

PATHOTYPES AND VIRULENCE MARKERS IN *ESCHERICHIA COLI* ASSOCIATED WITH DIARRHOEA AMONG HIV SEROPOSITIVE AND SERONEGATIVE CHILDREN BELOW FIVE YEARS IN WESTERN KENYA

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Abstract

Diarrhoea is among the leading causes of childhood morbidity and mortality worldwide. Diarrheagenic *Escherichia coli* (DEC) are responsible for 30% to 40% of acute diarrhea and chronic episodes in children in developing countries. This study sought to elucidate genotypic diversity and virulence markers of *Diarrheagenic Escherichia coli* associated with diarrhoea among HIV seropositive and seronegative children aged below five in Western Kenya. A total of 105 diarrheagenic E coli isolates obtained from children aged below five years with diarrhoea admitted at Moi Teaching and Referral Hospital , Kenya, were subjected to multiplex PCR using seven sets of primers. The study revealed that 36 (34.2%) pathotypes were identified as; EHEC 4 (11.1%), ETEC 2 (5.6%), EAEC 21 (58.3%), EIEC 3 (8.3%) and EPEC 6 (16.7%), EAEC. Seven virulence genes were identified including EHEC shigatoxin genes, *stx1* (0 vs 100%) and *stx2* (0 vs 100%), ETEC heat labile, *elt* (0 vs 100%), and heat stable toxin, *est* (0 vs 100%), EPEC, *bfp* (33.3% vs 66.7%), EAEC, *aatA* (71.4% vs 28.6%) and EIEC *ipaH* (66.7% vs 33.3%) among isolates from HIV seropositive and negative cases respectively. EPEC and ETEC were significantly associated with acute diarrhoea episodes in HIV negative cases while EAEC was

predominantly linked to persistent diarrhoea with higher incidence in HIV seropositive than seronegative cases, ($p < 0.05$). There was a significant difference in the expression of virulence genes in relation to age.

Keywords: Diarrhoea, Pathotypes, Diarrheagenic *Escherichia coli*, HIV

Introduction

Diarrhoeal diseases are a leading cause of childhood morbidity and mortality worldwide with over 2 million deaths annually (UNICEF, 2009). In Kenya, prevalence of diarrhoea is estimated at 16 % among children aged below five years and the third leading cause of paediatric admissions after malaria and pneumonia (MOH, 2007). Childhood Human immunodeficiency virus (HIV) infection in Kenya remains high despite government efforts to scale up prevention of mother to child transmission of HIV. Diarrhea in HIV is a major clinical problem associated with malabsorption, significant weight loss and extra-intestinal opportunistic infections particularly in cases of severe immuno-suppression (WHO, 2010). The gastrointestinal tract plays a key role in Acquired immune deficiency syndrome (AIDS) pathogenesis, due to suppressed immunologic responses at the mucosal level that tend to delay the intestinal nonspecific defense mechanisms (Gassama et al., 2005) Globally, the most common etiological agents of diarrhea include bacteria, viruses and parasites (Nguyen et al., 2005). However, some of the bacterial enteropathogens commonly associated with diarrhoea in children below five years include *E coli*, *Salmonella* and *Shigella*.

The diarrheagenic *Escherichia coli* (DEC) are responsible for 30% to 40% of acute diarrhea and chronic episodes in children in developing (Nataro et al., 2006) and developed countries (O’Ryan et al., 2005; Cohen et al., 2005) Six categories of DEC are recognized which include; *Enterohaemorrhagic Escherichia coli* (EHEC), shiga toxin producing *Enteropathogenic Escherichia coli* (EPEC), enteroinvasive *Escherichia coli* (EIEC), *Enteroaggregative Escherichia coli* (EAEC or EAaggEC), *Enterotoxigenic Escherichia coli* (ETEC) and *Diffusely Adherent E. coli* (DAEC), (Nguyen et al., 2005; Huang et al., 2006). These pathotypes have different virulence mechanisms (Weintraub, 2007) which are genetically coded for by chromosomal, plasmid and bacteriophage DNAs (Yang et al., 2007) and include heat-labile toxin (*lt*) and heat-stable toxin (*st*) in ETEC enteroaggregative mechanisms (Eagg) in EAEC and enteroinvasive mechanisms in EIEC (Nataro et al., 2006). EHEC is a highly infectious pathogen that colonizes the distal ileum and large bowel in humans and is associated with outbreaks of severe gastroenteritis in developed countries (Yang et al., 2007). Its main virulence factor is the phage-encoded shiga toxin (*stx_I* and *stx_{II}*), a defining characteristic of the shiga toxin-producing

