Emerging Problems in Infectious Diseases

Molecular characterization of virulence factors in diarrhoeagenic *Escherichia coli* isolates from children in Nairobi, Kenya

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Abstract

Introduction: Among the bacterial causes, diarrheagenic *Escherichia coli* (DEC) is the most important etiologic agent of childhood diarrhoea and represents a major public health problem in developing countries. New evidence suggests that major differences in virulence among groups of DEC pathotypes may be related to the presence of specific pathogenicity islands (PAIs).

Methodology: Multiplex and conventional PCR assays were used to identify the DEC pathotypes and PAIs respectively from 207 *E. coli* isolates.

Results: The predominant DEC pathotype isolated was EPEC 19.3% (40/207), followed by ETEC 7.25% (15/207), EAEC 3.86% (8/207), STEC 0.97% (2/207) and EIEC 0.48% (1/207). The PAIs detected were enteropathogenic secreted protein C (EspC) 12.2% (8/66), locus of enterocyte effacement (LEE) 62.1% (41/66), and high pathogenicity island (HPI) 57.6% (38/66). Six percent (4/66) expressed only *fyuA* gene, 12.2% (8/66) *irp2* only, and 39.4% (26/66) expressed both fyuA and *irp2* genes. SHI-2 39.4% (26/66), *she* 6% (4/66) and O island 33.3% (22/66), 19.8% (13/66) expressed only *efa/lifA* gene, 7.6% (5/66) *pagC* gene only and 6.1% (4/66) expressed both *efa/lifA* and *pagC* genes. Toxigenic invasion A (TIA) PAI was not detected.

Conclusion: This study revealed that in addition to *eaeA, stx, aat, einv, st* and *lt* virulence genes exhibited in the different DEC pathotypes there are numerous PAIs in the DEC pathotypes. The PAIs can increase gene mobility within various motile elements, which has implications for the spread of virulence factors from DEC to commensal *E. coli.*

Key words: DEC pathotypes; virulence genes; pathogenicity islands

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Introduction

Diarrhoea is one of the leading causes of morbidity and mortality among children under five years of age in the developing world [1]. Currently diarrhoea has been reported to account for 1.6 to 2.5 million deaths annually; Diarrhoea in most developing countries still remains one of the principal causes of morbidity in children with each child reported to experience an average of three episodes of diarrhoea per year [2,3]. Diarrhoeal diseases in Kenya are among the five main causes of mortality in children younger than five years. Bacterial diarrhoea has been reported to account for up to 30% of all cases of infantile diarrhoea and as the most common cause of travellers' diarrhoea (TD) [4]. The spread of different pathogenic *E. coli* is a major concern in developing countries where it is enhanced by factors such as harsh climatic conditions, poor sanitation, malnutrition and immunosuppression related to HIV and AIDS [5]. Evidence from studies indicates DEC is a potential public health risk, with EHEC O157: H7 causing life-threatening sequelae, including hemolytic uremic-syndrome (HUS) and thrombocytopenic purpura (TTP), which causes kidney failure, hemolytic anemia, and thrombocytopenia [6]. New evidence suggests that major differences in virulence between groups of DEC pathotypes might be related to the presence of specific pathogenicity islands (PAIs) [7]. The PAIs increase gene mobility within various mobile elements such as plasmids, and from chromosomal location to mobile elements, which has implications for the spread of virulence factors from DEC to commensal *E. coli*; thus the PAIs play a significant role in pathogenesis of DEC and increased virulence in disease presentation. This study was conducted to

establish the prevalence of DEC pathotypes causing diarrhoea among children in Mbagathi District Hospital and to identify pathogenicity islands in different DEC pathotypes.

Methodology

Study design

This was a laboratory based study on *E. coli* isolates collected between January 2005 and May 2008, from children treated at the Mbagathi District Hospital, Nairobi, Kenya. Mbagathi District Hospital serves mainly a population of medium to low socio-economic status. The *E. coli* isolates were collected from children younger than five years of age admitted with diarrhoea (defined as three or more loose movements in the previous 24 hours) before being treated with antibiotics. The study was ethically approved by the Kenya Medical Research Institute (KEMRI), Kenya Ethical Review Committee N0.1419.

Bacterial isolates

Two hundred and seven (207) *E. coli* isolates were randomly selected from archived *E. coli* isolates processed by previously described methods [8,9] and revived by inoculation on Brain Heart Infusion broth. The isolates were incubated at 37°C for 18 to 24 hours, then sub cultured onto lactose MacConkey and sorbitol MacConkey Agar and incubated at 37°C for 18 to 24 hours. Morphological characteristics on these media were used to re-confirm *E. coli* isolates.

