High prevalence and molecular characterization of extended-spectrum $\beta$-lactamase-producing *Proteus mirabilis* strains in southern Croatia

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The aim of this study was to determine the prevalence and antibiotic resistance rates of extended-spectrum $\beta$-lactamases (ESBLs) (Livermore, 1998). ESBL production confers resistance to all $\beta$-lactams except for cephemycins and carbapenems (Bradford, 2001). The prevalence of ESBL-producing bacteria is increasing worldwide (Canton & Coque, 2006). The ESBL producers most frequently isolated in the hospital setting are *Klebsiella pneumoniae* and *Escherichia coli*. Infections due to these organisms often occur during outbreaks and therefore ESBL-producing bacteria represent a serious problem for hospitalized patients (Fiett et al., 2000). In the last few years, ESBL-producing *Proteus mirabilis* strains have been isolated with increasing frequency, and infection caused by these strains is becoming a serious therapeutic issue, especially in hospital settings (Winokur et al., 2001). Among the *Enterobacteriaceae*, *P. mirabilis* is one of the commonest causes of urinary tract infections, particularly when a hospital stay is prolonged (Coker et al., 2000). Split University Hospital is a 1700-bed university hospital and the only hospital in the region of Split–Dalmatia County, Croatia, which has a population of 1 million. Data on the prevalence of the most common ESBL-producing bacteria in our hospital, *K. pneumoniae* and *E. coli*, are already known (Tonkić et al., 2005), but we recently observed an increase in ESBL-producing *P. mirabilis* strains as a causative agent of infection, especially in urinary tract infections. In our earlier report published in 2008, we indicated the emergence, increasing prevalence and clonal spread of ESBL-producing *P. mirabilis* strains in Split University Hospital (Tonkić et al., 2008), which also prompted other authors to research in this field (Sardelic et al., 2009).

The objective of this study was to determine the prevalence and antibiotic resistance rates of ESBL-producing *P. mirabilis* strains isolated from inpatients at Split University Hospital during a survey conducted between 2005 and 2008. A total of 2152 consecutive isolates of *P. mirabilis* were isolated. The prevalence was 0.5 % in 2005 and increased significantly to 20.9 % by 2008. Strains were most frequently isolated from urine (36.5 %) and bronchial aspirates and wound swabs (11.3 %). ESBL-producing *P. mirabilis* isolates showed very high resistance rates to the majority of non-$\beta$-lactam antibiotics and were susceptible to a $\beta$-lactam/$\beta$-lactamase inhibitor and carbapenems. The isolates were genotyped and their ESBLs were molecularly characterized. Strains originating from the intensive care unit and the surgery and neurosurgery wards were clonally related. All *P. mirabilis* isolates produced the TEM-52 type of ESBL. To the best of our knowledge, our work detailed here and summarized in an earlier communication is the first report of such isolates from southern Croatia. Increased monitoring and screening for ESBL production in this species at our hospital is mandatory.

INTRODUCTION

Many Gram-negative bacteria are resistant to antibiotics due to the production of plasmid-borne extended-spectrum $\beta$-lactamases (ESBLs) (Livermore, 1998). ESBL production confers resistance to all $\beta$-lactams except for cephemycins and carbapenems (Bradford, 2001). The prevalence of ESBL-producing bacteria is increasing worldwide (Canton & Coque, 2006). The ESBL producers most frequently isolated in the hospital setting are *Klebsiella pneumoniae* and *Escherichia coli*. Infections due to these organisms often occur during outbreaks and therefore ESBL-producing bacteria represent a serious problem for hospitalized patients (Fiett et al., 2000). In the last few years, ESBL-producing *Proteus mirabilis* strains have been isolated with increasing frequency, and infection caused by these strains is becoming a serious therapeutic issue, especially in hospital settings (Winokur et al., 2001). Among the *Enterobacteriaceae*, *P. mirabilis* is one of the commonest causes of urinary tract infections, particularly when a hospital stay is prolonged (Coker et al., 2000). Split University Hospital is a 1700-bed university hospital and the only hospital in the region of Split–Dalmatia County, Croatia, which has a population of 1 million. Data on the prevalence of the most common ESBL-producing bacteria in our hospital, *K. pneumoniae* and *E. coli*, are already known (Tonkić et al., 2005), but we recently observed an increase in ESBL-producing *P. mirabilis* strains as a causative agent of infection, especially in urinary tract infections. In our earlier report published in 2008, we indicated the emergence, increasing prevalence and clonal spread of ESBL-producing *P. mirabilis* strains in Split University Hospital (Tonkić et al., 2008), which also prompted other authors to research in this field (Sardelic et al., 2009).

