# Isolation of Ciprofloxacin Resistant *Elizabethkingia anopheles*

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## Abstract

*Elizabethkingia anophelis* is an extensively antibiotic resistant emerging pathogen that causes mortality in the most vulnerable populations. Because of it's high antibiotic resistance, there are very effective treatments for infections. The fluouroquinolone ciprofloxacin is an example of such treatment, which makes finding the cause of fluoroquinolone resistance in these organism of the upmost importance. Previous work in Gram-negative organisms, including *E. anophelis*, has shown that mutations in *gyrA* confers ciprofloxacin resistance. I hypothesized that laboratory-selected ciprofloxacin resistance in *E. anophelis* will result from mutation(s) in *gyrA* as well. To test this hypothesis, I isolated and sequenced five ciprofloxacin-resistant mutants of *E, anophelis*. All five strains exhibited increased ciprofloxacin minimal inhibitory concentrations (MICs) (8-16 mg/L) when compared to the parent strain (0.25 mg/L). After a population analysis, I found that each of the mutant strains had subpopulations the survived well beyond the normal MIC. Through DNA sequencing, I found eight unique and three shared mutations in *gyrA,* and no mutations in *gyrB, parC,* or *parE*. This demonstrated that even after a single exposure to ciprofloxacin, *E. anophelis gyrA* mutants emerged that were resistant to this drug.

## Introduction

*Elizabethkingia anophelis* was first isolated from the midgut of the mosquito, *Anopheles gambiae* in 2011[1]. This bacterium is ubiquitous in nature and has been found

in multiple countries and in multiple species[1-6]. *E. anophelis* mainly infects the immunocompromised, such as elderly or neonatal patients. The severity of this bacteria comes from it's noticeable multiple-antibiotic resistance phenotype[4]. *E. anophelis* has shown resistance to ampicillin, cefotaxime, ceftazidime, imipenem, amikacin, gentamicin, kanamycin, strepytomycin, trobromycin, tetracycline, and chloramphenicol.[7] As a result of the multiple-antibiotic resistant phenotype, this bacterium is particularly difficult to treat and can have a mortality rate ranging from 23.5%[8] -52%[9]. The aim of this study is to identify the gene mutation(s) that conferred ciprofloxacin-resistance in *E. anophelis.* Studying ciprofloxacin resistance in *E. anophelis* in the laboratory will provide insight into fluoroquinolone resistance in this newly emerging Gram-negative pathogen. Presently, ciprofloxacin resistance is very predominant in bacteria isolated from both hospitals[10] and healthy humans[11]. Understanding the mechanisms of antibiotic resistance in bacteria should allow us to find better therapeutic options for infections caused by resistant organisms.

*Elizabethkingia* spp.

*Elizabethkingia* spp. are genetically diverse and gaining important traction in the research community. *Elizabethkingia* was initially classified as *Flavobacterium*[12]. Because of how broadly defined this genus was, it was reclassified into *Chryseobacterium gen. nov* in 1994[13]. In 2005, *C. meningosepticum* and *C. miricola* were found to be genetically heterogeneous from other *Chryseobacterium* species through 16S rRNA sequencing. Therefore, after combined phylogenetic and phenotypic anakyses, these two species were transferred to two new species within *Elizavbethkingia*: *E. meningoseptica* and *E. miricola*, respectively[14].· *E. anophelis*[1] was added to the

species in 2011, and *E. endophytica* was later identified to be a sub-species of *E. anophelis* in 2016[15]. In 2017, Nicholson *et al.* proposed 3 more species, *E. bruuniana, E. occulta,* and *E. ursingii* [16].

