Original Article

High Prevalence of Diarrheagenic *Escherichia coli* among Children with Diarrhea in Kenya

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SUMMARY: Diarrheagenic *Escherichia coli* (DEC) is an important agent of endemic and epidemic diarrhea worldwide, particularly in developing countries. DEC cannot be differentiated from commensal *E. coli* on selective media, although there are a few exceptions. Most studies use the colony isolation method, which cannot detect low numbers of DEC, and therefore, these studies might underestimate the incidence of DEC. In the present study, we employed a colony sweep method with real-time PCR targeting virulence genes of 5 categories of DEC; this technique can detect very low numbers of DEC among hundreds of commensal *E. coli*. DEC was detected in 171 (55.9%) of 306 children with diarrhea in Kenya. The prevalence of DEC in Kenya was notably higher than that (30 in 143, 21.0%) in Indonesia. Occurrences of multiple DEC infection in Kenya were frequent (69 in 306, 23.2%), suggesting that the source of DEC infection may be related to grossly contaminated food and water. In contrast, only 9 (6.0%) of 150 healthy adults in Kenya carried DEC. Considering that healthy adults naturally harbor non-DEC, it is interesting how children exclude DEC but not non-DEC as they grow up. Several mechanisms, such as mucosal immunity and intestinal microbiota, might be involved in the exclusion of DEC.

INTRODUCTION

Diarrhea is one of the most common causes of morbidity and mortality among infants and children in developing countries (1). The etiological agents of diarrhea include a wide range of viruses, bacteria, and parasites. Among the bacterial pathogens, diarrheagenic *Escherichia coli* (DEC) is an important agent of endemic and epidemic diarrhea worldwide (2). *E. coli* is a common component of the intestinal microbiota in humans, but certain pathogenic strains can cause diarrhea. DEC can be classified into 5 categories on the basis of their specific virulence properties: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), and Shiga toxin-producing *E. coli* (STEC). In addition, diffusely adherent *E. coli* (DAEC) may represent a 6th category (2). Unlike other diarrheagenic bacteria, such as *Salmonella enterica*, *Shigella* spp., and *Vibrio* spp., which can be readily isolated using selective plating media, DEC cannot be differentiated from commensal *E. coli* on selective media, with the exception of some serotypes of STEC. Most studies on

DEC incidence have employed a colony isolation method, which cannot detect the presence of low numbers of DEC among commensal *E. coli* (3,4). Thus, the incidence of DEC infections might be underestimated. In the present study, we have examined DEC incidence in a Kenyan population using a colony sweep method, which can detect even small numbers of DEC among hundreds of commensal *E. coli*. We compared our results with those of previous study in Indonesia, in which DEC was detected using the same procedures employed in this study (5).

MATERIALS AND METHODS

Stool specimens and bacterial culture: The subjects were children under 5 years of age with diarrhea who visited Mbagathi District Hospital in Nairobi, Malindi District Hospital in Malindi, New Nyanza Provincial Hospital in Kisumu, or Alupe Leprosy Hospital in Busia, from September 2007 to December 2009. Malindi is an east coastal town in Kenya, while Kisumu and Busia are districts in western Kenya. Non-diarrheal adults who were 20–40 years old were enrolled as a control group. The national ethical review committee of the Kenya Medical Research Institute approved this study, including the ethical aspects. Verbal informed consent to use the stool samples for research purposes was obtained from the parents of children with diarrhea and the cooperating adults without diarrhea.

The stool samples were collected in sterile containers and were inoculated onto MacConkey agar plates to detect *E. coli*, thiosulfate citrate bile salts sucrose

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(TCBS) agar plates to detect *Vibrio* spp., and xylose lysine deoxycholate (XLD) agar plates to detect *S. enterica* and *Shigella* spp. The suspected colonies on TCBS agar and XLD agar were analyzed using morphological, biochemical, and serological methods. For detection of virulence genes of DEC, colony sweeps from confluent areas on MacConkey agar plates were suspended in half concentration of Luria-Bertani broth containing 10% glycerol, and frozen at -20°C until use for further characterization.

