Characterization of $\text{bla}_{\text{OXA-23}}$ gene regions in isolates of Acinetobacter baumannii

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Background/purpose: To investigate the characterization of $\text{bla}_{\text{OXA-23}}$ gene regions in isolates of Acinetobacter baumannii from Taizhou Municipal Hospital.

Methods: Fifty-nine non-repetitive, multiresistant (including imipenem-resistant) isolates of A. baumannii were recovered from clinical infections in hospitalized patients from January 2010 to August 2011 in Taizhou Municipal Hospital (affiliated with Taizhou University) in China. These isolates were genotyped using pulsed-field gel electrophoresis (PFGE). $\text{bla}_{\text{OXA-23}}$ β-lactamase and associated genetic structures were analyzed using polymerase chain reaction (PCR), and recombinant plasmids were analyzed by BamHI- or SacI- restriction enzyme digestion; predicted promoter structures of $\text{bla}_{\text{OXA-23}}$ genes were determined and compared using protein-protein BLAST analysis.

Results: Fifteen out of 59 isolates expressing imipenem-resistant A. baumannii clinical isolates acquired either a $\text{bla}_{\text{OXA-23}}$ β-lactamase gene. A new gene cluster (ISAAb1-$\text{bla}_{\text{OXA-23}}$-AMP) with three previously identified transposons (Tn2006, Tn2007, and Tn2008) and one previously identified gene cluster (ISAAb1-$\text{bla}_{\text{OXA-23}}$) was found in the isolates. Recombination plasmids were...
Acinetobacter baumannii, a typical opportunistic pathogen, is often involved in nosocomial outbreaks, and increasingly reported to be resistant to carbapenems. The strains may be linked not only to the production of Ambler class B metallo-β-lactamasases, but also to the production of carbapenem-hydrolyzing class D β-lactamasases (CHDLs).

Several studies have shown the geographically widespread occurrence of three identified multidrug-resistant A. baumannii strains (European clones I, II, and III), suggesting their clonal relatedness. According to literature reports, A. baumannii strains with increasing resistance to carbapenems have been observed worldwide in the past decade; the main cause of carbapenem resistance in A. baumannii clinical isolates worldwide is the production of CHDLs, also designated OXA-type carbapenemases. From the different groups of OXA-type enzymes defined on basis of sequence comparisons, OXA-23, OXA-24, and OXA-58 (which can be either plasmid- or chromosomally-encoded) have been most frequently associated to carbapenem-resistant clinical strains of A. baumannii, but OXA-23 has the higher dissemination worldwide. The β-lactamase OXA-23 was identified first in A. baumannii from Scotland and was found to confer transferable imipenem resistance in A. baumannii.

Since then, OXA-23 producers have been identified as the source of nosocomial outbreaks worldwide, including Brazil, Colombia, the United Kingdom, Korea, Tahiti (France), and China. Recently, transposons Tn2006, Tn2007, and Tn2008 have been described in A. baumannii isolates from Europe, and Tn2008 is a major vehicle carrying blaOXA-23 in A. baumannii from China. We describe here the characterization of the genetic structures in which blaOXA-23 genes are embedded in A. baumannii clinical isolates obtained in Taizhou Municipal Hospital of China, between January 2010 and August 2011. These structures included three transposons (Tn2006, Tn2007, and Tn2008) and two gene clusters (ISAba1-blaOXA-23-AMP and ISAba1-blaOXA-23). This is the first report that identifies blaOXA-23 genes harbored by Tn2006, Tn2007, and Tn2008 transposons in our region.
or SacI. The resulting fragments were ligated into either BamHI- or SacI-digested pBKCMV plasmid, and transformed into E. coli DH10B as previously described.23

Transformed bacteria were plated on trypticase soy agar plates containing amoxicillin (50 μg/mL) and kanamycin (30 μg/mL), and plasmids obtained from different colonies selected under these conditions were purified in order to sequence and analyze the cloned DNA fragments as described previously.24 The deduced genetic structures associated with \( \text{bla}_{OXA-23} \) gene obtained from these studies are depicted in Fig. 2. Finally, the cloned DNA fragments were sequenced and analyzed as described previously.24

Predicted promoter structures of \( \text{bla}_{OXA-23} \) genes and comparison of partial amino acid sequences for OXA-23-like enzymes

The different promoter regions of \( \text{bla}_{OXA-23} \) or \( \text{bla}_{OXA-23} \)-like genes identified in the \( \text{A. baumannii} \) isolates studied here were obtained by sequence analysis of PCR fragments obtained using primers described in Table 1, and the results are shown in Fig. 3. The deduced amino acid sequences of OXA-23 enzymes were subsequently compared using BLASTp. The sites of initiation of transcription of the \( \text{bla}_{OXA-23} \) gene were mapped also using real time PCR as the primer of pre-OXA-23 (Table 1).

