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Multiple Mutations in the Quinolone Resistance Determining Regions (QRDR) Detected Among Fluoroquinolone-Resistant *Haemophilus parainfluenzae* Collected in the USA

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ABSTRACT

Background: *H. parainfluenzae* (HPAR) is an oropharyngeal commensal that can cause respiratory tract and other infections. Fluoroquinolone (FQ) resistance (R) among HPAR is generally rare. We investigated QRDR mutations and susceptibility (S) results for 14 FQ-R HPAR collected in USA hospitals during 2012; an increasing occurrence.

Methods: Isolates were S tested by CLSI reference broth microdilution method against moxifloxaxin, levofloxacin, ciprofloxacin and comparators. Extended dilution ranges for the FQs were tested using Haemophilus testing media (HTM) or Mueller-Hinton F (MHF, EUCAST method). QC was performed using *H. influenzae* ATCC 49247. QRDR amplified by PCR, sequenced and compared to HPAR ATCC 33392. PFGE was performed using SmaI and/or ApaI.

Results: Among 153 HPAR collected in the USA during 2012, 14 (9.2%) isolates were R to at least one FQ according to CLSI breakpoints. When tested in HTM broth, moxifloxacin MIC results ranged from 2-64 µg/mL and ciprofloxacin and levofloxacin results ranged from 2-16 µg/mL. MIC results for HTM and MHF were within two-dilution steps, but endpoints were easier to determine with MHF. All isolates displayed GyrA S84F and D88Y alterations. Two to four ParC alterations were also observed and S73(F/Y/L) were noted in all strains. Eight isolates displayed M187L and five had S127T ParC alterations. Only one isolate (Tampa, FL) had a GyrB aminoacid substitution (G545S). Seven isolates had ParE mutation D420N, including the isolate harboring GyrB G545S which displayed the highest MIC values for all three fluoroquinolones. I437L and G467A ParE substitutions were also observed in one strain each. PFGE results demonstrated that all but two isolates were genetically distinct. The two identical isolates were collected in Hawaii and Florida, and had the same alterations in GyrA (S84F, D88Y) and ParC (S73Y, S127T, M187L), but only one had a ParE mutation (G467A). These isolates were non-typable using SmaI and results were based on the ApaI restriction pattern only.

Conclusions: We detected isolates with multiple QRDR mutations for two to four topoisomerase-encoding genes leading to elevated MIC values for the FQ agents. Continued surveillance of FQ-R in HPAR is warranted in the USA and globally as these FQ-R strains evolve.

INTRODUCTION

Haemophilus parainfluenzae is part of the upper respiratory tract biota and this is the main *Haemophilus* species to colonize the oral cavity in the pharynx. This organism can cause acute otitis media, acute sinusitis and acute exacerbation of chronic bronchitis and rarely, it has been reported as cause of subacute bacterial endocarditis.

Quinolones or fluoroquinolones are active against a wide range of Gram-positive and Gram-negative bacterial pathogens, have good absorption after oral administration and display long half-lives that allow once-daily dosing. All these characteristics make this antimicrobial class an attractive option for the empiric treatment of upper respiratory tract infections. Resistance against fluoroquinolones is mainly due to mutations in the fluoroquinolone binding regions in the DNA gyrase and topoismerases IV encoding genes, named quinolone resistance determining regions (QRDR).

Fluoroquinolone resistance among *H. parainfluenzae* seems rare and has been reported in a few isolates from Japan and Spain. We recently observed an increasing number of isolates of *H. parainfluenzae* displaying elevated fluoroquinolone MIC values in United States hospitals and in this study we characterize these isolates for QRDR mutations and genetic relatedness. Additionally, isolates were susceptibility tested using Haemophilus Testing Media (HTM) and Mueller-Hinton Fastidious (MH-F) as recommended by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) guidelines, respectively.

MATERIALS AND METHODS

Bacterial isolates. A total of 153 *H. parainfluenzae* isolates were collected during 2012 among 56 USA hospitals. These isolates were recovered from bloodstream (4 isolates), intra-abdominal (1), skin/soft tissue (46), urinary tract (1), respiratory tract (76) infections and other sites (25). Only clinically significant isolates were included in the study, one per patient episode according to defined protocols. Species identification was confirmed by standard biochemical tests and using the MALDI Biotyper (Bruker Daltonics, Billerica, Massachusetts, USA) according to the manufacturer instructions, where necessary. All 14 fluoroquinolone-resistant isolates had identifications confirmed by using the MALDI Biotyper.

