Molecular detection of enterotoxigenic *Escherichia coli* surface antigens from patients in Machakos District Hospital, Kenya

Juma BW[1,5], Waiyaki PG[1,3], Bulimo WD[1,2,4], Wurapa EK[2], Mutugi MM[1], Kariuki S[1,3]

[1]*Jomo Kenyatta University of Agriculture and Technology*
[2]*US Army Medical Research Unit, Kenya*
[3]*Centre for Microbiology Research, Kenya Medical Research Institute*
[4]*Department of Biochemistry, University of Nairobi*
[5]*Centers for Disease Control and Prevention-Kenya*

Corresponding author:
Dr BM Juma, P.O. Box 54840 – 00200, Mbagathi road, Nairobi, Kenya.
Email: xwl2@cdc.gov; bwachekone@yahoo.ca

Abstract

Introduction: Enterotoxigenic *Escherichia coli* (ETEC) is known for its public health importance globally, however, a protective vaccine is yet to be developed. Information regarding the immunology of ETEC's virulence proteins that can lead to studies on vaccine development such as the heat stable toxins (ST), heat-labile toxin (LT), colonization factors (CFs) and coli surface antigens (CS) from many regions of the world is available. In Kenya, specific CFAs and CS have not been adequately characterized. This study looked at the surface antigens of diarrhoeagenic *E. coli* in search of indicators for vaccine materials development.

Methodology: Multiplex polymerase chain reaction assay were employed to detect diarrhoeagenic *E. coli* pathotypes and enteroxigenic *Escherichia coli* surface antigens/ colonization factors antigens from 300 patients in Machakos Hospital, Kenya.

Results: Enteroaggrigative *Escherichia coli* was the most predominant (13.7%) followed by ETEC (11%), Enteroinvesive *E. coli* (8.3%) and Enteropathogenic *E. coli* (4.3%). Among the colonization factor antigens, CFAI was detected at 25 (23%), CS1, CSII 2(1.9%), CS3 1(0.9%), CS6 13(12%), CS7 2 (1.9%), CS12 1(0.9%), CS19 11 (10.25%) and those without colonization factor 37 (34.3%).

Conclusions: ETEC isolates carrying ST or STLT toxins had more recoverable CFs than those with LT alone ($P<0.05$). The CS6 is increasing and CS19 was detected for the first time in Kenya and shown to be persistent adhesins. These may be further investigated as possible candidates for the formulation of a novel vaccine for the prevention of diarrhoea in Kenya and the region.

Key words: Colonization factor antigens, Coli surface antigens, Multiplex Polymerase Chain Reaction, Enterotoxigenic *E. coli.*

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is one of *E. coli* pathotypes causing diarrhoea in children from developing countries and adults travelling from developed to developing countries [1]. ETEC is rated the second cause of diarrhoea after rotavirus in children under five years of age [2]. Ingestion of contaminated food and water is the main mode of transmission of ETEC [3]. The disease presents with vomiting and/ or diarrhoea with or without dehydration [4] . Enterotoxigenic *E. coli* adheres and colonizes the lining of the intestines by use of specific fimbriae referred to as colonization factors or coli surface antigens (CFs) [1].

More than 25 human ETEC CFs have been described based on differences in their primary amino acid sequences of the adhesive fimbrial structural subunits [5]. This puts them in distinct families based on distinct fimbriae on the basis of their genetic diversity [5]. CFs have been divided into three families: (i) a CFA/I-like group including CFA/I, CS1, CS2, CS4, CS14, CS17 and CS19; (ii) a CS5-like group including CS5, CS7, CS18, and CS20; and (iii) a unique group including CS3, CS6, and CS10 to CS12 [1]. The ETEC isolates may express a single detectable CF, for example, in CFAI or multiple CFs as seen in CS3 (CS3 alone or in combination with CS1 or CS2) and CS6 (CS6 alone or in combination with CS4 or CS5).

In Kenya several studies on the causes of diarrhoea have been carried out [6-8]. These studies have elaborated the existence of ETECs as causes of diarrhoea. However, they lacked the capability and tools to characterize the specific CFs within the ETEC isolates. To shed more light on circulating CFs, the current study sought to determine the prevalence of LT, ST, and ETEC CFs in patients from four months old and above in Machakos District Hospital.

