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Resistance to β-Lactamines by Gram Negative Bacteria, Producing Several Types of Enzymes, Isolated from Urines in Pediatric Center of Ouagadougou in Burkina Faso

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Abstract: Extended Spectrum β-Lactamases (ESBL) producing bacteria have been reported in several countries, but there is little information available on the different types of ESBL produced by Enterobacteria in Burkina Faso. For compensate for the lack of scientific information of extreme importance, this study deals with molecular characterization of several types of extended spectrum β-lactamases (ESBL) produced by Enterobacteria strains isolated in pediatric medium. Enterobacteria strains producing ESBL were identified by their profile of resistance to the cephalosporin of third generation (C3G) on Petri box. These strains consist of several bacterial species resistant to antibiotics. The production of ESBL by these strains was confirmed by kinetic approach. The genomes of the different strains were obtained by DNAZOL extraction. The molecular characterization of the ESBL was based on classical PCR and sequencing using specific primers of the *CTX-M*, *SHV* and *TEM* genes. *CTX-M*, *SHV* and *TEM* genes were searched in DNAs extracted from 30 isolates positives to ESBL production. *CTX-M-15* was found in the 30 isolates, *CTX-M-15* and *SHV-1,-11,-12,-28,-32,-38,-76* or-*99* in 11 isolates, *CTX-M-15* and *TEM-1* in 11 isolates , and *CTX-M-15*, *TEM-1* and *SHV-1,-11,-12,-32,- 76 or -99* in 5 isolates. *β-lactamases* types of *CTX-M-15*, *TEM-1* and *SHV-1,-11,-12,-28,-32,-38,-76* and-*99* were highlighted in the University Hospital center Pediatric Charles De Gaulle of Ouagadougou/BF.

Keywords: Extended Spectrum β-Lactamases, Genes, Resistance to Antibiotics

1. Introduction

The production of a β-lactamase is the main mechanism of resistance in Gram-negative bacteria. Since the early 1980s, the emergence of new extended-spectrum β-lactamases (ESBLs), conferring acquired resistance to third-generation cephalosporins, has been a major public health problem [1]. They are encoded by genes located on transferable elements (plasmids, transposons) and are easily disseminated by inter-species conjugation. The use of cephamycins (cefoxitin, cefotetan, and latamoxef), β-lactamase inhibitors (clavulanate, sulbactam, tazobactam) and carbapenems, which are not very sensitive to these enzymes, made it possible to find a therapeutic outcome. However, new transferable enzymes, insensitive to cephamycins and inhibitors, having a phylogenetic kinship with cephalosporinases or penicillinases β-lactamases produced naturally by many species of Enterobacteriaceae are appearing. The ever-increasing number of β-lactamases with varied characteristics has made it necessary to find a standardized classification, able to differentiate the new enzymes from those already listed. Therefore, a classification based on the molecular structure (amino acid sequence) has been proposed [2] and another one based on the functional properties (substrate profile and susceptibility to β-lactamase inhibitors) has been given [3]. In Burkina Faso, studies on the

different types of ESBL that circulate are scarce and recent [4, 5]. Here, the molecular characterization of extended-spectrum β-lactamases (ESBL) developed by strains of Gram negative bacteria isolated from urine in pediatric environments is investigated.

2. Material and Methods

2.1. The Bacterial Strains

Thirty (30) strains of Gram negative bacteria resistant to at least one third generation cephalosporin (C3G) were isolated from the urine of sick children between 2010 and 2012 at the University Hospital Pediatric Charles De Gaulle (CHUP-CDG) of Ouagadougou in Burkina Faso. These strains have been isolated from different departments of the hospital. Bacterial identification was performed by conventional techniques.

2.2. Criteria for the Selection of Strains

The antibiogram was performed by the Muller-Hinton agar (MH) diffusion method with according to the recommendations of the Committee of the antibiogram of the French Society of microbiology. The determination of the ESBL profile of the isolates focused on their resistance to at least one third generation cephalosporin (C3G). The ESBL profile has been made more convincing by the synergistic action test between augmentin (AMC) and a third generation cephalosporin (C3G) which gives a synergistic image of action in case of positive presumption of ESBL. The ESBL profile of the isolates has been made irrefutable by kinetic methods.

2.3. Extraction of Bacterial DNAs

DNA was extracted from of the different strains using DNAzol®Reagent (Invitrogen / DNA by life technologies). The bacterial DNA extraction was carried out on 1mL of bacterial suspension whose turbidity is compared to Mac Farland 0.5 following the instructions of the manufacturer.

