Genetic detection of quinolone resistance in *Haemophilus parainfluenzae*: Mutations in the quinolone resistance-determining regions of *gyrA* and *parC*

Dennis KS Law BA BSc¹, Michelle Shuel BSc¹, Sadjia Bekal PhD², Elizabeth Bryce MD³, Raymond SW Tsang PhD¹

DKS Law, M Shuel, S Bekal, E Bryce, RSW Tsang. Genetic detection of quinolone resistance in *Haemophilus parainfluenzae*: Mutations in the quinolone resistance-determining regions of *gyrA* and *parC*. Can J Infect Dis Med Microbiol 2010;21(1):e20-e22.

The quinolone resistance-determining regions of *gyrA* and *parC* of both quinolone-sensitive and quinolone-resistant *Haemophilus parainfluenzae* strains were amplified and sequenced. Similar to *Haemophilus influenzae*, resistance to quinolones in *H parainfluenzae* is associated with mutations in the quinolone resistance-determining regions of both *gyrA* and *parC*. The present study discusses the importance of this finding.

Key Words: Genetic detection; *Haemophilus parainfluenzae*; Quinolone resistance

Although generally not regarded as a pathogen, *Haemophilus parainfluenzae* does occasionally cause infections in humans, including pneumonia (1), peritonitis (2,3), biliary tract infection (4), endocarditis (5) and urinary tract infection (6). More recently, its role in chronic obstructive pulmonary disease has been discussed (7,8). Due to its potential role in the infectious process, susceptibility testing of *H parainfluenzae* is important to guide antibiotic treatment when required. This is especially relevant because beta-lactamase-mediated resistance to ampicillin has been observed more frequently in *H parainfluenzae* than in *Haemophilus influenzae* (9). Furthermore, fluoroquinolone-resistant *H parainfluenzae* has recently been reported in Japan (10). Of the 920 *H parainfluenzae* isolates recovered from respiratory specimens of patients admitted to outpatient clinics in Japan between 1998 and 2000, 31 isolates or 3.4% were resistant to fluoroquinolones based on the standard microbroth dilution method, and had a minimum inhibitory concentration (MIC) breakpoint of greater than 2 µg/mL for levofloxacin, according to the Clinical and Laboratory Standards Institute (USA) guidelines (11). The MIC required to inhibit the growth of 90% of organisms for five fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin, sitafloxacin and sparfloxacin) for the fluoroquinolone-sensitive *H parainfluenzae* isolates ranged from 0.015 µg/mL or less to 0.06 µg/mL, and was 100-fold higher for the fluoroquinolone-resistant *H parainfluenzae* isolates. However, the molecular basis of this resistance was not studied.

Mutations in both *gyrA* and *parC* have been documented to be associated with quinolone resistance in *H influenzae* and a number of other Gram-negative bacteria (12-15).

Le dépistage génétique de la résistance à la quinolone en cas d'*Haemophilus parainfluenzae* : Les mutations dans les régions du *gyrA* et du *parC* déterminant la résistance à la quinolone

Les auteurs ont amplifié et séquencé les souches d'*Haemophilus parainfluenzae* sensibles à la quinolone et résistantes à la quinolone des régions du *gyrA* et du *parC* déterminant la résistance à la quinolone. À l'instar de l'*Haemophilus influenzae*, la résistance du *H parainfluenzae* aux quinolones s'associe à des mutations des régions de *gyrA* et de *parC* déterminant la résistance à la quinolone. La présente étude expose l'importance de cette découverte.

METHODS

In the present study, the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* for both quinolone-sensitive and quinolone-resistant *H parainfluenzae* strains were amplified by polymerase chain reaction (PCR) and then sequenced. PCR primers described for amplifying *gyrA* and *parC* of *H influenzae* (14) did not amplify the corresponding genes of *H parainfluenzae*. Based on the complete genome sequence of *H parainfluenzae* strain T3T1 (www.sanger.ac.uk/Projects/H_parainfluenzae/), primers were designed to target the QRDRs of both *gyrA* and *parC* of this organism. For *gyrA*, the forward and reverse primers GYRAF2 (5'-TCG ACG TGT GCT TTT CTC CAT G-3') and GYRAR1 (5'-GTT GCC ATC CCC ACC GCA ATA CCG-3'), respectively, amplified a 405 base pair gene fragment. For *parC*, the forward and reverse primers PARCF1 (5'-TCT GAA CTG GGT TTA AAT GCC-3') and PARCR1 (5'-ACC ACG ACC GGT TTC ATA CAT-3'), respectively, amplified a 564 base pair gene fragment.

