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# Emergence of CTX-M-15 extended-spectrum $\beta$ -lactamase-producing *Klebsiella pneumoniae* isolates in Bosnia and Herzegovina

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## Abstract

Fifty-seven nosocomial *Klebsiella pneumoniae* isolates producing extended-spectrum  $\beta$ -lactamases (ESBLs) were collected between February 2007 and November 2007 in different wards of the Sarajevo (Bosnia-Herzegovina) reference hospital. These isolates comprise two major epidemic pulsed-field electrophoresis-defined clones plus two minor clones. In addition to the ESBL-mediated resistance, all strains uniformly showed resistance to ciprofloxacin, gentamicin and tobramycin. The  $\beta$ -lactamases involved in this resistance phenotype were TEM-1, SHV-1, and CTX-M-15, as demonstrated by isoelectric focusing, PCR amplification, and sequencing. TEM-1 and CTX-M-15  $\beta$ -lactamases, as well as the aminoglycoside resistance determinants, were encoded in plasmids that could be transferred to *Escherichia coli* by conjugation. In three of the infected patients with the predominant clone, ceftazidime resistance development (MICs >128 mg/L) was documented. The analysis of the outer membrane proteins of the ceftazidime-susceptible and ceftazidime-resistant isolates revealed that the former expressed only one of the two major porins, OmpK36, whereas in the latter, the expression of OmpK36 was altered or abolished. This is the first report of CTX-M-15-producing *K. pneumoniae* in Bosnia-Herzegovina. Furthermore, we document and characterize for the first time ceftazidime resistance development in CTX-M-15-producing *K. pneumoniae*.

## Introduction

Extended-spectrum  $\beta$ -lactamases (ESBLs) represent a major threat among multidrug-resistant bacterial isolates. The production of ESBLs in Enterobacteriaceae confers resistance to all penicillins and cephalosporins (with the exception of cephamycins, in some cases), with the organisms generally remaining susceptible only to  $\beta$ -lactam- $\beta$ -lactamase inhibitor combinations, such as amoxicillin-clavulanate, and the carbapenems, which are frequently the only therapeutic options available for treatment of hospital-acquired severe infections caused by these microorganisms [1]. Co-resistance to non- $\beta$ -lactam antibiotics is also frequent, either by the co-transfer of the resistance determinants in the same genetic elements (such as aminoglycoside resistance) or simply by the co-selection of both resistance mechanisms, as occurs with fluoroquinolones [2].

The classical ESBLs are those derived from the broadspectrum enzymes TEM-1, TEM-2 and SHV-1 by the acquisition of specific point mutations that expand their spectrum of hydrolysis to oxyimino-cephalosporins and aztreonam [3].

Nevertheless, the most widespread plasmid-mediated ESBLs nowadays are the CTX-M enzymes, which are directly derived from the chromosomal  $\beta$ -lactamases of several species of the genus *Kluyvera* [4]. Five different groups of CTXMs, containing a total of over 80 different variants, have been described so far, but CTX-M-2, CTX-M-3, CTX-M-14 and CTX-M-15 are the most widespread enzymes [4].

To date, only SHV-5  $\beta$ -lactamase has been reported in 14 *Klebsiella pneumoniae* community-isolated strains in Bosnia-Herzegovina [5], with no record of CTX-M ESBLs in any

species. Given the scarcity of data from this country, the aim of the present study was to characterize the ESBL-producing isolates of *K. pneumoniae* at the molecular level in the reference hospital of the capital of this country, to investigate whether the CTX-M-type ESBLs have appeared in *K. pneumoniae* from Bosnia-Herzegovina.

## **Materials and Methods**

### ***Clinical strains and antibiotic susceptibility testing***

The isolates included in this study comprised one from each adult patient admitted between February and November of 2007 to the clinical centre (1762 beds, reference public hospital from the city of Sarajevo, Bosnia and Herzegovina) and infected with an ESBL-producing *K. pneumoniae* strain.

Bacterial identification and initial susceptibility testing was performed with the VITEK-2 system (bioMérieux, Hazelwood, MO, USA). Additionally, the MICs of several antibiotics were determined by microdilution or by using Etest strips (AB Biodisk, Solna, Sweden), following the manufacturer's recommendations.

