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Full Length Research

Expression of some resistant genes on six *Elizabethkingia* spp. strains isolated in Vietnam

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ABSTRACT: This study focused on the assessment of the multiantibiotic-resistance and gene expression associated with multi-drug resistance in *Elizabethkingia.* Six *Elizabethkingia* spp. strains were isolated from patient samples, and the antibiotic-resistance of the six isolates with all 14 tested antibiotics was confirmed. MacConkey subculture and *16S rRNA* sequencing were used to identify the six isolates as; one *Elizabethkingia meningoseptica* (*E. meningoseptica*) isolate, and five *E. anophelis* isolates. Seven genes including four-resistant genes (*blaB, blaCME, ant6, lolD and catB* and two antibiotics target gene (*gyrA* and *parC*) were selected for evaluation of gene expression using Reverse Transcriptase PCR (RT-PCR). The resistant genotype–phenotype correlation of β-lactams (*blaB* and *blaCME*) and fluoroquinolones (*gyrA* and *parC*) groups was observed. The chloramphenicol resistant gene (*catB*) was also found in all six isolates. The gene expression level was different between isolates but lacked the relationship with high resistant phenotypes. The aminoglycoside (*ant6*) and macrolide (*lolD*) resistant genes were absent, which indicated that other genetic factors could be implicated. The multidrug-resistance of *Elizabethkingia* spp. is reported for the first time in Vietnam, demonstrating the presence of this drug-resistance bacterium in the Vietnamese hospital environment. The initial results in this study will be the basis for further research regarding *Elizabethkingia* spp.

Keywords: 16S rRNA, *Elizabethkingia* spp, multi-antimicrobial resistance, phylogeny, resistant gene expression, Reverse Transcriptase PCR.

Abbreviation: RT-PCR, Reverse Transcriptase PCR; **CLSI,** Clinical and Laboratory Standards Institute; **cDNA,** complementary DNA; **MUSCLE,** MUltiple Sequence Comparison by Log-Expectation; **ANOVA,** Analysis of variance; **MIC,** minimum inhibitory concentration.

INTRODUCTION

Elizabethkingia is Gram-negative, non-motile, aerobic, catalase positive, oxidase positive, and indole positive bacteria bacilli (Janda and Lopez, 2017). There are three species: *E. meningoseptica*, *E. miricola*, and *E. anophelis* that are known to infect humans (Jean et al., 2014) and are the cause of many outbreaks in the United States (Perrin et al., 2017), Taiwan (Lin et al., 2018), and Hong Kong (Lau et al., 2016). They can be located in water, soil and especially on hospital surfaces (Weaver et al., 2010). *Elizabethkingia* spp. is opportunistic bacteria and cause pneumonia, sepsis, meningitis, and fever, and can threaten the survival of infants and immunodeficient patients (Breurec et al., 2016; Lau et al., 2016). The treatment of *Elizabethkingia* spp. is challenging because of the resistance to multiple antibiotics, such as β-lactam, aminoglycosides, tetracyclines, and chloramphenicol, which are the most common antibiotics for Gram negative bacteria (Kirby et al., 2004). However, they showed

susceptibility to treatment with the antibiotics for Gram-Positive Bacteria (Ceyhan and Celik, 2011).

Elizabethkingia spp. are often resistant to β-lactam antibiotics due to class A extended-spectrum β-lactamases and class B metallo-β-lactamases (Lin et al., 2012). In recent years, reference genomes of *Elizabethkingia* spp. were analyzed and published in different countries. Many antibiotic-resistant genes were reported in all of the three species related to β-lactam, sulfonamide, tetracycline, macrolide, quinolone, aminoglycoside, and chloramphenicol (Chen et al., 2017; Wang et al., 2019). These studies showed the variety of genes related to antibiotic-resistance, and they can be reason for the different in susceptibility test results of published isolates (Lin et al., 2019).

