Detection of virulence-related genes by multiplex PCR in multidrug-resistant diarrhoeagenic *Escherichia coli* isolates from Kenya and Japan

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SUMMARY

We compared serotypes, drug susceptibility and presence of virulence-related genes in diarrhoeagenic *Escherichia coli* isolates from children <5 years from Kenya ($n=82$) and Japan ($n=47$). Multiplex PCR was used to detect genes coding for enteroaggregative adherence (*aggR*), heat-stable toxin (*st*), heat-labile toxin (*lt*), verotoxin (*vt*), attaching and effacing mechanism (*eaeA*), enteroaggregative *E. coli* heat-stable enterotoxin 1 (*astA*) and enteroinvasive mechanism (*invE*). Kenyan *E. coli* O-serotypes were more diverse than those from Japan (29 *vs.* 12 serotypes) and exhibited high level multidrug resistance to World Health Organization (WHO) recommended antibiotics. Resistance rates to tetracycline, ampicillin and sulphamethoxazole–trimethoprim were 70·7, 65·9 and 68·3% respectively, but resistance to sulphamethoxazole–trimethoprim among the *E. coli* isolates from Japan was low (2·1%). Kenyan isolates harboured virulence-related genes in high frequency (82·9%) compared to those from Japan (25·5%) with *aggR* and *astA* being the most frequently detected genes. The presence of multiple virulence genes was associated with multidrug resistance and this merits further investigation.

INTRODUCTION

Escherichia coli is one of the leading causes of acute diarrhoea in Kenya and other developing countries and may account for up to 80% of enteritis in children <5 years with significant morbidity and mortality [1–3]. Five different pathotypes of diarrhoeagenic *E. coli* are recognized based on their pattern of adherence to tissue culture cells (HEp-2 or HeLa cells), which include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAggEC) and enteroinvasive *E. coli* (EIEC). In most developing countries, EPEC, ETEC and EAggEC are the most common cause of infectious diarrhoea in young children [4].

In Kenya, differentiation of diarrhoeagenic *E. coli* from non-pathogenic normal flora is achieved by identification of the surface O-antigen. However, this method is often inconclusive as many strains do not belong to known O-serotypes and are non-reactive with O-antisera. Furthermore, the O-serotype does not correlate with the presence of virulence genes [5]. *In vitro* assays such as cell culture and cytotoxicity for the identification of virulent strains are expensive, time-consuming and require expertise which may be lacking in developing countries such as Kenya.

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Recently, the health consequences associated with diarrhoeagenic *E. coli* infection has been worsened by the emergence of multidrug-resistant *E. coli* (MDREC). Resistance to antibiotics recommended by the World Health Organization (WHO) as first-line treatment for diarrhoea including ampicillin, tetracycline, sulphamethoxazole–trimethoprim, chloramphenicol and augmentin has increased among diarrhoeagenic *E. coli* [3]. Selective antibiotic pressure associated with inappropriate use of antibiotics and their use in poultry farming is the key factor in the evolution of resistant strain phenotypes in the developing world [6, 7]. However, there have been no reports on the relationship between antibiotic resistance and virulence gene complement among diarrhoeagenic *E. coli* in Kenya.

Several virulence factors have been associated with diarrhoeagenic *E. coli*; these include the heat-labile toxin (LT), heat-stable toxin (ST), verotoxin (VT), attaching and effacing, enteroaggregative and entero-invasive mechanisms [8]. There is good correlation between the ability of a strain to cause diarrhoea and the properties of adherence to cultured cells or to produce attaching and effacing histopathological lesions in these cells [9–11]. These virulence-related genes can now be detected by multiplex PCR assay enabling their rapid identification in a single reaction with high specificity [12]. In this study we investigated the use of a multiplex PCR to detect the presence of seven virulence-related genes in diarrhoeagenic *E. coli* from children in Kenya and Japan.

