



In vitro activities of imipenem, vancomycin, and rifampicin against clinical *Elizabethkingia* species producing BlaB and GOB metallo-beta-lactamases

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Abstract

Elizabethkingia genus is emerging in hospitals and resistant to multiple antibiotics. The intrinsic imipenem resistance of *Elizabethkingia* genus is related to two chromosome-encoded metallo-beta-lactamases (MBLs), BlaB and GOB. This study was aimed to investigate the in vitro activity of imipenem, vancomycin, and rifampicin in clinical *Elizabethkingia* species. The distribution and heterogeneity of MBLs responsible for imipenem resistance were also evaluated. A total of 167 *Elizabethkingia* isolates from different patients were collected, including *E. anophelis* (142), *E. meningoseptica* (11), and *E. miricola* (14). All isolates were evaluated by the broth microdilution assay, ethylenediaminetetraacetic acid (EDTA) combination disk test, and EDTA-based microdilution test. The characteristics of BlaB and GOB were evaluated in phylogenetic analysis and heterologous expression experiments. Most of the isolates were susceptible to rifampin (94%), whereas none of the isolates were susceptible to imipenem. Vancomycin showed intermediate effectiveness. EDTA could reduce 4 folds or more minimum inhibitory concentrations (MICs) of imipenem in 105 isolates (62.9%). Of the isolates, the amino acid sequences of BlaB and GOB were divided into 22 and 25 different types, respectively. Phylogenetic analysis showed BlaB and GOB are species-specific proteins. Furthermore, GOB and BlaB from *E. anophelis* showed higher imipenem hydrolysis efficiency than those from the other two species. Rifampicin remained the most active agent in the current study. The mechanism of *Elizabethkingia* resistance to imipenem primarily stemmed from MBLs but other mechanisms could also exist, which requires further investigation.

Keywords *Elizabethkingia* · Vancomycin · Imipenem · Rifampin · Metallo-beta-lactamase

Tein-Yao Chang and Hsing-Yu Chen contributed equally to this work.

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Introduction

The *Elizabethkingia* species is an aerobic, non-motile, glucose non-fermenting Gram-negative bacilli (GNBs) frequently detected in the environment and is known to be widely resistant to many classes of antibiotics. In recent years, the incidence of *Elizabethkingia* infections shows an unexplained growing trend around the world [1]. To date, more than seven species are included in the *Elizabethkingia* genus [2, 3]. Among them, *E. anophelis*, *E. meningoseptica*, and *E. miricola* were reported to be associated with clinical infections. The mortality rate of the patients with *Elizabethkingia* infections ranges from 18.2 to 34.2% [4–6]. The three clinically related *Elizabethkingia* species could be identified by the specific peaks produced by the matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-

TOF MS), 16S ribosomal RNA (rRNA) gene sequencing, and RNA polymerase beta subunit (*rpoB*) gene sequencing [2, 7–9].

Metallo-beta-lactamases (MBLs) are a worldwide concern carbapenemase as they possess activities against carbapenems and all beta-lactam antibiotics (except aztreonam) [10]. MBL activities could be inhibited by chelating agents such as ethylenediaminetetraacetic acid (EDTA) [11, 12]. All *Elizabethkingia* species carry two chromosome-borne intrinsic MBLs, BlaB (subclass B1) and GOB (subclass B3). Both MBLs are related to the intrinsic resistance to carbapenems [4, 6, 8, 13]. The sequences of BlaB and GOB have revealed heterogeneity, with up to 15 BlaB and 18 GOB alleles identified and registered in the GenBank.

Most infections of *E. anophelis* and *E. miricola* were misidentified as *E. meningoseptica* [14, 15]. Vancomycin and rifampicin have been recommended as the empirical treatment for *E. meningoseptica* infections in previous research [16, 17]. However, only one study in the literature has evaluated the minimal inhibitory concentrations (MICs) of vancomycin and rifampicin, and the susceptibilities among different *Elizabethkingia* species [14]. There has been no study to determine the distribution of BlaB and GOB in clinical-related *Elizabethkingia* species. In this study, we focused on the in vitro activity of imipenem, vancomycin, and rifampicin against the three clinically related *Elizabethkingia* species. The genotypes and heterogeneity of the genes encoding MBLs were also analyzed.

