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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2   **ciprofloxacin and levofloxacin in *Elizabethkingia* species.**

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14   **Running title:** Quinolone resistance in *Elizabethkingia* species

Abstract

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24 infections associated with severe morbidity and high mortality rates worldwide. In this study, we

25 determined fluoroquinolone susceptibility profiles of clinical *Elizabethkingia* spp. isolates and

26 investigated the resistance mechanisms.

27 **Methods**. In 2017–2018, 131 *Elizabethkingia* spp. isolates were recovered from specimens

28 collected at tertiary care centers in northern Taiwan. Initial species identification using the Vitek

29 MS system and subsequent verification by 16S rRNA sequencing confirmed the presence of *E.*

30 *anophelis* (n=111), *E. miricola* (n=11), and *E. meningoseptica* (n=9). Fluoroquinolone

31 susceptibility was determined using the microbroth dilution method, and fluoroquinolone

32 resistance genes were analyzed by sequencing.

33 **Results**. Among *Elizabethkingia* spp. isolates, 91% and 77% were resistant to ciprofloxacin and

34 levofloxacin, respectively. The most prevalent alterations were two single mutations in GyrA,

35 Ser83Ile and Ser83Arg, detected in 76% of the isolates exhibiting fluoroquinolone MIC between

36 8 and 128 µg/ml. Another GyrA single mutation, Asp87Asn, was identified in two quinolone-

37 resistant *E. miricola* strains. None of the isolates had alterations in GyrB, ParC or ParE. We

38 developed a high-resolution melting assay for rapid identification of the prevalent *gyrA* gene

39   mutations. The genetic relationship between the isolates was evaluated by random amplified

40   polymorphic DNA PCR that yielded diverse pulsotypes, indicating the absence of any temporal

41   or spatial overlap among the patients during hospitalization.

42   **Conclusions**. Our analysis of fluoroquinolone-resistant *Elizabethkingia* spp. isolates provides

43   information for further research on the variations of the resistance mechanism and potential

44   clinical guidance for infection management.

45

46

47   **Introduction**

48   The genus *Elizabethkingia* has been recently revised to include several species based on whole-

49   genome sequencing analysis (Doijad et al. 2016; Nicholson et al. 2017). *Elizabethkingia* species

50   are non-motile, non-fastidious, and glucose-non-fermentative gram-negative bacilli (Janda &

51   Lopez 2017). Three species, *E. meningoseptica*, *E. miricola,* and *E. anophelis,* are known to

52   cause diseases in humans (Green et al. 2008; Jean et al. 2014a; Lau et al. 2016b). Recent studies

53   suggest that certain strains causing sporadic cases of meningitis and bacteremia, previously

54   identified as *E. meningoseptica,* belong to *E. anophelis* (Chew et al. 2017; Lin et al. 2017).

55   Several outbreaks of *E. anophelis*-associated infections have been reported, including two

56   outbreaks in the U.S. Midwest in 2016 with 65 confirmed cases (Coyle 2017; Janda & Lopez

57   2017). *Elizabethkingia* species also cause outbreaks in intensive care units as emerging

58   pathogens of nosocomial infections with a high mortality rate and severe morbidity in critically

59   ill patients (Jean et al. 2014a; Lau et al. 2016b; Opota et al. 2017; Shaohua et al. 2017). Because

60   of their ability to accumulate different resistance mechanisms and a growing number of more

61   vulnerable hosts, the prevalence of multidrug-resistant *Elizabethkingia* species has increased in

62   the past decades, limiting the options for treatment (Janda & Lopez 2017; Jean et al. 2014b). For

63   instance, resistance to carbapenems is mediated by metallo-β-lactamases (Breurec et al. 2016;

64   Chen et al. 2017; Colapietro et al. 2016). A previous report indicated that fluoroquinolones are

65   suitable for treating *E. meningoseptica* bacteremia (Huang et al. 2018), and empirical evidence

66   indicates that they are effective in treating *E. anophelis* and *E. miricola* infections (Coyle 2017;

67   Figueroa Castro et al. 2017; Green et al. 2008; Zdziarski et al. 2017). However, a detailed

68   analysis of fluoroquinolone-resistant *Elizabethkingia* spp. infections has not yet been performed.