METHODS

Bacterial strains

A total of 129 *E. coli* isolates from patients with diarrhoea resident in Kenya and Japan were used in the study. Eighty-two isolates were from Nairobi, Kenya and 47 isolates were from Kyorin University Hospital, Japan. All the isolates were recovered from stool specimens of children <5 years of age presenting with diarrhoea in which no other bacterial pathogens were detected. *E. coli* isolates were the predominant organism or grew in pure culture on primary isolation medium and were identified by their reactivity in biochemical tests [13] and API 20 E (bioMérieux, Marcy l'Etoile, France). The serotypes were determined with group O-antisera (Denka Seiken Co. Ltd, Tokyo, Japan). Susceptibility to antimicrobial drugs was performed on Mueller–Hinton agar (Beckton Dickinson, Sparks, MD, USA) and inhibition zone sizes were interpreted according to National Committee for Clinical Laboratory Standards [14]. Antibiotics were obtained from Showa Disk (Nissui, Tokyo, Japan) and the disc strengths were $30 \mu g$ ampicillin, $30 \mu g$ tetracycline, $30 \mu g$ gentamicin, $100 \mu g$ chloramphenicol, $381 \mu g$ sulphamethoxazole/$0.19 \mu g$ trimethoprim, $50 \mu g$ fosfomycin and $30 \mu g$ cefotiam. *E. coli* ATCC 25922 was used for quality control. HEp-2 cell adherence assay was performed as previously described [15].

Detection of virulence-related genes

Overnight broth bacterial suspensions were adjusted to 0·5 Macfarland in sterile deionized water, boiled for 10 min and centrifuged for 5 min at high speed, and $5 \mu l$ of supernatant was used for PCR. Seven sets of primers were used in two multiplex reactions tubes (Table 1). The first multiplex reaction included primers MK-1, MK-5 for the detection of verotoxin (*vt*), ST-1, ST-2 for heat-stable toxin (*st*), LT-1, LT-2 for heat-labile toxin (*lt*) and I-1, I-5 for enteroinvasive mechanism (*invE*) [16, 17]. The second multiplex included sets of primers, aggRks-1, aggRkas-2 for enteroaggregative mechanism (*aggR*) eaek-1, eaek-4 for attaching and effacing mechanism (*eaeA*) and EASTOSD1, EASTOAS2 for *astA* [18, 19]. PCR was performed in a PerkinElmer Gene Amp PCR system 9600-R (Roche Diagnostics GmbH, Mannheim, Germany) in a $50 \mu l$ reaction mixture containing $5 \mu l$ template DNA, $5 \mu l \times 10$ PCR buffer (20 mM Mg^{2+}), $4 \mu l$ of 2·5 mM dNTP mixture (dNTP, dCTP, dTTP, dGTP), $0.3 \mu l$ of each primer and $0.3 \mu l$ (5 U/μl) of ampliTaq (Takara, Shuzo Co. Ltd, Shiga, Japan) adjusted to $50 \mu l$ volume with deionized water. The primer sequences, PCR conditions and the expected amplicon size are shown in Table 1. Strains known to contain *st*, *vt*, *lt*, *eaeA*, *aggR*, *astA* and *invE* genes were used as positive controls.

The PCR products were separated by horizontal mini electrophoresis on 3% agarose gel at 100 V and stained with 1 μg/ml ethidium bromide. Products were sized against a 100-bp ladder marker.

RESULTS

E. coli (EAggEC) was the most frequently isolated pathotype among the isolates from Kenya accounting for 36·6% followed by ETEC (26·8%) (Table 2).

Table 1. *Specific oligonucleotide primers and conditions in multiplex PCR*

Expt	Primers	Locus	Primers sequence	Amplicon size
1*	I-1	*invE*	F: 5′-ATATCTCTATTTCCAATCGCGT-3	382
	I-5		R: 5′-GATGGCGAGAAATTATATCCCG-3′	
	MK-1	*vt*	F: 5′-TTTACGATAGACTTCTCGAC-3′	228
	MK-2		R: 5′-CACATATAAATTATTTCGCTC-3′	
	ST-1	*st*	F: 5′-TTTATTTCTGTATTGTCTTT-3′	171
	ST-2		R: 5′-ATTACAACACAGTTCACG-3′	
	LT-1	*lt*	F: 5′-AGCAGGTTTCCCACCGGATCACCA-3′	132
	LT-2		R: 5′-GTGCTCAGATTCTGGGTCTC-3′	
2†	eaek-1	*eaeA*	F: 5′-GCTTAGTGCTGGTTTAGGAT-3′	489
	eaek-4		R: 5′-CGCTAGAGACTTGCCGCT-3′	
	EASTOSD1	*astA*	F: 5′-GCCATCAACACAGTATATCCG-3′	109
	EASTOAS2		R: 5′-GATGTTTCGGCAGTGAGCGC-3′	
	aggRks-1	*aggR*	F: 5′-GTATACACAAAAGAAGGAAGC-3′	254
	aggRkas-2		R: 5′-ACAGAATCGTCAGCATCAGC-3′	

* PCR conditions; denature at 94 °C for 30 s, annealing at 47 °C for 60 s and extension at 72 °C for 90 s for 25 cycles.
† PCR conditions as above except annealing was carried out at 50 °C.