Ciprofloxacin

Fluoroquinolones are one of the most used antibiotics on the market, mainly because of their broad spectrum of activity and excellent pharmacokinetic properties[17]. The parent compound of fluoroquinolones is nalidixic acid[18], which was first discovered in 1962 and shows antibacterial activity in a variety of disease-causing microorganisms[19]. Quinolone resistance develops by either a mutation in the genes encoding DNA gyrase and topoisomerase IV resulting in alterations of the drug's target enzyme, or decreasing the concentration of the target within the cell[18]. The mutations of DNA gyrase are generally found in *gyrA* and *gyrB,* and the mutations of topoisomerase IV are generally found in *parC,* and *parE*[20]. Gram-negative resistance first occurs as an amino acid substitution in the quinolone-resistance-determining region (QRDR) of *gyrA*[21]. The QRDR of *Escherichia coli* occurs between positions 51 and 106, with specific mutations occurring at position 83 and 87 causing the greatest resistance[22]. Mutations in genes controlling efflux pumps have also been shown to help reduce the internal concentration of quinolones[23]. Chakrabarty *et al.* showed that in 18 of their Enterobacteriaceae isolates, the presence of *acrA, acrB,* and *tolC* (genes that encode for the AcrAB-TolC efflux pump complex) were associated with increased fluoroquinolone resistance[24]. Additionally, plasmid-mediated quinolone resistance has begun to show increasing prevalence among [Make Italics] *Enterobacteriaceae*[25]. *qnr*, the plasmid-mediated resistance gene in *E. coli*, produces a protein that protect DNA gyrase from the

quinolones[25]. Qnr protects DNA gyrase by reversing the gyrase-mediated DNA supercoiling caused by the quinolone, reducing gyrase binding to DNA, or by binding to gyrase directly to inhibit the gyrase-DNA interaction. Currently, *Elizabethkingia* has not been shown to exhibit plasmid-mediated resistance, but the genome of this organism does encode a number of multi-drug efflux pumps[26].

Ciprofloxacin is a second-generation fluoroquinolone that targets bacterial type II topoisomerases, enzymes which are essential during DNA replication[27]. Ciprofloxacin is effective against both Gram-negative and Gram-positive bacteria, and is used extensively in human and veterinary medicine[27]**.** Unfortunately, ciprofloxacin resistance is increasing in a multitude of medical cases, such as urinary tract infections[28] and *E. coli* infections[29]. In an *in vitro* study, Ghafur *et al.* (2013) found 29 *E. meningoseptica* isolates which were resistant to ciprofloxacin. These isolates were found in patients in a tertiary care oncology and stem cell transplant center that had received combination therapy with two or more antibiotics (cotrimoxazole, rifampicin, piperaillin-tazobactam, tigecycline, or cefepime-tazobactam). There was a 17% mortality rate among these patients[30]. Like other quinolones, conferred ciprofloxacin resistance is most likely attributed to mutations in *gryA, gyrB, parC,* and *parE.* Jorgensen *et al.* (2013 found nucleotide mutations in *gyrA* (T83I and D87Y) and *gyrB* (S465Y) in *Pseudomonas aeruginosa* strains with conferred ciprofloxacin resistance [31]. Dahiya *et al.* found mutations in *gyrA* gene (S83F and S83Y) and *parC* (S80I) in *Salmonella enterica* serovar Typhi ciprofloxacin-resistant mutants[32]. This demonstrates that ciprofloxacin resistance is prevalent in an array of different Gram-negative bacteria, and that these mutations cluster around amino acid 83 in *gyrA*.

*E. anophelis* + Ciprofloxacin

*Elizabethkingia* spp. have commonly been treated with ciprofloxacin worldwide, with varying mortality rates (Table 1). In a recent *E. anophelis* outbreak in Wisconsin, Perrin *et al.* (2017) reported a low prevalence (9%) of ciprofloxacin resistance by Kirby-Bauer assay[8]**.** This contrasts Lin *et al* (2017)*.,* who reported a much higher prevalence of resistance (78%) among strains isolated in Taiwan[2]. The results from these studies demonstrate that *E. anophelis* has the ability to develop ciprofloxacin resistance. While both of these studies reported mutations in *gyrA*, it is known that other genes are important for the development of ciprofloxacin resistance. However, what other mutations can lead to resistance, and where these mutations are, remains poorly understood.

Therefore, the objective of this project was to obtain ciprofloxacin-resistant isolates of *E. anophelis* and to characterize mutations in regions that are known to be important to ciprofloxacin resistance. Based on the literature, I hypothesized that *E. anophelis* ciprofloxacin resistance is likely to be associated with a mutation at position 83 of DNA gyrase subunit A, in which serine is replaced by isoleucine[2, 8]. This study is of the upmost important because of the increasing prevalence of antibiotic-resistant strains in Gram-negative bacteria[33].

