Molecular typing and profiling of topoisomerase mutations causing resistance to ciprofloxacin and levofloxacin in *Elizabethkingia* species

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Objectives. Several *Elizabethkingia* species often exhibit extensive antibiotic resistance, causing infections associated with severe morbidity and high mortality rates worldwide. In this study, we determined fluoroquinolone susceptibility profiles of clinical *Elizabethkingia* spp. isolates and investigated the resistance mechanisms.

Methods. In 2017–2018, 131 *Elizabethkingia* spp. isolates were recovered from specimens collected at tertiary care centers in northern Taiwan. Initial species identification using the Vitek MS system and subsequent verification by 16S rRNA sequencing confirmed the presence of *E. anophelis* (n=111), *E. miricola* (n=11), and *E. meningoseptica* (n=9). Fluoroquinolone susceptibility was determined using the microbroth dilution method, and fluoroquinolone resistance genes were analyzed by sequencing.

Results. Among *Elizabethkingia* spp. isolates, 91% and 77% were resistant to ciprofloxacin and levofloxacin, respectively. The most prevalent alterations were two single mutations in GyrA, Ser83Ile and Ser83Arg, detected in 76% of the isolates exhibiting fluoroquinolone MIC between 8 and 128 µg/ml. Another GyrA single mutation, Asp87Asn, was identified in two quinolone-resistant *E. miricola* strains. None of the isolates had alterations in GyrB, ParC or ParE. We developed a high-resolution melting assay for rapid identification of the prevalent gyrA gene mutations. The genetic relationship between the isolates was evaluated by random amplified polymorphic DNA PCR that yielded diverse pulsotypes, indicating the absence of any temporal or spatial overlap among the patients during hospitalization.

Conclusions. Our analysis of fluoroquinolone-resistant *Elizabethkingia* spp. isolates provides information for further research on the variations of the resistance mechanism and potential clinical guidance for infection management.
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**Running title:** Quinolone resistance in *Elizabethkingia* species
Abstract

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Methods. In 2017–2018, 131 *Elizabethkingia* spp. isolates were recovered from specimens collected at tertiary care centers in northern Taiwan. Initial species identification using the Vitek MS system and subsequent verification by 16S rRNA sequencing confirmed the presence of *E. anophelis* (n=111), *E. miricola* (n=11), and *E. meningoseptica* (n=9). Fluoroquinolone susceptibility was determined using the microbroth dilution method, and fluoroquinolone resistance genes were analyzed by sequencing.

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**Conclusions.** Our analysis of fluoroquinolone-resistant *Elizabethkingia* spp. isolates provides information for further research on the variations of the resistance mechanism and potential clinical guidance for infection management.
Introduction

The genus *Elizabethkingia* has been recently revised to include several species based on whole-genome sequencing analysis (Doijad et al. 2016; Nicholson et al. 2017). *Elizabethkingia* species are non-motile, non-fastidious, and glucose-non-fermentative gram-negative bacilli (Janda & Lopez 2017). Three species, *E. meningoseptica*, *E. miricola*, and *E. anophelis*, are known to cause diseases in humans (Green et al. 2008; Jean et al. 2014a; Lau et al. 2016b). Recent studies suggest that certain strains causing sporadic cases of meningitis and bacteremia, previously identified as *E. meningoseptica*, belong to *E. anophelis* (Chew et al. 2017; Lin et al. 2017).

Several outbreaks of *E. anophelis*-associated infections have been reported, including two outbreaks in the U.S. Midwest in 2016 with 65 confirmed cases (Coyle 2017; Janda & Lopez 2017). *Elizabethkingia* species also cause outbreaks in intensive care units as emerging pathogens of nosocomial infections with a high mortality rate and severe morbidity in critically ill patients (Jean et al. 2014a; Lau et al. 2016b; Opota et al. 2017; Shaohua et al. 2017). Because of their ability to accumulate different resistance mechanisms and a growing number of more vulnerable hosts, the prevalence of multidrug-resistant *Elizabethkingia* species has increased in the past decades, limiting the options for treatment (Janda & Lopez 2017; Jean et al. 2014b). For instance, resistance to carbapenems is mediated by metallo-β-lactamases (Breurec et al. 2016; Chen et al. 2017; Colapietro et al. 2016). A previous report indicated that fluoroquinolones are
suitable for treating *E. meningoseptica* bacteremia (Huang et al. 2018), and empirical evidence indicates that they are effective in treating *E. anophelis* and *E. miricola* infections (Coyle 2017; Figueroa Castro et al. 2017; Green et al. 2008; Zdziarski et al. 2017). However, a detailed analysis of fluoroquinolone-resistant *Elizabethkingia* spp. infections has not yet been performed.

