

# Resistance to extended-spectrum $\beta$ -lactams by the emergence of SHV-12 and the loss of OmpK35 in *Klebsiella pneumoniae* and *Escherichia coli* in Malaysia

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**Background and purpose:** In addition to  $\beta$ -lactamase production, loss of porins confers resistance to extended-spectrum  $\beta$ -lactams in *Klebsiella pneumoniae* and *Escherichia coli* infection. This study describes the detection of SHV-12 extended-spectrum  $\beta$ -lactamase (ESBL) subtype and the loss of OmpK35 porin in 4 strains of *K. pneumoniae* and *E. coli*.

**Methods:** Isoelectric focusing was performed to detect  $\beta$ -lactamases in 4 strains of *K. pneumoniae* and *E. coli*. The presence of the SHV gene in the 4 isolates was characterized by polymerase chain reaction, DNA sequencing, and DNA hybridization. Loss of porin in these strains was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis.

**Results:** The strains of *K. pneumoniae* and *E. coli* were confirmed to be ESBL producers and were resistant to cefoxitin, with minimal inhibitory concentration values of 512  $\mu$ g/mL. All 4 strains had  $\beta$ -lactamases with an isoelectric value of 8.2. The SHV gene from these strains was characterized to be the SHV-12 subtype and was plasmid-borne. The deduced amino acid sequence showed that the SHV-12  $\beta$ -lactamase was a derivative of the more common ESBL, SHV-5 subtype. All the strains showed absence of the OmpK35 porin.

**Conclusion:** Resistance of the strains towards extended-spectrum  $\beta$ -lactams was a result of a dual-mechanism — the production of SHV-12 enzymes and loss of the OmpK35 porin.

**Key words:** beta-Lactamase SHV-12; *Escherichia coli*; *Klebsiella pneumoniae*; OmpK35 porin, *Klebsiella pneumoniae*

## Introduction

The mechanism of resistance to extended-spectrum  $\beta$ -lactams in *Klebsiella pneumoniae* and *Escherichia coli* is predominantly due to extended-spectrum  $\beta$ -lactamases (ESBLs), mainly of the SHV family. The SHV gene has evolved from an ancestor gene by single point mutations, resulting in a wide range of approximately 63 variants. Extensive use of  $\beta$ -lactam antibiotics for therapeutic purposes has resulted in

stepwise mutations, with either single or multiple amino acid changes in the encoded enzymes resulting in a broader substrate profile [1]. In addition, non-enzymatic mechanisms that confer resistance to all cephalosporins, including the cephamycins, have been reported. These mechanisms include the loss of specific proteins within the outer membrane (porins) that results in decreased permeability to the  $\beta$ -lactams [2]. Two major porins, OmpF and OmpC, have been described in *E. coli*, both of which are homologous to the OmpK35 and OmpK36 porins, respectively, from *K. pneumoniae*. It is important for laboratories to be able to detect for such novel mechanisms of resistance since they also play a major role in the emergence of extended-spectrum  $\beta$ -lactam-resistant strains of *K. pneumoniae* and *E. coli*.

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This study evaluated resistance towards extended-spectrum  $\beta$ -lactams in *E. coli* and *K. pneumoniae* due to production of SHV-12 ESBL and the loss of the OmpK35 porin.

## Methods

Three strains of *K. pneumoniae* and 1 strain of *E. coli*, that were ESBL producers and ceftaxime-resistant, were isolated from routine clinical samples collected from inpatients at the University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia, between April 2001 and September 2002. Two of the *K. pneumoniae* strains (2/B14 and 2/F16) were isolated from blood and the other strain (1731) was isolated from urine. The *E. coli* strain (987) was isolated from a wound swab. All the strains were identified using the API 20E kit (bioMérieux SA, Marcy l'Etoile, France).

Screening for ESBL production was carried out by the double-disc synergy test [3] as described by the National Committee for Clinical Laboratory Standards (NCCLS) using ceftazidime, cefotaxime, ceftriaxone, and aztreonam in combination with an amoxicillin-clavulanate disc. Phenotypic confirmation was done by the ESBL screen test using ceftazidime in combination with clavulanate Etest strips (AB Biodisk, Solna, Sweden).

