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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

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Authors' contributions

This work was carried out in collaboration among all authors. Authors OMB and ASW design the study. Authors OMB, ASW, TAA, GMVC, TKB and KKF took part in the recruitment of participants and samples collection. Authors OMB, KKG and KKI analysed the samples and interpreted the results. Author OMB drafted the manuscript and Authors GKB, DSK, AA, Konate Ali, GNK, Kamenan Alphonse and DM revised it critically. Authors OMB, ASW and AAT revised the advanced manuscript. All authors read and approved the final manuscript.

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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 between 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

"Enterobacteriaceae are a group of Gramnegative, 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 pathogenic bacteria resistance amongst worldwide as well as in developing countries" [3-"Inappropriate use of antimicrobials 51. is considered to be one of the main factors responsible for the high prevalence of antibioticresistant pathogens in developing countries" [5]. "Colonization of the gastrointestinal tract plays a key role in the epidemiology and clinical significance of extended spectrum betalactamase (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 [17,18,19,20], relatively few studies from the African continent report on carriage of ESBL-producing organisms [21,22]. "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, and of particularly concern in healthcare settings, especially in developing countries where infection control is often inadequate" [12-14]. 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. MATERIALS 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 $+4^{\circ}C$ for storage for less than 24h and at - 20°C for storage for more than 24h.

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 [21] 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).

2.4 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)" [22]. "The double synergy test was used for detection ESBL-producing strains. The disks of 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 (B-lactams. quinolones, aminoglycosides, cyclins, polymixin and sulfamid) were tested. Clinical Laboratory Standards Institute (CLSI) guidelines were followed for inoculum medium incubation standardization, and 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" [23]. 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).

2.5 PCR Amplification of Beta-lactamase Genes

Plasmid DNA was used for detection of Betalactamases and was extracted using Mini prep K0502 kit (Fermentas, Vilnius, Lithuania). The ESBL gene was characterized by polymerase chain reaction as described by [24]. 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 MgCl₂, 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 thermalcycler UNOII (BIOMETRA®). а Amplification products were analyzed bv 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 cvcles 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.

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" [25-31]. "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 Abidian, 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 thirdgeneration of cephalosporins and 75 (14.6%) strains of enterobacteria were ESBL. Among 75 ESBL enterobacterial strains, 35 (46.7%) (17.3%) 13 Klebsiella Escherichia coli. 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%) [32], Mozambique University (20%) [33], and Norway (15.8%) [34]. However, it was lower than a report in Beirut (24.5%) [35], Southeast Asia (50.7%) [36], Venezuela (34.6%) [35], Turkey (30%) [37], Sweden (35%) [38]. The common species were Escherichia coli (46,7%), Klebsiella pneumoniae (17,3%), Enterobacter cloacae (13,3%) and to a lesser extent aerogenes (6,7%), Proteus Enterobacter mirabilis (6,7%), Klebsiella oxytoca (4%), Proteus vulgaris (2,7%), Citrobacter koseri (1,3%) and Citrobacter freundii (1,3%). Several studies have prevalence addressed the of resistant Escherichia coli and the genus Klebsiella spp isolated from the stools of children [39-42]. 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 oxvtoca and Citrobacter spp, 0,4% Proteus mirabilis [43]. This variation may be due to the difference in the study population and geographical location.

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 Madagascar that in showed 100% resistance to ceftazidime and cefotaxime [38], Addis Ababa ceftazidime (97%) and

| 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 | | | |

Table 1. Primers used in the study

| 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 | | |

Table 2. Diversity of ESBL strain isolated

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 | |

cefotaxime (98%) [36], and Turkey cefotaxime (96%) and ceftazidime (94%) [37], but it was higher than a study conducted in Venezuela ceftazidime (46%) and cefotaxime (68.7%) [35], and Guinea-Bissau ceftazidime (66%) and cefotaxime (65%) [44]. During the study, we did not find any resistance to carbapenem (Imipenem, meropenem and Ertapenem).

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% [45]. Another study on *Escherichia coli* and *Klebsiella pneumoniae* isoled from community showed respectively a rate of ciprofloxacin (25% and 78%) [46].

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

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 Klebsiella Escherichia coli, 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" [47]. 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 [35].

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

Table 4. Enterobacteria ESBL resistance rates to quinolones

| 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 | | |

Table 6. Distribution of Bla genes harboring by enterobacteria ESBL

| Enterobacteria species | Genes bla | | | | | | | | |
|------------------------|-----------|-----|-----|-----|-----|-----------|-----------|-----------|-----------|
| | TEM | SHV | PER | VEB | GES | CTX M1 | CTX M2 | CTXM 8 | CTXM 9 |
| Escherichia coli | + | + | - | - | - | + | + | + | + |
| Klebsiella pneumoniae | + | + | - | - | - | + | + | + | + |
| Enterobacter cloacae | + | + | - | - | - | + | + | + | + |
| Enterobacter aerogenes | + | + | - | - | - | + | + | + | + |
| Proteus mirabilis | + | + | - | - | - | + | - | - | - |
| Klebsiella oxytoca | + | + | - | - | - | + | + | + | + |
| Proteus vulgaris | + | + | - | - | - | + | - | - | - |
| Citrobacter koseri | + | + | - | - | - | - | - | - | - |
| Citrobacter freundii | + | + | - | - | - | - | - | - | - |

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

"Plasmid mediated resistance to cephalosporins was largely due to blaCTX-M -15 which is in keeping with other studies done in many countries [48,49]. 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" [48,49].

4. CONCLUSION

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 transmission of ESBL-PE poses a serious public health problem and emphasizes the urgent need for improved sanitation and the

implementation of antibiotic stewardship in African nations. Future research should examine the processes of plasmid transfer as well as the factors that influence the reported intestinal carriage.

ETHICAL APPROVAL

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

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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