Role of healthy human gut microbiota in the emergence and dissemination of extended-spectrum β-lactamase-producing enterobacteriaceae and genes associated with β-lactam resistance in community settings in Abidjan, Côte d'Ivoire

ABSTRACT

Overuse of β-lactam antibiotics in communities in developing countries has transformed healthy human intestinal flora into a reservoir of antibiotic-resistant organisms. The prevalence of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae in community settings remains undetermined. In order to obtain data on ESBL enterobacteria, 265 stool samples were collected from August 2019 to February 2020 from individuals residing in the urban districts of Abidjan and attending medical consultations at the Institut Pasteur de Côte d'Ivoire. Isolates belonging to family Enterobacteriaceae were isolated on MacConkey and identified using the API 20E galerie and antibiotic susceptibility was determined using Clinical Laboratory Standard Institute disc diffusion method. Detection of extended spectrum β-lactamases (TEM, SHV, GES, PER, VEB, CTXM 1, CTXM 2, CTXM 8 and CTXM 9) was done by simplex and multiplex PCR. The human stools strains consisted of 513 species of Enterobacteria multidrug resistants. Among the 513 strains, 75 (14.6%) of the enterobacterial strains produced ESBLs, while 438 (85.4%) produced high-level cephalosporinases. Enterobacteria producing extended-spectrum β-lactamase we dominated by the species *Escherichia coli* (46.7%), *Klebsiella pneumoniae* (17.3%), *Enterobacter cloacae* (13.3%), *Enterobacter aerogenes* (6.7%), *Proteus mirabilis* (6.7%), *Klebsiella oxytoca* (4%), *Proteus vulgaris* (2.7%), *Citrobacter koseri* (1.3%), and *Citrobacter freundii* (1.3%). Strains were resistant (100%) to antibiotics from beta-lactam family (penicillin with inhibitor, monobactam, cephalosporin) but low level resistant (1,3%) was observed to carbapenem (imipénème, méropénème, Ertapenem). The rate of resistance to quinolones and aminoglycosides were respectively betwenn 22.9% - 43.3% and 7.9-35.1%. The resistance genes TEM, SHV, CTXM 1, CTXM 2, CTXM 8 and CTXM 9 were detected. No GES and PER genes were not detected. The high fecal carriage rate of ESBL-PE associated with genes in community settings of Ivory Coast highlights the risk for transmission and dissemination because healthy people are potential patients on borrowed time.

Keywords: Enterobacteriaceae ESBL, genes, Fecal carriage, Ivory Coast

1. INTRODUCTION

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Enterobacteriaceae are a group of Gram-negative, rod-shaped facultative anaerobe, and their natural host is the human and animal intestine [1, 2]. Enterobacterial are commensal bacteria present in the intestinal tract of humans and various animals, are an important reservoir of resistance genes, leading to Extended-Spectrum β-Lactamase-Producing Enterobacterial (ESBL-PE) dissemination in communities [3]. Use of antibiotics plays a crucial role in the emergence of antibiotic resistance amongst pathogenic bacteria worldwide as well as in

developing countries [3–5]. Inappropriate use of antimicrobials is considered to be one of the main factors responsible for the high prevalence of antibiotic-resistant pathogens in developing countries [5]. Colonization of the gastrointestinal tract plays a key role in the epidemiology and clinical significance of extended spectrum beta-lactamase (ESBL) producing bacteria [6]. ESBL-PE have spread worldwide and have become endemic in several countries since their first description in 1983 [7, 8]. Their diffusion is mainly attributed to ESBL encoding genes that are often carried by mobile genetic elements, such as plasmids, that facilitate their dissemination [9].

