Role of healthyhumangutmicrobiota in the emergence and dissemination of extended-spectrum β-lactamase-producingenterobacteriaceae and genesassociatedwith β-lactamresistance in community settings in Abidjan, Côte d'Ivoire

ABSTRACT

Overuse of β-lactamantibiotics in communities in developing countries has transformedhealthyhuman intestinal flora into a reservoir of antibiotic-resistantorganisms. The prevalence of extended-spectrum β-lactamase (ESBL)-producing*Enterobacteriaceae* in community settings remainsundetermined.In order to obtain data on ESBL enterobacteria, 265 stoolsampleswerecollectedfrom August 2019 to February 2020 fromindividualsresiding in the urban districts of Abidjan and attendingmedical consultations at the Institut Pasteur de Côte d'Ivoire. Isolatesbelonging to family*Enterobacteriaceae*wereisolated on MacConkey and identifiedusingthe API 20E galerie and antibioticsusceptibilitywasdeterminedusingClinicalLaboratory Standard Institute disc diffusion method. Detection of extendedspectrum β-lactamases (TEM, SHV, GES, PER, VEB, CTXM 1, CTXM 2, CTXM 8 and CTXM 9) wasdone by simplex and multiplex PCR.The humanstoolsstrainsconsisted of 513 species of Enterobacteriamultidrugresistants. Among the 513 strains, 75 (14.6%) of the enterobacterialstrainsproducedESBLs, while 438 (85.4%)produced high-levelcephalosporinases.Enterobacteriaproducingextended-spectrumβ-lactamase wedominated 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%), *Citrobacterkoseri* (1.3%), and*Citrobacterfreundii*(1.3%).Strainswereresistant (100%) to antibioticsfrombeta-lactamfamily (penicillinwithinhibitor, monobactam, cephalosporin) but lowlevelresistant (1,3%)wasobserved to carbapenem (imipénème, méropénème, Ertapenem). The rate of resistance to quinolones and aminoglycosides wererespectivelybetween 22.9% - 43.3% and 7.9-35.1%. Theresistancegenes TEM,SHV, CTXM 1, CTXM 2, CTXM 8 and CTXM 9 weredetected. No GES and PER geneswerenotdetected.The high fecalcarriage rate of ESBL-PE associatedwithgenes in community settings of Ivory Coast highlights the risk for transmission and disseminationbecausehealthy people are potential patients on borrowed time.

Keywords:*Enterobacteriaceae* ESBL, genes, Fecalcarriage, Ivory Coast

1. INTRODUCTION

Enterobacteriaceae are a group of Gram-negative, rod-shaped facultative anaerobe, and theirnatural host is the human and animal intestine [1, 2].Enterobacterialare commensal bacteriapresent in the intestinal tract of humans and variousanimals, are an important reservoir of resistancegenes, leading to Extended-Spectrum β-Lactamase-ProducingEnterobacterial(ESBL-PE)dissemination in communities[3]. Use of antibioticsplays a crucial role in the emergence of antibioticresistanceamongstpathogenicbacteriaworldwide as

well as in developing countries [3-5].Inappropriate use of antimicrobialsisconsidered to be one of the main factorsresponsible for the high prevalence of antibiotic-resistantpathogens in developing countries [5].Colonization of the gastrointestinal tract plays a key role in the epidemiology and clinicalsignificance of extendedspectrumbeta-lactamase (ESBL)producingbacteria [6].ESBL-PE have spread worldwide and have becomeendemic in several countries sincetheir first description in1983 [7, 8]. Their diffusion ismainlyattributed to ESBL encodinggenesthat are oftencarried by mobile geneticelements, such as plasmids,thatfacilitatetheirdissemination [9].