Determination of the minimal inhibitory concentrations (MICs) of ceftazidime, cefotaxime, ceftriaxone, ceftaxime, cefepime, imipenem, and ceftazidime in combination with clavulanate were carried out using the NCCLS agar dilution method [4]. *E. coli* American Type Culture Collection (ATCC) 25922 and *E. coli* ATCC 35218 were used as the control organisms. All the antibiotics except for imipenem were in powder form with known potency and were purchased from Sigma-Aldrich Corp (St Louis, MO, USA) with the exception of cefepime and clavulanate, which were generously provided by Bristol-Myers Squibb (Rome, Italy) and SmithKline Beecham (Brentford, UK), respectively. The parenteral preparation of imipenem was obtained from Merck & Co. Inc. (Cologne, Germany).

Test strains were cultured in Luria Bertani (LB) medium 50 mL with overnight aeration at 37°C. Cells were disrupted by the standard sonication method [5] and the supernatant containing crude  $\beta$ -lactamase extract was analyzed by isoelectric focusing (IEF) on Phast Gels using the PhastSystem (Pharmacia, Uppsala, Sweden).  $\beta$ -Lactamase activity was visualized by nitrocefin. *E. coli* producing SHV-1 with an isoelectric

point (pI) value of 7.6 and SHV-5 with a pI value of 8.2 were used as controls.

Polymerase chain reaction (PCR) amplification of the complete coding region of the SHV gene was carried out using specific SHV primers as described by Babini and Livermore [6]. The SHV primer sequences used were: 5'-ATGCGTTATATTCGCCTGTG-3' (F) and 5'-GTTAGCGTTGCCAGTGCTCG-3' (R) producing an amplicon of 865 bp [6] in size. PCR products were sized using a 100-bp ladder (Promega, Madison, WI, USA). *E. coli* producing SHV-5 was used as the control.

DNA sequencing of the PCR products of the *bla*<sub>SHV</sub> gene were carried out using an automated DNA sequencer, ABI PRISM 377 (Perkin Elmer Applied Biosystems, Foster City, CA, USA) using the PCR primer.

Plasmids were extracted by the method of Kado and Liu [7]. *E. coli* 39R861 containing plasmids of known sizes was used to estimate the size of each of the large plasmids.

Nylon membrane containing the plasmids extracted from the strains, together with positive controls,  $\lambda$  DNA/*Hind*III marker (Promega) and the 475-bp PCR product of the SHV gene [8] (synthesized from the SHV-12 gene), were hybridized with biotin-labeled probes. Hybridization involved the reaction of the membrane with streptavidin-alkaline phosphatase conjugate (Sigma-Aldrich Corp), followed by the addition of BCIP/NBT (5'-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium) substrate (KPL, Gaithersburg, MD, USA) to detect the presence of plasmid-mediated SHV genes. Biotin-labeled probes included the PCR product of the SHV gene and  $\lambda$  DNA/*Hind*III marker.

Outer membrane proteins (OMPs) were extracted using the modified method of Matsuyama et al [9]. Briefly, 500 mL of exponential-phase bacterial cells grown in nutrient broth (Amersham Biosciences, Piscataway, NJ, USA) were harvested. The cells were resuspended in 20 mL of 10 mM phosphate buffer, pH 7.2 (Oxoid, Cambridge, UK), and subjected to 6 rounds of sonication at 30 KHz output for 30 sec, with intermittent 30-sec cooling periods. The resulting supernatant was treated with 2% Triton X-100 (Sigma-Aldrich Corp) before final centrifugation at 12,000 rpm for 90 min. The extracted OMPs were solubilized in Laemmli's sample buffer at 96°C. Samples were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% SDS gels, after which OMPs were visualized using Coomassie Blue.

Porins were tentatively identified on the basis of porin expression being suppressed by high-osmolarity media [10]. A single colony of bacteria was grown in a high-osmolarity medium (Mueller-Hinton broth) and a low-osmolarity medium (nutrient broth). The OMPs were extracted as described previously and separated on the SDS-PAGE gels. The OMP profiles expressed in both media were compared, and porins were identified from the gel and sized with reference to porins from *K. pneumoniae* ATCC 13883 — which expresses OmpK35 and OmpK36 porins, as well as OmpA, which is not a porin. In low-osmolarity media, both porins in the control strain are highly expressed when compared with their level of expression in high-osmolarity media. Based on the differences in osmolarity of the media from which the OMPs were extracted, the porins from clinical samples could be identified.

