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Ciprofloxacin Concentrations 100-Fold Lower than the MIC Can Select for Ciprofloxacin Resistance in *Neisseria subflava*: an *In Vitro* Study

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Abstract: Background: *Neisseria gonorrhoeae* can acquire antimicrobial resistance (AMR) through horizontal gene transfer (HGT) from other *Neisseria* spp. such as commensals like *Neisseria subflava.* The prevalence of *Neisseria subflava* in the oropharynx is close to 100%. Low doses of antimicrobials in food could select for AMR in *N. subflava,* which could then be transferred to *N. gonorrhoeae*. In this study, we aimed to determine the lowest concentration of ciprofloxacin that can induce ciprofloxacin resistance (minimum selection concentration – MSC) in *N. subflava.* **Methods:** *Neisseria subflava* Co000790/2 was serially passaged on GC agar plates containing ciprofloxacin concentrations ranging from 1:100- to 1:10,000-below its ciprofloxacin MIC (0.006 µg/ml) for six days. **Results:** After 6 days of serial passaging at ciprofloxacin concentrations 1/100th of the MIC, 24 colonies emerged on the 0.06 µg/ml ciprofloxacin plate. Their ciprofloxacin MICs were between 0.19 to 0.25 µg/ml, and whole genome sequencing revealed a missense mutation T91I in the *gyrA* gene, which has previously been found to cause reduced susceptibility to fluoroquinolones. **Conclusion:** The *N. subflava* $MSC_{de\ novo}$ was determined to be 0.06 ng/mL or 1:100 below the MIC. The implications of this finding are that the low concentrations of antibiotics found in certain environmental samples and even the food we eat may be able to select for ciprofloxacin resistance in *N. subflava.*

Keywords: minimum selection concentration; MSC; $MSC_{de\ novo}$; ciprofloxacin; *Neisseria subflava;* commensals; antimicrobial resistance

1. Introduction

The threat of antimicrobial resistance (AMR) is compromising the treatment of common infections, including sexually transmitted infections (STIs) such as gonorrhoea [1]. The minimum inhibitory concentration (MIC) is routinely used to measure the lowest concentration of an antibiotic that inhibits the growth of a microorganism. However, the selection of resistant bacteria is not limited to concentrations between the MIC of the susceptible wild-type population and that of the resistant bacteria [2]. The lowest concentration of an antimicrobial that can select for antimicrobial resistance in a particular bacterium is referred to as the minimal selection concentration (MSC) [2, 3].

The MSC encompasses two components. The MSC_{select} denotes the lowest concentration that provides a selection pressure for resistant mutants over susceptible strains, and the $MSC_{de\ novo}$ is defined as the lowest concentration that can induce *de novo* AMR [2, 3]. Gullberg et al. established the ciprofloxacin MSC_{select} and $MSC_{de\ novo}$ for *Escherichia coli* as 0.1 ng/ml and 2.3 ng/ml (1/230th and 1/10th the MIC), respectively. However, they did not assess if ciprofloxacin concentrations below 2.3ng/ml could induce *de novo* resistance [2]. Recent experiments with *Neisseria gonorrhoeae* revealed that ciprofloxacin concentrations of 0.004 ng/ml, or 1/1000th of the MIC could induce *de novo* resistance [4]. Once again lower concentrations were not tested in this study [4]. These MSCs are considerably

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lower than the maximum residue limits of fluoroquinolones allowed in various meat products by the European Medicines Authority and the Food and Agriculture Organization [4, 5].

These MSCs are also orders of magnitude lower than the concentrations of ciprofloxacin detected in samples of milk, eggs, and edible fish in certain East Asian countries (mean concentration: 8.5 μg/L, 16.8 μg/kg and 331.7 μg/kg, respectively) [6–8]. Of further concern is that these MSCs are higher than the ciprofloxacin concentration detected in the faeces of random individuals in three regions of China (median concentration 20 μg/kg) [9]. The ingestion of veterinary antimicrobials in food was thought to be responsible [10–12]. Low concentrations of antimicrobials in the soil and water may also select for AMR which may then be transmitted to humans or other animals. A global survey of pharmaceuticals in the world's rivers found that the concentration of ciprofloxacin exceeded 'safe levels' of 60 ng/L at 64 out of 135 sites [13]. These country-level ciprofloxacin concentrations in rivers were found to be positively associated with the prevalence of fluoroquinolone resistance in *E. coli* [14].