69   Fluoroquinolones, such as ciprofloxacin (CIP) or levofloxacin (LVX), have two bacterial drug

70   targets, DNA gyrase and DNA topoisomerase IV (Khodursky et al. 1995; Kreuzer & Cozzarelli

71   1979). Each enzyme is a heterotetramer, with gyrase composed of two GyrA and two GyrB

72   subunits and topoisomerase IV composed of two ParC and two ParE subunits. Mechanisms of

73   fluoroquinolone resistance include mutational alterations in drug target affinity, increased efflux

74   pump expression, and acquisition of resistance-conferring genes (Hooper & Jacoby 2016). Single

75   amino acid changes in either gyrase or topoisomerase IV can cause quinolone resistance. In

76   gram-negative bacilli, mutations have been typically localized to the amino-terminal region of

77   the primary target, GyrA (Yoshida et al. 1990), a region conserved among all potential quinolone

78   targets. Mutations in these conserved regions of GyrB, ParC, and ParE are also known to confer

79   fluoroquinolone resistance, like the amino-terminal GyrA region (Heisig 1996; Yoshida et al.

80   1991). Accordingly, the genomic DNA regions encoding the conserved protein regions of GyrA,

81   GyrB, ParC, and ParE have been termed quinolone resistance-determining regions (QRDRs).

82   In this study, we aimed to assess the relationship between the quinolone-resistant phenotype of

83   clinical *Elizabethkingia* spp. isolates in Taiwan and mutations in their DNA gyrase and DNA

84   topoisomerase IV genes.

85   **Materials & Methods**

86   **Bacterial isolates**

87   In 2017–2018, 131 isolates of *Elizabethkingia* spp. (*E. anophelis,* n=111; *E. meningoseptica,*

88   n=9; *E. miricola,* n=11) were recovered by bacterial culture from respiratory tract, urine, catheter

89   tip, and blood specimens collected at the Tri-Service General Hospital (TSGH), tertiary care

90   centers in northern Taiwan. The species were initially identified using the Vitek MS system with

91   the IVD 3.0 database (bioMérieux, Mercy l'Etoile, France). Isolates identified as *Elizabethkingia*

92   species using a previously reported study (Cheng et al. 2018). Briefly, MALDI-TOF spectral

93   analysis software identified significant species-specific peaks to create reference masses for

94   efficient and accurate identification of *Elizabethkingia* spp.. All bacterial isolates were kept

95   frozen until used in this study.

96   **Antimicrobial susceptibility**

97   MIC of CIP and LVX were determined using the broth microdilution method. The

98   susceptibilities were evaluated according to guidelines published by the Clinical and Laboratory

99   Standards Institute (CLSI) including antibiotic-specific breakpoints (CIP: susceptible ≤ 1 µg/ml,

100   resistant ≥ 4 µg/ml; LVX: susceptible ≤ 2 µg/ml, resistant ≥ 8 µg/ml)

101

102   **DNA extraction**

103   Genomic DNA was isolated using a previously reported protocol (Syn & Swarup 2000). Briefly,

104   cellular lysis is achieved by a combination of EDTA/SDS detergent lysis and brief heat

105   treatment. An additional phenol/chloroform step further deproteinates the preparation yielding

106   DNA of good quality. Using a picodrop spectrophotometer, purified genomic DNA

107   concentrations were determined by measuring the optical density at 260 nm, whereas the purity

108   was estimated by calculating the ratio of the optical densities measured at 260 nm and 280 nm.

109   DNA samples were stored at −20°C until PCR was performed.

110

111   **Bacteria species identification by 16S rRNA sequencing**

112   The microbial identification accuracy was verified by 16S rRNA sequencing using a pair of

113   specific primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-

114   GGYTACCTTGTTACGACTT-3′), as previously described (Chang et al. 2014). DNA

115   sequencing were compared to reference sequences using the Basic Local Alignment Search Tool

116   (BLAST) of the National Center for Biotechnology Information database.

117

118   **PCR and DNA sequencing of the topoisomerase gene**

119   Isolates were screened for mutations in the *gyrA, gyrB, parC,* or *parE* genes by PCR using

120   species-specific primers (Table 1). PCR products were sequenced for detection of nucleotide

121   polymorphism. Primers were commercially synthesized by Genomics (New Taipei city, Taiwan).