The PCR mixture consisted of 2.5 mM magnesium chloride, 0.2 mM deoxyribonucleotide triphosphates, 10 mM primers and 1.25 units of HotStarTaq DNA polymerase (Qiagen, Canada). The PCR cycle consisted of an initial 15 min at 95°C, followed by 35 cycles at 95°C for 1 min, 54°C for 1 min and 72°C for 1 min and a final phase of 72°C for 10 min. The PCR amplicons were purified with the QIAquick PCR purification kit (Qiagen, Canada) before being sequenced by the DNA analyzer 3730x1 (Applied Biosystems, USA). The sequences of both strands were edited, assembled and aligned for comparison with published sequences obtained from

¹Vaccine Preventable Bacterial Diseases, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba; ²Laboratoire de santé publique du Québec, Institut national de santé publique du Québec, Ste-Anne-de-Bellevue, Québec; ³Division of Medical Microbiology and Infection Control, Vancouver General Hospital, Vancouver, British Columbia

Correspondence: Dr Raymond SW Tsang, Vaccine Preventable Bacterial Diseases, National Microbiology Laboratory, 1015 Arlington Street, Winnipeg, Manitoba R3E 3R2. Telephone 204-789-6020, fax 204-789-2018, e-mail raymond.tsang@phac-aspc.gc.ca

TABLE 1
Amino acid compositions of the *gyrA* and *parC* quinolone resistance-determining regions (QRDRs) in fluoroquinolone-sensitive and quinolone-resistant *Haemophilus parainfluenzae* strains

Strain number	Fluoroquinolone MIC values by Etest (µg/mL)			Amino acid composition							
	Levofloxacin	Ciprofloxacin	Moxifloxacin	QRDR of <i>gyrA</i> *			QRDR of <i>parC</i> †				
ATCC 33392‡	<0.03	Not done	Not done	Asp	Ser	Asp	Gly	Asp	Ser	Tyr	
87-0092	0.016	0.023	0.125	Asp	Ser	Asp	Gly	Asp	Ser	Tyr	
88-0043	0.012	0.008	0.032	Asp	Ser	Asp	Gly	Asp	Ser	Tyr	
88-0194	0.023	0.012	0.064	Asp	Ser	Asp	Gly	Asp	Ser	Tyr	
88-0283	0.016	0.006	0.032	Asp	Ser	Asp	Gly	Asp	Ser	Tyr	
89-0518	0.012	0.006	0.094	Asp	Ser	Asp	Gly	Asp	Ser	Tyr	
89-0796	0.064	0.047	0.250	Asp	Ser	Asp	Gly	Asp	Ser	Tyr	
90-0572	0.032	0.008	0.094	Asp	Ser	Asp	Gly	Asp	Ser	Tyr	
95-0376	0.032	0.016	0.125	Asp	Ser	Asp	Gly	Asp	Ser	Tyr	
95-0377	0.023	0.016	0.094	Asp	Ser	Asp	Gly	Asp	Ser	Tyr	
08-013	0.023	0.016	0.125	Asp	Ser	Asp	Gly	Asp	Ser	Tyr	
07-020	2.0	4.0	>32	Asp	Phe§	Tyr§	Gly	Asp	Phe§	Tyr	
07-028	12	12	>32	Asp	Phe§	Tyr§	Gly	Asp	Phe§	Tyr	

Etest (bioMérieux, Canada). The minimum inhibitory concentration (MIC) of levofloxacin was determined by Sensititre plates (Trek Diagnostic Systems Inc, USA).

*QRDR of *gyrA* at amino acid positions 83, 84 and 88 (14); †QRDR of *parC* at amino acid positions 82, 83, 84 and 88 (14); ‡ATCC 33392 is a *H parainfluenzae*-type strain sensitive to fluoroquinolones; §Mutations in QRDRs. Asp Aspartate; Gly Glycine; Phe Phenylalanine; Ser Serine; Tyr Tyrosine

quinolone-sensitive and quinolone-resistant *H influenzae* strains (14) by using software from DNASTar Inc (USA).