EHEC O157:H7. 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). Moreover, EAEC is recognized as a cause of endemic and epidemic diarrhoea that is often watery but may be accompanied by mucus or blood. The characteristic phenotype of EAEC is aggregative adhesion, which involves the formation of a stacked-brick pattern of HEp-2 cells mediated by the genes encoded on virulence plasmids (Guerrant et al., 2005; Nataro et al., 2006). EAEC colonization occurs in the mucosa of both the small and large bowels leading to mild inflammation in the colon through secretion of cytotoxins (Kaper et al., 2004) Attachment to enterocytes occurs through aggregative adherence fimbriae (AAF) that stimulate a strong interleukin-8 (IL-8) response, allowing biofilms to form on the surface of cells. EPEC is a major cause of potentially fatal diarrhoea in infants in developing countries (Nataro et al., 2006). This pathotype forms attaching and effacing (A/E) lesions on intestinal epithelial cells. The initial attachment of EPEC to enterocytes in the small bowel is thought to involve the bundle-forming pilus (*bfp*) and *eae* gene on EPEC adherence factor (EAF) plasmid encoding the adhesin intimin responsible for the intimate attachment of the bacteria to the epithelial cells (Gassama et al., 2005) EIEC has similar pathogenic mechanisms as *Shigella* and is associated with bloody diarrhoea similar to shigellosis (Kaper et al., 2004). Virulence is due to a plasmid such as *ipaH* that encodes genes required for invasion, cell survival and apoptosis of macrophages (Ogawa et al., 2008; Schroeder & Hilbi, 2008). The virulence genes in DEC are often located on transmissible genetic elements that can be transferred to *E. coli* recipient strains (Dobrindt et al., 2002). These virulence determinants give each pathotype the capacity to cause a clinical syndrome with distinctive epidemiologic and pathologic characteristics (Robins-Browne et al., 2004) and are therefore ideal targets for the determination of the pathogenic potential of any given *E. coli* variant (Bekal et al., 2003).

Diagnosis of diarrhoea suspected bacterial etiology in reference laboratories is usually achieved by conventional methods such as culture, biochemical and serological tests (Murray et al., 2000). However, due to limited facilities in rural health centres in Kenya, stool samples are not routinely evaluated for these pathogens. The few reference and research laboratories located in urban settings identify diarrhoeagenic agents such as

E coli serologically based on their surface O-antigen and flagella H-antigens. Such methods are often inconclusive since many strains are non-reactive and in most cases, the serotype may not correlate appropriately with the presence of virulence genes (Sunabe & Honma, 1998). Currently, rapid and sensitive molecular techniques are available for identification of various serotypes from clinical samples (Zahraei et al., 2006). Multiplex PCR provides a specific and superior ability to detect genotypic diversity in the presence of other bacteria simultaneously (Yan et al., 2010; Malkawi & Gharaibeh 2004). Few studies have documented DEC as the main agent associated with bacterial diarrhoea in infants and children in Kenya [Bii et al., 2005; Sang et al., 1997; Sang et al., 2012 & Makobe et al., 2012). However, studies to identify molecular pathotypes and associated virulence genes especially at Moi Teaching and Referral Hospital (MTRH) and the Western Kenya region are limited. Moreover, in spite of the relevance of association between AIDS and diarrhea, few studies have been conducted to compare prevalence of DEC strains among HIV positive and negative infantile population. The aim of this study was to determine molecular pathotypes of DEC isolated associated diarrhoea among HIV infected and un-infected children with diarrhoea with a view to understanding their role in disease and guide in determining specific therapies and optimizing preventive interventions.

