RESEARCH LETTER

Emergence and spread of carbapenem-resistant *Acinetobacter baumannii* in a tertiary care hospital in Turkey

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Abstract

An intensive care unit (ICU)-based OXA-23-producing multiple-drug resistant *Acinetobacter baumannii* (MDRAB) outbreak was detected between October 2005 and October 2006. A total of 47 patients were infected/colonized with the outbreak strain. Clinical data were available from 37 patients. The all-cause mortality rate among the patients exposed to the epidemic strain was 35% (13/37). The outbreak strain and the resistance determinants were characterized both by microbiological methods and by molecular techniques. Cloning and sequencing experiments identified IS*AbaI*-associated bla_{oxa-23} on the chromosome. Screening of imipenem-resistant *Acinetobacter* isolated from the ICU during the outbreak period with PCR identified 97 isolates as positive for the IS*AbaI*-bla_{oxa-23} structure. Pulsed-field gel electrophoresis and plasmid analyses with selected nonrepetitive isolates revealed the clonality. Disk diffusion on cloxacillin-supplemented agar media and the real-time PCR experiments showed that outbreak isolates are overexpressing the *amp*C enzyme. This study highlights the occurrence of OXA-23-producing and *amp*C-overexpressing MDRAB in ICUs.

Introduction

Carbapenem-resistant *Acinetobacter baumannii* has emerged in hospitals as a significant therapeutic challenge in recent years (Bergogne-Berezin & Joly-Guillou, 1991; Richards *et al.*, 1999; Erbay *et al.*, 2003). Carbapenem resistance is predominantly caused by the occurrence and dissemination of β-lactamases, particularly OXA-carbapenemases, in the members of this genus (Poirel & Nordmann, 2006; Walther-Rasmussen & Hoiby, 2006; Yong *et al.*, 2006).

Sequence-based comparisons divide the OXA-carbapenemases into 8 subgroups (Walther-Rasmussen & Hoiby, 2006). OXA-23, the first member of this group of enzymes, was discovered in 1985 in Scotland before imipenem's wide availability in the market (Donald *et al.*, 2000). Since then, a number of outbreaks of OXA-23-producing *Acinetobacter* have been reported from various regions of the world (Dalla-Costa *et al.*, 2003; Naas *et al.*, 2005; Turton *et al.*, 2005; Jeong *et al.*, 2006; Koh *et al.*, 2007).

Herein, we report an intensive care unit (ICU)-based outbreak caused by IS*AbaI*-associated $bla_{\mathrm{OXA-23}}$-bearing *Acinetobacter* in a 450-bed tertiary care hospital in Turkey between October 2005 and October 2006. Molecular epidemiological studies were performed to identify the characteristics of both the resistance determinant and the outbreak strain.

Materials and methods

Hospital setting and clinical data

This study was performed in a 450-bed tertiary care university hospital (Kocaeli University, Turkey) with a 15-bed ICU (for adults). Nine beds in this ICU are equipped with mechanical ventilation devices and are reserved for severely ill patients who require ventilation support. The other beds are separated and reserved for postsurgical patients for 1–2 days of observation. An infection control team visits this ICU daily and recommends appropriate infection control procedures. However, infection rates remain high due to overcrowding, an inadequate number of medical staff, and the lack of isolation facilities.

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An outbreak of carbapenem-resistant *Acinetobacter* was detected in this ICU. A total of 94 isolates resistant to major antibiotics, including carbapenems, were recovered from 47 patients during their ICU stay between October 2005 and October 2006.

