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Diarrhoeagenic *Escherichia coli* from childhood diarrhoea

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A cross-sectional study was done to characterize the diarrhoeagenic *Escherichia coli* (DEC), isolated from stool samples of children under 5 years of age with diarrhoea who were admitted to the Paediatric Ward of North Okkalapa General Hospital from January to September, 2007. DEC was isolated from 16 stool samples among 60 samples collected. Characterization of DEC was done by serotyping, toxin assay and polymerase chain reaction (PCR). Serotyping identified 8 enteropathogenic *E. coli* (EPEC), 3 enterotoxigenic *E. coli* (ETEC), and one enteroinvasive *E. coli* (EIEC). Four isolates were not serotypable. The primers used in PCR assay were LTI-O1 and LTI-O2 for ETEC-Labile Toxin; ST-157 and ST-158 for ETEC-Stable Toxin; STX-O1, STX-O2, STX-101 and STX-102 for enterohaemorrhagic *E. coli* (EHEC). PCR assay result showed 3 EHEC and one ETEC. Latex agglutination test was done for verotoxin (VT) and heat-labile toxin (LT) and ELISA test for heat-stable toxin (ST). Toxin assays showed 4 VT, 4 ST and one with both VT and ST. In this study, serology results did not correspond to toxin assay and PCR results indicating that serotyping alone is not sufficient for diagnosis of pathogenic type for DEC.

INTRODUCTION

Diarrhoea caused by *Escherichia coli* infection is one of the major health problems for children in many developing countries. More than one billion episodes of diarrhoea occur every year among children under one year of age causing approximately 2.5 million deaths [1]. Diarrhoeagenic *E.coli* (DEC) are recognized as five major phenotypes: enteropathogenic *E.coli* (EPEC), enterotoxigenic *E.coli* (ETEC), enteroinvasive *E.coli* (EIEC), enterohaemorrhagic *E.coli* (EHEC) and enteroaggregative *E.coli* (EAEC). The most commonly reported DEC were EPEC, ETEC and EHEC [2].

EPEC strains cause diarrhoea mostly in infants which leads to significant morbidity and mortality. These organisms are recognized by serological testing and they belong to a limited group of specific serotypes. Between 1971 and 1978 many investigators tested classical serotypes of EPEC strains from patients with diarrhoea for their ability to invade epithelial cells and for the presence of heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) [3]. ETEC strains that cause diarrhoea in human, which is recognized as traveller's diarrhoea, produce heat-labile and/or stable enterotoxins. It also comprises a heterogenous array of O: K: H serotypes and enterotoxin phenotypes. These strains were found within a small number of O serotypes including O6, O8, O15, O20, O25, O63, O115, O128, O148 and O159 [4].

EHEC are also important pathogens around the world and it is mostly associated with foodborne outbreaks. It produces Shiga toxins (STX1 and STX2). It is associated with O157:H7 serotypes. Identification of DEC requires that these organisms be differentiated from nonpathogenic members of the normal flora. Serogrouping of O antigen is not sufficient to identify a strain as diarrhoeagenic, because it does not correlate,

in most cases, with the presence of virulence factors [5]. Standard methods currently used in the identification of major *E. coli* pathotypes are based on distinct sets of virulence markers such as toxins (heat-labile and heat-stable enterotoxins). Thus identification of DEC strains needs to detect factors that determine the virulence of these organisms.

Objectives

- To identify diarrhoeagenic *E. coli* isolated from stool samples of children under 5 years of age
- To characterize the isolated DEC by serotyping, toxin assay and polymerase chain reaction assays

MATERIALS AND METHODS

A hospital-based, cross-sectional study was carried out to characterize the diarrhoeagenic *Escherichia coli* (DEC) from under five children with acute diarrhoea who attended the Paediatric Ward of North Okkalapa General Hospital (NOGH) between 1st January and 30th September, 2007. Rectal swabs were collected from 64 children (less than 5 years of age) with acute diarrhoea (≥ 3 watery or loose stools within 24 hours for <14 days) [6], admitted to the Paediatric Ward of NOGH within 9 months period. Rectal swabs were transported in Cary-Blair transport media to the Bacteriology Research Division, Department of Medical Research (LM). Culture and biochemical tests were performed to isolate the *E. coli* colonies in accordance with the WHO standard procedure [7]. Rectal swabs in Cary-Blair transport media were inoculated directly onto the MacConkey agar for culture overnight (18 hours) at 37°C. One to three isolates with typical *E. coli* morphology were initially selected and examined biochemically following Cowan and Steel method [8]. Serotyping assay, polymerase chain reaction (PCR) assay, and toxin assays were performed for all *E. coli* isolates.