Materials and Methods

Study patients: Study participants comprised of children from four months old and above from Machakos County seeking medical attention due to diarrhoea illness at the Machakos District Hospital, Kenya from September 2010 - September 2013. The study participants who had reached the age of consent gave informed consent and parents / guardians gave consent for their children to participate in the study. Research ethical approval was granted by the Ethical Review Committee of Kenya Medical Research Institute (KEMRI) SSC Number 989.

After the informed consent was voluntarily obtained, patients were examined and a questionnaire administered to collect each patient's history and other demographic information.

Sample collection: Stool samples were collected in stool cups by the patients and immediately transferred to the Carry Blair medium by the technician and transported to Kenya Medical Research Institute's Centre for Microbiology Research for culture and detection of enterotoxins and CFs. Children who could not provide stool samples had their rectal swabbed and placed in the Carry-Blair medium immediately for shipment to the laboratory in Kenya Medical Research Institute (KEMRI).

The samples were examined under a microscope, plated on MacConkey agar, and allowed to develop colonies. Five colonies of lactose fermenters (*E. coli* like in morphology) were picked and further tested using Triple sugar iron, motility, indole ornithine, and stored at -80^{0}c for further analysis.

Bacteria identification: Samples were processed using standard microbiological techniques. Briefly, stool samples were plated on different freshly prepared and quality controlled media (MacConkey, Xylose lysine desoxycholate (XLD) and incubated at 37^{0}C for 18-24 hours. They were then sub-cultured on nutrient agar. Different colonies from each media were selected as follows: Two to three colonies (black, medium and small size pink or clear) from XLD for suspected *Salmonella* spp and *Shigella* spp; 5-6 colonies (5-lactose and one non- lactose fermenter) from Lactose MacConkey for different *E. coli* pathotypes; both yellow and green colonies from TCBS for *Vibrio cholerae* and *Vibrio parahemolyticus* respectively, and one clear colony from Sorbital MacConkey to detect *E. coli* 0157:H7. Isolates confirmed as *E. coli* using TSI (A/A, -H_2S, +- gas), Simmon's citrate (green), motile, and indole positive were confirmed for different *E. coli* pathotypes by multiplex polymerase chain reaction (mPCR).

Detection of E. coli pathotypes, ETEC CFAs and coli surface antigens: The DNA was extracted from *E. coli* by boiling. PCR was done in a 25-µL reaction mixture containing 5µL of boiled whole cell lysate, 100 µmol/L each deoxynucleoside triphosphate, 1× GoTaq Flexi DNA polymerase (Promega) as previously described [9].

Genetic relatedness of ETEC by PFGE: Genomic DNA of various strains of *ETEC*, were prepared in Agarose plugs as described by Kariuki *et al.* [10] from an overnight culture in Luria batani broth. For complete digestion of DNA, the *XbaI (*Life Technologies, Paisley, UK) enzymes were added following the manufacturer's instructions. PFGE of agarose plug inserts was performed in the contour clamped homogenous electric field CHEF-DRIII (Bio-Rad Laboratories, Hercules, CA, USA) on a horizontal 1% agarose gel for 22 hours at 120V hours and pulse time of 1 to 40 sec at 14^{o}C. A DNA (lambda ladder, Bio-Rad) consisting of 22 fragments of increasing size from 48kb -1000kb were used as the DNA size standard maker. The gels were stained with 0.05% ethidium bromide and observed on a gel Doc 2000 (Bio-Rad). Restriction endonuclease digest pattern of Xba*I* digest genomic DNA were compared and similarities as well as differences scored as described by Kariuki *et al* [11].

Statistical analysis: The data were analyzed using EpiInfo (version 3.4.3; Center for Disease Control and Prevention, Atlanta, Georgia). The Chi-square and Fishers' Exact tests were used for comparisons between the groups.

Results

Between September 2010 to September 2013, 1600 isolates were cultured from 301 specimens from patients with diarrhoea attending Machakos District Hospital who ranged from the ages of 4 months to 85 years.

A total of 98 ETECS isolates were recovered from 33/300 patients among them 13 children below five years of age, 2 participants aged between 6 and 10 years and 18 subjects above the age of 16 years. We did not isolate any ETEC from the ages between 11 and 15 years. The ETEC isolates represented 11% of all *E. coli* pathotypes detected (56 LTs, 17 ST1, 19 ST2 and 6 LT / ST1 combined). The frequency of ETEC infection and the associated virulence traits were compared across the study period, and there was no statistical significant (P> 0.05).

