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Research Article

FIRST DETECTION OF AMINOGLYCOSIDES RESISTANCE GENES *AAC*(6)-IB, *ANT*(2")-I AND *AAD* IN ENTEROBACTERIACEAE PRODUCING EXTENDED-SPECTRUM BETA-LACTAMASES IN ABIDJAN (CÔTE D'IVOIRE)

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Abstract- The aim of this study was to highlight the presence of aminoglycoside resistance genes in enterobacteriaceae producing extended-spectrum beta-lactamases isolated in Abidjan. The study involved 153 enterobacteriaceae of human origin and whose identification has been confirmed by Maldi Tof-type Mass Spectrometry. The antibiotic susceptibility testing was performed by diffusion on Mueller-Hinton E agar. The beta-lactams resistance genes were characterized by real-time PCR, conventional PCR and sequenced. While the aminoglycoside resistance genes were detected through conventional PCR directly. Of these strains 90 (58.8%) were producing broad-spectrum beta-lactamase. A high resistance rate to aminoglycosides (90%), cefotaxime (95.6%), ceftriaxone (96.7%), and cefoxitin (72.2%) was observed in enterobacteriaceae producing extended-spectrum beta-lactamases. The aminoglycoside resistance genes found were *aac (6) -Ib*, *ant (2 ") –I* and *aad* at the rate of 58.9%, 8.9% and 7.8% respectively. Resistance genes to β-lactams detected were bla_{CTX-M} (96.7%), bla_{TEM} (67.8 %) and bla_{SHV} (27.8%). This study is the first to describe the aminoglycoside resistance genes in clinical strains of enterobacteriaceae in Abidjan.

Keywords- *Enterobacteriaceae, ESBL, aminoglycosides, resistance, Abidjan*

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Introduction

Beta-Lactams, aminoglycosides and fluoroquinolones are the main antibiotics of choice prescribed in the treatment of bacterial infections, especially in enterobacteriaceae [1]. However, their abusive and uncontrolled use has led to the gradual development of resistance to these microorganisms [2]. The production of β-lactamases in enterobacteria is the major mechanism for β-lactam resistance. Starting from the first β-lactamases (TEM, SHV) described the extended-spectrum β-lactamases (ESBLs) have been derived and their spectrum of action now extends to third-generation cephalosporins [3]. These β-lactamases have diversified with the explosion of type CTX-M particularly CTXM-15, which is responsible for epidemics of colonization and nosocomial infections worldwide [4].
As regards to resistance to fluoroquinolones, it is chromosomal or plasmidic. Chromosomal resistance is manifested by the alteration in the target enzymes DNA-gyrase and topoisomerase IV or by reduction of the production of porins, which may lead to a decrease in the intracellular concentration of the antibiotic [5]. The genetic determinant of the plasmid resistance of enterobacteria to fluoroquinolones is the *qnr* gene whose main characteristic is to be carried by a class 1 integron, highly mobile between different plasmids, and which causes an acceleration of the diffusion of résistance [6]. As for aminoglycoside resistance, it has been attributed mainly to the inactivation of the target enzymes by the modifying enzymes (acetyl transferases, nucleotidyl transferases and phospho-transferases [7]. However, the methylation of 16S rRNA within the 30S sub-unit of bacterial strains by genes methylation has recently emerged as a mechanism of high resistance rate to aminoglycoside (Arbekacin, Amikacin, Tobramycin, And Gentamicin) [8]. The dissemination of resistance genes between bacteria has led to the appearance of bacteria that are resistant to several antibiotics (multidrug-resistant bacteria or MDR), particularly in broad-spectrum beta-lactamase-producing Enterobacteriaceae [9]. The presence of ESBLs is frequently associated with certain genes that confer resistance to other classes of antibiotics [10]. This situation is the cause of therapeutic failures and an increase in morbidity and mortality [11]. In Côte d'Ivoire the presence and spreading of ESBLs strains has been reported in enterobacteria of human origin [12, 13]. The ESBL of the type TEM, SHV, CTXM have been described and often associated with quinolone resistance genes [14]. However very little data are available on the resistance to aminoglycosides in Enterobacteriaceae, hence our interest. The objective of this study was to highlight the presence of aminoglycoside resistance genes in clinical strains of enterobacteriaceae producing Extended-Spectrum Beta-Lactamases isolated in Abidjan.