RESULTS AND DISCUSSION

Two *H parainfluenzae* isolates recovered from respiratory specimens were referred to the National Microbiology Laboratory (NML; Winnipeg, Manitoba) for further characterization: isolate 1, NML# 07-028, from the pleural fluid of a 52-year-old woman from British Columbia; and isolate 2, NML# 07-020, from the sputum of an 83-year-old man from Quebec. Both isolates were confirmed as *H parainfluenzae* by biochemical tests and 16S ribosomal RNA sequencing. Isolate 1 belonged to biotype I, and isolate 2 belonged to biotype II. Detection of beta-lactamase was performed using the BBL DrySlide nitrocefin (Becton Dickinson, Canada). Antibiotic susceptibility testing by the disk-diffusion method was performed according to the Clinical and Laboratory Standards Institute guidelines (16). Etest (bioMérieux, Canada) was used to determine the MIC of fluoroquinolones (ciprofloxacin, levofloxacin and moxifloxacin) to *H parainfluenzae*. Microbroth dilution method using the Sensititre plates (Trek Diagnostic Systems Inc, USA) was also performed to test the MIC of levofloxacin. Both isolates did not produce beta-lactamase and were found by disk-diffusion susceptibility testing to be resistant to ciprofloxacin, moxifloxacin, clarithromycin and azithromycin, but sensitive to ampicillin, amoxicillin-clavulanic acid, cefaclor, ceftriaxone, trimethoprim-sulfamethoxazole and chloramphenicol. In addition, 11 quinolone-sensitive *H parainfluenzae* isolates (sensitive to ciprofloxacin, moxifloxacin and levofloxacin) were included for comparison. These 11 isolates included ATCC 33392, three from respiratory sources, three from stool/appendix, and one each from cerebral spinal fluid, eye, gallbladder and one of unknown source.

Table 1 compares the MICs of fluoroquinolones and the amino acid compositions of the QRDRs of *gyrA* and *parC* in the *H parainfluenzae* strains examined in the present study. Similar to *H influenzae* (14), resistance to quinolones in *H parainfluenzae* is associated with mutations in both *gyrA* and *parC*. In the two resistant strains examined, the mutations involved substitutions of Ser84Phe and Asp88Tyr of *gyrA*, and

Ser84Phe of *parC*. The difference in the MICs (2 µg/mL versus 12.0 µg/mL) of levofloxacin in these two resistant strains suggest that strain 07-028 might possess additional resistance mechanisms, such as additional mutations in the drug targets GyrB and ParE, decreased permeation of the drug, or presence and/or over-expression of an endogenous efflux pump system that causes reduced accumulation of the drug in the target site. These possibilities have not been examined in the strains.

Besides being capable of causing a variety of infections, *H parainfluenzae* has been reported to be associated with acute exacerbations of chronic bronchitis (AECB) (17). Empirical antibiotic treatment of patients with AECB has been proven to be beneficial in those with severe airflow obstruction and presenting with at least two or all three cardinal symptoms (increased dyspnea, increased sputum volume and increased sputum purulence) (18). With increasing reports of resistance of many bacteria to multiple antibiotics including the beta-lactams, fluoroquinolones have become the antibiotic of choice to treat AECB (19).

SUMMARY

We report new primers for PCR amplification of the QRDRs of *gyrA* and *parC* of *H parainfluenzae* for molecular detection of quinolone resistance. We confirm that quinolone resistance in *H parainfluenzae*, similar to that reported for *H influenzae* and other Gram-negative bacteria, is associated with genetic mutations that lead to amino acid substitutions in the QRDRs of both *gyrA* and *parC*. Because these two random isolates were identified at the local hospital or provincial public health laboratories, the prevalence of quinolone-resistant *H parainfluenzae* strains in the general population is not available from the present study. Further studies are required to understand the extent of fluoroquinolone resistance in *H parainfluenzae*, and the potential involvement of other DNA gyrase and topoisomerase genes, such as *gyrB* and *parE*, in the resistance of these organisms to quinolones.

ACKNOWLEDGEMENT: The authors thank the staff of the National Microbiology Laboratory's DNA Core Facility in Winnipeg, Manitoba, for the DNA sequencing work.