Methods

DNA Extraction

The DEC isolates were previously obtained from diarrhoeic stool samples by culture, biochemical and serotypic analysis at MTRH Microbiology laboratories and stored in tryptic soy broth (TSB) at -20⁰ C. The frozen isolates were allowed to thaw at room temperature in preparation for DNA extraction. To revive the organisms, each sample was inoculated onto sorbitol MacConkey agar and incubated for 18-24 hours at 35-37⁰C. A loopful of bacterial colony from each sample was suspended in 200µl sterile deionized water in 1.5ml eppendorf tubes and adjusted to 0.5 McFarland. This was mixed thoroughly by vortexing, boiled at 95-100⁰C for 10 minutes and then cooled to room temperature (RT). The suspension was centrifuged at 10,000 rpm for 1min in RT and the supernatant DNA extract pipetted into 1.5ml microfuge tube and stored at -20. To confirm EHEC, ETEC, EIEC, EAEC and EPEC strains, multiplex PCR assays were performed using the AFRIMS Enteric Department protocol, SOP ETR-AD-000-F1, 2010. DNA standards (positive controls) with respective virulence genes *Escherichia coli*; ATCC 35401, PDAS 101, ATCC 933J, ATCC 933W, ATCC 43887 and MHK 00238 obtained from Kenya Medical Research Institute and negative controls without virulence genes were used. Multiplex PCR was used to detect seven virulence genes in *E coli* including ETEC, heat stable

toxin (st), heat labile toxin (lt), shiga toxin1 (stx1) and shigatoxin 2 (stx2) , EIEC invasive plasmid adhesin (ipaH), EAEC (aatA) and EPEC bundle forming pilus (bfp) factors. The list of primers, Oligonucleotide sequences and expected amplicon sizes in base pairs are indicated below (**Table 1**).

Table 1: Target virulence genes and Oligonucleotide sequences for DEC Multiplex PCR Primers, Expected Products and Control Strains

Target virulence factor / gene	Primer	Oligonucleotide sequence (5' -3')	Product size (bp)	Controls
Heat labile toxin, <i>elt</i>	ETEC 508F	CACACGGAGCTCCCTCCAGTC	508	ATCC 35401
	ETEC 508R	CCCCCAGCCTAGCTTAGTTT		
Heat-stable toxin, <i>est</i>	ETEC 147 F	GCTAAACCAGTAGGTCT	147	PDAS 101
	ETEC 147R	CCCGGTACARGCAGGATTACAACA		
<i>shigatoxin - 1, stx₁</i>	EHEC 348 F	CAGTTAATGTGGTGCGAAGG	348	ATCC 933J
	EHEC 348 R	CACCAGACAATGTAACCGCTG		
<i>shigatoxin - 2, stx₂</i>	EHEC 584 F	ATCCTATTCCCGGAGTTTACG	584	ATCC 933W
	EHEC 584 R	GCGTCATCGTATACAGGAGC		
Bundle forming pilus, <i>bfp</i>	EPEC 300F	GGAAGTCAAATTCATGGGGGTAT	300	ATCC 43887
	EPEC 300R	GGAATCAGACGCAGACTGGTAGT		
Invasive plasmid adhesin, <i>IpaH</i>	EIEC 423F	TGGAAAACTCAGTGCCTCT	423	ATCC 43893
	EIEC 423R	CCAGTCCGTAATTCATTCT		
Aggregative attachment adhesin, <i>aatA</i>	EAEC 650F	CTGGCGAAAGACTGTATCAT	650	
	EAEC 650R	MHK 00238-1		

Source: USAMRU protocol, 2010

Preparation of PCR mixture and Amplification

PCR mixture was prepared in 0.5ml micro-centrifuge tubes by thawing the DNA samples at room temperature, vortexing and then centrifuging for 10 seconds. Dilution of DNA template was done according to manufacturers' instructions (Bioserve Biotechnologies, Laurel, MD, USA). The sample tubes were aligned in an 80-well format rack on an ice block. 20µl aliquots of PCR master mix containing 2 ml of 2.5 mM dNTP

mixture (dNTP, dCTP, dTTP, dGTP), 2.5µl MgCl₂), 2.5µl 10X reaction buffer, 0.3 µl of each primer (reverse, R and forward, F), 5µl each of DNA template and *taq* polymerase (Applied Biosystems, Roche Molecular, Inc, and Branchburg, New Jersey, USA) were dispensed in polypropylene reaction tubes. These reaction tubes were sealed and mixture vortexed followed by centrifugation for 30 seconds and then loaded onto a DNA Gene Amp PCR system 9700 thermal cycler (Applied Biosystems) for amplification of the PCR end products. Amplification was set at 20 cycles of denaturation at 94 °C for 60 sec, annealing at 64°C for 90 sec, extension at 72°C for 90 sec and post-extension at 72°C for 10 min.

