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Short Communication

In vitro induction and selection of fluoroquinolone-resistant mutants in *Elizabethkingia anophelis*

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Abstract For 29 parent strains, recognized by pulsed-field gel electrophoresis, the MICs multiplied significantly in the ciprofloxacin group than levofloxacin group, following the first and third induction cycle. Ser83Arg in GyrA was the most common site of mutations. No mutation in ParC nor ParE was identified in the selected mutants.

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determining regions

Introduction

Elizabethkingia is a genus of Gram-negative, obligate aerobic, non-spore-forming, and glucose-nonfermenting microorganisms.[1] *Elizabethkingia anophelis* has recently been recognized as a crucial pathogen that causes lethal infections with substantial morbidity and mortality, particularly in immunocompromised individuals.[1,2] Traditionally, *E. anophelis* is frequently resistant to many antimicrobials clinically prescribed, such as the majority of penicillins and cephalosporins, carbapenems, beta-lactam-beta-lactamase inhibitor combinations, and aminoglycosides.[1,3] However, the susceptibility of *E. anophelis* to fluoroquinolones varies geographically.[1,3,4] The mechanisms of resistance development in fluoroquinolones include point mutations in quinolone resistance-determining regions (QRDRs) and the gene that induces the over-expression of efflux pumps or the under-expression of porins.[5] Recently, point mutations in QRDRs have been established as the principal mechanism of fluoroquinolone resistance.[6,7] However, the report comprehensively detailing the alterations of amino acid and point mutations for each resistant mutant throughout stepwise exposure to fluoroquinolones is limiting. Accordingly, we investigated the changes in minimum inhibitory concentrations (MICs) and the episode of mutations in the QRDRs following exposure to levofloxacin or ciprofloxacin.

Materials and methods

From 2005 to 2022, 142 levofloxacin-susceptible *E. anophelis* isolates (i.e., MICs $\leq$2 mg/L) were collected from E-Da Hospital (84 isolates), Kaohsiung Medical University Hospital (30), National Cheng Kung University Hospital (15), E-Da Cancer Hospital (10), and Taichung Veterans General Hospital (3). As previously described,[3,4] the species of these isolates were confirmed using 16S rRNA gene sequencing before processing the induction and resistance selection.

Of the total 142 *E. anophelis* isolates, bacterial clones were recognized by recognized by pulsed-field gel electrophoresis (PFGE), as previously described.[2] One to three isolates, simultaneously exhibiting the ciprofloxacin and levofloxacin MIC $\leq$1 mg/L, in the individual clone were randomly selected as the parent strains for the multicycle of induction and resistance selection (shown in Supplemental Material). Because our preliminary study showed no mutations in the QRDRs of *GyrA*, *GyrB*, *ParC*, and *ParE* in selected isolates with levofloxacin or ciprofloxacin MICs $\leq$32 mg/L,[7] only isolates with MICs $\geq$64 mg/L after fluoroquinolone exposure were subjected for sequence analyses to identify mutations in the QRDRs. Further amplification and sequencing for identifying mutations in QRDRs were consistent with previous descriptions.[7] Susceptibilities were determined based on the MIC breakpoints for "other non-Enterobacteriaceae" issued by the Clinical and Laboratory Standards Institute guidelines in 2022.[8]

Statistical analyses were performed using the Statistical Package for the Social Sciences for Windows (Version 23.0; Chicago, IL, USA). Continuous variables were analyzed for differences in mean values by the Student's *t*-test. The *Pearson* correlation coefficient (*r*) was calculated to assess the relationship between the MICs and the number of mutations. Categorical variables were compared by the *Chi*-square method or Fisher's exact test if an expected value $<$ 5. All *P* values were measured by 2-tailed, and a *P* value of $<$0.05 was considered statistically significant.

Results

Study isolates

Of 142 levofloxacin-susceptible *E. anophelis* clinically collected, twenty-seven PFGE patterns were recognized (Supplemental Fig. 1). Because the isolate, that manifested both ciprofloxacin and levofloxacin MICs $\leq$1 mg/L, was not disclosed among seven PFGE clones, only 29 parent strains from 20 clones were included for multicycle induction and mutant selection.

