Infection and Drug Resistance

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ORIGINAL RESEARCH

Carbapenemase OXA-423: A Novel OXA-23 Variant in *Acinetobacter baumannii*

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Purpose: A novel variant of OXA-23, named OXA-423, was identified in an *Acinetobacter baumannii* clinical isolate. The aim of this study was to analyse the resistance phenotype of OXA-423.

Methods: The *A. baumannii* strain WY-0713 was isolated from an intensive care unit patient. PCR was used to detect the *bla*$_{\text{OXA-23}}$-like genes. Amplifying, cloning and sequencing were performed for the complete *bla*$_{\text{OXA-23}}$-like. The novel *bla*$_{\text{OXA-423}}$ and its ancestor *bla*$_{\text{OXA-23}}$ were cloned into the expression vector pET-28b(+), and transformed into *E. coli* Rosetta (DE3) for antibiotic susceptibility testing. SDS-PAGE, modified Hodge test and CarbaNP test were used for detecting the expression of OXA-423 and OXA-23.

Results: PCR screening of *A. baumannii* WY-0713 was positive for *bla*$_{\text{OXA-23}}$-like genes. Sequencing of the PCR product identified a novel *bla*$_{\text{OXA-23-like,}}$ named *bla*$_{\text{OXA-423}}$ which encoding OXA-423. OXA-423 differed from OXA-23 by a crucial amino acid substitution (Val128Ala). The V128A substitution was located at the conserved active-site motifs SAV of OXA-23. Antibiotic susceptibility testing performed using isogenic *E. coli* showed that the MICs of *E. coli* Rosetta (pET-OXA-423) for penicillins and carbapenems were lower (reduced MICs 4-fold to 16-fold) than that of *E. coli* Rosetta (pET-OXA-23). The MICs of cefotaxime, ceftazidime and aztreonam for both transformants remained the same as the acceptor strain. Moreover, OXA-423 was slightly inhibited by sulbactam, clavulanic acid and tazobactam. SDS-PAGE analysis showed that OXA-423 and OXA-23 were conspicuously expressed. Modified Hodge test and CarbaNP test were positive demonstrated both of them were functional.

Conclusion: OXA-423, the first report of an amino acid substitution located at conserved active-site motifs of OXA-23, conferred lower MIC values of penicillins and carbapenems as compared with OXA-23, while without affecting the resistance profiles of expanded-spectrum cephalosporins and aztreonam.

Keywords: OXA-423, OXA-23, *Acinetobacter baumannii*, carbapenemase, CHDL

Introduction

Acinetobacter baumannii is one of the most troublesome opportunistic pathogens, especially for ICU patients.[1] Carbapenem antibiotics are the most important treatment strategies for infections caused by *A. baumannii*, due to their efficacy, safety and the increased rate of *A. baumannii* resistance to other antibiotics.[2] Anxiously, Carbapenem-resistant *A. baumannii* (CRAB) has been classified as one of the critical-priority pathogens that need investments in new antibiotics research and development by World Health Organization, due to its increasing prevalence and resistance.[3] Carbapenem-hydrolyzing class D β-lactamases (CHDLs) have been considered as

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the primary factors responsible for carbapenem resistance in *A. baumannii*.[4] Nowadays, six subclasses of CHDLs have been identified in *A. baumannii*: OXA-23-like, OXA-24/40-like, OXA-58-like, OXA-143-like, OXA-235-like, and the intrinsic chromosomal OXA-51-like.[5] Among them, OXA-23-like have spread to many locations worldwide through plasmid-mediated transfer mechanism.[5] So far, 40 OXA-23-like carbapenemases have been identified in *Acinetobacter* species (ftp://ftp.ncbi.nlm.nih.gov/pathogen/Antimicrobial_resistance/ Data, Accessed October 2, 2020). Previous studies on the structure of the CHDLs had showed that the active site of OXA-23-like is represented by three conserved motifs (STFK, SAV and KTG).[6] In addition, the Val128 residue has been defined as a crucial residue for carbapenem binding and hydrolytic cleavage.[7] Notably, all of the amino acid substitutions of the previously reported OXA-23-like variants were located outside the conserved active-site motifs. Here, we report a novel variant of OXA-23, named OXA-423, which differed from OXA-23 by a crucial amino acid substitution (Val128Ala) in the conserved active-site motif of SAV.

