International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2013): 4.438

Distribution of Colonization Factors and Toxins among Diarrheal Children below 5Years: A Case Study of Moi Teaching and Referral Hospital, Kenya

Faith Jeruto[1], Dr. Lizzy Mwamburi[2], Dr. James Wanga[3]

[1, 2, 3]Department of Biological Studies, University of Eldoret, Kenya

Abstract: ***Estimates suggest that approximately 200 million cases and 380,000 deaths occur in children below 5 years of age due to Enterotoxigenic Escherichia coli(ETEC) infections annually. The study aimed at molecular determination of ETEC toxins and colonization factors distribution among children below 5 years of age. Multiplex PCR was used in the identification of ETEC toxin and colonization factors. A total of 200 isolates of E.coli-like recovered from diarrheic children at Moi Teaching and Referral Hospital were collected and transported to Microbiology Hub Walter Reed Project-Kericho and tested for the presence of virulent factors using Multiplex PCR method. Positive isolates for ETEC were subjected to a panel of colonization factor antigens (CFA) using multiplex PCR for identification of colonization factors. Data was analyzed using statistical analysis system (SAS) and fisher's exact test was used for comparing 2 variables. Heat labile toxins (LTh) were more common (65.38%) than heat stable toxins (STh) (26.92%). LThETECtoxins were observed in younger and older children (42.3% and 23.08% respectively) while SThETECtoxins were more frequent in children below 2 years and were not observed in children above 2 years old. The most common colonization factor was coli surface antigens 6 (Cs6) and coli surface antigens 1 (Cs1) and was detected in children below 2 years of age. STh and LThETECtoxins had a detectable Colonization factor at higher frequency (19.23%). Children below 5years are susceptible to ETEC toxins and CFs. Use of molecular diagnostic technique to determine the distsribution of ETEC toxins and CFs should be adopted.***

Keywords: Enterotoxigenic *Escherichia coli*, ETEC toxins, colonization factors

1. Introduction

Enterotoxigenic*Escherichia coli (*ETEC)are bacterial pathogen that causes between1.6 to 2.5 million deaths annually amongst children in developing countries (Makobe*et al.,* 2011). Albert *et al.* (1995) reported that ETEC are the main causes of diarrhea morbidity and mortality among children below 5yrs of age.ETEC is a common cause of travellers' diarrhoea known to have fatal consequences in children aged below 5 years of age (Croxen& Brett, 2010). It usually anchors to enterocytes of the small bowel through colonization factors (CFs) encoding *eae*and *bfp*genes and secretes two toxins; heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) which are responsible increased intracellular levels of cyclic amino-phosphate (cAMP) and cyclic guano-monophosphate (GMP) resulting in impaired absorption of Na+ and H2O influx into the lumen leading to passage of copious quantities of watery stool (Turner *et al.,* 2006) Diagnosis of ETEC infections is done by detection of the major virulent determinants i.e. heat labile enterotoxins (LTh), heat stable Enterotoxins of human origin (STh), heat stable enterotoxins of porcine origin (STp) and colonization factor(CF)(Vidal *et al.,* 2009).Colonization factors (CFs) are specialized fimbrial structures present on the surface of the bacteria that aid in the adhesion to the luminal surface of epithelial cells and after binding, it secretes a heat-labile toxin (LTh) or a heat-stable toxin of the human (STh) or porcine(STp) that causes pediatric diarrhea (Steinsland*et al.,* 2002). Therefore the two variant of ST (STh and STP) can be present alone or in combination with LT.Colonization factor antigens that are commonly found in ETEC include; colonization factor antigen/1(CFA/1),CFA/II) and (CF1V) (Iseri*et al.,* 2011). CFA/II is composed of *coli* surface antigen 1(CS1),*coli* surface antigen 2(CS2), *coli* surface antigen 3 (CS3), and CFA/IV is composed of *coli* surface antigen 4 (CS4), *coli* surface antigen 5 (CS5) and *coli* surface antigen 6 (CS6) (Vilchez*et al.,* 2014).Also there islittle knowledge on the molecular characteristics and virulence profiles of ETEC in MTRH hence incidences of ETEC illness and epidemiological studies in these areas are lacking. Therefore, this paper aimed at determining the distribution of colonization factors and toxins among diarrheal children below 5 years.

