


MBLs, Rather Than Efflux Pumps, Led to Carbapenem Resistance in Fosfomycin and Aztreonam/Avibactam Resistant *Elizabethkingia anophelis*

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Objective: To assess the risk factors associated with infections and in-hospital mortality, antimicrobial susceptibility patterns and carbapenem resistance mechanisms in *E. anophelis*.

Methods: This retrospective case-control study was conducted to reveal the risk factors associated with *Elizabethkingia anophelis* (*E. anophelis*) infection and in-hospital mortality in a university tertiary hospital in southwest China, using multivariable logistic-regression analyses. Complete 16S rRNA gene sequencing was used to reconfirm the identity of all isolates. We employed the broth microdilution method to investigate the antimicrobial susceptibility profiles. The presence of resistance genes was confirmed by polymerase chain reaction and DNA sequencing. Full-length resistance genes were cloned into the pET-28a vector for further functional studies.

Results: Our multivariate analysis indicated that coronary artery disease, chronic obstructive pulmonary disease, surgery in the past 6 months, anemia and systemic steroid use were independent risk factors for the acquisition of *E. anophelis*. Additionally, anemia was the only independent risk factor associated with in-hospital mortality in patients with *E. anophelis* infections. *E. anophelis* isolates showed high in-vitro susceptibility towards minocycline (100%) and piperacillin/tazobactam (71.8%), but were resistant to colistin, fosfomycin, ceftazidime/avibactam and aztreonam/avibactam. The PCR revealed the presence of bla_{GOB} and bla_{BlaB} in 37 isolates, and bla_{CME} β-lactamase genes in 36 isolates out of 39 *E. anophelis* isolates. Additionally, we showed that two metallo-β-lactamases (MBLs) BlaB and GOB, were responsible for carbapenem resistance and the serine-β-lactamase, CME, was functionally involved in resistance to cephalosporins and monobactams. Interestingly, the various putative efflux pumps in *E. anophelis* were not responsible for resistance.

Conclusion: Our findings will help clinicians to identify high-risk patients and suggests that minocycline should be considered as a therapeutic option for *E. anophelis* infections. Additionally, carbapenem resistance in *E. anophelis* is mainly associated with the MBLs, BlaB and GOB, rather than various putative efflux pumps.

Keywords: *Elizabethkingia anophelis*, risk factors, antimicrobial susceptibility patterns, carbapenem resistance

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Introduction

Elizabethkingia anophelis (*E. anophelis*) is an aerobic, immotile, oxidase-positive, indole-positive, Gram-negative, non-fermenting bacillus, belonging to the genus *Elizabethkingia* and the family *Flavobacteriaceae*, which was first isolated from the midgut of the mosquito *Anopheles gambiae* in 2011.¹ The first clinically significant

E. anophelis infection was associated with a case of neonatal meningitis in Bangui, Central African Republic in 2011.²

Thereafter, within the genus *Elizabethkingia*, *E. anophelis* has emerged as a major nosocomial pathogen, which can cause severe pneumonia, meningitis, infections of the bloodstream, osteomyelitis, endocarditis, endophthalmitis, skin and soft tissue infection, urinary tract infection and abdominal infection.^{3–10,14,15} Whole genome sequencing (WGS) has recently uncovered a wide range of virulence factors contributing to the pathogenesis of *E. anophelis*, including products of the capsule, lipopolysaccharides, endopeptidases, lipid biosynthesis and metabolism, magnesium transport proteins, macrophage infectivity, heat shock proteins, catalase, peroxidases, superoxide dismutase, two-component regulatory system, and more.^{5,18–20}

Extensive research has shown that *E. anophelis* isolates are resistant to most β -lactams, carbapenems and aminoglycosides.^{4,5,14–17} The emergence of multidrug-resistant (MDR) bacterial pathogens is considered a potential public health hazard, they are widely detected in the environment and their transmission to humans is either by the food chain or via infected animals, poultry, and fish.^{26–29} Furthermore, MDR bacterial pathogens often pose a therapeutic dilemma for clinicians and are therefore associated with a high mortality rate and poor prognosis.

Recently, the incidence of infections caused by the genus *Elizabethkingia* has increased continuously worldwide, especially those caused by *E. anophelis* species. A single-hospital study from South Korea reported that there was an increase in the prevalence of *Elizabethkingia* genus infections among hospitalized patients from 0.02% in 2009 to 0.88% in 2017.⁹ The first recorded outbreak of *E. anophelis* infection was from Singapore in 2012, in which three out of five patients died of septicemia.³ Furthermore, during 2014–2016, several outbreaks have occurred in the Midwestern United States, including Wisconsin, Illinois, and Michigan, in which the patient fatality rate related to *E. anophelis* infections ranged from 30.8% to 70%.^{5–8} Therefore, *E. anophelis* infection is regarded as profoundly serious and important and should be taken seriously by clinicians.

A recent study indicated that *E. anophelis* has been continuously misidentified as *Elizabethkingia meningoseptica* (*E. meningoseptica*) using conventional methods (API/ID32, Phoenix 100 ID/AST, Vitek 2 and Vitek MS).¹³ Therefore, most of the previously reported data

regarding clinical characteristics, antimicrobial susceptibility patterns and carbapenem resistance mechanisms of *E. anophelis*, may be incorrect. To date, however, the susceptibility patterns of *E. anophelis* isolates have not been reported in Mainland China, especially with data collated using the more robust broth dilution method. Genome-wide analysis has revealed that this multidrug-resistant pathogen carries a class A serine- β -lactamase, CME, 2 metallo- β -lactamases, GOB and BlaB, in addition to numerous genes encoding for putative efflux pumps.^{5,18–20} However, no studies have focused on the function of these putative efflux pumps in *E. anophelis* isolates. In addition, data reporting on risk factors associated with infection and mortality in *E. anophelis* infected patients may potentially help clinicians identify high-risk patients and help guide future therapeutic strategies.

The present study was therefore initiated to: (i) identify the risk factors associated with *E. anophelis* infection and in-hospital mortality, (ii) investigate the antimicrobial susceptibility patterns and carbapenem resistance mechanisms of *E. anophelis* isolates and (iii) characterize the function of β -lactamases and putative efflux pumps expressed in *E. anophelis* isolates.

Methods

Isolation and Identification of Bacterial Strains

This study used the clinical microbiology database from a 3200-bed university-affiliated medical center (Chongqing, China) to retrospectively collect those strains that were identified as the genus *Elizabethkingia* between January 2015 and December 2019. Sampling and isolation of bacterial strains were a part of the routine hospital laboratory procedures and microbial identification was performed in the microbiology laboratory using the VITEK2 compact (bioMérieux, Inc., NC, USA) and the VITEK MS (bioMérieux, MO, USA) systems. All strains from the genus *Elizabethkingia* were stored at -80°C in 15% glycerol until use. Complete 16S rRNA gene sequencing was used to reconfirm the identity of all isolates. The primers used for amplification and sequencing of the 16S rRNA gene are listed in [Table S1](#) in [Additional file 1](#). The sequences were assembled using SeqMan (DNASTar) and compared with publicly available sequences in the NCBI (<http://www.ncbi.nlm.nih.gov>) using the BLAST algorithm. Strains were considered to be accurately identified when a strain shared >99.0% 16S rRNA sequence with

a type of strain in GenBank. The sequences of 16S rRNA were performed using ClustalW, and the phylogenetic trees were constructed in MEGA7 software using the Neighbor-Joining method.