Clinical data were examined from 37 patients by a retrospective chart review. Based on the records, 22 of these patients were diagnosed with pneumonia, seven with surgical site infection, three with bloodstream infection and one with central nervous system infection (meningitis); four were diagnosed with other infections according to Centers for Disease Control definitions of nosocomial infections. Of these 37 cases, 31 (21 of whom had pneumonia) were treated with a carbapenem (imipenem or meropenem) or with a sulbactam combination (ampicillin/sulbactam or cefoperazon/sulbactam), either as a monotherapy or as a combination therapy (a carbapenem plus sulbactam combination). Three of the 12 patients who received a monotherapy died, as did nine of the 19 who received a combination therapy (Fisher's exact test, $P = 0.23$). The all-cause mortality rate among the patients exposed to the epidemic strain AcKOU1 was 35% (13/37). During the same period, the overall mortality rate in this ICU was 22% (116/517).

Microbiology

Bacteria were isolated from clinical specimens, identified and stored at $-80\,^{\circ}C$ in the microbiology laboratory. Before the study, the stored isolates were reidentified using the API 20NE system (bioMérieux, Marcy-l'Étoile, France). Identification at the genomic species level was carried out by amplified rRNA gene restriction analysis (ARDRA) and bla_{OXA-51}-like PCR screening (Vaneechoutte *et al.*, 1995; Turton *et al.*, 2006).

Minimal inhibitory concentrations (MICs) were determined using the standard agar dilution method on Mueller—Hinton (MH) agar (Oxoid, Basingstoke, UK) following CLSI guidelines with inocula of 10^4 CFU per spot. End points were interpreted after 18 h of incubation at 37 °C. *Eschrichia coli* ATCC 25922 was the control strain. Antibiotic powders were provided by a number of companies: amikacin (Eczacibasi), cefepime (Bristol-Myers Squibb), cefoperazon/sulbactam (Pfizer), ceftazidime (Glaxo-SmithKline), ciprofloxacin (Bayer), imipenem (Merck), meropenem (Astra-Zeneca) and piperacillin/tazobactam (Wyeth).

To explore the chromosomal *amp*C β-lactamase's contribution to the resistance of cefotaxime, ceftazidime and imipenem, a disc diffusion test was performed in both the presence and in the absence of cloxacillin (500 μg mL^{-1})(Corvec *et al.*, 2003).

Plasmid isolation and genotyping by pulsed-field gel electrophoresis (PFGE)

Plasmids were isolated using the alkaline-lysis method, digested with EcoRV, run on 0.9% (ethidium bromide supplemented) agarose gels at a constant 8 V cm^{-1} for 1 h and visualized on a UV transilluminator.

PFGE with ApaI-digested genomic DNAs, which were prepared according to the Antibiotic Resistance Prevention and Contact (ARPAC) protocol (http://www.hpa.org.uk/hpa/inter/arpac/a_typingprotocol.pdf), was run on 1% agarose gel [in 0.5× TBE (Tris-borate-EDTA) buffer] for 20 h at 6 V cm^{-1} at 14 °C using a CHEF-DRII system (Bio-Rad). DNA fingerprints were compared visually according to the Tenover criteria (Tenover *et al.*, 1995).

PCR screening and Southern hybridization

DNAs for PCR screening were prepared by boiling a dense bacterial suspension in ddH_2O. The 50-μL PCR reaction mixture consisted of 1× buffer, 1.5 U Taq polymerase (Fermentas, Lithuania), 1.5 mM $MgCl_2$, 0.8 mM dNTPs and 50 pmol primers. The full-length bla_{OXA-23} gene was amplified with forward (5′-GATCTGGTGTTTAAAATGAA TAAATAT-3′) and reverse (5′-GGGCTTTTTATAAAATTAG AGTTTC-3′) primers. A 563-bp fragment of the IS*AbaI*–Oxa23 structure, flanking on the 5′ to the transposase and on the 3′ to bla_{oxa-23}, was amplified with forward (5′-TATTAAGCAATGTCCAAAGG-3′) and reverse (5′-CTCGGGGTTTCATTTATTAA-3′) primers. The annealing temperature was 52 °C for both primer pairs.