Materiel and methods

Bacterial isolates: The strains included in this study were enterobacteriaceae isolated from January 2012 to December 2015.

These strains were isolated from urine, blood, sputum, pleural fluid and ascites from different Hospital Centres in Abidjan. These strains were previously pre-identified and stored at the Biological Resources Centre of the Pasteur Institute of Côte d'Ivoire. Identification of strains was confirmed by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Brucker).

Antibiotic susceptibility test and phenotypic detection of ESBL

The susceptibility testing was performed using Mueller-Hinton E agar (BioMérieux SA, France) by the standard method of diffusion in agar medium described by the Antibiogram Committee of the French Society of Microbiology (CA-SFM, 2013). The antibiotics used to perform the antibiogram were: amoxicillin (25µg), amoxicillin + clavulanic acid (20µg + 10µg), ticarcillin + clavulanic acid (75µg + 10µg), cefotaxime (30µg), cefoxitin (30µg), ceftriaxone (30µg), aztreonam (30µg), imipenem (10µg), ertapenem (10µg), amikacin (30µg), gentamicin (15µg), ciprofloxacin (5µg), fosfomycin (50µg), colistin (50µg), cotrimoxazole (25µg), rifampicin (30µg). The phenotypic detection of ESBLs was carried out by the double synergy test comprising clavulanic acid, cefotaxime, ceftriaxone, aztreonam [15].

Detection of resistance genes

The kit "EZ1 DNA Tissue" (Qiagen) was used to extract the total bacterial DNA of each resistant strains. The beta-lactams resistance genes were detected through real-time PCR and conventional PCR. Aminoglycoside resistance genes were searched directly by conventional PCR.

Real-time PCR for detection of beta-lactams (*bla*) resistance genes

The reaction was carried out in a reaction volume of 20 µL composed of 10 µL of Master Mix (Qiagen Quantitect Probe PCR master mix), 2 µL of Forward and Reverse primers (Eurogentec), 2 µL of DNase-free water (Invitrogen), 1 µL probe (Life Technologies) and 5µL of total DNA diluted to 10%. The amplification procedure consisted of an initial denaturation step of the double-stranded DNA for 15 min at 95°C, followed by 35 cycles of amplification of the target DNA including denaturation at 95°C for 1s., hybridization and elongation at 60°C for 35s. Primers and specific probe for real-time PCR were summarized in [Table-1].

Conventional PCR amplification and electrophoresis

The target genes for beta-lactams were $bla_{CTXM\text{-}1}$, bla_{SHV}, bla_{TEM}, for the aminoglycoside *aac(6)-Ib, ant (2")-I, aad*. The reaction was carried out in a reaction volume of 25 µL composed of 12.5 µl of Master Mix (Quantitect Probe PCR master mix, Qiagen), 1 µL of Forward and Reverse primers (Eurogentec) [Table-2], 6.5 µL of DNase-free water (Invitrogen) and 5 µL of total DNA. The amplification consisted of an initial denaturation step of the DNA for 15 min at 95°C. This step was followed by 35 amplification cycles comprising denaturation at 94°C for 1 min, hybridization at 55°C for 50 sec, elongation at 72°C for 2 min and a final elongation step of 7 min at 72°C. The amplification products were analyzed by electrophoresis on 1.5% agarose gel prepared with 0.5% Tris-Borate-EDTA. The DNA bands of the amplicons were visualized on a transilluminator.

DNA sequencing

The purified amplification products were sequenced using the BigDye® kit (Life Technologies) in an automate ABI PRISM 3730xl DNA Analyzer. The same primers used for the detection of resistance genes by conventional PCR were used to sequence some resistance genes. The obtained nucleotide sequences were analysed by local BLAST in the ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) database of IHU-Marseille.

