Molecular Characterization Of Extended Spectrum Betalactamases Genes (Blactxm And Blashv) In Enterobacteria Isolates In Medical Specimens In Lomé (Togo)

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Abstract

Background Extendum Bêtalactamases genes spread throughout the world. Many informations are known about it in Europe, Asia and elsewhere. In Africa particularly in Togo, we have lack informations although their prevalence are still increasing. The aim of this study is to identify the blaSHV and blaCTXM genes on Escherichia coli and Klebsiella pneumoniae strains isolated in two medical bacteriology laboratories in Lomé. Material and method 46 strains (20 Klebsiella pneumoniae, 23 Escherichia coli and 3 Enterobacter cloacae) isolated at Sylvanus Olympio Teaching Hospital (n = 31) and at Institut d'Hygiène (n = 15) in Lomé were investigated in search of blaSHV and blaCTXM through gene amplification. The strains were isolated from various samples in 2015 and 2016. An amplification of blaTEM was carried out in case of negativity to both genes. A sequencing of the amplicons was carried out then the sequences identified through blastX on the basis of NCBI data. Results We found 97.9% resistance to amoxicillin + clavulanic acid, gentamicin, levofloxacin and to sulfamethoxazole + trimethoprim. 8.7% of the strains were resistant to ertapenem. All the strains carried blaCTXM-15. In Klebsiella pneumoniae, blaSHV-1 blaSHV-11, blaSHV-28, blaSHV-61, blaSHV-77 were identified. Associations were found (blaSHV-1 / blaCTXM-15, blaSHV-11 / blaCTXM-15, blaSHV-28 / blaCTXM-15). blaTEM was identified on a strain of Enterobacter cloacae. Conclusion There is a diversity of blaSHV genes with a dominance of blaCTXM-15. blaTEM remains the gene to search for in case of absence of the two previous genes in ESBL strains in Lomé.

Background

Beta-lactams are a class of antibiotic molecules widely prescribed worldwide in various bacterial infections. The resistance of bacteria to antibiotics is a public health issue. Indeed, the development by resistant bacteria especially to this class of antibiotics significantly reduces the therapeutic options. These include the production of extended-spectrum beta-lactamases (ESBL); enzymes which inactivate all cephalosporins and spare only cephamycins and carbapenems are quite frightening. They are quite widespread worldwide because most often their production is mediated by plasmids (1) and remains a concern at the hospital because responsible for healthcare associated infections (2). Most often, we find the enzymes of CTX-M and SHV types in enterobacteria. SHV is a constitutive enzyme in *Klebsiella pneumoniae* which first SHV-1 was described in 1972 and since,several variants have been described (3). CTX-M remains by far the most described in ESBL (1).

Data on these enzymes exist at the Asian and European levels, however, are lower at the African level and rare at the national level. However, prevalence of ESBL producing bacteria are mentioned across the continent (4-6). In order to have epidemiological data at the national level, we have initiated this work which purpose is to describe the types of CTX-M and SHV carried by clinical strains of enterobacteria in Lomé.

Methods

ESBL strains were routinely identified and we randomly selected 46 strains (20 *Klebsiella pneumoniae*, 23 *Escherichia coli* and 3 *Enterobacter cloacae*) including 31 strains isolated at Sylvanus Olympio Teaching Hospital (CHUSO) and 15 strains at InstitutNational d'Hygiène (INH).

- Sylvanus Olympio Teaching Hospital (CHUSO) is the reference center of the country and is located in Lomé, the capital city of Togo

- Institut National d'Hygiène (INH) is the national public health laboratory of Lomé.

These two centers are the ones that carry out the most acts of medical bacteriology in Lomé.

A total of 15 strains were isolated in 2015 (3 *Klebsiella pneumoniae* and 12 *Escherichia coli*) and the others in 2016. Strains came from various samples (urine, blood culture, pus, vaginal specimen).

Susceptibility testing

Bacteria were identified for the most part by conventional technique on the basis of their biochemical characters or by API 20E system. They were tested on various antibiotic discsthrough the method of Kirby Bauer and the interpretations made in accordance with the recommendations of the CA-SFM 2015 (Antibiogram Committee ofFrench Society for Microbiology). The antibiotics tested were: beta-lactams (amoxicillin, amoxicillin + clavulanic acid, ticarcillin, ticarcillin + clavulanic acid, cefoxitin, cefotaxime, cefepime, ceftriaxone, imipenem, ertapenem); monobactams (aztreonam), aminoglycosides (gentamycin, amikacin); quinolones (levofloxacin); sulfadoxine-pyrimethamine; fosfomycin.