The *amp*C enzyme's transcriptional expression in outbreak isolates was estimated relative to a ceftazidime-susceptible *A. baumannii* by quantitative real-time PCR as described elsewhere in detail (Savli *et al.*, 2003). Reverse transcriptase PCR (RT)-PCR reactions were set up in duplicate with cDNAs from three independently isolated RNA samples. The crossing-point (CP) values obtained during RT–PCR for *amp*C mRNA were normalized by the CP values obtained simultaneously for the corresponding isolate's 16S rRNA gene. The relative expression ratio and significance were estimated with the aid of an Excel applet (http://www.gene-quantification.info) (Pfaffl, 2001; Kolayli *et al.*, 2004). The primer pairs used for RT–PCR were as follows: for the *amp*C gene, forward (5′-GGCGTCAAATC GACACTACCC-3′) and reverse (5′-CAACCGAGTGCCT GATACATGG-3′); and for the 16S rRNA gene, forward (5′-ACGGGTGAGTAATGCTTAGG-3′) and reverse (5′-CGC AAGGTCCGAAGATCC-3′). An annealing temperature of 55 °C was used for both primer pairs. The association between the *amp*C gene and the IS*AbaI* transposase was analyzed by PCR screening, with the forward primer used to detect IS*AbaI* and the reverse primer used to detect the *amp*C gene as described above.

DNAs for Southern hybridization were prepared as reported elsewhere (Vahaboglu *et al.*, 2006). A probe internal to bla_{OXA-23} was generated by labeling PCR products with digoxigenin-dUTP (Roche)-supplemented master mixtures (Vahaboglu *et al.*, 2006). Southern hybridization and

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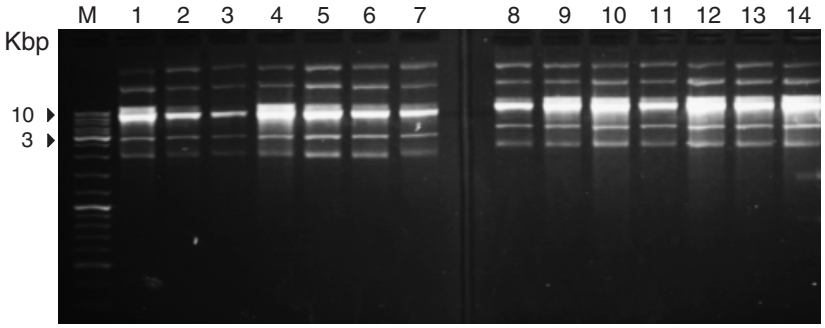


Fig. 1. EcoRV restriction fragment patterns of plasmids. Lane M, DNA ladder (GeneRuler™ mix, Fermentas); lanes 1–14, plasmid fragments from randomly selected nonrepetitive isolates. Plasmid restriction patterns were indistinguishable and did not give a positive hybridization signal with the bla_{OXA-23} probe (not shown).

detection were accomplished using the digoxigenin-dUTP detection kit (Roche) as recommended by the manufacturer.

Cloning

For shotgun cloning, genomic DNA partially digested with MboI was ligated to the dephosphorylated BamHI arms of the phagemid vector pBK–CMV (Stratagene) overnight at 16 °C and was then transformed to electrocompetent *E. coli* DH10B cells (Invitrogen) by electroporation (Eppendorf model 2510) at 1700 V in a 50 μL volume. Positive clones were selected on agar plates supplemented with ampicillin (50 μg mL^{-1}) and kanamycin (35 μg mL^{-1}). The insert's sequence was determined by the dye terminator cycle sequencing methodology (Agowa, Germany).

Results and discussion

Experiments in this study, if not otherwise indicated, were performed with 14 randomly selected nonrepetitive isolates from patients admitted to ICU in different months during the outbreak period.