In Vietnam, the understanding of the antibiotic-resistance of *Elizabethkingia* has been limited. There was not any research about *Elizabethkingia* and their resistant characteristics. Therefore, in this study, multiple antibiotic resistant *Elizabethkingia* isolates in a Vietnam hospital and screened for resistant genes were detected. However, because the previous database in Vietnam was lacked, the genes was selected from published resistant genes of *Elizabethkingia* (Chen et al., 2017; González and Vila, 2012; Jian et al., 2018). The *blaB* and *blaCME* genes were two of the β-lactamases family. In which, the *blaB* gene encodes carbapenemases enzymes (enzymes that catalyze the degradation of the carbapenem group) (Vessillier et al., 2002), while the CME enzyme encoded by *blaCME* is capable of hydrolyzing cephalosporin, including fourth-generation cephalosporin (Bellais et al., 2000). The *catB* gene encoded for Chloramphenicol acetyltransferase (CAT) enzymes. These enzymes play a role in chloramphenicol acetylation through the use of acetyl coenzyme A (Rogers et al., 2002). Both *gyrA* and *parC* were genes encoding enzymes regarded the targets for quinolones antibiotics and maintained quinolone-resistance-determining region (Fàbrega et al., 2009; Nouri et al., 2016). The *ant6* and *ants* family encoded for the Nucleoside triphosphate-dependent O-phosphotrans-ferases that catalyzed the phosphorylation, one of the most common mechanisms of aminoglycoside inactivation (Ramirez and Tolmasky, 2010). Even the expression of *lolD,* a macrolide resistance gene in the Gram-positive antibiotic group, also presented in many published isolates (Chen et al., 2017).

Thus, the objective of this study is to assess the multi-antibiotic-resistance and gene expression associated with multidrug-resistance in *Elizabethkingia*. The results of this test are going to lay the groundwork for further research concerning *Elizabethkingia* in Vietnam.

MATERIALS AND METHODS

Bacterial isolates

Six multiple antibiotic-resistant isolates of *Elizabethkingia* spp. were isolated from sputum, bronchial fluid, and blood samples during 2017 and stored in the Vietnam Military Medical Academy (Table 1) before this study was performed. The VITEK2 system (bioMerieux Vitek; bioMerieux, Marcy l'Etoile, France) was used to identify them as *Elizabethkingia* species and confirmed the antimicrobial susceptibility using MIC standards (µg/mL) for other non-*Enterobacteriaceae* in the Clinical and Laboratory Standards Institute (CLSI) guideline (Patel et al., 2017). The antimicrobial susceptibilities were tested for 14 different antibiotics.

Species identification

Six isolates were subcultured on MacConkey agar plates and incubated at $37^0C \pm 1^0C$ for 18 h ± 2 h. The subculture results were confirmed using *16S rRNA* gene phylogenic analysis. The *16S rRNA* gene of six isolates was sequenced in the ABI3500 system (Applied Biosystem, United States). The six isolate sequences in this study, and 18 published reference sequences including three *Elizabethkingia* and 15 other *Flavobacteriaceae* isolates were aligned using MUSCLE algorithms. The maximum likelihood tree with T92+G substitution model and bootstrap 1000 were built to determine the genetic relationship. All analysis processes were performed using MEGA6 software (Tamura et al., 2013).

RNA extraction

Total RNA was extracted using the TRIZOL RNA Isolation Protocol. RNA concentrations were determined using a NanoDrop™ One Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, United States) and diluted to a final concentration of 1 µg/µl.

Resistant gene expression screening using RT-PCR

cDNA synthesis was performed according to the Revert Aid First Strand cDNA Synthesis Kit manual (Thermo Fisher Scientific, San Jose, CA, US) with 1 µl of total RNA in a 20 µl reaction volume. One µl of cDNA was continuously used as template for 25 µl PCR reaction containing 12.5 µl Thermo Scientific DreamTaq PCR Master Mix (Thermo Fisher Scientific, San Jose, CA, US), 0.5 µl of each 10 pmol/µl primer, and 10.5 µl of nuclease free water.

All reactions were performed in triplicate with PCR conditions of 95^0C for 5 min, followed by 25 to 40 cycles of denaturation at 95^0C for 30 seconds, annealing of specific primers for 30 seconds, and extension at 72^0C for 1 min. The reaction finished at 72^0C for 7 min and was finally held at 4^0C. The primers and annealing temperatures for the amplification of seven resistant genes and the *16S rRNA* gene are listed in Table 2. The gel electrophoresis results

Table 1. Name and sample sources of six multidrug-resistant *Elizabethkingia* strains.