Materials and Methods

Bacterial Strain

Acinetobacter baumannii WY-0713 strain was isolated from the sputum of a hospitalized patient in ICU of Yijishan hospital in China on 13 July 2013. The isolate WY-0713 was initially identified as *Acinetobacter calcoaceticus-baumannii* complex using VITEK 2 Compact (bioMérieux, Durham, USA) and later species level was confirmed as *A. baumannii* by sequencing two hypervariable zone sequences of the *rpoB* gene, as previously published.[8] Multilocus sequence typing (MLST) was performed for the WY-0713 strain according to the Pasteur scheme.[9]

PCR, Gene Cloning and Sequencing

The *bla*$_{\text{OXA}}$ type genes (*bla*$_{\text{OXA-23}}$-like, $-2_{4/40}$-like, -5_{1}-like, -5_{8}-like) were detected by PCR, respectively, with internal primers showed in Table 1. PCR was conducted using the ExTaq mix (Takara, Dalian, China) with the conditions of 95°C for 5 min, 35 cycles (95°C for 30 s, 55°C for 20 s, 72°C for 50 s) and a final elongation step at 72°C for 10 min. Then, in order to amplifying the complete *bla*$_{\text{OXA-23}}$-like gene, the external primers were used (Table 1). The PCR conditions was 95°C for 5 min, 35 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 1 min) and a final elongation for 10 min at 72°C. The amplicons of a 1008-bp fragment comprising the complete *bla*$_{\text{OXA-23}}$-like and flanking regions (−46 to +131 bp) were purified using a gel extraction kit (AxyGen, Hangzhou, China) and then cloned into the cloning vector pMD18-T (ampicillin-resistance, Takara, Dalian, China). Subsequently, recombinant plasmids were reproduced in *E. coli* DH5α cells. The complete *bla*$_{\text{OXA-23}}$-like was sequenced using an ABI 3730 DNA Analyzer at GenScript Inc. (Nanjing, China). The nucleotide sequences of *bla*$_{\text{OXA-23}}$-like and deduced amino acid sequences were analyzed using BioEdit™ software and BLAST programs.

Construction of *E. coli* Transformants

To analyse the resistance phenotype, *bla*$_{\text{OXA-423}}$ and its ancestor *bla*$_{\text{OXA-23}}$ were cloned into the expression vector pET-28b(+) (kanamycin-resistant, Novagen, USA) and

Table 1 Primers Used in This Study

Primer	Sequence (5′-3′)	Target	Amplicon Size (bp)	Purpose
OXA-23-like F	ACCCCGAGTCAGATTGTTCAAGG	*bla*$_{\text{OXA-23}}$-like	606	Screening
OXA-23-like R	CCAGCCCACTTGTGGTTTTATAT			
OXA-24-like F	GTCCCTGCATCAACATTTAAGATGC	*bla*$_{\text{OXA-24}}$-like	477	Screening
OXA-24-like R	CACCCAACCAGTCAACCAACCTACCT			
OXA-51-like F	GCTATGGTAATGATCTTGCTCGTGC	*bla*$_{\text{OXA-51}}$-like	509	Screening
OXA-51-like R	TCCAGTTAACCAGCCTACTTGTGGG			
OXA-58-like F	ATATGGCACGCATTTAGACCGAG	*bla*$_{\text{OXA-58}}$-like	515	Screening
OXA-58-like R	CAACAAAACCCACATACCAACCC			
OXA-23-EXT-F	CTATTTTTGTCGTGTACAGAGC	*bla*$_{\text{OXA-23}}$-like	1008	Sequencing
OXA-23-EXT-R	GGATCACAACAACTAAAAGCACTG			
NdeI-OXA-23F[a]	GGAATTCCATATGTTTAAAATGAATAAATATTTTAC	*bla*$_{\text{OXA-23}}$-like	828	Expressing
XhoI-OXA-23R[a]	CCGCTCGAGTTAAATAATATTCAGCTGT			

Note: [a]The restriction sites for NdeI and XhoI are underlined.

