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MOLECULAR TYPING AND ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF ENTEROPATHOGENIC AND SHIGATOXIN PRODUCING *ESCHERICHIA COLI* ISOLATED FROM FOOD HANDLERS IN THREE AREAS OF KENYA

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ABSTRACT

Objectives: **To determine the aetiology, epidemiology and sanitary factors of carriage of enteropathogenic *Escherichia coli* (EPEC) and Shiga-toxin producing *E. coli* (STEC) in food-handlers working in tourist hotels in three popular tourist destinations in Kenya.**

Design: **Cross sectional laboratory based study.**

Setting: **Three tourist destinations of Nairobi, Malindi and Diani in Kenya.**

Subjects: **Food handlers who were working in hotels frequented by tourists in the three study sites.**

Results: **Overall, during the period of April 2003 to May 2004, a total of 1399 food handlers stool samples were collected and analysed. EPEC expressing the *eaeA* gene and STEC expressing the *stx2* gene were detected in 11/1399 (0.8%) and 2/1399 (0.1%) of the study subjects respectively. The mean age of the subjects from whom EPEC and STEC were isolated was similar (32.6 years) to those from whom no EPEC and STEC were isolated (32.5 years). Prior use of antibiotics, water source and toilet types were not significantly associated with the isolation of EPEC and STEC ($p>0.05$). There were 11 resistance patterns with six isolates (6/13, 46.2%) showing multidrug resistance. High prevalence of resistance was observed to co-trimoxazole (55.6%), chloramphenicol (33.3%), ampicillin (22.2%) and tetracycline (22.2%). High concentrations of antibiotics were required to achieve MIC_{90} for tetracycline, (>64 mg ml^{-1}) and ampicillin (>256 mg ml^{-1}). Cluster analysis of the Pulse Field Gel Electrophoresis profiles revealed that the EPEC and STEC isolates belonged to two main genotypes with 11 distinct DNA fragment profiles.**

Conclusion: **This is the first report in Africa on the isolation of STEC from food handlers working in tourist hotels. These food handlers who carry the STEC and EPEC could potentially infect tourists and other people through food or water contamination in the hotel settings and thus our findings are of great public health importance.**

INTRODUCTION

Infectious bacterial diarrhoea is increasingly common in travellers to tropical and subtropical countries (1) and affects about 40% of people travelling to these countries (2). The incidence of traveller's diarrhoea (TD) in various parts of the world has been reported to vary from 26% to over 50% (3-5). Food-borne illnesses which are commonly under reported (6-7) are the major causes of TD with about 76 million cases of illness and 325,000 hospitalisations reported annually in the United States alone (8). The bacterial pathogens causing these infections have been found to be multi-drug resistant especially those isolated from tourists (3). The possibility of hotel workers harbouring multi-drug resistant bacterial pathogens has not been investigated in Kenya. The present study was designed to investigate the aetiology and epidemiology of

enteropathogenic *Escherichia coli* (EPEC) and shiga-toxin-producing *Escherichia coli* (STEC) isolated from food handlers working in tourist hotels in three popular tourist destinations in Kenya.

MATERIALS AND METHODS

Study sites: The study was carried out in Nairobi, Malindi and Diani, which are some of the main tourist destinations in Kenya. Nairobi with a population of about three million people is the capital city and has the main international airport used for the arrivals of the greater majority of tourists coming to Kenya (9). Malindi is a small, northern coastal town in Kenya with a population of about 53,805 (10) and is one of the major tourist destinations in Kenya. There is a high concentration of tourist hotels within the town and its environs. Tourism is the main economic activity in this town. Diani, with a population of about 50,000 people in the south coast of Kenya has a high concentration of tourist facilities especially along the beach areas of Diani.

Study population: The subjects were all adult food handlers working in tourist hotels in the three study areas of Nairobi, Malindi and Diani. For the purposes of this study, food handlers were defind as any person who was involved in any way with handling, processing and serving of food such as waiters, cooks, chefs, barmen, butchers and delivery people.