**Table 1.** Cases in which *Elizabethkingia* spp. was treated with ciprofloxacin and the resulting mortality

| Species | Antibiotics used | # died/# treated with ciprofloxacin | Location (Reference) |
|---|---|---|---|
| *E. meningoseptica* | Vancomycin and ciprofloxacin | 0/1 | Brazil [34] |
| *E. meningoseptica* | Ciprofloxacin and Imipenem-cilastatin | 0/1 | Taiwan [35] |
| *E. meningoseptica* | Ciprofloxacin and piperacillin-tazobactam | 0/1 | Saudi Arabia [36] |
| *E. anophelis* | Ciprofloxacin (4), vancomycin and ciprofloxacin (2), ciprofloxavin and trimethoprim/sulfamethoxazole (2), or ciprofloxacin and piperacillin/tazobactam (1) | 2/11 | Wisconsin, USA [37] |
| *E. anophelis* | Ciprofloxacin (2/3) or Ampicillin-sulbactam then ciprofloxacin (1/3) | 0/3 | Hong Kong [5] |
| *E. anophelis* | Penicillin G then cefuroxime and metronidazole then ciprofloxacin | 0/1 | Hong Kong [7] |
| *E. anophelis* | β-lactams (41.8%), β-lactam/Lactamase inhibitors (23.9%), levofloxacin (34.4%), ciprofloxacin (13.4%), carbapenems (16.4%), aminoglycosides (9%), tigecycline (9%), vancomycin (9%), and colistin (3%) | (19/67 total morbidity)[a] | Taiwan [38] |
| *E. miricola* | Chloramphenicol and prednisolone, then ciprofloxacin | 0/1 | United Kingdom [39] |
| *E. miricola* | Benzodiazepines, then imipenem-cilastatin and fluconazole, then ciprofloxacin with imipenem/cilastin, then ciprofloxacin with piperacillin/tazobactam | 0/1 | Italy [40] |
| *E. miricola* | Peicillin and dicloxacillin, then cefuroxime and ciprofloxacin, then piperacillin and tazobactam, then dicloxacillin and ciprofloxacin | 0/1 | Denmark [41] |
| *C. meningosepticum* | Ciprofloxacin with no response after 6-7 days, switched to vancomycin and rifainpin | 1/4 | Turkey [42] |

[a] Mortality rate amongst cases treated with ciprofloxacin was not specified

**Methods**

Working stocks and growth conditions

Working stocks were maintained on heart infusion agar (HIA; Remel, Lenexa, KS) supplemented with 5% defibrinated rabbit blood (Hemostat, Dixon, CA). Overnight cultures were grown by inoculating 3 mL of Mueller Hinton broth (MHB; Becton Dickinson & Co, Cockeysville, MD) with a single isolated colony and incubated overnight with 200 rpm shaking at 37°C.

Minimum inhibitory and bactericidal concentration assays

Minimum inhibitory concentrations (MICs) and the minimal bactericidal concentrations (MBCs) were determined as previously described[26] using the broth microdilution procedure following standard CLSI[43] guidelines. Overnights were diluted to an optical density at 600nm ($OD_{600nm}$) of 0.010 using MHB and 1 mL was added to 13 mm X 100 mm screw top tubes containing two-fold serial dilutions of ciprofloxacin to yield final concentrations between 0.125 μg/ml and 128 μg/ml and incubated overnight at 37°C. MICs were recorded as the lowest concentration were no growth was observed. MBC was determined by plating 100 μl from each tube starting with the highest concentration of observable growth onto Mueller-Hinton agar (MHA) and incubated overnight at 37°C. The MBC was defined as the concentration of ciprofloxacin where no colonies were observed after incubation.