Data Collection and Clinical Definitions

Electronic medical records of the patients were collected retrospectively and we excluded subjects with the following characteristics: patients with polymicrobial infection and patients admitted for <48 hours. Only the first episode was considered for patients with more than one positive *E. anophelis* culture. To evaluate the risk factors associated with *E. anophelis* infection, controls were defined as randomly selected patients with non-*E. anophelis* infections during the same time (at a 3:1 ratio to the case group). Selected epidemiological, demographic, clinical, laboratory, treatment and outcome data were obtained from the electronic medical records. Empirically administering agents to isolates that were not susceptible was defined as inappropriate empirical antimicrobial therapy. Shock was defined as the coexistence of a systolic pressure of <90 mm Hg and organ dysfunction of the respiratory system, liver, or kidneys. Serum total protein content of <60 g/L or albumin content <25 g/L was the criteria used to define hypoproteinemia. Hypokalemia was diagnosed for a serum potassium level <3.5 mmol/L. Systemic steroid use was defined as oral or intravenous administration of at least 20 mg/day of a steroid (prednisone, hydrocortisone, methylprednisolone, or dexamethasone) within 1 month of infection. We defined anemia as a hemoglobin level of <130 g/L in men and <120 g/L in women according to the World Health Organization (WHO) guidelines. The primary clinical outcome was in-hospital mortality.

Antimicrobial Susceptibility Testing

The reference broth microdilution method was used to evaluate the minimum inhibitory concentrations (MICs) of all antibiotics in *E. anophelis* and recombinant strains according to the Clinical and Laboratory Standards Institute (CLSI) M07-Ed11 (2019). The criterion suggested by the CLSI for “other non-Enterobacteriaceae” was used to determine the susceptibility of isolates to antibiotics except for ceftazidime/avibactam, aztreonam/avibactam, vancomycin, tigecycline, rifampicin, colistin and fosfomycin. The US Food and Drug Administration (FDA) *Enterobacteriaceae* criteria were used to interpret isolate susceptibility to tigecycline (resistant MIC $\geq 8 \mu\text{g}/\text{mL}$, susceptible MIC $\leq 2 \mu\text{g}/\text{mL}$ and intermediate MIC = 4

$\mu\text{g}/\text{mL}$). The MIC breakpoint applied to vancomycin and rifampicin was adapted from the CLSI criteria for *Staphylococcus* spp. A MIC of $\geq 16/4 \mu\text{g}/\text{mL}$ was considered resistant for the combination of ceftazidime/avibactam and aztreonam/avibactam. MICs for colistin were interpreted at susceptible breakpoints of $\leq 2 \mu\text{g}/\text{mL}$ and resistant breakpoints of $>2 \mu\text{g}/\text{mL}$ according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) *Enterobacteriaceae* criteria. Likewise, for fosfomycin, we elected to use the susceptible breakpoint of $\leq 32 \mu\text{g}/\text{mL}$ and resistant breakpoint of $>32 \mu\text{g}/\text{mL}$ based on EUCAST *Enterobacteriaceae* criteria. The reference strains *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were used as negative controls and quality controls for antibiotic susceptibility testing.

Confirmation and Cloning of Drug Resistance Genes

The presence of the carbapenemase genes (*blaKPC*, *blaIMP*, *blaVIM*, *blaNDM*, *blaBlaB*, *blaGOB* and *blaOXA-48-like*) and extended-spectrum beta-lactamase (ESBL) genes (*blaTEM*, *blaSHV*, *blaCME* and *blaCTX-M*) were confirmed by touch-down PCR assays and sequencing. Touch-down PCR amplification was performed using a thermal cycler (Applied Biosystems VeritiPro PCR, CA, USA) under the following cycling conditions: Initial step of 95°C for 3 min, followed by 8 cycles of 92°C for 20 sec, with annealing temperatures starting at 68°C for 20 sec (decreasing 2°C/cycle), and with a final extension at 72°C for 30 sec; this step was followed by 21 cycles of 92°C for 20 sec, 55°C for 20 sec, 72°C for 30 sec, and finally, 72°C for 5 min. DNA sequencing was performed using an Applied Biosystems 3730 DNA Analyzer.

We amplified the full-length coding sequences (CDSs) of the genes using specific primers flanked by restriction sites (EcoRI, XhoI or BamHI). These included genes encoding for *blaCME*, *blaBlaB*, *blaGOB*, CzcABC family efflux RND transporter, Efflux ABC transporter (ATP-binding protein), the MATE family of MDR efflux pumps, small multi-drug resistance family (SMR) proteins and MFS-type transporter. Amplified PCR fragments were purified and cloned into the corresponding sites within the pET-28a plasmid and then electroporated into BL21 competent *E. coli*. Positive clones were verified by PCR and sequencing. The

sets of primers used for amplification and sequencing of target genes are provided in [Table S1](#) in [Additional file 1](#).

Statistical Analyses

The data were evaluated using SPSS statistical software (version 22.0, IBM). Data are presented as counts (proportions) for categorical variables. Direct comparisons between two groups were determined using the χ^2 test or Fisher's exact test. Means (\pm standard deviation) were used to express normally distributed continuous variables and the median (\pm inter-quartile range) was calculated for non-normally distributed variables. Comparisons between two groups were conducted using Student's *t*-test for normally distributed variables or a Mann–Whitney *U*-test for non-normally distributed variables. To evaluate independent risk factors for the infection and in-hospital mortality of *E. anophelis* isolates, we examined all plausible variables using a univariate analysis. Risk factors with a *P* value <0.1 as detected by the univariate analysis were included in a multivariate logistic-regression model with the enter method. Odds ratios (ORs) and 95% confidence intervals (CIs) were determined for each risk factor of infection and in-hospital mortality. A two-tailed *P* value <0.05 was considered statistically significant.

Ethical Considerations

The Institutional Review Board and Ethics Committee of Chongqing Medical University approved this study (approval number: 2020–703). The study was conducted in accordance with the Declaration of Helsinki. The collection of culture isolates and collation of anonymous clinical data was in accordance with the approved clinical practice guidelines. The need to give informed consent for this study was waived by the Institutional Review Board due to the nature of the retrospective analysis with no individual patient identifiers.

Results

Clinical Characteristics

A total of 59 non duplicated *Elizabethkingia* isolates were collected from the clinical microbiology laboratory for microbial trait investigation. Full 16S rRNA gene sequencing was performed on 59 *Elizabethkingia* clinical strains. After comparison with available sequences in the NCBI using BLAST, 16S rRNA sequencing showed that 39 (69.6%) isolates were identified as *Elizabethkingia anophelis* R26. The phylogenetic tree based on 16S rRNA

showed the genetic relationship among *Elizabethkingia anophelis* ([Figure 1](#)).