Fluoroquinolones, such as ciprofloxacin (CIP) or levofloxacin (LVX), have two bacterial drug targets, DNA gyrase and DNA topoisomerase IV (Khodursky et al. 1995; Kreuzer & Cozzarelli 1979). Each enzyme is a heterotetramer, with gyrase composed of two GyrA and two GyrB subunits and topoisomerase IV composed of two ParC and two ParE subunits. Mechanisms of fluoroquinolone resistance include mutational alterations in drug target affinity, increased efflux pump expression, and acquisition of resistance-conferring genes (Hooper & Jacoby 2016). Single amino acid changes in either gyrase or topoisomerase IV can cause quinolone resistance. In gram-negative bacilli, mutations have been typically localized to the amino-terminal region of the primary target, GyrA (Yoshida et al. 1990), a region conserved among all potential quinolone targets. Mutations in these conserved regions of GyrB, ParC, and ParE are also known to confer fluoroquinolone resistance, like the amino-terminal GyrA region (Heisig 1996; Yoshida et al. 1991). Accordingly, the genomic DNA regions encoding the conserved protein regions of GyrA, GyrB, ParC, and ParE have been termed quinolone resistance-determining regions (QRDRs).

In this study, we aimed to assess the relationship between the quinolone-resistant phenotype of...
clinical *Elizabethkingia* spp. isolates in Taiwan and mutations in their DNA gyrase and DNA topoisomerase IV genes.
Materials & Methods

Bacterial isolates

In 2017–2018, 131 isolates of *Elizabethkingia* spp. (*E. anophelis*, n=111; *E. meningoseptica*, n=9; *E. miricola*, n=11) were recovered by bacterial culture from respiratory tract, urine, catheter tip, and blood specimens collected at the Tri-Service General Hospital (TSGH), tertiary care centers in northern Taiwan. The species were initially identified using the Vitek MS system with the IVD 3.0 database (bioMérieux, Mercy l’Etoile, France). Isolates identified as *Elizabethkingia* species using a previously reported study (Cheng et al. 2018). Briefly, MALDI-TOF spectral analysis software identified significant species-specific peaks to create reference masses for efficient and accurate identification of *Elizabethkingia* spp.. All bacterial isolates were kept frozen until used in this study.

Antimicrobial susceptibility

MIC of CIP and LVX were determined using the broth microdilution method. The susceptibilities were evaluated according to guidelines published by the Clinical and Laboratory Standards Institute (CLSI) including antibiotic-specific breakpoints (CIP: susceptible ≤ 1 µg/ml, resistant ≥ 4 µg/ml; LVX: susceptible ≤ 2 µg/ml, resistant ≥ 8 µg/ml)

DNA extraction
Genomic DNA was isolated using a previously reported protocol (Syn & Swarup 2000). Briefly, cellular lysis is achieved by a combination of EDTA/SDS detergent lysis and brief heat treatment. An additional phenol/chloroform step further deproteinates the preparation yielding DNA of good quality. Using a picodrop spectrophotometer, purified genomic DNA concentrations were determined by measuring the optical density at 260 nm, whereas the purity was estimated by calculating the ratio of the optical densities measured at 260 nm and 280 nm.

DNA samples were stored at $-20^\circ\text{C}$ until PCR was performed.

Bacteria species identification by 16S rRNA sequencing

The microbial identification accuracy was verified by 16S rRNA sequencing using a pair of specific primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGYTACCTTGTGACTT-3′), as previously described (Chang et al. 2014). DNA sequencing were compared to reference sequences using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information database.

PCR and DNA sequencing of the topoisomerase gene

Isolates were screened for mutations in the $gyrA$, $gyrB$, $parC$, or $parE$ genes by PCR using species-specific primers (Table 1). PCR products were sequenced for detection of nucleotide
polymorphism. Primers were commercially synthesized by Genomics (New Taipei city, Taiwan).