Strains	Sample sources	MacConkey subculture
17 -13 M	Sputum	No-growth
17 -28D	Bronchial fluid	No-growth
17 -30M	Blood	No-growth
17 -35D	Bronchial fluid	No-growth
17 -84M	Blood	Growth
17 -88M	Blood	No-growth

Table 2. A list of primer pairs was used in this study.

No.	Primer		Sequence	Size (bp)	Tm (ºC)	Reference
1	*16S rRNA*	Fw Rv	GCCTACGGGAGGCAGCAG CCGTCAATTCMTTTGAGTTT	550	50	
2	*blaB*	Fw Rv	TTGTGGTTATAGACTGTCCGTGGG TATTCAAGACCTCCGGCACGAT	136	55	(González & Vila, 2012)
3	*blaCME*	Fw Rv	AAGAAAGCCACAGTAGCTGTTTC ACTGCAATTGCATAATGTTTACC	695	55	(González & Vila, 2012)
4	*catB*	Fw Rv	CCCCGGATTCTTTCTCGGAT AAGTCCTGTTTTTGCCACCG	338	55	NUHP1 (NZ_CP007547.1)
5	*parC*	Fw Rv	GCTCAGTATGGCAATGCTAAAA TTGCTCTTACCTTACCGCCG	785	50	(Jian *et al.*, 2018)
6	*gyrA*	Fw Rv	AGCCCGTTGTTTAAATCCTGAA CCCTGTTGGGAAGTCTGGTG	743	50	(Jian *et al.*, 2018)
7	*ant6*	Fw Rv	GAAAGAGGCGCAACGGGATA GCGGGCAAGACATTTTACCA	202	50	NUHP1 (NZ_CP007547.1)
8	*lolD*	Fw Rv	GAGCTTGCAGGAGTTCCGAT GTCTAATGCTCCGGTTGGCT	333	50	NUHP1 (NZ_CP007547.1)

were analyzed using the Quantity One program (Gel Doc EQ; Bio-Rad, Hercules, CA, US). The resistant gene expression level was normalized to the level of the *16S rRNA* gene.

Statistical analyses

Gene expression statistical comparison was performed using ANOVA one-way test in GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, US). A p-value of ≤ 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Susceptibility test

Six *Elizabethkingia* spp. isolates were tested with 14 antibiotics from nine groups. The antimicrobial susceptibility test mentioned that the multidrug-resistance appeared in six *Elizabethkingia* spp. isolates. All of the tested antibiotics were very common and widely used in the treatment of infection. Including the broad-spectrum antibiotics such as ciprofloxacin and levofloxacin, two of the most widely used antibiotics, were effective against both Gram-negative and Gram-positive bacteria (Fàbrega et al., 2009; Heeb et al., 2011), and trimethoprim/ sulfamethoxazole, which was a potential candidate for *Elizabethkingia* treatment (Gokce et al., 2012). After all, ciprofloxacin and levofloxacin were resistant by five of isolates (excepted 17-88M), while trimethoprim/ sulfamethoxazole also produced a resistant result in the 17-13M and 17-35D isolates and an intermediate result in the other four isolates (Table 3). These resistant phenotypes were similar with previous researches (Lin et al., 2019), even Piperacillin and Piperacillin/Tazobactam,

Table 3. Summary of susceptibility test.

