Detection of PER 1 Extended-Spectrum β-Lactamase among Nosocomial *Providencia stuartii* isolates in Tunisia

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Détection des β-lactamases à spectre étendu PER-1 Chez *Providencia stuartii* en Tunisie

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R É S U M É

Prérequis : l'émergence des bêtalactamases à spectre étendu (BLSE), conférant une résistance acquise aux céphalosporines de troisième génération chez les entérobactéries devient une préoccupation de plus en plus grande dans le monde

But : caractérisation d'isolats cliniques de Providencia stuartii producteurs de bêtalactamases à spectre étendu en milieu hospitalier.

Méthodes : Nous avons analysé 10 isolats de Providencia stuartii producteur de BLSE

Collecté à partir de sang au sein du service de réanimation. La présence du blaPER-1 a été realisée par Réaction en Chaîne de la Polymérase (PCR).

Résultats : PER-1 a été détecté seulement pour trois isolats de P.stuartii. Le gène codant pour cette enzyme est localisé sur un plasmide conjugatif de 100 kb et qui code pour une β-lactamase de pI de 5,3.

Conclusion : cette étude suggère la dissémination de ce gène chez les isolats de P.stuartii dans le futur en Tunisie.

M o t s - c l é s

béta-lactamase à spectre élargi ; Providencia stuartii; PER-1; Tunisie

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S U M M A R Y

Background: The Emergence of extended-spectrum beta-lactamases (ESBLs) in Enterobacteriace species imparting resistance to third-generation cephalosporins is a growing concern worldwide.

Aims: This study was undertaken to characterize ESBL producers from clinical isolates of Providencia stuartii in Tunisia.

Methods: We analysed ten non-duplicate ESBL positive, P. stuartii isolates collected in intensive care units of military Hospital in Tunisia from blood specimens and presented an extended-spectrum resistance phenotype . the presence of the blaPER-1 encoding genes was confirmed by polymerase chain reaction.

Results: We detected PER-1 in three of ten isolates of P. stuartii. The gene coding for this enzyme was located on a 100 kb conjugative plasmid that encoded a β-lactamase with a pI of 5.3.

Conclusion: This study suggests the spreading of this gene among P. stuartii. clinical strains in the next year in Tunisia.

K e y - w o r d s

Extended spectrum β-lactamases; Providencia stuartii; PER-1; Tunisia

The current emergence and dissemination of clavulanic acid inhibited, extended-spectrum ß-lactamase (ESBL)-producing Enterobacteriaceae represent a global threat, as they are difficult to trace and eradicate, and cause both nosocomial and community-acquired infections [1]. Most of these ESBLs have evolved from TEM and SHV types but the group also includes enzymes that are not TEM or SHV derivatives [2-4]. PER-1, one of these enzymes, was first identified in 1991, in a *Pseudomonas aeruginosa* strain isolated from a Turkish patient hospitalized in France [5]. PER-3, a point-mutant derivative of PER-1, was identifieded in Aeromonas caviae in france [6]. a related enzyme ,PER-2, which has 86% amino-acid homology with PER-1, was found among *S. enterica* seovar typhimuriumstrain in argentina in 1996 [7] . The blaPER-1 gene is widespread in Acinetobacter spp., *Salmonella enterica* seovar Typhimurium in Turkey [8] and has also been detected in *Providencia* spp in that country [9]. In PER-1-producing strains, blaPER-1 gene was found to be either inserted in the chromosome as a part of composite transposons [10,11] or carried on plasmids [12,13] .

In this Paper, we describe the first description of PER-1-ESBL in a Tunisian hospital from clinical isolates of *Providencia stuartii* .

MATERIALS AND METHODS

Bacterial strains. We investigated 10 multiresistant clinical strains of *P. stuartii* isolated from different patients hospitalized in intensive care unit (ICU) of military Hospital in Tunisia. It was identified as *P.stuartii* either by automated (GNI+; bioMerieux Vitek) and by manual biochemical analyses using the Api 20E system (bioMérieux SA, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

The antibiotic susceptibilities of these strains were first determined by disk diffusion test [14] and the MICs were performed by a dilution technique on Mueller-Hinton liquid medium with inoculums of 10^6 CFU. The double-disk synergy test between amoxicillin–clavulanic acid and extended-spectrum cephalosporins was used to detect production of extended-spectrum ß-lactamases [15] .The results were interpreted according to the Guideliness Clinical Laboratory Standards Institute[16] after incubation at 37°C for 18 h [16].

ß-lactamase preparation.

Culture was grown overnight at 37°C in Trypto–Caseine Soja broth (TCS). Bacterial suspension was disrupted by sonication in UP 400 S (five times for 45 s each time) and centrifuged (10,000 rpm, 15 min, 4°C (Universel 32 R, Hettich). The supernatant contained the crude enzyme extract.

Isoelectric focusing (IEF). Isoelectric focusing of extracts was carried out in polyacrylamide gels with a pH range of 3 to 10 at a voltage range of 100 to 300 [17] . Estimations of pI values were made by comparison with extracts of reference bacterial strains producing the PER-1 (5.3), TEM-1 (5.4), TEM-2 (5.6), TEM-3 (6.3), and SHV-1 (7.6). ß-lactamases activities were revealed by iodemetric method using benzylpenicillin to 1 mM as substrate and ceftazidime (3 mM) in phosphate buffer (25 mM; pH 7) [18].