Material and methods

Bacterial isolates and growth media

From May 2017 to Feb 2018, 167 non-duplicate clinical isolates of the *Elizabethkingia* species were collected for this study at the Tri-Service General Hospital in Taiwan. The isolates were collected from microbial cultures yielded from 124 respiratory tract specimens, 34 blood samples, 5 abscess samples, 3 urine samples, and 1 pleural fluid sample. The clinical samples were extracted in the microbiology laboratory for routine examination and culture. Cultured pathogens were identified using the Vitek MS matrix-assisted laser desorption ionization–time-of-flight mass spectrometry system (VITEK MS system) (bioMérieux, Mercy l’Etoile, France). The *Elizabethkingia* species were identified using the Vitek MS system with the IVD 3.0 database (bioMérieux, Mercy l’Etoile, France), sequence analysis of the 16S ribosomal RNA (rRNA) and *rpoB* gene as described previously [7]. This study also included 10 carbapenem-resistant *Klebsiella pneumoniae* (CRKP) as a control group for MBL detection. Among them, three were MBL producers (producing IMP-8 type enzyme) and seven were non-producers. Bacteria were

grown routinely in the Luria-Bertani (LB) broth (BD Difco, Franklin Lakes, NJ) at 35 °C with shaking. Plasmids were maintained and selected in *E. coli* DH5alpha and BL21(DE3) using a medium supplemented with kanamycin (Sigma–Aldrich) (25 mg/L).

Antimicrobial susceptibility tests

The minimum inhibitory concentration (MIC) values (mg/L) of imipenem, vancomycin, and rifampicin were evaluated by the broth microdilution assay and the *E*-test (AB Biodisk, Solna, Sweden). Antimicrobial susceptibility interpretation was based on the Clinical and Laboratory Standards Institute (CLSI) criteria (M100-S27, 2017) [18]. The breakpoint used for imipenem was adapted from that for non-*Enterobacteriaceae* and those for vancomycin and rifampicin were from *Staphylococcus* species. *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. To verify the performance of MBLs, we prepared the Mueller–Hinton broth with and without adding isopropyl -D-thiogalactopyranoside (IPTG) (Sigma–Aldrich) at a 0.025 mM final concentration.

EDTA-imipenem microdilution test

The MICs of imipenem in the presence or absence of EDTA were determined by the broth microdilution assay performed as described previously [11]. The MICs of EDTA alone were more than 1.6 mM for all *Elizabethkingia* isolates and CRKP isolates. All *Elizabethkingia* and CRKP isolates grew well in the MHB broth with one-fourth (0.4 mM) of the EDTA MIC. The final concentration of EDTA used in the EMT assay was 0.4 mM. Each isolate was tested in duplicates. In each series, the MICs of imipenem and the imipenem plus EDTA were either identical or differed by a twofold dilution at most. The EDTA effects on the MIC of imipenem were interpreted and reported as good effect when there was a four-fold or more reduction, and otherwise as poor effect [11].

EDTA combination disk test (EDT)

Together with imipenem disks, imipenem/0.1 M EDTA, imipenem/0.2 M, and imipenem/0.5 M EDTA combination disks were employed for detection in the combination disk tests (CDTs) as described previously [12, 19, 20]. In summary, four imipenem (10 µg) disks (BBL, France) were placed on a Mueller–Hinton agar for each tested isolate. Ten milliliters of each 0.1 M, 0.2 M, and 0.5 M EDTA solutions (pH 8.0) was added separately to three of the four imipenem disks. After incubation for 18–20 h at 35 °C, the inhibition zones of the imipenem disks with and without EDTA were compared. An increase of ≥ 4 mm in the zone diameter of the imipenem disk

in the presence of 0.1 M EDTA was interpreted as a positive result for MBL production [19]. For those with 0.2 M and 0.5 M EDTA in the imipenem disk, a ≥ 7 -mm zone diameter difference was interpreted as a positive result [12, 20].