Gel Electrophoresis

Agarose gel electrophoresis was performed in 2% agarose gel containing 1.6g of agarose powder in 80ml 0.5 X Ethidium bromides in TBE (44.5mM Tris, 44.5 mM Boric acid and 1mM EDTA) buffer. 0.5µg/ml 6X orange DNA loading dye (Thermo scientific, USA) was added to 10 µl of amplified PCR product on a parafilm and mixed briefly before loading onto agarose gel. 5-10 µl of 100-bp DNA ladder (Promega, Madison, Wisconsin, USA) was loaded onto the side lanes of gel for amplicon size estimation of PCR products. Electrophoresis was performed at 120 volts until the bands were fully formed. The gel was then rinsed twice with deionized water and amplified DNA bands visualized by exposure to UV light and using Gel Documentation System (Alpha Imager HP, 250v) attached to a digital monochrome printer (Mitsubishi Electric 100-240v) in which the gel picture was printed and virulence genes identified based on the size of the amplicons.

Statistical analysis

Data was coded and analyzed using STATA 10. Descriptive statistics including means and frequency listings were applied for discrete variables. Chi square test (X^2) was used to assess for associations between variables. The adopted significance level for statistical inference was 5%.

Results

The study revealed that out 105 DEC isolates, 36 (34.2%) pathotypes were identified as follows; EHEC 4 (11.1%), ETEC 2 (5.6%), EAEC 21 (58.3%), EIEC 3 (8.3%) and EPEC 6 (16.7%), EAEC. No EHEC and ETEC strains were detected from isolates of HIV positive cases while EAEC (71.4% vs 28.6%) EIEC (66.7% vs 33.3%) and EPEC (33.3% vs 66.7%) were detected in both cases.

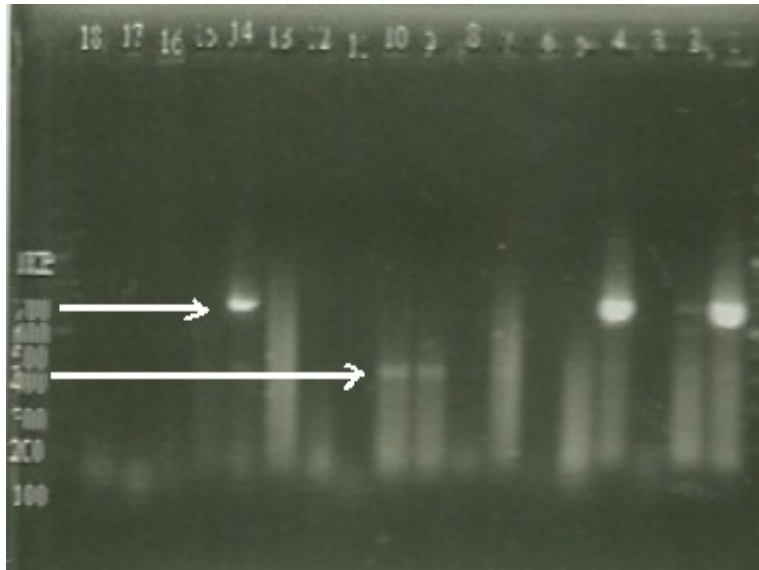


Figure 1: Agarose Gel Electrophoresis of multiplex PCR products of Diarrheagenic *E. coli* virulence genes from multiplex left to right; First Lane Positive control. Positive reactions with target genes are in lane 1, 5 and 15 are EAEC aatA 650 while lane 10 and 11 are EIEC ipaH 423. Last lane is Negative control.

