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# Molecular Characterization of Quinolone-Resistant *Neisseria gonorrhoeae* in Hong Kong

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**In Hong Kong, ParC changes among high-level quinolone-resistant *Neisseria gonorrhoeae* (QRNG) isolates at Ser-87→Arg were associated with a higher level of resistance than a Ser-87→Ile alteration. Two previously undescribed mutations in clinical isolates occurring in *gyrA*, conferring Ala-92→Pro and Asp-95→Tyr changes, were detected. Nine different outer membrane lipoprotein (Lip) repeat classes—11 to 19 repeats—were identified, with repeat lengths of 16 and 17 the most common, indicating considerable strain diversity.**

In Hong Kong, a dramatic increase in quinolone-resistant *Neisseria gonorrhoeae* (QRNG) isolates has been observed (2–4). These made up 24% of our isolates in 1996 and 50% of our isolates in 1998 and then reached a high level of endemicity of 80% in 2000. All *N. gonorrhoeae* isolates possess an outer membrane lipoprotein, designated Lip, which was first revealed by a monoclonal antibody designated H.8. The protein epitope is immunogenic in humans and conserved among pathogenic *Neisseria* species, including *N. gonorrhoeae* and *N. meningitidis*, but not most nonpathogenic *Neisseria* species. We report on the current genetic alteration patterns within the quinolone resistance-determining region (QRDR) in GyrA and ParC proteins from organisms with various levels of quinolone resistance in Hong Kong and also examined these strains by Lip subtyping (1) and assessed whether Lip subtyping is useful in a situation in which QRNG isolates are occurring at high levels of endemicity.

One hundred six clinical strains of *N. gonorrhoeae* were drawn randomly from isolates obtained consecutively from patients attending government sexually transmitted disease (STD) clinics during the year 2001. These strains were tested by a standard methodology (5) and classified into four categories of ofloxacin susceptibilities: (i) MIC of ≥8 μg/ml, 27 isolates; (ii) MIC of ≥2 but ≤4 μg/ml, 45 isolates; (iii) MIC of ≥0.125 but <2 μg/ml, 28 isolates; and (iv) MIC of <0.125 μg/ml, 6 isolates. These were grouped according to previously published data, which correlated MIC with treatment outcome (4). Four World Health Organization (WHO) quality assurance strains (GC1, GC4, GC5, and 94G395) and two controls (F28 and F45) from the Centers for Disease Control and Prevention (CDC), Atlanta, Ga., were used because the ofloxacin MICs for these strains are known, although their molecular characters have not been previously described.

The amino acid substitutions in GyrA or ParC protein, based on the nucleotide sequences of *gyrA* and *parC* gene fragments that include QRDRs of the respective proteins were investigated by methods previously described (8–10). Nucleotide sequencing was performed by a D-rhodamine dideoxynucleotide chain termination method by using the ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Foster City, Calif.). Sequence compilation and analyses were carried out with DNAstar software programs (DNASTAR, Inc., Madison, Wis.). For alignment, *gyrA* and *parC* genes were compared with U08817 and U08907, respectively (T. Deguchi, M. Yasuda, M. Nakano, S. Ozeki, E. Kanematsu, Y. Kawada, T. Ezaki, and I. Saito, Letter, Antimicrob. Agents Chemother. **40:**2437-2438, 1996). The *lip* gene was amplified by PCR after extraction of the chromosomal DNA by DNAzol (Molecular Research Center, Inc., Cincinnati, Ohio). Amplicons were sequenced, and the primers were used as described previously (12). Profiles of known Lip-type patterns, kindly supplied by D. L. Trees (STD Branch, CDC), were used for alignment and analysis.

Forty-nine percent of patients acquired the strains locally, while the source of contact of the other patients could be traced to other Chinese provinces, including Macau, in 47% of our isolates. A minority of patients originated from the Philippines and Pakistan, which together accounted for 2%, while the source of the remaining 2% was unknown.