The reaction mixture (50 μl) contained 10 mM Tris–HCl (pH 7.5), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 10 pmol of the forward and reverse primer, 50 ng template DNA, and 0.8 U of Taq DNA polymerase (Applied Biosystems, Foster City, CA). Amplification was carried out in a ProFlex PCR thermal cycler (Applied Biosystems, Foster City, CA) with one initial denaturation step of 2 min at 95°C; 40 cycles of a denaturing step of 15 sec at 94°C, an annealing step of 1 min at 48–50°C with corresponding genes, and an extension step of 1 min at 72°C; and a final elongation step of 5 min at 72°C. All PCR products were processed for DNA sequencing (Genomics, New Taipei city, Taiwan) with the same PCR primer sets. Sequencing results in candidate genes from each isolate were compared with the respective reference sequences in the GenBank database (NCBI reference sequences: *E. anophelis*, NZ_CP007547.1; *E. meningoseptica*, NZ_CP016376.1; *E. miricola*, NZ_CP023746.1).

High-resolution melting (HRM) assay for *gyrA* mutation screening

Three different reverse primers and one common forward primer with homology to *Elizabethkingia* spp. *gyrA* gene were designed (Table 1). PCR amplification was performed using the KAPA HRM FAST PCR Kit for preparing the following reaction: 20 μl reaction mix containing 1 μl template DNA (10 ng), 8 μl PCR grade nuclease-free H₂O, 10 μl KAPA HRM FAST Master Mix, 2 μl 25 mM MgCl₂, and 0.5 μl of forward/reverse primer mix (10 μM each). The amplification and HRM curve analyses were conducted on a LightCycler 96 instrument.
(Roche, Manheim, Germany) using the following cycling conditions: initial activation at 95°C for 2 min, 40 cycles at 95°C for 10 sec and at 60°C for 30 sec. The post-PCR melting curve was performed using temperatures between 65°C and 95°C in temperature increments of 0.3°C.

**RAPD-PCR and capillary gel electrophoresis analysis**

RAPD-PCR was performed using primers (Table 1) described previously (Hsueh et al. 1996, Chiu et al. 2000). The reaction mixture (25 μl) contained 10 mM Tris–HCl (pH 7.5), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM dNTPs, 15 pmol of the RAPD primer, 50 ng genomic DNA, and 0.8 U of DyNAzyme II DNA polymerase (ABI, Thermo Fisher Scientific, Foster City, CA, USA). For every sample, each RAPD reaction was performed at least twice for each DNA extract. Amplification was carried out in a ProFlex PCR thermal cycler (Applied Biosystems, Foster City, CA) with one initial denaturation step of 5 min at 95°C; 40 cycles of a denaturing step of 1 min at 94°C, an annealing step of 1 min at 36 °C, and an extension step of 2 min at 72°C, and a final elongation step at 72°C for 8 min.

After PCR amplification, the products were analyzed on Qsep100 DNA Analyzer (BiOptic, Taiwan) according to the manufacturer's instructions. PCR fragments were applied into a miniaturized single-channel capillary cartridge of the Qsep100 DNA-CE with separation buffer. The run was performed using a high-resolution cartridge with a sample injection protocol of 8 kV for 10 s and separation at 5 kV for 300 s. The DNA alignment markers (20 bp, 1.442 ng/μl, and 5000 bp, 1.852 ng/μl) and the DNA size marker (50–3000 bp, 10.5 ng/μl) were obtained from BiOptic. Sample peaks were visualized using Q-Analyzer software (BiOptic).

**Molecular pattern analysis**

Isolates were categorized as identical, similar or unrelated according to their PCR banding
patterns. The data were analyzed using GelCompar II software (Applied Maths NV, Belgium).

Dice similarity coefficients were calculated and clustering was done by unweighted pair group mean association (UPGMA).

**Data analysis**

Statistical significance was determined using Student's t-test (GraphPad Prism). Differences were considered statistically significant when $p < 0.05$. 
Results

CIP and LVX susceptibility profiles of *Elizabethkingia* spp. isolates and corresponding resistance mutations

The 131 *Elizabethkingia* spp. isolates differed in their susceptibility to CIP and LVX (Fig. 1 and Table 2); 91% and 77% were resistant to CIP and LVX, respectively. All *E. meningoseptica* isolates were resistant to CIP, whereas 44% were resistant to LVX; 73% of the *E. miricola* isolates were resistant to CIP and 27% were resistant to LVX. Most *E. anophelis* isolates were resistant to CIP and LVX (92% and 85%, respectively).

