High prevalence of OXA-51-type class D β-lactamases among ceftazidime-resistant clinical isolates of Acinetobacter spp.: co-existence with OXA-58 in multiple centres

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Objectives: This study was designed to demonstrate the prevalence of the newly discovered carbapenem-hydrolysing class D enzymes, OXA-51-type and OXA-58, among clinical isolates of Acinetobacter spp.

Methods: A total of 72 isolates from six centres were studied. Isolates were screened by PCR with specific primers for blaOXA-51-type and blaOXA-58. PCR products were sequence-analysed. Plasmids were digested with EcoRV and genomic DNAs were digested with PvuII. Hybridization experiments were done with digoxigenin-labelled specific probes. Macro-restriction analysis was done on SmaI-digested genomic DNAs.

Results: A total of 56 (77.8%) isolates were positive for blaOXA-51-type genes. Sequence analysis of the products from 23 selected isolates revealed the occurrence of multiple alleles in all contributing centres. The blaOXA-58 gene was detected among 10 isolates from five centres. All were also positive for blaOXA-51-type genes. Among the blaOXA-58-positive isolates, two from the same centre were positive for a novel OXA-51 allele (OXA-86). Southern hybridization of plasmids and of genomic DNAs suggested that blaOXA-51-type genes are located on chromosomes whereas blaOXA-58 genes are plasmid borne in these 10 isolates. Plasmid profiles and pulsed-field gel electrophoresis patterns indicated the spread of the blaOXA-58 gene among multiple clones. The blaOXA-51-type and blaOXA-58 co-carrier strains were mostly associated with a pandrug-resistant phenotype.

Conclusions: This study indicated that blaOXA-58-bearing plasmids are readily spreading among multiple clones of the blaOXA-51-type-bearing clinical isolates of Acinetobacter spp. Since these isolates are highly resistant to antibiotics this finding indicates the existence of a significant problem in Turkish hospitals.

Keywords: oxacillinases, carbapenemases, pandrug resistance

Introduction

Acinetobacter spp. are predominant pathogens in ICUs in Turkey.1 Antibiotic resistance rates to penicillins and cephalosporins among Acinetobacter spp. are high and therefore carbapenems are often the treatment of choice. However, resistance to this class of β-lactams is also increasing.

Resistance to carbapenems among Acinetobacter spp. is in many cases associated with reduced drug uptake due to porin deficiency and with reduced affinity to penicillin-binding proteins due to mutations.2,3 Recent data signify the responsibility of carbapenemases belonging to Ambler class B and Ambler class D among clinical isolates of Acinetobacter spp.4–9

In contrast to the relatively rare occurrence of class B metallo-enzymes, the carbapenem-hydrolysing members of class D are widespread over multiple continents.8,10 Class D carbapenemases from Acinetobacter spp. are currently classified under four subgroups. Subgroup I is composed of OXA-23, -27 and -49, 

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Materials and methods

Bacterial strains, identification and antibiotic resistance tests

Bacterial strains used were those Acinetobacter spp. recovered from the collection stored during a previous study. The collection was composed of ceftazidime-resistant clinical strains isolated in ICUs from seven University hospitals between April and June in 2003. This collection had been screened for (i) IMP and (ii) VIM-type metallo-β-lactamases and as well as for (i) OXA-23, (ii) OXA-25, (iii) OXA-48, (iv) OXA-48-type carbapenem-hydrolyzing oxacillinas with consensus primers, but failed to show the existence of such enzymes (F. Kolayli, G. Gacir and H. Vahaboglu, unpublished data).

The current study is designed to explore the prevalence of recently identified subgroups of class D carbapenemases: OXA-51-type and OXA-58, among the above-mentioned collection of Acinetobacter spp. strains.

Screenings were performed by outer-most primers, probes were generated by inner primers and sequencing was performed by both sets to obtain accurate sequence readings on both strands.

The sequence analyses were accomplished after product purification using High Pure PCR Product Purification Kit (Roche Diagnostics, Basel, Switzerland). The sequencing was performed with two sets of primers on both strands. The sequence analysis method used was dye terminator cycle sequencing with the ABI Prism BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA). The assay was carried out according to a standard protocol. Data were collected on an ABI 377 automated fluorescence sequencer.