Results

Bacteria source and antibiotic susceptibility

The majority of the 59 multiresistant (including imipenem-resistant) isolates of \( \text{A. baumannii} \) came from the ICU (52.2%), neurosurgery (22.0%), and respiratory unit (10.2%), with most samples isolated from sputum (74.6%). In addition, all of the imipenem-resistant isolates showed high resistance rates (> 99%) to ampicillin, cefoxitin, ceftazidime, levofloxacin, amikacin, and cefoperazone/sulbactam in 15 out of 59 isolates, respectively (Fig. 1).

Molecular typing

Fifteen out of 59 clinical isolates were grouped into five clonal patterns by PFGE. The two major patterns were pattern A represented by nine isolates and pattern B represented by three isolates (Fig. 1). Each of the patterns C, D, and E was represented by a single strain. Four molecular types, namely patterns A, B, D, and E, were from the ICU. Strains Ab34 and Ab37, both isolated from venous cannulas of patients, belonged to patterns C and A, respectively (Fig. 1). Meanwhile, strains Ab12 and Ab28, both from the blood of patients in neurosurgery, had patterns B and E, respectively (Fig. 1). Pattern A clonal lineage was shared by isolates Ab53, Ab22, Ab40, Ab9, Ab24, Ab31, Ab15, Ab28, and Ab19 based on PFGE analysis following Tenover et al.20 criteria (Fig. 1). According to the details, however, isolates Ab53, Ab22, Ab40, Ab9, and Ab24 were apparently identical on this basis, but isolates Ab31, Ab15, Ab28, and Ab19 were closely related to the above. Actually, isolates Ab31 and Ab15 were apparently identical among them, but differed in PFGE fragments of isolates Ab53, Ab22, Ab40, Ab9, and Ab24; simultaneously, isolates Ab28 and Ab19 were apparently identical among them but were different with PFGE fragments of isolates Ab31 or Ab15. Consequently, they were obviously of different subtypes of pattern A between isolate Ab28 or Ab19 and isolate Ab31 or Ab15, in the study.
Identification of genetic structures containing \textit{bla}_{OxA-23} genes in the \textit{A. baumannii} clinical isolates under study

Different genetic structures harboring \textit{bla}_{OxA-23} genes were identified in the \textit{A. baumannii} isolates under study by cloning BamH1- or SacI-digested DNA fragments in plasmid pBKCMV and selecting \textit{E. coli} transformants bearing \textit{bla}_{OxA-23} genes in amoxicillin-containing solid media. Further sequence analysis of different clones allowed the identification of five different genetic structures in which \textit{bla}_{OxA-23} genes were present (Figs. 1 and 2). These were transposons Tn2006, Tn2008, and IS\textit{Aba1}-\textit{bla}_{OxA-23}-AMP-acid ligase gene, and IS\textit{Aba1}-\textit{bla}_{OxA-23} (Fig. 2). The \textit{A. baumannii} isolates in which these structures were identified are depicted in Fig. 1. As seen in this figure, the IS\textit{Aba1}-\textit{bla}_{OxA-23} arrangement was the most frequently found (10/15 isolates: Ab31, 22, 40, 9, 14, 24, 41, 5, 17, and 19). Tn2008 was found in two isolates (Ab12 and Ab18), Tn2006 in Ab34, Tn2007 in Ab37, and IS\textit{Aba1}-\textit{bla}_{OxA-23}-AMP-acid ligase gene in Ab53.