A total of 101 (77%) *Elizabethkingia* spp. isolates had single-nucleotide mutations in the QRDR of the *gyrA* gene, whereas no mutations were found in the *gyrB, parC*, or *parE* gene of these isolates. In contrast, none of the 30 LVX-susceptible *Elizabethkingia* spp. isolates had mutations in the topoisomerase genes.

Among *E. anophelis* isolates with a *gyrA* gene mutation, 88 (93.6%) had a single-nucleotide mutation resulting in Ser83Ile amino acid substitution, whereas a different nucleotide mutation in 6 isolates resulted in Ser83Arg substitution. The most common single-nucleotide mutation encoding the Ser83Ile substitution was also found in *E. meningoseptica* and *E. miricola* isolates.

Another single-nucleotide mutation in the *gyrA* gene, encoding an Asp87Asn substitution, was found in two *E. miricola* isolates. Our results indicate a strong correlation between the antibiotic
susceptibility profiles of the clinical isolates and their mechanisms of fluoroquinolone resistance.

The resistance against CIP and LVX in *Elizabethkingia* spp. is mainly mediated by a single-nucleotide mutation in the QRDR of the *gyrA* gene.

The 29 isolates without any mutation in *gyrA, gyrB, parC*, or *parE* were completely susceptible or had intermediate susceptibility to CIP (MIC, 0.25–2.00 µg/ml) and LVX (MIC, 0.25–2.00 µg/ml), whereas 102 isolates were fully resistant to CIP with a corresponding MIC range of 32–128 µg/ml and an LVX MIC range of 4–128 µg/ml (Table 3).

Rapid detection of *gyrA* mutations using the HRM assay

The results of the *gyrA* gene sequence analysis of *Elizabethkingia* spp. isolates for the identification of mutations in the QRDR were used to develop an HRM assay that can be used to rapidly scan clinical isolates for typical *gyrA* gene mutations in 131 isolates of *Elizabethkingia* species. The HRM assay successfully detected all *gyrA* mutations in this study, encoding the Ser83Ile, Ser83Arg, and Asp87Asn substitutions. (Fig. 2). The HRM assay results for *gyrA* genotyping were in complete agreement with our DNA sequencing results without any exception (Table 3).

RAPD-PCR typing of *Elizabethkingia* spp. isolates
The 131 *Elizabethkingia* spp. isolates were clustered into multiple pulsotypes defined by a similarity of $\geq 85\%$ (Figure 3). The widespread pulsotype clusters indicated a lack of temporal or spatial overlap among the infected patients during hospitalization. Specifically, pulsotypes of *E. meningoseptica* and *E. miricola* isolates harboring a *gyrA* mutation were found to be distributed among wild type clusters.
Elizabethkingia spp. strains represent a group of emerging pathogens, causing infections that are associated with prolonged hospital stays and high mortality rates. In 2015–2016, there was an *E. anophelis* outbreak in Wisconsin, USA, that involved at least 63 patients and 18 deaths (Elbadawi et al. 2016). In addition, another outbreak in Illinois, USA, involving 10 cases with 6 deaths has also been reported in 2014–2016 (Navon et al. 2016). Globally, there are numerous sporadic *E. meningoseptica* nosocomial infection clusters and *E. miricola* infection case reports in medical centers including in Taiwan. Thus, pathogenic *Elizabethkingia* spp. strains appear to be opportunistic infectious agents associated with high mortality rates.

Quinolones underwent decades of development since the discovery of nalidixic acid in 1962, and quinolone resistance has also existed for decades. Recent studies described LVX-resistant *E. meningoseptica* bacteremia that is associated with an increase in mortality and prolonged hospital stays (Huang et al. 2017; Huang et al. 2018). Appropriate antibiotic use and an effective treatment regime are very important in fighting *Elizabethkingia* spp. infections. Using the broth microdilution method for MIC testing, we found differences in susceptibility to CIP and LVX among the *Elizabethkingia* spp. isolates. The discrepancy might be due to the different CLSI breakpoints, 4 μg/ml for CIP and 8 μg/ml for LVX. Previously reported susceptibility profiles of *E. anophelis* isolates, including from the outbreak in Wisconsin in 2016, indicated that most
isolates were susceptible to quinolones (Lau et al. 2016a; Perrin et al. 2017). In sharp contrast, among our 111 *E. anophelis* isolates, only 9 and 17 were found to be susceptible to CIP and LVX, respectively.