Sequence data were read and edited by two freeware programs: Chromas pro (http://www.technelysium.com.au) and BioEdit (version 7.0.5.2).

Enzyme extraction and IEF

Crude cell extracts were prepared by sonication. Analytical isoelectric focusing (IEF) was performed on 5% polyacrylamide gels containing ampholytes (pH range, 3–10; Fluka, Switzerland) with an inoculum of 10^6 cfu per spot as recommended by the Clinical and Laboratory Standards Institute (formerly NCCLS). Endpoints were read after 18 h of incubation at 37°C. E. coli ATCC 25922 was used as the control strain.

Plasmids were digested with MfeI and EcoRV (Fermentas) and the other set from inner sequences. For OXA-58, 58A-5'CTATAAAATACCTAATTGTTCT-3' and 58D-5'CTATAAAATACCTAATTGTTCT-3' amplified a 453 bp fragment (94% of the gene) whereas 58B-5'CTATAAAATACCTAATTGTTCT-3' and 58C-5'CTATAAAATACCTAATTGTTCT-3' amplified an 825 bp fragment (100% of the gene) and 58F-5'TTCGTTTGCTTAAGCATAAGTA-3' and 51C-5'GGTACCGATATCGCTTCATGTGC-3' amplified a 453 bp inner fragment. Additionally for sequencing the OXA-51-type genes 51B2-5'TTCGTTTGCTTAAGCATAAGTA-3' and 51B-5'TTCGTTTGCTTAAGCATAAGTA-3' was used.

Screenings were performed by outer-most primers, probes were generated by inner primers and sequencing was performed by both sets to obtain accurate sequence readings on both strands.

The sequence analyses were accomplished after product purification using High Pure PCR Product Purification Kit (Roche Diagnostics, Basel, Switzerland). The sequencing was performed with two sets of primers on both strands. The sequence analysis method used was dye terminator cycle sequencing with the ABI Prism BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA). The assay was carried out according to a standard protocol. Data were collected on an ABI 377 automated fluorescence sequencer.

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Enzyme extraction and IEF

Crude cell extracts were prepared by sonication. Analytical isoelectric focusing (IEF) was performed on 5% polyacrylamide gels containing ampholytes (pH range, 3–10; Fluka, Switzerland) with a Model 111 Mini IEF Cell (Bio-Rad Laboratories, USA). Enzymes were focused at constant 1 W for 45 min and detected by over-laying the gel with 1 mM nitrocefin solution. Known enzymes, SHV-1 (pl 7.6), OXA-14 (pl 6.2) and TEM-1 (pl 5.4) were used as references.

Plasmid isolation, restriction enzyme digestion of genomic DNA, Southern hybridization and genotyping with PFGE

Plasmids were isolated using alkaline-lysis method, run on a 0.9% agarose gel at a constant 8 V/cm for 1 h, stained with ethidium bromide and visualized on a UV transilluminator. For the Southern hybridization of genomic DNA restriction fragments the isolation method depended on guanidine thiocyanate lysis. This method is fully described in the literature.

Plasmids were digested with EcoRV (Fermentas) and the genomic DNAs were digested with PvuII (Fermentas) at 37°C. All were run on a 0.9% agarose gel at a constant 8 V/cm, stained with ethidium bromide and visualized on a UV transilluminator. For the Southern hybridization of genomic DNA restriction fragments the isolation method depended on guanidine thiocyanate lysis. This method is fully described in the literature. Plasmids were digested with EcoRV (Fermentas) and the genomic DNAs were digested with PvuII (Fermentas) at 37°C. All were run on a 0.9% agarose gel at a constant 6 V/cm, visualized using a UV lamp and later the EcoRV-digested plasmids and PvuII-digested genomic DNAs were transferred to positively charged nylon membranes (Roche) using capillary blotting method.

Probes were generated by labelling PCR products internal to the blaOXA-58 and blaOXA-51-type by supplementing the master mixture with Dig-dUTP (Roche). Probes were purified with High Pure PCR Product Purification Kit (Roche) before hybridization. Southern
hybridization and detection steps were accomplished using the Dig-dUTP detection kit as recommended by the manufacturer (Roche).