overexpressed in *E. coli* Rosetta (DE3) (chloramphenicol resistance), respectively. Both genes were amplified using primers OXA-F-NdeI and OXA-R-XhoI (Table 1). The products of PCR were purified and digested with NdeI and XhoI, and then inserted into pET-28b(+). The ligation was performed at 16°C for 18 h with a 1:1 insert/vector ratio with T4 DNA ligase (Thermo Fisher Scientific, Pittsburgh, USA). Recombinant plasmids (pET-OXA-423 and pET-OXA-23) were introduced into *E. coli* DH5α cells. Transformant strains were selected on LB agar plates containing 20 μg/mL of kanamycin and checked by PCR and sequencing. The recombinant plasmids were then separately transformed into competent *E. coli* Rosetta (DE3) cells using chemical method, producing two *E. coli* transformants. Cells were selected on MH agar plates containing 20 μg/mL of kanamycin and 20 μg/mL of chloramphenicol. Selected transformants were inoculated in 200 mL of fresh MH broth supplemented with the same antibiotics. The transformants were cultured at 37°C with shaking (225 rpm) until the OD_{600} reached to 0.6. Then, IPTG was added to the cells at a final concentration of 0.1 mM to induce the expression of OXA-423 and OXA-23.

Antibiotic Susceptibility Testing

The induced bacterial cells were used for antibiotic susceptibility testing. The minimum inhibitory concentrations (MICs) for the clinical strain WY-0713 and transformants were determined by the agar dilution method, according to the protocols of the CLSI.[10] The modified Hodge test, CarbaNP test and SDS-PAGE were used for detection of the expression of the two proteins.

Protein Modeling

The X-ray structure of OXA-23-meropenem complex (PDB code: 4JF4) was downloaded from the Protein Data Bank database. SWISS-MODEL server (http://swissmodel.expasy.org/) was employed to create the homology model of OXA-423 using OXA-23-meropenem complex structure as a template. Protein structural figures were conducted using PyMOL.[11]

Nucleotide Sequence Accession Number

The nucleotide sequence of $bla_{\text{OXA-423}}$ we reported has been submitted to the GenBank database under accession number KM433672.

Results

PCR, Sequencing and Expressing

The $bla_{\text{OXA-23}}$-like and $bla_{\text{OXA-51}}$-like were detected in the *A. baumannii* WY-0713, but not $bla_{\text{OXA-24/40}}$-like and $bla_{\text{OXA-58}}$-like. DNA sequencing of the 1008-bp PCR product identified a novel variant that had two mutations (A327G and T383C) compared with the $bla_{\text{OXA-23}}$ gene sequence. The nucleotide alteration at position 327 of the novel $bla_{\text{OXA-23}}$ variant was a synonymous mutation, while mutation at position 383 led to the deduced amino acid substitution of a valine by an alanine at position 128 (DBL numbering system) (Figure 1).[12] This novel OXA-23 variant was named OXA-423 by the Lahey clinic (https://www.lahey.org/). DNA sequencing of the amplicon of a 1008-bp fragment comprising the complete $bla_{\text{OXA-23}}$-like and flanking regions (−46 to +131 bp) showed that IS*Aba1* sequence was identified upstream of $bla_{\text{OXA-23}}$-like in the *A. baumannii* WY-0713 strain.

SDS-PAGE analysis of the crude extracts of the two transformants, *E. coli* Rosetta (pET-OXA-423 and pET-OXA-23), showed conspicuously expression of the two proteins. The modified Hodge test and CarbaNP test to carbapenemases were positive for both transformants, demonstrating that the expressed OXA-423 and OXA-23 were functional.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing performed using isogenic *E. coli* showed that the MICs of *E. coli* Rosetta (pET-OXA -423) for penicillins and carbapenems were lower (reduced MICs 4-fold to 16-fold) than that of *E. coli* Rosetta (pET-OXA-23), suggesting that OXA-423 conferred a weaker hydrolytic capacity towards those substrates, as compared to OXA-23 (Table 2). Moreover, OXA-423 was slightly inhibited by sulbactam, clavulanic acid, and tazobactam. Noteworthy, the MICs of cefotaxime, ceftazidime and aztreonam for both transformants remained the same as the acceptor strain, demonstrating the OXA-423 resistance profile to expanded-spectrum cephalosporins and aztreonam was similar to OXA-23 which do not hydrolyze expanded-spectrum cephalosporins or aztreonam (Table 2).