These considerations mean it is important to establish the MSCs of a wider range of bacteria. In the current study, we extend this investigation to Neisseria subflava to assess if ciprofloxacin concentrations as low as 1/10,000th the MIC can select for de novo resistance to ciprofloxacin. We chose *N. subflava,* as it is an important part of our normal oropharyngeal microbiota and can transfer DNA encoding antimicrobial resistance to the pathogenic *Neisseria* species, *N. gonorrhoeae and N. meningitidis* [15–20]. A number of studies have confirmed that this horizontal gene transfer from commensal *Neisseria* spp. has played a crucial role in the emergence of resistance to fluoroquinolones, cephalosporins, dihydrofolate reductase inhibitors and macrolides in *N. gonorrhoeae*/*N. meningitidis* [15–20].

A systematic review of AMR in *Neisseria* spp. found that resistance was typically higher in commensal than pathogenic *Neisseria* spp. [21] This is likely related to the fact that the prevalence of the commensal *Neisseria* spp. is close to 100%, whereas that of the pathogenic *Neisseria* spp. is one or two orders of magnitude lower [22, 23]. This higher prevalence means that the commensal *Neisseria* are exposed to antimicrobial selection pressure every time someone ingests an antimicrobial [23]. Their higher prevalence may also mean that the commensal *Neisseria* are more susceptible to the effects of chronic low-dose exposure to fluoroquinolones such as those in food [22]. This hypothesis is, however, dependent on the concentration of fluoroquinolones in foodstuffs being higher than the MSCs.

In the present study, we determined the *N. subflava* ciprofloxacin $MSC_{de\ novo}$ by passaging *N. subflava* in ciprofloxacin concentrations ranging from 1:100 to 1:10,000 below the MIC for 6 days.

2. Materials & Methods

2.1. Bacterial Strain

We used *N. subflava* Co000790/2, a clinical isolate collected in a previous community study performed at ITM [24]. This strain has a ciprofloxacin MIC of 0.006 μg/ml as ascertained by E-testing in triplicate.

2.2. $MSC_{de\ novo}$ Determination

The $MSC_{de\ novo}$ of *N. subflava* Co000790/2 was ascertained via exposure to a constant concentration of ciprofloxacin at 1:100, 1:1,000 and 1:10,000 of its ciprofloxacin MIC on GC agar plates (Difco GC medium, Becton Dickinson) with 1% isovitalex enrichment (Becton Dickinson) in 5% CO_2 incubator at 36°C. Control experiments were conducted simultaneously using identical conditions, except the GC agar plates did not contain ciprofloxacin. The experiments were conducted in quadruplicate. Every 24 to 48 hours, each lineage was passaged to a new plate with the same conditions by transferring a 1/4th loopful (Copan, 10 μl loop) to the next plate. This process was continued for 6 days.

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On day 7, the number of colonies of each lineage with reduced susceptibility to ciprofloxacin was established as follows: 100 µL of Phosphate Buffered Saline (PBS) solution containing the lawn of colonies (1.0 McFarland) was plated onto 2 GC agar plates with either no ciprofloxacin or 0.06 µg/mL ciprofloxacin (which we used to define resistance to ciprofloxacin according to EUCAST MIC breakpoints for *N. gonorrhoeae* [25]). The number of colonies was counted after 24 hours of incubation at 36°C. The lowest ciprofloxacin concentration with growth in the 0.06 µg/mL plates was defined as the $MSC_{de\ novo}$.

2.3. Characterization of Colonies that Grew on Ciprofloxacin-Containing Plates

The MICs of colonies that grew on the ciprofloxacin plates were ascertained via gradient diffusion strips (Etest™, bioMérieux, France), following EUCAST guidelines. The species identity of these colonies was confirmed via MALDI-TOF (Bruker, USA).