The objective of this study was to determine the prevalence and antibiotic resistance rates of ESBL-producing *P. mirabilis* strains isolated from inpatients at Split University Hospital during a survey conducted between
2005 and 2008. These strains were genotyped and their ESBLs were molecularly characterized.

METHODS

Bacterial isolates and antimicrobial susceptibility testing. During the study period of 2005–2008, a total of 2152 consecutive isolates of P. mirabilis from different clinical samples and from various clinical wards at Split University Hospital were isolated. Susceptibility of the strains to various antibiotics was determined using a disc diffusion test on Mueller–Hinton agar (Bio-Rad) (CLSI, 2008a).

Detection of ESBL production. The production of an ESBL was detected using a double-disc synergy test (Jarlier et al., 1998). Cefotaxime, ceftriaxone and cefotaxime discs were placed on Mueller–Hinton agar around an amoxicillin/clavulanic acid disc (distances between the discs were 30 mm from centre to centre). Synergy between amoxicillin/clavulanic acid and any one of the cephalosporins tested was taken as presumptive evidence of ESBL production. In isolates that were suspected of harbouring an ESBL but that tested negative using the standard distance of 30 mm between discs, the test was repeated with closer (20 mm) or more distant (40 mm) discs (Ho et al., 1998). ESBL production was confirmed using Clinical and Laboratory Standards Institute confirmatory double-disc tests with both cefotaxime and cefazidime (CLSI, 2008a). Thirty-eight ESBL-producing P. mirabilis isolates (29 urinary isolates, three isolates from bronchial aspirates, two isolates from tracheal aspirates and four from wounds) were further analysed and their ESBLs were molecularly characterized.

MIC determination. MICs were determined for the 38 ESBL-producing P. mirabilis isolates using a microbroth dilution method (CLSI, 2008b).

Statistical methods. A χ² test was used to evaluate differences between proportions. All differences in which the probability of the null hypothesis was P<0.05 were considered statistically significant.

PFGE. PFGE was used to investigate the clonal relationship among the ESBL-producing P. mirabilis strains. The method used for PFGE analysis was adapted from the protocol for E. coli O157:H7 established by the Centers for Disease Control and Prevention, Atlanta, GA, USA (CDC, 2004). Briefly, the plugs were lysed in cell lysis buffer [50 mM Tris/HCl (pH 8.0), 50 mM EDTA, 1% sarcosine and 0.1 mg proteinase K (Sigma) ml⁻¹]. Restriction of the DNA from lysed cells was performed with 50 U SfiI endonuclease (BioLabs) at 50 °C for 2.5 h. Electrophoresis was performed using a CHEF-DR III pulsed field electrophoresis system (Bio-Rad) at 14 °C and at 6 V cm⁻¹ for 20 h, using a pulse time ranging from 5 to 50 s. The gel was stained with ethidium bromide and visualized under UV illumination using a Gel Doc–XR apparatus (Bio-Rad). Molecular Analyst software (Bio-Rad) was used to analyse the DNA restriction patterns. Their similarity was calculated using the Dice similarity coefficient and unweighted pair group with arithmetic mean (UPGMA) algorithm. Isolates were considered to be genetically related when the Dice coefficient correlation was ≥0.80% (Pitout et al., 2008).