For pulsed-field gel electrophoresis (PFGE), SmaI-digested genomic DNA was prepared according to the instructions recommended by Bio-Rad (Hercules, CA, USA). Electrophoresis was performed in a 1% agarose gel [in 0.5·TBE (Tris–borate–EDTA) buffer] and fragments were separated for 20 h at 6 V/cm at 14°C by using a CHEF-DRII system (Bio-Rad), with initial and final pulse times of 5 and 30 s, respectively. DNA fingerprints were compared visually.

Results

A total of 70 Acinetobacter spp. from the 84 isolates stored during a previous study were recovered.13 Two strains, isolated in 1999 in Kocaeli University Hospital’s ICU, were included in the study as well. Altogether, 72 strains from six centres were studied. Since these strains had been identified previously, they were checked only by using classical methods such as oxidation–fermentation reaction in TSI agar, oxidase production and colony appearance and later the collection was PCR screened for the existence of bla\textsubscript{OXA-51-type} and bla\textsubscript{OXA-58}.

Among the studied 72 strains 56 (77.8%) were positive with primers specific to bla\textsubscript{OXA-51-type}. PCR screening with the bla\textsubscript{OXA-58}-specific primer set of the 72 isolates and later the sequence data from PCR products (95% of the gene) revealed the existence of this gene among 10 strains, which were also positive for bla\textsubscript{OXA-51-type}.

Identification and MICs for bla\textsubscript{OXA-58}-positive isolates

All of these 10 isolates were re-identified as A. baumannii by API20NE. This was further confirmed by the existence of 0.4 kb genomic species 2, 3 and 13TU (A. baumannii), specific PCR fragment in all strains. ARDRA patterns of the digested products were identical to each other and consistent with DNA group 2 according to a previously reported data.17

The MICs and the distribution of these strains between centres are shown in Table 2. All of these strains, except AO66 (OXA-58 plus OXA-68 producer), were pandrug-resistant.

Plasmid and Southern hybridization studies of bla\textsubscript{OXA-58}-positive isolates

Plasmid isolation from these strains revealed the existence of multiple plasmids (data not shown). Restriction patterns of plasmids with EcoRV were polymorphic, such that five patterns were observed (Figure 1a).

<table>
<thead>
<tr>
<th>OXA-51 alleles</th>
<th>(1) Istanbul</th>
<th>(2) Kocaeli</th>
<th>(3) Samsun</th>
<th>(4) Trabzon</th>
<th>(5) Izmir</th>
<th>(6) Kayseri</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA-51</td>
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<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
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<td>OXA-66</td>
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<td>2</td>
<td>2</td>
<td>3</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>3</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>OXA-86</td>
<td></td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>8</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>NSI\textsuperscript{b}</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>8</td>
<td>3</td>
<td>13</td>
<td>33</td>
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<tr>
<td>Total</td>
<td>6</td>
<td>2</td>
<td>9</td>
<td>12</td>
<td>9</td>
<td>18</td>
<td>56 (77.8%)</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Grand total</td>
<td>8</td>
<td>2</td>
<td>15</td>
<td>14</td>
<td>10</td>
<td>23</td>
<td>72</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Names of the cities of contributing centres.

\textsuperscript{b}NSI, sequence identification not obtained.

\emph{OXA-51-type $\beta$-lactamases}

\textbf{Table 1.} Occurrence of OXA-51-type $\beta$-lactamase-positive isolates of \emph{Acinetobacter} spp. in six ICUs

(n = 32), sequence data were obtained from nine isolates among which \textit{bla}\textit{OXA-66} ($n = 6$) was the most prevalent variant.

Two strains (AO14, AO28), both from the same centre, contained a novel variant of OXA-51 which was assigned OXA-86 (accession # DQ149247). This novel variant was confirmed with two independent amplifications from the strains AO14 and AO28. The distribution of OXA-51 variants among contributing centres is shown in Table 1.

Multiple amino acid alignment of \textit{bla}\textit{OXA-51-type} revealed that OXA-86 differs by four amino acids from the other members such as Thr replaced Ala at positions 12 and 31, Ile replaced Thr at position 74 and Ile replaced Val at position 101 (according to DBL numbering).20

\textbf{PCR screening with primers specific to \textit{bla}\textit{OXA-58}}

PCR screening with the \textit{bla}\textit{OXA-58}-specific primer set of the 72 isolates and later the sequence data from PCR products (95% of the gene) revealed the existence of this gene among 10 strains, which were also positive for \textit{bla}\textit{OXA-51-type}.