122   The reaction mixture (50 µl) contained 10 mM Tris–HCl (pH 7.5), 50 mM KCl, 1.5 mM MgCl$_2$,

123   0.2 mM dNTPs, 10 pmol of the forward and reverse primer, 50 ng template DNA, and 0.8 U of

124   Taq DNA polymerase (Applied Biosystems, Foster City, CA). Amplification was carried out in a

125   ProFlex PCR thermal cycler (Applied Biosystems, Foster City, CA) with one initial denaturation

126   step of 2 min at 95°C; 40 cycles of a denaturing step of 15 sec at 94°C, an annealing step of 1

127   min at 48–50°C with corresponding genes, and an extension step of 1 min at 72°C; and a final

128   elongation step of 5 min at 72°C. All PCR products were processed for DNA sequencing

129   (Genomics, New Taipei city, Taiwan) with the same PCR primer sets. Sequencing results in

130   candidate genes from each isolate were compared with the respective reference sequences in the

131   GenBank database (NCBI reference sequences: *E. anophelis,* NZ_CP007547.1; *E.*

132   *meningoseptica,* NZ_CP016376.1; *E. miricola,* NZ_CP023746.1)

133

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

135   Three different reverse primers and one common forward primer with homology to

136   *Elizabethkingia* spp. *gyrA* gene were designed (Table 1). PCR amplification was performed

137   using the KAPA HRM FAST PCR Kit for preparing the following reaction: 20 µl reaction mix

138   containing 1 µl template DNA (10 ng), 8 µl PCR grade nuclease-free H$_2$O, 10 µl KAPA HRM

139   FAST Master Mix, 2 µl 25 mM MgCl$_2$, and 0.5 µl of forward/reverse primer mix (10 µM each).

140   The amplification and HRM curve analyses were conducted on a LightCycler 96 instrument

141   (Roche, Manheim, Germany) using the following cycling conditions: initial activation at 95°C

142   for 2 min, 40 cycles at 95°C for 10 sec and at 60°C for 30 sec. The post-PCR melting curve was

143   performed using temperatures between 65°C and 95°C in temperature increments of 0.3°C.

144

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

146   RAPD-PCR was performed using primers (Table 1) described previously (Hsueh et al. 1996,

147   Chiu et al. 2000). The reaction mixture (25 μl) contained 10 mM Tris–HCl (pH 7.5), 50 mM

148   KCl, 2.5 mM MgCl$_2$, 0.2 mM dNTPs, 15 pmol of the RAPD primer, 50 ng genomic DNA, and

149   0.8 U of DyNAzyme II DNA polymerase (ABI, Thermo Fisher Scientific, Foster City, CA,

150   USA). For every sample, each RAPD reaction was performed at least twice for each DNA

151   extract. Amplification was carried out in a ProFlex PCR thermal cycler (Applied Biosystems,

152   Foster City, CA) with one initial denaturation step of 5 min at 95°C; 40 cycles of a denaturing

153   step of 1 min at 94°C, an annealing step of 1 min at 36 °C, and an extension step of 2 min at

154   72°C, and a final elongation step at 72°C for 8 min.

155   After PCR amplification, the products were analyzed on Qsep100 DNA Analyzer (BiOptic,

156   Taiwan) according to the manufacturer's instructions. PCR fragments were applied into a

157   miniaturized single-channel capillary cartridge of the Qsep100 DNA-CE with separation buffer.

158   The run was performed using a high-resolution cartridge with a sample injection protocol of 8

159   kV for 10 s and separation at 5 kV for 300 s. The DNA alignment markers (20 bp, 1.442 ng/μl,

160   and 5000 bp, 1.852 ng/μl) and the DNA size marker (50–3000 bp, 10.5 ng/μl) were obtained

161   from BiOptic. Sample peaks were visualized using Q-Analyzer software (BiOptic).

162

163   **Molecular pattern analysis**

164   Isolates were categorized as identical, similar or unrelated according to their PCR banding

165   patterns. The data were analyzed using GelCompar II software (Applied Maths NV, Belgium).

166   Dice similarity coefficients were calculated and clustering was done by unweighted pair group

167   mean association (UPGMA).

168

169   **Data analysis**

170   Statistical significance was determined using Student's t-test (GraphPad Prism). Differences were

171   considered statistically significant when $p < 0.05$.