2.4. Mutation Stability Assessment

Two strains (1/100-4.1 and 1/100-4.7) were randomly selected from the plates containing 0.06 µg/mL ciprofloxacin for further experimentation to determine the stability of acquired mutations. Each strain was retrieved from frozen skim milk stored at -80°C, replated on GC agar + 1% IV, and subcultured every 24 hours for 6 days. Finally, the cultures were subjected to E-testing following EUCAST guidelines.

2.5. Whole Genome Sequencing

Five isolates (1/100-4.1, 1/100-4.7, 1/100-4.14, 1/100-4.21 and 1/100-4.24) and one isolate from day 5 of the control experiment exposed to no ciprofloxacin were outsourced for DNA isolation, library preparation and whole genome sequencing (WGS) to Eurofins, Germany. Post DNA extraction, libraries were prepared using the TruSeq DNA library kit (Illumina Inc., San Diego, CA, USA), and multiplexing was performed using the Nextera DNA library kit (Illumina Inc., San Diego, CA, USA). Sequencing was carried out on NextSeq6000 v2 platform, generating 2×150 bp reads. Quality assessment of the raw reads was done using FASTQC [26]. The raw reads were then trimmed for quality (Phred ≥ 30) and length (≥32 bases) using Trimmomatic (v0.39) [27]. The processed reads were assembled with Shovill (v1.0.4) [28], which uses SPAdes for the *de novo* assembly (v3.14.0) [29] using the following parameters: —trim —depth 150 —opts —isolate. The quality of the assembled *de novo* contigs was evaluated using Quast (v5.0.2) [30]. Genome annotation of the draft genome was carried out using Prokka (v1.14.6) [31]. The quality-controlled reads were mapped to the reference draft genome (Ns_Ctrl) using Snippy (https://github.com/tseemann/snippy). Single nucleotide polymorphisms (SNPs) were determined using default parameters. The raw reads are deposited at PRJNA1107029

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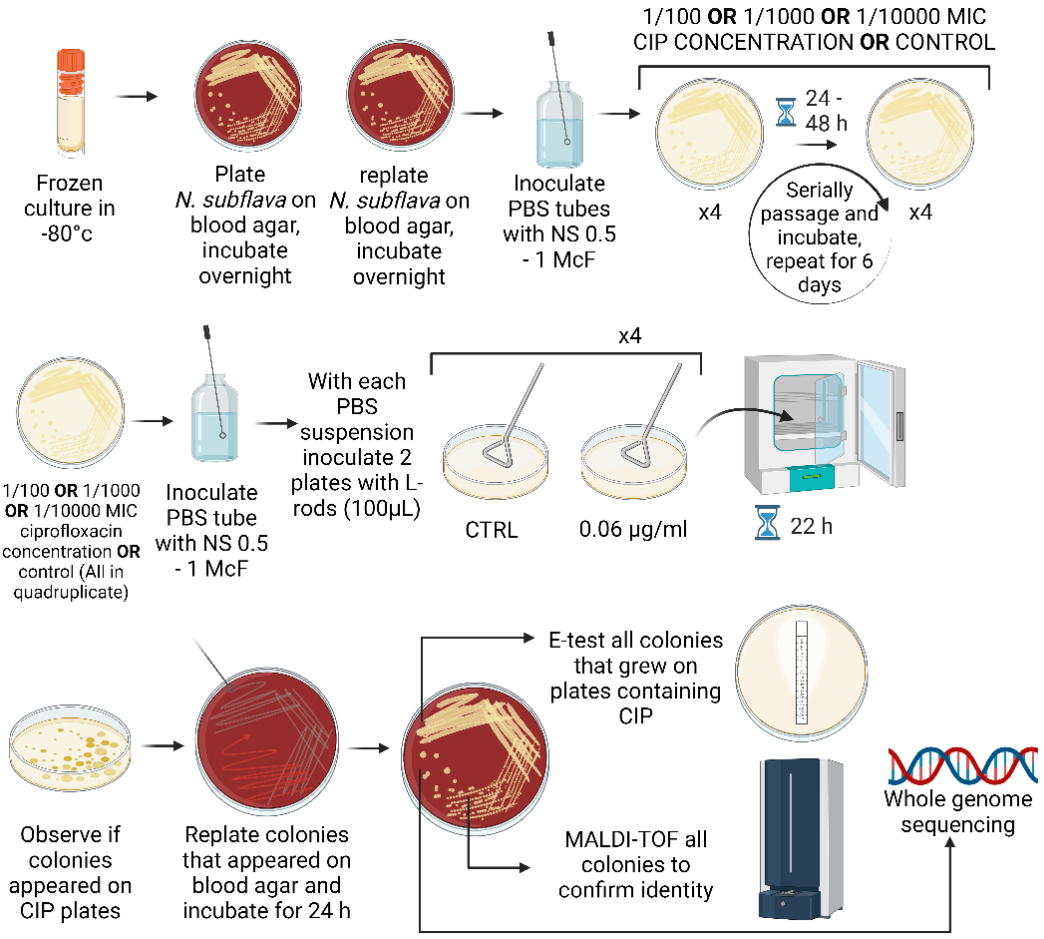