Antibiotics	Groups	Susceptible (MIC Breakpoints)	Intermediate	Resistant (MIC Breakpoints)
AN-Amikacin	Amino-glycosides	0 (≤16 µg/mL)	0	6 (≥64 µg/mL)
GM-Gentamicin		0 (≤4 µg/mL)	0	6 (≥16 µg/mL)
TM-Tobramycin		0 (≤4 µg/mL)	0	6 (≥16 µg/mL)
TCC-Ticarcillin/Clavulanic Acid	Beta-lactams	0 (≤16 µg/mL)	0	6 (≥128 µg/mL)
TIC-Ticarcillin		0 (≤16 µg/mL)	0	6 (≥128 µg/mL)
TZP-Piperacillin/Tazobactam		0 (≤16 µg/mL)	0	6 (≥128 µg/mL)
ATM-Aztreonam	Monobactams	0 (≤8 µg/mL)	0	6 (≥32 µg/mL)
IPM-Imipenem	Carbapenems	0 (≤4 µg/mL)	0	6 (≥16 µg/mL)
MEM-Meropenem		0 (≤4 µg/mL)	0	6 (≥16 µg/mL)
CAZ-Ceftazidime	Cephems (parenteral)	0 (≤8 µg/mL)	0	6 (≥32 µg/mL)
FEP-Cefepime		0 (≤8 µg/mL)	0	6 (≥32 µg/mL)
PIP-Piperacillin	Penicillins	0 (≤16 µg/mL)	0	6 (≥128 µg/mL)
CIP-Ciprofloxacin	Fluoroquinolones	0 (≤1 µg/mL)	1	5 (≥4 µg/mL)
LEV-Levofloxacin		0 (≤2 µg/mL)	1	5 (≥8 µg/mL)
SXT-Trimethoprim/ Sulfamethoxazole	Folate pathway inhibitors	0 (≤4 µg/mL)	4	2 (≥16 µg/mL)
CS-Colistin	Lipopeptides	0 (≤2 µg/mL)	0	6 (≥4 µg/mL)

which most published isolates were susceptible to, also observed the resistance in this study. This is a warning concerning the emergency status of resistance in Vietnam's environmental hospitals. The frequent use and misuse of antibiotics have led to the emergence of multidrug-resistant bacteria.

Species identification

Following the previous study, the *16S rRNA* sequence-based method to determine the species of each isolate was selected (Han et al., 2017). Base on the *16S rRNA* sequences, the phylogenetic tree showed the different genetic between 3 groups of the *Flavobacteriaceae* family. Six isolates in this study belonged to the *Elizabethkingia* groups as supported by 90 bootstrap percentage, and had a closer relationship with *E. meningoseptica* and *E. anophelis* than *E. miricola* (Figure 1). Unfortunately, the sequence similarity of our strains *16S rRNA* with public sequences to be: *E. miricola* (98.8-99%), *E. meningoseptica* (99.2-99.4%) and *E. anophelis* (99.6-99.8%) (data not shown) decreased the bootstrap percentage to less than70%, which is the supported level for species determination (Soltis and Soltis, 2003). Hence, the MacConkey subculture method was used as the parallel approach, based on the *E. anophelis* phenotype characteristic that lacks growth in MacConkey medium (Kämpfer et al., 2011).

After 18 hours of cultivation, only the developing of 17-84M on MacConkey agar petri was observed, similar to the phenotype characteristic of *E. meningoseptica* (Ratnamani and Rao, 2013), and *E. miricola* (Gupta et al., 2017). And 17-84M isolate was closer to *E. meningoseptica* than *E. miricola* in the phylogenetic tree, so it was concluded that 17-84M isolate was *E. meningoseptica.* Five other strains cannot grow in MacConkey medium and were close with *E. anophelis* in the phylogenetic tree, so it was concluded that they were *E. anophelis.* The *16S rRNA* sequences of the 17-13M, 17-28D, 17-30M, 17-35D, 17-84M, and 17-88M isolates were published with GenBank accession number MK156744, MK156750, MK156754, MK156757, MK156761, and MK156765 respectively.

Resistant gene expression screening using RT- PCR

Seven selected genes were recommended in many previous articles that are related to the resistance of *Elizabethkingia* spp. (Chen et al., 2017; González and Vila,