PCR amplification of β-lactamase-encoding (bla) genes. DNA from clinical isolates of P. mirabilis was isolated with a commercial kit for DNA isolation (DNeasy Blood & Tissue kit; Qiagen) according to the manufacturer’s instructions. Each PCR was performed in a final reaction volume of 50 µl containing 5 µl recombinant Taq DNA polymerase (Fermentas) and 3 µl sample. Amplifications were performed in a Mastercycler Personal (Eppendorf). Sequence amplification for three types of β-lactamase (SHV, TEM and CTX-M) was performed using different sets of primers. Amplification of the blaSHV sequence was performed with primers 5'-CGCCGGAATTCCTTTATGGAAGGC-3' (MN-1) and 5'-CTTTCCGATTCCGGCTG-3' (MN-2) (Sigma) (Nüesch-Inderbinen et al., 1996) using an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a final extension of 72 °C for 10 min. The size of the amplified PCR product was 1016 bp. Primers used for amplification of the blaTEM sequence were 5'-ATGATGATTCAATTTCCG-3' (OT-3) and 5'-CCATGCTTAATCAGTGAGG-3' (OT-4) (Sigma) (Arlet et al., 1995). The size of the amplified PCR product was 832 bp. PCR conditions for the OT-3 and OT-4 primers were the same as for MN-1 and MN-2, except that the annealing temperature was 51 °C for 1 min. Positive controls producing TEM and SHV β-lactamases were kindly provided by Professor B. Bedenic (Medical Faculty, Zagreb, Croatia). For amplification of the blaCTX-M sequence, primers 5'-SCATGCGACGCTTAACCA-3' (MA-1) and 5'-CCGCRATATGRTTTGTTG-3' (MA-2) (Sigma) were used (Woodford et al., 2004) with the following PCR conditions: initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 63 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 5 min. The size of the amplified PCR product was 554 bp. Additional primers in the multiplex PCR were also used for the detection of all five major CTX-M lineages (groups 1, 2, 8, 9 and 25) as described previously (Woodford et al., 2005). One positive control was used for each CTX-M ESBL group. The positive controls were kindly provided by Dr Neil Woodford (Health Protection Agency, London, UK). All positive controls produced the expected product size. Gel electrophoresis of the amplified PCR products was carried out on a 1.5% agarose gel (Sigma) with a 1000 bp DNA marker (Fermentas) and was visualized by ethidium bromide staining. PCR products were purified with a QIAquick Gel Extraction kit (Qiagen) according to the manufacturer’s instructions and were then sent to Macrogen for sequencing with a 3730xl DNA analyser (Applied Biosystems), using a specific reverse primer. The sequences were analysed using BLAST.

RESULTS AND DISCUSSION

Among the 38 ESBL producers, PCR amplification performed to detect alleles of bla gene sequences coding for SHV, TEM and CTX-M enzymes revealed the presence only of TEM enzymes. Sequence analysis confirmed that our isolates contain the TEM-52 ESBL. In recent years, the CTX-M group of ESBLs produced mainly by E. coli and K. pneumoniae has become the dominant type in countries such as the UK and Australia (Lau et al., 2008; Zong et al., 2008). As the majority of our isolates had higher MICs for cefotaxime than for ceftazidime (Table 1), we expected that some of them would be CTX-M producers, but none of the isolates were CTX-M or SHV producers. Polish researchers have found that their ESBL-producing P. mirabilis isolates are mainly CTX-M producers (Empel et al., 2008). Our results are in agreement with the results of Italian and French authors who have stated that their ESBL-producing P. mirabilis strains are mainly TEM-type ESBL producers (Spanu et al., 2002; Biendo et al., 2005). TEM-52 was detected for the first time in a K. pneumoniae isolate in France in 1998 (Poyart et al., 1998).
Table 1. Origin of and MIC values for the ESBL-producing *P. mirabilis* strains