Double-disk synergy testing (DDST) for the detection of ESBL production was performed using amoxicillin–clavulanate, cefotaxime, ceftazidime, cefepime and aztreonam disks that were applied 30 and/or 20 mm apart [6]. Phenotypic detection of AmpC was performed using the disk-based inhibitor assay, using boronic acid or cloxacillin as an AmpC inhibitor [7].

### ***Molecular strain typing***

The clonal relationship between the different isolates was studied by pulsed-field gel electrophoresis (PFGE). Agarose plugs containing total bacterial DNA were prepared as described elsewhere [8]. Plugs were then digested with XbaI and loaded into a 1% Megabase agarose (Bio-Rad, La Jolla, CA, USA) gel. DNA separation was performed in a CHEF-DRIII apparatus (Bio-Rad) under the following conditions: 6 V/cm<sup>2</sup> for 20 h at 14 °C, with initial and final pulse times of 2 s and 35 s, respectively. The results were interpreted following the criteria of Tenover et al. [9]. Comparison of sample profiles and generation of dendrograms was carried out by hierarchical clustering analysis using the unweighted pair group moving average method and the Dice coefficient for distance measure, using the software PAST-Paleontological Statistics v.1.29. Characterization of β-lactamases and their genes. The pIs of the β-lactamases were determined by isoelectric focusing, applying the supernatants of crude sonic cell extracts to Phast gels (Pharmacia AB, Uppsala, Sweden) with a pH gradient of 3–9 in a Phast system (Pharmacia AB). β-Lactamases with known pI values (TEM-1, TEM-2, TEM-4, TEM-3, SHV-1, CTX-M-10 and CTX-M-1) were included as controls. Gels were stained with 500 mg/L nitrocefin (Oxoid, Madrid, Spain) to identify the bands corresponding to β-lactamases.

PCRs for genes encoding TEM, SHV and CTX-M β-lactamases were performed using primers and conditions described previously [10,11]. PCR products were sequenced on both strands, using the BigDye terminator kit (PE-Applied Biosystems, Barcelona, Spain) for performing the sequencing reactions, which were analysed with the ABI Prism 3100 DNA sequencer (PE-Applied Biosystems).

### ***Conjugation and plasmid analysis***

Conjugation experiments were performed by filter mating, using a rifampin-resistant mutant of *Escherichia coli* strain HB101 as the recipient at a 1 : 1 ratio. Transconjugants were selected in Luria-Bertani agar plates containing 100 mg/L rifampin and 2 mg/L cefotaxime. Transconjugants were checked by DDST, PCR amplification, and sequencing of the appropriate ESBL-encoding gene, and Etest testing of susceptibility to all β-lactams and non-β-lactams to determine the resistance determinants co-transferred with the ESBL. For the analysis of the plasmids, plasmid DNA was obtained from the transconjugants using the QIAGEN plasmid Midi kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Plasmids were digested with EcoRI or BamHI, and the resulting restriction fragments were separated by electrophoresis in a 1% agarose gel. Isolation and analysis of outer membrane proteins (OMPs) Isolation of OMPs was performed as previously described [12]. Electrophoretic analysis of OMPs by SDS-PAGE was performed with 11% acrylamide–

0.35% bisacrylamide–0.1% SDS by using Laemmli's buffer and Coomassie blue staining. Western blot analysis of SDS-PAGE-separated OMPs was performed with the buffers and conditions described by Hernández-Allés et al. [12].

## Results

In 2007, the prevalence of ESBL-producing microorganisms at the Sarajevo clinical centre was 19.6%, *K. pneumoniae* being the most prevalent species (88.8%). In fact, 68.8% of the *K. pneumoniae* clinical isolates produced an ESBL. To characterize these isolates, all nosocomial ESBL-producing *K. pneumoniae* isolates infecting adult patients admitted to the clinical centre between February and November of 2007 were collected, and one randomly selected isolate from each patient was further characterized by PFGE.

Representative PFGE profiles of these isolates (n = 57) are shown in Fig. 1. Four distinct PFGE types, designated A, B, C and D, were identified. The prevalent PFGE types encountered were type A and type B, with 35 and 17 isolates, respectively. Altogether, these PFGE types represented 91.2% of the isolates.

