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Emergence of CTX-M-15 extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates in Bosnia and Herzegovina

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Abstract

Fifty-seven nosocomial *Klebsiella pneumoniae* isolates producing extended-spectrum β-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 β-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 β-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, cefoxitin resistance development (MICs >128 mg/L) was documented. The analysis of the outer membrane proteins of the cefoxitin-susceptible and cefoxitin-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 cefoxitin resistance development in CTX-M-15-producing *K. pneumoniae*.

Keywords: CTX-M-15, ESBL, *K. pneumoniae*, porin
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Introduction

Extended-spectrum β-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 β-lactam–β-lactamase inhibitor combinations, such as amoxycillin–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-β-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 broad-spectrum 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 β-lactamases of several species of the genus *Kluyvera* [4]. Five different groups of CTX-Ms, 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 β-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 amoxycillin–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 *Xba*I 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^2 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 *Eco*RI or *Bam*HI, 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. pnemoniae* 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,