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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

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Fluoroquinolone-resistant isolates of *Haemophilus influenzae,* obtained from a long-term care facility, were examined for nucleotide sequence differences in the quinolone-resistance-determining regions of *gyrA, gyrB, parC*, and *parE*. Similarities among the resistant isolates, plus multiple differences with susceptible isolates, suggest clonal dissemination involving two resistant subclones.

Over the last decade the use of fluoroquinolones for treatment of bacterial respiratory disease has increased (for example, in Canada the number of fluoroquinolone prescriptions increased sevenfold between 1988 and 1997 [5]). Concomitantly, the prevalence of resistant *Streptococcus pneumoniae* has increased (5, 9), and occasionally resistant isolates of *Haemophilus influenzae*, which is generally highly susceptible to fluoroquinolones, have been recovered (1–4, 6, 10–12, 14, 15). Recently, an outbreak of levofloxacin-resistant *H. influenzae* was observed in a long-term care facility (13). Epidemiological data and electrophoretic analysis of DNA fragments from resistant isolates of the outbreak suggested that the resistant isolates were clonally related (13). To characterize the DNA regions likely to be responsible for the resistance, we have examined isolates for nucleotide sequence changes within mutational hot spots, called quinolone resistance-determining regions (QRDRs), of genes encoding DNA gyrase and DNA topoisomerase IV. In the present work we describe sequence differences between susceptible and resistant isolates in the QRDRs. These differences are consistent with the resistant outbreak arising from a single clone that has evolved into two related subclones.

H. influenzae was grown as colonies on Haemophilus test medium agar (HTM) or as liquid cultures in HTM broth (BD BBL, Detroit, Mich.), using overnight incubation at 37°C in 5% CO_2. Chromosomal DNA was extracted from selected isolates grown as lawns on HTM agar. About 10^8 cells, suspended in a solution containing 200 μl of 100 mM NaCl, 10 mM Tris-HCl (pH 8.3), 1 mM EDTA (pH 8.0), and 1% Triton X-100, were incubated with 0.5 mg of lysozyme/ml and 0.1 mg of pancreatic RNase A/ml at 37°C for 10 min and then transferred to boiling water for 10 min. Cell debris was removed by centrifugation, and 4 μl of supernatant fluid was used as a source of template DNA in a 50-μl PCR volume. PCR primer sequences were determined from GenBank entry Rd at the following positions: *gyrA* (96 to 114; 549 to 567), *gyrB* (1194 to 1213; 1875 to 1894), *parC* (120 to 141; 499 to 520), and *parE* (945 to 966; 1637 to 1656). PCR products were purified using a PCR purification kit (QIAGEN, Valencia, Calif.) and were sequenced directly with an automated DNA sequencer using primers at the following positions: *gyrA* (121 to 141), *gyrB* (1250 to 1271), *parC* (152 to 172), and *parE* (1006 to 1023).

Fluoroquinolone susceptibility was determined by plating serial dilutions on agar containing fluoroquinolones that differed in concentration by linear, rather than standard twofold, increments. Colonies on each plate were counted after overnight incubation. The MIC at which 99% of isolates were inhibited (8) and the standard MIC were determined by interpolation of plots of fluoroquinolone concentration versus fraction of CFU recovered. Garenoxacin was obtained from Bristol-Myers-Squibb (Wallingford, Conn.), moxifloxacin and ciprofloxacin were obtained from Bayer Corp. (West Haven, Conn.), and levofloxacin was obtained from the R.W. Johnson Pharmaceutical Research Institute (Spring House, Pa.).

Clinical isolates of *H. influenzae* were recovered during a 1-year study (May 2001 to May 2002) in which 28 cases of levofloxacin-resistant *H. influenzae* were identified in a long-term health care facility in Queens, N.Y. (13). At the same time, levofloxacin-susceptible *H. influenzae* was recovered from seven patients in the facility. When DNA from the isolates was cut by restriction endonucleases and examined by pulsed-field gel electrophoresis, the resistant isolates appeared to be clonally related; the susceptible ones were not (13). To further examine relationships among the isolates, nucleotide sequences were determined for the QRDRs of the *gyrA*, *gyrB*, parC, and *parE* genes of six resistant and four susceptible isolates.

The six resistant isolates were obtained from three patients treated with fluoroquinolone plus three who received no quinolone (Table 1), consistent with clonal spread. The six isolates had amino acid substitutions in GyrA at codons 84 and 88, in ParC at position 138, and in ParE at positions 458 and 474

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TABLE 1. Relationship of *H. influenzae* isolates[a]

Gene	Codon	Nucleotide sequence (amino acid) for:						
		Rd	NYC isolate(s)					
			Susceptible				Resistant	
			KD2215	*KD2216*	*KD2217*	*KD2218*	*KD2219, KD2220, KD2223, KD2224*	*KD2221, KD2222*
gyrA	84	CCC (S)	CCA (S)	CCA (S)	CCA (S)	CCA (S)	TTC (F)*	TTC (F)*
gyrA	88	GAT (D)					TAT (Y)*	TAT (Y)*
gyrB	573	ACA (T)	GCA (A)	GCA (A)	GCA (A)	GCA (A)		
gyrB	601	AAC (N)	AGC (S)			GGT (G)	AGC (S)	AGC (S)
gyrB	606	GCC (A)		ACT (T)	GTG (V)	ACT (T)	ACC (T)	ACC (T)
gyrB	610	CAA (Q)	AAA (K)					
gyrB	619	ATT (I)	GTT (V)	GTT (V)		GTT (V)	GTT (V)	GTT (V)
parC	133	TCT (S)	GCT (A)					
parC	138	AAT (N)					AGT (S)*	AGT (S)*
parE	420	GAC (D)					AAC (N)*	
parE	458	TCA (S)					GCA (A)*	GCA (A)* Insert GCA (A)
parE	474	AGC (S)					AAC (N)	AAC (N)
parE	542	AAT (N)		AGT (S)	AGT (S)		AAC (N)	AAC (N)