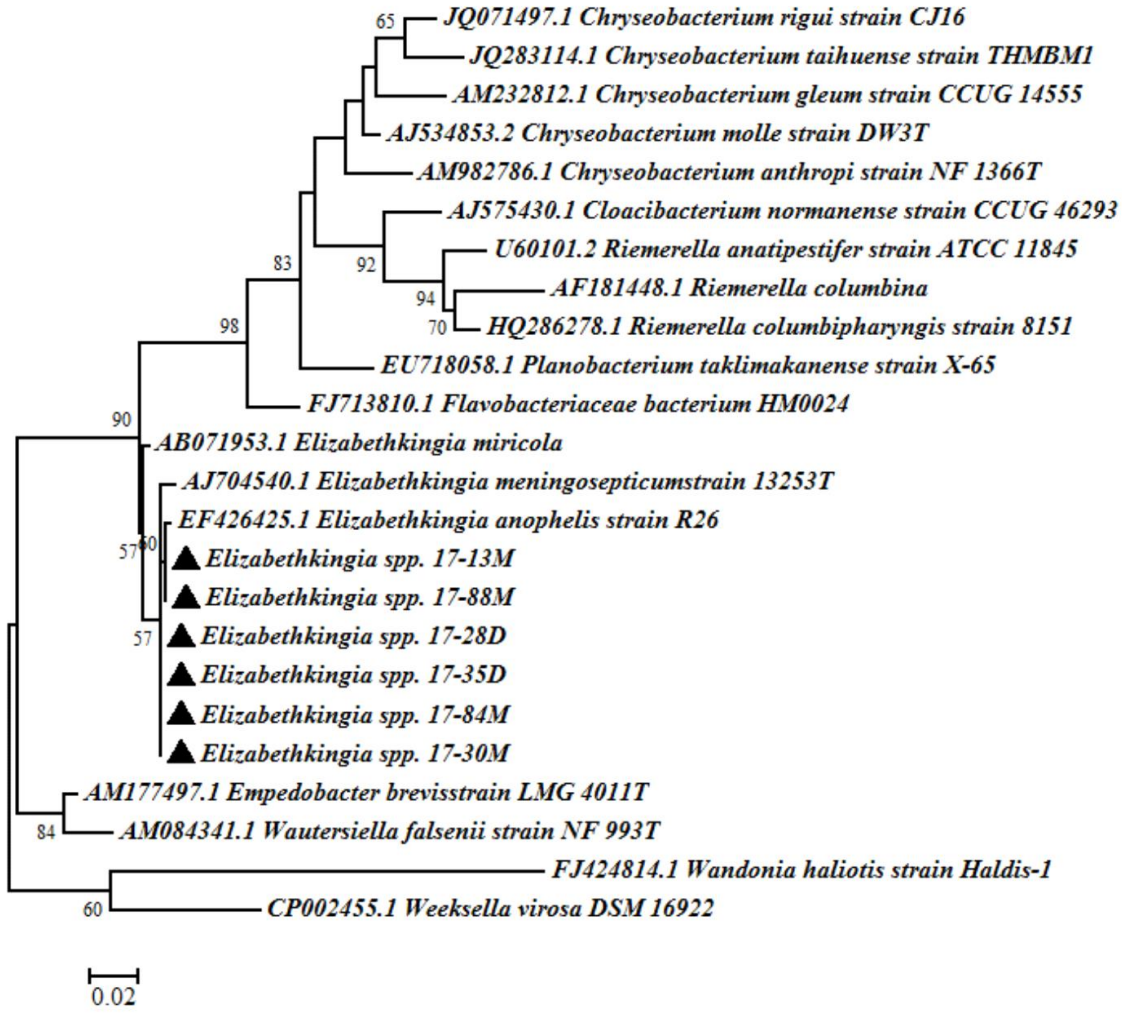

Figure 1. Phylogenetic tree was built using Maximum-Likelihood algorithm, bootstrap 1000. ▲: six Vietnam multidrug-resistance *Elizabethingia* strains.

2012; Jian et al., 2018) were selected to test the expression in the six isolates (Table 4). Besides, because of this commonality in previously published articles, *lolD* and *catB* were selected to test the gene expression without having results from the previous susceptibility test.