Fecal ESBL-producing Enterobacteriaceae in the community was first reported in Spain and Poland in 2001 and 2002, respectively [10]. Extended-spectrum beta-lactamase-producing Enterobacteriaceae have worldwide distributions with varying degrees of prevalence in the community and hospitals [10, 11]. In the community of developing countries, many people use antibiotic without prescriptions from a doctor and about a quarter obtain antibiotics from an informal dispenser [12, 13]. High prevalence of ESBL-producing bacteria has been reported worldwide [14–16]. While there are a number of publications on ESBL-producing bacteria causing clinical infections [17–20,25, 26], relatively few studies from the African continent report on carriage of ESBL-producing organisms [21–24]. While a better understanding of the impact on faecal carriage of ESBL-producing bacteria on subsequent development of infection is needed, carriage is a potential risk for transmission and infection [12–14], and of particularly concern in healthcare settings, especially in developing countries where infection control is often inadequate. Little is known about faecal carriage of ESBLs and antibiotic resistance in Ivory Coast. The aim of this study was to investigate the prevalence of faecal carriage of ESBL-producing Enterobacteriaceae and their gene in Abidjan, Ivory Coast.

2. MATERIAL AND METHODS

2.1. Period and area of stools collection

This study was carried out from August 2019 to February 2020 in Abidjan (Ivory Coast). 265 stools freshly emitted by heathly humain were obtained from the clinical bacteriology unit (CBU) of the Institut Pasteur of Côte d'Ivoire. These stools were collected in sterile jars containing saline solution. Inclusion criteria of stool samples in this study was stools must come from people who have not been hospitalized and who have not received antibiotic treatment in the last three months.

2.2. Conservation of samples in the laboratory

When the stool samples were not processed on the same day, they were stored at a temperature of +4°C for storage for less than 24-hours and at -20°C for storage for more than 24-hours.

2.3 Isolation and Identification of ESBL Enterobacteria Strains

All ESBL producing enterobacteria strains were isolated on MacConkey (Oxoîd, United Kindom) supplemented with 4 mg/ml of ceftazidime [27] and were identified using the API 20E galerie (bioMérieux, Marcy l'Etoile, France). Isolation and Identification of ESBL Enterobacteria Strains was done in the laboratory of clinical bacteriology unit (CBU).

2.3 Antibiotic Susceptibility Testing

Antibiotic Susceptibility Testing was done in the National Reference Center for Antibiotics of the Institut Pasteur of Côte d'Ivoire. The antimicrobial susceptibility of the extended spectrum enterobacteria β-lactamase isolates was determined by the Bauer-Kirby disk diffusion test using antibiotic disks (Bio-Rad, France) [28]. The double synergy test was used for detection of ESBL-producing strains. The disks of cefotaxime (30 µg), ceftazidime (30 µg), céfépime (30 µg) and ceftriaxone (30 µg) were placed around an amoxicillin/clavulanic acid disk (10/20 µg) on Mueller Hinton agar (BioMérieux, France). The distance between the discs, center to center was 20 mm. This test was performed when the strain was categorized resistant to third generation cephalosporins. Of these, sixteen antimicrobial agents from six antibiotic families (β-lactams, quinolones, aminoglycosides, cyclins, polymixin and sulfamid) were tested. Clinical Laboratory Standards Institute (CLSI) guidelines were followed for inoculum standardization, medium and incubation conditions, and internal quality control organisms (E. coli ATCC 25922). Isolates were screened for the ESBL-producing phenotype by the standard double-disc synergy test, as described previously [29]. Antimicrobial discs (concentration of antibacterial in µg) used were amoxycillin/clavulanic acid (10/20), ceftazidime (30), ceftriaxone (30), cefotaxime (30), cefepime (30), cefoxitin (30), imipenem (10), ertapenam (30), aztreonam (30), nalidixic acid (30), ciprofloxacin (5), pefloxacin (5), amikacin (30), gentamycin (15) and tobramycin (10). All the antibiotics were procured from Bio-rad (France).

2.4 PCR Amplification of Beta-lactamase Genes

Plasmid DNA was used for detection of βeta-lactamases and was extracted using Mini prep K0502 kit (Fermentas, Vilnius, Lithuania). The ESBL gene was characterized by polymerase chain reaction as described by [25]. PCR amplification was performed in a final reaction volume of 50 µl. Primers used in this study are given in Table 1. The reaction mixture contained a PCR Reaction Buffer, 10x concentrated with 20 mM $MgCl_2$, PCR Grade Nucleotide Mix (2.5 mM each), specific primers for each target (20 pmol) and a FastStart Taq DNA Polymerase, 5 U/µl (Roche). The PCR conditions were carried out in a thermalcycler UNOII (BIOMETRA®). Amplification products were analyzed by electrophoresis in a 2% agarose gel (Invitrogen) stained with syber green and visualized with GELDOC logiciel. The cycling conditions for amplification were as follows: for blaTEM, initial denaturation at 94°C for 1 min and 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, followed by 7 min at 72°C; for blaSHV, PER, VEB, GES et CTXM gene, initial denaturation of 1 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by 7 min at 72°C.