Serotyping assay

Colonies isolated from Triple Sugar Iron agar (of biochemical set) were tested for agglutination with 4 polyspecific antisera A, B, C, D. Strains that were agglutinated by the polyspecific antisera were retested by using 32 monospecific O:K antisera (Difco Laboratories, USA; Denka Seiken Co, Japan).

Identification of virulence markers by PCR

E. coli isolates were subjected to PCR assay using primer sets described in Table 1.

Table 1. Primer sets for detection of enterotoxigenic and enterohaemorrhagic *E. coli*

Category	Target	Nucleotide sequence (5'→ 3')	Size of amplified product (bp)
ETEC - LT	LTI-01	CAAGCTTGGAGAGAAGAACCC	203
	LTI-02	TCATCCCGAATTCTGTTATAT	
ETEC - ST	ST-157	TTTTCTTTCTGTATTATCTT	191
	ST-158	ATTACAACACAATTCACAGC	
EHEC	STX-01	ATCAGTCGTCACTCACTGGT	113
	STX-02	CTGCTGTCACAGTGACAAA	
EHEC	STX-101	CAACACTGGATGATCTCAG	349
	STX-102	CCCCCTCAACTGCTAATA	

ETEC = enterotoxigenic *E. coli*; EHEC = enterohaemorrhagic *E. coli*; LT= heat-labile enterotoxin; ST =heat-stable enterotoxin; STX = Shiga toxin; bp = base pair.

Boiled lysates from overnight-grown bacterial colonies were used as PCR templates. The reaction mixtures were run in a thermal cycler (PCR Sprint Thermal Cycler, Thermo Electron corporation) with the following cycling profile: 25 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 60 seconds and primer extension at 72°C for 60 seconds. Amplified products were resolved by 2% agarose gel electrophoresis and visualized under UV transillumination after ethidium bromide staining. DNA templates from positive and negative control strains for virulence markers and minus-template samples were included in each PCR [9].

Toxin assays

E. coli isolates were inoculated into 2 ml of sterile CAYE broth. The culture was incubated with continuous and vigorous shaking at 37°C for overnight (18 hours). After incubation, the culture broth was

centrifuged at 900g for 30 minutes at 4°C and supernatant was used as the test sample. *E. coli* ST EIA (Oxoid) was used for detection of heat-stable *E. coli* enterotoxin, VET-RPLA (Oxoid) for detection of heat-labile *E. coli* enterotoxin and VETC-RPLA (Oxoid) for detection of verotoxins VT1 and VT2 of *E. coli* were performed according to instruction in the test manuals accompanied in each test kit [10, 11, 12].

RESULTS

There are 16 *E. coli* isolates from 60 rectal swabs. Among them, 12 serotypes were identified by serotyping assay. Four isolates were not serotypable with antisera used in this study (Table 2).

Table 2. Result of serotyping assay, PCR and toxin assays for 16 *E. coli* isolates

No.	Lab. ID	Serotyping assay		PCR	Toxin assay
		Serotype	Pathotype		
1	1	O55:K59	EPEC	-	-
2	2	Non-typing		EHEC	-
3	5	O27:K+	ETEC	EHEC	-
4	8	O1:K51	EPEC	-	-
5	9	O157:K+	EPEC	-	-
6	13	O8:K40	ETEC	-	-
7	14	O8:K25	ETEC	EHEC	VT
8	18	O1:K51	EPEC	-	ST
9	16	O125:K70	EPEC	ETEC	ST
10	21	O127:K63	EPEC	-	ST
11	25	Non-typing		-	VT
12	19	Non-typing		-	VT
13	31	O112:K66	EIEC	-	ST
14	36	O44:K74	EPEC	-	VT, ST
15	38	O125:K70	EPEC	-	-
16	48	Non-typing		-	VT