SDS-PAGE gels containing OMPs from all strains and the positive control, *K. pneumoniae* ATCC 13883, were transferred onto nitrocellulose membrane by the modified method of Towbin et al [11] by using a semi-dry transfer system. The membrane was incubated overnight with anti-OmpK35 raised in rabbit (dilution, 1:1000). Following overnight incubation, the bound antiserum was reacted with biotin-labeled anti-rabbit immunoglobulin G (dilution, 1:400,000) [Sigma-Aldrich Corp]. Streptavidin–alkaline phosphatase (Sigma-Aldrich Corp) was added to the membrane, followed by the addition of BCIP/NBT substrate.

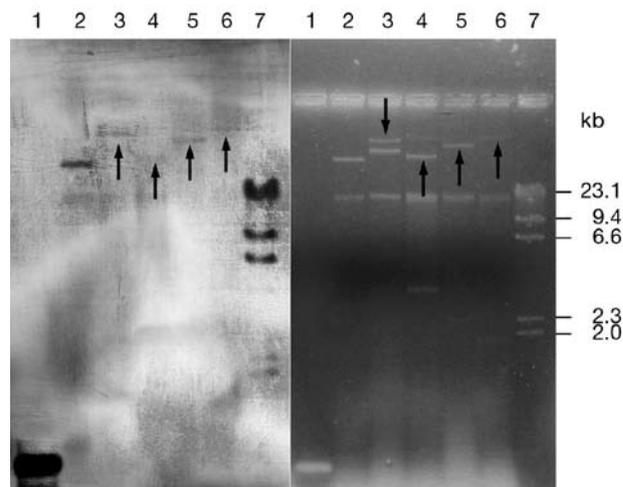
## Results

Three *K. pneumoniae* strains and 1 *E. coli* strain were confirmed to be ESBL producers by the double-disk synergy test and were resistant to cefoxitin, a marker for resistance towards 7- $\mu$ -methoxy-cephalosporins. In addition, the phenotypic confirmatory test, the ESBL screen test, showed a >4-fold reduction in the MIC values of ceftazidime in combination with clavulanate when compared with the MIC values of ceftazidime, confirming that all 4 strains were ESBL producers. All strains showed high levels of resistance to ceftazidime, ceftriaxone and cefotaxime (MIC values of >256  $\mu$ g/mL, 128  $\mu$ g/mL, and 128  $\mu$ g/mL, respectively). The strains were highly resistant to cefoxitin, with an MIC value of 512  $\mu$ g/mL, while the MIC value of cefepime was 32  $\mu$ g/mL.

The MIC value of imipenem for the 4 strains ranged from 0.5  $\mu$ g/mL to 1  $\mu$ g/mL. IEF analyses

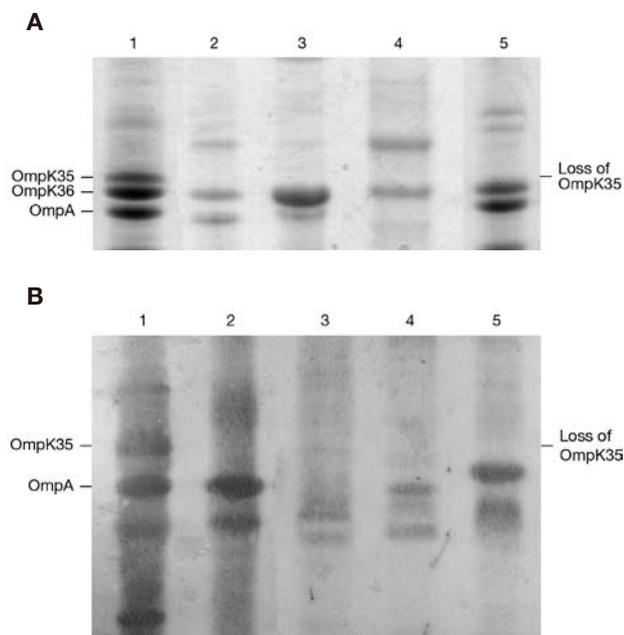
showed that the strains had  $\beta$ -lactamase, with a pI value of 8.2, correlating with the pI value of SHV-12. The 865-bp PCR product of the SHV gene was detected in all strains. The nucleotide sequence of the SHV gene obtained from strains 1731 (GenBank accession number, DQ408259), 987 (GenBank accession number, DQ408261), and 2/B14 (GenBank accession number, DQ408262) were homologous to the DNA sequence of *bla*<sub>SHV-12</sub> (GenBank accession number, AY826418) of *K. pneumoniae* strain Kp 2183 [12] with 100% homology. The SHV-12 gene of strain 2/F16 (GenBank accession number, DQ408260) matched the *bla*<sub>SHV-12</sub> gene of *K. pneumoniae* plasmid pK7746 strain (GenBank accession number, AY008838) [13] with 100% homology. Deduced amino acid sequence analyses revealed that the SHV-12 subtype was closely related to the SHV-5 [14] subtype, and differed by a single amino acid substitution from leucine to glutamine (L<sub>35</sub> to Q<sub>35</sub>).