Phylogenetic analysis of MBL genes

Genomic DNA was isolated using the standard phenol/chloroform protocol suggested in the literature [21]. All oligonucleotides used in the study are shown in Table S1. Primer pairs that can detect BlaB and GOB MBL genes were used for PCR amplification of the target genes. Standard PCR amplifications were performed by Phusion DNA polymerase with proofreading activity (Thermo Scientific). Nucleotide sequencing was performed by the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The nucleotide and protein sequences of BlaB and GOB MBLs were compared and grouped by multiple sequence alignments. All of the novel variants were confirmed by the Sanger sequencing of PCR-amplified segments in at least two independent experiments. Different BlaB and GOB MBL amino acid sequences were aligned using the Clustal W multiple sequence alignments. Phylogenetic trees were constructed using MEGA version X (20). The maximum-likelihood (ML) method, using a Jones–Taylor–Thornton (JTT) distance matrix, was used for the construction of BlaB and GOB MBL protein phylogenetic trees. A bootstrap consensus tree inferred from 1000 replicates was taken to deduce confidence levels for the ML trees.

MBL cloning and expression

The open reading frame (ORF) of BlaB and GOB genes was amplified by PCR using the primers pairs, respectively (see Table S1). The ORF amplicon was digested with *NotI* and then cloned in pET-41b(+). The expression vectors pET-41b(+) were digested with *NdeI* and made blunt-ended with the Klenow fragment of DNA polymerase I. The blunt-ended plasmid was then treated with *NotI* to generate a blunt-end/*NotI* digested pET-41b(+) plasmid. The PCR products of BlaB and GOB genes were digested by *NotI* before being ligated into the blunt-end/*NotI* digested pET-41b(+) plasmid to generate a series of expression plasmid pET-BlaB and pET-GOB. The plasmid was transformed into *E. coli* BL21(DE3) and selected a LB agar supplemented with kanamycin (Sigma–Aldrich) (25 mg/L).

Pulsed-field gel electrophoresis

Clinical *Elizabethkingia* species isolates with different BlaB and GOB patterns were genotyped by the pulsed-field gel electrophoresis (PFGE) following the digestion of genomic DNA with *ApaI* [22]. DNA fragments were separated on 1%

SeaKem Gold agarose gel (Lonza Rockland, USA) in 0.5 × tris/borate/ethylenediaminetetraacetic acid buffer with CHEF DRIII (BioRad, Hercules, CA, USA) with 6 V/cm, pulse time from 7 to 35 s for 21 h at 14 °C. The PFGE results of representative isolates were interpreted by a phylogenetic tree analysis (BioNumerics program, Applied Maths). Comparison of the patterns was performed by the unweighted pair group method with arithmetic mean clustering. A similarity coefficient of 85% was selected to define the pulsotype clusters.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under accession numbers MK360026 (BlaB_variant01), MK360027 (BlaB_variant02), MK360028 (BlaB_variant03), MK360029 (BlaB_variant04), MK360030 (BlaB_variant05), MK360031 (BlaB_variant06), MK360032 (GOB_variant01), MK360033 (GOB_variant02), MK360034 (GOB_variant03), MK360035 (GOB_variant04), MK360036 (GOB_variant05), MK360037 (GOB_variant06), MK360038 (GOB_variant07), and MK360039 (GOB_variant08).

Results

Demographic characteristics and microbial cultures

The *Elizabethkingia* species were isolated from the clinical isolates collected from a total of 167 different patients. There was no temporal or spatial overlap among the infected patients during hospitalization. After sequence analyses, the 167 isolates were identified as *E. anophelis* (142, 85%), *E. meningoseptica* (11, 6.6%), and *E. miricola* (14, 8.4%). The most frequently found source was the respiratory tract. Most of the cultures with *Elizabethkingia* isolates were polymicrobial (105, 62.9%). The major five co-isolated pathogens were *Acinetobacter baumannii* complex, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, *Candida* species, and *Klebsiella pneumoniae*. There was no significant difference in the demographic data and the sources of isolation among the infected patients.

Antimicrobial susceptibilities

The MICs of antibiotics against *Elizabethkingia* isolates are shown in Table 1. Rifampicin was active against *E. anophelis* (MIC_{50/90}, 0.5/1 mg/L; 94.4% susceptible), *E. meningoseptica* (MIC_{50/90}, 0.5/1 mg/L; 100% susceptible), and *E. miricola* (MIC_{50/90}, 0.75/6 mg/L; 94.4% susceptible). Vancomycin exhibited limited activity against *E. anophelis* (MIC_{50/90}, 16/

Table 1 Minimum inhibitory concentrations and susceptibilities of antibiotics against *Elizabethkingia* isolates