In the other five genes expressed, the result showed the similarities between resistant phenotype and genotype and indicated those genes relate to the resistance of *Elizabethkingia.* It was surprising that two genes; *ant6* and *lolD* were absent in all isolates, that make the difference of genotype between these isolates and published isolates (Chen et al., 2017; Jian et al., 2018). All the more, the expression levels of five genes were different for each. Some hypotheses suggest that the drug-resistant phenotype of multidrug-resistant bacteria can be predicted by the change in expression of a small number of genes (Ferrer et al., 2017). In this study, there was a bias of high expression in 17-84M, the only isolated *E. meningoseptica* strain in four out of five genes, except for the *parC* gene showed the highest expression in the 17-88M isolate. The 17-13M and 17-28D isolates seem to be weaker gene expression isolates than the other, even though 17-88M isolate also showed the lowest *catB* gene expression level (35.9% gene expression of 17-88M strain) (Figure 2). However, there was not a clear relationship between the gene expression and the high resistant phenotype. Thus, it was very hard to confirm the role of these genes to the resistant phenotype of *Elizabethkingia.*

The explanation for this result may come from the regulation of other genetic factors related to antibiotic-resistance. There are many different signaling pathways also relate to the linking mechanism between the genotype and phenotype of drug-resistant in bacteria (van Hoek et al., 2011). Therefore, the absence of aminoglycoside (*ant6*) and macrolide (*lolD*) resistant genes can be replaced by another factor. Some mutation genes also play a role in the complex relationship of genotypes to drug-resistant phenotypes. Many different types of mutations can cause