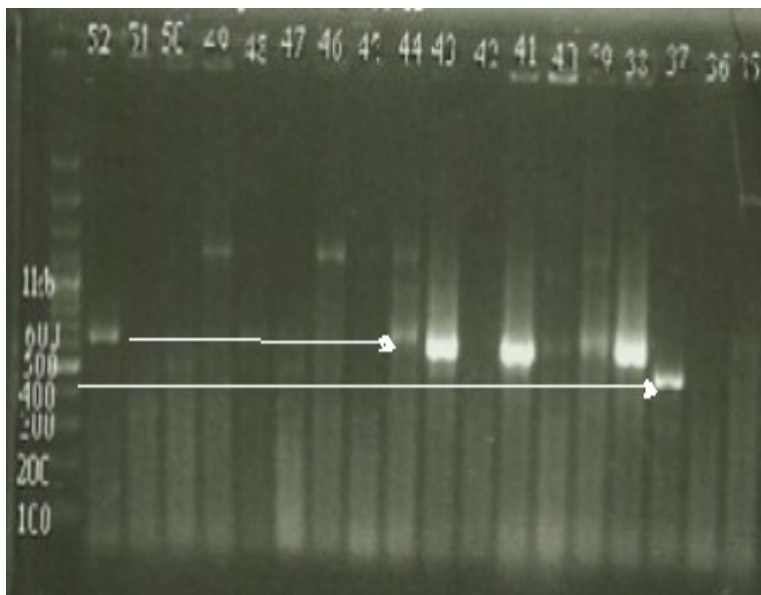


Figure 2: Agarose Gel Electrophoresis of PCR products left to right: First Lane is Negative control. Positive reactions with target genes in lane 37-39, 41, 43-44 are EAEC aatA 650 while lane 52 is EHEC 584. Last lane is Positive control.



Figure 3: Agarose Gel Electrophoresis of multiplex PCR products of Diarrheagenic *E coli* left to right; First Lane Positive control. Positive reactions with target genes in lane 68, 70, 72 and 75 are EAEC aatA 650 while lane 79 is EPEC bfp 300. Last lane is Negative control.

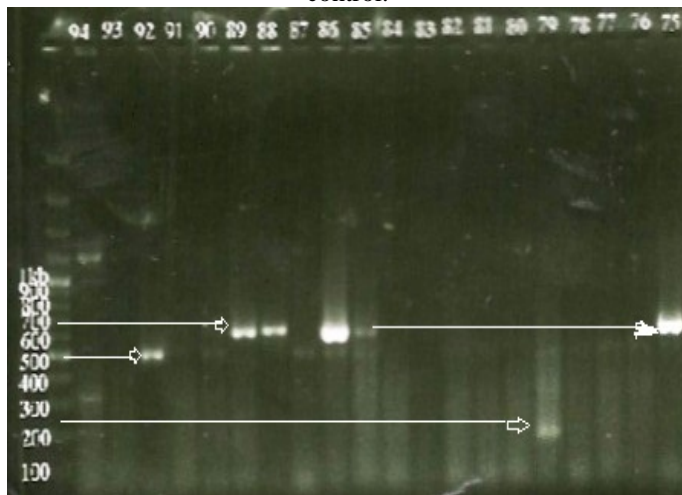


Figure 4: Agarose Gel Electrophoresis of Multiplex PCR products left to right: First Lane Positive control. Positive reactions with target genes are in lanes 84, 85, 88, 89 and 75 are EAEC aatA 650 while lane 92 is EHEC 584. Last lane is Negative control.

A total of seven virulence markers were identified which included EHEC shigatoxin genes, *stx1* (0 vs 100%) and *stx2* (0 vs 100%), ETEC heat labile *elt* (0 vs 100%), and heat stable toxin *est* (0 vs 100%), EPEC *bfp* (33.3% vs 66.7%), EAEC *aatA* (71.4% vs 28.6%) and EIEC *ipaH* (66.7% vs 33.3%) among isolates from HIV positive and negative cases respectively (**Table 2**). There were significant statistical differences in molecular pathotypes and virulence markers among HIV positive and negative

diarrhoea cases, (Kruskal Wallis test: $H= 23.66$; $p = 0.005$, $df = 4$, $P < 0.05$).