Selection of Ciprofloxacin Mutants

Mutants displaying reduced ciprofloxacin resistance were derived from *E. anophelis* OSUVM 1 by single step selection[26]. Briefly, overnight cultures were serially-diluted ten-fold in phosphate buffered saline and 100 μl of each dilution were spread onto MHA

plates containing 0.5-2 X the observed MIC of ciprofloxacin and incubated for 48 hr at 37°C. Colonies were counted and 5 isolated colonies were selected and passaged three times on drug-free MHA. The MICs for each colony was then determined as described above and DNA was extracted from isolates demonstrating an increased ciprofloxacin MICs. DNA was isolated from 3 ml heart infusion broth (HIB) cultures of each isolate using Qiagen Genomic-tip 100/G columns (Qiagen, Hilden, Germany) following the manufacturers protocol.

Sequencing

*gyrA, gyrB, parC,* and *parE* genes were amplified using PCR and primers described in Table 2 and sequenced at the Oklahoma State University Core Facility using an ABI biosystems 3730 DNA sequencer following manufacturers recommendations. Resulting sequences were aligned to the OSUVM-1 parent strain wildtype *gyrA, gyrB, parC,* and *parE* genes, using the BLAST-N and BLAST-P algorithms [44, 45].

**Table 2.** Primers for Sanger sequencing of targeted genes.

| Primer Name | Target | Sequence |
|---|---|---|
| EAgyrA-F | *gyrA* | CATGAAAGGTAACGCTAAGAACAC |
| EAgyrA-R | | GCTCTTGTACAGAAGGCTCTAAC |
| EAgyrB-F | *gyrB* | GGCCAGTAGTATTCAGTCGTTAG |
| EAgyrB-R | | CCAGAATTTCTTCCGGTCTTCT |
| EAparC-F | *parC* | ACCGGACAGACAGAACTTTATTC |
| EAparC-R | | GTTGTGCCGCTTCTTGTTTG |
| EAparE-F | *parE* | TTTCCTGCTGAGCCAACATAG |
| EAparE-R | | TCCAGAAACCAGTCCTGATAAAG |
| EAgyrACnf-F | *gyrA* | CGTCATTCTGCAAACCCTC |
| EAgyrACnf-R | | TCATTTCTCCACCAGCATAATC |
| EAgyrBCnf-F | *gyrB* | ATCGTTATTATGACCGATGCC |
| EAgyrBCnf-R | | ACGCATCGCTACTTCTACC |
| EAparCCnf-F | *parC* | AAACCAAGTGCCCGTATCC |
| EAparCCnf-R | | AAACACCTTCCTCCTTCATTTC |
| EAparECnf-F | *parE* | ATCCTTTACCCAATCATTCACC |
| EAparECnf-R | | GCCCATTTCGTTACTTCCC |

Population Analysis

Overnights were serial diluted ten-fold in HIB and plated onto HIA infused with ciprofloxacin concentrations ranging from 0.25 μg/ml to 20 μg/ml. The plates were incubated overnight at 37°C and the number of colonies were counted.

**Results**

Selection of Ciprofloxacin Mutants

Colonies were successfully isolated from all concentrations tested (Table 3). The five colonies selected for analysis demonstrated MIC's that ranged from 8-16 mg/L. Mutation frequencies ranged from $1.25 \times 10^{-7}$ to $9.04 \times 10^{-8}$ (Table 3).

**Table 3.** Ciprofloxacin MICs

| Strain | Ciprofloxacin Selection Concentrations (mg/L) | Number of Colonies Observed | Mutation Rate | MIC (mg/L) |
|---|---|---|---|---|
| OSUVM-1 | - | - | - | 0.25 |
| OSUVM-CRS1 | 4 | 2 | $6.25 \times 10^{-8}$ | 8 |
| OSUVM-CRS2 | 4 | 4 | $1.25 \times 10^{-7}$ | 8 |
| OSUVM-CRS3 | 5 | 3 | $9.04 \times 10^{-8}$ | 8 |
| OSUVM-CRS4 | 6 | 5 | $1.56 \times 10^{-7}$ | 16 |
| OSUVM-CRS5 | 7 | 1 | $3.13 \times 10^{-7}$ | 16 |

Sequencing

There were eight unique mutations, and three shared mutations (Table 4). All of the mutations were observed in the *gyrA* gene. OSUVM-CRS1 and OSUVM-CRS2 shared an amino acid mutation at K297R and V301L. OSUVM-CRS3 and OSUVM-CRS4 shared an amino acid mutation at V158E. OSUVM-CRS3 and OSUVM-CRS5 had a shared amino acid mutation at R162K (Table 3). OSUVM-CRS1 had a unique amino acid

mutation at I301M and E304D. OSUVM-CRS3 had a unique amino acid mutation at Q146L (Table 4).