Results

Identification and susceptivity to antibiotics

A total of 153 enterobacteriaceae composed of 71 *Escherichia coli*, 57 *Klebsiella pneumoniae*, 22 *Enterobacter cloacae*, 2 *Citrobacter freundii*, and 1 *Morganella morganii* were identified. Of these strains 90 (58.8%) were producing ESBL and the ESBL distribution by species was 44 *E. coli*, 31 *K. pneumoniae* and 15 *E. cloacae*. The susceptivity test showed a high third-generation cephalosporins resistance rate respectively 95.6% to cefotaxime, 96.7% to ceftriaxone and 72.2% to cefoxitin in enterobacteriaceae producing extended-spectrum beta-lactamases. The aztreonam and ertapenem resistance levels were 95.6% and 31.1% respectively. Among the ESBL 90% (81 strains) were resistant to gentamicin while 10% (9 strains) were resistant to amikacin [Table-3].

Types of β-lactamases genes

Detection of *bla* genes showed the presence of bla_{TEM} (67.8 %), bla_{SHV} (27.8%), $bla_{CTXM\text{-}1}$ (96.7%) genes. Sequencing of bla_{TEM} genes revealed the presence of $bla_{TEM\text{-}191}$ (13.3%), $bla_{TEM\text{-}104}$ (11.1%) and $bla_{TEM\text{-}198}$ (3.3%). The distribution of the bla_{SHV} gene as follows: $bla_{SHV\text{-}12}$ (2.2%), $bla_{SHV\text{-}27}$ (1.1%), $bla_{SHV\text{-}100}$ (8.9%), $bla_{SHV\text{-}83}$ (1.1%), $bla_{SHV\text{-}89}$ (2.2%), $bla_{SHV\text{-}106}$ (2.2%) and $bla_{SHV\text{-}150}$ (1.1%). For $bla_{CTX\text{-}M}$ gene, the distribution showed the presence of $bla_{CTX\text{-}M\text{-}15}$ (44.4%) and $bla_{CTX\text{-}M\text{-}1}$ (1.1%).

Distribution of aminoglycoside resistance genes

The detection of aminoglycoside resistance genes has shown the presence of the *aac (6')-Ib, ant (2")-I* and *aad* genes at variable rates in Enterobacteriaceae producing ESBL. The *aac(6')Ib* gene was detected in 53 strains (58.9%) against 8 strains (8.9%) for the *ant (2")-I* gene. The *aad* gene was detected in 7 strains (7.8%) and sequencing identified the *aad*1 gene in one strain (1.1%) and *aad*2 in 6 strains (6.7%).