Species (no. of isolates) and antimicrobial agents	Breakpoint (mg/L)		MIC (mg/L)			Interpretation of susceptibility, n (%)		
	Susceptible	Resistant	Range	MIC ₅₀	MIC ₉₀	Susceptible	Intermediate	Resistant
<i>E. anophelis</i> (142)								
Rifampin	≤ 1	≥ 4	0.25–64	0.25	1	134 (94.4)	0	8 (5.6)
Vancomycin	≤ 4	≥ 32	8–256	16	16	0	136 (95.8)	6 (4.2)
Imipenem	≤ 4	≥ 16	32–128	64	64	0	0	142 (100)
Imipenem + EDTA	≤ 4	≥ 16	8–64	16	32	0	98 (69)	44 (31)
<i>E. meningoseptica</i> (11)								
Rifampin	≤ 1	≥ 4	0.125–1	0.25	1	11 (100)	0	0
Vancomycin	≤ 4	≥ 32	8	8	8	0	11 (100)	0
Imipenem	≤ 4	≥ 16	16–32	32	32	0	0	11 (100)
Imipenem + EDTA	≤ 4	≥ 16	4–16	8	16	1 (9.9)	10 (90.9)	0
<i>E. miricola</i> (14)								
Rifampin	≤ 1	≥ 4	0.25–8	0.63	6	12 (85.7)	0	2 (14.3)
Vancomycin	≤ 4	≥ 32	8–128	16	128	0	11 (78.6)	3 (21.4)
Imipenem	≤ 4	≥ 16	32–128	64	128	0	0	14 (100)
Imipenem + EDTA	≤ 4	≥ 16	8–32	16	32	0	10 (71.4)	4 (28.6)

16 mg/L; 4.2% resistance), *E. meningoseptica* (MIC_{50/90}, 8/8 mg/L; 0% resistance), and *E. miricola* (MIC_{50/90}, 16/128 mg/L; 21.4% resistance). Among the three species, *E. meningoseptica* showed a lower MIC of vancomycin (MIC = 8 mg/L) than the other two. In contrast, imipenem exhibited ineffective activity in all three species (100% resistant).

Activity of MBLs

To test whether the activity of MBLs contributed to imipenem resistance, all *Elizabethkingia* isolates were tested by the EDTA-imipenem microdilution test (EMT) and EDTA combination disk test (ECT). Using EMT experiments, all of the *Elizabethkingia* isolates exhibited a notable reduction of imipenem MICs in the presence of the mixture of EDTA (Fig. 1). The median MIC value for the isolates was 64 mg/L (range, 16–128 mg/L). This value dropped to 16 mg/L (range, 4–64 mg/L) in the presence of EDTA. When comparing results between different species, the magnitude of reduction ranged from 2 to 8 folds (Table 2). One hundred five (62.9%) of the 167 isolates showed a 4-fold or greater reduction in the MIC of imipenem when EDTA was added. The contribution of MBLs to imipenem resistance was correlated by ECT experiments using different concentrations of EDTA (see Table S2). The inhibitory zones of the imipenem disks increased as the EDTA concentration increased. These data indicate that the MBL activity is involved in imipenem resistance.

Amino acid sequence diversity of MBLs

PCR experiments detected both genes encoding BlaB and GOB MBLs in all clinical isolates. By analyzing the protein sequences, we found that BlaB and GOB contained 248–249 amino acids and 290–292 amino acids, respectively. Amino acid analysis revealed that *Elizabethkingia* isolates possessed 22 types of BlaB, including 6 novel variants, and 25 types of GOB, including 8 novel variants (see Table S3). The most common combination of MBLs was BlaB-01 (sequence ID: WP_029729112) plus GOB (sequence ID: WP_009089555) ($n = 106$). Pulsed-field gel electrophoresis (PFGE) was performed on 32 representative isolates with different BlaB and GOB patterns