According to toxin assays, there were 4 isolates positive for heat-stable enterotoxin (ST) and 4 isolates positive for verotoxin (both VT1 and VT2). One isolate was positive for both ST and VT. Two pathotypes were identified in 4 isolates on the basis of virulence marker profile by PCR assay. There were three isolates which showed pathotype EHEC (positive for STX-01, STX-02, STX-101, STX-102) and only one isolate was ETEC pathotype (positive for ST-157, ST-158). There was no isolate positive for LTI-01, LTI-02 marker in this assay. The detected heat-stable toxin gene and verotoxin gene of *E. coli* by PCR in this study are shown in Fig. 1 and 2.

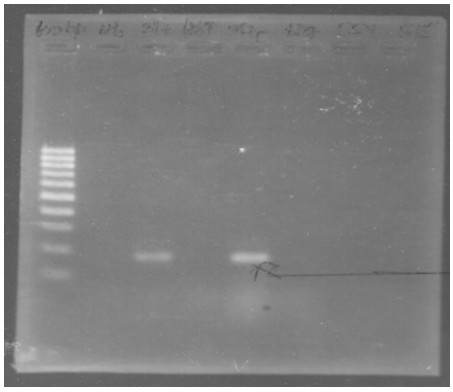

Fig. 1. Detection of heat-stable toxin gene of *E. coli* by PCR (191 bp)

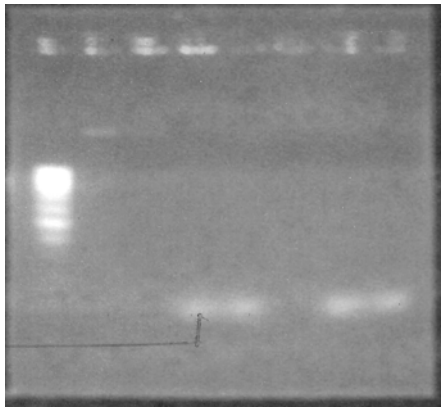

Fig. 2. Detection of verotoxin gene of *E. coli* by PCR (113 bp)

In this study, pathotypes identified according to serogroups did not correspond with the PCR identified pathotypes and with toxin assays. There were two isolates, (Lab ID 14 and 16 in Table 2) which were EHEC and ETEC according to PCR assay and these two isolates showed corresponding results in toxin assays as VT and ST respectively.

DISCUSSION

Diarrhoea remains one of the main causes of morbidity and mortality in children and a large proportion is caused by diarrhoeagenic *E. coli*. Many of the virulence determinants

have been determined and some DEC produce toxins. The virulence factors of some DEC have yet to be fully determined and in the meantime they remain large and emerging problems without the availability of effective vaccine [13].

O, H and K antigens of *E. coli* are used to classify the isolates for epidemiologic purposes. Specific antigenic serogroups are also associated with greater virulence [14]. In this study, 12 serotypes were identified among 16 isolates from 60 rectal swabs collected during study period. These serotypes were identified as 8 EPEC, 3 ETEC and one EIEC according to manufacturer of antisera.

The results of toxin assays showed that 9 isolates produced toxins, among them 4 produced heat-stable toxin, 4 produced verotoxin and one produced both verotoxin and ST toxin. None of the isolates produced heat-labile toxin. When the serogroup defined pathotypes were compared with toxin assays, there was one ETEC serotype (O8:K25) that produced verotoxin and three EPEC (O1:K51, O125:K70, O127:K63) which produced heat-stable toxin and one EPEC (O44:K74) that produced verotoxin and heat-stable toxin. One EIEC (O112:K66) produced heat-stable toxin and three non-serotypable isolates produced verotoxin. This finding shows pathotypes determined by serotyping assays are not compatible with toxin assay results. Toxin production is not associated with specific serogroups and tissue cultures, animal model assays or *in vitro* assays must be performed if toxigenic strains are to be detected. Nucleic acid probes have also been used to detect the toxin genes [14]. In this study nucleic acid assay was performed by using primers targeting virulent genes for ETEC and EHEC. The primers for EPEC and EIEC were not included in this study. Results of PCR assay showed three EHEC and one ETEC, which were different from results obtained by serotyping.