Out of 100 isolates, the ofloxacin MIC for 28 isolates was ≥0.125 μg/ml but ≤1 μg/ml, while the ofloxacin MICs for 17, 28, 14, and 13 isolates were 2, 4, 8, and ≥16 μg/ml, respectively. Table 1 shows the frequency distribution of various GyrA/ParC alteration patterns of quinolone-resistant strains against the respective ofloxacin MICs. Isolates for which the ofloxacin MIC was >0.06 but ≤1 μg/ml had 11 patterns, mainly possessing a single-site alteration in GyrA (64%). Simultaneous mutations at single sites in both *gyrA* and *parC* were mainly found in this category. In contrast, isolates for which the ofloxacin MICs ranged from 2 to ≥16 μg/ml did not possess alterations at a single site in QRDR. They had double and triple mutations in both *gyrA* and *parC*. Isolates for which the ofloxacin MIC was >1 μg/ml had 18 patterns, and 2 patterns predominated—GyrA (Ser91→Phe, Asp95→Gly) with ParC (Ser87→Arg) and GyrA (Ser91→Phe, Asp95→Gly) with ParC(ϕ) (where Φ represents the QRDR without mutation)—both in ParC, accounting for 24 and 21%, respectively. Isolates

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TABLE 1. Distribution of GyrA and ParC alteration patterns against respective ofloxacin MICs for sample isolates from Hong Kong in 2001[a]

| Ofloxacin MIC (μg/ml) | GyrA alteration | ParC alteration (frequency) | Total frequency |
|---|---|---|---|
| <0.1 | Φ | Φ (6) | 6 |
| 0.1–0.5 | Ser-91 → Phe | Φ (7), Glu91 → Ala (1), Glu91 → Gly (1) | 14 |
| | Ser91 → Tyr | Φ (4) | |
| | Asp95 → Asn | Φ (1) | |
| 1 | Ser91 → Tyr | Φ (4) | 14 |
| | Ser91 → Phe | Φ (2), Glu91 → Ala (1), Asp86 → Asn (1) | |
| | Ser91 → Phe and Asp95 → Gly | Φ (2), Glu91 → Gln (1) | |
| | Ser91 → Phe and Asp95 → Ala | Φ (1), Glu91 → Ala (1), Asp86 → Asn (1) | |
| 2 | Ser91 → Phe and Asp95 → Asn | Φ (3), Glu91 → Gly (1) | 17 |
| | Ser91 → Phe and Asp95 → Gly | Φ (3), Ser87 → Arg (2), Asp86 → Asn (1) Glu91 → Gln (1) | |
| | Ser91 → Phe and Asp95 → Ala | Glu91 → Ala (2), Φ (1), Ser87 → Asn (1) | |
| | Ser91 → Phe and Ala92 → Pro | Φ (1) | |
| | Ser-91 → Phe | Glu91 → Ala (1) | |
| 4 | Ser91 → Phe and Asp95 → Gly | Φ (8), Ser87 → Arg (5), Ser88 → Pro (1) | 28 |
| | Ser91 → Phe and Asp95 → Ala | Φ (7), Glu91 → Ala (4), Asp86 → Asn (1) | |
| | Ser91 → Phe and Asp95 → Tyr | Ser87 → Arg (1) | |
| | Ser91 → Phe and Asp95 → Asn | Φ (1) | |
| 8 | Ser91 → Phe and Asp95 → Gly | Φ (3), Ser87 → Arg (3), Asp86 → Asn (3) | 14 |
| | Ser91 → Phe and Asp95 → Ala | Ser87 → Arg (2), Glu91 → Ala (1) | |
| | Ser91 → Phe and Asp95 → Asn | Asp86 → Asn (2) | |
| 16 | Ser91 → Phe and Asp95 → Gly | Ser87 → Arg (8), Ser87 → Ile (1), Φ (1) | 13 |
| | Ser91 → Phe and Asp95 → Asn | Ser87 → Ile (1), Asp86 → Asn (1), Glu91 → Gln (1) | |
| Controls | | | 6 |
| <0.1 (GC1) | Φ | Φ | |
| <0.1 (F28) | Φ | Φ | |
| <0.1 (F45) | Φ | Φ | |
| 0.25 (GC5) | Ser91 → Tyr | Φ | |
| 8 (GC4) | Ser91 → Phe and Asp95 → Gly | Asp86 → Asn | |
| >8 (94G395) | Ser91 → Phe and Asp95 → Gly | Glu91 → Gly | |