MIC changes in induction cycles

The MIC changes of levofloxacin or ciprofloxacin in each cycle were exhibited in Table 1. Irrespective of levofloxacin (Table 1A) or ciprofloxacin (Table 1B) exposure, the MICs of levofloxacin or ciprofloxacin apparently increased in the mutant isolates by the induction step. Notably, a higher proportion of ciprofloxacin-resistant isolates was observed than that of levofloxacin-resistant isolates after one cycle (29/29, 100% *vs.* 14/29, 48.3%; $P < 0.001$). After three cycles, the majority (25/29, 86.2%) of mutant isolates reached a ciprofloxacin MIC $\geq$256 mg/L, but the proportion of mutant isolates with a levofloxacin MIC $\geq$256 mg/L only accounted for 44.8% (13/29; $P = 0.001$). As shown in Fig. 1, the MICs multiplied significantly vaster in the group of ciprofloxacin exposure than in that of levofloxacin exposure following the first induction cycle ($P = 0.011$) and third cycle ($P = 0.031$).

Mutations in QRDRs by levofloxacin induction

Following levofloxacin exposure, non-synonymous mutations were detected in *GyrA* and/or *GyrB* of 28 *E. anophelis* isolates and 24 isolates with $\geq$2 times of point mutants were recognized (Supplemental Table 1). No mutation was discovered in *ParC* and *ParE* throughout the multicycle of induction and selection by levofloxacin.

Along with the elevation of MICs after levofloxacin induction, the number of mutation points in *GyrA* increased

Table 1 The minimum inhibitory concentrations (MICs) of levofloxacin and ciprofloxacin against *E. anophelis* in each step of multicycle induction and mutant selection by levofloxacin and ciprofloxacin, respectively*.

	Levofloxacin MIC (mg/L) of mutant in steps							
Strains	0	1	2	3	4	5	6	7
EM98-05	0.5	1	64	>256	-	-	-	-
EM151-25	0.5	32	256	>256	-	-	-	-
EM458-14	0.5	64	256	256	>256	-	-	-
EM703-45	0.5	2	2	8	256	>256	-	-
EM730-50	1	4	8	8	8	>256	-	-
EM749-74	0.5	4	8	64	64	256	>256	-
EM755-100	0.5	4	16	16	16	32	>256	-
EM770-49	0.5	8	8	32	32	>256	-	-
EM993-93	0.5	0.5	0.5	8	256	>256	-	-
EM1064-93	0.5	8	256	>256	-	-	-	-
EDC46-69	1	8	8	>256	-	-	-	-
EDC52-15	0.5	16	>256	-	-	-	-	-
EDC53-89	0.5	8	32	32	>256	-	-	-
VGHTC3	0.5	64	256	>256	-	-	-	-
KMUH21	0.5	8	8	8	8	8	8	256
KMUH41	0.5	2	32	>256	-	-	-	-
NCKU67	0.5	2	16	64	128	256	>256	-
EM87-63	1	4	64	>256	-	-	-	-
EM265-62	0.5	4	64	128	128	>256	-	-
EM356-57	1	4	4	8	8	8	8	8
EM861-69	0.5	4	4	32	256	256	>256	-
EM1059-55	0.5	32	256	>256	-	-	-	-
EDC43-35	0.5	8	128	128	128	>256	-	-
EDC58-23	1	32	256	>256	-	-	-	-
KMUH25	0.5	8	8	8	32	32	>256	-
KMUH28	0.5	4	32	256	>256	-	-	-
KMUH57	0.5	16	128	128	256	>256	-	-
NCKU62	0.5	2	4	32	256	256	>256	-
NCKU63	0.5	4	256	>256	-	-	-	-