DEC detection: DEC was detected as described in a previous study (5). In brief, DNA extracts from colony sweep suspensions on MacConkey agar were obtained using the boiling method. DNA extracts were analyzed using the real-time PCR with primers and probes targeting *elt*, *estp*, *esth*, and *estII* for ETEC (4,5), *ipaH* for EIEC (6), *aggR* for EAEC (4), *eae* for EPEC (7), and *stx1*, *stx2*, and *eae* for STEC (7).

When virulence genes of 5 categories of DEC were detected using real-time PCR with a threshold cycle (Ct) value of 35 or less, DEC isolation was attempted from particular stool specimens. Twenty of the lactose-fermenting colonies with typical *E. coli* morphology on MacConkey agar plates were randomly selected and transferred onto trypticase soy agar (TSA) plates using sterile toothpicks. For *ipaH*-positive samples, the non lactose-fermenting colonies were also collected. The colonies grown on TSA plates were analyzed using the primers and probes that can detect the virulence genes. Even if no DEC colonies were detected among the 20 colonies, the samples with a Ct value of 35 or less were defined as positive for each DEC.

For EPEC, the presence or absence of the *bfpA* gene in addition to the *eae* gene was also analyzed using real-time PCR (5). EPEC with and without the *bfpA* gene were designated as typical and atypical EPEC, respectively. For EAEC, the presence or absence of the *astA* gene was also examined using real-time PCR (5). If samples tested positive for virulence genes of ETEC such as *elt*, *estp*, *esth*, and *estII*, we investigated whether these genes belonged to single or multiple organisms using colony isolation.

Comparison with samples in Indonesia: The prevalence of DEC in Kenya was compared with the results of a previous study in Indonesia (5), in which DEC was detected using the same procedures employed in this study. Age-matched samples (0–5 years old) were chosen and re-calculated because children at 1–10 years old were chosen in the study in Indonesia (5). The data of children younger than 1 year old were included and children at 6–10 years old were omitted.

RESULTS

A total of 264 DEC were detected in the stool samples of 306 children with diarrhea in Kenya (Table 1). EAEC was detected in 138 samples (45.1%). Typical EPEC, ETEC, and EIEC genes were detected in 51 samples (16.7%), 50 samples (16.3%), and 25 samples (8.2%), respectively. No STEC was detected in the samples used in this study. At least 1 diarrheagenic bacteria and at least 1 DEC were detected in 178 (58.2%) and 171 (55.9%) samples, respectively. The prevalences of EAEC, EPEC, ETEC, and EIEC in Kenya were sig-

nificantly higher than those in Indonesia (P , <0.0001 – 0.0049).

Interestingly, out of the 171 DEC infections, multiple DEC were detected in 69 samples (40.4%). Two or more DEC were sometimes detected in the same sample, and this was observed more than would be expected by chance, except the combination of EIEC and EAEC (P , <0.0001 – 0.0011 ; $\chi^2 = 10.67$ – 19.96 ; based on the data in Table 1).

A total of 58 ETEC strains were detected from 50 diarrheal children in Kenya (Table 2). Among 58 ETEC strains, ETEC harboring *elt* alone were the most prevalent (41 strains, 70.7%), followed by ETEC harboring *estp* alone (6 strains, 10.3%), both *elt* and *estp* (5 strains, 8.6%), *esth* alone (3 strains, 5.2%), and *elt* and *esth* (3 strains, 5.2%). No ETEC harboring *estII* was detected in this study. Of the 138 EAEC, there were 99 (71.7%) and 39 (28.3%) with and without *astA* gene, respectively. Of the 90 EPEC, there were 51 (56.7%) typical EPEC harboring *bfpA* gene and 39 (43.3%) atypical EPEC without the *bfpA* gene. No significant difference in DEC prevalence was found between the 4 hospitals in Nairobi, Malindi, Kisumu, and Busia (data not shown).