The SHV-12 gene was located on large plasmids ranging in size from 60 to 150 kb, as shown by DNA hybridization with an SHV probe (Fig. 1). Comparison of the porin profiles of these strains with the porin profile of *K. pneumoniae* ATCC 13883 strain, which expresses OmpK35 and OmpK36 porins, showed that all test strains had the constitutively expressed OmpA protein, but the strains showed absence of OmpK35 on polyacrylamide gel (Fig. 2A) and Western blot (Fig. 2B).



**Fig. 1.** DNA hybridization of the SHV-12 gene on to large plasmids of all strains suggests plasmid mediation.

Lane 1: SHV polymerase chain reaction product; Lane 2: plasmid-mediated SHV-5 producing *Escherichia coli*; Lane 3: 1731; Lane 4: 987; Lane 5: 2/F16; Lane 6: 2/B14; Lane 7:  $\lambda$ DNA/*Hind*III marker.



**Fig. 2.** Outer membrane protein (OMP) profiles by (A) sodium dodecyl sulfate polyacrylamide gel electrophoresis and (B) Western blot analysis of the 4 strains suggest cefoxitin resistance due to loss of OmpK35 porin.

Lane 1: *Klebsiella pneumoniae* ATCC 13883; Lane 2: 1731; Lane 3: 2/F16; Lane 4: 2/B14; Lane 5: 987.

## Discussion

SHV-12  $\beta$ -lactamase is the most prevalent ESBL in Asia, notably in Singapore, Thailand, Taiwan, and Japan [15]. Therefore, it is not surprising that this ESBL is emerging in Malaysia. SHV-12 ESBLs have been detected in 2 major species of enteric pathogens, *K. pneumoniae* and *E. coli*, at the UMMC, Kuala Lumpur, Malaysia. Previously, the SHV-5 ESBL enzyme was the prevalent enzyme identified in *E. coli* and *K. pneumoniae* isolated from inpatients at the UMMC [16,17]. Since the SHV gene may evolve by a single point mutation resulting in other variants, it is likely that the SHV-12 enzyme characterized in this study was a derivative of the previously identified SHV-5 enzyme among these organisms.

In addition to the production of ESBLs, resistance to the extended-spectrum cephalosporins among enteric pathogens may also be due to porin loss, as was observed in this study. All the strains showed absence of the OmpK35 porin by SDS-PAGE analysis, which may be a contributing factor to their resistance to the ESBLs. Moreover, OmpK35 is an important porin for  $\beta$ -lactam penetration [18]. This suggests that, apart from ESBL production, absence of the OmpK35

porin, which contributed to cefoxitin resistance, also resulted in extended-spectrum  $\beta$ -lactam resistance among these isolates.

Dual-mechanism  $\beta$ -lactam resistance, with loss of OmpK35 and ESBL production in *E. coli* and *K. pneumoniae*, is an emerging phenomenon at the UMMC. The multiple mechanisms of  $\beta$ -lactam resistance determinants involved in these organisms may pose a challenge for detection and selection of therapy. This is the first report of the detection of SHV-12 ESBL-producing organisms from UMMC that has been shown to be a derivative of SHV-5 that was previously identified at UMMC [16,19].

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