They were labeled carriers of ESBL in presence of synergy between amoxicillin+ clavulanic acid discs and aztreonam or between amoxicillin+ clavulanic acid and one of the third generation cephalosporin discs.

Molecular Characterisation of ESBL genes

The DNA was extracted by boiling extraction from an isolated colony suspended in 100µlof water at 95 ° C for 15 minutes followed by a centrifugation step. The bla_{CTXM} and bla_{SHV} genes were identified through gene amplification using well defined primers(SHV-A: 5'-atg-cgt-tat-wtt-cgc-ctg-tgt-3 '; SHV-B : 5'-tta-gcg-ttg-cca-gtg-ctc-g-3'; CTXM-A : 5'-scs-atg-tcg-agy-acc-agt-aa-3'; CTXM-B : 5'- ccg-cra-tat-grt-tgg-ttg-tg3' ; TEM-1 : 5'-gta-tcc-gct-cat-gag-aca-ata-3' ; TEM-2 : 5'-tct-aaa-gta-tat-atg-agt-aaa-ctt-ggt-ctg-3')according to the following program: 95 ° C for 3min, 95 ° C for 30s, 55 ° C for 30s, 72 ° C for 60seconds, then 72 ° C for 7 minutes with 50 µl of reaction mixture (25 µl of GreenTaq, 2.5 µl of each primer;18 µl of H2O and 2 µl of DNA extract). The search for bla_{SHV} was carried out only on the strains of *K. pneumoniae*, that of bla_{CTXM} on all the strains without any exception. The gene amplification of bla_{TEM} was carried out only in case of negativity to the two desired genes. The primers used were (TEM-1: 5'-gta-tcc-gct-cat-gag-aca-ata-3 '; TEM-2: 5'-tct-aaa-gta-tat-atg-agt-aaa- ctt-ggt-ctg-3 '). For all the desired genes, the gene amplification was done over 30 cycles. Positive controls for each gene were used as controls. The products of amplification were

revealed by a UV reader after electrophoretic migration on a 1% agarose gel using ethidium bromide as intercalating agent. The migration was performed over 60 minutes at 100V with a 100bp size marker (Promega, USA).

Sequencing

All the positive amplicons were purified by a Quiagen Kit, QIAquick PCR Purification Kit (Roche laboratories) and a sequencing PCR for each primer was performed with a 10µltotal mix (2µlof Big Dye, 3µl of primer 1mM, 3µlof Water,2µ1 of Purified extract) according to the following program: 96 ° C for 3 minutes, 96 ° C, 55 ° C for 15sec, 60 ° C for 4 minutes, 60 ° C for 10min over 25 cycles. The resulting PCR products underwenta membrane filtration using the Edge BIO kit and then through the Hitachi ® 3130 Genetic Analyzer Applied Biosystems Sequencer. The sequences obtained were transferred to a computer for analysis. The sequencesediting was done using 4peaks® software and the sequences were subjected to comparison by blastx (www.ncbi.nim.nih). The comparison results were selected on the basis of 97-100% identity.

Molecular characterization was performed at Bacteriology-Hygiene Laboratory of Bicêtre Hospital in Paris.

Results

Antibiotic susceptibility

The different bacteria were all resistant to amoxicillin. Only a few (2.1%) were susceptible to levofloxacin and sulfamethoxazole-trimetroprime beta-lactamase inhibitors. Cefepime and cefoxitin resistance rates were respectively 95.6% and 19.5%. The resistance to gentamicin was 72% (Figure 1). Some cases of resistance to imipenen (6.5%) and ertapenem (8.7%) were observed.

Extendum Spectrum Beta-lactamase genes

All bacteria produced at least one desired beta-lactamase gene. Indeed 97.9% (n = 45) carried bla_{CTXM} and / or bla_{SHV}. bla_{CTXM} was carried by 33 strains mean 72%; all *E. coli* strains were positive to bla_{CTXM}. All the strains of *K. pneumoniae* (n = 20) carried one type of bla_{SHV} and for eight (08) of them, which is 40% it was associated with bla_{CTXM}. One strain of *E. cloacae* was negative to both genes despite the phenotypic presence of a synergistic image between the molecule of aztreonam and that of amoxicillin + clavulanic acid. For the latter we performed an amplification of the bla_{TEM}gene which was positive (Figure 2). At sequencing, all bla_{CTXM} genes identified were $bla_{CTXM\text{-}15}$ for the 33 positive bla_{CTXM} strains.