Stool samples collection and initial processing: Stool samples were collected in sterile wide mouth containers after obtaining informed written consent from the workers. The study was approved by the scientific steering committee of the Kenya Medical Research Institute (KEMRI) and the National Ethical Review Committee. Socio-economic and demographic data on age, sex, water source, sanitation disposal, and antibiotic use in the preceding two weeks, and residence was also obtained from each individual. The stool samples from workers from Nairobi hotels were transported to the KEMRI, Centre for Microbiology Research laboratory in chilled boxes. These were processed within one hour of collection. For hotels in Malindi and Diani the stool samples were collected in containers as before and transported chilled to the KEMRI laboratories in Malindi and Diani for initial processing.

Microbiological procedures

Isolation and real-time PCR for identification of EPEC and STEC: Stool samples were inoculated onto MacConkey agar and incubated aerobically at 37°C for 18 to 24 hours. Five lactose fermenting colonies were picked at random from the MacConkey plate and inoculated into vials containing trypticase soy broth with 15% glycerol and kept at -80°C until analysed. Other enteropathogens and enteroparasites were identified using standard microbiological methods.

Real-time PCR was done as previously described (11). Briefly, bacteria were grown on Luria-Bertani (LB) agar and suspended at a concentration of approximately $1x10^9$ ml^{-1} in Milli-Q water and boiled at 100°C for five minutes. After centrifugation at 10,000 x g for five minutes, the supernatants were diluted 10-fold with Milli-Q water and applied to the real-time PCR assay. Primers and TaqMan probes sequences to detect STEC and EPEC are shown in Table 1. Isolated STEC and EPEC were serotyped by anti-diarrhoegenic *E. coli* that recognises 50 serotypes (Denka Seiken, Tokyo, Japan).

Table 1
Primer and fluorogenic probe sequences for detection of EPEC and STEC

E. coli strain	Gene	Sequence (5′ - 3′)	
STEC		Forward	TCTCGACTGCAAAGACGTATGTAGA
	stx1	Reverse	TCCTGATGAAATAGTCTGTAATGGAGTAC
		Probe	FAM- TCGCTGAATGTCATTCGCTCTGCAATA- TAMRA
		Forward	ACCCCACCGGGCAGTT
	stx2	Reverse	GGTCAAAACGCGCCTGATA
		Probe	FAM- TTTTGCTGTGGATATACGAGGGCTTGATGT- TAMRA
EPEC		Forward	TGTTGCTTTGTTTAATTCYGATAAGC
	eaeA	Reverse	GGAATCGGAGTATAGTTTACACCAA
		Probe	FAM-AGTCGAATCCTGGTGCGGC-TAMRA

Determination of antibiotic minimum inhibitory concentrations: The minimum inhibitory concentration (MIC) of antibiotics against the test isolates were determined by the agar dilution technique as described by the American Society for Microbiology and revised by the National Committee for Clinical Laboratory Standards (12,13). This was defined as the lowest concentration of the antibiotic to prevent visible growth of the bacteria. Pure antibiotic powders of ampicillin, chloramphenicol, gentamicin, cefuroxime, ciprofloxacin, tetracycline and amoxycillin-clavulanic acid were used to prepare doubling dilutions of the antibiotics in Mueller-Hinton agar (13). The concentrations to be tested were determined by the interpretative breakpoints as provided by NCCLS. *E. coli* ATCC 25922 was used to control for drug potency and growth of bacteria.

Pulsed-field gel electrophoresis (PFGE): PFGE was performed using the contour-clamped homogenous electric field (Chef DRII) apparatus from Bio-Rad Laboratories according to the procedures described earlier (14-15) in one per cent pulsed-field certified agarose in 5x TBE buffer for 40 hours at 200V at a temperature of 14°C with the following modified pulse times: 1-10 seconds for ten hour, 3-28 seconds for ten hour, 3-35 seconds for five hour, and 5-70 seconds for fifteen hour, a gradient of 6.0 V per second and a 120 degrees switch angle. The gels were then stained with ethidium bromide, destained and photographed under UV illumination on a gel documentation system. The DNA size standards used were the bacteriophage lambda ladder ranging from 50 kb to 1,000 kb (BioRad).