Figure 1. Overview of the study methodology.

3. Results

3.1. Minimal Selective Concentration

3.1.1. *N. subflava*

After 6 days of serial passaging at ciprofloxacin concentrations 1/100th of the MIC, equivalent to 0,06 ng/ml, 24 colonies emerged after 22 hours of incubation on a single 0.06 µg/ml ciprofloxacin plate (Plate 1/100-4; Table 1). MALDI-TOF MS analysis verified that these colonies were *N. subflava*.

E-testing of these colonies revealed a MIC of 0.19 to 0.25 µg/ml for all the colonies, which represents a minimal 31-fold increase in ciprofloxacin MIC. No colonies with resistance (0.06 µg/ml) were observed on the control or other plates passaged at 1/100, 1/1,000, and 1/10,000 of the ciprofloxacin MIC.

Table 1. Minimum inhibitory concentrations of all resistant colonies and subsequent MALDI-TOF results.

Colony	Ciprofloxacin MIC (µg/mL)	MALDI-TOF-MS ID	MALDI-TOF score	Whole genome sequencing
1/100-4.1	0.19	*N. flavescens subflava group*	2.16	✓
1/100-4.2	0.19	*N. flavescens subflava group*	2.17	x
1/100-4.3	0.19	*N. flavescens subflava group*	2.26	x
1/100-4.4	0.19	*N. flavescens subflava group*	2.17	x
1/100-4.5	0.25	*N. flavescens subflava group*	2.21	x
1/100-4.6	0.19	*N. flavescens subflava group*	2.06	x

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1/100-4.7	0.25	*N. flavescens subflava group*	2.01	✓
1/100-4.8	0.19	*N. flavescens subflava group*	2.12	x
1/100-4.9	0.19	*N. flavescens subflava group*	2.27	x
1/100-4.10	0.19	*N. flavescens subflava group*	2.3	x
1/100-4.11	0.19	*N. flavescens subflava group*	2.28	x
1/100-4.12	0.19	*N. flavescens subflava group*	2.19	x
1/100-4.13	0.19	*N. flavescens subflava group*	2.27	x
1/100-4.14	0.19	*N. flavescens subflava group*	2.25	✓
1/100-4.15	0.25	*N. flavescens subflava group*	2.07	x
1/100-4.16	0.25	*N. flavescens subflava group*	2.05	x
1/100-4.17	0.25	*N. flavescens subflava group*	2.11	x
1/100-4.18	0.25	*N. flavescens subflava group*	2.28	x
1/100-4.19	0.19	*N. flavescens subflava group*	2.28	x
1/100-4.20	0.25	*N. flavescens subflava group*	2.32	x
1/100-4.21	0.19	*N. flavescens subflava group*	2.31	✓
1/100-4.22	0.19	*N. flavescens subflava group*	2.25	x
1/100-4.23	0.19	*N. flavescens subflava group*	2.13	x
1/100-4.24	0.25	*N. flavescens subflava group*	2.27	✓

3.1.2. Mutations in Fluoroquinolone Target Gene (gyrA)

WGS analysis of 5 randomly selected isolates that grew on the ciprofloxacin plate, with a MIC ranging from 0.19 to 0.25 μg/ml, revealed a missense mutation T91I in the *gyrA* gene, the known resistant associated mutation. Additionally, all four isolates had the missense mutation A385V in the *spoT* gene, which encodes the bifunctional (p)ppGpp synthase/hydrolase), and a synonymous mutation T828C (A276) in the *nnr* gene, which encodes a bifunctional NAD(P)H-hydrate repair enzyme).