The genetic determinants of quinolone resistance have never been studied in *Elizabethkingia* spp. Our results revealed that certain single-nucleotide substitutions in *gyrA* conferred resistance to CIP and LVX in *Elizabethkingia* spp. The Ser83Ile substitution in GyrA protein was caused by the most prevalent mutation among all isolates, followed by the Ser83Arg or Asp87Asn amino acid substitutions caused by single-nucleotide mutations in *E. anophelis* or *E. miricola*. To our knowledge, this is the first report discussing genetic quinolone resistance determinants in *Elizabethkingia* spp.

Bacterial isolates carrying single alterations in QRDRs of DNA gyrase and topoisomerase IV typically exhibit reduced susceptibility to fluoroquinolones, which is considered as the first step in the development of full resistance (Hooper & Jacoby 2017). The genetic basis for fluoroquinolone resistance appears to be additive, different combinations of distinct resistance mechanisms may result in different MIC (Conley et al. 2018). Other resistant mechanisms such as plasmid-mediated quinolone resistance (PMQR) might also be involved in the quinolone resistance mechanism (Yugendran & Harish 2016). In our study, resistance to CIP and LVX was associated with single-nucleotide mutations in the QRDR of the *gyrA* gene in all *Elizabethkingia*
spp. isolates causing low-level to high-level fluoroquinolone resistance. The level of fluoroquinolone resistance did not correlate with the type of mutation found in the \textit{gyrA} gene. Other mechanisms typically implicated in fluoroquinolone resistance might be responsible for the differences in CIP and LVX MIC observed among the isolates. Changes in permeability and increased efflux pump activity along with plasmid-encoded resistance determinants cannot be excluded.

In this study, we also established a novel rapid HRM assay for detecting \textit{gyrA} mutations in \textit{Elizabethkingia} spp. The HRM results were in complete agreement with the DNA sequencing results, indicating that we developed a potentially useful adjunct test for the rapid detection of CIP and LVX resistance in \textit{Elizabethkingia} spp.

Conclusions

Our findings demonstrated that the quinolone resistance in \textit{Elizabethkingia} spp. is associated with mutations in the QRDR of the \textit{gyrA} gene. However, the level of resistance to quinolones of \textit{Elizabethkingia} spp. isolates could not be predicted based on the mutations identified in the \textit{gyrA} gene. This study provided information for further research on the variations of the fluoroquinolone resistance mechanism and potential clinical guidance for infection management.
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No.

Ethical approval:

This article does not contain any studies with human participants performed by any of the authors.

Informed consent:

For this type of study formal consent is not required.
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Figure legends

Figure 1. Fluoroquinolone MIC values of Elizabethkingia species. (A) E. anophelis isolates (n=111). (B) E. meningoseptica isolates (n=9). (C) E. miricola isolates (n=11). Each symbol (◇, □, Δ, ○) represents one isolate.

CIP, ciprofloxacin; LVX, levofloxacin; S, susceptible; I/R, intermediate/resistant

Susceptibility (≤ value), intermediate and resistance (≥ value) breakpoints defined by CLSI (2016): 1 µg/ml, 2 µg/ml and 4 µg/ml for CIP; 2 µg/ml, 4 µg/ml and 8 µg/ml for LVX.

**: P < 0.01; ***: P<0.001; ****: P<0.0001.

Figure 2. Representative HRM analysis of gyrA mutation and wild-type in Elizabethkingia species isolates. (A) gyrA wild-type (n=2) and gyrA mutation (n=28) Elizabethkingia anophelis isolates. (B) gyrA wild-type (n=5) and gyrA mutation (n=4) Elizabethkingia meningoseptica isolates. (C) gyrA wild-type (n=8) and gyrA mutation (n=3) Elizabethkingia miricola isolates.

WT: wild-type. Blue lines represent gyrA wild-type isolates, red lines and green lines represent gyrA mutation isolates, orange lines represent no template control.
Figure 3. RAPD PCR dendrogram of the *Elizabethkingia* spp. isolates investigated in this study.