\textbf{Identification and MICs for \textit{bla}\textit{OXA-58}-positive isolates}

All of these 10 isolates were re-identified as \textit{A. baumannii} by API20NE. This was further confirmed by the existence of 0.4 kb genomic species 2, 3 and 13TU (\textit{A. baumannii}), specific PCR fragment in all strains. ARDRA patterns of the digested products were identical to each other and consistent with DNA group 2 according to a previously reported data.17

The MICs and the distribution of these strains between centres are shown in Table 2. All of these strains, except AO66 (OXA-58 plus OXA-68 producer), were pandrug-resistant.

\textbf{Plasmid and Southern hybridization studies of \textit{bla}\textit{OXA-58}-positive isolates}

Plasmid isolation from these strains revealed the existence of multiple plasmids (data not shown). Restriction patterns of plasmids with EcoRV were polymorphic, such that five patterns were observed (Figure 1a).
Southern hybridization of EcoRV-digested plasmids was positive with an OXA-58-specific probe and revealed that the genes were located on (approx.) 11 and 12 kbp fragments (Figure 1b).

On the other hand, Southern hybridization with an OXA-51-type-specific probe of plasmids was negative (data not shown) whereas the genomic DNAs were positive (Figure 2). This indicated the plasmidic spread of OXA-58 among those strains bearing blaOXA-51-type genes on their chromosomes.

IEF and macro-restriction analysis of blaOXA-58-positive isolates

IEF patterns of crude enzyme extracts were identical (data not shown). All isolates were hyperproducers for pI > 8.5 enzymes, which were consistent with those chromosomal enzymes of Acinetobacter spp., and additionally all displayed two almost identical activities at pI points 6.2 and 7.2. The enzymatic activity located to pI approx. 7.2 is consistent with OXA-58. However, we observed this band in OXA-58-negative strains as well. IEF, at least in our conditions, was not discriminative between OXA-51 variants. Although in silico analysis with deduced protein sequences suggested highly different pI values for various OXA-51 variants, the enzymes in crude extracts failed to migrate readily and to separate from each other on the gel. This imperfect migration of enzymes in acrylamide–polyacrylamide gels might be due to the electrophoresis conditions in our laboratory or due to the multimeric forms of these enzymes.21

Macro-restriction analysis, with at least three different patterns, revealed a multiclonal spread of blaOXA-58 gene (Figure 3).

Table 2. MICs (mg/L) and co-existence with OXA-51 alleles of OXA-58-positive strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Location</th>
<th>OXA-51-type</th>
<th>IPM</th>
<th>MEM</th>
<th>FEP</th>
<th>CAZ</th>
<th>SUL</th>
<th>CFP/SUL</th>
<th>PIP</th>
<th>TZP</th>
<th>ATM</th>
<th>CIP</th>
<th>AMK</th>
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<td>6</td>
<td>OXA-86</td>
<td>32</td>
<td>8</td>
<td>16</td>
<td>32</td>
<td>8</td>
<td>8</td>
<td>&gt;512</td>
<td>128</td>
<td>256</td>
<td>4</td>
<td>&gt;512</td>
<td>4</td>
</tr>
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<td>6</td>
<td>OXA-86</td>
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<td>8</td>
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<td>&gt;512</td>
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<td>256</td>
<td>4</td>
<td>&gt;512</td>
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<td>16</td>
<td>16</td>
<td>&gt;512</td>
<td>&gt;128</td>
<td>512</td>
<td>64</td>
<td>128</td>
<td>4</td>
</tr>
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<td>128</td>
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</tr>
</tbody>
</table>

IPM, imipenem; MEM, meropenem; FEP, cefepime; CAZ, ceftazidime; SUL, sulbactam; CFP/SUL, cefoperazone/sulbactam; PIP, piperacillin; TZP, piperacillin/tazobactam; ATM, aztreonam; CIP, ciprofloxacin; AMK, amikacin; TIG, tigecycline.

Locations of centres are such that the numbers represent cities cited in Table 1.