	Ciprofloxacin MIC (mg/L) of mutant in steps							
Strains	0	1	2	3	4	5	6	7
EM98-05	1	8	8	64	256	>256	-	-
EM151-25	1	16	128	256	>256	-	-	-
EM458-14	1	64	>256	-	-	-	-	-
EM703-45	1	32	128	>256	-	-	-	-
EM730-50	1	8	16	16	256	>256	-	-
EM749-74	0.5	4	64	256	>256	-	-	-
EM755-100	1	16	16	64	64	128	128	128
EM770-49	1	64	>256	-	-	-	-	-
EM993-93	1	32	>256	-	-	-	-	-
EM1064-93	1	64	>256	-	-	-	-	-
EDC46-69	1	16	>256	-	-	-	-	-
EDC52-15	1	128	>256	-	-	-	-	-
EDC53-89	1	16	128	>256	-	-	-	-
VGHTC3	1	256	>256	-	-	-	-	-
KMUH21	1	64	>256	-	-	-	-	-
KMUH41	1	32	32	256	>256	-	-	-
NCKU67	1	32	32	256	>256	-	-	-
EM87-63	1	16	256	>256	-	-	-	-
EM265-62	0.5	4	128	>256	-	-	-	-
EM356-57	1	8	128	>256	-	-	-	-
EM861-69	1	4	32	256	256	>256	-	-
EM1059-55	1	16	256	>256	-	-	-	-
EDC43-35	1	32	>256	-	-	-	-	-
EDC58-23	1	256	>256	-	-	-	-	-
KMUH25	1	16	16	16	>256	-	-	-
KMUH28	0.5	8	128	>256	-	-	-	-
KMUH57	0.5	8	32	>256	-	-	-	-
NCKU62	0.5	4	32	256	>256	-	-	-
NCKU63	0.5	16	>256	-	-	-	-	-

* The color background is as follows: green indicates susceptibility, yellow indicates intermediate resistance, and orange indicates resistance.

($r = 0.9743$). For example, 34 point mutations in *GyrA* were identified when the MIC was >256 mg/L, but eight mutations were recognized when the MIC was 128 mg/L (Supplemental Table 2). Amino acid alterations occurred most frequently at position 87 in *GyrA*, followed by position 83, 81, and 119. Overall, Ser83Arg in *GyrA* is the most common amino acid alteration after levofloxacin exposure.

The relationship between the MIC elevation and the number of mutations in *GyrB* also demonstrated a high correlation ($r = 0.9856$). Mutants with MICs >256 mg/L possessed 20 mutations in *GyrB*, but those with MICs of 64 mg/L only had two mutations. The most frequent mutation of amino acid was at position 431, followed by position 439, 451, 471, 470, and 450. Asp431Asn in *GyrB* occurred most frequently following levofloxacin exposure.

Mutations in QRDRs by ciprofloxacin induction

Non-synonymous mutations were detected in *GyrA* and/or *GyrB*, but no mutations were found in *ParC* and *ParE* in the multicycle of ciprofloxacin induction (Supplemental Table 3). In detail, 18 isolates experiencing one point mutant and 11 with ≥2 times of point mutants were disclosed.

Eleven mutations in *GyrA* occurred in mutants with MICs of 256 mg/L; however, 36 mutations in *GyrA* were discovered in isolates with MICs >256 mg/L (Supplemental Table 4). The MICs were well correlated to the number of mutations in *GyrA* ($r = 0.9733$). Amino acid replacement in *GyrA* was detected most frequently at position 83, followed by position 87, 119, 81, and 82. Like levofloxacin exposure, Ser83Arg in *GyrA* was the most common amino acid change after ciprofloxacin exposure.

For mutations in *GyrB* after ciprofloxacin exposure, only 15 episodes of amino acid alterations were detected. The MICs were not well correlated with the number of mutations in *GyrB* ($r = 0.723$). The most common mutations were Leu449Trp, Asn469Asp, and Glu471Lys.

Discussion

Traditionally, *E. anophelis* is frequently resistant to most beta-lactams and other antimicrobials commonly prescribed in clinical practice.[1,3,4] On account of the multidrug-resistant characteristics of *E. anophelis*, fluoroquinolones could potentially be regarded as a reasonable choice of antimicrobial agents in treating patients with *E. anophelis*