The first clonal ESBL-producing isolate appeared in February, and it was a PFGE type B strain. PFGE type A and type B strains were isolated throughout the year, reaching a maximum in August and February, respectively. They were isolated from urine infections (30 type A and eight type B), blood (four type A and five type B), and wound infections (one type A and one type B). Three PFGE type B strains were isolated from respiratory infections. Both types were isolated in at least seven different wards of the hospital.

All of the *K. pneumoniae* isolates had the same pattern of multiresistance, showing, in addition to the ESBL-mediated resistance to penicillins and cephalosporins, resistance to ciprofloxacin, gentamicin, and tobramycin (Table 1). All isolates with this pattern of multiresistance were uniformly susceptible to imipenem.

In order to identify the  $\beta$ -lactamases present in these isolates, we amplified the DNA from all ESBL-producing isolates, using the specific primers described previously [10,11]. Sequencing of the PCR products confirmed the presence of TEM-1, SHV-1 and CTXM-15 in all *K. pneumoniae* isolates, independently of the PFGE type exhibited. Isoelectrofocusing confirmed the presence of three  $\beta$ -lactamase bands with pIs of 5.4, 7.6 and 8.6, co-focusing with TEM-1, SHV-1 and CTXM-15, respectively.

We investigated whether the multiresistant phenotype exhibited by these isolates was due to the transference of a unique plasmid encoding the  $\beta$ -lactamases. Plasmid location of these  $\beta$ -lactamases was demonstrated by conjugation experiments. Plasmid DNA from two randomly selected isolates from each PFGE type was transferred to *E. coli*, and transconjugants growing on Luria–Bertani agar plates containing 2 mg/L cefotaxime were checked by DDST, followed by PCR amplification and sequencing with the specific primers.

The MICs for the *E. coli* transconjugants are shown in Table 1. As can be observed, in addition to the ESBL-mediated resistance pattern, aminoglycoside (gentamicin and tobramycin) resistance was co-transferred in the same plasmid. PCR and sequencing analysis of transconjugated plasmids showed that TEM-1 co-transferred with the CTX-M-15 gene in all of the isolates, independently of the PFGE type.

DNA plasmid analysis showed that transconjugants derived from clinical isolates with the same PFGE type harboured the same plasmid. However, they differed from transconjugants derived from isolates with different PFGE types. The size of the plasmids determined by the analysis of the EcoRI and BamHI restriction enzymes were estimated to range between 50 kb and 100 kb for all PFGE types.

Interestingly, three isolates of PFGE type A were resistant to ceftazidime (MIC 128 mg/L). In order to investigate whether ceftazidime resistance was caused by alterations of the outer membrane permeability, we analysed the OMPs of susceptible and resistant *K. pneumoniae* clinical isolates of PFGE type A. SDS-PAGE analysis of the OMP of *K. pneumoniae* showed only two major proteins, of

about 33 kDa and 36 kDa, in the 31–45-kDa range for the cefoxitin-susceptible isolate (Fig. 2a, lane 1), corresponding to OmpA and OmpK36, respectively. On the other hand, the expression of the 36-kDa protein was reduced or absent in two cefoxitin-resistant isolates (Fig. 2a, lanes 2 and 3, respectively). Interestingly, both OmpK36-deficient strains were also resistant to ertapenem. By contrast, one of the cefoxitin-resistant isolates did not present alterations in the expression of OmpK36 (Fig. 2a, lane 4). The porin nature of the 36-kDa protein present in the cefoxitin-susceptible isolates and absent or showing reduced expression in two of the resistant isolates was confirmed by western blot analysis, using specific antibodies (Fig. 2b).

## Discussion

The dissemination of ESBL-producing *K. pneumoniae* in the hospital setting is a major threat with important therapeutic and epidemiological consequences, particularly when it affects wards caring for critically ill patients. Nowadays, CTX-M-2, CTX-M-3, CTX-M-14 and CTX-M-15 are the most widespread CTX-M enzymes in different European countries [4]. However, data on ESBL epidemiology in Bosnia-Herzegovina are scarce.