Discussion

The aim of this study was to highlight the presence of aminoglycoside resistance genes in clinical strains of enterobacteriaceae producing Extended-Spectrum Beta-Lactamases isolated in Abidjan. We determined the prevalence of ESBL producing enterobacteria at 58.8%. This rate is higher than those of previous studies that reported prevalences of 9 and 56.2% respectively in ESBL-producing enterobacteria in Côte d'Ivoire [13, 16]. The significant increase in the prevalence of ESBL could be explained due to the abuse and inappropriate use of antibiotics, the main cause of the emergence of antibiotic resistance [17]. Detection of β-lactam resistance genes has also enable detection of several resistance genes. The $bla_{CTX\text{-}M}$ gene was the most represented with predominance of the $bla_{CTX\text{-}M\text{-}15}$. The latter is involved in many epidemiological situations and nosocomial infections worldwide as a result of epidemic plasmid transfer [18]. Its strong presence in the strains could be the origin of the high resistance to C3G observed in this study. Indeed, a recent study conducted in Tunisia in 2014 revealed that 88% of *E. coli* strains, isolated from the urine of patients in a Tunisian hospital, were resistant to cefotaxime, they harbored the $bla_{CTX\text{-}M\text{-}15}$ gene. The increased consumption of cefotaxime may have contributed to the emergence of ESBL, and in particular CTX-M [19]. Earlier work in Côte d'Ivoire has also reported the presence of this gene [20]. Moreover, the ESBLs genes of the type TEM ($bla_{TEM\text{-}191}$, $bla_{TEM\text{-}104}$, $bla_{TEM\text{-}198}$) obtained in this study are new genes described in Côte d'Ivoire. They were detected in Klebsiella pneumoniae strains in Switzerland and Iran [21,22]. In addition to these genes, several variants of the bla_{SHV} gene have been highlighted in this study. The $bla_{SHV\text{-}12}$ gene was first identified in Switzerland in 1997 [23] and later reported in various continents, including Africa in Mali and Nigeria [24,25], indicating a high endemicity of Enterobacteriaceae in West Africa. In Côte d'Ivoire, $bla_{SHV\text{-}12}$ has been described in previous work and associated with *qnr* genes [14]. The other genes detected namely $bla_{SHV\text{-}27}$, $bla_{SHV\text{-}83}$, $bla_{SHV\text{-}89}$, $bla_{SHV\text{-}100}$, $bla_{SHV\text{-}106}$, $bla_{SHV\text{-}150}$ are new genes described in Côte d'Ivoire. The $bla_{SHV\text{-}27}$ gene was detected in different plasmids isolated from *E. coli, K. pneumoniae* and *Enterobacter cloacae* and associated with the resistance genes of certain antibiotics ($bla_{DHA\text{-}1}$, $bla_{TEM\text{-}1}$, $bla_{TEM\text{-}1b}$, $bla_{CMY\text{-}2}$, bla_{IMP}, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}12}$, $bla_{SHV\text{-}45}$, $bla_{OXA\text{-}1}$, dfr_{A5}, ere_{A2}) [26-28]. The $bla_{SHV\text{-}89}$, $bla_{SHV\text{-}100}$, $bla_{SHV\text{-}150}$, $bla_{SHV\text{-}83}$ and $bla_{SHV\text{-}106}$ genes were also detected in *Klebsiella pneumoniae* strains in various parts of the world, in China, Algeria, USA and Portugal [29-32]. The β-lactamases of the type SHV-83, SHV-89 belong to phenotype 2b and are capable of hydrolyzing the penicillin and cephalosporins (cephaloridine and cephalothin) while the beta-lactamases of the type SHV-12, SHV-27, SHV-100, SHV-106, belonging to phenotype *2be* are capable of hydrolysing third-generation cephalosporins (cefotaxime, ceftazidine) and aztreonam [33].

Table-1 *Primers used in this study for real-time PCR*

Gene name	Primer name	Primer sequence (5'->3')	Amplicon size (bp)
Bla_{TEM}	TEM_RT_F	TTCTGCTATGTGGTGCGGTA	213
	TEM_RT_R	GTCCTCCGATCGTTGTCAGA	
	TEM_RT_Probe	AACTCGGTCGCCGCATACACTATTC	
Bla_{CTX-M}	CTXM_groupA_RT_F	CGGGCRATGGCGCARAC	105
	CTXM_groupA_RT_R	TGCRCCGGTSGTATTGCC	
	CTXM_groupA_RT_Probe	CCARCGGGCGCAGYTGGTGAC	
	CTXM_groupB_RT_F	ACCGAGCCSACGCTCAA	221
	CTXM_groupB_RT _R	CCGCTGCCGGTTTTATC	
	CTXM_groupB_RT_Probe	CCCGCGYGATACCACCACGC	
Bla_{SHV}	SHV_RT_F	TCCCATGATGAGCACCTTTAAA	105
	SHV_RT_R	TCCTGCTGGCGATAGTGGAT	
	SHV_RT_Probe	TGCCGGTGACGAACAGCTGGAG	