2. Materials and Methods

Study location

Isolates of enterotoxigenic coli from stool samples were obtained from Moi Teaching and Referral Hospital (MTRH). This is the second National Referral Hospital in Kenya after Kenyatta National Hospital (KNH). The Hospital is located along Nandi Road in Eldoret town (310 kilometers Northwest of Nairobi the capital city of Kenya), UasinGishu County, in the North Rift region of Western Kenya. The hospital serves a population of approximately16 million.

Study Design

The study was a laboratory based.A total of 200 isolates of enterotoxigenic coli stool samples were used based on Makobe*et al.*(2011). *Escherichia coli*like archived isolates recovered from diarrheal stool samples of children at MTRH. Systematic samplingwas used to choose the isolates from the archives (Makobe*et al.,* 2011). Samples were then transported to Walter Reed Project-Kericho (WRP-K) Microbiology hubfor analysis using multiplex PCR assay for

detection of ETEC toxin and colonization factors as per MHK SOP (2013).

Sample processing

Samples of *E.coli*like processed that were previously using standard microbiological methods were revived by sub-culturing on lactose macCkonkey and incubated at 37^{o}C for 18 to 24hrs. Morphological characteristics on the media was used to re-confirm *E. coli* isolates and tested immediately for the presence of toxins using multiplex PCR assay. Positive isolates for ETEC were subjected to the multiplex PCR ETEC colonization factor assayto assess colonization factors profiles.Multiplex PCR for detection of the colonization factors (CFs) provide a time efficient method that is easy to perform and interpret (Rodas*et al.,* 2009).

Identification of colonization factors using Multiplex PCR.

ETEC isolates were identified according to Rodas*et al.* (2009). Test for genes encoding different types of colonization factor antigens (CFAs) by three part Multiplex PCR assay was done. Each Multiplex PCR test was performed in 25ul reaction mixture containing: 1ul of 10mM deoxyribonucleotide triphosphate (dNTP) mix, 2ul of $mgcl_2$ (25mM), 5ul of Promega Go Taq Flexi 5x PCR Buffer, 0.25ul Promega Go Taq Flexi DNA polymerase, 5ul of DNA template and 0.13ul each of *rrsA*(3.8pmol).

Primers for colonization factor antigen (CFA) Multiplex PCR 1 which targeted CFA/I, CS4, CS6 and CS14, Multiplex PCR 2 primers that targeted CS3, CS5 and CS7 and Multiplex PCR 3 primers that targeted CS1/PCFO71, CS2, CS17/19 and CS21 were used at 30pmol (appendix 1). Positive control strains for Multiplex PCR 1 was BANG10-SP (CS6) and H10407 (CFA/1), B2C (CS3) control strain was used in Multiplex PCR 2 and finally B2C (CS2) and E24377A (Cs1/PCF071) controls strain was used in Multiplex PCR 3. PCR products for Multiplex PCR mix 1, Multiplex PCR mix 2 and Multiplex PCR mix 3 were subjected to thermo cycling program as follows: Initial denaturation at 95°C for 2 min followed by 30 cycles of amplification (95°C for 1 min, 60°C for 30 sec and 72°C for 1 min) and finally primer elongation at 72°C for 10 minutes. Electrophoresis were done using 20ul of PCR reaction on 1.5% Agarose gel stained with Ethidium bromide and illuminated using UV transilluminator. A molecular size marker (100bp) was included in each agarose gel run to estimate the size of the amplicon.