Of the *E. anophelis* isolates with a documented site of infection, 18 (46.2%) were from the respiratory tract, eight (20.5%) were from the urinary tract, six (15.4%) from blood, three (7.7%) from cerebrospinal fluid, and four were from peritoneal fluid, amniotic fluid, conjunctival sac, and the cornea ([Table 5](#)). From January 2015 to December 2019, 39 *E. anophelis* samples were isolated from 39 consecutive patients who were enrolled in the study. These patients consisted of 21 males (53.8%) and 18 females (46.2%) with a median age of 61.

Notably, chronic obstructive pulmonary disease was the most frequent comorbidity (76.9%), followed by anemia (66.7%). The empirical antibiotics used in the 39 patients included carbapenems (38.5%), β -lactam/lactamase inhibitors (33.3%), β -lactams (30.8%), teicoplanin (25.6%), levofloxacin (17.9%), aminoglycosides (17.9%), minocycline (12.8%) and antibiotics administered either alone or in combination ([Table 5](#)), and 84.6% of these empirical antibiotic therapies were deemed as inappropriate antibiotic use. Overall, the in-hospital mortality rate of patients with *E. anophelis* infection was 51.3%. Furthermore, β -lactam/lactamase inhibitor antibiotics were used significantly more in patients who did not survive (*P* = 0.041).

Risk Factors and Clinical Outcomes Associated with *E. anophelis* Infections

Compared to the non-*E. anophelis* infection controls, the potential risk factors for the acquisition of *E. anophelis* infections are shown in [Tables 1](#) and [2](#). Univariate analysis indicated that hypertension, cerebrovascular diseases, chronic obstructive pulmonary disease, renal diseases, surgery in the past 6 months, anemia, hypoproteinemia, and hypokalemia were significantly more frequent in patients with *E. anophelis* infections (*P* < 0.05). There was also a positive correlation between patient age and their likelihood of acquiring an *E. anophelis* infection (*P* < 0.05). In the multivariate analysis, coronary artery diseases (OR 5.81, 95% CI: 1.09–30.93, *P* = 0.039), chronic obstructive pulmonary disease (OR 6.71, 95% CI: 1.55–28.99, *P* = 0.011), surgery in the past 6 months (OR 18.04, 95% CI: 3.29–98.87, *P* = 0.001), anemia (OR 6.72, 95% CI: 1.12–40.42, *P* = 0.038) and systemic steroid use (OR 9.87, 95% CI: 1.30–74.94, *P* = 0.027) were independent risk factors for the acquisition of an *E. anophelis* infection.

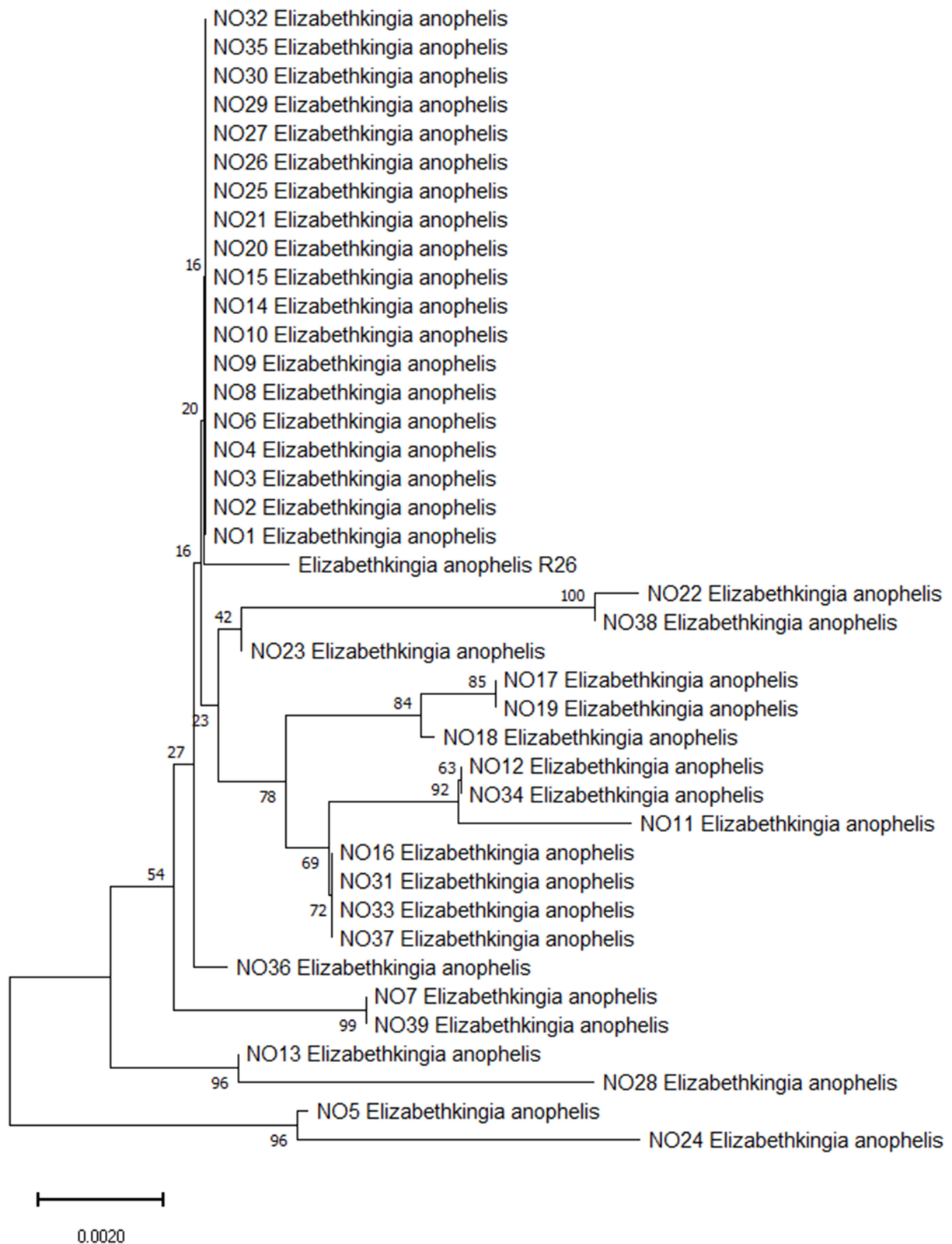


Figure 1 Phylogenetic tree showing the relationship of the 39 *E. anophelis* isolates using 16S rRNA gene sequence. Bootstrap support value above branches, the scale bar indicates the number of substitutions per site.