ARDRA and OXA-51-like PCR screening revealed that the isolates belong to the *Acinetobacter* genomic species 2. The MICs of studied antibiotics were as follows: [median (min–max), μg mL^{-1}]; amikacin, >256; cefepime, 64 (16–128); ceftazidime, 64 (32–128); cefoperazon/sulbactam, 64 (32–64); ciprofloxacin, 64 (64–128); imipenem, 64 (64–128); meropenem, 32 (32–64); and piperacillin/tazobactam, >256. Colistin and tigecycline were unavailable.

Cloning and sequencing of a 3171-bp fragment from an imipenem-resistant clinical isolate confirmed the presence of bla_{OXA-23} associated with an IS*AbaI* transposase (GenBank accession, EF120622). PCR screening revealed the existence of IS*AbaI*–bla_{OXA-23} structure in all 94 isolates recovered during the outbreak period. Detection of a highly mobile genetic element such as IS–OXA23 does not necessarily show that a unique strain is responsible for this epidemic, because epidemic clones with few differences spread throughout geographically distinct areas (van Dessel *et al.*, 2004).

However, a comparison showed that both EcoRV restriction patterns of plasmids (Fig. 1) and the ApaI macrorestriction patterns of genomic DNAs were indistinguishable (Fig. 2a), suggesting a clonal spread of the outbreak strain *A. baumannii* (AcKOU1) in the ICU. A digoxigenin-labeled probe internal to bla_{OXA-23} hybridized with a 4.5-kb fragment of EcoRV digested genomic DNAs (not shown), and also with one or (in one isolate) two ApaI fragments of PFGEs (Fig 2b), but did not hybridize with *Acinetobacter* plasmids (not shown). These hybridization experiments suggest that IS-OXA23 is located on the AcKOU1 chromosome. On the other hand, the presence of multiple copies of bla_{OXA-23} may affect the MICs as was shown with bla_{OXA-58} in another study (Bertini *et al.*, 2007).

The sequence analysis of the recombinant plasmid pBK-AcKOU1 revealed that the IS*AbaI*-bla_{OXA-23} structure (IS-OXA23) lay between two *Acinetobacter* genes. Immediately upstream of the left inverted repeat of IS*AbaI* was a disrupted ORF that is 99% identical to the gene that encodes a major facilitator superfamily (MFS) metabolite protein (GenBank accession ABO10595) of *A. baumannii* ATCC 17978. In addition, downstream of the 125-bp flanking region of bla_{OXA-23} was a truncated ORF encoding the protein acyl–CoA synthetase/AMP–acid ligase of *Acinetobacter* (99% identity with accession ABO10599 of *A. baumannii* ATCC 17978) (Fig. 3). On the *Acinetobacter's* (ATCC 17978) chromosome, the gene that encodes Acyl-CoAS is located *c.* 4100 bp downstream of the gene that encodes MSF. The configuration of these genes is reversed on the cloned fragment, which suggests that this part of the genome might have reorganized during the recombination of the IS–OXA23 structure.

To search for the right extremity of a composite transposon, we set up PCR reactions with several combinations of sense primers that were specific for bla_{OXA-23} and for the truncated acyl–CoA synthetase genes with antisense primers that were specific for both the transposase gene and the MSF gene's missing 5′ fragment. These experiments failed to confirm the existence of a composite transposon as was shown recently on the plasmids of various *Acinetobacter* isolates (Corvec *et al.*, 2007).