[a] Nucleotide sequences were determined for the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* for four susceptible isolates and six resistant isolates. The isolates were grouped according to nucleotide sequence differences and similarities. Below the isolate numbers are listed nucleotide sequences for codons expected to cause an amino acid difference in at least one isolate of the set (the encoded amino acid is indicated by single-letter abbreviation in parentheses). Where no codon is listed, it is identical to that shown for reference strain Rd. Strains KD2221 and KD2222 contain an insertion of Ala between Gly- 457 and Ala-458. Asterisks indicate that the indicated amino acid change has been associated previously with fluoroquinolone resistance (2, 3, 7, 10, 11, 14). Strains KD2219, KD2220, and KD2221 were obtained from patients treated with quinolone; KD2222, KD2223, and KD2224 were from patients that received no quinolone. NYC, New York City.

(Table 1). Many of these changes had previously been associated with fluoroquinolone resistance (Table 1). Resistant isolates KD2221 and KD2222 had an alanine inserted between amino acids 457 and 458 of the ParE protein that was not seen with resistant isolates KD2219, KD2220, KD2223, and KD2224. The latter four contained an Asp-420-to-Asn change in ParE that was not observed in KD2221 and KD2222 (Table 1). These data suggested that the resistant isolates had evolved into two subclones.

All susceptible isolates differed from the prototype strain Rd and the resistant isolates by a GyrB Thr-573-to-Ala change. However, several amino acid sequence differences (Table 1) and many noncoding differences (data not shown) were seen among the four susceptible isolates, consistent with these isolates having independent origins. Surprisingly, all four were identical in the region of *gyrA* examined. However, they differed from the resistant isolates, which were also identical, at 17 nucleotide positions. Such data are consistent with the *gyrA* mutations being acquired by horizontal transfer; however, a search of GenBank failed to reveal a perfect match to another bacterial species.

When fluoroquinolone susceptibility was determined, isolates KD2221 and KD2222 were less susceptible to moxifloxacin, garenoxacin, levofloxacin, and ciprofloxacin than the other resistant isolates (Table 2), consistent with formation of two groups. The MIC for susceptible isolates was about 500- to 1,000-fold lower than that of resistant isolates (Table 2); the new quinolone garenoxacin exhibited activity similar to that of ciprofloxacin and greater than that of levofloxacin or moxifloxacin with susceptible isolates (Table 2).

The presence of several resistance mutations in the clinical isolates is consistent with stepwise resistance arising from multiple rounds of fluoroquinolone challenge (14). GyrA changes, which have been observed with other sets of clinical isolates (2,

TABLE 2. Fluoroquinonolone susceptibility of clinical isolates

Isolate no.	MIC_{99}[a] (standard MIC), μg/ml			
	Cipro	Levo	Garen	Moxi
ATTC 49247	0.007 (0.008)	0.019 (0.02)	0.0018 (0.022)	0.023 (0.035)
KD2215	0.007 (0.008)	0.012 (0.013)	0.0058 (0.007)	0.036 (0.05)
KD2216	0.008 (0.009)	0.012 (0.013)	0.0061 (0.008)	0.039 (0.05)
KD2217	0.007 (0.008)	0.012 (0.013)	0.0065 (0.0085)	0.04 (0.05)
KD2218	0.006 (0.008)	0.012 (0.013)	0.0072 (0.01)	0.048 (0.06)
KD2219	19 (21)	11 (12.5)	17 (20)	20 (22)
KD2220	11 (14)	4.8 (6)	7.5 (11)	14.6 (16)
KD2221	42 (48)	22 (26)	18 (28)	31 (35)
KD2222	40 (44)	21 (26)	18.5 (28)	33 (35)
KD2223	14 (15.5)	4.5 (6.5)	6.4 (9)	15 (17.5)
KD2224	11.5 (13.5)	4.5 (6)	4.5 (6)	14 (16)

[a] MIC at which 99% of isolates are inhibited (MIC_{99}) was determined as described in the text; standard MIC using linear increments of fluoroquinolone concentration is indicated in parentheses. Abbreviations: Levo, levofloxacin; Garen, garenoxacin; Cipro, ciprofloxacin; Moxi, moxifloxacin.

3, 7, 10, 11, 14, 15), may be the first to be enriched, since that is the case in vitro (X. Li, et al., unpublished observations) and since clinical isolates have been found that have QRDR mutations in *gyrA* but not in *parC* (2, 10, 11, 14). In the present case, *gyrA*-mediated resistance may have been acquired by horizontal transfer from an undefined source. The *parE* mutations that distinguish strains KD2221 and KD2222 from the four other resistant isolates are most easily explained if they were the most recently acquired.

In summary, previous epidemiological and DNA restriction fragment analyses (13) suggested that fluoroquinolone-resistant *H. influenzae* can be clonally disseminated. This conclusion was supported by nucleic acid sequence analysis of portions of the genes encoding the quinolone targets.

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