Table 1 Univariate Analysis of Clinical Features of Patients Infected with *E. anophelis* Isolates

Variables	<i>E. anophelis</i>	Controls	Univariable	
	n=39 (%)	n=117 (%)	OR (95% CI)	P value
Gender (male)	21 (53.8)	73 (62.4)	0.70 (0.34–1.46)	0.352
Age, median, y (IQR)	66 (52–76)	54 (41–67)	–	0.003
Elderly (≥ 60 y)	26 (66.7)	58 (49.6)	2.03 (0.95–4.34)	0.067
ICU admission	18 (46.2)	47 (40.2)	1.28 (0.62–2.65)	0.575
Underlying diseases and conditions				
Diabetes	9 (23.1)	20 (17.1)	1.46 (0.60–3.53)	0.476
Hypertension	18 (46.2)	31 (26.5)	2.38 (1.12–5.04)	0.029
Shock	8 (20.5)	17 (14.5)	1.52 (0.60–3.86)	0.450
Solid malignancy	9 (23.7)	32 (27.4)	0.82 (0.35–1.93)	0.833
Hematologic malignancy	6 (15.4)	12 (10.3)	1.59 (0.55–4.57)	0.393
Peripheral vascular diseases	1 (2.6)	16 (13.7)	0.17 (0.02–1.30)	0.073
Coronary artery diseases	13 (33.3)	22 (18.8)	2.16 (0.96–4.86)	0.076
Cerebrovascular diseases	15 (38.5)	20 (17.1)	3.03 (1.36–6.78)	0.008
Chronic obstructive pulmonary diseases	30 (76.9)	28 (23.9)	10.60 (4.50–24.98)	0.000
Gastrointestinal diseases	14 (35.9)	26 (22.2)	1.96 (0.89–4.30)	0.096
Hepatobiliary diseases	13 (33.3)	21 (17.9)	2.29 (1.01–5.17)	0.071
Renal diseases	16 (41.0)	26 (22.2)	2.44 (1.12–5.27)	0.036
Immune system diseases	0 (0.0)	14 (12.0)	0.73 (0.66–0.80)	0.022
Invasive procedures				
Mechanical ventilation	9 (23.1)	35 (29.9)	0.70 (0.30–1.63)	0.538
Central venous catheterization	14 (35.9)	27 (23.1)	1.87 (0.85–4.08)	0.142
Drainage tube	11 (28.2)	35 (29.9)	0.92 (0.41–2.05)	1.000
Nasogastric tube insertion	17 (43.6)	48 (41.0)	1.11 (0.53–2.31)	0.852
Urinary catheter	16 (41.0)	59 (50.4)	0.68 (0.33–1.42)	0.357
Surgery in the past 6 months	27 (69.2)	31 (26.5)	6.24 (2.82–13.81)	0.000
Immunosuppressive state				
Anemia	26 (66.7)	14 (12.0)	14.71 (6.17–35.08)	0.000
Hypoproteinemia	36 (92.3)	60 (51.3)	11.40 (3.32–39.09)	0.000
Hypokalemia	14 (35.9)	14 (12.0)	4.12 (1.74–9.74)	0.002
Chemoradiotherapy	8 (20.5)	10 (8.5)	2.76 (1.00–7.60)	0.078
Systemic steroid use	7 (17.9)	34 (29.1)	0.53 (0.22–1.33)	0.210

Note: Bold face indicate values that are significant ($P < 0.05$).

Abbreviations: OR, odds ratio; CI, confidence interval; IQR, interquartile range; ICU, intensive care unit; *E. anophelis*, *Elizabethkingia anophelis*.

Univariate and multivariate logistic-regression analysis results for the factors associated with in-hospital mortality are shown in Table 3. Univariate analysis showed that cerebrovascular disease ($P = 0.035$), chronic obstructive pulmonary disease ($P = 0.020$), nasogastric tube insertion ($P = 0.008$) and anemia ($P = 0.002$) were associated with a higher mortality rate. Using further multivariate analysis, anemia (OR 86.38, 95% CI: 1.42–5251.29; $P = 0.033$) was identified as the only independent risk factor for in-hospital mortality in patients with *E. anophelis* infections.

Antimicrobial Susceptibility

The susceptibility of the 39 *E. anophelis* isolates to the antimicrobial agents tested in this study is shown in Table 4. The

isolates showed high in-vitro susceptibility towards minocycline (100%), and piperacillin/tazobactam (71.8%) but with lower in-vitro susceptibility towards levofloxacin (38.5%), ciprofloxacin (30.8%), rifampicin (20.5%), piperacillin (17.9%) and tigecycline (10.3%). The MIC₅₀, MIC₉₀ and MIC range for minocycline on the isolates were 0.5, 1, and 0.25–1 $\mu\text{g/mL}$, respectively, whereas the MIC₅₀, MIC₉₀ and MIC range for piperacillin/tazobactam were 16, 32, and 4–64 $\mu\text{g/mL}$, respectively. All isolates displayed resistance to vancomycin, ceftazidime, cefepime, aztreonam, ceftazidime/clavulanic acid, cefepime/clavulanic acid, colistin and fosfomycin according to the breakpoints used. It is worth noting that while all isolates produced the MBLs BlaB and GOB, aztreonam/avibactam could not further inhibit growth.

Table 2 Multivariate Analysis of Clinical Features of Patients Infected with *E. anophelis* Isolates

Variables	Multivariable	
	OR (95% CI)	P value
Coronary artery diseases	5.81 (1.09–30.93)	0.039
Chronic obstructive pulmonary diseases	6.71 (1.55–28.99)	0.011
Surgery in the past 6 months	18.04 (3.29–98.87)	0.001
Anemia	6.72 (1.12–40.42)	0.038
Systemic steroid use	9.87 (1.30–74.94)	0.027

Note: Bold face indicate values that are significant ($P < 0.05$).

Abbreviations: OR, odds ratio; CI, confidence interval.

Table 3 Risk Factors Associated with In-Hospital Mortality

Variables	Non-Survival	Survival	Univariable		Multivariable	
	n=20 (%)	n=19 (%)	OR (95% CI)	P value	OR (95% CI)	P value
Gender (female)	8 (40.0)	13 (68.4)	0.31 (0.08–1.15)	0.080		
Elderly (≥ 60 y)	16 (80.0)	10 (52.6)	3.60 (0.87–14.87)	0.077		
Drinking history	2 (10.0)	7 (36.8)	0.19 (0.04–1.08)	0.061		
ICU admission	12 (60.0)	6 (31.6)	3.25 (0.87–12.14)	0.080		
Cerebrovascular disease	11 (55.0)	4 (21.1)	4.58 (1.12–18.80)	0.035		
Central nervous system disease	13 (65.0)	7 (36.8)	3.18 (0.86–11.79)	0.083		
Chronic obstructive pulmonary disease	19 (95.0)	11 (57.9)	13.82 (1.52–125.65)	0.020		
Central venous catheterization	10 (50.0)	4 (21.1)	3.75 (0.92–15.34)	0.066		
Nasogastric tube insertion	13 (65.0)	4 (21.1)	6.96 (1.66–29.26)	0.008		
Anemia	19 (95.0)	7 (36.8)	32.57 (3.55–298.83)	0.002	86.38 (1.42–5251.29)	0.033
Systemic steroid use	5 (25.0)	2 (10.5)	2.83 (0.48–16.81)	0.252		

Note: Bold face indicate values that are significant ($P < 0.05$).