173 **Results**

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

175 **resistance mutations**

176 The 131 *Elizabethkingia* spp. isolates differed in their susceptibility to CIP and LVX (Fig. 1 and

177 Table 2); 91% and 77% were resistant to CIP and LVX, respectively. All *E. meningoseptica*

178 isolates were resistant to CIP, whereas 44% were resistant to LVX; 73% of the *E. miricola*

179 isolates were resistant to CIP and 27% were resistant to LVX. Most *E. anophelis* isolates were

180 resistant to CIP and LVX (92% and 85%, respectively).

181 A total of 101 (77%) *Elizabethkingia* spp. isolates had single-nucleotide mutations in the QRDR

182 of the *gyrA* gene, whereas no mutations were found in the *gyrB, parC.* or *parE* gene of these

183 isolates. In contrast, none of the 30 LVX-susceptible *Elizabethkingia* spp. isolates had mutations

184 in the topoisomerase genes.

185 Among *E. anophelis* isolates with a *gyrA* gene mutation, 88 (93.6%) had a single-nucleotide

186 mutation resulting in Ser83Ile amino acid substitution, whereas a different nucleotide mutation in

187 6 isolates resulted in Ser83Arg substitution. The most common single-nucleotide mutation

188 encoding the Ser83Ile substitution was also found in *E. meningoseptica* and *E. miricola* isolates.

189 Another single-nucleotide mutation in the *gyrA* gene, encoding an Asp87Asn substitution, was

190 found in two *E. miricola* isolates. Our results indicate a strong correlation between the antibiotic

191   susceptibility profiles of the clinical isolates and their mechanisms of fluoroquinolone resistance.

192   The resistance against CIP and LVX in *Elizabethkingia* spp. is mainly mediated by a single-

193   nucleotide mutation in the QRDR of the *gyrA* gene.

194   The 29 isolates without any mutation in *gyrA, gyrB, parC*, or *parE* were completely susceptible

195   or had intermediate susceptibility to CIP (MIC, 0.25–2.00 µg/ml) and LVX (MIC, 0.25–2.00

196   µg/ml), whereas 102 isolates were fully resistant to CIP with a corresponding MIC range of 32–

197   128 µg/ml and an LVX MIC range of 4–128 µg/ml (Table 3).

198

199   **Rapid detection of *gyrA* mutations using the HRM assay**

200   The results of the *gyrA* gene sequence analysis of *Elizabethkingia* spp. isolates for the

201   identification of mutations in the QRDR were used to develop an HRM assay that can be used to

202   rapidly scan clinical isolates for typical *gyrA* gene mutations in 131 isolates of *Elizabethkingia*

203   species. The HRM assay successfully detected all *gyrA* mutations in this study, encoding the

204   Ser83Ile, Ser83Arg, and Asp87Asn substitutions. (Fig. 2). The HRM assay results for *gyrA*

205   genotyping were in complete agreement with our DNA sequencing results without any exception

206   (Table 3).

207

208   **RAPD-PCR typing of *Elizabethkingia* spp. isolates**

209   The 131 *Elizabethkingia* spp. isolates were clustered into multiple pulsotypes defined by a

210   similarity of ≥85% (Figure 3). The widespread pulsotype clusters indicated a lack of temporal or

211   spatial overlap among the infected patients during hospitalization. Specifically, pulsotypes of *E.*

212   *meningoseptica* and *E. miricola* isolates harboring a *gyrA* mutation were found to be distributed

213   among wild type clusters.

214

215 **Discussion**

216 *Elizabethkingia* spp. strains represent a group of emerging pathogens, causing infections that are

217 associated with prolonged hospital stays and high mortality rates. In 2015–2016, there was an *E.*

218 *anophelis* outbreak in Wisconsin, USA, that involved at least 63 patients and 18 deaths

219 (Elbadawi et al. 2016). In addition, another outbreak in Illinois, USA, involving 10 cases with 6

220 deaths has also been reported in 2014–2016 (Navon et al. 2016). Globally, there are numerous

221 sporadic *E. meningoseptica* nosocomial infection clusters and *E. miricola* infection case reports

222 in medical centers including in Taiwan. Thus, pathogenic *Elizabethkingia* spp. strains appear to

223 be opportunistic infectious agents associated with high mortality rates.