PCR detection of diarrhoeagenic E. coli pathotypes and PAIs

Bacterial colonies from MacConkey (MAC) and sorbital MacConkey (SMAC) plates were inoculated into 5 ml phosphate-buffered saline (PBS) tube to a density of MacFarland 4 (10^9 $5X10^9$ bacterial/ml). The 5 ml bacterial suspension was boiled for 20 minutes and then centrifuged at 2,500 X g for 10 minutes to pellet cell debris. The supernatant was used for PCR assays.

Preparation of DNA template and primers

The DNA templates were subjected to multiplex PCR for the detection of the virulence markers shown in Table 1. Positive controls containing template DNA of the following reference strains were used in every amplification round: EHEC ATCC 43890, EHEC ATCC 43887, EPEC ATCC 43887, ETEC ATCC 35401, EIEC ATCC 43893, EAEC 97R and *E. coli* ATCC 11775 (negative control without virulence genes). Conventional PCR assay was used for detection of pathogenicity islands (PAIs) in the diarrhoeagenic *E. coli* isolates. The following distinct genes in particular islands were targeted *she* PAI (*pic* gene), HPI PAI (*irp2* and f*yu A* gene), LEE PAI (*eae* gene), TIA PAI (*tia* gene), SHI-2 PAI (*iutA gene*), EspC PAI (*espC gene*) and O-island PAI (*efa/lifA* gene and *pagC* gene). Primers used in this study are shown in Table 2.

PCR conditions

Each PCR test was performed in 0.5 ml microcentrifuge tubes using a 25-µl-reaction mixture containing: 2 µl of 10 mM mix deoxynucleotide triphosphate (dNTP); 2.5 µl of $MgCl_2$ (25mM); 2.5 µl 10X buffer solution; 1.25 µl of PCR primer (forward and reverse primer each) with concentration of pmol/ml (Bioserve Biotechnologies, Laurel, MD, USA); 0.3 µl of *Taq* polymerase (Applied Biosystems, Roche Molecular, Inc, and Branchbury, New Jersey, USA); and 2 µl of DNA template and water to the final volume of 25 µl. PCR amplifications were performed on a PTC-200 thermal cycler (MJ Research Inc, Watertown, Massachusetts, USA). A multiplex PCR cycle was used as previously described [10] in amplification of DEC pathogenic genes. PCR conditions for the detection of PAIs were used as earlier described for *she* PAI (*pic* gene) [11], HPI PAI (*irp2* and f*yu A* gene) [12], LEE PAI (*eae* gene) [13], TIA PAI (*tia* gene) [14], SHI-2 PAI (*iutA gene*) [13], EspC PAI (*espC gene*) [15] and O-island PAI (*efa/lifA* gene and *pagC* gene) [16]. The amplified PCR products were separated by electrophoresis in 2% agarose gels stained with ethidium bromide in Tris Borate (TBE) buffer at 100 V for one and half hours. The DNA in the gel was visualized on a UV transilluminator and photographed under ultraviolet light using a black-and-white instant Polaroid camera. A molecular size marker (100-bp DNA ladder; Promega, Madison, Wisconsin, USA) was included in each agarose gel run to estimate the size of the amplicons.

Statistical analysis

Data was entered using excel (Microsoft, Redmond, WA, USA)) and checked for consistency and integrity. Statistical analysis was performed with Statistical Package for Social Scientists (SPSS) version 11.5 (IBM Chicago, IL, USA); categorical variables were analysed using frequency distributions to establish prevalence of pathotype and pathogenicity islands.

Table 1. Sequences for DEC Multiplex PCR primers and their respective product size

Pathotype	Marker Virulence Factors (Genes)	Oligonucleotide Sequence (5'-3')	Product size bp	Reference
STEC	Verotoxin-1 *mVT1*	fp: ACGTTACAGCGTGTTGCRGGGATC rp: TTGCCACAGACTGCGTCAGTRAGG	121	[10]
STEC	Verotoxin- 2 *mVT2*	fp: TGTGGCTGGGTTCGTTAATACGGC rp: TCCGTTGTCATGGAAACCGTTGTC	102	[10]
EPEC	Attaching and effacing *meae*	fp: TGAGCGGCTGGCATGAGTCATAC rp: TCGATCCCCATCGTCACCAGAGG	241	[10]
ETEC	Heat-labile toxin 1 *mLT1*	fp: TGGATTCATCATGCACCACAAGG bp: CCATTTCTCTTTTGCCTGCCATC	360	[10]
ETEC	Heat-stable toxin -1 *mST1*	fp: TTTCCCCTCTTTTAGTCACTCAACTG rp: GGCAGGATTACAACAAAGTTCACAG	160	[10]
ETEC	Heat-stable toxin-2 *mST2*	fp: CCCCCTCTCTTTTGCACTTCTTTCC rp: TGCTCCAGCAGTACCATCTAACCC	423	[10]
EIEC	Invasive *meinv*	fp: TGGAAAAACTCAGTGCCTCTGCGG rp: TTCTGATGCCTGATGGACCAGGAG	140	[10]
EAEC	*aat secretion* CVD432	fp: AGACTCTGGCGAAAGACTGTATC rp: ATGGCTGTCTGTAATAGATGAGAAC	194	[10]