Statistical Analysis

Results obtained from PCR multiplex were subjected to Fisher's exact test for analysis using Statistical Analysis System (SAS) to compare the variables and test for significance. P value less than 0.05 was considered statistical significant

Results and Discussion

Table1 represents distribution of ETEC toxins in ETEC isolates from children below 5 years of age with diarrhea. Fromthe table, ETEC toxins decrease with age thus the number of ETEC toxins in children below 3 years is 25 ETEC strains and 1 ETEC toxins in children age 4 and 5 years old. This supports earlier findings by Mansour *et al.*(2013) that children below 3 years of age are more susceptible to ETEC toxins. A significant difference ($p<0.05$) was seen between the presence of toxins and ages of children with diarrhea.

In this present study, ETEC toxins were more prevalent in children with diarrhea who were below 2years of age than in children with diarrhea who were 3 to 5 years of age (figure1). This is in line with a study of diarrheagenic*E.coli*in Bolivia where ETEC toxins were more prevalent in children below 2 years of age than in children who were 2 years to 5years old (Gonzales *et al.,* 2013).

Table 1: Distribution of ETEC toxins in ETEC isolates in different age groups.

Number of subjects aged						
Toxins	Age1	Age2	Age3	Age4	Age5	TOTAL
Toxins negative	187 18.70%	194 19.40%	194 19.40%	199 19.90%	200 20.00%	974 97.40
Enterotoxins	13 1.30%	6 0.60%	6 0.60%	1 0.10%	0 0.00%	26 2.60%
TOTAL	200 20.00%	200 20.00%	200 20.00%	200 20.00%	200 20.00%	1000 100%

The most common toxins for ETEC isolates were LTh (65.38%) in the age group 1year to 4 years old and STh (26.92%) in the age group 1-2 years old (Figure 1). LTh/STh (3.85%) and STp (3.85%) ETEC toxins were identified less frequently. About 61.53% LThtoxins were found in the age group between 1-3 years old and STh (26.92%) toxins were common in the age group 1-2 years old.This finding is consistent with a research in Egypt by Mansour *et al.* (2013) which evaluated children below 3yrs of age with diarrhea for presence of ETEC toxins. Children below 3 years are susceptible to ETEC infections. LThETEC toxins increased in the group 1-2 years old and decreased with age while SThETEC strains were more frequent in children below 2 years and were not observed in children above 2 years old (figure 1). LTh/STh and STpETEC toxins were less common in children below 2 years of age (3.85%). This is in line with a study of diarrheagenic*E.coli*in Bolivia by Gonzales *et al.* (2013) where ETEC was more prevalent in children below 2 years of age than in children who were 2-5years old. ETEC toxins decrease after the age of 3 years probably due to acquired immunity among individuals which prevent ETEC infections (Qadri*et al.,* 2000). Also increased immune responses due to repeated episodes of ETEC infection in age below 2years prevent ETEC infections (Nataro*et al.,* 1998).

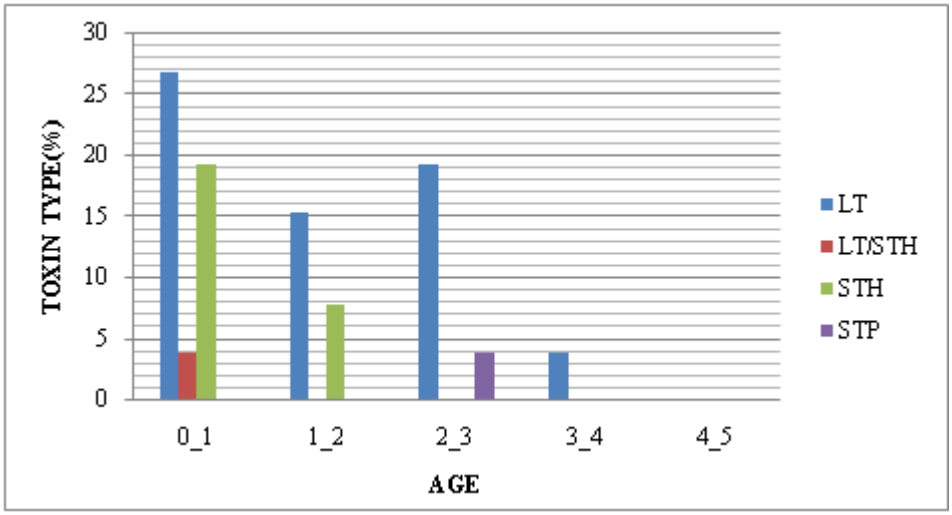

Figure 1: Distribution of different types of toxins in ETEC isolates in different age group