Abbreviations: OR, odds ratio; CI, confidence interval; ICU, intensive care unit.

Identification of β -Lactamases Present in *Elizabethkingia anophelis* Isolates

PCR experiments were used to detect the presence of *blaGOB* and *blaBlaB* in 37 isolates, and *blaCME* β -lactamase genes in 36 isolates from the original 39 *E. anophelis* isolates (Table 5 and Figure 2). Sequence alignments showed that *E. anophelis* strains harbored two types of the *blaCME* gene, *blaCME-1* ($n = 34$) and *blaCME-2* ($n = 2$), eight types of the *blaBlaB* gene, including *blaBlaB-29* (17), *blaBlaB-2* (7), *blaBlaB-1* (5), *blaBlaB-17* (3), *blaBlaB-3* (1), *blaBlaB-9* (1), *blaBlaB-33* (1) and *blaBlaB-34* (1), and eight types of the *blaGOB* gene, including *blaGOB-38* (19), *blaGOB-20* (8), *blaGOB-32* (3), *blaGOB-50* (3), *blaGOB-39* (1), *blaGOB-4* (1), *blaGOB-40* (1) and *blaGOB-45* (1). The most detected combination of β -lactamases was *CME-1 + BlaB-29 + GOB-38* ($n = 17$).

We also investigated the presence of other β -lactamase genes, however, none of the 39 *E. anophelis* isolates harbored carbapenemase genes (*blaKPC*, *blaIMP*, *blaVIM*, *blaNDM* and *blaOXA-48-like*) or ESBL genes (*blaTEM*, *blaSHV* and *blaCTX-M*).

MICs of Recombinant Strains

To further evaluate the function of β -lactamases, the most prevalent forms of *blaCME*, *blaBlaB* and *blaGOB* genes from *E. anophelis* isolates were cloned into a pET28a(+) plasmid vector. We also amplified and cloned genes encoding putative efflux pump proteins including the CzcABC family efflux RND transporter, Efflux ABC transporter (ATP-binding protein), MATE family members of the MDR efflux pump, small multidrug resistance family (SMR) proteins and MFS-type transporter. These plasmids were transformed into BL21 (DE3) *E. coli* and the MICs of the common antibiotics were tested in the resultant strains. The strain transformed with pET-CME displayed an increased MIC for ampicillin, piperacillin, cefazolin, cefuroxime, ceftazidime, ceftriaxone and aztreonam when compared to the pET28a(+) vector construct. This suggests that the extended-spectrum serine- β -lactamase CME is functionally involved in cephalosporin and monobactam resistance (Table 6). The MIC for imipenem increased 32-fold (from 0.125 to 4 $\mu\text{g/mL}$) in the presence of the pET-

Table 4 Antimicrobial Susceptibilities of *E. Anophelis* Isolates Determined by the Broth Microdilution Method

Antimicrobial Agents	Breakpoint ($\mu\text{g/mL}$)		MIC ($\mu\text{g/mL}$)			Susceptibility (%)		
	Susceptible	Resistant	Range	50%	90%	Susceptible	Intermediate	Resistant
Piperacillin	≤ 16	≥ 128	16–128	32	64	17.9	74.4	7.7
Piperacillin-TZB	≤ 16	≥ 128	4–64	16	32	71.8	28.2	0
Ceftazidime	≤ 8	≥ 32	128 to >256	>256	>256	0	0	100
Ceftazidime-CLA	≤ 8	≥ 32	32 to >256	>256	>256	0	0	100
Ceftazidime-AVI	≤ 8	≥ 16	64 to >256	>256	>256	0	0	100
Cefepime	≤ 8	≥ 32	32 to >256	>256	>256	0	0	100
Cefepime-CLA	≤ 8	≥ 32	32 to >256	256	>256	0	0	100
Aztreonam	≤ 8	≥ 32	>256	>256	>256	0	0	100
Aztreonam-AVI	≤ 8	≥ 16	>256	>256	>256	0	0	100
Imipenem	≤ 4	≥ 16	16 to >256	64	128	0	0	100
Meropenem	≤ 4	≥ 16	16 to >256	64	256	0	0	100
Levofloxacin	≤ 2	≥ 8	0.5–32	8	16	38.5	2.6	60.0
Ciprofloxacin	≤ 1	≥ 4	0.5 to >256	64	256	30.8	2.6	66.6
Minocycline	≤ 4	≥ 16	0.25–1	0.5	1	100	0	0
Tigecycline	≤ 2	≥ 8	2–8	4	4	10.3	82.1	7.6
Fosfomycin	≤ 32	>32	>256	>256	>256	0	0	100
Colistin	≤ 2	>2	>256	>256	>256	0	0	100
Vancomycin	≤ 2	≥ 16	8–32	16	32	0	20.5	79.5
Rifampicin	≤ 1	≥ 4	1–32	2	16	20.5	33.3	46.2

Abbreviations: MIC, minimum inhibitory concentration; TZB, tazobactam at a fixed concentration of 4 $\mu\text{g/mL}$; AVI, avibactam at a fixed concentration of 4 $\mu\text{g/mL}$; CLA, clavulanic acid at a fixed concentration of 2 $\mu\text{g/mL}$.

BlaB construct and 16-fold (from 0.125 to 2 $\mu\text{g/mL}$) in the presence of the pET-GOB construct. This suggests that the MBLs BlaB and GOB, are responsible for increased imipenem resistance (Table 6). Along with the increase in imipenem resistance, the pET-BlaB and pET-GOB constructs also conferred an increased MIC for ampicillin, piperacillin, cefazolin, cefuroxime, and ceftazidime. This indicates that the MBLs BlaB and GOB can also degrade ampicillin, piperacillin, cefazolin, cefuroxime, and ceftazidime (Table 6). All the efflux pump transformants tested, including pET-ABC, pET-MFS, pET-MATE, pET-SMR and pET-RND did not result in increased MICs for any of the antibiotics tested (Table 6).

Discussion

Infection with *E. anophelis* in humans is increasing in many countries and there have been several reports of *E. anophelis* outbreaks in the community and nosocomial environment in Singapore and the Midwestern United States, including Wisconsin, Illinois, and Michigan.^{3–10} However, as suggested previously, *E. anophelis* is commonly misidentified as *E. meningoseptica* when using biochemical identification methodologies or automated identification systems in clinical settings. Therefore, data regarding the clinical features, clinical prognosis, and the

antimicrobial susceptibility profiles of *E. anophelis* could be skewed. For these reasons, we have, for the first time identified the risk factors associated with the acquisition of *E. anophelis* and we have found that anemia is an independent risk factor for in-hospital mortality in patients with *E. anophelis* infections. Moreover, we demonstrate for the first time that various putative efflux pumps found in *E. anophelis* do not alter antimicrobial resistance and therefore, do not possess a drug efflux function. β -Lactamases were commonly found in *E. anophelis* isolates and the MBLs BlaB and GOB, are responsible for carbapenem resistance, whereas the ESBL, CME is functionally involved in resistance to cephalosporins and monobactams.