In contrast, among 150 healthy adults in Kenya who were 20–40 years old, only 9 (6.0%) tested positive for any gene related to diarrheagenic bacteria (ETEC, 1; EIEC, 0; EAEC, 8; typical EPEC, 0; STEC, 0). No multiple infections were detected among them. There were significant differences in DEC prevalence between children with diarrhea and adults without diarrhea ($P < 0.0001$).

When DEC isolation was attempted from 20 *E. coli* and *E. coli*-like colonies in each of 74 samples harboring 140 of DEC virulence genes, only 64 isolates (45.7%) were successfully obtained. When the Ct values were <20 , 20 to <25 , 25 to <30 , and 30 to <35 . Each virulence gene was successfully detected in 13/13 (100%), 27/35 (77.1%), 17/46 (37.0%), and 7/46 (15.2%) of cases, respectively. The higher the Ct value, the lower the success of DEC isolation.

DISCUSSION

PCR is a powerful molecular biology technique for the detection of target DNA in various samples. Of all PCR techniques, real-time PCR using TaqMan probes is particularly specific, rapid, quantitative, and advanced for analyses of multiple samples. In this study, we used real-time PCR with TaqMan probes to detect the virulence genes of DEC from colony sweeps. We identified DEC-positive samples base on the detection of virulence genes of DEC. Bacterial isolation is important for studying the characteristics of the bacterial strain, including drug-resistance and genomic diversity, although isolation or culture is impossible or difficult for some pathogens. In such cases, the detection of pathogens using PCR may be a substitute for colony isolation. Because isolation of low numbers of DEC among commensal *E. coli* was also very difficult using bacterial isolation, we adopted real-time PCR.

Indeed, DEC isolation was successful in only 45.7% of samples, when 20 colonies from each sample were analyzed. The result suggested that 54.3% of stool

Table 1. Bacterial findings in stool samples from diarrheal children in Kenya and Indonesia

	Kenya <i>n</i> = 306 (%)	Indonesia ¹⁾ <i>n</i> = 143 (%)	<i>P</i> value	χ^2
DEC detection				
ETEC ²⁾	50 (16.3)	5 (3.5)	0.0001	15.0
EIEC	25 (8.2)	2 (1.4)	0.0049	7.9
EAEC ³⁾	138 (45.1)	26 (18.2)	<0.0001	30.5
EPEC ⁴⁾	51 (16.7)	0	<0.0001	26.9
STEC	0	0	—	
Bacterial findings from individual children ⁵⁾				
At least one diarrheagenic bacteria	178 (58.2)	NA		
Single diarrheagenic bacteria	107 (35.0)	NA		
Multiple diarrheagenic bacteria	71 (23.2)	NA		
No diarrheagenic bacteria	128 (41.8)	NA		
At least one DEC	171 (55.9)	30 (21.0)	<0.0001	48.0
Single DEC	100 (32.7)	27 (18.9)	0.0025	9.1
Multiple DEC	69 (23.2)	3 (2.1)	<0.0001	30.3
ETEC alone	8 (2.6)	3 (2.1)		
EIEC alone	4 (1.3)	1 (0.7)		
EAEC alone	77 (25.2)	23 (16.1)		
EPEC alone	11 (3.6)	0		
STEC alone	0	0		
<i>Shigella</i> sp. alone	1 (0.3)	NA		
<i>Salmonella enterica</i> alone	6 (2.0)	NA		
ETEC + EIEC	4 (1.3)	0		
ETEC + EAEC	16 (5.2)	2 (1.4)		
ETEC + EPEC	4 (1.3)	0		
EIEC + EAEC	4 (1.3)	1 (0.7)		
EIEC + EPEC	2 (0.7)	0		
EAEC + EPEC	17 (5.6)	0		
EAEC + <i>Shigella</i>	2 (0.7)	NA		
ETEC + EIEC + EAEC	3 (1.0)	0		
ETEC + EIEC + EPEC	0	0		
ETEC + EAEC + EPEC	7 (2.3)	0		
EIEC + EAEC + EPEC	3 (1.0)	0		
ETEC + EAEC + <i>Shigella</i>	2 (0.7)	NA		
EAEC + EPEC + <i>Shigella</i>	1 (0.3)	NA		
ETEC + EIEC + EAEC + EPEC	5 (1.6)	0		
ETEC + EAEC + EPEC + <i>Shigella</i>	1 (0.3)	NA		

NA, not available.

