Role of SHV β-lactamase variants in resistance of clinical *Klebsiella pneumoniae* strains to β-lactams in an Algerian hospital

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Three clinical *Klebsiella pneumoniae* strains, KpARG74, KpARG220 and KpARG185, isolated from a hospital in Algeria, carried the novel β-lactamases SHV-98, SHV-99 and SHV-100, respectively, and co-expressed TEM-1 and either CTX-M-3 or CTX-M-15. In contrast, transformed cells possessing the genes for these novel β-lactamases, i.e. EcDH5α-SHV-98, EcDH5α-SHV-99 and EcDH5α-SHV-100, respectively, carried unique sequence features of bla_{SHV} gene variants, enabling oxyimino-cephalosporin susceptibility and confirming that none of the transformants exhibited extended-spectrum β-lactamase (ESBL) properties. SHV-100 is apparently functional, despite differing from the SHV-1 sequence by duplication of 13 amino acids. The SHV-99 enzyme differed from the parental SHV-1 by the amino acid substitution Asp104→Gly, which is an important position in the development of the ESBL phenotype in TEM β-lactamases. This is the first time, to our knowledge, that this mutation has been reported in clinically occurring isolates. Thus, kinetic characterization of the SHV-99 enzyme was performed. The SHV-99 enzyme showed higher affinity (K_m of 196 μM), catalytic activity (k_{cat} of 0.5 s^{-1}) and catalytic efficiency (k_{cat}/K_m of 0.003 μM^{-1} s^{-1}) than SHV-1 β-lactamase against aztreonam. These results showed that the neutral glycine at residue 104 increased the affinity of the enzyme to aztreonam, but was unable to develop the ESBL phenotype in SHV enzymes. As the emergence of new threatening combinations of resistance determinants among nosocomial pathogens is further possible, this study has highlighted the need to reverse the spread of initial mutations.

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INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen with clinical importance among members of the family *Enterobacteriaceae* responsible for various community and hospital infections (Paterson & Bonomo, 2005). β-Lactamase production is the most frequent antibiotic resistance mechanism in this pathogen. In Algerian hospitals, β-lactamases are also emerging, with CTX-M enzymes being the most described extended-spectrum β-lactamases (ESBLs) (Messai *et al.*, 2008; Ramdani-Bouguessa *et al.*, 2006).

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Abbreviations: ESBL, extended-spectrum β-lactamase; IC_{50}, 50 % inhibitory concentration.

The GenBank/EMBL/DDBJ accession numbers for the gene sequences of SHV-98, SHV-99 and SHV-100 are AM941844, AM941845 and AM941846, respectively.

K. pneumoniae is intrinsically resistant to amoxicillin due to the production of a chromosomal enzyme, the SHV-1 β-lactamase (Babini & Livermore, 2000). However, the SHV β-lactamase family confers resistance to β-lactamase inhibitors/β-lactam combinations or to expanded-spectrum cephalosporins and monobactams, when one to five amino acid substitutions occur in the structural gene (http://www.lahey.org/studies). More frequent substitutions related to clavulanic acid resistance are in Met69 and Ser130, and those associated with the ESBL phenotype are at positions Gly238 and Glu240 (Paterson & Bonomo, 2005).

In this work, phenotypes expressed by three clinical *K. pneumoniae* strains producing SHV enzymes, first isolated in an Algerian hospital, are reported. Molecular characterization demonstrated that SHV-98 and SHV-99 have one novel substitution and that SHV-100 has an amino acid duplication when compared with SHV-1. Biochemical characterization of the SHV-99 enzyme is also described as

this is the first time, to our knowledge, that the mutation Asp104→Gly has appeared in clinical SHV β-lactamase-producing isolates.

METHODS

Bacterial isolates. During the months of February, May and June 2005, three *K. pneumoniae* isolates (KpARG74, KpARG220 and KpARG185) were collected from male patients attending the Centre Hospitalo-Universitaire Mustapha Pacha, a 1800-bed facility located in Algiers, Algeria. These strains were collected from distinct clinical departments (orthopaedics, neurosurgery and intensive care unit, respectively) and from several biological sources (pus, cerebrospinal fluid and urine, respectively).

Antimicrobial susceptibility testing. The MICs of antibiotics tested against clinical isolates and respective transformants were each determined by an agar dilution method according to the French Society of Microbiology (CA-SFM; Bonnet *et al.*, 2010). The MICs of the β-lactam antibiotics tested were each determined alone and four antibiotics were tested in combination with fixed concentrations of β-lactamase inhibitors: amoxicillin, cefotaxime and ceftazidime were tested with 2 μg clavulanic acid ml^{-1} and piperacillin was tested with 4 μg tazobactam ml^{-1}. Clinical isolates that were not susceptible to one or more extended-spectrum β-lactams (cefotaxime, ceftazidime and/or ceftriaxone) and showing synergy between these antibiotics and clavulanic acid were inferred to produce ESBLs.

