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Reduced Levofloxacin Susceptibility in Clinical Respiratory Isolates of *Haemophilus Influenzae* Is Not yet Associated with Mutations in the DNA Gyrase and Topoisomerase II Genes in Korea

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Among 155 clinical respiratory isolates of *Haemophilus influenzae* in Korea, 6 (3.9%) isolates had reduced levofloxacin susceptibility (MICs ≥ 0.5 μg/mL). These six isolates had no significant quinolone resistance-determining region (QRDR) mutations in *gyrA, gyrB, parC,* or *parE*. This phenomenon suggests that neither evolution nor spread of any significant QRDRs mutations in clinical isolates occurred in Korea. Therefore, continued surveillance is necessary to observe the evolution of antibiotic-resistance and take measures to avoid the spread of drug-resistant clones.

Key Words: *Haemophilus influenzae*, levofloxacin, quinolone, drug resistance, DNA gyrase, DNA topoisomerases, Type II

Phenotypic resistance to fluoroquinolones is rarely seen in *Haemophilus influenzae*, but recent studies suggest that reduced susceptibility due to mutational hot spots in the quinolone resistance-determining regions (QRDRs) of genes encoding gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*) may be common.[1-4] The mutational hot spots in *gyrA* (Ser84Thr), *parC* (Ser84Ile), *gyrA* (Asp88Asn), and *parC* (Glu88Lys) with reduced susceptibility generally remain undetected when fluoroquinolone susceptibility tests are interpreted using current breakpoints.[3,5] However, it is important to detect these mutations because they may be associated with clinical failures, and high-level resistance may emerge during fluoroquinolone therapy.[4,5] Tentatively proposed breakpoints for levofloxacin, ciprofloxacin, moxifloxacin (MICs ≥ 0.5 μg/mL), and nalidixic acid (MICs ≥ 32 μg/mL) could be used to detect decreased fluoroquinolone susceptibility in *H. influenzae* strains.

We sought to define the relationship between amino acid changes in the QRDRs of *gyrA, gyrB, parC*, and *parE*, and decreased susceptibility to levofloxacin (MICs ≥ 0.5 μg/mL) among respiratory infection-inducing 155 *H. influenzae* isolates

from a tertiary hospital in Korea. We retrospectively tested all *H. influenzae* cultured from clinical respiratory isolates obtained between 2001 and 2005. The MICs of levofloxacin were measured using the Etest method.[6,7] Previously described or modified primers were used to define mutations in the *gyrA, gyrB, parC*, and *parE* genes.[3] PCR primer sequences were determined from Genebank entry Rd KW20 at the following positions *gyrA* (96 to 114; 549 to 567), *gyrB* (1194 to 1213; 1875 to 1894), *parC* (120 to 141; 699 to 720), and *parE* (945 to 966; 1637 to 1656). These primer sequences included the known mutational hot spots in QRDRs (for example, Ser84Thr and Asp88Asn in *gyrA*, Ser467Thr and Asn601Ser in *gyrB*, Ser84Ile and Glu88Lys in *parC*, and Asp420Asn, Ser458Ala, and Ser474Asn in *parE*). Direct sequencing of the PCR products was carried out using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). All sequences were compared to those of *H. influenzae* RD KW20 and nontypeable *H. influenzae* 86-028NP. These two strains have traditionally been considered as the reference sequences of *H. influenzae*. In addition, these two strains are susceptible to fluoroquinolone. β-lactamase production was determined using the nitrocefin test (Oxoid, Basingstoke, UK). Serotype b primers were used to amplify the portion of the gene encoding the serotype b capsule.[8] We used quality control strains *H. influenzae* ATCC 49247 and ATCC 49766 as controls for Etest and direct sequencing.