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Specimen</th>
<th>Ward</th>
<th>Date of isolation</th>
<th>MIC (μg ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DG 6499</td>
<td>Wound swab</td>
<td>Internal medicine</td>
<td>16/07/2005</td>
<td>AMC 1; TZP 0.5; CTX 16; CAZ 32; AN 32; GN 2; NET 0.016; CIP 64; FEP 4; IPM 4; MEM 0.25; ERT 0.016</td>
</tr>
<tr>
<td>UR 12495</td>
<td>Urine</td>
<td>Internal medicine</td>
<td>03/12/2005</td>
<td>AMC 8; TZP 0.5; CTX 4; CAZ 64; AN 4; GN 2; NET 2; CIP 2; FEP 2; IPM 4; MEM 0.125; ERT 0.008</td>
</tr>
<tr>
<td>UR 580</td>
<td>Urine</td>
<td>Internal medicine</td>
<td>23/01/2006</td>
<td>AMC 4; TZP 1; CTX 2; CAZ &gt;128; AN 32; GN 2; NET 2; CIP 2; FEP 2; IPM 4; MEM 0.064; ERT 0.008</td>
</tr>
<tr>
<td>UR 6959</td>
<td>Urine</td>
<td>ICU</td>
<td>06/07/2006</td>
<td>AMC 4; TZP 256; CTX 8; CAZ &gt;128; AN 128; GN 32; NET 2; CIP 4; FEP 2; IPM 4; MEM 0.125; ERT 0.016</td>
</tr>
<tr>
<td>UR 7189</td>
<td>Urine</td>
<td>Neurosurgery</td>
<td>14/07/2006</td>
<td>AMC 8; TZP 512; CTX &gt;1024; CAZ 32; AN 2; GN 4; NET 0.032; CIP &gt;1024; FEP 2; IPM 4; MEM 0.064; ERT 0.008</td>
</tr>
<tr>
<td>DG 6746</td>
<td>Wound swab</td>
<td>Surgery</td>
<td>19/08/2006</td>
<td>AMC 4; TZP 2; CTX 32; CAZ &gt;128; AN 128; GN 16; NET 1024; CIP 2; FEP 2; IPM 0.064; MEM 0.016; ERT 0.004</td>
</tr>
<tr>
<td>DG 6874</td>
<td>Wound swab</td>
<td>ICU</td>
<td>26/08/2006</td>
<td>AMC 8; TZP 256; CTX 16; CAZ &gt;128; AN 128; GN 16; NET 1024; CIP 2; FEP 2; IPM 0.064; MEM 0.008; ERT 0.004</td>
</tr>
<tr>
<td>UR 9798</td>
<td>Urine</td>
<td>Surgery</td>
<td>27/09/2006</td>
<td>AMC 4; TZP 1024; CTX 32; CAZ &gt;128; AN 128; GN 32; NET &gt;1024; CIP 2; FEP 2; IPM 0.25; MEM 0.032; ERT 0.003</td>
</tr>
<tr>
<td>DG 7808</td>
<td>Bronchial aspirate</td>
<td>ICU</td>
<td>28/09/2006</td>
<td>AMC 8; TZP 128; CTX 4; CAZ 128; AN 8; GN 0.016; NET &gt;1024; CIP 1; FEP 0.064; IPM 0.008; MEM 0.125; ERT 0.016</td>
</tr>
<tr>
<td>UR 10655</td>
<td>Urine</td>
<td>ICU</td>
<td>20/10/2006</td>
<td>AMC 4; TZP 128; CTX 16; CAZ &gt;128; AN 128; GN 32; NET 2; CIP 2; FEP 2; IPM 4; MEM 0.064; ERT 0.016</td>
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<tr>
<td>UR 11118</td>
<td>Urine</td>
<td>Surgery</td>
<td>02/11/2006</td>
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<tr>
<td>UR 12467</td>
<td>Urine</td>
<td>Neurosurgery</td>
<td>06/12/2006</td>
<td>AMC 8; TZP 2; CTX 16; CAZ 64; AN 128; GN 16; NET &gt;1024; CIP 2; FEP 2; IPM 0.125; MEM 0.016; ERT 0.004</td>
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<tr>
<td>DG 8966</td>
<td>Tracheal aspirate</td>
<td>Neurosurgery</td>
<td>05/11/2007</td>
<td>AMC 8; TZP 512; CTX &gt;1024; CAZ 32; AN 32; GN 2; NET 2; CIP 2; FEP 2; IPM 0.064; MEM 0.008; ERT 0.004</td>
</tr>
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**Characterization of ESBL-producing *Proteus mirabilis***
clonally related strains. In the second cluster, there were 12 clonally related strains isolated mainly in 2006 and 2007. The third group of related strains comprised eight strains isolated in 2007. One urine isolate (strain UR 7189) obtained from a patient in neurosurgery in 2006 did not fall into any of these lineages. The ESBL-producing *P. mirabilis* strain was initially isolated at our institution from a patient from the intensive care unit (ICU) and later from patients from six additional wards. The ESBL-producing *P. mirabilis* isolates that originated mainly from the ICU and the surgery and neurosurgery wards were clearly clonally related, indicative of intra-hospital spreading of ESBL-producing *P. mirabilis* among these wards.