Table 2. Sequences for PAIs PCR primers and their respective product size

PAIs	Marker Gene(s)	Oligonucleotide Sequence (5'-3')	Product size bp	Reference
She	*Pic*	fp: ATTCTTCTGGCTGGCATTCC rp: CGGGATTAGAGACTATTGTTGC	606	[11]
HPI	*Irp2* (P242)	fp: AAGGATTCGCTGTTACCGGAC rp: TCGTCGGGCAGCGTTTCTTCT	287	[12]
	FyuA	fp: GCGACGGGAAGCGATGAC rp: CGCAGTAGGCACGATGTTGTA	774	[12]
LEE	*Eae* (Attaching and effacing)	fp: GACCCGGCACAAGCATAAGC rp: CCACCTGCAGCAACAAGAGG	384	[13]
SHI-2	*iutA* (Aerobactin receptor)	fp: GGC TGG ACA TCA TGG GAA CTG G rp: CGT CGG GAA CGG GTA GAA TCG	301	[13]
Tia	*Tia*	fp: CGGGATCCGATGAGAGCAAAACAGGCTT rp: GGGGTACCGAAATGATAAGTTACCCC	756	[14]
EspC	*Espc*	fp: GCTCAACTAAATATTGATAATGTATG rp: CCCAGCCCCAACCCTGAAAC	453	[15]
O-islands	*efa/lifA* gene	fp: GAACAA A GAA CAT TTT CAC CAG TTC rp: CTTTCAGGTGGGGAACCCGC	742	[16]
	PagC (Z4321)	fp: ATGAGTGGTTCAACACTG rp: CCAACTCCAACAGTAAATCC	521	[16]

Results

Prevalence of diarrhoeagenic E. coli (DEC) pathotypes

Of the 207 *E. coli* isolates used in this study 50.7% had been isolated from male subjects while 49.3% were from female subjects. Overall the most predominant diarrhoeagenic *E. coli* (DEC) pathotypes was EPEC (19.3%) followed by ETEC (7.2%), EAEC (3.86%), STEC (0.96%) and EIEC (0.48%).

Pathogenicity islands detected in DEC

The pathogenicity islands are shown in Table 3. TIA PAI was not detected in any of the DEC pathotypes.

Discussion

Diarrhoeagenic *Escherichia coli* is a potential public health risk in children in developing countries, causing persistent diarrhoea. DEC pathotypes such as EHEC O157: H7 and ETEC have been reported to cause severe forms of diarrhoea in children. In this study EPEC was the predominant DEC pathotype followed by ETEC, EAEC, STEC and EIEC. The study further established that DEC pathotypes carried a number of PAIs with the huge majority of the pathogenic isolates possessing at least two PAIs systems. According to Schmidt and Hensel, genomic plasticity in bacteria is largely enhanced by horizontal gene transfer where large DNA fragments containing virulence-associated genes, or PAIs, can be exchanged between different bacterial species, and their acquisition can generate new pathogenic variants [7].

The majority (71%**)** of the DEC pathotypes possessed at least HPI and SHI-2 PAIs iron-uptake systems, a feature which may contribute to higher pathogenicity and facilitate colonization of the host [17,18,19]. The ability to acquire iron is crucial for bacteria for growth and during infection for their ability to form biofilms, slime-encased colonies of microbes that cause many chronic infections such as those that occur in wounds, on medical devices, and in the lungs of people with cystic fibrosis [20]. Other researchers have also observed the presence of the HPI in genomes of *E. coli* as seen from a study by Xu *et al.* [21] which isolated HPI-harboring *E. coli* strains from patients in all age groups and was most frequently detected in association with diarrhoea in children under 10 years of age. This observation shows HPI could play a major role in the pathogenesis of diarrhoea.