¹⁾: Age-matched samples were chosen from reference (5) and re-calculated.²⁾: Infection with two types of ETEC was dealt as an ETEC infection.³⁾: EAEC including *astA*-positive and -negative.⁴⁾: Typical (*bfpA*-positive) EPEC only.⁵⁾: Diarrheagenic bacteria except DEC were not studied in Indonesia.

specimens contained less than 1 (5%) DEC among 20 *E. coli*. This was similar to the results of a previous study in Japan showing that 59.3% of stool specimens from patients with DEC infections harbored 10% DEC or less among their *E. coli* population (4). This sensitive method might result in the observed high prevalence of DEC among children with diarrhea in Kenya. In other words, the prevalence of DEC might be underestimated because colony-based investigations cannot detect low numbers of DEC.

Among DEC infections, ETEC infection was the most widely studied in developing countries. A total of 51 studies employing the colony isolation method showed that an average of 13% (median value; range, 3–39%) of specimens from children with diarrhea tested positive for ETEC (8). In contrast, 50 of 308 speci-

mens (16.2%) tested positive for virulence gene(s) of ETEC using our colony sweep method, which targeted hundreds of colonies at a time. PCR screening for virulence genes of DEC using a colony sweep might be a better approach to understand the actual incidence of DEC.

In many cases in this study, 2 types of DEC were detected alongside each other, suggesting that the source of DEC infection may be grossly contaminated food and water. Improved hygiene in water supplies and kitchens, and better sanitation is necessary to reduce the incidence of gastroenteric infections, including DEC infections, in Kenya.

There are some studies in developing countries that show no significant difference in DEC prevalence between children with and without diarrhea, although

Table 2. Incidence of DEC in diarrheal children in Kenya and Indonesia

DEC	Kenya <i>n</i> = 306	Indonesia <i>n</i> = 143	<i>P</i> value	χ^2
ETEC ¹⁾	58	5	<0.0001	19.31
<i>elt</i> +	41	3	<0.0001	19.51
<i>esth</i> +	3	0	NS	
<i>estp</i> +	6	0	NS	
<i>estII</i> +	0	0	—	
<i>elt/esth</i> +	3	1	NS	
<i>elt/estp</i> +	5	1	NS	
EIEC	25	2	0.0003	12.97
EAEC	138	26	<0.0001	73.12
<i>astA</i> +	99	16	<0.0001	40.92
<i>astA</i> –	39	10	<0.0001	20.91
EPEC	90	2	<0.0001	39.11
<i>bfpA</i> +	51	0	<0.0001	28.15
<i>bfpA</i> –	39	2	0.0033	8.66
STEC	0	0	—	
<i>stx1</i> +	0	0	—	
<i>stx2</i> +	0	0	—	
Total	311	35		

NS, not significant.

¹⁾: In Kenya, a total of 58 ETEC were detected from 50 children with diarrhea.

there are exceptions (5,9–12). Since age-matched control samples were unfortunately not obtained, we could not show the difference in DEC prevalence between children with and without diarrhea in this study. However, we showed a remarkably high prevalence of DEC among children with diarrhea in Kenya in comparison to Indonesia as well as in comparison to adults without diarrhea. It is interesting that children exclude DEC but not non-DEC as they grow up. Several mechanisms, such as mucosal immunity and intestinal microbiota, might be involved in the exclusion of DEC (13,14).

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Conflict of interest None to declare.

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