner RC, et al. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. Lancet Infect Dis 2017;17(9):909–48.
Wang L, Xiao L, Duan L, Ye J, Guo Y, Guo M, et al. Concurrent infections of *Giardia duodenalis*, *Enterocytozoon bieneusi*, and *Clostridium difficile* in children during a *Cryptosporidiosis* outbreak in a pediatric hospital in China. PLoS Negl Trop Dis 2013;7(9):e2437.
Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, et al. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet 2005;366(9491):1079–84.
Wendt JM, Cohen JA, Mu Y, Dumyati GK, Dunn JR, Holzbauer SM, et al. *Clostridium difficile* infection among children across diverse US geographic locations. Pediatrics 2014;133(4):651–8.