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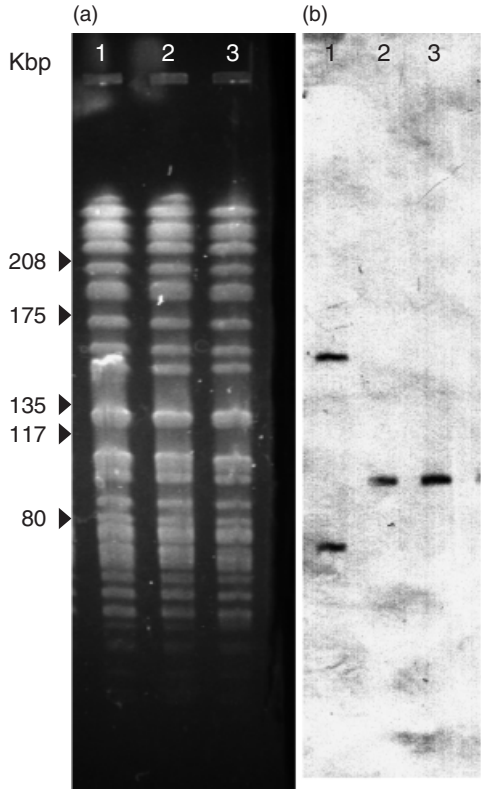


Fig. 2. A representative picture of the PFGE experiment (a) and Southern hybridization of corresponding lanes with a probe specific to bla_{oxa-23}(b). PFGE and Southern hybridization were performed with 14 nonrepetitive isolates. All exhibited the same pattern in PFGE and all but one (lane 1) the same in Southern hybridization with the probe specific to bla_{oxa-23}. Interestingly, in one isolate (lane 1), bla_{oxa-23} probe produced two signals indicating the existence of two copies of the *bla* gene.

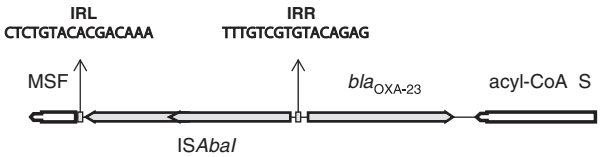


Fig. 3. Schematic map of the cloned fragment. Inverted repeats bracketing the IS*abai* transpose are indicated by vertical lines. IS*abai*-bla_{oxa23} genes are shaded.

The disk diffusion test on MH agar supplemented with an AmpC enzyme inhibitor (cloxacillin) showed an obvious inhibition of resistance against cefotaxime (12 mm zone diameter vs. no-zone), a less striking inhibition against ceftazidime (8 mm zone diameter vs. no zone) and no inhibition against imipenem in the epidemic strain, which indicated that the chromosomal *amp*C enzyme plays a role in the high MIC values of at least some of the β-lactam antibiotics. Accordingly, RT-PCR showed that the transcriptional expressions of the bla_{ampC} gene in the outbreak isolates are eight to 10 times ($P < 0.001$) 'up-regulated' relative to the ceftazidime-susceptible strain. The PCR screening for a potential IS*AbaI*-bla_{ampC} structure with specific primers detected a positive amplification (*c.*1400 bp) in isolates of the outbreak but failed to detect this band in the susceptible control strain. This finding may be related to the fact that when IS*AbaI* is located immediately upstream of the bla_{ampC} gene, it provides a strong promoter and thus up-regulates transcriptional expression (Corvec *et al.*, 2003; Heritier *et al.*, 2006).

OXA-23 was first identified in 1985 in an *Acinetobacter* strain from Scotland. The gene encoding this enzyme, named ARI-1, was plasmid born and was associated with the IS*AbaI* transposase (Donald *et al.*, 2000). Since then, the IS–OXA23 structure has been found among *Acinetobacter* isolates from various countries (Corvec *et al.*, 2007; Valenzuela *et al.*, 2007; Wang *et al.*, 2007) and among multiple clones of *Acinetobacter* (Valenzuela *et al.*, 2007). Interestingly, a 7-bp sequence (CTCTTTT) immediately downstream of the right IR, which is common to all these submitted sequences, is absent from both the sequence from Scotland and the one in this study, which may indicate a diversion in the IS-OXA23 structure's evolution (Valenzuela *et al.*, 2007).

In conclusion, this study indicates that bla_{OXA-23} bearing and *amp*C-overexpressing MDRAB is a significant pathogen that deserves attention, especially in settings where infection control measures cannot be implemented properly.

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