Fecal ESBL-producingEnterobacteriaceae in the communitywas first reported in Spain and Polandin 2001 and 2002, respectively [10]. Extended-spectrumbeta-lactamase-producingEnterobacteriaceae have worldwide distributions withvaryingdegrees of prevalence in the community and hospitals [10, 11]. In the community of developing countries, many people use antibioticwithoutprescriptions from a doctor and about a quarterobtainantibioticsfrom an informal dispenser [12, 13].High prevalence of ESBL-producingbacteria has been reportedworldwide [14-16]. Whilethere are a number of publications on ESBL-producingbacteriacausingclinical infections [17, 18,21,22], relatively few studiesfrom the African continent report on carriage of ESBL-producingorganisms [19, 20]. While a betterunderstanding of the impact on faecalcarriage of ESBL-producingbacteria on subsequentdevelopment of infection isneeded, carriageis a potentialrisk for transmission and infection [12-14], and of particularlyconcern in healthcaresettings, especially in developing countries where infection control isofteninadequate. Little isknown about faecalcarriage of ESBLs and antibioticresistance in Ivory Coast. The aim of thisstudywas to investigate the prevalence of faecalcarriage of ESBL-producingEnterobacteriaceae andtheirgene in Abidjan, Ivory Coast.

2. MATERIAL AND METHODS

2.1. Period and area of stoolscollection

This studywascarried out fromAugust 2019 to February 2020 in Abidjan (Ivory Coast).265 stoolsfreshlyemitted by heathly humain wereobtainedfrom the clinicalbacteriology unit (CBU) of the Institut Pasteur of Côte d'Ivoire. Thesestoolswerecollected in sterile jars containing saline solution.Inclusioncriteria of stoolsamples in thisstudywasstools must come from people who have not been hospitalized and who have not receivedantibiotictreatment in the last threemonths.

2.2.Conservation of samples in the laboratory

When the stoolsampleswere not processed on the sameday, theywerestored at a temperature of +4°C for storage for lessthan 24h and at -20°C for storage for more than 24h.

2.3 Isolation and Identification of ESBL EnterobacteriaStrains

All ESBL producingenterobacteriastrainswereisolated on MacConkey (Oxoîd, United Kindom) supplementedwith 4 mg/ml of ceftazidime[21] and wereidentifiedusing the API 20E galerie (bioMérieux, Marcy l'Etoile, France). Isolation and Identification of ESBL EnterobacteriaStrainswasdone in the laboratory of clinicalbacteriology unit (CBU).

2.3 AntibioticSusceptibilityTesting

AntibioticSusceptibilityTestingwasdone in the National Reference Center for Antibioticsof the Institut Pasteur of Côte d'Ivoire. The antimicrobialsusceptibility of the extendedspectrumenterobacteria β-lactamase isolateswasdetermined by the Bauer-Kirby disk diffusion test usingantibioticdisks (Bio-Rad, France) [22]. The double synergy test wasused for detection of ESBL-producingstrains. The disks of cefotaxime (30 µg), ceftazidime (30 µg), céfépime (30 µg) and ceftriaxone (30 µg) wereplacedaround an amoxicillin/clavulanicaciddisk (10/20 µg) on Mueller Hinton agar (BioMérieux, France). The distance between the discs, center to center was 20 mm. This test wasperformedwhen the strainwascategorizedresistant to thirdgenerationcephalosporins. Of these, sixteenantimicrobial agents fromsixantibioticfamilies(β-lactams, quinolones,aminoglycosides, cyclins, polymixinand sulfamid) weretested. ClinicalLaboratory Standards Institute (CLSI) guidelines werefollowed for inoculum standardization, medium and incubation conditions, and internalquality control organisms (E. coli ATCC 25922). Isolateswerescreened for the ESBL-producingphenotype by the standard double-disc synergy test, as describedpreviously[23].Antimicrobial discs (concentration of antibacterial in µg) usedwereamoxycillin/clavulanicacid (10/20), ceftazidime (30), ceftriaxone (30), cefotaxime (30), cefepime (30), cefoxitin (30), imipenem (10), ertapenam (30), aztreonam (30), nalidixicacid (30), ciprofloxacin (5), pefloxacin (5), amikacin (30), gentamycin (15) and tobramycin (10). All the antibioticswereprocuredfromBio-rad (France).

2.4 PCR Amplification of Beta-lactamase Genes

Plasmid DNAwasused for detection of βeta-lactamases and wasextractedusing Mini prep K0502 kit (Fermentas, Vilnius, Lithuania). The ESBL genewascharacterized by

polymerasechainreaction as described by [24]. PCR amplification wasperformed in a final reaction volume of 50 µl. Primersused in thisstudy are given in Table 1. The reaction mixture containeda PCR Reaction Buffer, 10x concentratedwith 20 mM $MgCl_2$, PCR Grade Nucleotide Mix (2.5 mM each), specificprimers for eachtarget (20 pmol) and a FastStart Taq DNA Polymerase, 5 U/µl (Roche). The PCR conditions werecarried out in a thermalcycler UNOII (BIOMETRA®). Amplification productswereanalyzed by electrophoresis in a 2% agarose gel (Invitrogen) stainedwithsyber green and visualizedwithGELDOC 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.