Table 2: Molecular Pathotypes of Diarrheogenic *E coli*

DEC Strain	No. (%)	Virulence gene	HIV Seropositive	HIV Seronegative	P value
			No. (%)	No. (%)	
EHEC	4 (11.1)	<i>stx1</i>	0	2 (100)	0.005
		<i>stx2</i>	0	2 (100)	
ETEC	2 (5.6)	<i>Elt</i>	0	1 (100)	
		<i>Est</i>	0	1 (100)	
EAEC	21 (58.3)	<i>aatA</i>	15 (71.4)	6 (28.6)	
EIEC	3 (8.3)	<i>ipaH</i>	2 (66.7)	1 (33.3)	
EPEC		<i>Bfp</i>	2 (33.3)	4 (66.7)	
Total	36 (100)		19 (52.8)	17 (47.2)	36 (100)

²Kruskal Wallis test: $H= 23.66$; $p = 0.005$; Level of significance $p < 0.05$

Table 3 shows association between molecular pathotypes of DEC versus type of diarrhoea. Three pathotypes of DEC including EHEC shigatoxigenic genes *stx1* and *stx2*, 2 (0 vs 100%) each and ETEC *elt* and *est* 1(0 vs 100%) each were associated with acute diarrhoea in HIV negative cases only. However, EAEC *aatA* was associated with acute and persistent diarrhoea in both HIV positive 3 (14.2 %) vs 12 (57.1%) and negative cases 2 (9.5%) vs 4(19.0%) respectively.

Table 3: Virulence Genes in Diarrheogenic *E coli* versus Type of Diarrhoea

DEC Strain	No.	Virulence Genes	HIV Seropositive			HIV Seronegative		P value	
			Acute Diarrhoea No. (%)	Pro AD No. (%)	Sub-Total No. (%)	Acute Diarrhoea No. (%)	Pro AD No. (%)		Sub-total No. (%)
EHEC	4	<i>stx1</i>	0	0	0	2 (100)	0	2 (100)	
		<i>stx2</i>	0	0	0	2 (100)	0	2 (100)	
ETEC	2	<i>elt</i>	0	0	0	1 (100)	0	1 (100)	0.995
		<i>est</i>	0	0	0	1 (100)	0	1 (100)	
EAEC	2	<i>aatA</i>	3 (14.2)	12 (57.1)	15 (71.4)	2 (9.5)	4 (19.0)	6 (28.6)	
EIEC	3	<i>ipaH</i>	0	2 (66.7)	2 (66.7)	1 (33.3)	0	1 (33.3)	
EPEC	6	<i>bfp</i>	1 (16.7)	1 (16.7)	2 (33.3)	4 (66.7)	0	6 (66.7)	
Total	36		4 (11.1)	15 (41.7)	19 (52.8)	13 (36.1)	4 (11.1)	17 (47.2)	

Kruskal Wallis test, $H= -26.95$; $p = 0.995$; Level of significance, $P < 0.05$

However, EIEC *ipaH* was associated with persistent diarrhoea only in both cases (66.7% versus 33.3%) respectively. There was no significant difference in association of virulence markers with diarrhoea among HIV positive and negative cases, (Kruskal Wallis test: $H= 26.95$; $p = 0.995$, $df = 4$; $P < 0.05$).

elsewhere. A recent study in Kenya demonstrated EAEC (8.9%), as the most frequent followed by ETEC (1.2%) and EIEC (0.6%) (Sang et al., 2012a). Similarly, a study to compare virulence genes in diarrheagenic *E. coli* of isolates from Kenya and Japan identified EAaggEC as the most frequent pathotype in (36.6% versus 17%) diarrhoea cases respectively (Bii et al., 2005). A further study on shigatoxigenic *Escherichia coli* from in Maasailand, Kenya, revealed a different scenario with ETEC (29.8%), EHEC (24%), EAEC (14.2%) and EPEC (3.5%) being identified (Sang et al., 2012 b). In Nairobi, Kenya, Makobe et al., 2012, identified EPEC (19.3%) as the most prevalent followed by ETEC (7.25%) and EAEC (3.86%) [30]. In Tanzania, 64.1% EAEC, 20.3% EPEC and 15.6% ETEC were also detected (Moyo et al., 2011). Studies elsewhere have shown variations in prevalence of DEC among infants and children owing to differences in immune status (Samie et al., 2012). Several studies have established higher rates of EAEC infection among HIV positive patients (Gassama et al., 2005) ; Gassama et al., 2001; Kelly et al., 2003). However, in Zambia EAEC genes were detected both HIV cases and controls with no evidence of long-term carriage (Crump et al., 2011). Although *E. coli* is a normal flora of the human gastrointestinal tract, pathogenic *E. coli* with typical virulence factor gene profiles have been associated with severe outbreaks of diarrhea (Schierack et al., 2006). From our study, it is evident that virulence genes in DEC occur in both HIV positive and negative patients and may manifest differently depending on host immunity.