Results on fisher's exact test are summarized in Table 2. Statistical analysis showed no association (P>0.05) between age and CF profile or restriction to age in the prevalence of CFs on ETEC. Colonization factorswere detected more frequently in children below 2 years of age and above 2 years old(Table 2).Five out of 13 CFs positive were CS6 (38.46%) and were observed in children below 2years of age. Also CS1 ETEC isolates were detected more frequently in children below 2 years of age (15.23%) and absent in children above 2 years old. Expression of multiple CF was notable were CS6 plus CS1 was found in one child with diarrhea (Plate 1).

Toxin profiles and CFs profiles of ETEC isolates are variable and differ among different geographical regions and populations (Vidal *et al.*, 2009). Among the wide range of CFs present on diarrheagenic strains includes CFA/1, CS1-CS6 and recently CS14 and CS21 have been found on ETEC strains in various frequencies (Qadri*et al.*, 2005). This suggests that there is a continuous circulation of similar strains among populations. However, CFs has not been detected on all ETEC and on roughly 30 to 50% of strains in different countries lack detectable CFs. This could be due to the absence of CFs, to loss of CF properties on subculture of strains or to lack of specific tools for their detection (Qadri*et al.*, 2005).

Table 1: Distribution of colonization factors (CFs) in ETEC isolates from diarrheal children in different age groups

	Number of subjects aged				
CF profile	Age1	Age2	Age3	Age4	Total
Cf-negative	3 23.08%	5 19.23%	4 15.23%	1 3.85%	13 50%
CFA/1	1 3.85%	1 3.85%	0 0.00%	0 0.00%	2 7.69%
CS1	4 15.23%	0 0.00%	0 0.00%	0 0.00%	4 15.23%
CS2	0 0.00%	0 0.00%	1 3.85%	0 0.00%	1 3.85%
Cs3	0 0.00%	1 3.85%	0 0.00%	0 0.00%	1 3.85%
Cs6	4 15.23%	0 0.00%	1 3.85%	0 0.00%	5 19.23%
Total	12 46.15%	7 46.15%	6 23.08%	1 3.85%	26 100.0%

Cohort studies in children below 2 years old in Egypt have shown that Pathogenicity increases when multiple CFs are expressed by an individual (Mansour *et al.*, 2013). CF negative was high in frequency among the ETEC strains isolated. The reason for low prevalence of CFs on ETEC isolates could be due to loss of CF antigens on subculture and other CFs requires specific conditions for growth (Qadri*et al.*, 2000). Furthermore there was high percentage of LTh only strains not expressing a known CFs. Out of 17 LThETEC producing strains only 7 LTh (41.2%) presented known CFs (table 4.2). This support the notion that LT producing ETEC strains are less virulent than ST or LT/ST ETEC strains according to a cohort study done by Mansour *et al.*(2013).