Table 1. Primersused in the study

Genesbla	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		

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3. RESULTS AND DISCUSSION

Antimicrobialresistance in commensal flora is a seriousthreatbecause a veryhighlypopulatedecosystem, such as the gut, maybecome a source of additional intestinal infections at a later stage. These infections maysubsequently spread to other hosts or transfergeneticresistanceelements to othermembers of the microbiotaincludingpathogens [25-31].During the last decade, an alarmingworldwideincrease in the incidence ofcommunityacquired infections withpathogensresistant to multiple antibiotics ofcommon use has been observed [28].

To the best of ourknowledge, thisis the frststudy to document the prevalenceand riskfactors for faecalcarriage of ESBL-EP in Abidjan, Ivory Coast. In thisstudy, The humanstoolsstrainsconsisted of 513 species of Enterobacteriamultidrugresistants. Among the 513 strains, 438 (85.4%) wereresistant to third-generation of cephalosporins and 75 (14.6%) strains of enterobacteriawere ESBL. Among 75 ESBL enterobacterialstrains, 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%) *Citrobacterkoseri* and 1 (1.3%) *Citrobacterfreundii* (Table 2).The overallprevalence of ESBL-producingEnterobacteriaceae group of bacteriawas14.6%,whichwas concordant with a report in France (17.7%) [37], Mozambique University (20%) [43], and Norway (15.8%) [42]. However, itwaslowerthan a report in Beirut (24.5%) [38], Southeast Asia (50.7%) [41], Venezuela (34.6%) [38], Turkey (30%) [39], Sweden (35%) [40],. The commonspecieswere*Escherichia coli* (46,7%), *Klebsiella pneumoniae* (17,3%), *Enterobacter cloacae* (13,3%) and to a lesserextent*Enterobacter aerogenes* (6,7%), *Proteus mirabilis* (6,7%), *Klebsiella oxytoca* (4%), *Proteus vulgaris* (2,7%), *Citrobacterkoseri* (1,3%) and*Citrobacterfreundii*(1,3%). Severalstudies have addressed the prevalence of resistant*Escherichia coli*and the genus*Klebsiella* spp isolatedfrom the stools of children [32-35]. However, a study on high prevalence of faecalcarriage of ESBL ProducingEnterobacteriaceaeamongchildren in Dar es Salaam, Tanzaniashowed 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* [36]. This variation maybe due to the difference in the study population and geographical location.

Table 2. Diversity of ESBL strainisolated

ESBL species	Number of strainstested	Rates of

	(N=75)	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
Citrobacterkoseri	01	1.3
Citrobacterfreundii	01	1.3

The averagelevels of resistance to second generation of cephalosporins (FOX), thirdgeneration, and fourthgenerationcephalosporins (CAZ, CRO, FEP, CTX) monobactam (ATM) and penicillinwithinhibitor (AMC) for all strainsrangedfrom 99 to 100%. Carbapenems (IPM, MEM and ETP) level of resistancewas 1,3% (Table 3).Hundred percent resistance to ceftazidime and cefotaximewasobserved in all ESBL-PE, whichis compatible with a studyconducted in Madagascar thatshowed 100% resistance to ceftazidime and cefotaxime [40], Addis Ababaceftazidime (97%) and cefotaxime (98%) [41], and Turkeycefotaxime (96%) and ceftazidime (94%) [39], but itwashigherthan a studyconducted in Venezuela ceftazidime (46%) and cefotaxime (68.7%) [38], and Guinea-Bissauceftazidime (66%) and cefotaxime (65%) [44].During the study, wedid not findanyresistance to carbapenem(Imipenem, meropenem and Ertapenem).

