Evaluation of Quinolone Resistance Determining Region Mutations and Efflux Pump Expression in *Neisseria meningitidis* Resistant to Fluoroquinolones

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AMENDED ABSTRACT

Objectives: To evaluate additional fluoroquinolone resistance (R) mechanisms in *N. meningitidis* (NMEN) strains carrying a GyrA T91I alteration and displaying elevated ciprofloxacin (CIP) MIC values (0.06 and 0.25 mg/L). CIP-R in NMEN is rare in North America, and to date only three isolates with this phenotype have been described in the United States (USA).

Methods: Serotype B NMEN strains (3; courtesy of Dr. Henry M. Wu, CDC-USA) collected from USA hospitals (North Dakota [1 strain] and Minnesota [2 strains]) displaying elevated CIP MICs and *gyrA* mutation (T91I) were susceptibility tested by CLSI (M07-A8, 2009) reference broth microdilution method. Quinolone R determining region (QRDR) sequencing analysis of *gyrA*, *gyrB*, *parC* and *parE* was performed. Mapping of the *mtrCDE* efflux system was carried out using primers anchoring in the components of the pump, coverage included intergenic regions. mRNA expression for pump components was evaluated by quantitative reverse-transcriptase real-time PCR (qRT-PCR) and comparing to NMEN ATCC 13102 control.

Results: Two strains showed CIP and levofloxacin MIC values at 0.25 mg/L and one strain had a CIP MIC at 0.06 mg/L. *parC* mutations causing alterations H141N and P186S were detected in all strains. In addition to T91I, the two strains displaying higher MIC values also possessed a T173A alteration on GyrA. All components of the efflux pump *mtrCDE* (also associated to rifampin-R) were intact and had the correct amplicon size, excluding the presence of insertions/deletions and the Correia element within the pump operon. The promoter region (*mrtR*) was fully sequenced and was distinct from the susceptible control. Isolates 7782J and 7783J had identical promoter region, whereas isolate 7784J showing lower ciprofloxacin MIC values possessed a different sequence. Expression experiments showed discrepancies in the mRNA in pump components. The most remarkable difference was for the outer membrane protein (OMP) encoded by *mtrE* that was hyperexpressed (>3600X elevated compared to control) on strain 7784J showing a 0.06 mg/L CIP MIC.

	NMEN strains		
Parameters	7782J	7783J	7784J
MIC (mg/L)			
Ciprofloxacin	0.25	0.25	0.06
Levofloxacin	0.25	0.25	0.06
Rifampin	0.03	0.03	≤0.008
QRDR mutations	*gyrA* T91I, T173A *parC* H141N, P186S	*gyrA* T91I, T173A *parC* H141N, P186S	*gyrA* T91I *parC* H141N, P186S
Expression[a]			
mtrC (fusion protein)	0.7	9.0	0.9
mtrD (transmembrane protein)	0.3	2.6	4.0
mtrE (OMP)	992.6	161.0	3651.9
mtrF (pump component)	57.1	62.5	26.5
mtrR (regulator)	13.6	70.1	89.0

a. Relative expression compared to *N. meningitidis* ATCC 13102.

Conclusions: Our results indicate that T91I alteration on GyrA had an important role in elevated CIP MIC values observed in these NMEN strains. Additional R determinants were present, including other *gyrA* and *parC* QRDR mutations. Alterations in expression of *mtrCED* pump appear to have minimal contribution to CIP-R. This finding was supported by low rifampin susceptibility results (MIC, ≤0.008 to 0.03 mg/L).

INTRODUCTION

Meningococcal strains with reduced susceptibility to ciprofloxacin are relatively rare and the resistance mechanism in these isolates have been mostly associated with point mutations in the quinolone resistance determining regions (QRDR) of the target sites for the fluoroquinolones, and particularly with changes in GyrA subunit of DNA gyrase. Additionally, alterations in the structure and expression of the efflux system *mtr* (multiple transferable resistance) has been reported to increase ciprofloxacin and rifampin MIC values. The finding of a 155-159 bp insertion sequence, named "Correia Element", immediately downstream of the *mtrC* promoter region was found to modulate the expression of this gene and encode to ciprofloxacin and rifampin resistances.

