Journal of Antimicrobial Chemotherapy (2000) **45**, 349–351

JAC

Brief reports

First identification of an SHV-12 extended-spectrum β-lactamase in *Klebsiella pneumoniae* isolated in Italy

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A clinical isolate of *Klebsiella pneumoniae* highly resistant to third- and fourth-generation cephalosporins, cephamycins and aminoglycosides, was isolated in 1991 from urine. Analysis of a crude extract showed the presence of three β-lactamases with isoelectric points of 6.6, 7.5 and 8.2. The enzyme with pI 8.2 was transferred by conjugation into *Escherichia coli* K-12 J53 and was responsible for the resistance to third-generation cephalosporins and monobactams, but not to other antibiotics. Kinetic studies of partially purified β-lactamase from the transconjugant strain confirmed that the enzyme was able to hydrolyse ceftazidime, cefotaxime and aztreonam but not cephamycins. Analysis of the transconjugant showed the presence of two small non-conjugative plasmids of 14 and 6 kb. A polymerase chain reaction was performed using primers specific for the bla_{SHV} gene and a fragment of the expected size (about 961 bp) was obtained with both the *K. pneumoniae* clinical isolate and the transconjugant. Nucleotide sequence analysis of the fragment showed that it encoded the enzyme SHV-12, derived from SHV-5 (with Gln-35 to Leu). This is the first report of an SHV-12-like enzyme isolated in Italy.

Introduction

The excessive use of expanded-spectrum cephalosporins in clinical practice is the main factor responsible for the appearance of extended-spectrum β-lactamases (ESBLs) in enteric bacteria. Several TEM and SHV enzymes have emerged by mutation of the genes encoding parental TEM and SHV enzymes,[1] and are able to hydrolyse stable antibiotics such as ceftazidime, cefotaxime, ceftriaxone and aztreonam but not carbapenems and cephamycins.

Since 1983, the year in which ESBLs were first described,[2] these enzymes have increased in number and variety. Currently, there are over 60 derivatives of TEM-1 and TEM-2 and 12 derivatives of SHV-1 β-lactamases. These enzymes are distributed worldwide,[3] but so far there is no significant information on the prevalence of ESBL-producing strains in Italy.

ESBL genes are generally located on large transferable plasmids that often carry other resistance determinants such as those for aminoglycosides, tetracycline, sulphonamides and chloramphenicol.[4] Dissemination of these plasmids is responsible for the spread of β-lactam resistance in Enterobacteriaceae, particularly in *Klebsiella pneumoniae*.

The present study was undertaken to characterize an ESBL enzyme obtained from a clinical isolate of *K. pneumoniae* in Italy.

Materials and methods

Bacterial strains

K. pneumoniae 4940 (Kp4940) was isolated in 1991 from urine and identified using the API-20E system (bioMérieux, Marcy l'Etoile, France). *Escherichia coli* K-12 J53 (rifampicin-resistant) and *E. coli* HB101 (lac$^+$, streptomycin-resistant) were used as recipients for conjugation experiments. *E. coli* 39 R861 (containing plasmids of 98, 42, 24 and 4.6 MDa) was used as a standard for plasmid analysis.

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Susceptibility testing

The MICs of amoxycillin, piperacillin, ceftazidime, cefotaxime, aztreonam, cefotetan, cefepime, imipenem, amikacin, gentamicin, tetracycline and chloramphenicol were determined by the agar dilution method using an inoculum of 10^4 cfu/spot according to NCCLS guidelines.[5] Clavulanic acid and tazobactam were used at fixed concentrations of 2 and 4 mg/L, respectively.

Conjugation experiments

The transfer of β-lactam resistance from Kp4940 to *E. coli* K-12 J53 was performed as previously described,[6] with the modification that IsoSensitest agar containing 2 mg/L ceftazidime and 250 mg/L rifampicin was used as the transconjugant selective medium. Identification of the transconjugant (Kp1) was confirmed by API 20E and that of β-lactamase by isoelectric focusing (IEF).

Analytical IEF

IEF was performed on polyacrylamide gels containing ampholines (pH range 3.5–9.5) with the supernatants of Kp4940 and Kp1 transconjugant sonicates, as previously described.[7] The gel was stained for β-lactamase activity with 0.5 mM nitrocefin solution.

β-Lactamase preparation and purification

β-Lactamase extraction was performed as previously described.[7] The clarified supernatant containing the β-lactamase was extensively dialysed against 50 mM Tris–HCl buffer pH 5.5 and loaded on to a Sepharose S FF column (2.5 × 30 cm) (Pharmacia Biotech, Milan, Italy) equilibrated with the same buffer. The enzyme was eluted with a linear gradient (0–1 M NaCl) in the same buffer; the fractions containing β-lactamase activity were pooled and concentrated. Hydrolytic activity against β-lactams was evaluated by spectrophotometry at 37°C in sodium phosphate buffer pH 7.0 containing 0.2 M KCl. Kinetic parameters were calculated from a Lineweaver–Burk plot.

