Original Article

Prevalence and antibiotic resistance of bacterial pathogens isolated from childhood diarrhoea in four provinces of Kenya

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Abstract

Introduction: Diarrhoea is one of the main causes of morbidity and mortality among children in sub-Saharan Africa, and one of the main causes of hospital admissions in rural areas of Kenya. In Kenya, antimicrobial resistance surveillance has been conducted only at the institutional levels, with limited sharing of information and analysis of data. As a result, the actual scale of regional or national antimicrobial drug resistance is not well defined.

Methodology: Stool samples were collected between 1 October 2007 and 30 September 2008 from a total of 651 outpatients with diarrhoea who were under five years of age in four provinces of Kenya. Conventional, biochemical methods, multiplex PCR and antimicrobial susceptibility were conducted to identify the bacterial causes and virulence factors in the isolates, respectively.

Results: Of the 651 patients screened, we identified the causes of 115 cases (17.7%) as follows: *Pathogenic E. coli* (11.2%) [*enteroaggregative* (8.9%), *enterotoxigenic* (1.2%), *enteroinvasive* (0.6%), *shigatoxigenic* (0.5%)], *Salmonella* (3.5%), *Shigella* (2%) and *Vibrio cholera* O1 (0.7%). The highest levels of resistance among the *E. coli* isolates were observed in ampicillin and trimethoprim/sulphamethoxazole each at 95% followed by tetracycline at 81%. *Shigella* isolate levels of resistance ranged from 80% to 100% for ampicillin, tetracycline and trimethoprim/sulphamethoxazole.

Conclusion: The highest prevalence of antimicrobial resistance was to ampicillin followed by trimethoprim/sulphamethoxazole and tetracycline. Though still at low levels, the major concern from our findings is the emerging resistance of enteric pathogens that was observed to quinolones (ciprofloxacin, nalidixic acid, norfloxacin) and gentamycin.

Key words: antimicrobial drug resistance; enteric bacterial pathogens; *E. coli*; *Shigella*; *Salmonella*; *Vibrio cholera*

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Introduction

Diarrhoea is a significant health problem worldwide, especially in the developing world where adequate sanitation facilities are lacking [1]. Globally diarrhoeal diseases account for almost a fifth of all deaths of children below five years of age, with an estimated 2.2 million deaths annually [2]. Epidemiological studies of diarrhoea have been reported from several African countries including South Africa [3], Gabon [4], Egypt [5] and Kenya [6].

In the year 2001, diarrhoea was the most common illness reported by the United States military service members deployed to Africa for strategic training and contingency operations. Out of 15,000 US military personnel who participated, more than 500 service members were affected by acute diarrhoea [7]. The service members represent an immunologically naïve group to the various enteric pathogens and are likely to be at higher risks for contracting acute infectious diarrhoea.

The causes of diarrhoea include a wide array of viruses, parasites and bacteria. *Shigella, Salmonella, Cryptosporidium* species and *Giardia lamblia* are found throughout the world while *Campylobacter jejuni* and cytotoxigenic *Clostridium difficile* are seen with increasing frequency in developed countries [8]. The bacterial pathogen most commonly associated with childhood diarrhoea is *Escherichia coli* and at least six categories have been described: enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroinvasive *E. coli* (EIEC); enterohemorrhagic *E. coli* (EHEC), also known as shigatoxigenic *E. coli* (STEC); diffusely adherent *E. coli* (DAEC); and enteroaggregative *E. coli* (EAEC). The associated clinical pictures comprise childhood

and traveller's diarrhoea (ETEC), bloody diarrhoea and hemolytic uremic syndrome (EHEC), infantile diarrhoea (EPEC), and bacillary dysentery-like diarrhoea (EIEC). Enteroaggregative *E. coli* have been associated with acute and persistent diarrhoea in children and adults in industrial and developing countries in Europe, America, Asia and Africa [9].

New virulent enteric pathogens are emerging throughout the world, Africa included. A multi-drug resistant enteroaggregative *E. coli*, O44, which is associated with acute and persistent diarrhoea, has been reported in Kenyan children [10]. Very recently, *E. coli* O157 was reported for the first time as the etiologic cause of a large dysentery outbreak in Swaziland [11]. Also, during a study on bacterial diarrhoeal diseases involving children below five years of age in Kenya, the KEMRI/JICA Research and Control of Infectious Diarrhea Project (between 1990 and 1995) reported the first confirmed case of hemorrhagic colitis due to *E. coli* serotype O157:H7 in Kenya [12]. This particular isolate produced only vero toxin II (VT2). In the same study, enterotoxigenic *E. coli* (ETEC) strains that elaborated at least one member of two defined groups of enterotoxins, heat-stable (ST) and heat-labile (LT) toxins, were isolated [6].