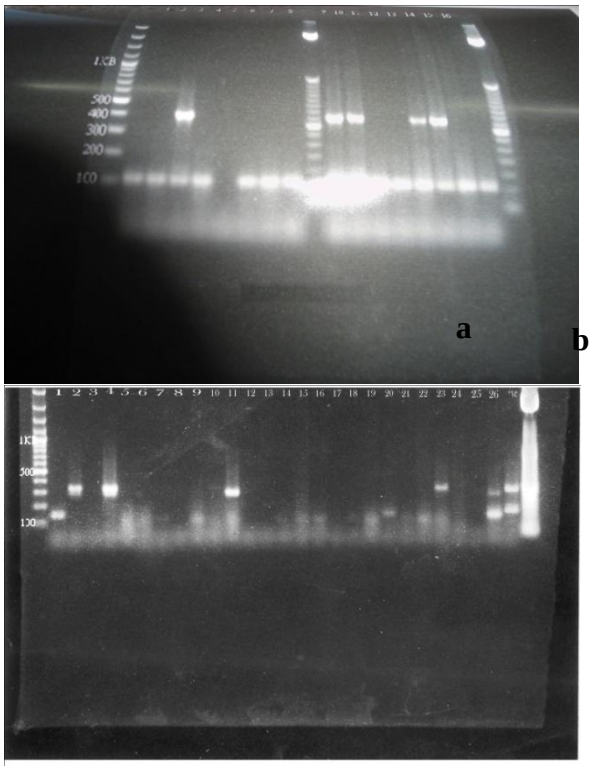

Plate 1: Agarose gel electrophoresis of ETEC multiplex PCR mixture representing CS6 (a) and CS1 (b). Lanes without bands did not contain any target gene.

References

[1] Albert M,.Faruque S., Faruque A., Neogi P., Ansaruzzaman M., Bhuiyan N., Alam K. and Akbar M. (1995).Controlled study of *Escherichia coli* diarrheal infections in Bangladeshi children.*Clin Microbial* 33(4): 973-977.

[2] Gonzales L., Sanchez S., Zambrana S., Iniquez V., Wiklund G., Svennerholm M. andSjöling A. (2013). Molecular characterization of Enterotoxigenic*Escherichia coli* isolates Recovered from children with Diarrhea during a 4 year period (2007 to 2010) in Bolivia. *Clin. Microbial**51***:1219.

[3] Iseri L., Apan T., Aksoy A., Koc F., Göcmen J. and Nuristani[II] D. (2011). The prevalence of Enterotoxigenic*E.coli* isolated from the stools of children aged 0-10yrs with diarrhea in mid-anatolia region, Turkey. Braz. J. microbial 42:1.

[4] Makobe K., Sang K., Kikuvi G. and Kariuki S (2011). Molecular characterization of virulence factors in diarrheagenic*Escherichia coli* isolates from children in Nairobi, Kenya. *Emerging Problems in Infectious Diseases.***6**: 598-604

[5] Mansour A., Shaleen L., Amine M., Hassan K., Sanders W., Riddle S., Armstrong W., Svennerholm m., Sebeny J., Klena D., Young N. and Frenck W. (2013). Pathogenicity and phenotypic characterization of Enterotoxigenic*E.coli* isolates from a Birth cohort of children in Rural Egypt. *Clin. Microbial***52**: 587

[6] MHK SOP (2013)., Multiplex PCR Assay for Identification of ETEC Toxins and Colonization Factors. MHK-BAC 030: 6-7

[7] Nataro P. and Kaper B. (1998).Diarrheagenic*Escherichia coli*.*Clin.Microbial*.**11**:142-201

[8] Qadri F., Das K., Faruque G., Fuchs J., Albert J., Sack B. and Svennerholm M. (2000). Prevalence of toxin types and colonization factors in Enterotoxigenic*Escherichia coli* isolated during a 2-year period from diarrheal patients in Bangladesh. *Clin.Microbial*.**38**:27.

[9] Rodas C., Iniquez V., Qadri F., Wiklund G., Svenneholm M. and Sjöling A. (2009).Development of multiplex PCR Assay for detection Enterotoxigenic*Escherichia coli* colonization factors and toxins.*Clin.Microbial*.**47**:1218-1220.

[10] Vidal M., Valenzuela P., Baker K., Lagos R., Esparza M., Livio S., Farfan M., Nataro P., Levine M. and Prado V. (2009).Characterization of the most prevalent colonization factor antigens present in Chilean clinical enterotoxigenic*Escherichia coli* strains using a new multiplex polymerase chain reaction. *Diagnostic microbiology and infectious Disease***65**: 217-223

[11] Vilchez S., Becker-dreps S., Amaya E., Perez C., Paniagua M., Reyes D., Espinoza F. and Weintreub A. (2014). Characterization of Enterotoxigenic*Escherichia coli* strains isolated from Nicaraguan children in hospital, primary care and community settings. *Medical microbial***63**:729-734.,