Three ciprofloxacin non-susceptible *Neisseria meningitidis* strains were recovered from patients hospitalized in United States (USA) hospitals from January 2007 to January 2008. These isolates showed elevated ciprofloxacin MIC results and belonged to serotype B and sequence type (ST) 162. In the initial report, all isolates were found to have a T91I alteration on GyrA.

In this extended study, we evaluated mutations in QRDR and the expression and integrity of *mtrCDE* efflux pump system components among three *N. meningitidis* strains collected in USA hospitals displaying elevated ciprofloxacin MIC values.

MATERIALS AND METHODS

Organism collection. Three serotype B *N. meningitidis* strains collected from USA hospitals displaying elevated ciprofloxacin MIC results (0.06-0.25 mg/L) and a T91I aminoacid substitution in the DNA gyrase subunit A, encoded by *gyrA* were evaluated. These isolates were collected in hospitals located in North Dakota (1 strain) and Minnesota (2 strains) and were kindly supplied by Dr. Henry M. Wu (CDC-USA).

Susceptibility testing. Isolates were tested using the Clinical and Laboratory Standards Institute (CLSI) reference broth microdilution (M07-A8, 2009) methodology in cation-adjusted Mueller-Hinton broth (CA-MHB) panels supplemented with 2 to 5% lysed horse blood. Direct colony suspensions from a 20 to 24 hour growth plate (chocolate agar) were used to obtain a 0.5 McFarland standard suspension. After inoculation, panels were incubated at 35ºC in 5% CO_2 for 20 to 24 hours. Antimicrobial agents tested included ciprofloxacin, levofloxacin and rifampin among others.

QRDR sequencing. Oligonucleotides previously described were used to amplify and sequence the hot spot regions on *gyrA*, *gyrB*, *parC* and *parE*. PCR amplicons were sequenced on both strands and the nucleotide sequences and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, Wisconsin, USA). Sequences were compared to others available via internet sources (http://www.ncbi.nlm.nih.gov/blast/).

Mapping of the *mtrCDE* efflux system. Custom oligonucleotides were designed to amplify *mtrC*, *mtrD*, *mtrE*, *mtrF* and *mtrR* and intergenic regions. The promoter region between the regulator gene (*mtrR*) and *mtrC* was sequenced and analysis was performed as described above.

Expression of *mtrCDE* components. DNA-free RNA preparations were tested using quantitative real-time PCR (qRT-PCR). Relative quantification of target gene expression was performed in duplicate by normalization to an endogenous reference (*ctrA*). The critical threshold cycle (C_T) numbers were determined by the detection system software as the amount of target was given as $2^{-\Delta\Delta T}$, where ΔΔT is the difference between the target and reference gene C_T values. *N. meningitidis* ATCC 13102 was used as a control for the expression experiments.

RESULTS

- Among the three *N. meningitidis* strains tested, two displayed ciprofloxacin MIC values of 0.25 mg/L (7782J and 7783J) and one (7784J) had a MIC of 0.06 mg/L (Table 1).
- All three isolates were very susceptible to other compounds tested, including rifampin (MIC, ≤0.008 to 0.03 mg/L; Table 1).
- The alteration T91I was confirmed in all three strains. A secondary GyrA alteration (T173A) was detected in the two strains displaying ciprofloxacin MIC values of 0.25 mg/L. This alteration was not detected in the strain showing ciprofloxacin MIC of 0.06 mg/L (Table 1).
- No *gyrB* or *parE* mutations were found in any of the three strains; however, *parC* mutations causing alterations H141N and P186S were detected in all strains (Table 1).
- Mapping of the efflux pump components demonstrated that they corresponded to their expected sizes and the "Correia Element" (insertion of 155-159 bp) was not detected within the *mtrC* promoter region nor other pump components.
- The promoter region (*mrtR*) was sequenced and was distinct from the susceptible control for all three strains. Isolates 7782J and 7783J had identical promoter region, whereas isolate 7784J showing lower ciprofloxacin MIC values possessed a different sequence, showing greater homology to the reference susceptible strain sequence (Figure 1).
- Relative expression ratios showed discrepancies in the mRNA in pump components when compared to the expression of these genes in the ATCC 13102 strain; however, none of the values correlated to the observed MIC differences.
- The *mtrE* encoding the outer membrane protein (OMP) component of the pump system was hyperexpressed on strain 7784J displaying ciprofloxacin MIC of 0.06 mg/L (>3600X elevated compared to control).