Antimicrobial resistance surveillance has been conducted only at the institutional levels (*e.g.*, referral and private hospitals), with limited sharing of information and analysis of data. As a result, the actual scale of regional or national antimicrobial drug resistance is not well defined. This study identified the bacterial causes of diarrhoea, the virulence properties associated with pathogenic *E. coli* isolates, and the antimicrobial susceptibility patterns of the enteric pathogens that were associated with diarrhoeal illnesses in children under five years of age from the selected study sites. These strains were tested for susceptibility to commonly used antimicrobials in Kenya for the management of diarrhoeal illness.

Methodology

This protocol for this study was approved by the KEMRI Scientific Steering Committee as well as the National Ethical Review Committee and the International Review Board (IRB) at Walter Reed Army Institute of Research (WRAIR, USA). Upon obtaining informed consent from either parent or guardian, stool samples were collected between 1 October 2007 and 30 September 2008 from a total of 651 outpatients (349 male and 302 female) with diarrhoea who were under five years of age. All the children enrolled in the study were eligible at district, provincial and mission hospitals selected for their geographical diversity namely, Malindi (141), Alupe (174), New Nyanza (134), and Mbagathi (174). Diarrhoea was defined as at least three loose stools in 24 hours, or any number of watery stools. Stool samples were collected on the day of presentation at the outpatient clinics, and were inoculated into Cary-Blair transport Media (MML Diagnostics Inc, Troutdale, Oregon, USA) and transported on ice bags to the laboratory at Kenya Medical Research Institute, Centre for Microbiology Research, and processed within 24 hours. Enteric pathogens were cultured and identified by standard methods [13]. *E. coli* isolates were subjected to multiplex PCR for detection of virulence genes [14]. DNA standards were extracted from bacteria known to contain the relevant genes. Bacteria containing ATCC 35401 (LT/ST), pEWD299 (LT), pDAS100 (STp), pDAS101 (STh), ATCC 43893 (EIEC), ATCC43887 (*BfpA*/EAE), 933J (SLTI), 933W (SLTII), ATCC1175 negative control and pCVD432 (*Eagg*) were obtained from the Armed Forces Research Institute of Medical Sciences in Bangkok. These isolates were grown on MacConkey agar plates to check purity and later cultured on nutrient agar plates for PCR analysis. Antimicrobial susceptibilities of pathogenic *E.coli*, *Shigella* and *Salmonella* were determined by disc diffusion method of Bauer and co-workers [15]. The breakpoints used were those recommended by the NCCLS (National Committee for Clinical Laboratory Standards) on guidelines for susceptibility testing [16]. All these isolates {Pathogenic *E. coli* (73/651), *Salmonella* (23/651), *Shigella* (15/651) and *Vibrio cholera* (4/651)} were tested for resistance to the following antimicrobials: ampicillin, chloramphenicol, ciprofloxacin, tetracycline, trimethoprin/sulphamethoxazole, nalidixic acid, norfloxacin and gentamycin. Standard *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 of known susceptibility were used as control organisms.

Statistical analysis

Statistical analysis was performed for the quantitative study data. Univariate descriptive and exploratory analyses were done by use of proportions. Bivariate analysis was performed using Pearson's chi-square or Fisher exact test to determine the difference in distribution of antimicrobial resistance by geographical region.

Table 1. Regional distribution of enteric pathogens by geographical regions

Isolates	Geographical region	Number of patients	Frequency	% Isolates	*P value
E. coli	New Nyanza	134	14	60.7 (14/23)	0.388
	Mbagathi	174	21	67.7 (21/31)	
	Malindi	141	7	33.4 (7/21)	
	Alupe	202	31	77.5 (31/40)	
Salmonella	New Nyanza	134	4	17.3 (4/23)	0.169
	Mbagathi	174	4	12.1 (4/31)	
	Malindi	141	9	42.9 (9/21)	
	Alupe	202	6	14.5 (6/40)	
Shigella	New Nyanza	134	3	12.9 (3/23)	0.497
	Mbagathi	174	4	12.1 (4/31)	
	Malindi	141	5	23.9 (5/21)	
	Alupe	202	3	7.2 (3/40)	
Vibrio	New Nyanza	134	2	8.7 (2/23)	1.000
	Mbagathi	174	2	6.0 (2/31)	
	Malindi	141	-	-	
	Alupe	202	-	-	

Enteric pathogens from New Nyanza, Mbagathi, Malindi and Alupe District Hospitals * Indicates significance between geographical regions of individual pathogens.