In the present study, we initially explored risk factors associated with *E. anophelis* infection and using multivariate analysis, we found that coronary artery diseases, chronic obstructive pulmonary disease, surgery in the past 6 months, anemia and systemic steroid use were independently associated with *E. anophelis* infection. Previous studies have suggested that patients with *E. anophelis* infection could have greater underlying comorbidities and this study is the first to provide a statistical analysis to support this hypothesis.^{3–10} Moreover, surgery in the past 6 months, anemia and systemic steroid use are known

Table 5 Empirical Antimicrobial Therapy in Patients Infected with *E. anophelis* Isolates

Isolates No.	Specimen	ESBL+MBLs	Empirical Antimicrobial Therapy	Prognosis
NO1	Blood	CME-1+ BlaB-2+ GOB-20	Ceftazidime+ Levofloxacin+ Teicoplanin	Survival
NO2	Sputum	CME-1+ BlaB-2+ GOB-20	Cefoperazone-sulbactam+ Minocycline	Non-survival
NO3	Sputum	CME-1+ BlaB-29+ GOB-38	Cefoperazone-sulbactam+ Teicoplanin	Non-survival
NO4	Urine	CME-1+ BlaB-29+ GOB-38		Survival
NO5	Sputum	CME-1+ BlaB-29+ GOB-38	Cefoperazone-sulbactam+ Minocycline+ Levofloxacin+ Teicoplanin	Non-survival
NO6	Sputum	CME-1+ BlaB-34+ GOB-32	Piperacillin-tazobactam+ Imipenem	Non-survival
NO7	Urine	CME-1+ BlaB-29+ GOB-38	Meropenem	Survival
NO8	Urine	CME-1+ BlaB-29+ GOB-38	Imipenem+ Cefoperazone-sulbactam	Non-survival
NO9	Sputum	CME-1+ BlaB-29+ GOB-38	Imipenem+ Clindamycin+ Teicoplanin+ Cefepime	Survival
NO10	Blood	CME-1+ BlaB-29+ GOB-38	Imipenem+ Gentamicin+ Teicoplanin	Non-survival
NO11	Sputum	CME-1+ BlaB-33+ GOB-4	Cefazolin	Non-survival
NO12	Urine	CME-1+ BlaB-2+ GOB-50	Teicoplanin	Non-survival
NO13	Sputum	CME-1+ BlaB-29+ GOB-38	Piperacillin-tazobactam+ ceftriaxone+ Ornidazole	Survival
NO14	Cerebrospinal fluid		Levofloxacin	Survival
NO15	Blood	CME-1+ BlaB-3+ GOB-45	Teicoplanin	Survival
NO16	Sputum	CME-1+ BlaB-9+ GOB-32	Levofloxacin+ Cefazolin+ Amikacin	Non-survival
NO17	Sputum	CME-1+ BlaB-1+ GOB-32	Imipenem+ Teicoplanin	Non-survival
NO18	Amniotic fluid	CME-1+ BlaB-1+ GOB-38	Cefuroxime	Survival
NO19	Blood		Ceftriaxone + Vancomycin	Survival
NO20	Sputum	CME-1+ BlaB-2+ GOB-20	Cefazolin	Non-survival
NO21	Sputum	CME-1+ BlaB-2+ GOB-50	Cefoperazone-sulbactam+ Levofloxacin+ Imipenem+ Teicoplanin	Survival
NO22	Sputum	CME-1+ BlaB-17+ GOB-40	Amikacin	Survival
NO23	Cerebrospinal fluid	CME-1+ BlaB-17+ GOB-20	Levofloxacin	Survival
NO24	Conjunctival sac	CME-1+ BlaB-29+ GOB-38	Cefazolin	Survival
NO25	Sputum	CME-2+ BlaB-2+ GOB-39	Imipenem+ Teicoplanin+ Ornidazole	Survival
NO26	Peritoneal fluid	CME-1+ BlaB-29+ GOB-38	Meropenem+ Vancomycin	Non-survival
NO27	Urine	CME-1+ BlaB-29+ GOB-38	Meropenem+ Vancomycin+ Amikacin+ ceftazidime	Non-survival
NO28	Cerebrospinal fluid	CME-1+ BlaB-29+ GOB-38	Ceftriaxone	Non-survival
NO29	Sputum	CME-1+ BlaB-29+ GOB-38	Linezolid+ Imipenem	Survival
NO30	Corneal	CME-1+ BlaB-29+ GOB-38	Amikacin	Survival
NO31	Blood	CME-1+ BlaB-29+ GOB-38	Imipenem + Levofloxacin+ Amikacin+ Teicoplanin	Non-survival
NO32	Sputum	BlaB-1+ GOB-20	Cefoperazone-sulbactam+ Minocycline	Non-survival
NO33	Urine	CME-1+ BlaB-1+ GOB-20	Cefepime+ Cefoperazone-sulbactam	Non-survival
NO34	Sputum	CME-1+ GOB-38	Imipenem	Survival
NO35	Sputum	CME-1+ BlaB-29+ GOB-38	Cefoperazone-sulbactam+ Minocycline	Survival
NO36	Urine	CME-1+ BlaB-17+ GOB-50	Cefoperazone-sulbactam+ Amikacin+ Metronidazole+ Ceftriaxone	Non-survival
NO37	Blood	CME-2+ BlaB-1+ GOB-20	Meropenem+ Cefoperazone-sulbactam+ Piperacillin-tazobactam	Non-survival
NO38	Sputum	CME-1+ BlaB-1+ GOB-20	Imipenem+ Teicoplanin+ Piperacillin-tazobactam	Non-survival
NO39	Urine	CME-1+ BlaB-29+ GOB-38		Survival

Abbreviations: ESBL, extended-spectrum beta-lactamase; MBLs, metallo-beta-lactamases; CME, extended-spectrum serine-beta-lactamase CME (class A); BlaB, metallo-beta-lactamase BlaB (subclass B1); GOB, metallo-beta-lactamase GOB (subclass B3).

to compromise the immune status of the patients. Therefore, these patients are more vulnerable to the acquisition of *E. anophelis* infection in the same hospital environment.

In previous reports, the case fatality rate of patients with *E. anophelis* infection ranged from 24% to 60% in different countries.^{4–14} In line with this, our study showed that the in-hospital mortality rate of patients with an *E. anophelis* infection was 51.3%. We, therefore, went on to explore the factors influencing this mortality.