β-Lactamase characterization. β-Lactamases were characterized by isoelectric focusing of ultrasonicated bacterial extracts and β-lactamase genes (bla_{TEM}, bla_{SHV}, bla_{OXA-1}, *ampC* and bla_{CTX}) were detected and identified by PCR and sequencing using previously described protocols (Caniça *et al.*, 1997; Mendonça *et al.*, 2007).

β-Lactamase gene transfer experiments. The bla_{SHV-99} gene was cloned into plasmid pBK-CMV and transferred into electrocompetent *Escherichia coli* DH5α Δ*ampC* by electroporation as described previously (Mendonça *et al.*, 2008). Transformants were selected on LB agar supplemented with 30 μg kanamycin ml^{-1} and 50 μg amoxicillin ml^{-1}. To confirm the presence of the inserted genes and to determine their orientation, PCR was performed as described previously (Mendonça *et al.*, 2008).

Biochemical characterization of the SHV-99 enzyme. After electroporation of the SHV-encoding plasmid (pBK-SHV-99), the β-lactamase was extracted from an overnight culture by sonication and purified by ion exchange and gel filtration according to previously described protocols (Labia *et al.*, 1973). SHV-99 total protein concentration was estimated by the BCA protein assay kit (Pierce). The purity of enzyme extracts was estimated by SDS-PAGE. The kinetic constants of purified extracts of SHV-99 were obtained by a computerized microacidimetric method using a 702 SM Titrino pH-stat apparatus (Metrohm) (Mendonça *et al.*, 2008). The complete hydrolysis time-courses were analysed and kinetic progress curves were fitted by non-linear least-squares regression. These parameters were determined and compared to those of the SHV-1 enzyme, which had been previously purified and characterized (Mendonça *et al.*, 2008), for eight β-lactams.

RESULTS AND DISCUSSION

Antimicrobial susceptibility testing revealed that the clinical isolates were resistant to the penicillins, cephalosporins and monobactam tested. β-Lactamase inhibitors only partially restored activity of the penicillins when tested in combination (Table 1).

Genotypic characterization showed that clinical isolates KpARG74, KpARG220 and KpARG185 had the bla_{SHV-98}, bla_{SHV-99} and $bla_{SHV-100}$ genes, respectively. These genes were cloned and transferred to an isogenic system to evaluate their phenotypic contributions. The SHV-98-, SHV-99- and SHV-100-producing transformants exhibited a β-lactam resistance phenotype similar to that of the clinical isolates with respect to penicillins. However, in contrast to the clinical isolates, EcDH5α-SHV-98, EcDH5α-SHV-99 and EcDH5α-SHV-100 were susceptible to oxyimino-cephalosporins, thus confirming that none of the transformants exhibited ESBL properties. Indeed, SHV-type β-lactamases contributed poorly to the resistance profile of the clinical isolates, which was mostly reflected by the expression of a CTX-M-type enzyme. Both clinical isolates and transformants were susceptible to imipinem and ciprofloxacin. Only the transformants were susceptible to gentamicin and trimethoprim, in contrast to the clinical isolates (Table 1).

Isoelectric focusing of KpARG74, KpARG220 and KpARG185 showed two bands that were not identified in the transformants, one cofocusing with TEM-1 (pI 5.4) and the other with CTX-M-3 or CTX-M-15 β-lactamases (pI 8.9 and 8.4, respectively); the presence of CTX-M-3 or CTX-M-15 β-lactamases explains the ESBL phenotype of the clinical isolates, with CTX-M-15 β-lactamase disseminated worldwide (Messai *et al.*, 2008; Paterson & Bonomo, 2005; Ramdani-Bouguessa *et al.*, 2006). Additionally, β-lactamases SHV-98, SHV-99 and SHV-100 produced by both clinical isolates and transformants had pIs of 7.6, 7.8 and 7.2, respectively.

Sequencing enabled us to identify the novel amino acid substitution Ser271→Ile in SHV-98, the substitution Asp104→Gly in SHV-99 and the amino acid duplication (n=13, SESQLSGRVGMIE) between positions 36 and 48 in the SHV-100 β-lactamase. As shown by MIC data, SHV-100 is apparently functional despite the insertion. As repeated attempts to detect the presence of these SHV-encoding genes in plasmid DNA failed, it is presumed that they have a chromosomal location (data not shown).