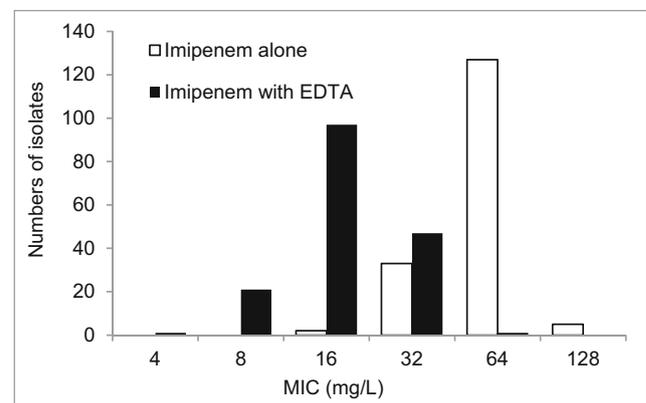


Fig. 1 Imipenem MIC profile of the clinical *Elizabethkingia* isolates. MIC to imipenem alone (white bar) and to imipenem/EDTA combination (black bar) for the 167 clinical *Elizabethkingia* isolates

Table 2 EDTA-based microdilution test (EMT) for minimal inhibitory concentrations against *Elizabethkingia* isolates

Species (no. of isolates)	MIC (mg/L)		Isolates showing a MIC reduction of, n (%)				
	IMP	IMP +EDTA	> 128-fold	8-folds	4-folds	2-folds	0-folds
<i>Elizabethkingia</i> species							
<i>E. anophelis</i> (142)	32–128	8–64		1 (0.7)	88 (62)	53 (37.3)	
<i>E. meningoseptica</i> (11)	16–32	4–16		1 (9.1)	4 (36.4)	6 (54.5)	
<i>E. miricola</i> (14)	32–128	8–32		2 (14.3)	9 (64.3)	3 (21.4)	
Carbapenem resistant <i>Klebsiella pneumoniae</i> (CRKP)							
CRKP without MBLs (7)	8–16	8–16				2 (28.6)	5 (71.4)
CRKP with MBLs (3)	8–16	0.06–0.12	3 (100)				

(see Figure S1). All the representative isolates were classified into 3 clusters and the other 12 unique types. The main cluster had 16 isolates containing 3 different species. The phylogenetic tree of BlaB and GOB based on amino acid sequences is shown in Fig. 2. The phylogenetic analysis separated BlaB proteins into four groups. *E. anophelis* was divided into two groups (groups 1 and 2). *E. miricola* and *E. meningoseptica* were clustered into the 2 separate groups. The phylogenetic analysis separated GOB proteins into four distinct isoform groups. *E. anophelis* was classified into the largest group (group 1). *E. miricola* was divided into two groups (groups 2 and 3). *E. meningoseptica* was clustered into group 4. The BlaB and GOB sequences of *Elizabethkingia* isolates revealed heterogeneity, which is associated with species types.

Cloning and heterologous expression of MBLs

To further assess the function of species-specific MBLs, the predominant type of BlaB and GOB genes from the three different species was cloned into pET41b(+) plasmid to construct pET-BlaB and pET-GOB, respectively. The recombinant plasmids were transformed respectively into *E. coli* BL21(DE3) to obtain the transformed strains. The transformed strains were then tested for imipenem MIC (Table 3). All transformed strains with either pET-BlaB or pET-GOB showed 2–8 folds increases in the MIC of imipenem in comparison with the host strain. Regardless of IPTG stimulation, the BlaB and GOB from *E. anophelis* showed higher imipenem hydrolysis efficiency than those from *E. meningoseptica*. The relationship between MBLs and imipenem resistance was verified indirectly by successfully reducing imipenem resistance with EDTA. These results suggested that both MBLs are functionally involved in imipenem resistance in the three clinical-related *Elizabethkingia* species.