Table 3. Enterobacteria ESBL resistancerates to bêta-lactamine

ESBL species	Number of	Rates

	strainstested(N=75)	(%)
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 averagelevels of resistancefor somestrainsto quinolones nalidixicAcid (NA), Ciprofloxacin (CIP) and Pefloxacin (PEF) wererespectively 43,3% ; 31,2% and 22,9% (Table 4). This rate werelowerthan rates observed in the study on prevalence and riskfactors for faecalcarriage of multidrugresistant*Escherichia coli*amongslaughterhouseworkerswhere the rates of ciprofloxacin and nalidixicacidwererespectively 52% and 75% [45]. Anotherstudy on *Escherichia coli* and *Klebsiella pneumoniae*isoledfromcommunityshowedrespectively a rate of ciprofloxacin (25% and 78%) [46].

Table 4. Enterobacteria ESBL resistancerates to quinolones

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

In ourstudy, the rates of aminoglycosides wereGentamicin (35,1%), Tobramicin (26%), Kanamicin (27,8%) and Amikacin (7,9%) (Table 5).Some of the earlierstudies have reportedthat a high level rate of resistance to gentamycin (86%), Tobramycin (89%) and amikacin (2%) [64]. This variation maybe due to the difference in the study population, geographical locationand the politic of antibioticconsumption.

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Table 5. Enterobacteria ESBL resistancerates to aminosides

ESBL species	Number of strainstested(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 genescharacterized in ESBL enterobacteriawere TEM, SHV, CTX M1, CTX M2, CTX M8 and CTX M9. Co-expression of thesegeneswasdetected in strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*and *Enterobacter aerogenes*. However, the PER, VEB and GES geneswere not detected in the isolated ESBL strains (Table 6). Some of the earlierstudies have reportedplausible correlativebetweenphenotypicalyresistance and genesresistance. In ourstudy, sevaralblagenessuch as bla CTX-M, blaSHV, blaTEMwhich confer resistance to bêta-lactamin have been detected. Howeverthe specie*Escherichiacoli* and the genus*Klebsiellaspp* and *Enterobacterspp*wereharboring the most of thisgenus. Therefore, under the pressure of excessive antibiotic use, genes, such as blaCTX-M, spread amongstdifferentbacterialspecies and strainsthrough horizontal genetransfer and thuscontribute to the rapid dispersal of antibioticresistance in the community [47]. It has been documentedthat multiplestudiesreported the high prevalence of CTX-M, blaSHV and blaTEMharboring by *Escherichia coli*isoledfrompoultryfarmersworkers [38].

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Plasmidmediatedresistance to cephalosporinswaslargely due to blaCTX-M -15 whichis in keepingwithotherstudiesdone in many countries [48,49]. The blaTEM and bla SHVare lessincrinatednot been subtypedtherefore no comment can be made for itscorelationwith ESBL production. It isinteresting to note thatblaSHVwas not detected. The presence of genescoding for extendedspectrum of beta lactamases and plasmidmediated quinolone resistance in commensal *E. coli*isdisconcerting[48,49].

Table 6. Distribution of Blagenesharboring by enterobacteria ESBL

Enterobacteriaspecies	Genesbla								
(number)	TEM	SHV	PER	VEB	GES	CTXM1	CTXM2	CTXM8	CTXM9
Escherichia coli(35)	+	+	-	-	-	+	+	+	+
Klebsiella pneumoniae(13)	+	+	-	-	-	+	+	+	+
Enterobacter cloacae(10)	+	+	-	-	-	+	+	+	+
Enterobacter aerogenes(35)	+	+	-	-	-	+	+	+	+
Proteus mirabilis	+	+	-	-	-	+	-	-	-
Klebsiella oxytoca	+	+	-	-	-	+	+	+	+
Proteus vulgaris	+	+	-	-	-	+	-	-	-
Citrobacterkoseri	+	+	-	-	-	-	-	-	-
Citrobacterfreundii	+	+	-	-	-	-	-	-	-

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

4. CONCLUSION

To ourknowledge, thisis the first study on the intestinal carriage of ESBL-PE in healthycommunityvolunteers in Ivory Coast, and shows high carriage rate associatedwith the geneblaCTX-M, blaSHV and blaTEM enzyme. The intestinal carriage of ESBL-PE is a significantchallenge for public health, and highlights the urgent necessity to improvesanitation and implementantibioticstewardship in African countries. Future studiesshould explore mechanismsinvolved inplasmidtransfer and the determinants of the observed intestinal carriage.

EthicalApproval

This studywasapproved by the Research and EthicsCommittee of Pasteur Institute.

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