2.4. Amplification and Sequencing of Genes

PCR was made in a reaction volume of 25 µL and consisted of 35 cycles followed by a final extension at 72°C for 10 minutes. The *CTX-M* gene (cycles of 96°C for 5 minutes, 50°C for 1 minute and 72°C for 1 minute) and the *TEM* gene amplification programs (cycles of 96°C for 5 minutes, 58°C for 1 minute and 72°C for 1 minute) allowed to amplify a 1 kb fragment. The *SHV* gene amplification program (cycles of 96°C for 5 minutes, 60°C for 1 minute and 72°C for 1 minute) allowed to amplify a 875 bp fragment. The PCR amplified DNA fragments were electrophoretically separated on a 1.5% agarose gel prepared in 0.5X tris - borate - EDTA tris solution and containing ethidium bromide. These fragments were visualized under UV light.

The sequences of the primers used in this study, provided by Eurogentec, are TEM-F 5'-ATA-AAA-TTC-TTG-AAG-ACG-AAA-3' and TEM-R 5'-GAC- AGT-TAC-CAA-TGC-TTA-ATC-A-3 'for blaTEM, SHV-F 5'-ATG-CGT-TAT-ATT-CGC-CTG-TG-3' and SHV-R 5'- TTA-GCG-TTG-CCA-GTG-CTC-3' for blaSHV, CTX-MF 5'-GTT-ACA-ATG-TGT-GAG-AAG-CAG-3' and CTX-MR 5'-CCG- TTT-CCG-CTA-TTA-CAA-AC-3' for blaCTX-M [6].

The PCR products comprising the coding regions of the different genes were sequenced. The different sequences obtained were analyzed with the BLAST software (Basic Local Alignment Search Tool) available on the National Center for Biotechnology Information website (http://www.ncbi.nih.gov), the 'Reverse Complement' software available on the website http://www.bioinformatics.org/sms/rev_comp.html, the Clustalw2 software available on the website http://www.ebi.ac.uk/Tools/msa/clustalw2/ and with ApE (A plasmid editor Enterprise) version 8.5.2.0

3. Results

3.1. Third Generation Cephalosporin Sensitivity (C3G) Study

All thirty (30) strains were resistant to at least one third generation cephalosporin (C3G). These strains have a high resistance to cefuroxime (CXM), cefotaxime (CTX) and ceftriaxone (CRO) and are less resistant to ceftazidime (CAZ) and cefepime (CFM). All the strains studied were sensitive to imipenem.

3.2. Confirmation of the ESBL Profile of Strains

The production of ESBL has been confirmed by kinetic methods. The extracts of the 30 strains responded positively to the production of extended spectrum β-lactamases (ESBL). In the experimental conditions, none of our isolates hydrolyzed imipenem (IPM).

3.3. Gene Amplification and Sequencing

The search for genes coding for CTX-M, TEM or SHV by PCR was positive for all 30 strains. Pure PCR products were directly sequenced (Table 1). Examination of the different sequences made it possible to identify 10 types of beta-lactamases and to note that the *CTX-M* genes from all treated strains have the same nucleotidic sequence and correspond to *CTX-M-15*, on the other hand a great diversity is observed for the *SHV* genes. The *TEM* gene carried by the analyzed isolates is the *TEM-1* type. All 30 ESBL-producing isolates harbor the *CTX-M-15* gene, 11 isolates harbor both *CTX-M-15* and *SHV-1, -11, -12, -28, -32, -38, -76* or *-99*; 11 isolates harbor *CTX-M-15* and *TEM-1*; and 5 isolates harbor *CTX-M-15*, *TEM-1* and *SHV-1, -11, -12, -28, -32, -38, -76* or *-99*.

Table 1. *Molecular characteristics of the genes harbored by the treated isolates.*