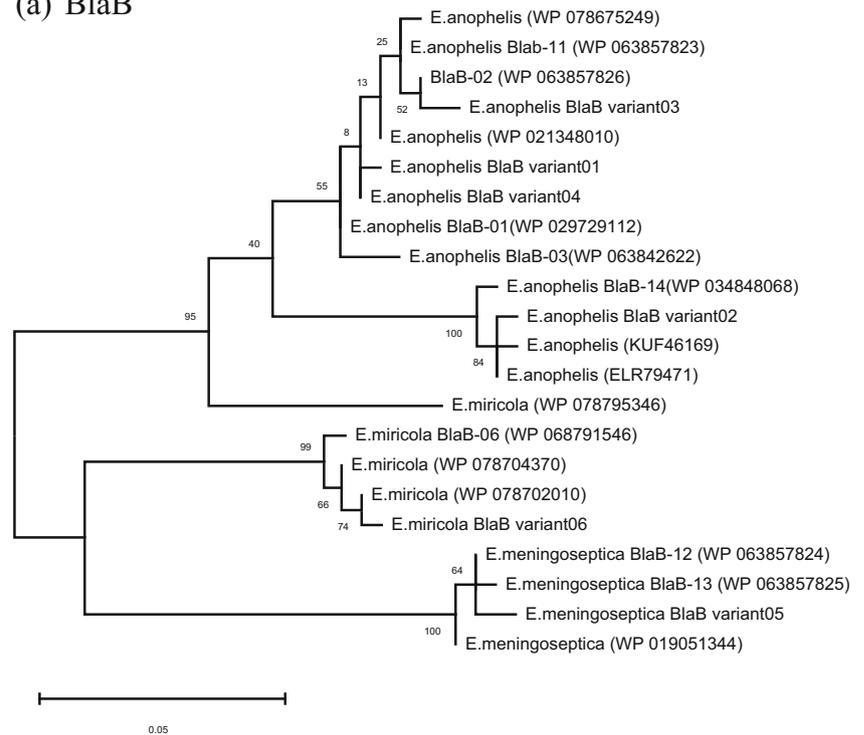
Discussion

E. anophelis was found to be the predominant species in the *Elizabethkingia* genus in clinical settings [5, 7–9, 14]. The older age (≥ 65 years) appears to be a risk factor for clinically related *Elizabethkingia* species infection as suggested in previous studies [4–6, 25]. Senile populations are generally more prone to be hospitalized for infections and thus require antimicrobial treatments. *Elizabethkingia* species infections are difficult to treat because of intrinsic antimicrobial resistance, and their incidence has recently increased in a global trend [1, 5, 9, 25]. Previous studies showed that the *Elizabethkingia* species was highly sensitive to piperacillin/tazobactam, doxycycline, minocycline, and rifampin [5, 7, 14]. We found that 94% (157/167) of the *Elizabethkingia* isolates were sensitive to rifampin as suggested in the literature [14]. Previous studies also indicated that vancomycin was successfully used to cure several patients with bacteremia and meningitis caused by the *Elizabethkingia* species [6, 16]. Vancomycin, which targets the cell wall peptidoglycan, is bactericidal against Gram-positive bacteria. It is inactive against Gram-negative bacteria due to its inability to penetrate the protective outer membrane [26]. The MIC of vancomycin in the *Elizabethkingia* species was between 8 and 256 mg/L in our study. This result suggested that a conventional dosage of vancomycin alone might not be sufficient to treat *Elizabethkingia* infections.

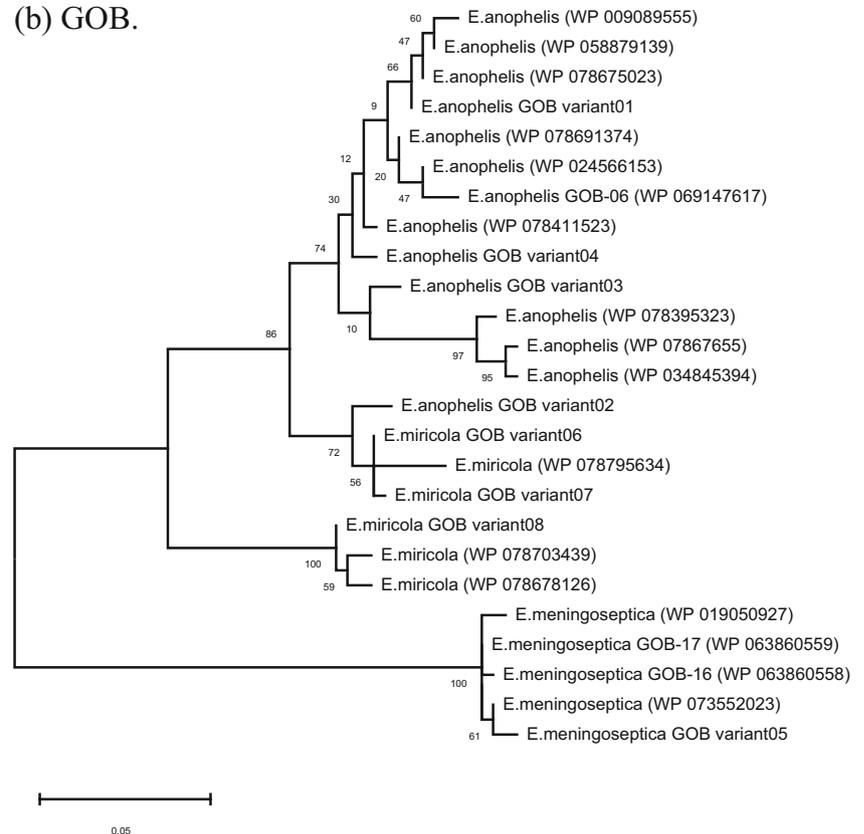
All *Elizabethkingia* genus harbors two types of chromosomal MBLs (BlaB and GOB) simultaneously [27, 28]. However, another survey in China reported that only 32.4% (55/170) of clinical *E. meningoseptica* isolates harbors both types of MBLs and 22.4% (38/170) of them harbors a single type of MBL [29]. This might be attributable to the misidentification of the *Chryseobacterium* species as the *Elizabethkingia* species in biochemistry tests [9]. Our findings and previous literature have revealed high genetic variability in both MBLs [27, 29]. However, we further point out that the variation of two MBLs is related to the *Elizabethkingia*