224 Quinolones underwent decades of development since the discovery of nalidixic acid in 1962, and

225 quinolone resistance has also existed for decades. Recent studies described LVX-resistant *E.*

226 *meningoseptica* bacteremia that is associated with an increase in mortality and prolonged

227 hospital stays (Huang et al. 2017; Huang et al. 2018). Appropriate antibiotic use and an effective

228 treatment regime are very important in fighting *Elizabethkingia* spp. infections. Using the broth

229 microdilution method for MIC testing, we found differences in susceptibility to CIP and LVX

230 among the *Elizabethkingia* spp. isolates. The discrepancy might be due to the different CLSI

231 breakpoints, 4 µg/ml for CIP and 8 µg/ml for LVX. Previously reported susceptibility profiles of

232 *E. anophelis* isolates, including from the outbreak in Wisconsin in 2016, indicated that most

233   isolates were susceptible to quinolones (Lau et al. 2016a; Perrin et al. 2017). In sharp contrast,

234   among our 111 *E. anophelis* isolates, only 9 and 17 were found to be susceptible to CIP and

235   LVX, respectively.

236   The genetic determinants of quinolone resistance have never been studied in *Elizabethkingia* spp.

237   Our results revealed that certain single-nucleotide substitutions in *gyrA* conferred resistance to

238   CIP and LVX in *Elizabethkingia* spp. The Ser83Ile substitution in GyrA protein was caused by

239   the most prevalent mutation among all isolates, followed by the Ser83Arg or Asp87Asn amino

240   acid substitutions caused by single-nucleotide mutations in *E. anophelis* or *E. miricola*. To our

241   knowledge, this is the first report discussing genetic quinolone resistance determinants in

242   *Elizabethkingia* spp.

243   Bacterial isolates carrying single alterations in QRDRs of DNA gyrase and topoisomerase IV

244   typically exhibit reduced susceptibility to fluoroquinolones, which is considered as the first step

245   in the development of full resistance (Hooper & Jacoby 2017). The genetic basis for

246   fluoroquinolone resistance appears to be additive, different combinations of distinct resistance

247   mechanisms may result in different MIC (Conley et al. 2018). Other resistant mechanisms such

248   as plasmid-mediated quinolone resistance (PMQR) might also be involved in the quinolone

249   resistance mechanism(Yugendran & Harish 2016). In our study, resistance to CIP and LVX was

250   associated with single-nucleotide mutations in the QRDR of the *gyrA* gene in all *Elizabethkingia*

251   spp. isolates causing low-level to high-level fluoroquinolone resistance. The level of

252   fluoroquinolone resistance did not correlate with the type of mutation found in the *gyrA* gene.

253   Other mechanisms typically implicated in fluoroquinolone resistance might be responsible for

254   the differences in CIP and LVX MIC observed among the isolates. Changes in permeability and

255   increased efflux pump activity along with plasmid-encoded resistance determinants cannot be

256   excluded.

257   In this study, we also established a novel rapid HRM assay for detecting *gyrA* mutations in

258   *Elizabethkingia* spp. The HRM results were in complete agreement with the DNA sequencing

259   results, indicating that we developed a potentially useful adjunct test for the rapid detection of

260   CIP and LVX resistance in *Elizabethkingia* spp.

261   **Conclusions**

262   Our findings demonstrated that the quinolone resistance in *Elizabethkingia* spp. is associated

263   with mutations in the QRDR of the *gyrA* gene. However, the level of resistance to quinolones of

264   *Elizabethkingia* spp. isolates could not be predicted based on the mutations identified in the *gyrA*

265   gene. This study provided information for further research on the variations of the

266   fluoroquinolone resistance mechanism and potential clinical guidance for infection management.

268   **Acknowledgments**

269   No.

270   **Ethical approval :**

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

272   authors.

273   **Informed consent:**

274   For this type of study formal consent is not required.

275

276

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418   **Figure legends**

419   **Figure 1.** Fluoroquinolone MIC values of *Elizabethkingia* species.   (A) *E. anophelis* isolates

420   (n=111). (B) *E. meningoseptica* isolates (n=9). (C) *E. miricola* isolates (n=11). Each symbol (◇,

421   □, Δ, ○) represents one isolate.

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

423   Susceptibility (≤ value), intermediate and resistance (≥ value) breakpoints defined by CLSI

424   (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.