In class A β-lactamases, Ambler position 104 shows some amino acid variability (Guo *et al.*, 1999; Ambler *et al.*, 1991); however, previous investigators have shown that amino acid substitution at this position is important in the development of the ESBL phenotype in TEM β-lactamases (Petit *et al.*, 1995). To evaluate the role of this substitution, which has not to our knowledge been previously reported in naturally occurring isolates, in SHV enzymes the kinetic parameters of the purified SHV-99 enzyme (purity rate ⩾98 %) were determined for eight β-lactams (Table 2). SHV-99 showed comparable K_m values for penicillins to SHV-1 (K_m of 5–13 μM and 11–31 μM, respectively). For cefalotin, kinetic parameters were slightly different for the two enzymes: the SHV-99 enzyme had diminished affinity

Table 1. MICs of antibiotics for the clinical *K. pneumoniae* KpARG74, KpARG220 and KpARG185 isolates, SHV-1-, SHV-98-, SHV-99- and SHV-100-producing transformants and the recipient *E. coli* EcDH5α Δ*ampC*

Strains: 1, *E. coli* EcDH5α Δ*ampC*; 2, *E. coli* EcDH5α-SHV-1 (pBK-SHV-1); 3, *K. pneumoniae* KpARG74 (SHV-98/TEM-1/CTX-M-3); 4, *E. coli* EcDH5α-SHV-98 (pBK-SHV-98); 5, *K. pneumoniae* KpARG220 (SHV-99/TEM-1/CTX-M-15); 6, *E. coli* EcDH5α-SHV-99 (pBK-SHV-99); 7, *K. pneumoniae* KpARG185 (SHV-100/TEM-1/CTX-15); 8, *E. coli* EcDH5α-SHV-100 (pBK-SHV-100). MIC values for *E. coli* EcDH5α Δ*ampC* and *E. coli* EcDH5α-SHV-1 are from Mendonça *et al.* (2008).

Antibiotic	1	2*	3	4*	5	6*	7	8*
Amoxicillin	8	2.048	>4.096	1.024	>4.096	>4.096	>4.096	2.048
Amoxicillin + CLA†	8	8	8	≤0.5	>64	8	16	8
Ticarcillin	4	1.024	>4.096	4.096	>4.096	>4.096	>4.096	>4.096
Piperacillin	2	64	>64	>64	>64	>64	>64	64
Piperacillin + TAZ‡	≤16	≤16	≤16	≤16	256	≤16	≤16	≤16
Cefalotin	8	16	64	8	>1.024	32	>1.024	8
Cefuroxime	4	4	>256	128	>256	8	>256	8
Ceftazidime	≤0.5	≤0.5	4	≤0.5	64	1	16	≤0.5
Ceftazidime + CLA†	≤0.5	≤0.5	≤0.5	≤0.5	1	≤0.5	≤0.5	≤0.5
Ceftriaxone	≤0.25	≤0.25	>512	≤0.25	>512	≤0.25	512	≤0.25
Cefotaxime	0.06	0.06	>256	≤0.25	>256	0.06	256	0.06
Cefotaxime + CLA†	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	≤0.25	≤0.25
Aztreonam	≤0.5	≤0.5	64	≤0.5	512	≤0.5	32	≤0.5
Imipenem	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Ciprofloxacin	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Gentamicin	≤2	≤2	>8	≤2	>8	≤2	>8	≤2
Trimethoprim	≤0.125	≤0.125	>64	≤0.125	>64	≤0.125	>64	≤0.125

**E. coli* DH5α-SHV-1, *E. coli* DH5α-SHV-98, *E. coli* DH5α-SHV-99 and *E. coli* DH5α-SHV-100 were the transformants producing SHV-1, SHV-98, SHV-99 and SHV-100, respectively.

†CLA, Clavulanic acid at a fixed concentration of 2 μg ml^{-1}.

‡TAZ, Tazobactam at a fixed concentration of 4 μg ml^{-1}.

(K_m, 2.5-fold), catalytic activity (k_{cat}, 3.5-fold) and catalytic efficiency (k_{cat}/K_m, 9-fold) compared to SHV-1, with no impact on the MIC value. Neither enzyme exhibited significant enzymic activity against oxyimino-cephalosporins. Nevertheless, the Asp104→Gly mutation of SHV-99 generated a considerably higher affinity (K_m of 196 μM), catalytic activity (k_{cat} of 0.5 s^{-1}) and catalytic efficiency (k_{cat}/K_m of 0.003 μM^{-1} s^{-1}) against aztreonam than the parental enzyme, whose values were not determinable because the hydrolysis rate was too low. Fifty per cent

Table 2. Kinetic constants and IC_{50} values of SHV-99 and SHV-1 β-lactamases

Values for kinetic enzyme parameters are means ± SD. ND, Not determinable because the hydrolysis rate was too low.