DNA techniques

Plasmid DNA was prepared from wild-type and transconjugant strains as reported by Kado & Liu.[8] SHV genes were amplified by polymerase chain reaction (PCR) using the primers 5′-CGCCGGAGCTGCCTTTATCGGCCCTC-3′ and 5′-CGCGGGTACGTTGCCAGTGCTCGATCA-3′. The conditions employed were as follows: 95°C for 12 min, then 25 cycles consisting of 30 s denaturation at 95°C, 1 min annealing at 52°C and 1 min extension at 72°C, finally 72°C for 7 min. Sequence determination of the SHV gene was performed on both strands of amplimers with a dideoxy-chain termination method using an automated DNA sequencer ABI PRISM 377 (Perkin Elmer, Milan, Italy).

Results and discussion

The Table shows the susceptibility of Kp4940. This strain showed an unusual resistance phenotype, being highly resistant to third- and fourth-generation cephalosporins, monobactams, cephamycins and aminoglycosides. The transconjugant (Kp1) acquired resistance only to penicillins, ceftazidime, cefotaxime and aztreonam; the frequency of conjugation was 10^{-4}. The addition of clavulanic acid drastically reduced the MIC values of third-generation cephalosporins in Kp4940 and Kp1 transconjugant, suggesting the presence of a plasmid-encoded extended-spectrum β-lactamase (ESBl).

The crude extract of Kp4940 showed a predominant β-lactamase band which co-focused with SHV-5 at a pI of 8.2 and two other enzymes at pIs of 6.6 and 7.5 (data not shown). The enzyme with pI 6.6 was possibly an isoform of the enzyme with pI 7.5 (which could be the species-specific chromosomal β-lactamase of *K. pneumoniae*) as it showed the same hydrolytic profiles (data not shown). The β-lactamase with pI 8.2 was the only β-lactamase present in the transconjugant Kp1, confirming that it was responsible for the resistance to third-generation cephalosporins and aztreonam. The partially purified β-lactamase with pI 8.2 extracted from Kp1 hydrolysed ceftazidime, cefotaxime

Table. *In vitro* activity of various antibiotics against Kp4940 and its transconjugant Kp1

	MIC (mg/L)	
Antibiotics	Kp4940	Kp1
Amoxycillin	>512	512
Co-amoxiclav	128	4
Piperacillin	>256	32
Piperacillin–tazobactam	>128	1
Ceftazidime	>256	64
Ceftazidime–clavulanate	4	0.06
Cefotaxime	>256	8
Cefotaxime–clavulanate	0.5	<0.015
Aztreonam	>256	128
Aztreonam–clavulanate	0.5	<0.015
Cefotetan	64	<0.5
Cefepime	>128	<0.25
Amikacin	4	2
Gentamicin	>32	4
Tetracycline	4	1
Chloramphenicol	16	16
Imipenem	0.06	0.06

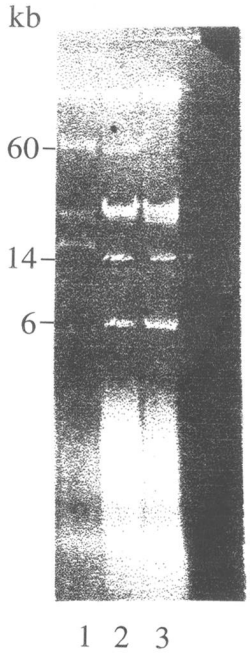

Figure. Plasmid profile of Kp4940 and Kp1. Lane 1, standard 39R 861; lane 2, Kp4940; lane 3, Kp1 transconjugant.

and aztreonam as well as penicillin and cephaloridine. This behaviour was similar to that of the previously described enzyme SHV-12.[9]

Plasmid analysis of Kp4940 and Kp1 indicated that the resistance gene was carried by one of two small plasmids with estimated sizes of 6 and 14 kb, respectively. A large plasmid (60 kb) was also present in Kp4940 but was not transferred to Kp1 (Figure). The small size of the Kp1 plasmids suggested that they were not self-transferable, but probably mobilized by the 60 kb plasmid. A second conjugation experiment between Kp1 and *E. coli* HB101 failed, adding weight to this suggestion.

The 961 bp fragment of the SHV gene was amplified and directly sequenced on both strands demonstrating that the ESBL gene was a $bla_{\text{SHV-12}}$ with characteristic mutations[10] (glutamine-35, serine-238 and lysine-240, according to Ambler's numbering).

In conclusion, the enzyme studied was identified as SHV-12 which was encoded by a non-self-transferable plasmid, which did not carry other resistance determinants. SHV-12 had not been reported previously in Italy.

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Received 12 July 1999; returned 14 October 1999; revised 29 October 1999; accepted 15 November 1999