Cefoperazone/sulbactam (CFP/SUL) and piperacillin/tazobactam (TZP) drug-to-inhibitor ratio is 2:1.
OXA-51-type β-lactamases

Figure 3. PFGE patterns of bla\textsubscript{OXA-58} positive isolates. Three patterns observed. Pattern 1, lanes 1 and 2 (bla\textsubscript{OXA-58} plus bla\textsubscript{OXA-68} detected strains (AO14 and AO28, respectively)); pattern 2, lanes 3–8 and 10 (bla\textsubscript{OXA-58} plus bla\textsubscript{OXA-51} detected strains); pattern 3, lane 9 (bla\textsubscript{OXA-58} plus bla\textsubscript{OXA-68} detected strain (from Kocaeli)).

Discussion

This study demonstrated that (i) OXA-51-type enzyme-producing Acinetobacter spp. is highly prevalent and scattered around different parts of the country; (ii) among the clonally variable strains bearing chromosomally located bla\textsubscript{OXA-51-type} genes, the plasmidic bla\textsubscript{OXA-58} is readily disseminating and (iii) isolates with OXA-51-type plus OXA-58 enzymes are often associated with pandrug resistance.

OXA-51 was first detected in Argentina among genetically distinct clinical isolates of A. baumannii,\textsuperscript{11} whereas OXA-58 was detected among some isolates in Toulouse (France) in 2003.\textsuperscript{6,14} However, more recent studies revealed that both genes exist in carbapenem-resistant clinical isolates of Acinetobacter spp. from various continents and for even more than 10 years in some countries.\textsuperscript{5,10} In this study we could not detect bla\textsubscript{OXA-51-type} genes in all strains as was the case in one other study.\textsuperscript{11} It is not clear, therefore, whether these resistance genes are acquired or occur naturally in Acinetobacter spp.\textsuperscript{12}

In one recent study, bla\textsubscript{OXA-51-type} genes were suggested to be chromosome borne, as was suggested among those bla\textsubscript{OXA-58} positive isolates in this study.\textsuperscript{11} Some of the OXA carbapenemases other than OXA-51 variants were shown to be plasmid borne and mostly associated with insertion sequences but not integrons.\textsuperscript{14,22,23} However, from one strain—not mentioned in this study—we were able to transfer another OXA-51 variant (OXA-87, GenBank accession # DQ348075) to E. coli DH5α (currently, more data are not available), indicating that bla\textsubscript{OXA-51} variants might jump to plasmids as well.

On the other hand, bla\textsubscript{OXA-58} was originally found to be plasmidic and shown to be located on a 11 kb EcoRV fragment.\textsuperscript{9} In this study the bla\textsubscript{OXA-58} gene was found to be located on different-sized (11 and 12 kb) fragments.

Since IEF was not discriminative between the alleles of OXA-51-type enzymes we selected representative strains for sequence analysis according to imipenem–meropenem resistance phenotypes. Interestingly, OXA-66 was the most prevalent type among IMP-MEM\textsuperscript{6} phenotype. This is in contrast with previous data, where MICs of IMP for several OXA-66 producers were above the breakpoint.\textsuperscript{21} However, beyond β-lactamas, especially for those OXA-type enzymes, some porin-efflux barriers play a decisive role in the resistance phenotypes of Acinetobacter.

OXA-51-type plus OXA-58 double enzyme producers were found to be pandrug-resistant. This finding is consistent with the previous data.\textsuperscript{10} Of note was that the MICs of ticarcillin, a new antibiotic on the market, were above the acceptable level (susceptible, MIC ≤ 2 mg/L; FDA, 2005) and therefore disappointing.\textsuperscript{24,25}

Data from the plasmid maps (data not shown), Southern hybridization and PFGE revealed that bla\textsubscript{OXA-58} gene has been disseminated among multiple clones of Acinetobacter on different gene fragments. This indicated a high dissemination potential. High antibiotic consumption in ICUs will probably continue to provide enough pressure to maintain these highly contagious resistance genes as a growing problem.

This study further indicated that OXA-carbapenemases, especially those multiple-OXA carbapenemase producers, deserve attention as a significant health problem.

Acknowledgements

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Transparency declarations

We have no conflicts to declare.

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Vahaboglu et al.