Diarrhoeagenic *E. coli* isolates from 217 children (aged under five years) who had been admitted to Yangon Children's Hospital in Myanmar between 1999 and 2003 were studied by Eizo Takahashi and group [15]. In that study, 47 isolates possessed virulence genes characteristics of diarrhoeagenic *E. coli* among which 30 were EAEC, 12 were EPEC and 5 were ETEC.

Serotyping has classically been used as the standard assay to detect strains of diarrheagenic *E. coli*. Although serotyping is conventional, most serogroups of diarrheagenic *E. coli* are not restricted to only one pathotype. Also the serogroups that had been considered to represent diarrhoeagenic *E. coli* have also been considered to represent among non-diarrhoeagenic *E. coli*. For these reasons, serotyping is not considered to be a reliable method for detection of diarrhoeagenic *E. coli* [16, 17].

This study also shows that serotyping assay alone is not sufficient to determine the pathogenic type of *E. coli* and the role of PCR assay and toxin assays are more important in determining the pathogenic type of *E. coli*. In future, more sensitive and specific tests using nucleic acid probes should be established to characterize the diarrhoeagenic *E. coli* that causes childhood diarrhoea in Myanmar.

REFERENCES

1. O'Ryan M, Prado V & Pickering LK. A millennium update on paediatric diarrhoea illness in the developing world. *Seminar on Pediatrics Infectious Diseases* 2005; 16 (2): 125-136.
2. Brooks GF, Butel JS & Morse SA. The Enterobacteriaceae. In: *Jawetz, Melnick and Adelberg's Medical Microbiology*. Twenty-third edition. Lange Medical Book/ Mc Graw-Hill Medical Publishing Division 2004.
3. Levine MM, Berquist EJ & Narlin DI. *E. coli* strain that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. *Lancet* 1978; i (8074):1119-1122.
4. Levine MM, Kaper JB & Black RE. New knowledge on pathogenesis of bacterial enteric infection as applied to vaccine development. *Microbiological Reviews* 1983; 47(4): 510-550.

5. Sunable T & Honma Y. Relationship between O serogroup and presence of pathogenic factor genes in *E. coli. Microbiology and Immunology* 1998; 42:845-849.
6. World Health Organization. The treatment of diarrhoea: a manual for physicians and other senior health workers.WHO/CAH/03.7; 2003; 10/03.
7. World Heath Organization. Manual of diagnostics laboratory procedure for acute enteric infection. Simplified methods. Geneva: World Health Organization. 1983; 3.
8. Cowan & Steel. Manual for the identification of medical bacteria: 2nd ed. Cambridge University Press 1974.
9. Toma C, Lu Y, Higa N, Nakasone N *et al*. Multiplex PCR Assay for identification of human diarrhoeagenic *Escherichia coli. Journal of Clinical Microbiology* June 2003; 2669-2671.
10. Operation Manual for E. Coli ST EIA (Oxoid) TD 700, November 2003.
11. Operation Manual for VET-RPLA (OXOID) TD 0920A, November 2003.
12. Operation Manual for VTEC-RPLA (OXOID) TD 960, November 2003.
13. Clarke SC. Diarrhoeagenic *Escherichia coli*- an emerging problem. *Diagnostic Microbiology and Infectious Disease* 2001; 41(3):93-8.
14. Murray PR, Rosenthal KS, Kobayashi GS & Pfaller MA. Enterobacteriaceae. In: *Medical Microbiology*. Fourth Edition. Mosby, Inc. 2002.
15. Takahashi E, Sultan Z, Shimada S, Aung WW *et al.* Studies on diarrhoeagenic *E coli.* isolated from children with diarrhoea in Myanmar. *Microbiology and Immunology* 2008; 52: 2-8.
16. Ratchtrachenchai OA, Subpasu S, Hayashi H & Ba Thein W. Prevalence of childhood diarrhoea-associated *Escherichia coli* in Thailand. *Journal of Medical Microbiology* 2004; 53: 237-243.
17. Nataro JP & Kaper JB. Diarrhoeagenic *E coli. Clinical Microbiology Review* 1998; 11:142-201.