Fig. 2 Phylogenetic inferences of MBL proteins among *Elizabethkingia* species. An phylogenetic tree was constructed from the BlaB (a) and GOB (b) amino acid sequences by using the maximum likelihood method and JTT matrix-based model [23]. Within the brackets is the sequence ID available in the NCBI database. Evolutionary analyses were conducted in MEGA X [24]

(a) BlaB



(b) GOB.



species. Chromosome-encoded MBLs may not transmit between *Elizabethkingia* species and this requires further investigation.

All of our clinical isolates were detected to carry both genes and exhibit high MIC values for imipenem (MIC, 16–128 mg/L). In our EMT experiment, only 62.9% (105/167) of the

Table 3 Imipenem susceptibilities of *E. coli* BL21Star(DE3) expressing BlaB, GOB, or the pET41b(+) vector

BL21 with plasmid (species, sequence ID)	Imipenem MIC (mg/L) for MHB		
	Alone	With IPTG	With IPTG and EDTA
pET41b(+)	0.25	0.25	0.125
pET-BlaB (<i>E. anophelis</i> , WP_029729112)	2	4	0.125
pET-BlaB (<i>E. meningoseptica</i> , WP_019051344)	0.5	2	0.125
pET-BlaB (<i>E. miricola</i> , WP_078702010)	1	4	0.125
pET-GOB (<i>E. anophelis</i> , WP_009089555)	2	4	0.125
pET-GOB (<i>E. meningoseptica</i> , WP_019050927)	1	2	0.125
pET-GOB (<i>E. miricola</i> , WP_078703439)	1	2	0.125

Elizabethkingia isolates met this criterion. Previous research showed that the EDTA mixture reduced 4-fold or more of the imipenem MICs for MBL producers [11]. These results implied the presence of another imipenem resistant mechanism that was not affected by EDTA. However, in the ECT experiment, the zone size of the imipenem with EDTA was found to be related to the concentration of EDTA added. This phenomenon suggests that high molarity EDTA may induce the membrane permeabilization effect and the zinc ion chelation MBL inhibition effect at the same time [12, 19, 30]. The reductions of outer membrane permeability and carbapenemase hydrolysis are the two main mechanisms of imipenem resistance in Gram-negative bacteria [31]. Accordingly, the high MICs of imipenem against *Elizabethkingia* isolates may be associated with both MBL hydrolysis and low membrane permeability.

One limitation of this study is that small sample sizes of *E. meningoseptica* and *E. miricola* may not represent the antimicrobial susceptibility pattern and MBL gene diversity of these strains in the world. In conclusion, the *Elizabethkingia* species have emerged in recent years, mainly found in senile patients. Rifampicin remained the most active agent against the *Elizabethkingia* species. Additionally, our findings suggest that all *Elizabethkingia* isolates harbor species-specific BlaB and GOB enzymes. To the best of our knowledge, our study is the first one that finds the two MBLs are present in all clinical *Elizabethkingia* strains. MBL diversity is associated with three clinical *Elizabethkingia* species and cannot be transferred between species. MBLs are therefore a promising candidate for unique species markers and therapeutic drug development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed. This work has gone through the ethics committee of our hospital without receiving any kind of objection.

Informed consent There were no requirements for informed consent.

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