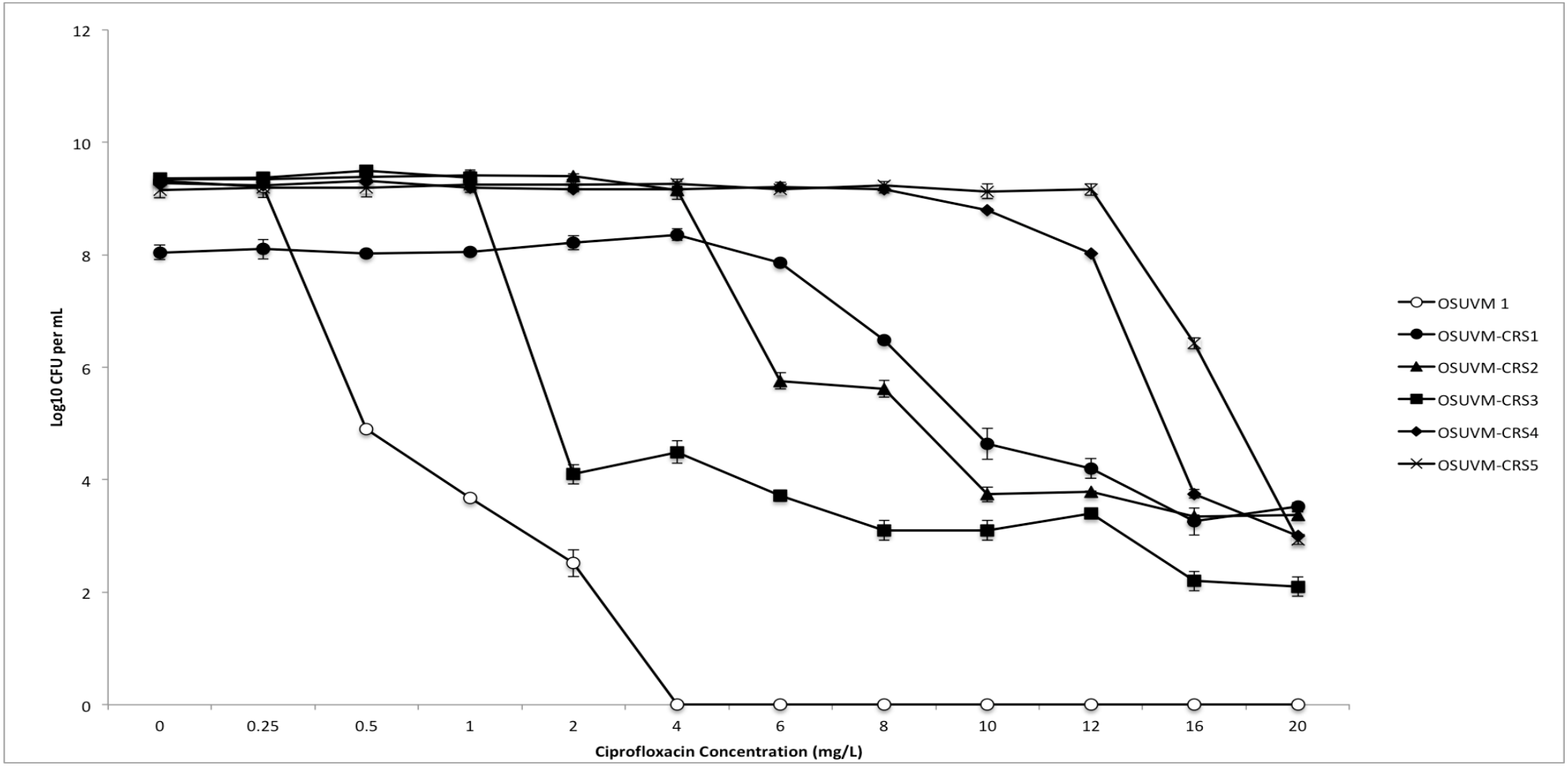
**Table 4**. Nucleotide and amino acid mutations from reduced susceptibility mutants.