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Table 1. Primers used in the study

Genes bla	Primers	Sequence (5'->3')	Position	PCR product size (pb)	Accession number
TEM	a216 (+)	ATAAAATTCTTGAAGACGAAA	1-21	1079	AB282997
	a217 (-)	GACAGTTACCAATGCTTAATCA	1080-1059		
SHV	os-5 (+)	TTATCTCCCTGTTAGCCACC	23-42	795	X98098
	os-6 (-)	GATTTGCTGATTTCGCTCGG	818-799		
PER	per (+)	CCTGACGATCTGGAACCTTT	465-485	716	721957
	per (-)	GCAACCTGCGCAAT(GA)ATAGC	1181-1161		
VEB	veb (+)	ATTTCCCGATGCAAAGCGT	351-370	542	AF010416
	veb (-)	TTATTCCGGAAGTCCCTGT	893-875		
GES	ges (+)	ATGCGCTTCATTCACGCAC	1332-1350	863	AF156486
	ges (-)	CTATTTGTCCGTGCTCAGGA	2195-2176		
CTXM-1	ctxM1(+)	GGTTAAAAAATCACTGCGTC	65-84	863	X92506
	ctxM1(-)	TTGGTGACGATTTTAGCCGC	928-909		
CTXM-2	ctxM2(+)	ATGATGACTCAGAGCATTCG	6-25	865	X92507
	ctxM2(-)	TGGGTTACGATTTTCGCCGC	871-852		
CTXM-8	CtxM8(+)	GCGGCGCTGGAGAAAAGCAG	712-731	608	AF189721
	CtxM8(-)	GCTGCCGGTTTTATCCCGA	6336-6355		
CTXM-9	ctxM9(+)	ATGGTGACAAAGAGAGTGCA	6336-6355	869	AF174129
	ctxM9(-)	CCCTTCGGCGATGATTCTC	7205-7187		

3. RESULTS AND DISCUSSION

Antimicrobial resistance in commensal flora is a serious threat because a very highly populated ecosystem, such as the gut, may become a source of additional intestinal infections at a later stage. These infections may subsequently spread to other hosts or transfer genetic resistance elements to other members of the microbiota including pathogens [29-31]. During the last decade, an alarming worldwide increase in the incidence of community acquired infections with pathogens resistant to multiple antibiotics of common use has been observed [28].