When compared with the only previous study investigating risk factors for mortality,¹⁰ our study demonstrated that anemia was the only independent predictor of mortality in patients infected with *E. anophelis*, a factor which has not been reported previously and these patients may present as anemic because of the hemolytic activity of *E. anophelis*. Several studies have reported that this bacterium can lyse erythrocytes to access essential nutrients (such as amino acids) using hemolysins and heme-degrading proteins.^{20–22} This process may alter the host's physiological status and

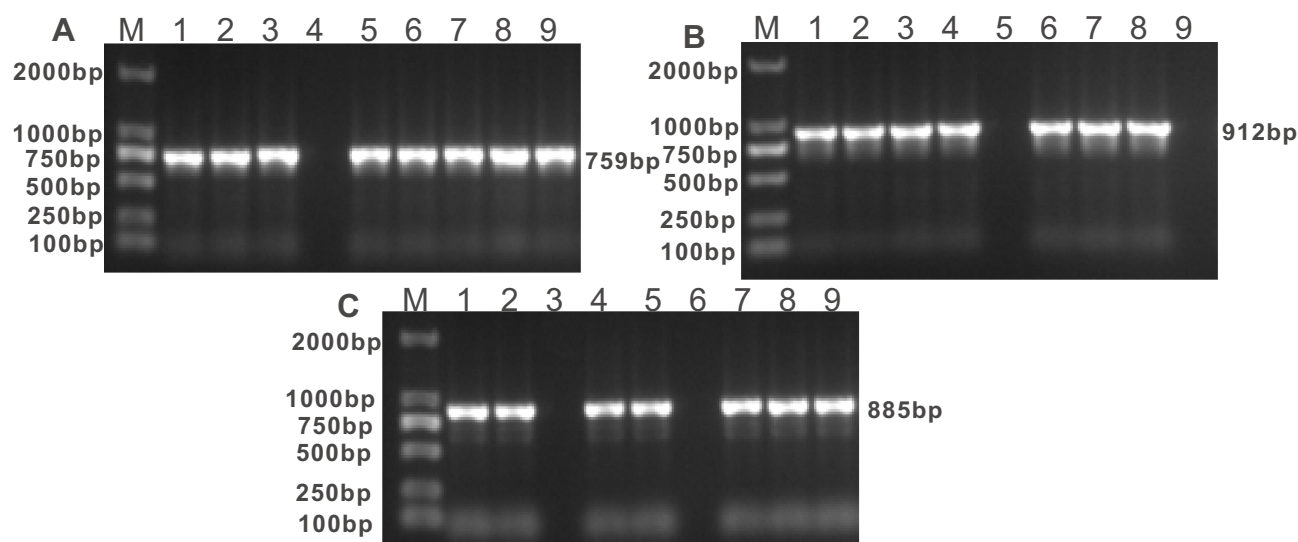


Figure 2 (A) Electrophoretic pattern of BlaB gene (759 bp); M: 100–2000 bp DNA ladder; Lanes 1, 2, 3, 5, 6, 7, 8, 9: positive *E. anophelis* strains; Lanes 4: negative *E. anophelis* strain. (B) Electrophoretic pattern of CME gene (912 bp); M: 100–2000 bp DNA ladder; Lanes 1, 2, 3, 4, 6, 7, 8: positive *E. anophelis* strains; Lanes 5, 9: negative *E. anophelis* strains. (C) Electrophoretic pattern of GOB gene (885 bp); M: 100–2000 bp DNA ladder; Lanes 1, 2, 4, 5, 7, 8, 9: positive *E. anophelis* strains; Lanes 3, 6: negative *E. anophelis* strains.

compromise the immune system, thereby worsening the patient prognosis with *E. anophelis*. Therefore, anemic patients with a confirmed *E. anophelis* infection should be considered as higher risk and should be given greater scrutiny and special care. Unexpectedly, inadequate antibiotic therapy was not associated with mortality, this is probably because of the small sample size of infected patients in our study.

Published information concerning the antimicrobial susceptibility patterns of *E. anophelis*, identified using

reliable methods is limited. Studies from Singapore and Taiwan have demonstrated that *E. anophelis* was resistant to carbapenems, β -lactams, β -lactam/ β -lactam inhibitor and aminoglycosides.^{14–16} However, previous research showed that the susceptibility of *E. anophelis* to fluoroquinolones, tigecycline, piperacillin, piperacillin-tazobactam and trimethoprim-sulfamethoxazole was variable.^{4–6,10,17} Using a disk diffusion or agar dilution test, studies from Hong Kong, South Korea and the USA reported the following susceptibilities of *E. anophelis* to

Table 6 The Antibiotic Susceptibilities of BL21 (DE3) *E. coli* Expressing CME, BlaB, GOB, Putative Efflux Proteins or the pET28a(+) Vector

BL21 with Recombinant Plasmid	MIC ($\mu\text{g/mL}$)											
	AMP	PIP	CFZ	CXM	CAZ	CRO	FEP	AZT	IPM	AK	CIP	TGC
pET28a(+) vector	0.5	0.5	1	1	1	<0.125	<0.125	<0.125	0.125	8	<0.125	4
pET-CME	32	4	32	32	64	1	<0.125	8	0.25	8	<0.125	4
pET-BlaB	256	128	2	16	4	<0.125	<0.125	<0.125	4	4	<0.125	4
pET-GOB	4	2	4	8	16	<0.125	<0.125	<0.125	2	4	<0.125	4
pET-ABC	0.5	1	1	1	1	<0.125	<0.125	<0.125	0.25	8	<0.125	4
pET-MFS	1	0.5	2	1	1	<0.125	<0.125	<0.125	0.25	8	<0.125	4
pET-MATE	0.5	0.5	2	1	1	<0.125	<0.125	<0.125	0.25	8	<0.125	4
pET-SMR	1	0.5	2	1	1	<0.125	<0.125	<0.125	0.25	8	<0.125	4
pET-RND	0.5	0.5	2	1	1	<0.125	<0.125	<0.125	0.25	8	<0.125	4

Note: Bold face indicate MIC values that increase at least 4-fold as compared to the pET28a(+) vector.

Abbreviations: MIC, minimum inhibitory concentration; AMP, ampicillin; PIP, piperacillin; CFZ, cefazolin; CXM, cefuroxime; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; AZT, aztreonam; IPM, imipenem; AK, amikacin; CIP, ciprofloxacin; TGC, tigecycline; CME, extended-spectrum serine-beta-lactamase CME (class A); BlaB, metallo-beta-lactamase BlaB (subclass B1); GOB, metallo-beta-lactamase GOB (subclass B3); ABC, efflux ABC transporter (ATP-binding protein); MFS, MFS-type transporter; MATE, MATE family of MDR efflux pump; SMR, small multidrug resistance family (SMR) protein; RND, CzcABC family efflux RND transporter.

ciprofloxacin (22%–100%), levofloxacin (29%–96%), piperacillin (41.1%–100%), piperacillin-tazobactam (92%), vancomycin (0%–100%) and trimethoprim-sulfamethoxazole (22%–70.6%).^{4,5,17} However, when using the broth microdilution test, researchers from Taiwan and Singapore showed the following susceptibilities of *E. anophelis* to ciprofloxacin (1%–21.5%), levofloxacin (16%–78.5%), piperacillin (19.4%), piperacillin-tazobactam (30.6%–92.4%), vancomycin (0%), tigecycline (5.1%–26.4%), minocycline (97.5%–100%) and trimethoprim-sulfamethoxazole (4%–92.4%).^{14–16} As shown above, there are huge discrepancies in the susceptibility levels of *E. anophelis* to ciprofloxacin, levofloxacin, piperacillin, and vancomycin when comparing the disk diffusion or agar dilution tests and the broth microdilution test. This suggests that the susceptibility of *E. anophelis* seen using the disk diffusion test or agar dilution test may be unreliable and inaccurate, as the broth microdilution test represents the gold-standard method for antimicrobial susceptibility testing.