| Isolate | Gene | Nucleotide Mutations | Amino Acid Mutations |
|---|---|---|---|
| OSUVM-CRS1 | *gyrA* | A890G; T903G; G904C; A912C | K297R; I301M; V302L; E304D |
| OSUVM-CRS2 | *gyrA* | A890G; G904C | K297R; V302L |
| OSUVM-CRS3 | *gyrA* | G485A; T473A; A437T | R162K; V158E; Q146L |
| OSUVM-CRS4 | *gyrA* | T473A | V158E |
| OSUVM-CRS5 | *gyrA* | G485A | R162K |

Population Analysis

OSUVM 1 had a large drop in population at 0.25 mg/L, whereas the five mutants showed decreases at higher concentrations (Figure 1). OSUVM-CRS1 did not have a clear break point, but started decreasing at 4 mg/L. OSUVM-CRS2 had a break point at 4 mg/L. OSUVM-CRS3 had a break point at 1 mg/L. OSUMV-CRS4 had a break point at 12 mg/L. OSUVM-CRS5 had a break point at 12 mg/L. OSUVM-CRS5 did not demonstrate a notable decrease in survival until ~12 mg/L. All of the mutant strains showed survival even at 20 mg/L, while no surviving colonies were detecting for the parent strain above 4 mg/L (Figure 1).

**Figure 1.** Ciprofloxacin resistance population analysis. OSUVM1 is our parent strain of *E. anophelis*. OSUVM-CRS1-5 are our ciprofloxacin-resistant mutants. We exposed each strain to varying ciprofloxacin concentrations, ranging from 0-20 mg/L. We measured colony forming units (CFU) per mL.



## Discussion

Selection of Ciprofloxacin Mutants

I successfully selected, isolated, and characterized mutants of *E. anophelis* with reduced susceptibility to ciprofloxacin (Table 3). The MIC of these mutants were increased when compared to the parent strain. OSUVM-CRS1, OSUVM-CRS2, and OSUVM-CRS3 had MICs of 8 mg/L, a 32-fold increase from the parent strain. OSUVM-CRS4 and OSUVM-CRS5 had MICs of 16 mg/L, a 64-fold increase from the parent strain. Han *et al.* (2017) found ciprofloxacin MICs ranging from 1 to >64 by agar dilution in their 51 *E. anophelis* isolated. They found that 22% of the isolates were susceptible, 6% had intermediate susceptibility, and 72% were resistant to ciprofloxacin as interpreted by the CLSI non-*Enterobactericiae* standards[46]. Comparatively, Lin *et al*. (2018) found that their 67 *E. anophelis* isolates showed ciprofloxacin MICs between <1 mg/L and >32 mg/L. Four point five % of the isolates were susceptible, 50.7% had intermediate susceptibility, and 44.8% were resistant to ciprofloxacin by microtiter interpreted according to the non-*Enterobactericiae* breakpoints established by the CLSI. This is a fairly large frequency, especially since only 13.4% of the patients were empirically treated with ciprofloxacin [38]. Both of these reports compare with my recorded MIC values for my ciprofloxacin-resistant mutants. While Perrin *et al.* reported high rates of susceptibility (89.7% susceptible, 6.9% intermediate susceptibility, and 3.4% resistant), they used the Kirby Bauer disk diffusion method interpreted using EUCAST breakpoints for *Pseudomonas spp.*, and therefore direct comparison is not acceptable. I only found a few ciprofloxacin-resistant mutants, as expected given the low mutation rates ranging from $1.25 \times 10^{-7}$ to $9.04 \times 10^{-8}$ (Table 3). However, a single step selection procedure was used to select for

mutants. This demonstrates that even a single exposure to ciprofloxacin can select for mutants demonstrating elevated resistance to ciprofloxacin, albeit at a low frequency.