Table 2. *Frequency of virulence related genes in* E. coli *isolates from Kenya and Japan*

Category	No. of isolates (%)		Detected genes	No. of positive	
	Kenya	Japan		Kenya	Japan
EAggEC	30 (36·6)	8 (17·0)	*aggR*	13	7
			aggR + astA	17	1
ETEC	22 (26·8)	0 (0·0)	*st*	1	0
			st + astA	2	0
			lt	2	0
			lt + astA	2	0
			st + lt	4	0
			st + lt + astA	1	0
			astA	10	0
EPEC	10 (12·2)	3 (6·4)	*eaeA*	5	2
			eaeA + astA	2	1
			eaeA + lt	1	0
			eaeA + st + lt + astA	2	0
EIEC	6 (7·3)	0 (0·0)	*invE*	6	0
EHEC	0 (0·0)	1 (2·1)	*vt*	0	1
Others*	14 (17·1)	35 (74·5)		14	35
Total	82 (100)	47 (100)			

EAggEC, Enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; EIEC, enteroinvasive *E. coli*; EHEC, enterohaemorrhagic *E. coli*.
* No virulence-related genes detected.

Similarly among the isolates from Japan, EAggEC was the most frequent (17·0%) pathotype but unlike the Kenyan isolates neither ETEC nor EIEC was detected among Japanese isolates although one EHEC strain was identified. *E. coli* isolates from Kenya were classified into 29 different O-serotypes compared to only 12 O-serotypes from Japan. However, almost half of the Kenyan isolates failed to react with the O-antisera. The serotypes from Kenya were mostly different from those in Japan but eight serotypes were common to both sets of isolates (Table 3).

Antimicrobial resistance

E. coli isolates from Kenya were more resistant to antimicrobials than those from Japan (Table 4). Over

Table 3. *Presence of virulence related genes and O-serotype distribution of* E. coli *from Kenya and Japan*

	O serotypes	
Category	Kenya	Japan
EAggEC having *aggR*	15, 44, 55, 63, 78, 115, 136, 142, 146, 152, 166, 167, 168, NR	1, 25, 26, 86a
ETEC having enterotoxin-related genes	6, 18, 25, 26, 28ac, 44, 55, 78, 111, 125, 153, 164, 169, NR	—
EPEC having *eaeA*	6, 55, 111, 115, 144, NR	1,
EIEC having *invE*	143, 146, 166, NR	—
EHEC having *vt*	—*	157,
Others†	8, 15, 25, 28ac, 29, 136, 144, 157, NR	1, 6, 8, 15, 18, 25, 128, 146, 153

EAggEC, Enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; EIEC, enteroinvasive *E. coli*; EHEC, enterohaemorrhagic *E. coli*; NR, non-reactive.
* No strains isolated.
† No targeted virulence genes detected.

Table 4. *Drug susceptibility profile of* E. coli *isolates from Kenya and Japan*

Antimicrobial agent	Conc (μg/ml)	Resistant (%)	Intermediately susceptible (%)	Susceptible (%)
Kenya				
Ampicillin	30	54 (65·9)	1 (1·2)	27 (32·9)
Cefotiam	30	3 (3·7)	2 (2·4)	77 (93·9)
Fosfomycin	50	0 (0·0)	3 (3·6)	79 (96·4)
Gentamicin	30	5 (6·1)	1 (1·2)	76 (92·7)
Tetracycline	30	58 (70·7)	17 (20·7)	7 (8·6)
Chloramphenicol	100	18 (22·0)	0 (0·0)	64 (78·0)
Norfloxacin	10	0 (0·0)	0 (0·0)	82 (100)
Sulphamethoxazole–trimethoprim	381; 0·19	56 (68·3)	1 (1·2)	25 (30·5)
Japan				
Ampicillin	30	18 (38·3)	1 (2·1)	28 (59·6)
Cefotiam	30	5 (10·6)	1 (2·1)	41 (87·3)
Fosfomycin	50	2 (4·3)	0 (0·0)	45 (95·7)
Gentamicin	30	0 (0·0)	0 (0·0)	47 (100)
Minocycline	30	5 (10·6)	0 (0·0)	42 (89·4)
Levofloxacin	100	2 (4·3)	0 (0·0)	45 (95·7)
Sulphamethoxazole–trimethoprim	381; 0·19	1 (2·1)	0 (0·0)	46 (97·9)