Susceptibility testing. Isolates were initially susceptibility tested by the reference broth microdilution method according to CLSI guidelines (M07-A9, 2012) using HTM supplemented with 2 to 5% lysed horse blood (LHB). Categorical interpretations for all antimicrobials were those found in the CLSI document M100-S24 and the EUCAST website, and quality control (QC) was performed using *H. influenzae* ATCC strains 49247 and 49766. All QC results were within specified ranges as published in CLSI documents. Ciprofloxacin, levofloxaxin and moxifloxacin MIC values were confirmed using extended dilution ranges (up to 256 µg/mL) using the reference methodologies described by the CLSI and EUCAST. EUCAST method testing was performed used MH-F, MH supplemented with 5% LHB and β-nicotinamide adenine dinucleotide (βNAD), as recommended by EUCAST guidelines. All panels were incubated at 35ºC in 5% CO_2 for 20 to 24 hours.

QRDR sequencing. Oligonucleotides HPAR GyrA-F (TCGACGTGTGCTTTTCTCCATG), HPAR GyrA-R (GTTGCCATCCCCACCGCAATACCG), HPAR ParC-F (TCTGAACTGGGTTTATTAGCC), HPAR ParC-R (ACCACGACCGGTTTCATACAT), HPAR GyrB-F (CCAGCACTTTCAGAACTTTAC), HPAR GyrB-R (CCATCTAACGCAAGCATTAATTC), HPAR ParE-F (TAGTGAGTGGTCCTGCTCTAC), HPAR ParE-R (AAAGAGGGCACAGAGTAGGGT) previously described were used to amplify and sequence the QRDR 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 those of *H. parainfluenzae* ATCC 33392 and fluoroquinolone-susceptible *H. parainfluenzae* T3T1 reference strain.

Molecular typing. Fluoroquinolone-resistant *H. parainfluenzae* were epidemiologically typed by pulsed-field gel electrophoresis (PFGE). Genomic DNA was prepared in agarose blocks, digested with *Sma*I and *Apa*I (New England Biolabs, Beverly, Massachusetts, USA) in separate reactions and resolved in the CHEF-DR II apparatus (BioRad, Richmond, California, USA). Results were analyzed by GelCompar II software (Applied Math, Kortrijk, Belgium). Percent similarities were identified on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.2% and 0.5%, respectively.

RESULTS

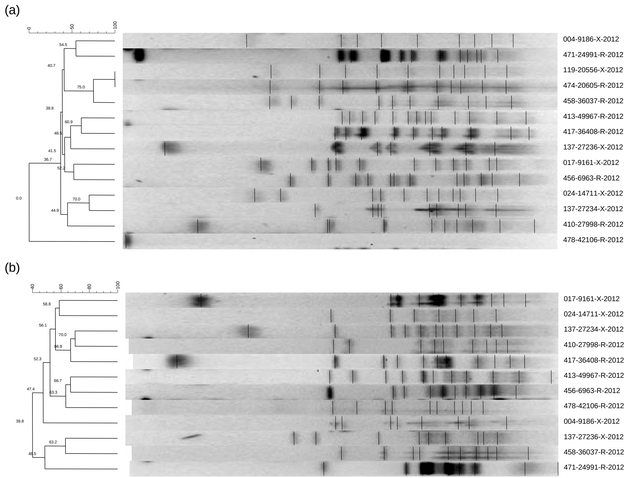
- Fourteen *H. parainfluenzae* isolates collected in 12 USA hospitals displayed elevated MIC values according to CLSI breakpoint criteria for at least one of the three fluoroquinolones tested: ciprofloxacin, moxifloxacin and levofloxacin.
- Susceptibility results ranged for moxifloxacin from 2-64 µg/mL and for ciprofloxacin and levofloxacin from 2-16 µg/mL tested according to CLSI methodology, using HTM broth supplemented with LHB (**Table 1**). Using the EUCAST method (MH-F), moxifloxacin and levofloxacin results ranged from 2-32 µg/mL and ciprofloxacin results ranges from 2-16 µg/mL. EUCAST MIC values were usually two-dilution steps lower than CLSI.
- All isolates displayed GyrA S84F and D88Y alterations when compared to both fluoroquinolone susceptible strains *H. parainfluenzae* ATCC 33392 and T3T1 reference strain (**Table 1**).
- Two to four ParC alterations were also observed and S73 (F/Y/L) were noted in all strains. Eight isolates displayed M187L and five had S127T ParC alterations. When compared to *H. parainfluenzae* ATCC 33392, all isolates had V194A substitution, but not compared to sequences fluoroquinolone-susceptible for *H. parainfluenzae* T3T1 reference strain (**Table 1**).
- Only one isolate (Tampa, FL) had a GyrB aminoacid substitution (G545S). Seven isolates had ParE mutation D420N, including the isolate harboring GyrB G545S, which displayed the highest MIC values for all three fluoroquinolones. I437L and G467A ParE substitutions were also observed in one strain each (**Table 1**).
- PFGE results demonstrated that all but two isolates were genetically distinct (**Figure 1**). The two identical isolates were collected in Hawaii and Florida, and had the same alterations in GyrA (S84F, D88Y) and ParC (S73Y, S127T, M187L), but only one had a ParE mutation (G467A). These isolates were non-typable using *SmaI* and results were based on the *ApaI* restriction pattern only.
- Two isolates collected in the one hospital from Cleveland, OH and another two collected from Tampa, FL were genetically distinct and carried different QRDR alterations (**Table 1** and **Figure 1**).