Sequencing

Mutations in *gyrA* were found to cause alteration in GyrA at amino acid residues 146, 158, 162, 297, 301, 302, and 304 (Table 4). Contrary to previous reports[2, 8, 38], I found no mutation at amino acid position 83. This shows that a mutation at this position is common but not necessary for conferred ciprofloxacin resistance. No mutations were found in *gyrB, parC,* or *parE.* The lack of mutations in these genes is consistent with all previously reported sequencing of ciprofloxacin-resistant mutants[2, 8, 38].

To date, there is no known characterized QRDR for *E. anophelis*. The QRDR of *E. coli* is between positions 51 and 106 of *gyrA*[47]. Additionally, mutations that also confer ciprofloxacin resistance in *E. coli* are commonly found in amino acid positions 426 and 447 of GyrB, 78, 80, and 84 of ParC, or 445 of ParE[47]. Some of the mutations are close to the known QRDR of *E. coli* (positions 146, 158, and 162) but some are not. Mutations at amino acid position 297, 301, and 304 were not close to the known QRDR.

Population Analysis

After a single exposure to ciprofloxacin, I found subpopulations of both the parent strain and the mutant strains that survived past the recorded MICs (Fig. 1). OSUVM-CRS5 consistently had the largest number of CFU/mL until the final concentration, in which OSUVM-CRS1 had the highest CFU/mL (Fig. 1). This poses concern for treatment, because even after one exposure of ciprofloxacin, resistant subpopulations arose. This can cause complications because ciprofloxacin is commonly used to treat *E. anophelis*

(Table 1). Moreover, Lin *et al.* (2018) showed rapid emergence of resistance in a large number of isolates. Subpopulations of ciprofloxacin-resistant mutants also commonly appear in in both *E. coli*[48] and *P. aeruginosa*[49]. Olofsson *et al.* (2006) found several *E. coli* subpopulations showing varying levels of ciprofloxacin resistance while evaluating mutant prevention concentration of 3 ciprofloxacin reduced susceptibility strains[48]. Similar results were observed by Hansen *et al.* (2006) who exposed 6 strains of *P. aeruginosa* to varying concentrations of ciprofloxacin or levofloxacin, observing that the fraction of colonies recovered compared to the input number of colonies decreased sharply near the MIC, although colonies continued to be recovered at concentrations up to five times the MIC[50]. Furthermore, mutants displaying elevated ciprofloxacin resistance were selected from these plates displaying mutations in *gyrA* only. I observed similar results with both the parent and mutant strains, all of which displayed subpopulations that survived well past the nominal MIC.

**Conclusion and future directions**

I have found that ciprofloxacin-resistance can emerge rapidly in *E. anophelis* after a single exposure to the drug. In contrast to the original hypothesis, no mutations were found that affected amino acid position 83. While a mutation that confers increased ciprofloxacin resistance is frequently found in position 83 of *gyrA*[8, 38], I found that this is not the only position where a mutation can confer increased resistance, raising the possibility that the QRDR for *Elizabethkingia* covers a larger portion of the protein than those in other organisms. Moreover, it seems that mutations are most likely to occur in the *gyrA* gene, as I found no other mutations in *gyrB, parC,* or *parE.* There are some possible confounding variables in this study. Only a small number of isolates were

sequenced, and the selection of those isolates was done *in vitro*. I did not look for mutations that suggested an increase in efflux pump activity. While the OSUMV-1 genome did show efflux pumps, whether those were drug-resistance efflux pumps is still to be determined. Additionally, plasmid-mediated resistance is unlikely because OSUVM-1 did not show any *qnr* genes, nor were any sequences related to known plasmids detected[26]. Whole genome sequencing will be required to identify any additional mutations that are contributing to the multiple-antibiotic resistance phenotype selected by ciprofloxacin exposure. Since *E. anophelis* has rapidly emerging worldwide significance, I hope that these findings help to spark further research into *E. anophelis* antibiotic resistance mechanisms, which hopefully can lead to more effective treatments for infections.

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