\textbf{IS-derived hybrid promoters enhancing \textit{bla}_{OxA-23} expression}

Using BLASTp analysis, although the \textit{bla}_{OxA-23}-like amino acid sequence was approximately 99.9\% identical to that of \textit{bla}_{OxA-23} with a difference at site 104 (K104X), however, the later experiment proved that \textit{bla}_{OxA-23}-like was identified with \textit{bla}_{OxA-23}. \textit{IS\textit{Aba1}} is an insertion sequence located upstream of \textit{bla}_{ampC} and \textit{bla}_{OxA-23} genes in \textit{A. baumannii} associated with overexpression of these genes.\textsuperscript{25,26} As seen in Fig. 3, in isolates Ab31, 22, 40, 9, and 17 we found that an \textit{IS\textit{Aba1}} insertion upstream of the \textit{bla}_{OxA-23} gene generated a hybrid promoter (-35 sequence, TTAGAA; -10 sequence, TTATT) identical to that identified previously\textsuperscript{26} that directs expression of this gene. Moreover, we also found in strain Ab37 an \textit{IS\textit{Aba4}} insertion upstream of the \textit{bla}_{OxA-23} gene, also generating a hybrid promoter directing expression of this gene. In this case, the -1 transcription start was located 31 bp upstream of the \textit{bla}_{OxA-23} start codon, and inside \textit{IS\textit{Aba4}} IRL. The identified -35 sequence (TAACTA) and -10 (TTTCTT) promoter sequences which were separated by 17 bp (Fig. 3) were identical to those described previously by other authors.\textsuperscript{26}

\textbf{Discussion}

In this study, the 59 non-repetitive pan-resistant (including imipenem-resistant) isolates of \textit{A. baumannii} were from the ICU unit (52.2\%) and neurosurgery (22.0\%). Fifteen out of 59 clinical isolates were grouped into five clonal patterns by PFGE: the two major patterns were pattern A (9 isolates) and pattern B (3 isolates); patterns A, B, D, and E were from the ICU unit (8 isolates) and pattern C was from the respiratory unit (1 isolate). It was suggested that pattern A might be prevalent in our hospital, and pattern B happened to appear in the ICU unit of our hospital. However, further investigation showed that pattern B also appeared in the neurosurgery and respiratory units in latter experiments.

Carbapenems have the most extended antimicrobial spectrum of antibacterial drug among all the \beta-lactams. However, reports of carbapenem-resistance have been increasing, especially in \textit{P. aeruginosa} and \textit{A. baumannii}.\textsuperscript{13} A limited number of antimicrobial agents, including polymyxin, sulbactam, and minocycline, maintain a high activity against \textit{OXA-23}-producing \textit{A. baumannii}.\textsuperscript{10} In particular, sulbactam has a direct antimicrobial and synergistic effect with ampicillin against \textit{A. baumannii}.\textsuperscript{27}

In silico analyses of GenBank databases and personal observations have indicated that an \textit{IS\textit{Aba1}} element, which belongs to the IS4 family, is constantly identified upstream of \textit{bla}_{OxA-23}, regardless of the geographical origin of the isolate. In addition, an \textit{IS\textit{Aba4}} element, which belongs to the IS982 family, has been identified recently in \textit{A. baumannii} clinical isolates from France and Algeria.\textsuperscript{26} The presence of these IS elements upstream of \beta-lactamase genes suggests that they may play a role in \textit{bla}_{OxA-23} expression by providing strong hybrid promoter sequences, and perhaps participate in the acquisition of carbapenem resistance.\textsuperscript{1,28}