Results

Stool samples from four study sites collected between 1 October 2007 and 30 September 2008 from a total of 651 patients with diarrhoea were analyzed. All patients were under five years of age. The stool samples were analyzed by conventional biochemical methods, antimicrobial susceptibility and multiplex PCR. The results showed that bacterial diarrhoea was present in 115 out of 651 patients (17.7%). Among these isolates, pathogenic *E. coli* comprised 73/651 (11.2%), *Salmonella* 23/651 (3.5%), *Shigella* 15/651 (2.3%) and *Vibrio cholera* 4/651 (0.6%). The multiplex PCR detected ETEC strains producing both LT and ST in 5/8 (62.5%), while those expressing LT alone were 2/8 (25%) and ST alone were 1/8 (12.5%).

The 22 strains which carried Intimin gene (*EAE*) only were grouped as typical EPEC while the 15 strains that harbored *BfpA*, *EAF* and *EAE* genes were grouped as atypical EPEC. Four strains that harbored the invasive gene (*invE*) were grouped as EIEC (Figure 1).

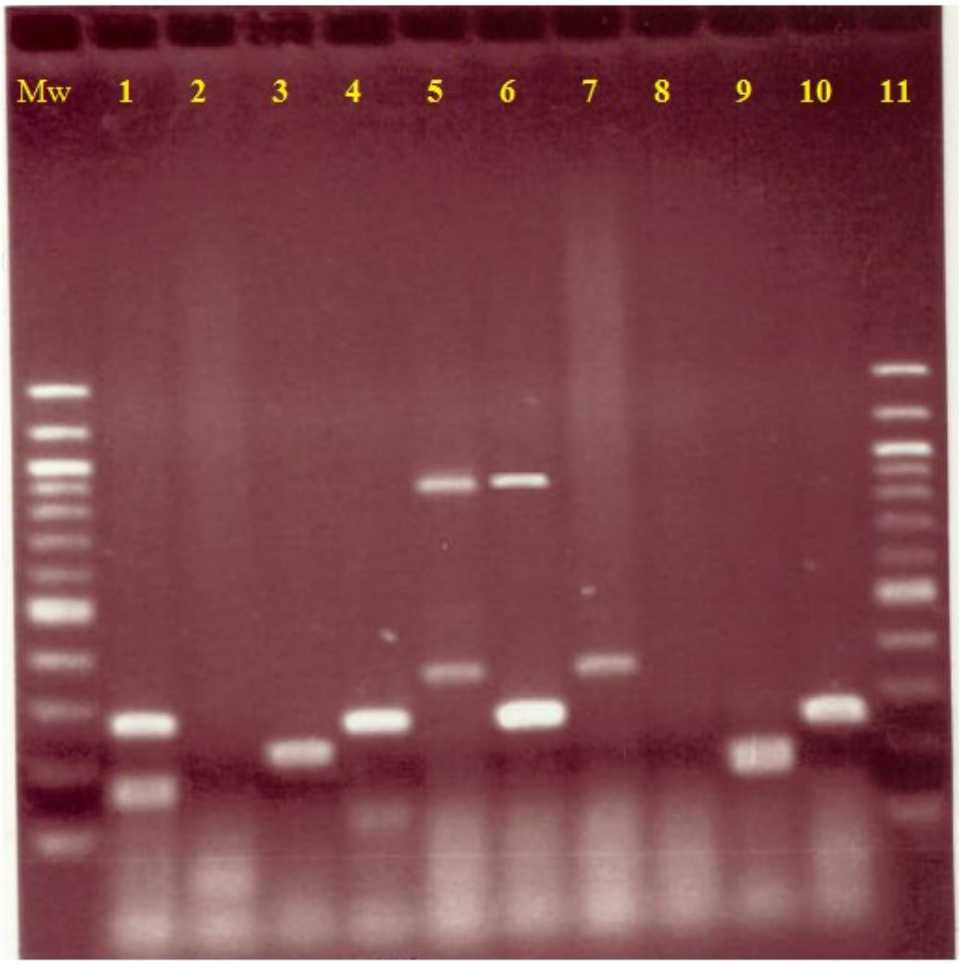
There was no significant difference the in distribution of isolated pathogens or in the antimicrobial resistance by geographical region (as shown in Table 1).