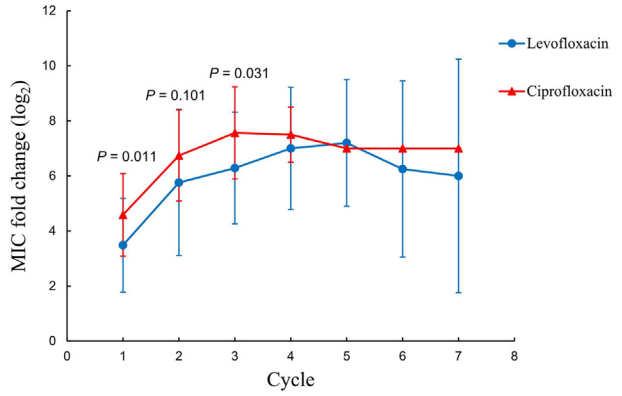

Figure 1. Fold changes in fluoroquinolone minimum inhibitory concentrations (MICs) in selected mutants of *E. anophelis*. Twenty-nine isolates (both levofloxacin and ciprofloxacin MICs $\leq$1 mg/L) were used as parent strains to be exposed to levofloxacin or ciprofloxacin in a stepwise manner. The x-axis numbers represent the cycles of induction and selection. The y-axis numbers indicate the average (lines) and standard deviation (vertical bars) of MIC fold changes (log2) in each cycle. The MICs multiplied significantly following ciprofloxacin exposure than after levofloxacin exposure in the first induction cycle ($P = 0.011$) and the third cycle ($P = 0.031$), but not significantly in the second cycle ($P = 0.101$).

infections. Nevertheless, extensive fluoroquinolone administration in clinical practice has led to the development of fluoroquinolone-resistant microorganisms and this issue has become a crucial dilemma of global public health.[5] Like the fact that a higher prevalence of ciprofloxacin-resistant *E. anophelis* than that of levofloxacin-resistant *E. anophelis* in numerous studies,[3,4,7] this study discerned that MICs increased more rapidly after ciprofloxacin exposure than after levofloxacin exposure. To prevent the widespread emergence of fluoroquinolone resistance and to optimize therapeutic strategies for *E. anophelis* infections, our findings reveal a clear understanding in the development of resistant mutants under selective pressure.

Fluoroquinolone resistance caused by target enzyme gene mutations has been well established.[5] The acquisition of mutations in the QRDRs has been studied through the *in vitro* exposure to increasing concentrations of fluoroquinolones in specific microorganisms, such as *Staphylococcus aureus*[9] and *Streptococcus pneumoniae*.[10] However, a study detailing the acquisition of point mutations in the QRDRs of target enzymes in *E. anophelis* is limited. Although the effect of porin loss and the contribution of efflux pumps on increasing MICs was not studied in the present study, mutations in *GyrA* and *GyrB* but not *ParC* or *ParE* were identified under the selective pressure of fluoroquinolones. Except for one clinical strain with a preexisting Pro134Thr mutation in *ParC*,[7] many studies have described amino acid alterations in QRDRs in clinical *E. anophelis* isolates that occurred only in *GyrA* but not in *GyrB*, *ParC* and *ParE*.[3,6] In addition to *GyrA* mutations, this *in vitro* study indicated numerous amino acid changes in *GyrB* after repeated fluoroquinolone exposure. According to the results of this study, we believe that *GyrB* mutations in clinical *E. anophelis* isolates will soon be encountered if fluoroquinolones are continued to be extensively administered in clinical practice.

Conclusions

Rapid induction of fluoroquinolone-resistant mutants in corresponding to increased MICs was recognized in *E. anophelis*. Of the QRDRs, the most frequent mutation was exhibited in *GyrA*, followed by *GyrB*; and mutations in *ParC* and *ParE* were not identified. Despite the absence of information regarding porin loss and efflux pumps, this study principally demonstrated that the selection of *E. anophelis* mutants occurred fast after fluoroquinolone exposure. Therefore, the strategies for the judicious use of fluoroquinolones, such as administration in combination with other antimicrobials, might be necessary to avoid the rapid emergence of drug resistance and treatment failure in patients infected by *E. anophelis*.

Ethics approval and consent to participate

The study protocol was approved by the Institutional Review Board of E-Da Hospital (EMRP-110-177) in accordance with the tenets of the Declaration of Helsinki and the national standards of Taiwan. The need for informed consent was waived by the Institutional Review Board of E-Da Hospital because only a retrospective analysis of clinical isolates was performed in this study. All designed methods were performed in accordance with the Declaration of Helsinki.

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Authors' contributions

JL conceived the study idea and designed the study. CHL and YH provided the data collection and chart reviews. JL and CY supervised the data collection. CHL provided the data of microbiologic analyses. JL provided methodological and statistical advice on study design and data analysis. CCL and JL provided expertise in infectious disease. CCL drafted this manuscript and prepared Fig. 1. JL revised it carefully from a professional point of view. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding authors upon reasoned request.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.05.011.