This study demonstrated that transposons Tn2006, Tn2007, and Tn2008, as well as genetic structures IS\textit{Aba1}-\textit{bla}_{OxA-23}-AMP and IS\textit{Aba1}-\textit{bla}_{OxA-23} were present in the clinical strains of \textit{A. baumannii} studied here. There is an association between the presence of these \beta-lactamase-
containing genetic elements and the increased MIC for imipenem, implying higher drug-resistance levels. Increased resistance to imipenem suggests that clinical isolates may contain one or more of the above transposons and structures. We hypothesize that carbapenem-hydrolyzing class D $\beta$-lactamase genes, belonging to the blaOxA-23, serve as the actual resistance genes in the study. An ISAba4 element was identified in the upstream region of a blaOxA-23 gene in A. baumannii isolate Ab37. This ISAba4 element belongs to the ISA982 family, is 975 bp in length, possesses two 18 bp IRs, and encodes a 292 amino acid putative transposase. No target site duplication was observed on either end of ISAba4. PCR mapping by different sets of primers did not detect any extra copy of ISAba4 downstream of the blaOxA-23 gene. Instead, a predicted AAA ATPase gene truncated at its 5’ terminus is located downstream of the blaOxA-23 gene. Detailed analysis of the truncation site identified a 7 bp sequence with an AT-rich content (TAATATA) at the extremity of the IRR of ISAba4 (Fig. 2), which most likely represented a transposition process mediated by ISAba4 that occurred at the origin of acquisition of the blaOxA-23 gene. This potential transposon, termed Tn2007, is 2471 bp long and included ISAba4 and blaOxA-23 genes. The ISAba4-mediated mobilization process may be based on a one-ended transposition mechanism. This is unlike what has been observed for ISEcpl1, which uses a wide range of DNA sequences such as that of IRR, which are not absolutely random.24,26,27 These data were consistent with previous findings.26 The Tn2006 insertion isolated from isolate Ab34 occurred inside a gene encoding a putative sulfonamide resistance protein sharing 89% amino acid identity to a protein identified in A. baumannii AYE (accession no.CAJ31116). Sequence analysis of strain Ab34 revealed a 2445 bp sequence containing the blaOxA-23 gene, as well as part of a putative AAA ATPase gene,29 and part of a putative DEAD helicase gene.26 These two latter genes were truncated at the exact same position, suggesting that a recombination event had occurred.26 Simultaneously, ISAba1 has been shown to be very prevalent in A. baumannii and might be “customized” for that species.30 It was described as an ISAba1-based putative composite transposon in a previous reference,26 and it was also proved to be identical in isolate Ab34 (Fig. 2A). Tn2008, identified from isolate Ab12 or Ab28, was the major vehicle carrying blaOxA-23 and had been inserted into
However, in recent studies, three different AT-rich locations (Fig. 2B). It is likely that this 16-bp sequence at the left end of Tn2008 served as IRL of ISAba1. A single copy of ISAba1 could transpose blaOXA-23 and ATPase with itself by recognizing IRL as a new left boundary. The 16 bp sequence resembled the inverted repeat of ISAba1, suggesting that ISAba1 might have utilized an alternative boundary to mobilize blaOXA-23. ISAba1 seems to be quite an important factor of genetic plasticity in A. baumannii and may facilitate the creation of composite transposons. Together with our study that ISAba1 could be considered widespread in A. baumannii and a previous report, this could suggest that blaOXA-23 originated from an Acinetobacter species (A. radioresistens). However, in recent studies, the genetic backgrounds of this enzyme were characterized, supporting its horizontal acquisition. It could also be hypothesized that the Tn2008 putative composite transposon might have been formed in A. baumannii after a previous acquisition process not related to ISAba1.

To our knowledge, the genetic cluster ISAba1-blaOXA-23-AMP was first to be identified in isolate Ab53 in the study. Its structure is similar to that of strain AcKOU1, but with slight differences: the left side of structure ISAba1-blaOXA-23 lacks a H+ symporter gene, and the right side contains the full-length AMP-acid ligase gene. Meanwhile, our study also expresses that the ISAba1-blaOXA-23 arrangement can be found in A. baumannii clonal lineages, namely patterns A, B, and D; Tn2008 can be found in clonal lineages, namely patterns A and C, and one group B or E isolate harbors Tn2008 or Tn2007. These findings probably suggest that different clones of A. baumannii might be widespread with the majority of ISAba1-blaOXA-23 clonal lineages in our hospital.

The corresponding promoter structures for the blaOXA-23 gene in strains Ab31, 22, 40, 9, and 17, were made up of a -35 sequence (TTAGAA) and the -10 sequence (TTATTTT), with a 16 bp spacer distance between them using bioinformatics procedures. These results agreed with previous work on ISAba1, but were not consistent with findings from other studies describing a 17 bp spacer region and a -10 promoter sequence of “TTATTT”. Further studies are needed to investigate the nature of these inconsistencies.

Although all the strains were recovered from patients, drug-resistant bacteria are also found in hospital spots such as bed sheets, desks, floors, and cisterns. Numerous studies have shown that the hospital environment was a preferred habitat for A. baumannii isolates. A. baumannii appears to have unique characteristics among nosocomial Gram-negative bacteria with enhanced environmental persistence. Our current findings warrant more extensive investigations into the occurrence of drug resistant bacteria.

Conflicts of interest
All authors declare no conflicts of interest.

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