3.2. Mutation Stability

Cross-plating of two strains (1/100-4.1 and 1/100-4.7) on GC agar + 1% IV was performed every 24 hours for 6 days. E-testing at day 6 revealed an unchanged ciprofloxacin MIC for 1/100-4.7 and a slightly higher MIC for 1/100-4.1 – from 0.19 μg/mL to 0.25μg/mL.

4. Discussion

Exposure to low ciprofloxacin concentrations (0.06 ng/ml) that were 100-fold lower than the MIC for six days resulted in the emergence of fluoroquinolone resistance in *N. subflava*. This resistance was associated with the T91I substitution in GyrA. This mutation has been shown to be associated with an intermediate fluoroquinolone resistance phenotype in *N. meningitidis* [32]. Using similar methodologies, Gonzalez et al. found that exposure to lower ciprofloxacin concentrations (0.004ng/ml) or 1000-fold lower than the MIC could induce *de novo* ciprofloxacin resistance in *N. gonorrhoeae* [4]. In contrast, Gullberg et al. found that the ciprofloxacin $MSC_{de\ novo}$ in *E. coli* was higher (2.3ng/ml), although lower concentrations were not tested[2]. These findings suggest that concentrations of ciprofloxacin as low as 0.004ng/ml can select for ciprofloxacin resistance.

This finding suggests the need to reconsider the definition of 'safe' concentrations of fluoroquinolones in environmental and food samples. For example, in their global survey of the world's rivers, Wilkinson et al. found alarming levels of pharmaceutical pollution [13]. One of their concerning findings was that the concentration of ciprofloxacin exceeded 'safe' levels of 0.06 ng/ml at 64 sites. This threshold of 0.06 ng/ml was determined by Bengtsson-Palme et al. by ascertaining what the lowest 1% minimum inhibitory

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concentration (MIC) was for a range of bacteria with available susceptibility data in the EUCAST dataset [33]. To adjust for the fact that the MSC may be an order of magnitude lower than the MIC, Bengtsson-Palme et al. set the safe concentration of ciprofloxacin at 10-fold lower than the lowest 1% MIC. The MSCs of *Neisseria* spp. are, however, 100- to 1000-fold lower than their MICs. Applying a 10-fold safety factor to these MSCs, would mean that the safe concentrations of ciprofloxacin could not 10-fold, but up to 10,000-fold lower than the lowest 1% MIC. While this hypothesis will require experimental validation, it does suggest that measured concentrations of ciprofloxacin in a much larger proportion of the world's rivers may be selecting for AMR.

We have only considered the ciprofloxacin MSC_{denovo} of a single strain of *N. subflava* in a very simple *in vitro* model. All the resistant isolates emerged on a single agar plate. The in vitro MSC_{select} is typically lower than the MSC_{denovo} [2]. MSCs will likely be different in complex environmental and microbial matrices. For example, MSCs may be lower in polymicrobial communities [34]. On the other hand, the presence of other compounds, such as heavy metals and selective serotonin receptor inhibitors, can reduce the MSC [35]. Our experiment only ran for 6-days. We cannot exclude the possibility that longer exposures may have resulted in a lower ciprofloxacin MSC.

These limitations mean that further experiments are required to determine MSCs in complex environments such as in vivo. Only a single study has assessed the MSC in vivo. This study found that single doses of the lowest dose of ciprofloxacin concentration tested (0.6ng/g) could induce ciprofloxacin resistance in *Klebsiella pneumoniae* [36]. This finding is concerning as this concentration was 10-fold lower than the ciprofloxacin food concentration classified as safe by the Food and Agriculture Organization [36]. As reviewed in the introduction, this concentration is also considerably lower than that of fluoroquinolones detected in food stuffs in various countries [6–13].

A recent study estimated that AMR infections are responsible for between 1 and 5 million deaths per year [37]. Combating AMR requires a one-health approach whereby all antimicrobial exposures are kept within safe thresholds [38]. This study contributes to a growing body of evidence that suggests that ciprofloxacin concentrations below those classified as safe in food and environmental samples may select for AMR.

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