Different *bla*$_{SHV}$were identified. The most found type was *bla*$_{SHV-11}$ in a proportion of 45% (n = 9). The other types were *bla*$_{SHV-1}$ (15%; n = 3), *bla*$_{SHV -28}$ (20%; n = 4), *bla*$_{SHV-61}$ (10%; n = 2), *bla*$_{SHV-77}$ (10%; n = 2). On the CTXM-15 combinations, we found 37.5% *bla*$_{SHV-11}$ (n = 3), 37.5% *bla*$_{SHV-28}$ (n = 3) and 25% *bla*$_{SHV-1}$ (n = 2). The strains carrying the *bla*$_{SHV-1}$ / *bla*$_{CTXM-15}$ combination were from INH and had almost the same susceptibility profile except that one was susceptible to gentamicin. Among the three strains carrying the *bla*$_{SHV-11}$ / *bla*$_{CTXM-15}$combination, two were from INH, one of which was resistant to cefoxitin and the strain isolated from CHUSO was resistant to ertapenem. The strains carrying the *bla*$_{SHV-28}$ / *bla*$_{CTXM-15}$ combination all came from INH and only one was susceptible to cefalotin. All these strains were isolated between July and August 2016. Among all the SHVs, only SHV-11 and SHV-77 were identified on strains of both years.

The strain of *E. cloacae* showing a synergistic image but showing neither *bla*$_{SHV}$ gene nor *bla*$_{CTXM-15}$ was positive for the amplification of the *bla*$_{TEM}$ gene. At sequencing, *bla*$_{TEM-1b}$ was found with 99% identity with strain S86b (GenBank Accession number JF910132)

The different distributions of the genes are in Table 1.

Discussion

In this study, different antibiotic susceptibilities were noted. Indeed ESBL confers resistance to all beta-lactams except cefoxitin and carbapenems and their action is inhibited by beta-lactamase inhibitors such as clavulanic acid. We found that our strains show relatively little susceptibility to amoxicillin + clavulanic acid combination and 19.5% of the strains were resistant to cefoxitin, suggesting a combination of mechanisms. Indeed for some (8.5%), we have a resistance to carbapenems which may explain the insensitivity to inhibitors. Resistance to cefoxitin to some extent may be explained by overexpression of the natural cephalosporinase in *K. pneumoniae* and *E. cloacae*. In all cases, these strains are multidrug-resistant, as most, 72%, and 98% are resistant to gentamicin, to sulfamethoxazole-trimetoprim and to levofloxacin, respectively. This multi-resistance was also observed in Senegal (5), Nigeria (7) on strains producing an ESBL and even here in Togo since 2009 (6). Extended spectrum beta-lactamases are disseminated throughout the world. So it is not surprising that we constantly find it in our laboratories of medical bacteriology.

The exclusive presence of *bla*$_{CTXM-15}$ on all *E. coli* and 40% of *K. pneumoniae* strains, and two-thirds of *E. cloacae* strains over these two years suggest a dissemination at the level of the city. Indeed, INH is a laboratory that receives samples from both the urban community and some private clinics in the city and the laboratory of Sylvanus Olympio Teaching Hospital deals exclusively with samples from inpatients or outpatients. *bla*$_{CTXM-15}$ is a common gene in Enterobacteriaceae especially *E. coli* and Klebsielles. It was already identified on enterobacterial strains in Senegal, Angola, Ghana, Nigeria and Burkina Faso (8-11), which are countries of the sub-region. Additional studies are needed to determine if this is the same clone circulating in the sub-region.

SHV is a constitutive beta-lactamase in *K. pneumoniae*. The first to be described is SHV-1 (3) and since then several variants exist that are either ESBL or not (12). All variants described in our study were all ESBL and some associated with CTXM-15 (www.bldb.eu; https://www.card.ca). The identification of different types of SHV shows us a diversity of circulation of *K. pneumoniae* strains in Lomé. Two types remain dominant: bla_{SHV-1} and bla_{SHV-11} identified in total over 60% of the strains. Indeed bla_{SHV-11} is a variant of bla_{SHV-1} with a slight amino acid substitution (12). These two types were also prevalent in Korean hospitals in 2002 (13). In Senegal, however, they had identified SHV-2 and SHV-12 on Salmonella strains from 1999 to 2001 (8).

In our study, two other types: SHV-1 and SHV-77 were described on strains in both years suggesting a probable expansion of the same strains.