65 % of *E. coli* isolates from Kenya were resistant to sulphamethoxazole–trimethoprim, ampicillin and tetracycline. Only 8·6 % of the isolates were fully susceptible to tetracycline and 70·7 % were resistant; 68·3 and 65·9 % of the isolates were resistant to sulphamethoxazole–trimethoprim and ampicillin respectively. In contrast, only 2·1 and 38·3 % of Japanese isolates showed resistance to sulphamethoxazole–trimethoprim and ampicillin respectively while resistance to cefotiam (10·6 %) and fosfomycin (4·3 %) was uncommon. No resistance to fosfomycin and norfloxacin was detected among the Kenyan isolates. Only 6·1 % of the latter isolates were resistant to gentamicin, whereas all the *E. coli* isolates from Japan were susceptible to this agent.

Virulence-related genes

Virulence-related genes were detected in over 80 % of the *E. coli* from Kenya compared to only 25·5 % from Japan (Table 2). The most common genes identified among the Kenyan group were *aggR* and *astA*; *eaeA* and *vt* were relatively rare. All EAggEC isolates which were positive for the a*ggR* gene exhibited the classical stacked-brick-like aggregative adherence pattern on Hep-2 cells. Among the ETEC isolates, *st* and *lt* were

the most frequently detected genes while in EPEC and EIEC isolates *eaeA* and *invE* predominated. The *eaeA* gene was present in all EPEC isolates and in half of them it was the sole positive gene in the panel tested. In two isolates, the *eaeA* gene was detected in the presence of *astA* but in another two isolates it was accompanied by *lt*, *st* and *astA* genes. Overall 31/82 (37·8%) Kenyan isolates harboured more than one virulence-related gene compared with only 2/47 (4·2%) from Japan. No virulence-related genes were detected in 74·5% of the Japanese isolates. Seventy per cent of EPEC isolates from Kenya with the *eaeA* gene were resistant to sulphamethoxazole–trimethoprim and ampicillin, and 60% of EAggEC isolates with the *aggR* gene were also multiresistant.

DISCUSSION

EAggEC was the most common pathotype of diarrhoegenic *E. coli* isolates from Kenya (36·6%) and Japan (17%). This is in accord with several studies showing that EAggEC is commonly associated with persistent diarrhoea in children in Kenya [2] and is a major cause of infant morbidity and mortality in developing countries [8]. Similarly EAggEC has a global distribution and is associated with diarrhoea both in young children and adults [20, 21]. The Kenyan isolates exhibited more O-serotypes compared to those from Japan confirming the increased diversity of the former. Although O-serotyping is still widely used in Kenya to indicate *E. coli* pathogenicity, it is inadequate for this purpose as half of the isolates investigated were non-reactive with O-antisera.

 E. coli isolates from Kenya were multidrug resistant (70%) while no significant resistance was found in those from Japan (2·1%). In Kenya, evidence of multidrug resistance among diarrhoeagenic *E. coli* and other Enterobacteriaceae clearly emerged in 1997 [2]. This resistance in the developing countries has been attributed to the indiscriminate use of antibiotics and poor prescription practices [3, 22]. Sulphamethoxazole–trimethoprim is the first-line drug for the empiric treatment of diarrhoea and enteric infections including *E. coli* recommended by the Kenyan Ministry of Health and WHO [23] but the study of Leibovici *et al.* [6] identified the used of this agent to be a key factor in the development of resistance in *E. coli*. Moreover, widespread resistance to sulphamethoxazole has been shown to be an indicator of the presence of class I integrons that confer resistance to

multiple antibiotics [24, 25]. Sulphamethoxazole–trimethoprim is rarely prescribed for infectious diseases in Japan because of its toxicity and the availability of alternative drugs and this may explain the low (2·1%) resistance observed for this agent.