Distribution of ETEC colonization factor antigens by phenotype: The proportions of ETEC isolates expressing ST and LT in study subjects with diarrhoea were similar over the study period, with no seasonal variation. ETEC isolates expressing LT were isolated from similar proportions of cases during the first three quarters of the study. Similarly, ETEC isolates expressing ST were isolated from comparable proportions of ETEC diarrhoea episodes during the second quarter (40%; n = 48) and third quarter (43%; n = 47) but from a lower proportion during the fourth quarter of the study (20.1%; n = 21). The ETEC isolates expressing both LT and ST were recovered at the lowest frequency throughout the study.

To appreciate the diversity of the ETEC CFs obtained in our study, the expression of CFs for which specific oligonucleotide primer sequences are available were examined. In total, 46.9% (46/98) of the ETEC isolates expressed a detectable CF (Table 1). The most commonly expressed CF types were CFA/I (14.1%),

CS6 (5.0%), CS14 (5%), CS7 (2%), and CS19 (9%). Many of CFs expressed by ETEC isolates expressing a CF were ST -associated. Minimal numbers CFs from LT ETECS were observed. A total of 48% of ETEC isolates that expressed an ST or LTST expressed a detectable CF (Table 1).

Table 1: Distribution of ETEC colonization factor antigens by phenotype

CF type	ETEC toxin			
	LT	LT ST	ST	Mixed
	n = 56(%)	n = 6 (%)	N=36(%)	n = 1 0 (%)
CFAI	9	50	42	20
CSI	0	0	0	0
CS1+CS3	0	0	5.6	0
CS2+CS3	0	0	0	0
CS3	0	0	2.8	0
CS4+CS6	0	0	0	0
CS5	0	0	0	0
CS5+CS6	18	0	8.3	30
CS6	7	16.7	13.9	30
CS7	0	16.7	(2.8	0
CS12	0	0	2.8	0
CS17	0	0	0	0
CS19	5	0	19.4	10
CS20	0	0	0	0
No CFs	61	16.7	2.7	10

Clinical presentation associated with ETEC isolates isolates expressing CFA/I, CS5 plus CS6 and CS 19.

expressing CFA/I, CS5 plus CS6, CS6, and CS19.

The demographic and clinical data for the subjects with ETEC-associated diarrhoea were grouped according to the CF that the isolate expressed. Overall, ETEC isolates expressing a CF were more commonly recovered from children less than five years of age (Table 2). In addition, CS6 and CS19-expressing isolates were more frequently identified in the stools of children ranging between one and five years of age ($X^2 = 21.65$; $P < 0.05$, Table 2).

Table 2: Age range of patients infected with ETEC isolates expressing CFA1, CS5 plus CS6, CS6, CS14 and CS 19

CF type	Age (Years)			
	3mn-≤5	>5 - ≤10	>10 - ≤15	>15
CFAI	8(33.3)*	5(20.8)	0	1(4.2)
CS5+CS6	3(12.5)	2(8.3)	0	1(4.2)
CS6	4(16.7)	1(4.2)	0	0
CS14	2(8.3)	0	0	0
CS19	7(29.2)	2(8.3)	0	0

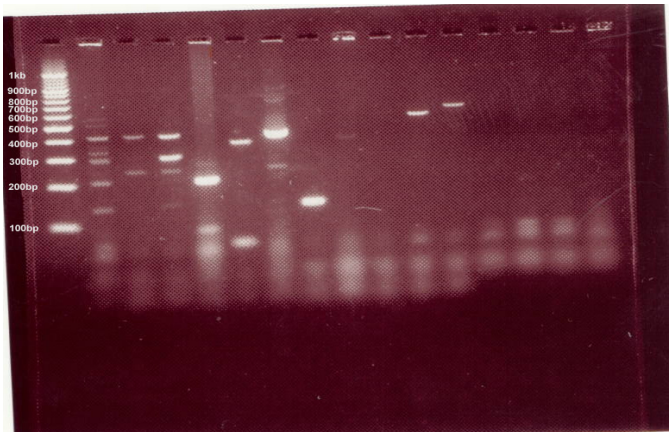
Most CFs were recovered from participants below 5 years of age.
(x) = %