Table 1. QRDR and susceptibility results of 14 fluoroquinolone-resistant *H. parainfluenzae* recovered from USA hospitals during 2012.

	QRDR amino acid alterations				CLSI MIC results tested in HTM + 3.5% LHB (µg/mL)[a]			EUCAST MIC results tested in MH-F 5% LHB + βNAD (µg/mL)[a]		
City, State	GyrA	ParC	GyrB	ParE	Moxi	Levo	Cipro	Moxi	Levo	Cipro
Birmingham, AL	S84F, D88Y	S73F, M187L, V194A[b]	WT	D420N	16	16	8	16	16	4
Rochester, NY	S84F, D88Y	S73Y, S127T, V194A[b]	WT	D420N	32	16	8	16	16	16
Akron, OH	S84F, D88Y	S73Y, S127T, M187L, V194A[b]	WT	WT	2	1	2	2	2	2
Cleveland, OH	S84F, D88Y	S73F, M187L, V194A[b]	WT	D420N	32	16	8	16	16	8
Cleveland, OH	S84F, D88Y	S73Y, S127T, V194A[b]	WT	WT	16	4	4	8	4	4
Houston, TX	S84F, D88Y	S73F, M187L, V194A[b]	WT	D420N	8	8	8	8	8	8
Ewa Beach, HI[c]	S84F, D88Y	S73Y, S127T, M187L, V194A[b]	WT	WT	4	2	2	4	2	2
Jacksonville, FL[c]	S84F, D88Y	S73Y, S127T, M187L, V194A[b]	WT	G467A	8	4	4	4	2	4
Tampa, FL	S84F, D88Y	S73F, M187L, V194A[b]	G545S	D420N	64	32	16	32	32	8
Tampa, FL	S84F, D88Y	S73F, M187L, V194A[b]	WT	D420N	16	16	8	8	8	8
Atlanta, GA	S84F, D88Y	S73L, S127T,M187L, V194A[b]	WT	I437L	64	16	16	32	8	16
Wauwatosa, WI	S84F, D88Y	S73Y, S127T, M187L, V194A[b]	WT	WT	16	8	8	8	4	4
Sun City, AZ	S84F, D88Y	S73Y, S127T, V194A[b]	WT	WT	16	8	4	16	4	4
Seattle, WA	S84F, D88Y	S73F, M187L, V194A[b]	WT	D420N	16	8	4	32	16	8

a. Moxi= moxifloxacin, Levo=levofloxacin, Cipro=ciprofloxacin.
b. When compared to *H. parainfluenzae* ATCC 33392 all isolates had V194A substitution, but not compared to fluoroquinolone-susceptible sequences for *H. parainfluenzae* T3T1 reference strain.
c. Genetically related isolates are highlighted in red.

Figure 1. PFGE restriction patterns of fluoroquinolone-resistant *H. parainfluenzae* digested with (a) *ApaI* or (b) *SmaI*. Two enzymes were used due to non-typable isolates with one or another method.



CONCLUSIONS

- As a pathogen in the normal biota of the pharynx, *H. parainfluenzae* isolates are often overlooked as cause of infections of the upper respiratory tract. Overall, these isolates are more susceptible to antimicrobial agents used to treat respiratory infections, but reports in the literature have documented the emergence of fluoroquinolone-resistant *H. parainfluenzae* in Japan and Spain.
- EUCAST and CLSI methodologies generated similar MIC values; however endpoints are much easier to be determined using MH-F instead of HTM.
- In this study, we document the emergence of fluoroquinolone-resistant *H. parainfluenzae* in various USA hospitals with an overall percentage of 9.2% of resistant isolates. Due to the convenient dosage and broad spectrum of activity, fluoroquinolones have been largely used for the treatment of respiratory and other infections and antimicrobial selective pressure could influence resistance in commensal bacteria, such as *H. parainfluenzae*.

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