bla<sub>OXA-23-like</sub> and bla<sub>TEM</sub> rather than bla<sub>OXA-51-like</sub> contributed to a high level of carbapenem resistance in <i>Acinetobacter baumannii</i> strains from a teaching hospital in Xi’an, China

Lei Han, PhD<sup>a,b</sup>, Jine Lei, MMEd<sup>a,b,c</sup>, Jiru Xu, PhD<sup>a,b</sup>, Shaoshan Han, MD, PhD<sup>d,*</sup>

Abstract

<i>Acinetobacter baumannii</i> is one of the leading causes of nosocomial infections, and is responsible for increasing number of infections in intensive care units (ICUs) worldwide.<sup>11</sup> It is resistant to a number of commonly used antimicrobial agents intrinsically, and has a notable capacity to develop antibiotic resistance via diverse mechanisms.<sup>2,3</sup>

As atypical β-lactam antibiotics, carbapenems also show their bactericidal effects by inhibiting penicillin-binding proteins (PBPs), which are involved in cell-wall synthesis.<sup>10</sup> Carbapenems are widely used in the treatment of multidrug-resistant <i>A. baumannii</i> infections. However, resistance to these antibiotics has remarkably increased due to β-lactamase mediated and non-β-lactamase mediated mechanisms.<sup>11,12</sup> The production of Ambler class A, class B, and class D β-lactamases is the main reason for β-lactamase mediated resistance.<sup>16</sup>

In this study, the resistance patterns to imipenem and meropenem were investigated for 51 nonduplicate <i>A. baumannii</i> clinical strains collected from the First Affiliated Hospital of Xi’an Jiaotong University in Xi’an, China. The molecular determinants responsible for carbapenem resistance (CR), and their relationship with CR phenotype were further analyzed. Moreover, the expressions of positive CR genes were determined to reveal the particular genes playing a key role in CR.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Fifty-one <i>A. baumannii</i> clinical strains were collected from the First Affiliated Hospital of Xi’an Jiaotong University in 2013. The isolates were identified by VITEK 2 system (bioMérieux, Marcy l’Etoile, France), and further confirmed using specific PCR
with primers sp2F, sp4F, and sp4R.17 A. baumannii standard stain ATCC 19606 was kept in our laboratory and used as a positive control. The bacteria were grown on Mueller-Hinton agar (MHA; OXOID, Hampshire, UK) plates and stored in Muller-Hinton broth (MHB; OXOID, Hampshire, UK) containing 20% glycerol at −80°C.

2.2. Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using VITEK 2 system. Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used as quality control strains. The minimal inhibitory concentrations (MICs) of imipenem and meropenem were determined by Etest strips (BIO-KONT, Wenzhou, China), which had antibiotic concentrations of 0.008, 0.016, 0.032, 0.064, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 μg/mL. The susceptibility results were interpreted according to the M100-S27 guidelines of Clinical and Laboratory Standards Institute (CLSI).18

2.3. DNA extraction and detection of carbapenem resistance genes

Genomic DNAs of 51 A. baumannii strains were extracted using TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China) according to the manufacturer’s instruction. A total of 15 β-lactamase encoding genes were screened in which blaKPC, blacES, blbTIM, blabSM1, blabCTX-M-1,1,3,9,12,15 were class A β-lactamases genes, blabIMP-1,4,6,9,10,18, blabIMP-2,8,13,19,20, blabTEM-1,2,4,5, blabVIM-2,6,8,11, blabNDM-1, blabOXA-23-like, blabOXA-24-like, blabOXA-51-like, blabOXA-18-like were class D β-lactamases genes.19 The primer sequences are summarized in Table 1. PCR was performed using Golden Easy PCR System (TIANGEN, China) in a total volume of 25 μL containing 12.5 μL of 2 × Reaction Mix, 0.5 μmol/L of each primer, 0.5 U of Golden DNA Polymerase, and 1 μL of DNA template. The amplification reaction consisted of a pre-denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 35 seconds, and extension at 72°C for 1 minute, followed by a final elongation at 72°C for 10 minutes. Positive PCR products were purified and sequenced by Sangon Biotech (Shanghai, China). In addition, the existence of ISAba1 in the upstream of blabOXA-23-like and blabOXA-51-like genes was also investigated using primer pairs ISAba1F/OXA-23-likeR and ISAba1F/OXA-51-likeR respectively according to Turton et al.10

2.4. Correlation analysis between CR genes and CR phenotype

The correlation of CR genes with CR phenotype was statistically analyzed by Chi-square test using SPSS 22.0 (SPSS Inc, Chicago, IL.). P value and contingency coefficient were obtained for each gene. The gene was considered correlated with CR phenotype if P value < .01. A higher contingency coefficient suggested stronger correlation.