[a] Φ represents QRDR without mutation. GC1, GC4, GC5, and 94G395 were QAP strains from WHO, while F28 and F45 were strains from CDC.

with higher-level resistance (MIC of ≥4 μg/ml) had an increased tendency toward three mutations in *gyrA* and *parC* ($P < 0.01$). The Ser-87→Arg alteration in ParC was associated with a higher level of resistance (MIC of ≥8 μg/ml) ($P < 0.001$). Two isolates containing the Ser-87→Ile substitution displayed the same high level of resistance (MIC of ≥16 μg/ml). Two new mutations among clinical isolates occurring in *gyrA*, conferring an Ala-92→Pro change (MIC = 2 μg/ml) and Asp-95→Tyr (MIC = 4 μg/ml) were detected. Double alterations in both GyrA and ParC were not found, although they have been reported elsewhere (6, 7, 10). Substitutions in *parC* alone were not found.

TABLE 2. Frequencies of Lip sequence subtypes in Hong Kong isolates in 2001

| Lip repeat class | Frequency of sequence subtype[a]: | | | | | | | | | | Frequency |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | a | b | c | d | e | f | g | h | i | j | |
| 11 | *0* | **1** | | | | | | | | | 1 |
| 12 | *1* | *4* | *0* | *0* | *0* | | | | | | 5 |
| 13 | *0* | *1* | *7* | **5** | | | | | | | 13 |
| 14 | *0* | *0* | *0* | *7* | *1* | **1** | **1** | **3** | **1** | **1** | 15 |
| 15 | *1* | *1* | *0* | *0* | **1** | **5** | **3** | **1** | | | 12 |
| 16 | *0* | *11* | *0* | *0* | *1* | *13* | | | | | 25 |
| 17 | *0* | *0* | *25* | **0** | **1** | **1** | | | | | 27 |
| 18 | *0* | **2** | **4** | | | | | | | | 6 |
| 19 | *0* | *0* | **2** | | | | | | | | 2 |
| 20 | *0* | | | | | | | | | | 0 |

[a] Numbers in italics represent subtypes provided and designated by D. L. Trees (CDC). Numbers in boldface represent new subtypes found in Hong Kong and not reported before.

Among the 106 quinolone-susceptible and -resistant isolates, 9 different Lip repeat classes were identified, ranging from 11 to 19 repeats, but repeat length 20 was not found (12). The respective ofloxacin susceptibility categories [i.e., (i) MIC of <0.125 μg/ml, (ii) MIC of ≥0.125 but <4 μg/ml, and (iii) MIC of ≥4 μg/ml] belonged to four (13 to 16 repeats), seven (12 to 18 repeats), and nine (11 to 19 repeats) Lip repeat classes, respectively. Repeat lengths of 16 and 17 were the most predominant in the current sample, comprising 23.6 and 25.5%, respectively. Even though they were predominant, they were found distributed in all ofloxacin MIC categories (except for the group for which an ofloxacin MIC was <0.125 μg/ml) as well as with the MICs of the other antibiotics tested, including penicillin, tetracycline, erythromycin, spectinomycin, and ceftriaxone (data not shown). After sequencing, the number of

TABLE 3. Lip subtypes in controls

| Controls | Lip subtype[a] |
|---|---|
| 94G395 | *13c* |
| GC1 | *14d* |
| F45 | *15b* |
| GC5 | **15e** |
| GC4 | *16a* |
| F28 | **17d**[b] |

[a] Numbers in italics represent subtypes provided and designated by D. L. Trees (CDC). Numbers in boldface represent new subtypes found in Hong Kong and not reported before.