In the analyses of the clinical data and CF types, ETEC cases associated with other bacterial (S*higella* spp, *Salmonella* spp), infections were excluded to avoid the analysis of data for subjects with overlapping symptoms due to the presence of other enteric pathogens. The lower proportion of hospitalization among subjects infected with ETEC isolates expressing CS6 and CS19 was not significant compared to the percentage of children infected with ETEC isolates expressing other CF types ($X^2 = 1.21$; P>0.05) (Table 3). Among the subjects infected with ETEC isolates expressing CFA/I, the percentage with dehydration (43%) noted was higher compared to the percentage among those infected with ETEC isolates expressing other CF types (Table 3). Visible mucus in the stool was also reported in subjects infected with ETEC

Table 3: Clinical presentation and ETEC colonization factor antigens expression

Clinical data	ETEC-CF expression				
	CFA1 n=14(%)	CS5/6 n=6(%)	CS6 n=5(%)	CS19 n=9(%)	Non CF n= 60(%)
Hospitalisation	36	16	0	22	12
Dehydration	13	0	20	11	33
Vomiting	21	33	80	66	63
Temp>38^{0}C	71	66	60	86	33
Mucus in stool	36	67	80	58	55
Stomach cramps	21	33	10	66	67

With the use of highly specific primers, a variety of CFs were amplified, and one CS type (CS19) that had neither been detected nor reported in Kenya was identified (Figure 1).

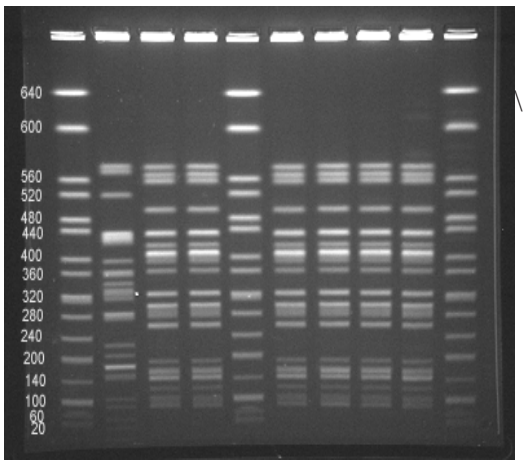


M 1 2 3 4 5 6 7 8 9 10 11

Figure 1: Amplification of enterotoxigenic *E. coli* CFAs and CS by mPCR

Lanes: M: 100bp ladder, Lane 1 pooled positive control, Lane 2: CS 5&6, Lane 3: CS5, 7&6, Lane 4: CS 19 and 20, Lane 5: CS5, Lane 6: CS 5&6,
Lane 7: CS 9, Lane 8: negative control Lane 10 &11 CS 17 and CS18 respectively.

The ETEC isolates were shown to be similar as indicated by PFGE bands which were within one to three band variations (Figure 2).



M 1 2 3 **M** 4 5 6 7 **M**

Figure 2: *Xba*1 digest pattern variations in ETEC isolates.

Lanes: M indicates 50-kb lambda molecular size ladder; lanes 1, 2, 3, 4, 5, 6, and 7 indicates ETEC isolates that were shown to be within one to three bands difference.

Discussion

Enterotoxigenic *E. coli* is one of the common causes of diarrhoea in the developing countries. We detected ETEC at 11% of diarrhoeal cases which is significantly higher than in previous studies in Kenya where the prevalence was reported at 3% [6,13,14]. Considering the diversity of ETEC CFs obtained in studies by Zhi-Dong' *et al*, [6] it is surprising that no CS6 and CS19 were detected. In Nigeria [12], ETEC was detected at a lower rate compared with those detected in previous Kenyan studies [8,13]. In Peru, a detection rate of 5.3% was realized [13]. Because of differences in the occurrences of ETEC, the implementation of immunoprophylactic measures for the control of ETEC diarrhoea, should first be assessed and variations in the phenotypic, genotypic, and pathogenic properties of the bacterium at hand be identified. It is worth noting that this is the first time that the presence of CS19 in ETEC in Kenya has been documented. Studies done elsewhere also showed that the detection of CS6 is increasing throughout the world [6,13-16]. In the present study, it was also noted that a significantly low recovery of ETEC strains expressing CS6 and CS19 compared with the frequency of isolation of ETEC strains expressing other CF types from children below five years was obtained. Similarly, the current study noted that the detection of CFA/I-expressing ETEC isolates appeared to be more associated with children below 5 years of age and this finding agrees with those obtained from studies conducted by Claudia in Brazil and Ireland [14], Shaheen *et al*. [5] in Egypt, Walker *et al*. [17] in Zambia, Hall *et al*. [18] in England and Peruski *et al*. [19] in Bangladesh.