Evaluation of the of DEC pathotypes and virulence genes in relation to type of diarrhoea revealed that both groups harboured virulence genes responsible for varied clinical presentations of diarrhoea ranging from acute to persistent diarrhoea. Acute diarrhoea in HIV positive cases was associated with EAEC *aatA* and EPEC *bfp* while EIEC *ipaH*, EHEC *stx₁*, *stx₂* and ETEC *elt* and *est* was noted among HIV negative cases. EAEC *aatA* was responsible for a high proportion of persistent diarrhoea in both cases. Several studies have indicated that *E. coli* pathotypes require multiple genes to be fully / highly virulent (Gassama et al, 2005). For instance ETEC with heat-labile toxin *elt*, heat-stable toxin *est* and colonization factor antigens (CFAs); EPEC with *bfp* and *eae* gene and Shiga-toxin-producing *E. coli* (STEC or EHEC) with shiga-like toxin *stx* and *eaeA*, are considered the most virulent (Ochoa et al., 2010; Turner et al., 2006). Although we did not detect multiple genes in our isolates, the dual existence of multiple virulence genes as viz-a-viz single gene in other studies raises questions as to whether or not these genes act in synergy to produce to induce acute disease. EAEC isolates have been found to be genetically heterogeneous containing various virulence genes including *aggR* (Ramamurthy et al., 2004). However, the presence of multiple genes has not been demonstrated in EAEC

pathogenesis. This may explain why EAEC is predominantly associated persistent diarrhoea while strains with multiple genes such as EPEC, ETEC and EHEC are significantly linked to acute diarrhoea. Studies elsewhere have described different markers of pathogenesis in EAEC infections including fecal cytokines such as IL-8 and IL-1R, lactoferrin, and occult blood (Greenberg et al., 2002). Although several studies have demonstrated heterogeneity in virulence mechanisms of EAEC isolates, there have been no clear associations between EAEC and diarrhea (Bouckennooghe,2000). Samie et al., (2005), demonstrated elevated levels of lactoferrin, IL-8, occult blood in diarrhea.

Studies in sub-Saharan Africa have shown that DEC were the main causes of diarrhea in children aged below two years (Moyo et al., 2011; Villamor et al., 2004). In our study, analysis of DEC virulence in relation to age of patients revealed a skewed distribution towards the younger age groups (0-24 months) in both categories except EAEC which was registered in all cohorts among HIV positive cases. This difference was statistically significant, ($p < 0.05$) and the pattern may reflect the lack of active immunity in the child, cessation of breastfeeding, introduction of weaning foods that may be contaminated with faecal bacteria, or direct contact with dirt and other infectious particles as the child starts to explore. HIV infected children are known to experience diarrhoea episodes that are more severe, prolonged and recurrent than the HIV negative whose decline in diarrhoea incidence beyond two years relates to acquisition of protective immunity (Nweze, 2010).

Conclusion

This study documented molecular pathotypes of DEC associated with childhood diarrhoea in Western Kenya. EPEC *bfp*, EHEC *stx₁* and *stx₂* and ETEC *elt* and *est* virulence markers were significantly associated with acute diarrhoea among HIV negative cases while EAEC *aatA* were linked to persistent bacterial diarrhoea in both categories but more significantly in HIV positive cases. There was a significant difference in distribution of DEC virulence genes among the various cohorts with a greater expression in age 0-24 months and among HIV positive cases in all age groups.

Acknowledgements

The authors recognize University of Kabianga who partially funded this research and Maseno University School of Graduate studies for guiding proposal development and approval. wish to thank Moi University/ MTRH Institutional Research and Ethics Committee for ethical review and approval of the study and Management of Microbiology Laboratories of Moi Teaching & Referral Hospital and KEMRI- Walter Reed Project, Microbiology Hub,

Kericho for availing laboratory facilities and equipment for this study. We express our sincere gratitude to Dr. Brett, Dr. Brooke, Clifford, Janet, Erick, Robert and Sheila for their co-operation and technical support and Dr. Ann Mwangi for data analysis.

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