Discussion

Currently, 40 further variants of OXA-23 have been identified in *Acinetobacter spp.* of which differed by one or several amino acids. Previous studies on the structure of

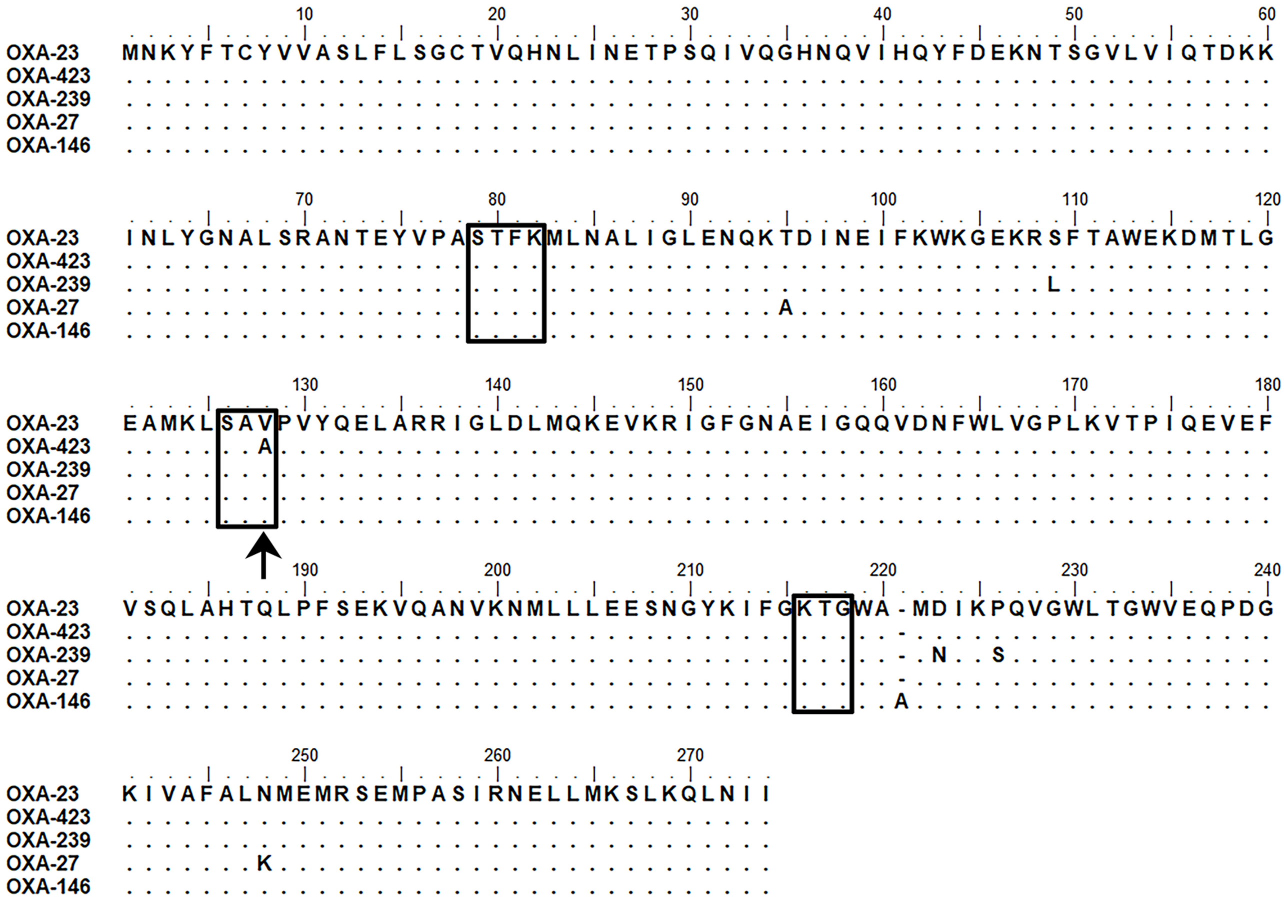

Figure 1 Sequence alignment of OXA-423 with several OXA-23-like carbapenemases. Conserved motifs are boxed. The Val128Ala substitution in OXA-423 was indicated by a black arrow.