Resistance to tetracycline and ampicillin among the isolates from Kenya was also higher (70·7 and 59·6% respectively) compared with Japan (38·3% to ampicillin). Recent studies have linked drug resistance in enteric bacteria to the widespread use of antibiotics in poultry farming [7, 26]. Tetracycline accounts for 55% of all the antibiotics consumed in poultry farming in Kenya [27] and it is noteworthy that the tetracycline resistance level observed in this study was similar (60–72%) to that reported for *E. coli* isolates from poultry farms in Kenya some years previously [3]. Domestic animals have been suggested as a significant reservoir of MDREC and horizontal transfer of bacterial genetic material from food animals to humans has been implicated as a driver for antimicrobial resistance. The fuelling role of antibiotic use for poultry farming in antibiotic resistance in Kenya cannot, therefore, be underestimated. On the other hand, evolution of microbial populations via exposure to selective forces such as antibiotics has been repeatedly documented among *E. coli* and other Enterobacteriaceae [28–30].

Gentamicin resistance was absent among *E. coli* isolates from Japan compared to 6·1% of Kenyan isolates which were resistant to gentamicin. In Japan, gentamicin is rarely prescribed for gastrointestinal infections but in Kenya it is still widely used for respiratory and other infections. Nevertheless, the great majority of isolates remain fully susceptible to the agent.

There was no difference in susceptibility to fosfomycin and cefotiam between the two groups of isolates. Due to the high cost of third-generation cephalosporins, quinolones and fluoroquinolones, these groups of drugs are not readily available in Kenya and are, therefore, unlikely to be used inappropriately. However, a small number of isolates were resistant to these drugs and most of them possessed virulence-related genes.

Diarrhoeagenic *E. coli* from Kenya harboured virulence-related genes in high frequency compared with those from Japan (82·9% *vs.* 25·5%). The low frequency of these genes among the latter is inconsistent with that reported in Saitama Prefecture in Japan where such genes were found in 17·1% of diarrhoeagenic *E. coli* [31]. *aggR* and *astA* genes were

the most frequently detected among the members of the EAggEC and ETEC isolates. Over half of the EAggEC isolates were positive for *astA* genes, and all contained the *aggR* gene. The aggregative adherence phenotype is encoded by a 60-MDa plasmid which also encodes for the EAggEC heat-stable enterotoxin 1 (EAST 1) [32]. Aggregative adherence fimbrial expression requires two unlinked plasmid regions which are known to be produced in 40% of isolates of this pathotype associated with persistent diarrhoea [11]. However, it is not restricted to this group of *E. coli* alone [33]. Among the ETEC from Kenya, the *astA* gene was detected in over two-thirds and just under half of the isolates, and in the absence of other virulence genes this suggests a pathophysiological significance. In 22·7% of ETEC, the *astA* gene was expressed with other toxins particularly with *lt* and *st*. ETEC strains can express multiple virulence genes including *lt* and *st* [33]. Although the pathogenic role of the *astA* gene is not yet clear, *E. coli* positive for this gene has been implicated in diarrhoea cases and a pathophysiological role is highly suggestive [34]. Unlike the isolates from Kenya, the *astA* gene was not common among *E. coli* isolates from Japan which is consistent with reports elsewhere of a high frequency of this gene among atypical strains of EPEC [35]. All the EPEC strains from Kenya were positive for the *eaeA* gene. In EPEC, *eaeA* encodes for the protein intimin responsible for attaching and effacing which results in colonic lesions leading to diarrhoea. Three EPEC isolates unusually hybridized with LT and/or ST probes, however, their significance was disregarded as a cytotoxicity assay was not performed.

Virulence-related genes were absent from over 70% diarrhoeagenic *E. coli* isolates from Japan, suggesting that other genetic factors were responsible for the pathogenicity of these isolates. Apart from the seven genes screened here, several other virulence mechanisms have been associated with diarrhoeagenic *E. coli* infection [8]. Data on the clinical or immunological factors of the patients were not available so it is difficult to identify specific factors which may have predisposed these patients to infections. *E. coli* isolates from Kenya harboured multiple virulence genes and were associated with a high frequency of multidrug resistance. Studies elsewhere have shown that widespread occurrence of integrons is associated with multidrug resistance especially to ampicillin [25, 36]. Although *E. coli* strains with virulence genes were more resistant to ampicillin than those without the genes, the factors responsible for this association were unclear and merit further investigation. Studies are underway to determine whether these phenomena are exhibited by *E. coli* isolates from healthy individuals.

ACKNOWLEDGEMENTS

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