To the best of our knowledge, this is the frst study to document the prevalence and risk factors for faecal carriage of ESBL-EP in Abidjan, Ivory Coast. In this study, The human stools strains consisted of 513 species of Enterobacteria multidrug resistants. Among the 513 strains, 438 (85.4-%) were resistant to third-generation of cephalosporins and 75 (14.6-%) strains of enterobacteria were ESBL. Among 75 ESBL enterobacterial strains, 35 (46.7%) *Escherichia coli*, 13 (17.3%) *Klebsiella pneumoniae*, 10 (13.3%) *Enterobacter cloacae*, 5 (6.7%) *Enterobacter aerogenes*, 5 (6.7%) *Proteus mirabilis*, 4 (4%) *Klebsiella oxytoca*, 2 (2,7%) *Proteus vulgaris*, 1 (1.3%) *Citrobacter koseri* and 1 (1.3%) *Citrobacter freundii* (Table 2). The overall prevalence of ESBL-producing Enterobacteriaceae group of bacteria was 14.6-%, which was concordant with a report in France (17.7%) [49], Mozambique University (20%) [60], and Norway (15.8%) [57]. However, it was lower than a report in Addis Ababa (52%) [58], Egypt (65%) [59], Morocco (42.8%) [61], Tanzania (34.3%) [50], Beirut (24.5%) [51], Southeast Asia (50.7%) [56], Venezuela (34.6%) [52], Turkey (30%) [53], Sweden (35%) [54], and Korea (28%) [55]. The common species were *Escherichia coli* (46,7%), *Klebsiella pneumoniae* (17,3%), *Enterobacter cloacae* (13,3%) and to a lesser extent *Enterobacter aerogenes* (6,7%), *Proteus mirabilis* (6,7%), *Klebsiella oxytoca* (4%), *Proteus vulgaris* (2,7%), *Citrobacter koseri* (1,3%) and *Citrobacter freundii* (1,3%). Several studies have addressed the prevalence of resistant *Escherichia coli* and the genus *Klebsiella* spp isolated from the stools of children [32–47]. However, a study on high prevalence of faecal carriage of ESBL Producing Enterobacteriaceae among children in Dar es Salaam, Tanzania showed a rate of 48,9-% *Klebsiella pneumoniae*, 45,4-% *Escherichia coli*, 3,9-% *Enterobacter cloacae*, 0,7 % *Klebsiella oxytoca* and *Citrobacter spp*, 0,4 % *Proteus mirabilis* [48]. This variation may be due to the difference in the study population and geographical location.

Comment [PM2]: Add table

Table 2. Diversity of ESBL strain isolated

ESBL species	Number of strains tested (N=75)	Rates of identification (%)
Escherichia coli	35	46.7
Klebsiella pneumoniae	13	17.3
Enterobacter cloacae	10	13.3
Enterobacter aerogenes	05	6.7
Proteus mirabilis	05	6.7
Klebsiella oxytoca	04	4
Proteus vulgaris	02	2.7
Citrobacter koseri	01	1.3
Citrobacter freundii	01	1.3

The average levels of resistance to second generation of cephalosporins (FOX), third generation, and fourth generation cephalosporins (CAZ, CRO, FEP, CTX) monobactam (ATM) and penicillin with inhibitor (AMC) for all strains ranged from 99 to 100%. Carbapenems (IPM, MEM and ETP) level of resistance was 1,3-% (Table 3). Hundred percent resistance to ceftazidime and cefotaxime was observed in all ESBL-PE, which is compatible with a study conducted in Madagascar that showed 100% resistance to ceftazidime and cefotaxime [43], Addis Ababa ceftazidime (97%) and cefotaxime (98%) [58], and Turkey cefotaxime (96%) and ceftazidime (94%) [53], but it was higher than a study conducted in Venezuela ceftazidime (46%) and cefotaxime (68.7%) [52], and Guinea-Bissau ceftazidime (66%) and cefotaxime (65%) [62]. During the study, we did not find any resistance to carbapenem (Imipenem, meropenem and Ertapenem).

Comment [PM3]: Must be improve. Add table

Table 3. Enterobacteria ESBL resistance rates to bêta-lactamine

ESBL species	Number of strains tested (N=75)	Rates (%)
Amoxicilline + acide clavulanique	75	100
Ceftazidime (CAZ)	75	100
Ceftriaxone (CRO)	75	100
Cefepime (FEP)	75	100
Aztreonam (ATM)	75	100
Cefotaxime (CTX)	75	100
Cefoxitine (FOX)	66	87
Imipenème (IPM)	0	0
Meropenème (MEM)	0	0
Ertapeneme (ETP)	0	0

Apart from beta lactams, The average levels of resistance for some strains to quinolones Nalidixic Acid (NA), Ciprofloxacin (CIP) and Pefloxacin (PEF) were respectively 43,3% ; 31,2% and 22,9% (Table 4). This rate were lower than rates observed in the study on Prevalence and risk factors for faecal carriage of multidrug resistant Escherichia coli among slaughterhouse workers where the rates of Ciprofloxacin and Nalidixic Acid were respectively 52–% and 75% [63]. Another study on *Escherichia coli* and *Klebsiella pneumoniae* isoled from community showed respectively a rate of ciprofloxacin (25% and 78%) [64].