REFERENCES

- Pillai A, Mitchell JL, Hill SL, Stockley RA. A case of *Haemophilus parainfluenzae* pneumonia. *Thorax* 2000;55:623-4.
- Betriu C, Coronel F, Martin P, Picazo JJ. Peritonitis caused by *Haemophilus parainfluenzae* in a patient undergoing continuous ambulatory peritoneal dialysis. *J Clin Microbiol* 1998;37:3074-5.
- Mayer MP, Schweizer P. Primary peritonitis in a child caused by *Haemophilus parainfluenzae*. *Pediatr Surg Int* 2002;18:728-9.
- Frankard J, Rodriguez-Villalobos H, Struelens MJ, Jacobs F. *Haemophilus parainfluenzae*: An undiagnosed pathogen of biliary tract infections? *Eur J Clin Microbiol Infect Dis* 2004;23:46-8.
- Choi D, Thermidor M, Cunha BA. *Haemophilus parainfluenzae* mitral prosthetic valve endocarditis in an intravenous drug abuser. *Heart Lung* 2005;34:152-4.
- Hansson S, Svedhem A, Wennerstrom M, Jodal U. Urinary tract infection caused by *Haemophilus influenzae* and *Haemophilus parainfluenzae* in children. *Pediatr Nephrol* 2007;22:1321-5.
- Hill SL, Mitchell JL, Stockley RA, Wilson R. The role of *Haemophilus parainfluenzae* in COPD. *Chest* 2000;117:293S.
- Mitchell JL, Hill SL. Immune response to *Haemophilus parainfluenzae* in patients with chronic obstructive lung disease. *Clin Diagn Lab Immunol* 2000;7:25-30.
- Scheifele DW, Fussell SJ. Frequency of ampicillin-resistant *Haemophilus parainfluenzae* in children. *J Infect Dis* 1981;143:195-8.
- Takemura H, Terakubo S, Yamamoto H, Shimada J, Nakashima H, Kanemitsu K. Susceptibilities of fluoroquinolone-resistant *Haemophilus parainfluenzae* isolates from Japanese patients with respiratory infections to five fluoroquinolones and other antimicrobial agents. *Antimicrob Agents Chemother* 2003;47:3996-7.
- NCCLS. Performance standards for antimicrobial susceptibility testing, 11th informational supplement. NCCLS document M100-S11, Wayne, Pennsylvania.
- Deguchi T, Yasuda M, Nakano M, et al. Quinolone-resistant *Neisseria gonorrhoeae*: Correlation of alterations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV with antimicrobial susceptibility profiles. *Antimicrob Agents Chemother* 1996;40:1020-3.
- Li X, Mariano N, Rahal JJ, Urban CM, Drlica K. Quinolone-resistant *Haemophilus influenzae* in a long-term care facility: Nucleotide sequence characterization of alterations in the genes encoding DNA gyrase and DNA topoisomerase IV. *Antimicrob Agents Chemother* 2004;48:3570-2.
- Perez-Vazquez M, Roman F, Aracil B, Canton R, Campos J. Laboratory detection of *Haemophilus influenzae* with decreased susceptibility to nalidixic acid, ciprofloxacin, levofloxacin, and moxifloxacin due to *gyrA* and *parC* mutations. *J Clin Microbiol* 2004;42:1185-91.
- Yoshizumi S, Takahashi Y, Watanabe Y, Okezaki E, Ishii Y, Tateda K. In vitro antibacterial activities of new fluoroquinolones against clinical isolates of *Haemophilus influenzae* with ciprofloxacin-resistance-associated alterations in GyrA and ParC. *Chemotherapy* 2004;50:265-75.
- Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing: Eighteenth information supplement, 2008: M100-S18. Wayne, Pennsylvania.
- Sethi S, Murphy TF. Bacterial infection in chronic obstructive pulmonary disease in 2000: A state-of-the-art review. *Clin Microbiol Rev* 2001;14:336-63.
- Saint S, Bent S, Vittinghoff E, Grady D. Antibiotics in chronic obstructive pulmonary disease exacerbations: A meta analysis. *JAMA* 1995;273:957-60.
- Grossman RF. Cost-effective therapy for acute exacerbation of chronic bronchitis. *Semin Respir Infect* 2000;15:71-81.