In this article, we document for the first time the presence of CTX-M-15 in one of the most important hospitals of this country. We show evidence that most of the CTX-M-15-producing isolates belonged to two predominant clones, indicating that, at least in part, dissemination of CTX-M-15 through different wards of the hospital was due to the spread of these clones. Nevertheless, we also demonstrate that, in both clones, CTX-M-15 genes were found to be encoded on large transferable plasmids that might contribute to the endemic situation that is facing this hospital. Furthermore, TEM-1 and the aminoglycoside resistance genes were co-transferred with CTX-M-15. This phenomenon has been previously reported; molecular characterization of plasmids encoding CTX-M-15 from *E. coli* strains involved in outbreaks in different countries have demonstrated that they additionally carried other antibiotic resistance genes, such as blaOXA-1, blaTEM-1, tetA, aac(6 $\phi$ )-Ib-cr, and aac(3)-II [13–15].

Given that  $\beta$ -lactamases degrading cefoxitin are uncommon, cefoxitin is frequently an alternative therapeutic option for the treatment of hospital-acquired severe infections caused by multiresistant ESBL-producing *K. pneumoniae* strains, such as those described in this work. Nevertheless, universal susceptibility to this antimicrobial agent in *K. pneumoniae* is no longer guaranteed. Indeed, cefoxitin resistance development in strains producing ESBLs due to the selection of mutants with reduced permeability to these antimicrobials is being increasingly reported [16]. Previous work has shown that the expression of both major *K. pneumoniae* porins, OmpK35 and OmpK36, plays a role in the susceptibility to cefoxitin antibiotics, and that mutants lacking or having reduced expression of both OMPs show reduced susceptibility to this antimicrobial agent. Nevertheless, in this work, we document and characterize, for the first time, development of in vivo resistance to cefoxitin in CTX-M-15-producing *K. pneumoniae* due to the selection of mutations leading to the lack of porin expression. As frequently occurs in clinical ESBL-producing *K. pneumoniae* isolates [12], the epidemic strains described in this work did not express OmpK35, favouring cefoxitin resistance development through the inactivation of OmpK36. Overall, CTX-M-15-producing *K. pneumoniae* isolates from three of the 36 adult patients infected with the prevalent clone type A (8.3%) developed cefoxitin resistance in clinical samples. Two of them were deficient in the expression of OmpK36, but one did not show altered expression of this porin, suggesting that another resistance mechanism is involved in the observed phenotype. Given that the disk-based inhibitor phenotypic assay using boronic acid or cloxacillin excluded the presence of an AmpC-type enzyme (data not shown), we hypothesized that cefoxitin resistance may be caused by mutations in the pore structure [17] or expression of an efflux pump [18].

In summary, we have described for the first time the emergence of CTX-M-15-producing *K. pneumoniae* isolates in Bosnia-Herzegovina, and have characterized the mechanisms leading to in vivo cefoxitin resistance development due to porin expression deficiency in two of the CTX-M-15-producing *K. pneumoniae* epidemic strains.

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## Transparency Declaration

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## Figures and Tables

FIGURE 1. Dendrogram obtained following pulsed-field gel electrophoresis (PFGE) of XbaI-digested genomic DNA from representative ESBL-producing *Klebsiella pneumoniae* PFGE types. Capital letters below the lanes refer to PFGE types, and numbers above the lanes refer to the number of isolates of each type. Molecular size markers (in kb) are indicated on the left.