Table 4. Enterobacteria ESBL resistance rates to quinolones

ESBL species	Number of strains tested (N=75)	Rates (%)
Acide nalidixique (NA)	33	43.3
Ciprofloxacine (CIP)	24	31.2
Pefloxacine (PEF)	18	22.9

In our study, the rates of aminoglycosides were Gentamicin (35,1%), Tobramicin (26%), Kanamicin (27,8%) and Amikacin (7,9%) (Table 5). Some of the earlier studies have reported that a high level rate of resistance to gentamycin (86%), Tobramycin (89%) and amikacin

(2%) [64]. This variation may be due to the difference in the study population, geographical location and the politic of antibiotic consumption.

Table 5. Enterobacteria ESBL resistance rates to aminosides

ESBL species	Number of strains tested (N=75)	Rates (%)
Gentamicine (GMN)	27	35.1
Tobramicine (TMN)	20	26
Kanamicine (KAN)	21	27.8
Amikacine (AKN)	6	7.9

Most of the genes characterized in ESBL enterobacteria were TEM, SHV, CTX M1, CTX M2, CTX M8 and CTX M9. Co-expression of these genes was detected in strains of *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae* and *Enterobacter aerogenes*. However, the PER, VEB and GES genes were not detected in the isolated ESBL strains (Table 6). Some of the earlier studies have reported plausible correlative between phenotypicaly resistance and genes resistance. In our study, sevaral bla genes such as bla CTX-M, blaSHV, blaTEM which confer resistance to bêta-lactamin have been detected. However the specie *Escherichia coli* and the genus *Klebsiella spp* and *Enterobacter spp* were harboring the most of this genus. Therefore, under the pressure of excessive antibiotic use, genes, such as blaCTX-M, spread amongst different bacterial species and strains through horizontal gene transfer and thus contribute to the rapid dispersal of antibiotic resistance in the community [65]. It has been documented that multiple studies reported the high prevalence of CTX-M, blaSHV and blaTEM harboring by *Escherichia coli* isoled from poultry farmers workers [39, 44, 52].

Plasmid mediated resistance to cephalosporins was largely due to blaCTX-M -15 which is in keeping with other studies done in many countries ~~[66, 67, 68]~~. The blaTEM and bla SHV are less incrinated not been subtyped therefore no comment can be made for its corelation with ESBL production. It is interesting to note that blaSHV was not detected. The presence of genes coding for extended spectrum of beta lactamases and plasmid mediated quinolone resistance in commensal E. coli is disconcerting [66~~, 67, 68~~].

Table 6. Distribution of Bla genes harboring by enterobacteria ESBL

Enterobacteria species (number)	Genes bla								
	TEM	SHV	PER	VEB	GES	CTX M1	CTX M2	CTXM8	CTXM9
Escherichia coli (35)	+	+	-	-	-	+	+	+	+
Klebsiella pneumoniae (13)	+	+	-	-	-	+	+	+	+
Enterobacter cloacae (10)	+	+	-	-	-	+	+	+	+
Enterobacter aerogenes (35)	+	+	-	-	-	+	+	+	+
Proteus mirabilis	+	+	-	-	-	+	-	-	-
Klebsiella oxytoca	+	+	-	-	-	+	+	+	+
Proteus vulgaris	+	+	-	-	-	+	-	-	-
Citrobacter koseri	+	+	-	-	-	-	-	-	-
Citrobacter freundii	+	+	-	-	-	-	-	-	-

(+) : gene detected **(-) : gene not detected**

4. CONCLUSION

Comment [PM4]: Can be improve

To our knowledge, this is the first study on the intestinal carriage of ESBL-PE in healthy community volunteers in Ivory Coast, and shows high carriage rate associated with the gene blaCTX-M, blaSHV and blaTEM enzyme. The intestinal carriage of ESBL-PE is a significant challenge for public health, and highlights the urgent necessity to improve sanitation and implement antibiotic stewardship in African countries. Future studies should explore mechanisms involved in plasmid transfer and the determinants of the observed intestinal carriage.

Ethical Approval

This study was approved by the Research and Ethics Committee of Pasteur Institute.

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