The rates of recovery of ETEC and the identities of the CFs reported in this study are similar to those from longitudinal community-based epidemiological studies of ETEC in northern Egypt and hospital-based studies in other disparate locations, such as Indonesia [20,21] but differ with findings in Peru [13,16] where the recovery rate was 5.3%. The rates of identification of ETEC isolates expressing CS6 and CS19 were similar in the entire study implying that these two CFs are persistent in the population. This makes them ideal vaccine candidates for prevention of diarrhoea due to ETEC. CS1 and CS3 were detected at a rate of 5.6%. These findings indicate that ETEC isolates expressing CS6 are more likely to be recovered from children below 5 years of age. They were however, significantly less recovered than ETEC isolates expressing CFA/I in children less than 5 years of age. Other ETEC CF types, such as CS3 alone were infrequently recovered at 2.8%. A similar observation was reported in Argentina and Brazil [15,22]. On the other hand, this finding differs from the results obtained in Mexico [23], Bangladesh [24,25] and Egypt [3] where CS6 component was identified at a higher percentage. It should be noted that the prevalence of infection due to ETEC isolates expressing CS6 or CS19 has been increasing in the world [3,26-29].

The highest rate of recovery of ETEC isolates expressing a detectable CF was during the warm months. This finding is in agreement with observations made in Egypt [30,31]. It should also be noted that the recovery of ETEC isolates expressing CS6 or CS19 did not appear to

vary by season as other CFs. From Studies carried out in the Judea area of the West-Bank of Israel [32] and by Sizemore in Malawi [33] similar observations were made, implying that CS6 and CS19 are stable and are viable candidates for the development of a vaccine for prevention of diarrhoea due to ETEC.

In the present study, there were ETEC isolates without detectable CF. This observation is in agreement with findings from work done in Egypt [5,28] and the Asian region [34]. In the latter studies, approximately 50% of the ETEC isolates identified lacked detectable CF expression based on either dot blot analysis or DNA hybridization assays [5,31,34]. The percentage of ETEC isolates where a CF was not detectable expressed LT and these results were in agreement with those obtained from studies that used genotypic detection methods [13,35-37]. The lack of an identifiable CF in ETECs may have been due to the loss of the plasmid harboring the CF genetic element, down regulation of the CF genes, a mutation within the genetic locus, or expression of a CF not covered by the mPCR panel [15]. To account for differences due to age or diarrhoea markers associated with ETEC isolates expressing one of the five major CFs found, the corresponding patient's demographic and clinical data were related according to the ETEC CF identified.

Although ETEC infection can cause profuse watery diarrhoea with little or no fever or vomiting, previous reports of ETEC isolates recovered from hospitalized infants elsewhere found the infection to be commonly associated with fever and vomiting [5,14,38,45].

In the current study, 5.3% of the children below three years of age with ETEC infection suffered from dehydration. In addition, vomiting and fever were reported in children shedding ETEC strains. The observation of severe diarrhoea markers for example, dehydration and the number of stools per day in some of the children may be attributed to the presence of strains that are more virulent due to the presence of additional virulence factors [39-41]. It could also be that the presence of severe diarrhoeal symptoms may be the result of an immunologically naïve child's first encounter with an ETEC pathogen.

An age-dependent isolation rate has been observed for ETEC isolates expressing CFA1 and CSs, suggesting a naturally acquired immunity in older children [31] and this was reflected in the current study. Establishing that CFA/I-expressing isolates also induce an age-dependent immunity in older children would corroborate the concept of immunoprophylaxis to ETEC infection [42-45]. In the current study, the decline in the rate of infection with ETEC isolates expressing CFA/I that was seen from the age of 10 years and above supports this idea and underlies the importance of developing an ETEC vaccine since the risk factors for acquiring ETEC infection are present among children up to one year of age [23,30,46]. Further studies are needed to obtain a more in-depth understanding of the naturally acquired ETEC antifimbrial immune responses. *In vitro* experiments have demonstrated that environmental components have an impact on the expression of ETEC virulence factors [47].

In summary, ETEC was isolated more from children below the age of five years compared to those aged above 5 years. The increasing rates of ETEC strains expressing CS6 and the first time detection of CS19 was noted in Machakos County, Kenya. This calls for the need to increase surveillance of ETEC CFs. The information obtained will provide more understanding and characterization of more ETECs and ETECs CFs/CS that may be included in a potential Kenyan-based vaccine development for the control of diarrhoea.