RESULTS

Demographic data and other characteristics of the study subjects: The hotels sampled varied from low budget backpackers hotels to deluxe five star hotels. There were a total of 19 hotels sampled from Nairobi, and sixteen from Malindi and seven from Diani respectively. The demographic data and other characteristics of the study subjects are shown in Tables 2 and 3. The study subjects from whom EPEC and STEC were recovered ranged in age from 24 years to 50 years with a mean age of 32.6 and a standard deviation of 17.2 years. This was only slightly different than for the general study population (mean 32.5 years, SD of8.1 and range 15-63 years). These differences were not statistically significant ($p>0.05$). The ratio of men to women mirrored that of the general population. Significantly more loose stools were associated with isolation of EPEC/STEC than with those from whom no pathogen was isolated ($X^2=67.76$, $p<0.001$). The number of subjects who admitted to having used any form of antibiotic in the last 14 days was 2/13 from whom EPEC/STEC were isolated who responded to the question on taking antibiotics. This, however, was not statistically significant ($p>0.1$). Only two subjects responded to the question of sanitation disposal so this question could not be analysed. No enteroparasites were observed from the stool samples of subjects from whom EPEC/STEC were isolated.

Table 2
Age characteristics of the study subjects by study site

Variable	Nairobi	Malindi	Diani	Total
Age range	19-63	15-56	18-55	15-63
Mean age	30.4	20.0	28.7	28.2
SD	10.9	16.0	15.2	13.3
Median age	39.0	33.0	36.0	38.0
IQR	29.0-49.0	25 - 42	27-46	27-49
Age not known	51/ (5.8%)	89 (35.2%)	46 (17.6%)	185 (13.2%)
Males	651 (73.7%)	199 (78.7%)	239 (91.2%)	1089 (78.0%)
Females	230 (26.1%)	54 (21.7)	23 (8.8%)	307 (22.0%)
Total	885*	253	262	1399

SD= Standard deviation

IQR- Inter-quartile range

*4 Sex not indicated

Table 3
Demographic data and other characteristics of the food handlers from whom EPEC and STEC was isolated (n=1399)

Parameter		NPI* (n=1316)	EPEC (n=13)	P-value
	Range	15.0-63.0	24.0-50.0	
Age (years)	Mean	32.5	32.6	
	SD	8.1	17.2	
Sex	Males	1021 (77.6%)	9/13 (69.2%)	X^2= 0.51
	Females	295 (22.4%)	4/13 (30.8%)	p>0.05
Stool Type	Formed	563 (62.4%)	0	
	Loose	82 (9.0%)	10	
	Mucoid	27 (3.0%)		
	Semi-formed	222 (24.7%)	1	X^2= 67.76
	Watery	1 (0.1%)	0	
				p<00.1
	Mucoid/semi-formed	1(0.1%)	0	
	Loose/watery	5 (0.5%)	0	
	Loose/mucoid/blood stained	1 (0.1%)		
	Not indicated	1 (0.1%)	0	
Antibiotic use in	Yes	62/1316 (4.7%)	2/13	X^2= 1.87
the last 14 days	No	1254/1316 (95.3%)	0	
				p>0.05
Water source	Rain water/Borehole	0 (0.0%)	0	
	Tap water	447 (80.8%)	1	X^2=0.43
	Borehole	84 (14.5%)	0	p>0.2
	Well water	5 (0.9%)		
Sanitation disposal	Pit latrine	282 (49.6%)	1	
	Flush toilet	262 (46.1%)	1	X^2-0.006
	Other	25 (4.4%)	0	p>0.2
Parasites		31/1316 (2.4%)	No O/C seen	