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

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428   **Figure 2.** Representative HRM analysis of *gyrA* mutation and wild-type in *Elizabethkingia*

429   species isolates. (A) *gyrA* wild-type (n=2) and *gyrA* mutation (n=28) *Elizabethkingia anophelis*

430   isolates. (B) gyrA wild-type (n=5) and *gyrA* mutation (n=4) *Elizabethkingia meningoseptica*

431   isolates. (C) *gyrA* wild-type (n=8) and *gyrA* mutation (n=3) *Elizabethkingia miricola* isolates.

432   WT: wild-type. Blue lines represent *gyrA* wild-type isolates, red lines and green lines represent

433   *gyrA* mutation isolates, orange lines represent no template control.

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436   **Figure 3.** RAPD PCR dendrogram of the *Elizabethkingia* spp. isolates investigated in this study.

437   (A) Clustering dendrogram of *E. anophelis* isolates (n=111). (B) Clustering dendrogram of *E.*

438   *meningoseptica* isolates (n=9). (C) Clustering dendrogram of *E. miricola* isolates (n=11). E.A: *E.*

439   *anophelis* E.M: *E. meningoseptica*; E.m: *E. miricola*. Black triangles represent clusters with

440   multiple isolates possessing the same *gyrA* mutations. Black circles represent monoisolate

441   clusters with *gyrA* mutations. The dashed line represents the similarity level (85%) used in the

442   clusters definition.

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Primer sequences used in this study

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

2 Primer sequences used to amplify *gyrA, gyrB, parC,* and *parE* genes in *Elizabethkingia* spp.

Primer name	Sequence (5′→3′)	Annealing temperature (℃)	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	48	905 896 903
gyrB-E.anophelis-R	CAT TGC CAT ACT GAG CTT GT		
gyrB-E.menigospetica-R	TCG AAG TGT TTG CTT TGT CA		
gyrB-E.miricola-R	GCG TTG TCA TAC TGA ACT TG		
parC-E.species-F[**]	GCT CAG TAT GGC AAT GCT AAA A	50	785
parC-E.species-R	TTG CTC TTA CCT TAC CGC CG		
parC-E.menigospetica-F	TGA CCG GAT CAA CCG AAG TC		
parC-E.menigospetica-R	CAG GTC GCC TGT TGT TTT GG		
parE-E.species-F	GTA TTC AGT TTA AAA GGT AAA CC	48	694 657 683
parE-E.anophelis-R	GAA TAT ATT GGG CTT CGA CA		
parE-E.menigospetica-R	ACT GAA CTT AGT TTG CCA TAA G		
parE-E.miricola-R	AGA AAT CGA CAT ATT CAG AGG T		

3 [*] gyrA-E.species-F and gyrA-E.species-R could amplify all *Elizabethkinga* species *gyrA* gene (including *E. anohpelis, E. menigospetica and E. miricola*)

4 [**] parC-E.species-F and parC-E.species-R could amplify both *E. anohpelis* and *E. miricola parC* gene

6 Primer sequences used for RAPD PCR.

Primer name	Sequence	Target
OPA-10	GTG ATC GCA G	*E. anophelis*
OPB-15	GGA GGG TGT T	*E. menigospetica* *E. miricola*

7   Primer sequences used for fluoroquinolone HRM analysis assays.

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

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

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

2   ***Elizabethkingia* species isolates**

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

5   * 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

6   levofloxacin.