All 73 *E. coli* isolates (58 *enteroaggregative*, 8 *enterotoxigenic*, 4 *enteroinvasive* and 3 *shigatoxigenic*, including 29 *Salmonella;* 15 *Shigella* isolates; and 4 *Vibrio cholera* O1) were tested for susceptibility to the commonly used antimicrobials.

The *E. coli* and *Shigella* isolates had a high prevalence of resistance to commonly used antimicrobials such as tetracycline, ampicillin and trimethoprin/sulphamethoxazole. The results of antimicrobial susceptibility testing of pathogenic *E.coli* revealed that from the four study regions, 63/73 (86%) *E. coli* isolates were resistant to ampicillin, whereas 64/73 (87.6%) were resistant to trimethoprin/sulphamethoxazole and 50/73 (68%) were resistant to tetracycline. Emergence of resistance, though still at low levels, was observed in ciprofloxacin, nalidixic acid, norfloxacin and gentamycin.

Among *Shigella* isolates, high levels of resistance to ampicillin, trimethoprin/sulphamethoxazole and tetracycline were noted in Malindi, New Nyanza, and Mbagathi. However, one strain of *Shigella* from Mbagathi was resistant to ciprofloxacin and nalidixic acid. Among the *Shigella* strains, none was resistant to gentamycin and norfloxacin.

Figure 1. Multiplex PCR amplification of reference strains and isolated pathotypes of diarrheagenic *E. coli*



Lane 1, LT/ST; Lane 2, Negative control; Lane 3, Stx1; Lane 4, Stx2; Lane 5, eaeA/bfpA; Lane 6, Stx2/eaeA; Lane 7, bfpA,; Lane 9, Eagg; Lane 10 EIEC

Salmonella isolate resistance patterns to all tested antimicrobials had lower levels of resistance compared to those of *E. coli* and *Shigella*. Moreover, resistance of *Salmonella* to nalidixic acid was 44% for Malindi. None of the isolates were resistant to ciprofloxacin. It was also noted that there is an emerging resistance of *Salmonella* isolates to gentamycin, norfloxacin and nalidixic acid in Nyanza and Mbagathi, which is not the case with Alupe as indicated. *Vibrio cholera* isolates were 100% resistant to chloramphenicol, nalidixic acid and trimethoprin/sulphamethoxazole. None among these isolates was resistant to ciprofloxacin, gentamycin, norfloxacin and tetracycline. There were two strains, one each from New Nyanza and Mbagathi, which were resistant to ampicillin, as indicated in Table 2.

Discussion

Previous studies in Kenya have documented the prevalence of some traditionally recognized agents of diarrhoea [17-19]. The present study provides results of prevalence and antimicrobial resistance patterns among enteric bacterial pathogens from children younger than five years, from four diverse regions of Kenya. Furthermore, this study highlights pertinent virulence factors associated with pathotypes of *E. coli* such as shigatoxin, heat labile, and heat stable toxins.

Surprisingly, EPEC was the most frequently identified potential pathogen in this study. Among pathogenic *E. coli* isolates (73 in total), 37 were EPEC and 8 were ETEC. These results are not in agreement with previous studies in which ETEC and *Shigella* species were found to be the most common

Table 2. Regional antibiotic resistance patterns of enteric pathogens

		Amp		Chlo		Cip		Gen		Nal		Nor		Sxt		Tet	
Pathogen	**Site**	N	%R	N	%R	N	%R	N	%R	N	%R	N	%R	N	%R	N	%R
Pathogenic *E. coli*	Malindi (7)	6	85.7	1	14.3	0		0		1	14.3	0		6	85.7	4	57.1
	New Nyanza (14)	13	92.9	5	35.7	0		0		1	7.1	0		13	92.9	10	71.4
	Alupe (31)	24	77.4	10	32.3	1	3.2	2	6.4	1	3.2	1	3.2	25	80.6	19	61.3
	Mbagathi (21)	20	95.2	7	33.3	0		0		3	14.3	0		20	95.2	17	81.0
Salmonella species	Malindi (9)	4	44.4	2	22.2	0		0		4	44.4	0		4	44.4	6	66.7
	New Nyanza (4)	3	75.0	2	50.0	0		1	25.0	1	25.0	1	25.0	3	75.0	2	50.0
	Alupe (6)	3	50.0	2	33.3	0		0		0		0		2	33.3	2	33.3
	Mbagathi (4)	3	75.0	2	50.0	0		1	25.0	1	25.0	1	25.0	3	75.0	2	50.0
Shigella species	Malindi (5)	4	80.0	2	40.0	0		0		0		0		4	80.0	4	80.0
	New Nyanza (3)	1	33.3	1	33.3	0		0		0		0		3	100.0	2	66.7
	Alupe (3)	1	33.3	0		0		0		0		0		0		0	
	Mbagathi (4)	3	75.0	2	50.0	1	25.0	0		1	25.0	0		3	75.0	3	75.0
Vibrio	New Nyanza (2)	1	50.0	2	100.0	0		0		2	100.0	0		2	100.0	0	
	Mbagathi (2)	1	50.0	2	100.0	0		0		2	100.0	0		2	100.0	0	

bacterial pathogens in childhood diarrhoea [18]. However, ETEC was more prevalent 9/14 in New Nyanza region.