Incidence of EPEC and STEC and the respective virulence genes: A total of two STEC and 11 EPEC were isolated and the distribution of these is shown in Table 4. None was isolated in Diani and only one EPEC was isolated in Malindi. The rest were all isolated from Nairobi samples. The incidence of EPEC and STEC combined was 1.4% in Nairobi and 0.4% in Malindi respectively. The overall incidence of EPEC and STEC combined for all the three study sites was found to be 0.9% and as a proportion of all the DEC it was 13/83 (15.7%). The EPEC had the *eaeA* gene while the STEC expressed the *atx2* and *eaeA* genes. One of the STEC was serotyped as 0159, but the other was non-serotypable. Of 11 EPEC, two were 0157 and one was 063. Others were non-serotypable. The two 0157 isolates were from two separate five star hotels in Nairobi.

Table 4
Distribution of EPEC and STEC and the respective toxin genes by study site

Pathogen	Nairobi (n=885)	Malindi (n=253)	Diani (n=262)	Total (n=1399)
STEC (*stx2*)	2	0	0	2
EPEC (*eaeA*)	10	1	0	11
Total	12	1	0	13

Antibiotic resistance profiles of the isolates: Nine of the thirteen isolates were tested by use of the E-test MIC method. Resistance was seen against co-trimoxazole (55.6%), chloramphenicol (33.3%), ampicillin (22.2%) and tetracycline (22.2%). MIC_{90} was elevated above the resistance range for ampicillin, tetracycline and

co-trimoxazole. However, the MIC_{50} and MIC_{90} of the other antibiotics were at low levels (Table 5). There were 11 resistance patterns with MDR being seen in six isolates (6/13, 46.2%). All the resistance types consisted of one isolate for each type. Only two isolates (2/13, 15.4%) were sensitive to all tested antibiotics. The two STEC isolates showed resistance to ampicillin, co-amoxyclav, ciprofloxacin and tetracycline (amp, amc, cip, te) and ampicillin, cefuroxime, cefotaxime (amp, cxm, ctx) respectively.

Clonal relatedness of the isolated EPEC and STEC: The genetic relatedness among the EPEC/STEC isolates from food handlers working in tourist hotels in Kenya was determined by the use of pulsed-field electrophoresis method. The PFGE analysis revealed that eight EPEC and two STEC strains produced 11 distinct DNA fragment profiles. Each PFGE profile displayed several bands from below 50 kb to about 600 kb within the 50 to 1000 kb range (Figure 2). The cluster analysis of the PFGE profiles revealed that the ETEC tested belonged to two major genetic groups (Figure 3). Cluster one comprised seven isolates whereas cluster II consisted of three isolates. Isolates IC 107 and NSC05 did not reveal any bands.

Table 5

MIC resistance range, MIC_{50} and MIC_{90} of the EPEC/STEC isolates

Antibiotic tested	Resistant No. (%)	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
Chloramphenicol	3 33.3	2-12	4	8
Co-amoxyclav	0 0.0	2-16	4	16
Gentamicin	0 0.0	0.5-1	1	1
Co-trimoxazole	5 55.6	0.047≥32	>32	>32
Ciprofloxacin	0 0.0	0.012-0.023	0.023	0.023
Ampicillin	2 22.2	2≥256	6	>256
Cefuroxime	0 0.0	4-8	6	8
Tetracycline	2 22.2	1-32	2	32
Cefotaxime	0 0.0	0.064-0.5	0.125	0.125

Figure 2

PFGE patterns of the EPEC and STEC isolated from the food handlers Lane 1 and Lane 15 are Lambda DNA 50 Kb step molecular weight markers. Lane 2 to lane 14 represent isolates IC107, NSC05, WGC033, UT137, NAS60, WGC016, TB012, IC03107, IC136, NSC36, UT37, NAS36, and MLD014 respectively. Isolates TB012 and MLD014 are EAEC isolates used as controls. All the strains were different except strain numbers IC107 and NSC05 which showed 100% similarity to each other. All the isolates tested above were from Nairobi