the CHDLs had showed that the active site of OXA-23-like is represented by three conserved motifs (STFK positions 79 to 82, SAV positions 126 to 128 and KTG positions 216 to 218) (Figure 1).[6] Notably, for the previously reported OXA-23-like variants, all of the amino acid substitutions were located outside the conserved active-site motifs. However, for OXA-423, the Val128Ala substitution was located at the conserved active-site motif SAV (Figure 1). In addition, the Val128 residue has been defined as a crucial residue for carbapenem binding and hydrolytic cleavage.[7] Therefore, the Val128Ala substitution will possibly affect the activity to hydrolyze carbapenems, and thus affects the resistance level. This speculation was supported by susceptibility tests, OXA-423 conferred lower MIC values of carbapenems in comparison to OXA-23 when expressed in an isogenic *E. coli*. Furthermore, DNA sequencing showed that IS*Aba1* sequence was identified upstream of *bla*$_{\text{OXA-23}}$ in the *A. baumannii* WY-0713 strain. Previous studies have demonstrated that the presence of IS*Aba1* upstream of the *bla*$_{\text{OXA-23}}$ can increase the expression of OXA-23 and contributes to a high level of carbapenem resistance in *A. baumannii*.[13] Surprisingly, despite other resistance mechanisms existed in the *A. baumannii* WY-0713 strain, the MIC of imipenem for this clinical strain was still relatively lower when compared with the *A. baumannii* ATCC 15,151 which harboring IS*Aba1*-*bla*$_{\text{OXA-23}}$ structure.[14] Overall, the strain harboring OXA-423 displayed lower MIC values of carbapenems than that harboring OXA-23, which were both observed in clinical strain and in vitro studies. For the compromised resistance to carbapenems by OXA-423, the most likely explanation was that the Val128Ala substitution conferred reduced carbapenemase hydrolytic activity. To illustrate the potential effect of the substitution, we built a structural model of OXA-423 using the solved OXA-23-meropenem complex structure (PDB code: 4JF4) as a template. Comparison of the homology model of OXA-423 with OXA-23 suggested that the Val128Ala mutation increased the distance of the active site for the substrate as Ala has a smaller side chain than Val (Figure 2). Therefore, the Val128Ala substitution most

Table 2 Antibiotic Susceptibilities of *A. baumannii* WY-0713 and *E. coli* Transformants

Antibiotic(s)	**MIC (µg/mL)**			
	***A. baumannii* WY-0713**	***E. coli* Rosetta(DE3)**		
		pET-OXA-23	**pET-OXA-423**	**pET[a]**
Ampicillin	>512	128	8	2
Ampicillin+SUL[b]	256	32	4	2
Amoxicillin	>512	128	16	2
Amoxicillin+CLA[b]	128	32	8	2
Piperacillin	256	64	8	1
Piperacillin+TAZ[b]	128	16	2	0.5
Cefotaxime	256	≤0.06	≤0.06	≤0.06
Ceftazidime	256	≤0.06	≤0.06	≤0.06
Aztreonam	512	≤0.06	≤0.06	≤0.06
Imipenem	8	4	1	0.12
Meropenem	16	2	0.25	0.06
Ertapenem	16	8	2	≤0.06
Doripenem	8	1	0.12	≤0.06

Notes: [a]*E. coli* Rosetta (DE3) harboring solely pET-28b(+) as negative control. [b]SUL, sulbactam.
Abbreviations: CLA, clavulanic acid; TAZ, tazobactam were used with a fixed concentration of 4, 2, 4 µg/mL, respectively.

likely reduces the affinity of OXA-423 for the substrate, and thus weakens the hydrolytic capacity.

It is worth noting that as it was described in other OXA-23 variants, such as OXA-239 and OXA-27, decreased hydrolytic activity against some β-lactams may be compensated by increased activity against other β-lactams.[15,16] OXA-239, an OXA-23 subfamily variant differed from OXA-23 by three amino acid substitutions (Figure 1), was been identified and horizontally transferred among *Acinetobacter* spp. in Mexico.[17,18] OXA-239 exhibited lower activity against carbapenems, but higher activity against a number of extended-spectrum cephalosporins and aztreonam compared to OXA-23.[15] For OXA-27, another OXA-23 variant differed from OXA-23 by two amino acid substitutions (Figure 1), was more active than OXA-23 against imipenem, whereas cephaloridine-hydrolyzing activity was relatively weaker.[16] Furthermore, maybe mutation impairs the hydrolytic activity not only against carbapenems but also against other β-lactams drugs, even they were not classic substrates of OXA-23-like enzymes. One example was OXA-146, another OXA-23 variant, differed from OXA-23 by a single residue insertion (A220). This insertion