(A) Clustering dendrogram of *E. anophelis* isolates (n=111). (B) Clustering dendrogram of *E. meningoseptica* isolates (n=9). (C) Clustering dendrogram of *E. miricola* isolates (n=11). E.A: *E. anophelis* E.M: *E. meningoseptica*; E.m: *E. miricola*. Black triangles represent clusters with multiple isolates possessing the same *gyrA* mutations. Black circles represent monoisolate clusters with *gyrA* mutations. The dashed line represents the similarity level (85%) used in the clusters definition.
**Table 1** (on next page)

Primer sequences used in this study
Table 1. Primer sequences used in this study

Primer sequences used to amplify \textit{gyrA}, \textit{gyrB}, \textit{parC}, and \textit{parE} genes in \textit{Elizabethkingia} spp.

| Primer name               | Sequence (5′→3′)                          | Annealing temperature (°C) | Product Size (bp) |
|---------------------------|------------------------------------------|----------------------------|------------------|
| gyra-E.species-F*         | AGC CCG TTG TTT AAA TCC TGA A            | 50                         | 743              |
| gyra-E.species-R          | CCC TGT TGG GAA GTC TGG TG               |                            |                  |
| gyrb-E.species-F          | GAT AAT TTC CTT CAT AAA GAG CC           |                            |                  |
| gyrb-E.anophelis-R        | CAT TGC CAT ACT GAG CTT GT               | 48                         | 905              |
| gyrb-E.menigospetica-R    | TCG AAG TGT CTT GTT CTT TGT CA           |                            | 896              |
| gyrb-E.miricola-R         | GCG TTG TCA TAC TGA ACT TG               |                            | 903              |
| pcar-E.species-F**        | GCT CAG TAT GGC AAT GCT AAA A           | 50                         | 785              |
| pcar-E.species-R          | TTG CTC TTA CCT TAC CGC CG               |                            |                  |
| pcar-E.menigospetica-F    | TGA CCG GAT CAA CCG AAG TC               |                            |                  |
| pcar-E.menigospetica-R    | CAG GTC GCC TGT TGT TTT GG               |                            |                  |
| pcar-E.species-F          | GTA TTC AGT TTA AAA GGT AAA CC           |                            |                  |
| pcar-E.anophelis-R        | GAA TAT ATT GGG CTT CGA CA               |                            |                  |
| pcar-E.menigospetica-R    | ACT GAA CTT AGT TGG CCA TAA G            | 48                         | 694              |
| pcar-E.miricola-R         | AGA AAT CGA CAT ATT CAG AGG T            |                            | 657              |

* \textit{gyrA-E.species-F} and \textit{gyrA-E.species-R} could amplify all \textit{Elizabethkinga} species \textit{gyrA} gene (including \textit{E. anophelis}, \textit{E. menigospetica} and \textit{E. miricola})

** \textit{parC-E.species-F} and \textit{parC-E.species-R} could amplify both \textit{E. anophelis} and \textit{E. miricola} \textit{parC} gene
## Primer sequences used for fluoroquinolone HRM analysis assays.

| Primer name                          | Sequence (5′→3′)                  | Annealing temperature (°C) | Product Size (bp) |
|--------------------------------------|-----------------------------------|-----------------------------|-------------------|
| gyrA-HRM-E.species-F                 | TGC CAG AAT TGT TGG AGA TG        | 50                          | 102               |
| gyrA-HRM-E.anophelis-R               | TAG CGC AGA GAC CAT GAC TG        | 50                          | 83                |
| gyrA-HRM-E.menigospetica-R           | GTG CCA TAC GCA CCA TAG CA        | 50                          | 85                |
| gyrA-HRM-E.miricola-R                | CTG TGC CAT ACG CAC CAT AG        | 50                          | 85                |
**Table 2** (on next page)