2.7. Plasmid profile and transfer of resistance

Plasmid DNA was extracted from clinical isolates by the method of Birnboim and Doly [19]. The resulting DNA preparation was analyzed to electrophoresis at 90 v for 2 h in a 0,8% agarose gel .

Transfer of resistance phenotypes by conjugation was performed with Escherichia coli J53 Azide^R as the recipient strain. Transconjugants were selected on Mueller-Hinton agar supplemented with 100 mg/l sodium azide to inhibit the growth of the donor strain, and with 2,5 mg/l ceftazidime to inhibit the growth of the recipient strain [20] . The resulting transconjugants were purified and identified with API 20 E

PCR amplification of bla-PER genes

Total DNA extraction was carried out for all samples using the heat-shock technique [21] and subjected to polymerase chain reaction (PCR) amplification with primers specific for the blaPER-1 genes. The DNA amplification programs consisted of an initial denaturing at 94 °C for 10 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. A final extension was performed at 72 °C for 7 min .The PCR products were separated in 1% agarose gel and visualised with UV.

DNA sequencing.

DNA sequencing was performed with an automated Applied Biosystems sequencer (ABI PRISM 3100). The nucleotide and deduced protein sequences were analyzed with BLAST at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

RESULTS

ß-Lactam resistance phenotype Susceptibility:

Synergies between amoxicillin-clavulanic acid and expanded-spectrum cephalosporins, such as cefotaxime and ceftazidime, were observed in a double disk diffusion test , suggesting ESBL production for all isolates . Minimal inhibitory concentrations (MICs) of ß-lactams showed resistance to penicillin and extended-spectrum cephalosporins for all isolates with higt-level resistance to cefotaxime , ceftazime and aztreonam only for three isolates which were retained for further analysis (Table.1). The transconjugants showed also significant degree of resistance with ticarcillin , ceftazidime, cefotaxime, and aztreonam (Table I).

Isoelectric focusing these three isolates were characterized by IEF ant revealed the production of an identical ß-lactamase band of approximately pI 7, 3 which corresponds to the pI of PER-1 ß-lactamase .

Table 1 : MICs of various antimicrobial Agents obtained for the clinical isolate P.stuartii , transconjugants and the E. coli J53 recipients.

Antibiotics	MIC (μg/ml) P.stuartii blaPER-1	Transconjugants Positive	E. coli J53
Ticarcillin	>512	>512	2
Cefotaxime	>512	256	<1
Ceftazidime	>512	64	<1
Cefoxitin	32	8	4
Cefpirome	8	8	<1
Aztreonam	>512	512	<1
Imipenem	<2	<2	<1

ß-Lactamase characterization PER–mediated resistance was confirmed by amplification and sequencing , using the primers PER-1. The three isolates tested were found positive for the PER gene .deduced amino acid sequence analysis revealed a 100% homology with that of blaPER-1.

Plasmid profiles and Transfer of resistance:
Conjugation studies, with ceftazidime selection and Escherichia coli J53 (Azide resistant) as the recipient, showed that the blaPER-1 gene was transferable and located on a 100 kb plasmid.

DISCUSSION

Ten isolates of *P. stuartii* were collected in intensive care units of military Hospital in Tunisia from blood specimens and presented an extended-spectrum resistance phenotype with a marked synergistic effect between association amoxicillin–clavulanic acid and cephalosporins. The results of antibiotic susceptibilities, IEF and sequencing result showed three of these 10 isolates produced PER-1 ESBL enzyme.
The PER-1 enzyme is a class A type ESBL , its three-dimensional structure reveals that it forms a subgroup in the class A superfamily, with a new fold in the Ω-loop a region , considered to be a canonical motif in class A enzymes [6] .this enzyme confers resistance to penicillin, cefotaxime, ceftazidime, and monobactams (aztreonam) but spares resistance to carbapenems and cephamycins [22].
The first PER-1 ß-lactamase-producing *P.aeroginosa* was isolated in 1991 from a turkish patient in paris [23], and the enzyme was present in 15 *P.aeroginosa* isolates collected in turkey between 1991 and 1993. In later studies; PER-1 was also identified in *Salmonella typhimurium* in 1995 and in *Providencia rettgeri* and *Klebsiella pneumonia* in 2003 [8, 9, 24, 25] .
To date, this ESBL has been identified in several countries such as France, Italy, Poland and South Korea and is particularly widespread in Turkey [1] .
In all of the study isolates, *bla*PER-1 resided in the same transposon named Tn*1213* [26] . This transposon was formed by two structurally related but significantly different insertion sequences , IS*Pa*12 and IS*Pa*13. An 8-bp duplication of the insertion site was noticed at the left-hand extremity of ISPa12and at the right-hand extremity of IS*Pa*13, clearly showing that this structure had transposed. Analysis of Tn*1213*-surrounding sequences in *P.aeroginosa, A. Baumanii* and *P.stuartii* isolates revealed that the transposition had occurred inside an IS element named IS*Pa14,* by interrupting its transposase gene [27] .
This is the first report of PER-1-producing *P. stuartii* in Tunisia indicates that the blaPER-1 gene has spread south of the Mediterranean and perhaps even to Africa. This case highlights the importance of spreading of the ß-lactamase-mediated resistance mechanisms between countries and continents, showing the importance of careful screening and the isolation of patients arriving from a different country.

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