Acknowledgements

The authors would like to thank the employees of Centre for Microbiology Research, Kenya Medical Research Institute (KEMRI) for their cooperation during the study period. We would also like to thank the Director, KEMRI for allowing us to publish this work. Thanks to the US Army Medical Research Unit-Kenya for providing funds to carry out the project.

Conflict of interest: No conflict of interest is declared.

References

1. Estrada-Garcia T, Lopez-Saucedo C, Thompson-Bonilla R, Abonce M, Lopez-Hernandez D, *et al.* Association of diarrheagenic Escherichia coli pathotypes with infection and diarrhea among Mexican children and association of atypical enteropathogenic E. coli with acute diarrhea. *J Clin Microbiol*. 2009; **47**: 93-98.
2. Goldsmid JM and Leggat PA. The returned traveller with diarrhoea. *Australian Fam Phys*. 2007; **36**: 322-327.
3. Qadri F, Das SK, Faruque A, Fuchs GJ, Albert MJ, *et al.* Prevalence of toxin types and colonization factors in enterotoxigenic Escherichia coli isolated during a 2-year period from diarrheal patients in Bangladesh. *J Clin Microbiol*. 2000; **38**: 27-31.
4. Harris AM, Chowdhury F, Begum YA, Khan AI, Faruque AS, *et al*. Shifting prevalence of major diarrheal pathogens in patients seeking hospital care during floods in 1998, 2004, and 2007 in Dhaka, Bangladesh. *The Amer J Trop Med Hyg.* 2008; **79**: 708.
5. Shaheen HI, Khalil SB, Rao MR, Elyazeed RA, Wierzba TF, *et al*. Phenotypic profiles of enterotoxigenic Escherichia coli associated with early childhood diarrhea in rural Egypt. *J Clin Microbiol*. 2004; **42**: 5588-5595.
6. Zhi-Dong JLB, Verenkar P, Ashley D, Steffen R, Tornieporth N, *et al.* Prevalence of enteric pathogens among international travelers with diarrhea acquired in Kenya (Mombasa), India (Goa), or Jamaica (Montego Bay). *J Infect Dis*. 2002; **32**: 1234 -1239.