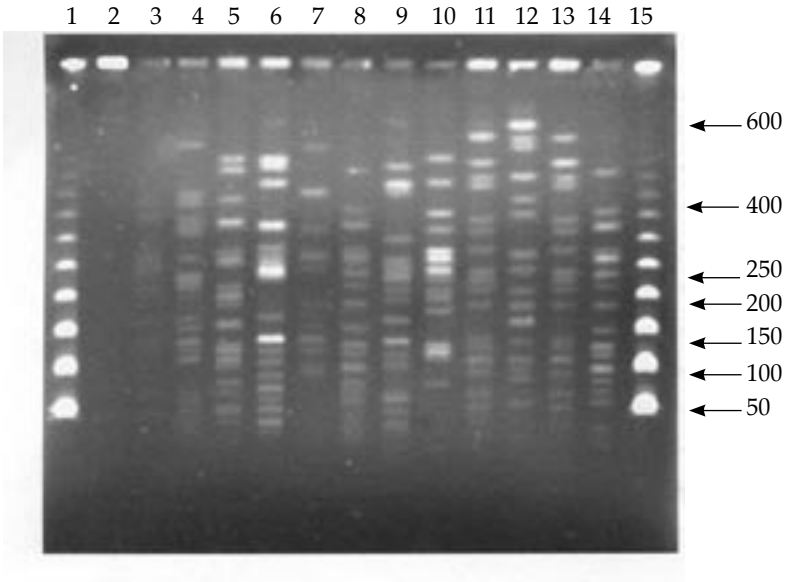
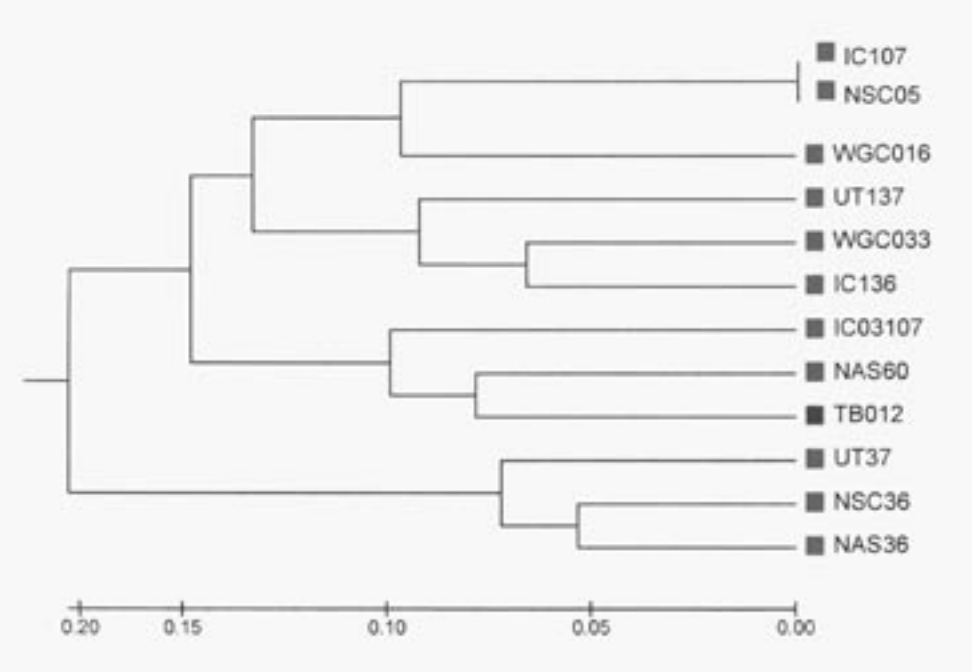


Figure 3
The dendrogram outlining the clonal relationship of the EPEC isolates. The genetic distance between isolates is shown on the scale



There were three sub-clusters in cluster 1 with very close genetic distance from each other. In cluster 2, there were two sub-clusters with one sub-cluster consisting of one isolate (UT37). The second sub-cluster consisted of two isolates, which were closely related with an equal genetic distance (NAS36 and NSC036).