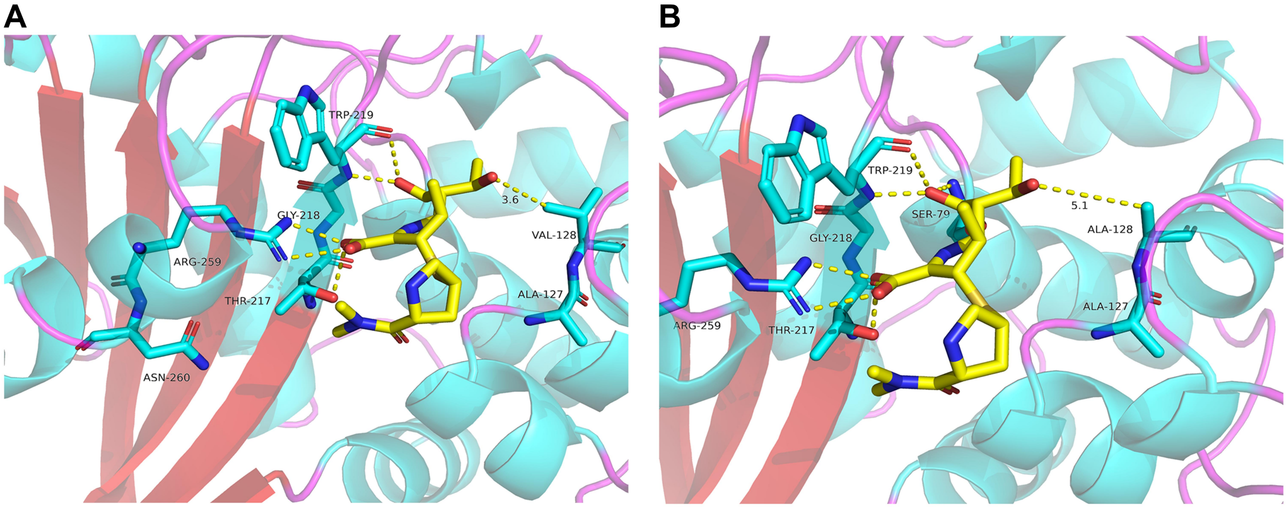

Figure 2 Active-site tertiary structure comparison of OXA-23-meropenem complex (**A**) with the model of OXA-423-meropenem complex (**B**). Distances (Å) are displayed as yellow dashed lines.

conferred OXA-146 to hydrolyze extended-spectrum cephalosporins and aztreonam more efficiently than OXA-23, while preserving activity to carbapenems.[19] Hence, in order to thoroughly understand the resistance phenotype of OXA-423, we determined the MICs of some other drugs: penicillins and β-lactamase inhibitors. In addition, we also detected the MICs of extended-spectrum cephalosporins and aztreonam, although they were not classic substrates of OXA-23-like enzymes. Interestingly, according to the antibiotic susceptibility testing results, the decreased hydrolytic activity against carbapenemases conferred by OXA-423 seemed not been compensated by increased activity against other β-lactams. A particular interest is rapid carbapenemase detection tests such as Modified Hodge test and CarbaNP test are able to detect this variant, although OXA-423 conferred a weaker hydrolytic capacity towards those substrates. It is unknown whether the OXA-423 variant was caused by the selective pressure under carbapenem therapy, as the strain WY-0713 was isolated after received treatment with imipenem (1 g q8h, iv) for 7 days. Curiously, no additional OXA-423 was been detected among *Acinetobacter* spp. (n=39) during a 2-month investigation period in our hospital since this first identification. Furthermore, database searches carried out through the National Center for Biotechnology Information using the BLAST program showed that OXA-423 was not present in other *A. baumannii* strains and other species. The reason for no additional OXA-423 has been detected might be because it is an evolutionary dead-end as it does not confer a resistance advantage over OXA-23, which is by large the primary factor responsible for carbapenem resistance in CRAB, as well as being intrinsic to *A. radioresistens*.[20] MLST analysis revealed the strain WY-0713 belonged to ST2, the dominant of International Clone 2 which is distributed worldwide.[21] Although only a single case of OXA-423 was identified, ongoing surveillance is important because mutations located at conserved amino-acid motifs are very rare.

Conclusion

OXA-423 reported in this study is a novel variant of OXA-23, conferred lower MICs values of penicillins and carbapenems as compared with OXA-23, while without affecting the resistance profiles of expanded-spectrum cephalosporins and aztreonam. This conclusion needs further studies on catalytic properties and crystal structure of OXA-423. Furthermore, from an evolutionary perspective, OXA-423 might be an evolutionary dead-end as it does not confer a resistance advantage over OXA-23.

Ethics Statement

The isolation of clinical strain was part of the routine hospital laboratory procedure, without the need for informed consent and ethics committee approval. The study complied with the Declaration of Helsinki.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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