2.5. RNA extraction and quantitative real-time PCR (RT-qPCR)

RNA templates were extracted with RNaprotect Bacteria Reagent (QIAGEN, Hilden, Germany) and RNeasy Mini Kit (QIAGEN) following the manufacturer’s protocol. Residual DNA was removed by DNase I (Thermo Scientific, Vilnius, Lithuania). One microgram of DNA-free total RNA from each sample was subsequently reverse transcribed to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with random hexamer primer. The expression levels of PCR-positive genes TEM (TEM-1), OXA-23-like (OXA-23), and OXA-51-like (OXA-66 and OXA-69) were analyzed by RT-qPCR using the Agilent Mx3005P QPCR System (Agilent Technologies, Santa Clara) and SYBR Select Master Mix (Applied Biosystems, Austin) in a final volume of 20 μL. The primers used for analysis are listed in Table 1. Cycling condition was carried out at 50°C for 2 minutes and 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 1 minute. Triplicate samples were analyzed and standardized against 16S rRNA gene expression.11

2.6. Ethical review

This study was approved by the Ethics Committee of Xi’an Jiaotong University Health Science Center.
3. Results

3.1. Bacterial strains and antibiotic susceptibility testing

All 51 bacterial strains were confirmed as A. baumannii using the methods of VITEK 2 and specific PCR amplification. Antibiotic susceptibility testing showed that strains 1 to 45 were resistant to both imipenem and meropenem, and strains S1 to S6 were sensitive to these 2 antibiotics (Table 2). The resistance ratio reached to 88.2%.

3.2. Carbapenem resistance gene analysis

Among 15 resistance-related genes, 3 were positive in bacterial genomic DNAs checked by PCR (Table 2). In which, blatem and blaOXA-23-like genes only showed up in CR strains, with the appearance ratio of 91.1% (41/45) and 97.8% (44/45), respectively. It was noteworthy that blaOXA-51-like gene was positive in all 51 A. baumannii strains including both CR and carbapenem-sensitive isolates. Moreover, ISAba1 was found upstream to blaOXA-23-like rather than blaOXA-51-like. Thus, the resistance gene profile in this study was divided into 4 types: TEM + OXA-23-like + OXA-51-like, TEM + OXA-51-like, OXA-23-like + OXA-51-like, and only OXA-51-like (Table 3). Sequencing of positive PCR products revealed that the resistance genes were subtypes TEM-1 for TEM, OXA-23 for OXA-23-like, and OXA-66 and OXA-69 for OXA-51-like.

3.3. Correlation analysis between CR genes and CR phenotype

blatem and blaOXA-23-like genes (both P values were <.001) rather than blaOXA-51-like gene were statistically associated with CR phenotype analyzed by Chi-square test. The contingency coefficient of 0.675 for OXA-23-like and 0.595 for TEM showed that they were highly correlated with CR. There was no statistic relationship between OXA-51-like and CR phenotype, as both resistant and sensitive strains harbored this gene.

3.4. Expression of resistance-related genes

In order to ascertain the genes that played a key role in leading our A. baumannii strains to the CR phenotype, the expressions of 3 PCR-positive genes were evaluated by RT-qPCR. TEM-1 and OXA-23 showed positive results, but no expression was detected for OXA-66/69 in either susceptible or resistant strains (Fig. 1).

Table 2

Antibiotic resistance patterns of A. baumannii strains.

| Strain | Imipenem MIC, μg/mL | Meropenem MIC, μg/mL | β-lactamase encoding genes content |
|--------|---------------------|----------------------|----------------------------------|
| 1      | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 2      | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 3      | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 4      | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 5      | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 6      | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 7      | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 8      | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 9      | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 10     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 11     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 12     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 13     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 14     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 15     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 16     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 17     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 18     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 19     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 20     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 21     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 22     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 23     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 24     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 25     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 26     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 27     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 28     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 29     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 30     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 31     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 32     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 33     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 34     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 35     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 36     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 37     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 38     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 39     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 40     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 41     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 42     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 43     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 44     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| 45     | ≥32                 | ≥32                  | TEM, OXA-23-like, OXA-51-like    |
| S1     | 0.125               | 0.125                | OXA-51-like                      |
| S2     | 0.125               | 0.125                | OXA-51-like                      |
| S3     | 0.25                | 0.25                 | OXA-51-like                      |
| S4     | 0.125               | 0.125                | OXA-51-like                      |
| S5     | 0.25                | 0.25                 | OXA-51-like                      |
| S6     | 0.125               | 0.125                | OXA-51-like                      |

* TEM = blaTEM, OXA-23-like = blaOXA-23-like, OXA-51-like = blaOXA-51-like.