7. Oundo JO, Kariuki SM, Boga HI, Muli FW and Iijima Y. High incidence of enteroaggregative Escherichia coli among food handlers in three areas of Kenya: a possible transmission route of travelers' diarrhea. *J Travel Med*. 2008; **15**: 31-38.
8. Sang W, Oundo J, Mwituria J. and Waiyaki P. Multidrug-resistant enteroaggregative escherichia coli associated with persistent diarrhoea in Kenyan Children. *J Diarrh Dis Res*. 1997; **15**: 190 - 192.
9. Nada RA, Shaheen HI, Touni I, Fahmy D, Armstrong AW, *et al*. Design and validation of a multiplex polymerase chain reaction for the identification of enterotoxigenic Escherichia coli and associated colonization factor antigens. *Diag Microbiol Infect Dis*. 2010; **67**: 134-142.
10. Kariuki S, Oundo JO, Muyodi J, Lowe B, Threlfall EJ, *et al*. Genotypes of multidrug-resistant Salmonella enterica serotype typhimurium from two regions of Kenya. *FEMS Immunol Med Microbiol*. 2000; **29**: 9-13.
11. Kariuki S, Revathi G, Gakuya F, Yamo V, Muyodi J, *et al*. Lack of clonal relationship between non-typhi Salmonella strain types from humans and those isolated from animals living in close contact. *FEMS Immunol Med Microbiol*. 2002; **33**: 165-171.
12. Okeke IN, Ojo O, Lamikanra A. and Kaper JB. Etiology of acute diarrhea in adults in southwestern Nigeria. *J Clin Microbiol*. 2003; **41**: 4525-4530.
13. Rivera F, Ochoa T, Maves R, Bernal M, Medina A, *et al.* Genotypic and phenotypic characterization of enterotoxigenic Escherichia coli strains isolated from Peruvian children. *J Clin Microbiol*. 2010; **48:** 3198-3203.
14. Rodas C, Klena JD, Nicklasson M, Iniguez V. and Sjöling Å. Clonal relatedness of enterotoxigenic Escherichia coli (ETEC) strains expressing LT and CS17 isolated from children with diarrhoea in La Paz, Bolivia. *PloS One.* 2011; **6:** e18313.
15. Rodas C, Mamani R, Blanco J, Blanco JE, Wiklund G, *et al*. Enterotoxins, colonization factors, serotypes and antimicrobial resistance of enterotoxigenic Escherichia coli (ETEC) strains isolated from hospitalized children with diarrhea in Bolivia. *Brazilian J Infec Dis.* 2011; **15**: 132-137.
16. Paniagua M, Espinoza F, Ringman M, Reizenstein E, Svennerholm A, *et al*. Analysis of incidence of infection with enterotoxigenic Escherichia coli in a prospective cohort study of infant diarrhea in Nicaragua. *J Clin Microbiol*. 1997; **35**: 1404-1410.
17. Walker RI, Steele D and Aguado T. Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic E. coli(ETEC) disease. *Vaccine*. 2007; **25**: 2545-2566.
18. Hall ER, Wierzba TF, Åhrén C, Rao MR, Bassily S, *et al*. Induction of systemic antifimbria and antitoxin antibody responses in Egyptian children and adults by an oral, killed enterotoxigenic Escherichia coli plus cholera toxin B subunit vaccine. *Infection and Immunity.* 2001; **69**: 2853-2857.
19. Peruski LF, Kay BA, El-Yazeed RA, El-Etr SH, Cravioto A, *et al.* Phenotypic diversity of Enterotoxigenic Escherichia coli strains from a community-based study of pediatric diarrhea in periurban Egypt. *J Clin Microbiol*. 1999; **37**: 2974-2978.
20. Subekti D, Lesmana M, Tjaniadi P, Machpud N, Daniel J, *et al*. Prevalence of enterotoxigenic Escherichia coli(ETEC) in hospitalized acute diarrhea patients in Denpasar, Bali, Indonesia. *Diag Microbiol Infect Dis*. 2003; **47**: 399-405.
21. von Sonnenburg F, Tornieporth N, Waiyaki P, Lowe B, Peruski Jr LF, *et al*. Risk and aetiology of diarrhoea at various tourist destinations. *The Lancet* 2000; **356**: 133-134.
22. Viboud GI, Jouve MJ, Binsztein N, Vergara M, Rivas M, *et al.* Prospective cohort study of Enterotoxigenic Escherichia coli infections in Argentinean children. *J Clin Microbiol*. 1999; **37**: 2829-2833.
23. Taxt A, Aasland R, Sommerfelt H, Nataro J and Puntervoll P. Heat-stable enterotoxin of enterotoxigenic Escherichia coli as a vaccine target. *Infection Iimmunity*. 2010; **78**: 1824-1831.
24. Blackburn D, Husband A, Saldaña Z, Nada RA, Klena J, *et al.* Distribution of the Escherichia coli common pilus among diverse strains of human enterotoxigenic E. coli. *J Clin Microbiol.* 2009; **47**: 1781-1784.
25. Mazariego-Espinosa K, Cruz A, Ledesma MA, Ochoa SA. and Xicohtencatl-Cortes J. Longus, a type IV pilus of enterotoxigenic Escherichia coli, is involved in adherence to intestinal epithelial cells. *J Bacteriol*. 2010; **192**: 2791-2800.
26. Li X, Mariano N, Rahal JJ, Urban CM and Drlica K Quinolone-resistant Haemophilus influenzae in a long-term-care facility: nucleotide sequence characterization of alterations in the genes encoding DNA gyrase and DNA topoisomerase IV. *Antimicrobial Agents Chemotherapy.* 2004; **48**: 3570-3572.
27. Sherlock O, Vejborg RM and Klemm P. The TibA adhesin/invasin from enterotoxigenic Escherichia coli is self recognizing and induces bacterial aggregation and biofilm formation. *Infection Immunity.* 2005; **73**: 1954-1963.
28. Steinsland H, Valentiner-Branth P, Gjessing HK, Aaby P, Mølbak K, *et al*. Protection from natural infections with enterotoxigenic Escherichia coli: longitudinal study. *The Lancet*. 2003; **362:** 286-291.
29. Jiang Z-D, Mathewson JJ, Ericsson CD, Svennerholm A-M, Pulido C, *et al*. Characterization of Enterotoxigenic Escherichia coli strains in patients with travelers' diarrhea acquired in Guadalajara, Mexico, 1992–1997. *J Infec Dis*. 2000; **181**: 779-782.
30. Sullivan CB, Diggle MA and Clarke SC. Multilocus sequence typing. *Molecular Biotech*. 2005; **29**: 245-254.