isolats	blagène PCR			Sequencing Types de beta-lactamases
	CTX-M	TEM	SHV	
K.pneumoniae 774	+	+	+	CTX-M-15 ; TEM-1 ; SHV-12
K.pneumoniae778	+	+	+	CTX-M-15 ; TEM-1 ; SHV-32
K.pneumoniae 538	+	+	+	CTX-M-15 ; TEM-1 ; SHV-76
Enterobacter sp1012	+	+	+	CTX-M-15 ; TEM-1 ; SHV-11
K.pneumoniae736	+	+	+	CTX-M-15 ; TEM-1 ; SHV-1
Enterobacter sp25	+	+	-	CTX-M-15 ; TEM-1
K.oxytoca362	+	+	-	CTX-M-15 ; TEM-1
E.coli 932	+	+	-	CTX-M-15 ; TEM-1
K.pneumoniae130	+	+	-	CTX-M-15 ; TEM-1
K.pneumoniae44	+	+	-	CTX-M-15 ; TEM-1
Enterobacter sp741	+	+	-	CTX-M-15 ; TEM-1
E.coli649	+	+	-	CTX-M-15 ; TEM-1
E.coli777	+	+	-	CTX-M-15 ; TEM-1
Enterobacter sp 678	+	+	-	CTX-M-15 ; TEM-1
Enterobacter sp 745	+	+	-	CTX-M-15 ; TEM-1
E.coli515	+	+	-	CTX-M-15 ; TEM-1
K.pneumoniae534	+	-	+	CTX-M-15 ; SHV-1
Enterobacter sp556	+	-	+	CTX-M-15 ; SHV-11
K.pneumoniae292	+	-	+	CTX-M-15 ; SHV-1
Klesiella sp715	+	-	+	CTX-M-15 ; SHV-11
K.pneumoniae46	+	-	+	CTX-M-15 ; SHV-99
Klesiella sp203	+	-	+	CTX-M-15 ; SHV-1
K.oxytoca613	+	-	+	CTX-M-15 ; SHV-1 1
K.pneumoniae 120	+	-	+	CTX-M-15 ; SHV-38
K.pneumoniae 213	+	-	+	CTX-M-15 ; SHV-11
K.pneumoniae466	+	-	+	CTX-M-15 ; SHV-28
Klesiella sp336	+	-	+	CTX-M-15 ; SHV-11
Pseudomonas sp555	+	-	-	CTX-M-15 ;
Enterobacter agglomerans29	+	-	-	CTX-M-15 ;
Pseudomonas aeruginosa54	+	-	-	CTX-M-15 ;

4. Discussion

The bladder is a site in the human body where high concentrations of certain antibiotics (quinolones and β-lactam antibiotics) are observed. As a result, resistance is easily acquired by the bacteria in the urine. Several multiresistant strains have been observed. This multidrug resistance can be explained by the fact that the genes responsible for these resistances can be carried by the same plasmid, by the coexistence of several resistance mechanisms, or by the production of several enzymatic types [7].

The sequencing of the PCR products of the 30 strains made it possible to demonstrate the production of ten types of β-lactamases by the bacteria isolated at CHUP-CDG. These are SHV-1, -11, -12, -28, -32, -38, -76 and -99, TEM-1 and CTX-M-15 which is produced by all 30 strains. The ESBL *SHV-12* appears to be a particularly migratory gene. ESBL and especially SHV type are very rarely found in certain *Enterobacter*, *Serratia*, *Citrobacter*, in which chromosomal cephalosporinase is predominant [8]. Nevertheless, ESBL SHV-12 has been demonstrated in *C. freundii* and *E. cloacae* [9].

CTX-M-15 ESBL such as CTX-M-16 and 19 ESBLs confer strong resistance to ceftazidime than other CTX-M ESBLs [10, 11]. However, it observed that the strains producing this ESBL were sensitive or moderately resistant to ceftazidime. It have described for the first time this enzyme in Burkina [4]. The *blaCTXM-15* genes we described were either associated with the *blaTEM-1* genes and / or one of the other types of *SHV*, as is often the case in the other studies [9]. The *blaCTX-M* genes would be carried by different plasmids that would facilitate their dissemination. Geographical and temporal groups of identical *blaCTX-M* genes carried by apparently different plasmids have been reported in a number of studies [9]. The prevalence and diversity of extended-spectrum ß-lactamases (ESBLs) of the CTX-M family is increasing worldwide [12]. These enzymes commonly have a preferential activity on cefotaxime and ceftriaxone compared to ceftazidime [13] which is in agreement with the results of this work which reveals that the *CTX-M-15* gene dominates the profile of the ESBL genes identified and that the different isolates harboring these genes have high resistance to cefotaxime (CTX) and ceftriaxone (CRO). In contrast to the metallo-β-lactamase of *Cryseobacterium indolegenes* discovered in Burkina Faso [14] which hydrolyzes imipenem, no extract of the analyzed strains hydrolyzed imipenem in the experimental conditions. This work identified many bacterial species harboring the different genes sought. The *K. pneumoniae* species is the majority species harboring the desired ESBL genes. It is therefore irrefutable that molecular characterization is the best method for detecting and identifying ESBLs.

5. Conclusion

To our knowledge, this study is the first to characterize ESBL coexisting in the same bacterium in Burkina Faso. It showed that CTX-M-15, and SHV-12, -28, -32, -38, -76 and -99 ESBLs with a CTX-M-15 predominance circulate at the University Hospital center Pediatric Charles De Gaulle (CHUP-CDG) of Ouagadougou. *K. pneumoniae* is the main species harboring the ESBL genes encountered. Rigorous application of hygiene rules and the rational use of antibiotics would limit the spread of such multidrug-resistant organisms.

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We have no conflicts of interest

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