During the 4-year study period, a total of 2152 *P. mirabilis* strains were isolated and 272 (12.6 %) were ESBL-positive. The prevalence was 0.5 % in 2005 and had increased significantly to 20.9 % by 2008 ($\chi^2=122; P<0.001$). ESBL-producing *P. mirabilis* strains were isolated most frequently from urine (36.5 %), bronchial aspirates and wound swabs (11.3 %) and blood (3.8 %). Samples that revealed ESBL-positive isolates originated from patients from ICUs (30 %), neurosurgery (10.7 %) and surgery (8.8 %). To the best of our knowledge, our work detailed here and summarized in an earlier communication (Tonkic*´ et al., 2008) is the first report of such isolates from southern Croatia. In the last decade, data have been published from various countries on ESBL-producing *P. mirabilis* strains. The first report on such isolates originated in France (Bonnet *et al.*, 1999). According to the SENTRY Antimicrobial Surveillance Program for the period 1997–1998, the prevalence of such isolates was 1.3 % in Spain and 28.9 % in Italy (Nijssen *et al.*, 2004). The overall prevalence of ESBL-producing *P. mirabilis* in our study was 12.6 %, similar to the prevalence found in Poland (14.5 %) in a survey conducted in 2003–2004 (Empel *et al.*, 2008) and was higher than that found in France (3.3 %) or Greece (5.9 %) (Nijssen *et al.*, 2004). A prevalence of ESBL-producing *P. mirabilis* of 20.9 % in 2008 at our hospital is concerning and puts our region in the group of countries with a high ESBL-producing *P. mirabilis* prevalence, which also includes Italy and Israel with a prevalence of >20 % (Nijssen *et al.*, 2004). In concordance with other studies, the majority of our ESBL-producing *P. mirabilis* isolates originated from urine samples of ICU patients (Jean *et al.*, 2009). It is interesting that all the ESBL-producing *P. mirabilis* isolates in our study were obtained from adult patients, whilst in a previous survey conducted in our hospital during 2001–2002 the highest proportion of ESBL-producing *E. coli* and *K. pneumoniae* strains were isolated from specimens obtained from the neonatal ICU and paediatric ward (Tonkic *et al.*, 2005). During the period of that survey, there were no ESBL-producing *P. mirabilis* isolates in our hospital.

The results of the antibiotic susceptibility testing of ESBL-producing *P. mirabilis* isolates showed high resistance rates to the majority of non-β-lactam antibiotics. Resistance rates for ciprofloxacin, trimethoprim/sulfamethoxazole,
gentamicin, netilmicin and amikacin were 73.4, 89.5, 86.2, 75.8 and 4.1 %, respectively. All the strains were susceptible to antibiotic combinations of a β-lactamase inhibitor, cefoxitin and carbapenems.

Among the cephalosporins tested, the highest MIC values were found for cefepime (Table 1). The cefotaxime MICs were higher than the ceftazidime MICs for a high proportion of the isolates. The lowest MICs were found for ertapenem (0.008–0.064 μg ml⁻¹).

This study showed that, at our institution, the emerging resistance of P. mirabilis strains to β-lactam antibiotics is a consequence of ESBL enzyme production.

In conclusion, both the increasing prevalence and the antibiotic resistance of ESBL-producing P. mirabilis strains at Split University Hospital are concerning and represent serious therapeutic and epidemiological issues. Further monitoring and screening for ESBL production as well as for AmpC production or for combinations of the β-lactamase types in this species is mandatory.

REFERENCES