That no recognized pathogen was identified from the larger portion of diarrhoea cases (a total of 536/651; 82%) raises a number of issues. First, many different organisms may cause diarrhoea and it is not possible in a study such as this to screen specimens for the entire range of potential enteropathogens such as *Yersinia enterocolitica*, *Aeromonas hydrophila*, *Entamoeba histolytica*, *Giardia lambia*, *Trichomonas hominis*, *Trichuris trichiura*, *Cryptosporidium species* and rotavirus. The study investigated the prevalence and drug susceptibility of enteric bacterial pathogens.

The widespread use of antimicrobial agents in the treatment of infections in the tropics has led to serious problems of antimicrobial resistance. The emergence and spread of antimicrobial resistance in bacteria of medical importance imposes serious constraints on the options available for treatment of many infections, and this raises a concern among general practitioners and pediatricians in developing countries [20].

The resistance of enteric pathogens to currently used antimicrobial agents has increased the world over as a result of the widespread use of antimicrobials. There are several reports on multiple antimicrobial resistance among strains of pathogenic *E. coli* in Kenya [10,21,22]. All *E. coli* isolates from this study displayed resistance to one or more antimicrobials including gentamicin, ampicillin, chloramphenicol, tetracycline and trimethoprim/sulphamethoxazole.

The high levels of antimicrobial resistance among *Shigella* isolates have been observed in previous studies in Kenya [23,24]. Although all isolates were susceptible to nalidixic and ciprofloxacin, chromosomal resistance to these agents is easily transferred [24,25]. Experience in other parts of the world has confirmed that resistance to these agents arises rapidly when selective pressure is exerted

through intensive use of quinolones and flouroquinolones. However, this study points out a rising prevalence of antimicrobial resistance among enteric pathogens in the four regions of Kenya where we noted, though at low levels, the emergence of *E. coli* resistance to nalidixic acid, norfloxacin and ciprofloxacin. This has not been the case in the previous studies in Kenya [22,26,27].

Evidence from studies in other countries demonstrates a high prevalence of multiple antimicrobial resistance in normal bowel flora, which suggests that they may act as a reservoir for resistance available to enteric pathogens. A study of commensal gut flora of children in Sudan found that 39% of children had strains resistant to six antimicrobials and over 70% of the children had strains resistant to at least 4 out of 6 antimicrobials commonly used in the country [28].

In Kigali, Rwanda, resistance of *Shigella* species to nalidixic acid emerged in 1984 [29]. One *Shigella dysenteriae* strain from this study showed resistance to nalidixic acid and also ciprofloxacin.

The *Shigella*, *Salmonella* and *Vibrio cholera* isolates in this study revealed high levels of multidrug antimicrobial resistance to the antimicrobials most frequently used to treat diarrhoeal illnesses in Kenya, such as ampicillin, trimethoprim/sulphamethoxazole and chloramphenicol.

Our findings show that a high percentage of cases of diarrhoea is caused by antimicrobial-resistant bacteria, thus illustrating the effect of long-standing unregulated antimicrobial use. Most enteric pathogens easily share genes for antimicrobial resistance, and the continuous selective pressure applied by the over-the-counter availability of these agents, as well as the prescription of those agents at most clinic visits, has potentially lethal consequences for a region plagued by epidemics of *Shigella dysenteriae 1* (Sd1) and cholera. Judicious use of antimicrobial therapy requires education of health workers and patients, adequate laboratory diagnostic capabilities, and government regulations. Antimicrobial susceptibilities must be monitored, to effectively treat pathogens such as *Sd1* and *Vibrio cholera* 01. Finally, emphasis should be placed on primary preventive measures such as ensuring sewerage management and safe drinking water in Kenya. The long-term benefits of such investment in infrastructure are highlighted by the failure of antimicrobial therapy to effectively treat diarrhoeal diseases in the developing world. Overall, there is no major geographical distribution of enteric pathogens as well as antimicrobial resistance in these areas.

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