Detection of the *pic* gene in this study significantly shows acquisition of virulence factors, which aid in intestinal colonization of the host and is vital for successful infection; however, the low isolation of the *pic* gene in this study may indicate that the *she* island could be one of but not a great contributor to diarrhoeal pathogenesis in the DEC pathotypes. Presence of the *pic* gene, which is used as a marker for *she* PAI, may indicate conferment of *set1A* and *set1B* encoding the two subunits of shET1 enterotoxin and *sigA* that encodes a cytopathic, autotransported protease. This process may aid in further pathogenesis of diarrhoea to the DEC pathotypes [11,22,].

The *eaeA* gene, which is a marker for LEE PAI, was detected in all EPEC pathotypes and only one of the STEC pathotypes. This is in agreement with findings by Morabito *et al*. [23], who showed that LEE PAI is mainly found in EPEC and STEC pathotypes. The *eaeA* gene, which is necessary for attaching and effacing activity, encodes a 94 to 97-KDa outer membrane protein termed intimin [24]. The LEE PAI plays a major role in the colonization process, particularly in STEC, which is reported to contribute to its higher virulence and development of hemolytic uremic syndrome (HUS) in patients [25].

EspC gene was detected only in the EPEC pathotype. This result concurs with observations by Navarro-García *et al*. [15] who determined that it mainly confers virulence properties to EPEC. The presence of EspC PAI shows significance of acquisition of other virulent PAIs in aiding pathogenesis of diarrhoeal illness.

This study also established that among the STEC strains one isolate contained both *efa/lifA* and *pagC* genes, while another contained the *efa/lifA* gene. *efa/lifA* and *pagC* genes were also detected in the other DEC pathotypes, however, at low rates. Studies by Morabito *et al.* [23] on *efa/lifA* virulence gene show that it is involved in the capability of EHEC to adhere to cells and in the repression of the host lymphocyte activation response of EPEC. *pagC* gene virulence is indicated as a putative adhesion and hemeagglutinin [23,26]. Detection of other virulence markers in the O-island of EHEC apart from LEE and *stx/vt* may help to explain the increase in pathogenicity of EHEC strains. Wickham *et al.* [27] proposed that the additive effect of *efa/lifA* and *pagC* contributes significantly to causing HUS while the *pagC* locus may contribute to pathogenicity in more virulent STEC.

Table3. Pathogenicity islands detected in DEC pathotypes

DEC Pathotypes	**PATHOGENICITY ISLANDS**												
	LEE	**HPI**				**SHI-2**	**O-island**				**EspC**	**she**	**TIA**
	eae	*fyuA & irp2*	*fyua*	*Irp2*	Total HPI	*iutA*	*eEfa/lifa & pagC*	*efa/lifA*	*pagC*	Total O-island	*espC*	*pic*	*tia*
EPEC (n = 40)	40 (100%)	15	2	4	21 (52.5%)	10 (25%)	7	3	2	12 (30%)	8(20%)	2 (5%)	0
ETEC (n = 15)	0	5	1	4	10 (66.7%)	10 (66.7%)	0	3	1	4 (26.7%)	0	1 (6.7%)	0
EAEC (n = 8)	0	4	0	0	4 (50%)	4 (50%)	0	1	2	3 (37.5%)	0	1 (12.5%)	0
STEC (n = 2)	1 (50%)	1	1	0	2 (100%)	2 (100%)	1	1	0	2 (100%)	0	0	0
EIEC (n = 1)	0	1	0	0	1 (100%)	1 (100%)	0	1	0	1 100%)	0	0	0

In this study the *tia* gene was not detected although other investigations show that it aids Enterotoxigenic *Escherichia coli* (ETEC) in adherence and invasion of epithelial cells originating from the human ileum or colon, and also may aid in maximum secretion of the LT enterotoxin. This function may add pathogenicity to the ETEC virulent strains [7,28].

Conclusion and recommendations

Results from this study showed that the DEC pathotypes carried a number of PAIs with the vast majority of the pathogenic isolates possessing at least two PAIs systems. Acquisition of the virulence gene to all the DEC pathotypes through horizontal gene transfer was observed by detection of PAIs documented from *Yersinia spp.* (HPI PAI), *Shigella spp.* (*she* and SHI-2 PAI) and EHEC (O-islands PAI). This feature may contribute to higher pathogenicity. Moreover, the virulence associated traits may also contribute to the persistence in the intestinal micro flora as well as increase in their pathogenic capability in the urinary tract.

Based on the results of this study, the authors make the following recommendations for further studies: 1) to confirm the role of the PAIs in severity of diarrhoeal disease presentation in Kenya; 2) to target the presence of other genes in the PAIs investigated in this study; 3) to investigate other PAIs and genes which can enhance persistence of diarrhoea; and 4) to initiate a correlation study of the different PAIs that exist and their virulence presentation in diarrhoea.

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