The association of two types of ESBL as described in our study indicates an exogenous acquisition by transfer of genetic elements. Since most ESBL plasmids are known to carry other antibiotic resistance genes, it is easy to understand the multi-resistance of the strains in this study. bla_{SHV-28} / $bla_{CTXM-15}$was described on strains of *K. pneumoniae* isolated in Copenhagen, Denmark as being an epidemic clone (14). This association was found in 3 strains isolated between July and August 2016 also making us think of this same hypothesis. However this is to be confirmed by genotypic techniques.

Two of the *K. pneumoniae* strains carried the bla_{SHV-61} gene, which was first described in 2009 in Portugal. They were isolated in June and July of the same year in pus from different patients. Without further information on patients, we were unable to investigate the notion of recent or past travel in this country.

bla_{TEM} is a beta-lactamase that was already described in isolates of *Escherichia coli* and *K. pneumoniae* in our country (15,16). Its presence on an isolate of *Enterobacter cloacae* remains a first in our country.

The prevalence of carbapenem resistance in this study is low. Indeed, carbapenems are molecules used as a last resort for the treatment of infections due to ESBL or multidrug-resistant bacteria. The existence of some carbapenem-resistant bacteria requires a better understanding of the mechanisms underlying this resistance.

Conclusions

During our study, we noticed a multi-resistance of the various strains isolated. The extended spectrum beta-lactamase genes carried by the strains in the two laboratoriesin Lomé are quite diverse (bla_{TEM}, bla_{SHV}, bla_{CTXM}). Indeed, there is a diversity of bla_{SHV} on strains of *Klebsiella pneumoniae*; on the other hand, $bla_{CTXM-15}$ remains dominant on the strains of *Escherichia coli* as well as on those of *K. pneumoniae*; bla_{TEM} would seem to be the ESBL to look for in case of negativity of the two previous ones.

The existence of certain genes combinations on several isolates of our study suggests a clonal expansion and therefore strategies must be put in place to avoid probable epidemics.

Abbreviations

ESBL: Extendum Spectrum Betalactamase,CHU: Centre Hospitalier Universitaire,CHUSO : Centre Hospitalier Universitaire Sylvanus Olympio,INH : Institut National d'Hygiène.

Declarations

Ethics approval and consent to participate: Not applicable
Consent for publication: Not applicable

Available of data and material: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interest: Authors declare that they have no competing interests

Funding: Not applicable

Authors contributions: This is a collaborative study done with all authors: DS design and conducted the study, wrote protocol and manuscript; IWM, PDM, DAY, SM helped in protocol writing, read and approved final manuscript; GAM, KBA, BB, AE gave assistance for having strains for each facility.

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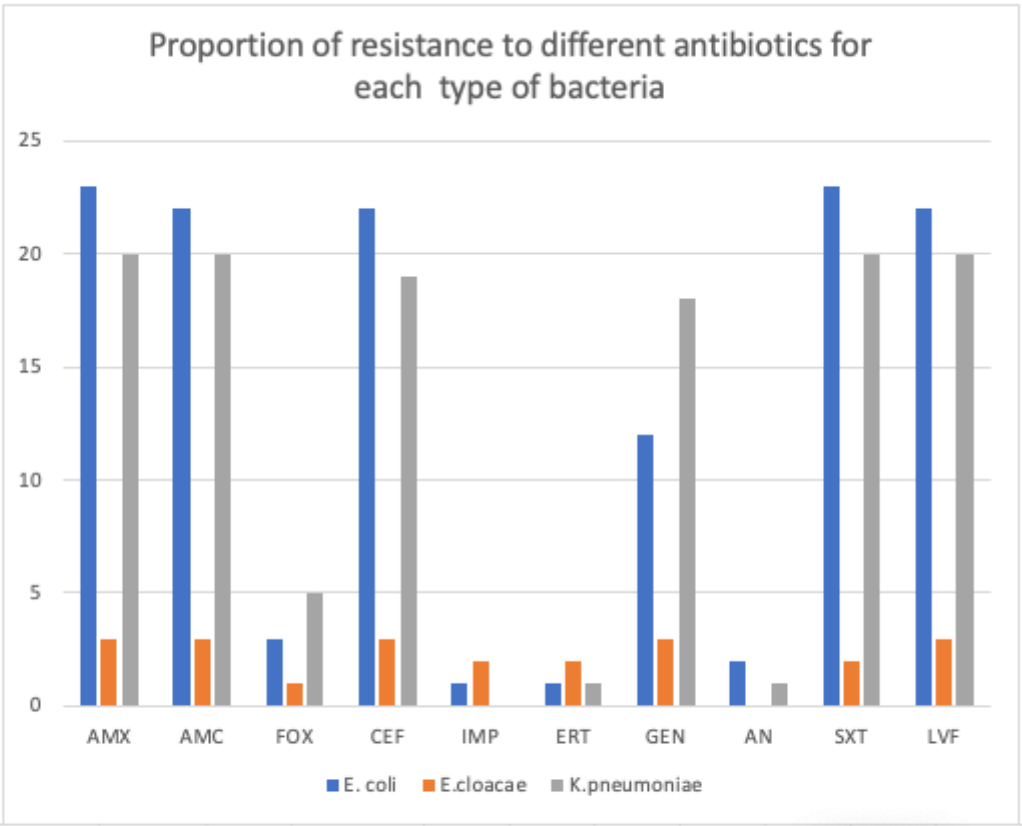
Tables