There are also obvious differences in the susceptibility of *E. anophelis* to antibacterial agents when the standard broth microdilution test is used. A possible explanation for this inconsistency is that natural geographical differences cause variation in the susceptibility patterns observed in previous studies. It is therefore necessary to investigate the antimicrobial susceptibility of *E. anophelis* in local areas as a guide to antibiotic selection. Our study showed the following susceptibility of *E. anophelis* to various treatments: minocycline (100%), piperacillin-tazobactam (71.8%), levofloxacin (38.5%), ciprofloxacin (30.8%), piperacillin (17.9%), rifampicin (20.5%) and tigecycline (10.3%). All isolates displayed resistance to ceftazidime, cefepime, aztreonam, ceftazidime/clavulanic acid, ceftazidime/clavulanic acid, colistin and fosfomycin, according to the breakpoints used. Most of the antimicrobial susceptibility results in this study are consistent with those of previous studies performed using the broth microdilution test. These results suggest that antimicrobial therapy for *E. anophelis* should prioritize minocycline or piperacillin-tazobactam. However, in our study, patients in the non-survival group were treated with significantly more β -lactam/lactamase inhibitor antibiotics as compared to the survival group ($P = 0.041$). This observation indicates that piperacillin-tazobactam is not an effective treatment for *E. anophelis* infections. This study was limited by the fact that antimicrobial susceptibility in-vitro does not equate to in-vivo clinical efficacy. Therefore, large

prospective clinical trials are urgently needed to validate therapeutic recommendations.

In this study, we screened for the presence of carbapenemase genes (*blaKPC*, *blaIMP*, *blaVIM*, *blaNDM*, *blaBlaB*, *blaGOB* and *blaOXA-48-like*) and ESBL genes (*blaTEM*, *blaSHV*, *blaCME* and *blaCTX-M*) in all 39 *E. anophelis* isolates. No carbapenemase genes (*blaKPC*, *blaIMP*, *blaVIM*, *blaNDM* and *blaOXA-48-like*) or ESBL genes (*blaTEM*, *blaSHV* and *blaCTX-M*) were detected in any isolates. However, we identified 35 (89.7%) isolates co-harboring *blaGOB*, *blaBlaB* and *blaCME* β -lactamase genes. The most detected combination of β -lactamases was CME-1, BlaB-29, and GOB-38 ($n = 17$).

To further evaluate the function of β -lactamases, recombinant strains harboring either *blaCME*, *blaBlaB* or *blaGOB* were constructed. The transformed strain expressing CMEs displayed an increased MIC for ampicillin, piperacillin, cefazolin, cefuroxime, ceftazidime, ceftriaxone and aztreonam as compared to the pET28a(+) vector construct. This suggests that the ESBL CME is functionally involved in resistance to cephalosporins and monobactams. The MIC for imipenem increased 32-fold (from 0.125 to 4 $\mu\text{g/mL}$) in the presence of the BlaB-expressing construct and 16-fold (from 0.125 to 2 $\mu\text{g/mL}$) in the presence of the GOB-expressing construct. This suggests that the metallo- β -lactamases BlaB and GOB are responsible for the observed carbapenem resistance.

Aztreonam/avibactam is a novel class of combinational β -lactamase-inhibitor, designed to treat serious infections of metallo- β -lactamase (MBL)-producing Gram-negative bacteria, which is currently in Phase I clinical trials (NCT01689207). Aztreonam is relatively stable against MBL hydrolysis, however, it is easily inactivated by class A (eg, KPC), class C (eg, AmpC) and certain class D (eg, OXA-48) serine- β -lactamase enzymes.²³ Avibactam potently inhibits class A, class C and certain class D serine- β -lactamase enzymes and displays a broader β -lactamase inhibition profile than other β -lactamase inhibitors.²³ When in combination, aztreonam/avibactam is effective against isolates co-producing ESBLs and MBLs with porin loss/deficiency.²⁴ However, it was quite unexpected that *E. anophelis* is resistant to aztreonam/avibactam according to our experimental results.

Genomic annotation of all *Elizabethkingia* spp. reveals that besides β -lactamases, there are also numerous putative efflux pump proteins including CzcABC family efflux RND transporter, Efflux ABC transporter (ATP-binding protein), MATE family of MDR efflux pumps, small

multidrug resistance family (SMR) proteins and MFS-type transporter. Interestingly, however, none of these transporters have been phenotypically characterized.^{5,18–20} It was, therefore, critical to investigate the function of these putative efflux pumps. Our data showed that all recombinant efflux pump strains including pET-ABC, pET-MFS, pET-MATE, pET-SMR and pET-RND did not result in increased MICs for β -lactam and non- β -lactam antibiotics. These results suggest that the putative efflux pump genes from *E. anophelis* are not responsible for antimicrobial drug resistance. Similarly, Schindler et al cloned and expressed 21 putative efflux pump genes in *Staphylococcus aureus* which had no effect on any of the antibiotics tested.²⁵ In summary, we demonstrate for the first time that the various putative efflux pumps found in *E. anophelis* do not possess antimicrobial drug efflux function.

There were some limitations to our study; however, firstly, the small sample size from the single-center study prevented its translation to the wider population. However, the identification of both a carbapenem resistance mechanism and the susceptibility profile of the drug-resistant *E. anophelis* are of great clinical importance and warrant an urgent, wider, in-depth study. Secondly, no further investigation into the clonality of these isolates was performed, so that the possibility of infection outbreaks cannot be ruled out.

Conclusions

In conclusion, this study provided a detailed report of risk factors, antimicrobial susceptibility patterns and carbapenem resistance mechanisms in *E. anophelis* clinical isolates from one medical center in Southwest China. Our data showed that patients with anemia, coronary artery diseases, chronic obstructive pulmonary disease or patients who have received systemic steroids or surgery in the past 6 months are more likely to acquire an *E. anophelis* infection. Furthermore, patients with anemia have a worse prognosis and therefore require more attention and special care from clinicians. The collected clinical isolates exhibited remarkable multidrug resistance to colistin, fosfomycin, aztreonam/avibactam and tigecycline, which are all regarded as last-resort treatments for carbapenem-resistant Enterobacteriaceae, while minocycline is the most effective antibiotic against *E. anophelis* in-vitro. Mechanistic analysis revealed that carbapenem resistance is associated with the hydrolytic activity of the MBLs BlaB and GOB and is not associated with various putative

efflux pumps expressed in *E. anophelis*. Future in-vivo and prospective clinical trials are urgently needed to determine optimal antimicrobial agent efficacies based on in-vitro drug susceptibility testing results and resistance mechanisms.

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Disclosure

The authors report no conflicts of interest in this work.

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