Antibiotic	SHV-1*				SHV-99			
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	IC_{50} (μM)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	IC_{50} (μM)
Clavulanic acid				0.17				0.02
Tazobactam				0.11				0.03
Penicillin G	23 ± 0.42	1937 ± 82	84.2 ± 2.0		12 ± 0.11	778 ± 61	62.3 ± 4.4	
Amoxicillin	31 ± 1.29	1044 ± 10	33.7 ± 1.1		11 ± 0.26	563 ± 8	49.6 ± 1.8	
Ticarcillin	11 ± 3.40	220 ± 49	20.0 ± 1.7		5 ± 0.93	58 ± 2	13.0 ± 2.4	
Piperacillin	24 ± 0.53	1490 ± 96	62.1 ± 2.7		13 ± 1.43	563 ± 13	43.5 ± 6.5	
Cefalotin	40 ± 1.46	128 ± 33	3.2 ± 0.8		102 ± 11.38	37 ± 2	0.37 ± 0.04	
Ceftazidime	142 ± 3.18	<0.1	<0.001		136 ± 4.09	<0.1	<0.001	
Cefotaxime	257 ± 20.65	<0.1	<0.001		183 ± 0.72	<0.1	<0.001	
Aztreonam	ND	<0.1	<0.001		196 ± 0.60	0.5 ± 0.001	0.003	

*The kinetic constants for SHV-1 are from Mendonça *et al.* (2008).

inhibitory concentrations (IC_{50}) indicated that SHV-99 was susceptible to clavulanic acid (IC_{50} of 0.02 μM) and tazobactam (IC_{50} of 0.03 μM), as observed with SHV-1 (IC_{50} values of 0.17 and 0.11 μM, respectively) (Table 2). The possibility that this novel β-lactamase is an inhibitor-resistant SHV enzyme, examples of which have been found by other workers, is therefore excluded (Mendonça *et al.*, 2008).

The hydrophilic, negatively charged side-chain of residue Asp104, together with Tyr105, is located on the left side of the active site pocket, being one of the active site loops of serine β-lactamases. This residue is hydrogen-bonded to Asn132, which may therefore stabilize the catalytic Ser130 in the conserved Ser-Asp-Asn (SDN) loop from positions 130 to 132 (Kuzin *et al.*, 1999). The increased affinity of the enzyme to aztreonam and, given its location in the catalytic pocket, the interaction with residue 104 seems to be the first step in the substrate binding and recognition of oxyimino-β-lactams (Bethel *et al.*, 2006).

Variations in the MICs of penicillins and cefalotin reveal that substitutions at position 104 are tolerated in both SHV and TEM enzymes (Bethel *et al.*, 2006; Guo *et al.*, 1999; Petit *et al.*, 1995; Knox, 1995). However, according to our results, the substitution of an Asp by a neutral Gly at residue 104 in SHV has a poor impact on the properties of the enzyme, at least in the absence of any other substitutions. These are interesting data since the structurally equivalent position in TEM β-lactamases is known to have more relevance (Guo *et al.*, 1999; Petit *et al.*, 1995; Knox, 1995). Our kinetic study confirmed that the Asp104→Gly substitution alone is unable to develop the ESBL phenotype in SHV enzymes, as suggested by phenotypic characterization, unlike in TEM β-lactamases (Petit *et al.*, 1995). The impact of this mutation might be more relevant if additional ESBL-conferring mutations are present (Bethel *et al.*, 2006).

CTX-M-type β-lactamases are the ESBLs most commonly produced by isolates in Algerian hospitals (Messai *et al.*, 2008; Ramdani-Bouguessa *et al.*, 2006), as in isolates from our study. However, their co-production with other β-lactamases is frequent, such as SHV and/or TEM, conferring resistance phenotypes which are additive, with the respective consequences at clinical and therapeutic levels (Paterson & Bonomo, 2005; Ramdani-Bouguessa *et al.*, 2006). In parallel, the importance of enzymes that do not contribute to β-lactamase inhibitor resistance or to the ESBL phenotype is an open issue. However, as already demonstrated, non-ESBL enzymes may facilitate the evolution to ESBLs under selection pressure (Hammond *et al.*, 2008).

The easy horizontal and vertical spread of β-lactamases worldwide will therefore dictate future research. Thus, considering the diversity of the enzymic characteristics of β-lactamases, the biochemical characterization of novel enzymes is essential to understand their individual contribution to resistance phenotypes of pathogens with an impact on public health (Bush & Jacoby, 2010). This characterization will also have an important role as the emergence of new threatening combinations of resistance determinants among nosocomial pathogens is further possible. Overall, this study has highlighted the need to reverse the spread of initial mutations.

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