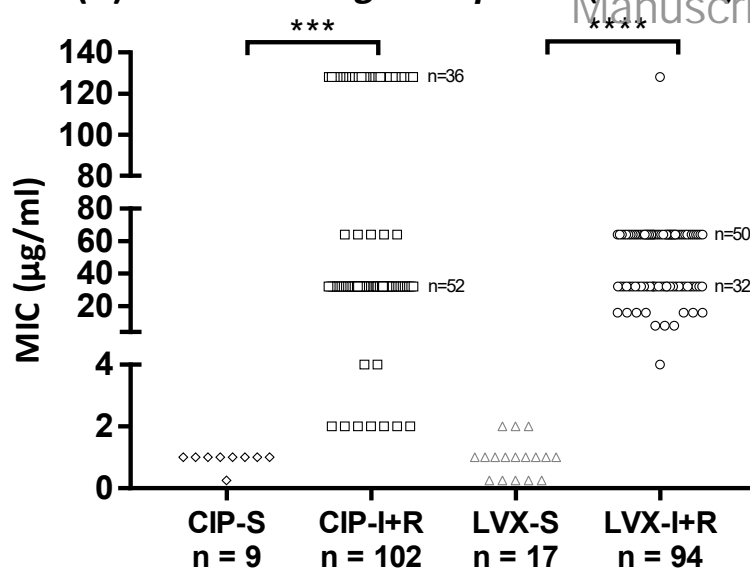
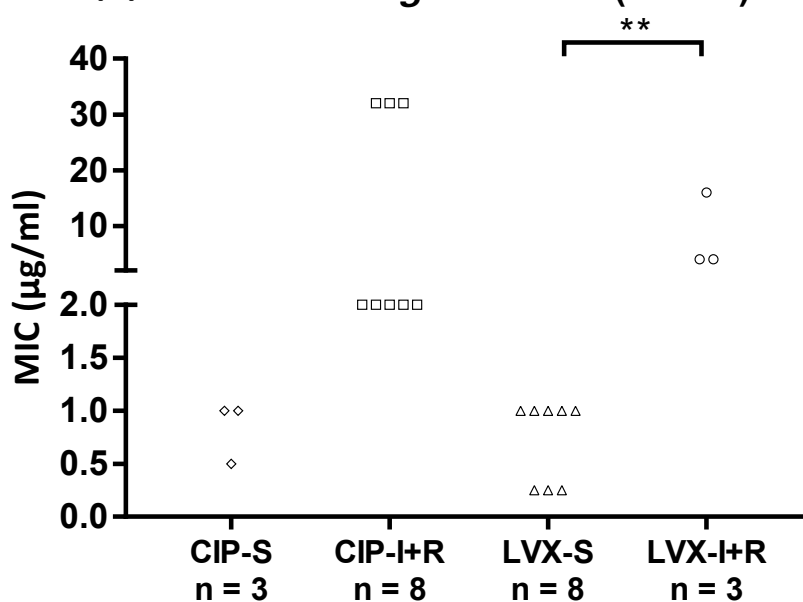
Alterations in *gyrA* genes detected by HRM assay and confirmed by DNA sequence analysis in *Elizabethkingia* spp. isolates.

1   **Table 3. Alterations in *gyrA* genes detected by HRM assay and confirmed by DNA sequence analysis in *Elizabethkingia* spp. isolates.**

Species	Number of isolate	Mutation detected by HRM		Confirmation by sequencing
		*gyrA*83	*gyrA*87	*gyrA* gene
E. anophelis	94	Mutation	None	Ser83Ile/Ser83Arg
	17	None	None	No mutation
E. menigoseptica	5	Mutation	None	Ser83Ile
	4	None	None	No mutation
E. miricola	1	Mutation	None	Ser83Ile
	2	None	Mutation	Asp87Asn
	8	None	None	No mutation

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)