31. Rao MR, Abu-Elyazeed R, Savarino SJ, Naficy AB, Wierzba TF, *et al*. High disease burden of diarrhea due to enterotoxigenic Escherichia coli among rural Egyptian infants and young children. *J Clin Microbiol.* 2003; **41**: 4862-4864.
32. Wolk M, Ohad E, Shafran R, Schmid I and Jarjoui E. Enterotoxigenic Escherichia coli(ETEC) in hospitalised Arab infants from Judea areaā€" West Bank, Israel. *Public Health*. 1997; **111**: 11-17.
33. Sizemore DR, Roland KL and Ryan US. Enterotoxigenic Escherichia coli virulence factors and vaccine approaches. *Expert Review of Vaccines*. 2004; **3**: 585-595.
34. Steinsland H, Valentiner-Branth P, Grewal H, Gaastra W, Mølbak Kå, *et al*. Development and evaluation of genotypic assays for the detection and characterization of enterotoxigenic Escherichia coli. *Diag Microbiol Infectious Dis*. 2003; **45**: 97-105.
35. Mandomando IM, Macete EV, Ruiz J, Sanz S, Abacassamo F, *et al*. Etiology of diarrhea in children younger than 5 years of age admitted in a rural hospital of southern Mozambique. *Amer J Trop Med Hyg.* 2007; **76**: 522-527.
36. Nishimura LS, Girón JA, Nunes SL and Guth BE. Prevalence of enterotoxigenic Escherichia coli strains harboring the longus pilus gene in Brazil. *J Clin Microbiol.* 2002; **40**: 2606-2608.
37. Merz AJ, So M, and Sheetz MP. Pilus retraction powers bacterial twitching motility. *Nature.* 2000; **407**: 98-102.
38. Nweze E. Aetiology of diarrhoea and virulence properties of diarrhoeagenic Escherichia coli among patients and healthy subjects in southeast Nigeria. *J Health, Population, and Nutrition*. 2010; **28:** 245.
39. Long KZ, Rosado JL, Santos JI, Haas M, Al Mamun A, *et al.* Associations between mucosal innate and adaptive immune responses and resolution of diarrheal pathogen infections. *Infection Immunity*. 2010; **78**: 1221-1228.
40. Diemert DJ. Prevention and self-treatment of traveler's diarrhea. *Clin Microbiol Reviews.* 2006; **19**: 583-594.
41. Alam M, Ahsan S, Pazhani GP, Tamura K, Ramamurthy T, *et al*. Phenotypic and molecular characteristics of Escherichia coli isolated from aquatic environment of Bangladesh. *Microbiol Immunol*. 2006; **50**: 359-370.
42. Chattopadhyay S, Tchesnokova V, McVeigh A, Kisiela DI, Dori K, *et al*. Adaptive evolution of class 5 fimbrial genes in enterotoxigenic Escherichia coli and its functional consequences. *J Biol Chem*. 2012; **287:** 6150-6158.
43. Harro C, Sack D, Bourgeois AL, Walker R, De Nearing B, *et al*. A combination vaccine consisting of three live attenuated enterotoxigenic Escherichia coli strains expressing a range of colonization factors and heat-labile toxin subunit B is well tolerated and immunogenic in a placebo-controlled double-blind phase I trial in healthy adults. *Clin Vaccine Immunol*. 2011; **18**: 2118-2127.
44. Turner AK, Terry TD, Sack DA, Londoño-Arcila P and Darsley MJ. Construction and characterization of genetically definedaro omp mutants of Enterotoxigenic Escherichia coli and preliminary studies of safety and immunogenicity in humans. *Infection Immunity*. 2001; **69**: 4969-4979.
45. Turner AK, Stephens JC, Beavis JC, Greenwood J, Gewert C*, et al.* Generation and characterization of a live attenuated enterotoxigenic Escherichia coli combination vaccine expressing six colonization factors and heat-labile toxin subunit B. *Clin Vaccine Immunol*. 2011; **18**: 2128-2135.
46. Qadri F, Ahmed F, Ahmed T, Svennerholm A-M. Homologous and cross-reactive immune responses to enterotoxigenic Escherichia coli colonization factors in Bangladeshi children. *Infection Immunity.* 2006; **74**: 4512-4518.
47. Fleckenstein JM, Roy K, Fischer JF and Burkitt M. Identification of a two-partner secretion locus of enterotoxigenic Escherichia coli. *Infection and Immunity*. 2006; **74**: 2245-2258.