

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 macConkey and incubated at 37°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 assay to 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 (Rodaset *et al.*, 2009).

Identification of colonization factors using Multiplex PCR.

ETEC isolates were identified according to Rodaset *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 mgcl₂ (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/PCF071, 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/I), 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. From the 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 2 years of age than in children with diarrhea who were 3 to 5 years of age (figure 1). 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 5 years 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 1 year 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% LTh toxins 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 3 yrs of age with diarrhea for presence of ETEC toxins. Children below 3 years are susceptible to ETEC infections. LTh ETEC toxins increased in the group 1-2 years old and decreased with age while STh ETEC strains were more frequent in children below 2 years and were not observed in children above 2 years old (figure 1). LTh/STh and STp ETEC 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 2-5 years 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 2 years prevent ETEC infections (Nataro *et al.*, 1998).

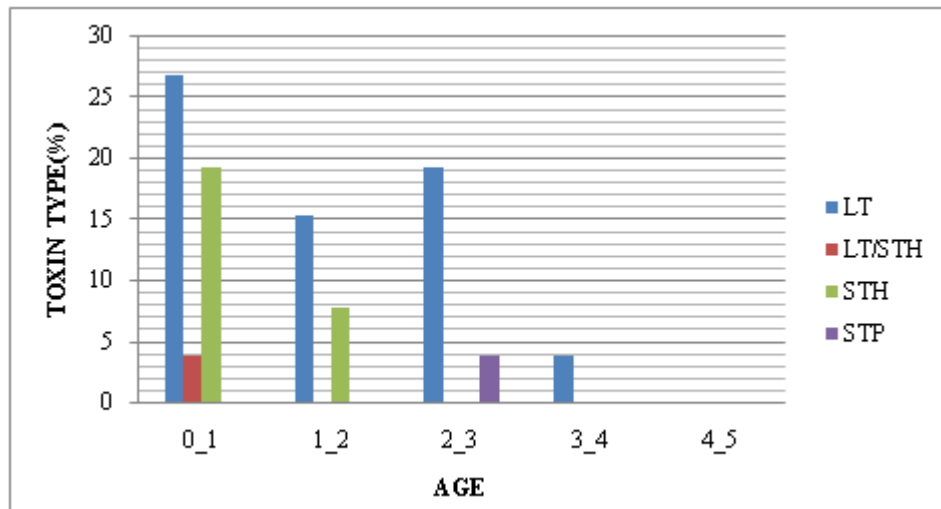


Figure 1: Distribution of different types of toxins in ETEC isolates in different age group

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 factors were 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 2 years 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

CF profile	Number of subjects aged				
	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 LTh ETEC 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).

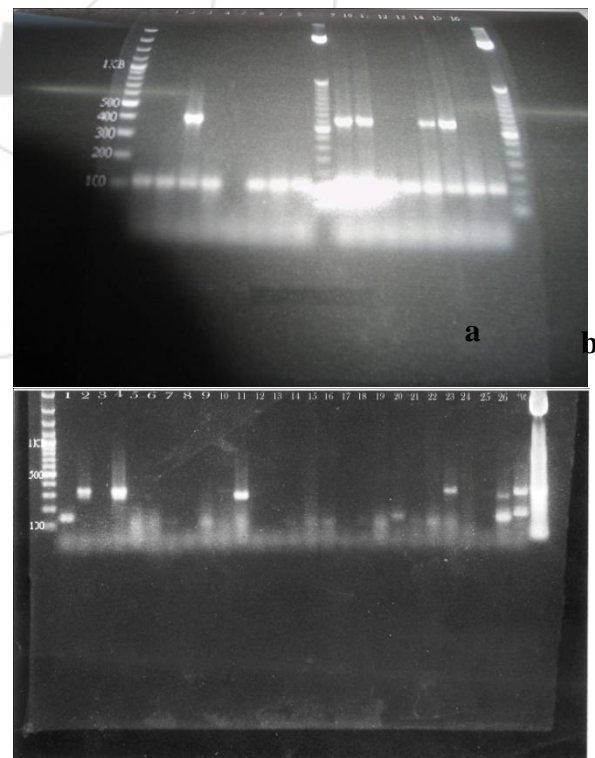


Plate 1: Agarose gel electrophoresis of ETEC multiplex PCR mixture representing CS6 (a) and CS1 (b). Lanes without bands did not contain any target gene.

References

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