Figure 1. Alignment of the promoter region between *mtrC* and *mtrR* for three ciprofloxacin non-susceptible *N. meningitidis* strains. Differences in sequences are highlighted in red and the sequences distinct from the control are also underlined.

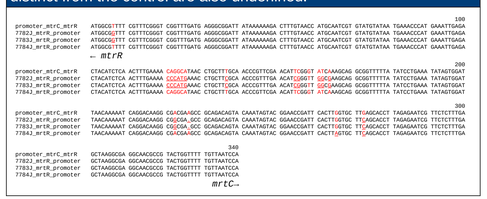

Table 1. Antimicrobial susceptibility results and QRDR mutation profiles of ciprofloxacin non-susceptible *N. meningitidis* strains.

	MIC (mg/L):[a]									QRDR alterations			
Strain	CIP	LEV	RIF	PEN	CTX	ERY	CLI	T/S	GEN	GyrA	GyrB	ParC	ParE
7782J	0.25	0.25	0.03	≤0.06	≤0.015	0.25	4	≤0.5	2	T91I, T173A	negative	N141H, P186S	negative
7783J	0.25	0.25	0.03	≤0.06	≤0.015	0.25	8	≤0.5	4	T91I, T173A	negative	N141H, P186S	negative
7784J	0.06	0.12	≤0.008	≤0.06	≤0.015	≤0.06	1	≤0.5	1	T91I	negative	N141H, P186S	negative
ATCC 13102	≤0.03	≤0.05	0.012	≤0.06	≤0.06	≤0.25	1	≤0.5	1	negative	negative	N141H, P186S	negative

a. CIP= ciprofloxacin, LEV= levofloxacin, RIF= rifampicin, PEN= penicillin, CTX= ceftriaxone, ERY= erythromycin, CLI= clindamycin, T/S= trimethoprim/sulfamethoxazole, GEN= gentamicin

Table 2. Expression results for the components of efflux pump *mtrCDE* for three clinical ciprofloxacin non-susceptible *N. meningitidis* strains and the ATCC 13102 used as control for the experiments.

		mtrC (membrane fusion protein)		*mtrD* (transmembrane protein)		*mtrE* (outer membrane protein)		*mtrF* (efflux pump component)		*mtrR* (transcriptional regulator)	
Strain	CIP[a] MIC (mg/L)	Exp[b]	Rexp[c]	Exp[b]	Rexp[c]	Exp[b]	Rexp[c]	Exp[b]	Rexp[c]	Exp[b]	Rexp[c]
7782J	0.25	9.28E-03	0.68	2.82E-02	0.27	4.05E-02	992.65	5.88E-03	57.09	5.21E-01	44.91
7783J	0.25	1.23E-01	9.04	2.69E-01	2.59	6.57E-03	161.03	6.44E-03	62.52	9.13E-02	7.87
7784J	0.06	1.22E-02	0.90	4.23E-01	4.07	1.49E-01	3651.96	2.73E-03	26.50	3.36E-02	2.90
ATCC 13102	≤0.03	1.36E-02	1.00	1.04E-01	1.00	4.08E-05	1.00	1.03E-04	1.00	1.16E-02	1.00

a. CIP = ciprofloxacin.
b. Exp = expression level that corresponds to the critical threshold (C_T) numbers determined by the detection system software as the amount of target was given as $2^{-\Delta\Delta T}$, where ΔΔT is the difference between the target and reference gene C_T values.
c. Rexp = Relative expression that corresponds to the ratio of the expression result for the clinical isolate and the ATCC 13102.

CONCLUSIONS

- The GyrA T91I alteration was previously reported in these ciprofloxacin non-susceptible strains; however the differences in the ciprofloxacin MIC levels on two strains indicated the presence of a secondary resistance mechanism.
- The additional GyrA alteration T173A was likely to contribute to the greater ciprofloxacin MIC levels (0.25 mg/L) in two *N. meningitidis* strains.
- Differences in expression and structure of *mtrCED* pump components were observed; however, these alterations were not likely to contribute to ciprofloxacin resistance on these isolates. Rifampin MIC results were distinct among the isolates; however the low values (≤0.008 to 0.03 mg/L; similar to ATCC 13102) support this conclusion.

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