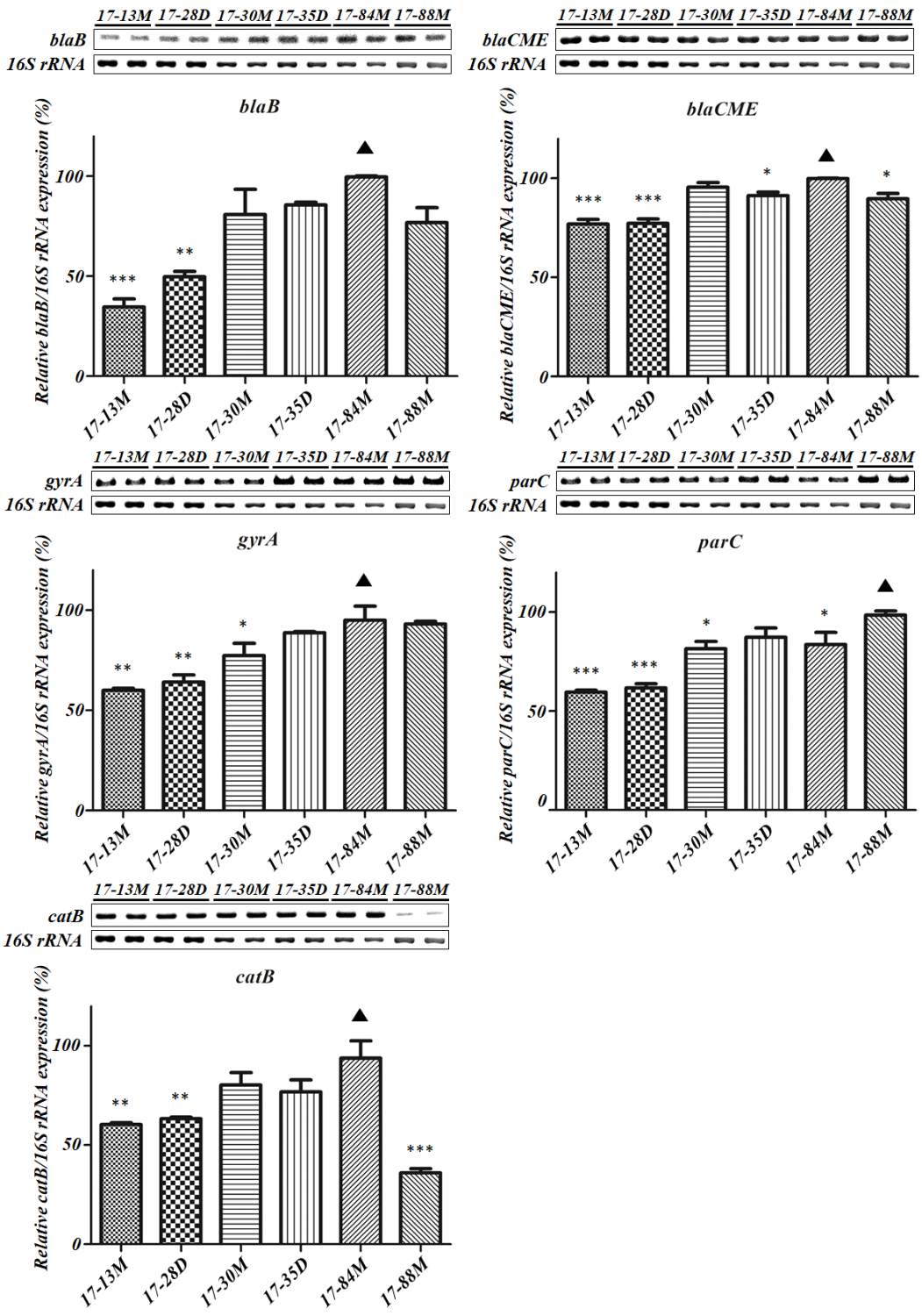

Figure 2. Expression of five multidrug-resistance related genes. ANOVA one-way was performed to compare the expression between the highest expression strain (▲) and the other strains. * $p \leq 0.05$; ** $p \leq 0.01$; $p \leq 0.001$.

the same resistant phenotype and also cause the change of many different phenotypes that affect the resistance and sensitivity to many antibiotics (Toprak et al., 2012). Previous studies have shown that a large number of mutant genes affect susceptibility and antibiotic-resistance, including genes that are not directly related to known drug-

Table 4. Antibiotic resistant genes among *Elizabethkingia* strains.

Groups	Gene	17-13M	17-28D	17-30M	17-35D	17-84M	17-88M
Beta-lactams	*blaB*	+	+	+	+	+	+
	blaCME	+	+	+	+	+	+
Quinolones	*gyrA*	+	+	+	+	+	+
	parC	+	+	+	+	+	+
Amino-glycosides	*ant6*	-	-	-	-	-	-
Chloramphenicol	*catB*	+	+	+	+	+	+
Macrolide	*lolD*	-	-	-	-	-	-

resistance in bacteria. Overall, the complex relationship between drug-resistance, genetic changes, and phenotypic changes remains unclear (Ferrer et al., 2017). Therefore, the combination of gene expression and gene sequencing analysis allows us to understand major phenotypic changes that produce drug-resistance. To get the conclusions, comprehensive screening tests for all relevant genes should be conducted for other resistances that appear on multidrug-resistant isolates in Vietnam.

On the other hand, the emergence of multidrug-resistant opportunistic strains from hospital environment such as *Elizabethkingia* is still a serious concern. Besides being difficult for treatment, the drug-resistant phenotype of *Elizabethkingia* can be transmitted to other microorganism isolates through horizontal gene transfer. Antibiotic sensitive isolates received the resistant genes can become resistant isolates (Brown-Jaque et al., 2015). Antibiotic-resistance is an important issue for public health, and the control of drug-resistant bacteria from different environments is an important part of any country's strategy for preventing drug-resistant bacteria. This study showed the status of drug-resistance of several bacteria found in the hospital environment, and the necessity of further research to understand the mechanism of drug-resistance and to find effective treatments for *Elizabethkingia.*

Conclusions

In conclusion, the multidrug-resistance of *Elizabethkingia* spp. was reported for the first time in Vietnam, showing the status of drug-resistance of bacteria in the Vietnam's hospital environment. The species identification mentioned the circulation of both *E. meningoseptica* and *E. anophelis* in Vietnam. The result also showed the genotype–phenotype correlation of β-lactams (*blab* and *blaCME*) and fluoroquinolones (*gyrA* and *parC*) resistance. But the expression level was not commensurate with the high resistant phenotype and the absence of aminoglycoside (*ant6*) and macrolide (*lolD*) resistant genes indicate that other genetic factors could be implicated. Therefore, further research is required to confirm the resistant genes and may be the molecular marker for antibiotic-resistance *Elizabethkingia* detection.

COMPLIANCE WITH ETHICAL STANDARDS

Not applicable. The study was based on laboratory work performed with screening strains isolated from human. No data from human patient research was used.

COMPETING INTERESTS

The authors declare that they have no conflict of interests.

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