Table 1: Distributions of the different genes identified

		bla $_{CTXM-15}$	bla $_{TEM-1b}$	Total
	Effectif	25	1	26
bla_{SHV-1}	1	2	0	3
bla_{SHV-11}	6	3	0	9
bla_{SHV-28}	1	3	0	4
bla_{SHV-61}	2	0	0	2
bla_{SHV-77}	2	0	0	2
Total	12	33	1	46

Figures



	AMX	AMC	FOX	CEF	IMP	ERT	GEN	AN	Chart Area	LVF
E. coli	23	22	3	22	1	1	12	2	23	22
E.cloacae	3	3	1	3	2	2	3	0	2	3
K.pneumoniae	20	20	5	19	0	1	18	1	20	20
Pourcentage (%)	100	97.9	19.5	95.6	6.5	8.7	71.7	6.5	97.9	97.9

Figure 1

Proportion of resistance to different antibiotics for each type of bacteria AMX : amoxicillin, AMC : amoxicillin+acid clavulanic, FOX : cefoxitin, CEF : cefepime, IMP : imipenem, ERT: ertapenem, GEN: gentamicin, AN: amikacine, SXT: suldadoxin+trimethoprim, LVF: levofloxacin.

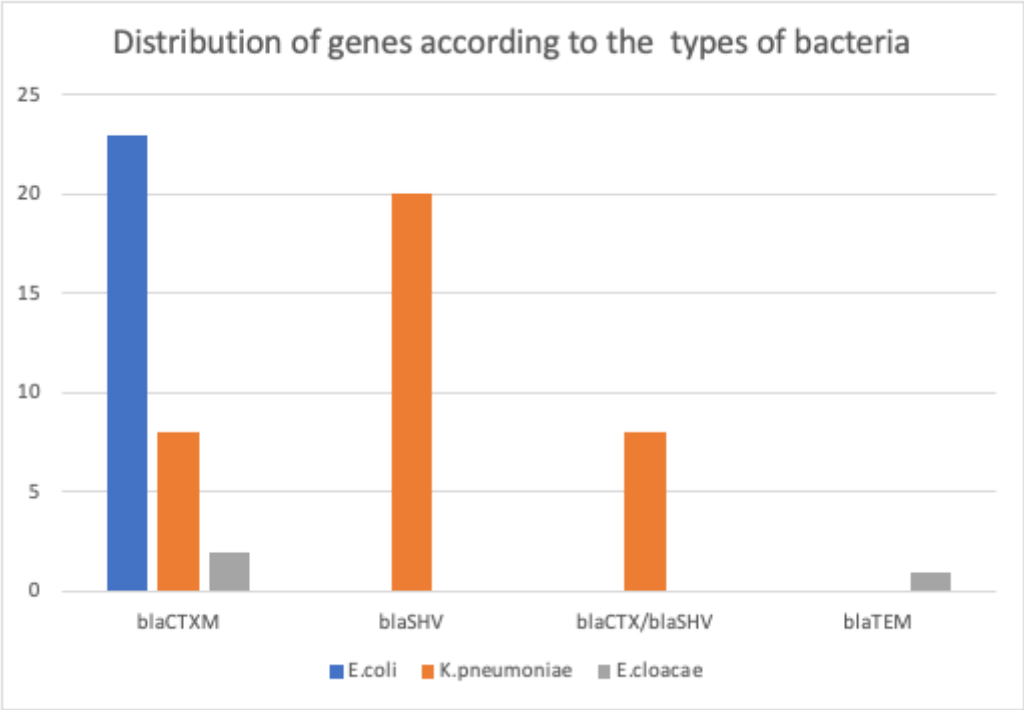


Figure 2

Distribution of the genes identified according to the types of bacteria.