Table-2 *Primers used for conventional PCR*

Gene name	Primer name	Primer sequence (5'->3')	Amplicon size (bp)
Bla_{TEM}	TEM_F	ATGAGTATTCAACATTTCCGTG	861
	TEM_R	TTACCAATGCTTAATCAGTGAG	
Bla_{CTX-M}	CTX-M1_F	CCCATGGTTAAAAAATCACTGC	944
	CTX-M1_R	CAGCGCTTTTGCCGTCTAAG	
Bla_{SHV}	SHV_F	ATTTGTCGCTTCTTTACTCGC	1051
	SHV_R	TTTATGGCGTTACCTTTGACC	
aac(6´)-Ib	Aac6-1B_F	TATGAGTGGCTAAATCGAT	395
	Aac6-1B_R	CCCGCTTTCTCGTAGCA	
ant(2´´)-I	Ant(2´´)-I_F	GACACAACGCAGGTCACATT	524
	Ant(2´´)-I_R	CGCATATCGCGACCTGAAAGC	
aad	Aad_F	TTGTACGGCTCCGCAGTG	812
	Aad_R	CCCAATTTGTGTAGGGCTTA	

Table-3 *Resistance profile of Enterobacteriaceae producing ESBL to antibiotics*

Antibiotics	Critical diamètres	Resistance rate n (%)
Amoxicillin/ clavulanic acid	16-21	90 (100)
Cefotaxime	23-26	86 (95.6)
Ceftriaxone	23-26	87 (96.7)
Cefoxitin	15-22	65 (72.2)
Ertapenem	26-28	28 (31.1)
Aztreonam	21-27	86 (95.6)
Amikacin	15-17	9 (10)
Gentamicin	16-18	81 (90)

In our study, aminoglycoside resistance was described with the detection of *aac (6') - Ib, ant (2' ') - I*, *aad*1 and *aad*2 genes. It is the first report of these genes in Côte d'Ivoire. The *aac (6 ')-Ib* gene is a plasmid and chromosomal gene that confers resistance to amikacin and gentamicin [34]. It was most represented in aminoglycoside-resistant strains (56%), which is superior to the results of a study conducted in the United States where *aac (6 ')-Ib* was found in 50.5% of enterobacteria [35]. This gene has also been detected in some countries like Belgium, Greece, France, India [36- 38]. The *ant (2 '') -I* gene induces resistance to Gentamicin, Tobramycin, Dibekacin, Sisomicin, Kanamycin, and is generally transported by plasmids and transposons [39]. It was found in 8.9% of the aminoglycoside-resistant strains, this rate is lower than that found in Turkey where 46.2% of the aminoglycoside resistant strains had the *ant(2'')-I* gene [40].

The *aad*1 and *aad*2 genes encode an aminoglycoside-3 "adenylyltransferase labeled *ant (3'')-I* or *aad (3'')* (9) [41]. The *aad*1 gene was detected for the first time in Shigella Isolated flexneri in Japan in the late 1950s [42]. In 1989 when integrons were first described, this gene was found to be associated with a class 1 integron [43]. The *aad*2 gene was first detected in Japan in 1965 in a clinical strain of Pseudomonas aeruginosa [44]. In addition to the class 1 integron, the *aad*2 gene is also present in Class 1 complex integrons [45, 46].

Conclusion

The aminoglycoside resistance shown in this study, the first of its kind to be carried out in this country, showed a high rate of the *aac (6) -1b, ant (2 '')-I* and *aad* genes among clinical strains of Enterobacteriaceae producing ESBL. New resistance genes to β-lactamase have been described and associated with resistance to aminoglycosides. Since these antibiotics (beta-lactams, aminoglycosides) are used in the treatment of many bacterial infections, the presence of resistance genes gives a cause for concern. Therefore, monitoring the prescription of these antibiotics is very necessary given the easy spreading of resistance genes between bacteria.

Application of research: This study should lead the authorities and health workers to a relevant policy of monitoring the prescription of antibiotics such as beta-lactams and aminoglycosides as well as a continuous monitoring of the resistance for a better control of the circulation of the resistant strains.

Research Category: Aminoglycosides Resistance

Abbreviation:
ESBL: Extended-Spectrum Beta-Lactamases; Bla : beta-lactam

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