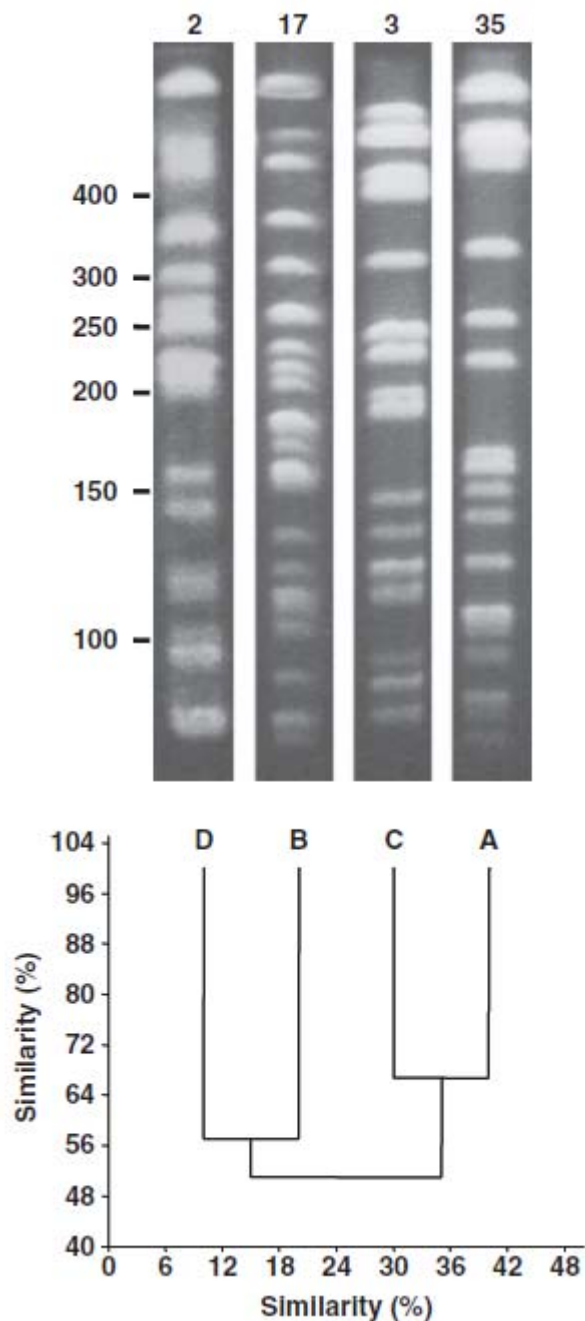


FIGURE 2. SDS-PAGE (a) and western blotting with anti-OmpK36 serum (b) of outer membrane proteins (OMPs) from the cefoxitinsusceptible *Klebsiella pneumoniae* clinical isolate (lane 1), and their respective *in vivo*-selected cefoxitin-resistant mutants (lanes 2, 3 and 4). The bands corresponding to OmpK36 and OmpA are indicated by arrows on the left of both panels. Only the relevant parts of the gel and western blot are shown.

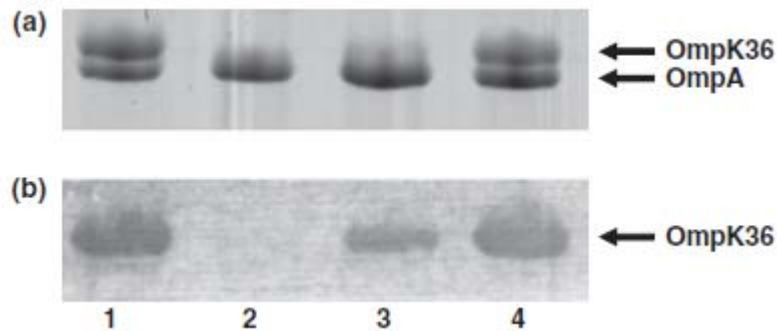


TABLE 1. MICs of several  $\beta$ -lactam and non- $\beta$ -lactam antibiotics for the extended-spectrum  $\beta$ -lactamase (ESBL)-producing *Klebsiella pneumoniae* isolates and the *Escherichia coli* transconjugants harbouring the ESBL-encoding plasmids

Antibiotic	MIC (mg/L) for:									
	<i>K. pneumoniae</i> isolates from patients of PFGE type <sup>a</sup> :				<i>E. coli</i> Hb101	<i>E. coli</i> Hb101 transconjugants harbouring plasmid from PFGE types:				
	A	B	C	D		A	B	C	D	
Amoxicillin-clavulanate	8 to >32	16 to >32	16 to >32	>32	<2	4	4	8	4	
Piperacillin-tazobactam	32 to >128	<4-64	<4-64	<4-64	<4	<4	<4	<4	<4	
Cefoxitin	<4-128	<4-8	<4-16	<4	<4	<4	<4	<4	<4	
Ceftazidime	16 to >64	>64	16-64	16 to >64	<1	16	4	4	4	
Cefotaxime	>64	>64	>64	>64	<1	>64	>64	>64	>64	
Cefepime	2 to >64	8 to >64	>64	<1-32	<1	<1	<1	<1	<1	
Imipenem	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	
Ciprofloxacin	>4	>4	>4	>4	<0.25	<0.25	<0.25	<0.25	<0.25	
Gentamicin	>16	8 to >16	>16	>16	<1	>16	8	8	8	
Tobramycin	8 to >16	>16	>16	>16	<1	8	8	8	>16	

PFGE, pulsed-field gel electrophoresis.

<sup>a</sup>Range of MICs for all ESBL-producing isolates, each recovered from different patients.