[b] 17d is a new subtype, only found in control strain F28, but not in isolates from Hong Kong.

subtypes has been extended to 28 in Hong Kong and up to 48 when the patterns from D. L. Trees (CDC) and the control strain F28 were included. The 14-repeat class was most diversified and consisted of seven subtypes (Tables 2 and 3). The use of Lip protein typing revealed that QRNG isolates are polyclonal in Hong Kong.

Based on the sequence differences in the repeats that resulted in amino acid alterations, the nine repeat patterns could be further classified into 28 subtypes. There were three subtypes in the proportion of 13:11:1 in the 16-repeat class and three subtypes in the proportion of 25:1:1 in the 17-repeat class as shown in Table 4. Repeat classes 11 and 19 had a low occurrence, and it is probable that these are more conserved during the course of evolution of quinolone resistance, although there is no genetic linkage between *lip*, *gyrA*, and *parC*.

A variety of QRDR patterns were present in Hong Kong, and the most prevalent ones were GyrA (Ser91→Phe, Asp95→Gly) with ParC (Ser87→Arg) (25%) and GyrA (Ser91→Phe, Asp95→Gly) with ParC (ϕ) (21%) in ParC. In a recent report, the former alteration pattern was also present in a single strain isolated from mainland China (6). Further studies will be needed to compare cross-country differences, as well as longitudinal changes, in QRDR patterns among QRNG strains isolated from different localities in the region.

Silent mutations in codons 104 (TAT to TAC), 129 (GCG to GCA), and 131 (CTC to CTG) of *parC* genes were detected. The former two did not occur in the absence of the latter. These silent mutations occurred in 75% of QRNG strains, but there was no association between these mutations and the alterations in QRDR of GyrA and ParC.

The ParC alterations reported to date have occurred in amino acids Gly-85, Asp-86, Ser-87, Ser-88, Glu-91, Ala-92, and Arg-116 (8, 10). The Asp86→Asn alteration was the most prominent pattern seen in the Philippines (11). Ser88→Pro was more commonly found in Japan (8, 9), while Ser87→Ile, which confers high-level resistance (MIC of ≥16 μg/ml), was reported in Hawaii, the Philippines, and Japan (11, 13; Deguchi et al., Letter). In Hong Kong, since ofloxacin had been used as the first-line drug for a long period of time, and given the large numbers of travelers passing through from other countries, we have now seen the emergence of a wide variety of alterations in *N. gonorrhoeae*, and types have been identified that are different from those previously described.

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TABLE 4. Amino acid sequences of subtypes in 16-repeat class and 17-repeat class in Hong Kong isolates

| Repeat class[a] | Sequence of repeat no.: 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | No. of cases |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 repeat | | | | | | | | | | | | | | | | | | |
| 16f | AAEAP | AAEAS | STEAP | AAEAP | AAEAP | AAEAP | AAEAP | AAEAA | ATEAP | AAEAA | ATEAP | AAEAA | ATEAP | AAEAP | AAEAP | AAEAA | | 13 |
| 16b | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----P | -A--- | ----P | -A--A | -T---P | -A--A | -T--- | ----- | ----- | | 11 |
| 16e | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----P | -A--A | -T--P | -A--A | -T---P | -A--P | ----- | ----- | ----- | | 1 |
| 17 repeat | | | | | | | | | | | | | | | | | | |
| 17c | AAEAP | AAEAS | STEAP | AAEAP | AAEAP | AAEAP | AAEAP | AAEAP | AAEAP | AAEAP | AAEAP | AAEAA | ATEAP | AAEAA | ATEAP | AAEAP | AAEAA | 25 |
| 17e | ----- | ----- | ----- | ----- | ----- | ----- | ----A | -T--- | ----A | -T--- | ----A | -T---P | -A--- | ----P | -A--- | ----- | ----- | 1 |
| 17f | ----- | ----- | ----- | ----- | ----- | ----- | ----A | -T--- | ----- | ----A | -T---P | -A--A | -T--P | -A--- | ----- | ----- | ----- | 1 |

[a] 17*e* and 17*f* are new subtypes.

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