DISCUSSION

STEC is an important cause of gastrointestinal disease in humans and which can result in life-threatening sequelae of haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (16). It has been suggested that STEC strains producing Shiga toxin type 2 encoded by the *stx2* gene is more virulent than *stx1* type and it is more likely to result in HUS disease (16). In our study we obtained from diarrhoegenic stools two STEC strains that contained the *stx2* gene and hence could potentially have resulted in severe disease in the food handlers from whom they were isolated. An incidence of 13/1399 (0.9%) was observed for the EPEC/STEC isolates in this study. Most of the isolates were from loose stools with only one being from semi-formed stool. These results are in agreement with other previous studies where the STEC was either absent (17) or was at much lower proportion than the isolated EPEC (18).

In a study on the epidemiology of infectious diarrhoea in children in Kenya (19) the incidence of EPEC/STEC was found to be 8.8% while in Taiwanese children it was 4% (20). In Nigeria EPEC was isolated from 1.6% of adult controls without diarrhoea and STEC from 20.4% of diarrhoea cases and 6.4% from controls. The isolation of STEC in Kenya is very rare with the first and only report being in 1996 (21). This is the second report of isolation of STEC in Kenya.

STEC produce potent toxins and cause a particularly severe form of disease; haemorrhagic colitis (HC). About 10% of patients with HC can go on to develop HUS, a life-threatening complication of STEC infection that is characterised by acute renal failure, haemolytic anaemia, and thrombocytopenia. These sequelae are particularly serious in young children and older people. On average, 2-7% of patients with HUS die, but in some outbreaks among the elderly, the mortality rate has been as high as 50% (22). The finding of STEC from the stools of food handlers has got major implications for food safety not only in TD but also for the community at large. There is a likelihood of the spread of this very virulent pathogen to food consumers and the general community resulting from contamination of food and water. Currently there are no specific and sensitive biochemical tests that can be used to identify conclusively EPEC and STEC from non-toxigenic strains of *E. coli* except for the non-sorbitol fermentation test for 0157 and serotyping. Rapid identification of EPEC and STEC is important for patient management and for prompt epidemiological investigations. The use of real-time PCR is an improvement in the classical PCR method and has scope for use in clinical microbiological laboratories.

From the antibiotic susceptibility experiments high resistance rates were exhibited against ampicillin 22.2%, tetracycline 22.2%, chloramphenicol 33.3% and co-trimoxazole 55.6%. In other work done in Kenya, Bii *el al,* (23) found resistance against tetracycline of 70.7%, co-trimoxazole of 68.3% and to ampicillin at 65.9%. Similar results were also obtained in studies from West Africa (24) where an increasing resistance to tetracycline from 34.9% to 100%, resistance to sulphonamides from 25.4% to 74.3% and 30% to 78% against ampicillin between 1986 and 1998 was observed. Similarly, in a study of antibiotic resistance of faecal *E. coli* from healthy volunteers from eight developing countries (25), resistance to tetracycline was seen at 92% and to ampicillin of 89%. There were 11 resistance types with MDR being seen in six isolates (6/13, 46.2%). All the resistance types consisted of one isolate each type. Only two isolates (2/13, 15.4%) were sensitive to all tested antibiotics. The high rate of untypability of the isolates might suggest that serotyping is not useful to detect diarrhoegenic *E. coli*. Genetic analysis might be necessary to detect diarrhoegenic *E. coli*. Cluster analysis of the PFGE profiles revealed heterogeneous EPEC and STEC spread in Kenya. This is the first report from sub-Saharan Africa on the isolation of EPEC 0157 and STEC from food handlers working in tourist hotels in Africa.

In resource poor settings especially, it is difficult to routinely test for diarrhoegenic *E. coli* and there is a scarcity of data from this region of the world. The use of basic isolation media, antibiotic susceptibility testing, real-time PCR and PFGE profiling has enabled the establishment of the status of carriage of EPEC and STEC by food handlers in Kenya. It should be emphasised that only a small population of food handlers was studied so the carriage rates may be different in the general population.

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