Table 3

Carbapenem resistance gene profile of 51 A. baumannii strains.

| Carbapenem resistance gene profile | Type of isolates | Positive rate, % (n=51) |
|------------------------------------|------------------|------------------------|
| TEM + OXA-23-like + OXA-51-like    | Carbapenem resistance | 78.43 (40) |
| TEM + OXA-51-like                  | Carbapenem resistance | 1.96 (1)    |
| OXA-23-like + OXA-51-like          | Carbapenem resistance | 7.84 (4)      |
| OXA-51-like                        | Carbapenem susceptible | 11.76 (6)   |

Figure 1. Expression of TEM-1, OXA-23, and OXA-66/69 as assessed by PCR and RT-qPCR in clinical isolates.
4. Discussion

Since the 1990s, strains from *A. baumannii* show a dramatic escalation in antimicrobial resistance, even with the appearance of isolates resistant to all antimicrobials except colistin.\[2\] Surveillance carried out in China annually demonstrates an ascending tendency of imipenem and meropenem resistance in *A. baumannii* from 2005 to 2014. The resistance rate ranges from 31.0% to 62.4% for imipenem, and 39.0% to 66.7% for meropenem.\[11\] *A. baumannii* strains in our observation had a higher resistance level at 88.2% (45 out of 51 isolates) for both imipenem and meropenem. This difference might be caused by the survey spectrum. The surveillances of China were carried out in 17 teaching hospitals including various departments, whereas our strains were mostly obtained in ICUs, for example, central ICU, respiratory ICU, and surgical ICU. In addition, an outbreak caused by extensively drug-resistant *A. baumannii* was covered during the period of sample collection.\[13\] Third, as our hospital is a tertiary care teaching hospital in Xi’an, many patients with severe diseases have been infected by drug-resistant *A. baumannii* before admitted in. Similar and even higher ratios of CR in *A. baumannii* have been reported.\[14,15\] In those studies, except of medical and surgical wards, samples are mainly collected from the ICUs.

Bacteria can overcome β-lactam antibiotics by 4 primary mechanisms: changes in the active site of PBPs, modification of porin proteins in the outer membrane of Gram-negative bacterial cell walls, overexpression of multicomponent drug efflux pump systems, and production of β-lactamases. Among which, the last one is the most common and important mechanism of resistance in Gram-negative bacteria.\[8\] In this study, β-lactamase encoding genes from class A, B, and D, which are responsible for CR, were investigated from both CR strains and carbapenem-susceptible strains.

Three (*bla*TEM, *bla*OXA-23-like, and *bla*OXA-51-like) out of 15 genes were found positive in the samples collected from our hospital. And coexistence of resistance genes was detected in most isolates, especially in CR strains. From these 3 genes, OXA-23-like was closely related to CR phenotype according to the correlation analysis, followed by TEM. OXA-23, the subtype of OXA-23-like, belongs to class D oxacillinase, and also named oxacillinase. It is the first reported acquired oxacillinase with appreciable carbapenem-hydrolyzing activity isolated from *A. baumannii*,\[16\] and subsequently showing expression globally through both plasmid and chromosomal carriage.\[17\] The positive expression results confirmed its relationship with CR phenotype. The high possession rate (97.8%) of ISAba1–OXA-23 indicated its leading role in causing CR in this study. TEM-1 encodes a class A β-lactamase, and has been found in many CR *A. baumannii* strains in different regions.\[18−21\] It also contributed to CR occurrence in our study, as all the CR isolates carrying this gene showed positive expression.

It was worth noting that OXA-51-like gene existed in all 51 clinical isolates, no matter they were CR or susceptible. OXA-51-like genes have been reported to be present in *A. baumannii* chromosomes,\[12,23\] and control the production of so-called intrinsic carbapenemases.\[24\] Nonetheless, they are commonly expressed at a low level, and show weak hydrolytic activity toward carbapenems, unless an upstream insertion of the ISAba1 sequence.\[10,25\] ISAba1 has been shown to be associated with *bla*OXA-23-like- and *bla*OXA-51-like-related CR of *A. baumannii*.\[26,27\] Its absence in the upstream of OXA-51-like but presence in that of OXA-23-like revealed that OXA-51-like was not as important as OXA-23-like in contributing to the CR phenotype in our study. This was proven by the null expression of OXA-66/-69 in the strains.

In conclusion, a high rate of CR in *A. baumannii* from our hospital was noticed. A coexistence of β-lactamases encoding genes *bla*TEM, *bla*OXA-23-like, and *bla*OXA-51-like was the primary resistance profile. And the drug resistance was closely associated with the expression of *bla*OXA-23-like and *bla*TEM, *bla*OXA-51-like was found in the chromosome of all clinical strains, but neither ISAba1 in its upstream nor the expression of this gene was found, thus *bla*OXA-51-like did not contribute to the CR phenotype.

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