Antimicrobial susceptibility of ciprofloxacin/levofloxacin and mutation position detected in the gyrase or topoisomerase IV genes of *Elizabethkingia* species isolates
Table 2. Antimicrobial susceptibility of ciprofloxacin/levofloxacin and mutation position detected in the gyrase or topoisomerase IV genes of *Elizabethkingia* species isolates

| Species                          | Number of isolate | CIP (µg/ml) | LVX (µg/ml) | gyrase | gyrB | parC | parE |
|---------------------------------|------------------|-------------|-------------|--------|------|------|------|
|                                 |                  | CIP         | LVX         | gyrase | gyrB | parC | parE |
| *Elizabethkingia anophelis*     | 88               | 32-128      | 16-128      | Ser83Ile | No mutation | No mutation |
|                                 | 6                | 32          | 8-64        | Ser83Arg | No mutation | No mutation |
|                                 | 17               | 0.25-2      | 0.5         | No mutation | No mutation | No mutation |
| *Elizabethkingia meningoseptica*| 5                | 32-64       | 32-64       | Ser83Ile | No mutation | No mutation |
|                                 | 4                | 2           | 0.25-2      | No mutation | No mutation | No mutation |
| *Elizabethkingia miricola*      | 1                | 32          | 16          | Ser83Ile | No mutation | No mutation |
|                                 | 2                | 32          | 4           | Asp87Asn | No mutation | No mutation |
|                                 | 8                | 0.5-2       | 0.5         | No mutation | No mutation | No mutation |

* CIP, ciprofloxacin; LVX, levofloxacin. Susceptibility (≤ value) and resistance (≥ value) breakpoints defined by CLSI (2016): 1 µg/ml and 4 µg/ml for ciprofloxacin, 2 µg/ml and 8 µg/ml for levofloxacin.
Table 3 (on next page)

Alterations in gyrA genes detected by HRM assay and confirmed by DNA sequence analysis in *Elizabethkingia* spp. isolates.
Table 3. Alterations in \textit{gyr}A genes detected by HRM assay and confirmed by DNA sequence analysis in \textit{Elizabethkingia} spp. isolates.

| Species               | Number of isolate | Mutation detected by HRM | Confirmation by sequencing |
|-----------------------|-------------------|---------------------------|---------------------------|
|                       | gyra83     | gyra87        | gyra4 gene               |
| \textit{E. anophelis} | 94         | Mutation       | None                      | Ser83Ile/Ser83Arg         |
|                       | 17         | None           | None                      | No mutation               |
| \textit{E. menigoseptica} | 5          | Mutation       | None                      | Ser83Ile                  |
|                       | 4          | None           | None                      | No mutation               |
| \textit{E. miricola}  | 1          | Mutation       | None                      | Ser83Ile                  |
|                       | 2          | None           | Mutation                  | Asp87Asn                  |
|                       | 8          | None           | None                      | No mutation               |
Figure 1 (on next page)

Fluoroquinolone MIC values of Elizabethkingia species.

(A) *E. anophelis* isolates (n=111). (B) *E. meningoseptica* isolates (n=9). (C) *E. miricola* isolates (n=11). Each symbol (◇, □, △, ○) represents one isolate. CIP, ciprofloxacin; LVX, levofloxacin; S, susceptible; I/R, intermediate/resistant Susceptibility (≤ value), intermediate and resistance (≥ value) breakpoints defined by CLSI (2016): 1 µg/ml, 2 µg/ml and 4 µg/ml for CIP; 2 µg/ml, 4 µg/ml and 8 µg/ml for LVX. **: P < 0.01; ***: P<0.001; ****: P<0.0001.
(A) *Elizabethkingia anophelis* (n = 111)

(B) *Elizabethkingia miricola* (n = 11)

(C) *Elizabethkingia meningoseptica* (n = 9)
Figure 2

Representative HRM analysis of *gyrA* mutation and wild-type in *Elizabethkingia* species isolates.

(A) *gyrA* wild-type (n=2) and *gyrA* mutation (n=28) *Elizabethkingia anophelis* isolates. (B) *gyrA* wild-type (n=5) and *gyrA* mutation (n=4) *Elizabethkingia meningoseptica* isolates. (C) *gyrA* wild-type (n=8) and *gyrA* mutation (n=3) *Elizabethkingia miricola* isolates. WT: wild-type. Blue lines represent *gyrA* wild-type isolates, red lines and green lines represent *gyrA* mutation isolates, orange lines represent no template control.
Manuscript to be reviewed

(A) gyraseA Ser83Arg or Ser83Ile

E. anophelis

(B) gyraseA Ser83Arg

E. menigoseptica

(C) gyraseA Ser83Arg or Asp87Asn

E. miliicola
Figure 3

RAPD PCR dendrogram of the *Elizabethkingia* spp. isolates investigated in this study.

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