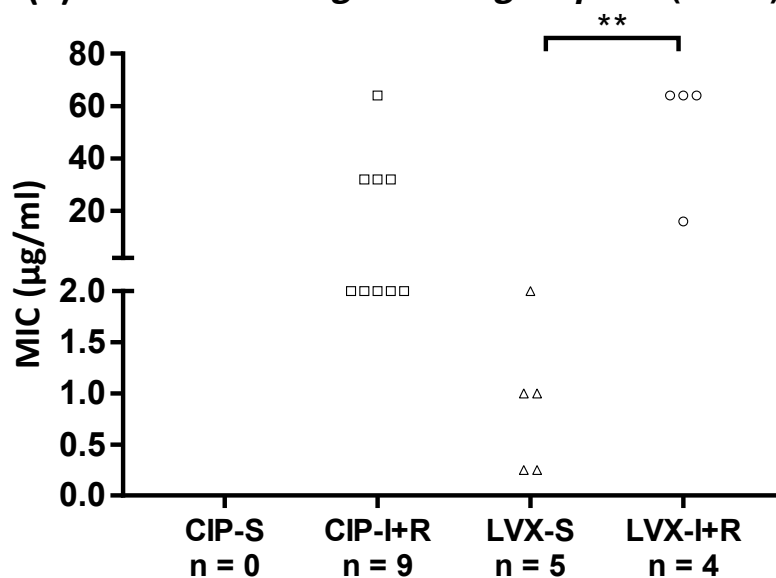
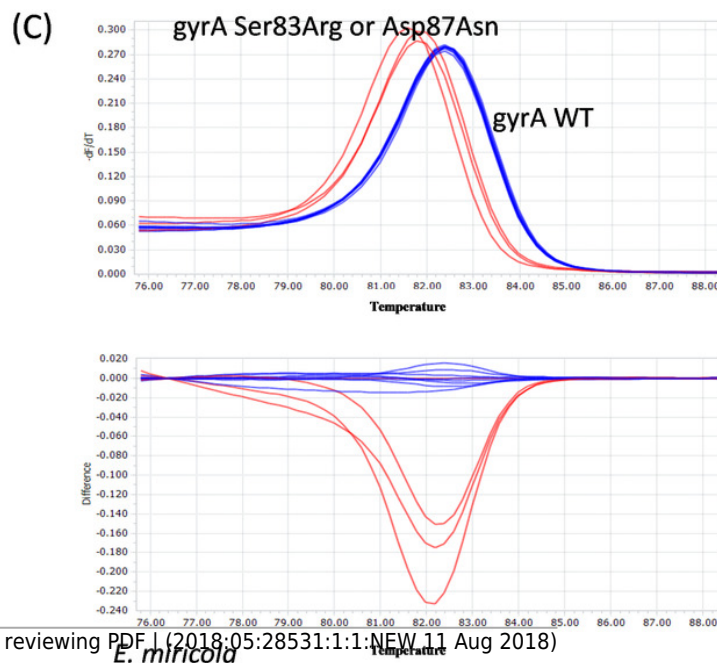
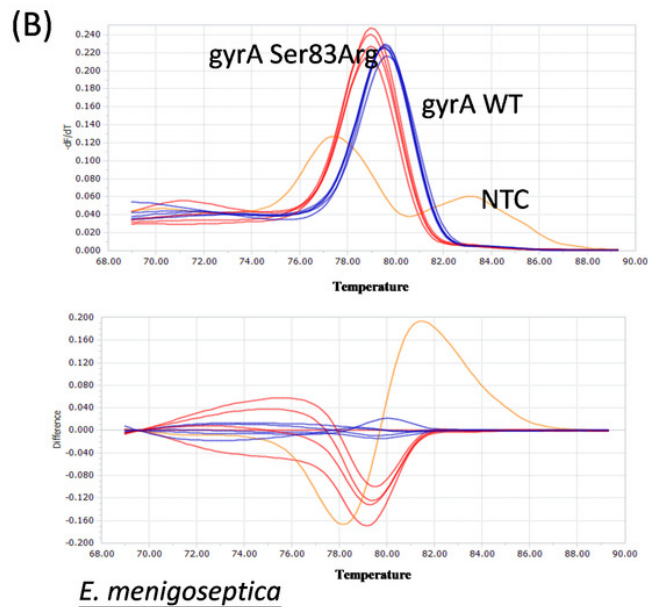
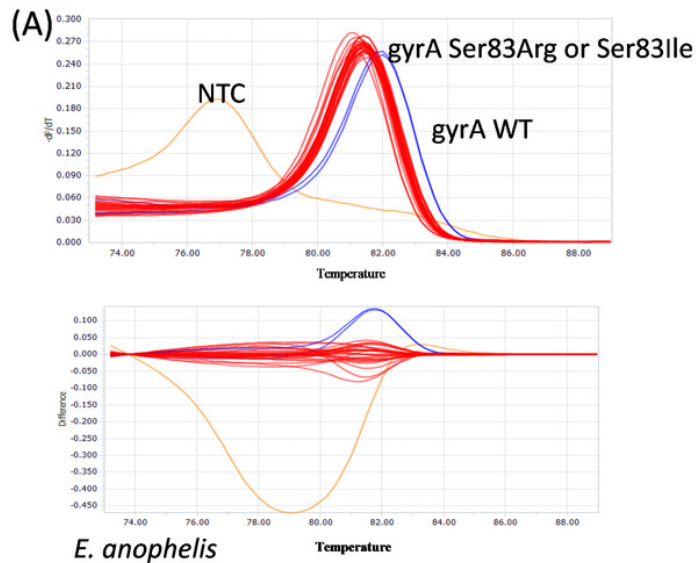



Figure 2

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.

(A)

NTC

gyrA Ser83Arg or Ser83Ile

gyrA WT

E. anophelis

(B)

gyrA Ser83Arg

gyrA WT

NTC

E. menigoseptica

(C)

gyrA Ser83Arg or Asp87Asn

gyrA WT

E. miricola

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.