Six of the 155 clinical respiratory isolates (3.9%) had decreased susceptibility to levofloxacin (MICs ≥ 0.5 μg/mL). Of the 6 isolates with reduced susceptibility, 1 was serotype b and and 2 were β-lactamase producers. The QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* were sequenced in the six isolates, and no significant substitutions in *gyrA*, *gyrB*, and *parE* were found (Table 1). Although new substitutions of Ala120Ser, Lyn131glu, and Val210Leu in *parC* were revealed in this study, the biological relevance of these *parC* substitutions is unknown. The association between the Asn138Ser substitution in *parC* and fluoroquinolone resistance had been previously reported;[3] however, this substitution has been found in susceptible *H. influenzae*[2] Furthermore, Ser133Thr and Gly206Arg substitutions in *parC* have been found in susceptible *H. influenzae* strains. These substituted sequences at codon 133, 138, and 206 of *parC* are identical to those in the non-typeable *H. influenzae* strain 86-028NP. The new substitutions in *parC* Ala120Ser, Lyn131glu, and Val210Leu were not noted in ATCC 49247 or in ATCC 49766 *H. influenzae*. The clinical significance

Table 1. Levofloxacin MICs and Quinolone Resistance-Determining Region Mutations in DNA Gyrase and Topoisomerase IV in *H. influenzae* Isolates with Reduced Levofloxacin Susceptibility

Strain	Age (yrs)	Sex	Material	Diagnosis	Levofloxacin MIC (μg/mL)	Mutation (s)			Beta-lactamase production	Serotype B
						gyrA	*gyrB*	*parC*	*parE*		
1	64	F	Sputum	Pneumonia	2	No	No	Gly206Arg*, Val210Leu?	No	Negative	Negative
2	6	M	Rhinorrhea	Chronic sinusitis	1.5	No	No	Gly206Arg*, Val210Leu?	No	Negative	Negative
3	5	M	Rhinorrhea	Chronic sinusitis	1	No	No	Ser133Thr*, Asn138Ser*	No	Negative	Negative
4	69	F	Transtracheal aspirates	Pneumonia	1	No	No	Ala120Ser?, Lyn131glu?, Ser133Thr*, Asn138Ser*	No	Positive	Negative
5	38	F	Sputum	Bronchiectasis	1	No	No	No	No	Positive	Positive
6	4	F	Rhinorrhea	Chronic serous otitis media	0.5	No	No	Ser133Thr*, Asn138Ser*	No	Negative	Negative

M, male; F, female.
*Mutations do not contribute to resistance; ?, mutations with unknown contribution to resistance.

of these new substitutions is uncertain because they are found outside the QRDRs located between residues 64 and 106 in the *parC* sequence. Therefore, additional studies are needed to determine whether substitutions Ala120Ser, Lyn-131glu, and Val210Leu in *parC* contribute to resistance.

Although new substitutions in *parC* with uncertain significance were discovered, no definitive significant QRDR mutations were found in respiratory isolates of *H. influenzae* in the present study. Our study suggests that neither spontaneous acquisition nor the spread of any significant QRDR mutations has occurred in respiratory *H. influenzae* isolates in Korea. Further study should be directed at strains with no QRDR mutations so the mechanism behind reduced fluoroquinolone susceptibility can be determined through analyzing the level of AcrAB efflux pump expression, or through analyzing porin protein loss.[9] However, no mechanism of fluoroquinolone resistance has been previously detected, and the specific contribution of higher *acrAB* cluster expression to the level of quinolone resistance has not been demonstrated in *H. influenzae* carrying no QRDR mutations.[10]

There are some limitations to this study. First, nalidixic acid, not levofloxacin using this study, has been proposed for use in screening for decreased susceptibility in *H. influenzae*.[5] Second, there are no isolates of high level resistant to levofloxacin (MIC > 2 μg/mL). Third, the antibiotic susceptibility test was done using the Etest method instead of the broth microdilution method recommended by the CLSI guidelines. However, cross-resistance to a large number of fluoroquinolones has been observed in *H. influenzae*, suggesting that testing for susceptibility to one fluoroquinolone is sufficient to describe susceptibility in the entire antibiotic family in the majority of cases.[11] Almost all strains with decreased susceptibility to levofloxacin (MICs > 0.5 μg/mL) had have at least one *gyrA* or *parC* mutation in various studies.[5,9,12,13] Although the Etest is not a reference method for antimicrobial susceptibility tests in *H. influenzae*, there is a good correlation between the levofloxacin broth microdilution and Etest results.[7]

In conclusion, clinical respiratory isolates of *H. influenzae* with reduced levofloxacin susceptibility in Korea had no significant QRDR mutations in *gyrA*, *gyrB*, *parC*, or *parE*. This study suggests that now is an ideal time to prevent spontaneous acquisition and horizontal spread of QRDR mutations in *H. influenzae*. Also, further study is required concerning other mechanisms of fluoroquinolone resistance.

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