Real-Time PCR Detection of gyrA and parC Mutations in Streptococcus pneumoniae

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Fluoroquinolone resistance in Streptococcus pneumoniae mainly involves stepwise mutations predominantly in the parC and gyrA genes. We have developed a single-run real-time PCR assay for detection of the four most common mutations in the quinolone resistance-determining regions of these genes. This assay provides a useful tool for both clinical and epidemiological use.

Streptococcus pneumoniae is a major cause of community-acquired infections ranging from otitis media to pneumonia or meningitis (12). Strains resistant to multiple antibiotics have recently been reported and pose a risk of therapeutic failure (1). This observation has encouraged the use of newer fluoroquinolones against pneumococci (8) and has thus led to the emergence of fluoroquinolone-resistant clones. Prevalence rates have increased in Canada and have reached 14.3% in Hong Kong, while they have remained below 1 to 2% in the United States and Europe (3). The development of resistance to fluoroquinolones in pneumococci is a stepwise mutational process (7) that mainly occurs in the quinolone resistance-determining regions (QRDRs) of the parC and gyrA genes (11). Several reports have noted that a significant proportion of isolates harboring first-step mutations had low or no phenotypic expression but had the potential to develop higher levels of resistance to fluoroquinolones when they suffered a second mutation, resulting in treatment failure (10). This evolution implies that microbiologists must be able to detect first-step, or “preresistant,” mutants. The use of a nonmolecular test implies that microbiologists must be able to detect first-step, or “preresistant,” mutants. The use of a nonmolecular test scheme for the detection of low-level resistance has been proposed, but this type of test lacks sensitivity and specificity (13). A powerful and highly specific technique for the detection of mutations within a DNA sequence is real-time PCR with fluorescent resonance energy transfer (FRET) hybridization probes. We report on the development of a real-time PCR assay that uses FRET probes for the single-run detection of the four most common resistance mutations within the QRDRs of the parC and gyrA genes of S. pneumoniae.

The S. pneumoniae strains were isolated, cultured, and identified as described previously (9). A total of 58 strains whose parC and gyrA genes had been sequenced previously were studied. They consisted of strain R6 and 3 R6 derivatives kindly provided by E. Varon (13), 10 mutants generated in vitro, reference strains CIP104485 and CP 1000, and 42 clinical isolates (37 from New Caledonia, 3 from Australia, and 2 from Tahiti). Table 1 summarizes the known mutations, the results of disk agar diffusion (Bio-Rad), and the MICs determined by agar dilution, as described previously (13). DNA was extracted with a QIAamp DNA mini kit (Qiagen). The primers and probes were designed by using LightCycler probe design software (version 2) and were ordered from Proligo Singapore Pty. Ltd. The wild-type sequences of gyrA (GenBank accession number DQ175176) and parC (GenBank accession number DQ176507) were used for oligonucleotide design. By using the parC sequence, an LC-Red705-labeled sensor probe was designed to detect the Ser79Tyr (C-to-A substitution) and the Asp83Ala (A-to-C substitution) mutations. On the basis of the gyrA sequence, the LC-Red640-labeled probes were designed to detect the Ser81Phe (C-to-T substitution) and the Glu85Lys (G→A substitution) changes. Probes for gyrA were designed so that each probe covered a mutation (Ser81 or Glu85) and had the same melting temperature ($T_m$), thus behaving as an anchor for the other probe. Table 2 summarizes the sequences of the primers and probes, the concentrations, and the reaction conditions. The primers led to 219-bp and 209-bp amplitons for gyrA and parC, respectively, which allowed the detection of mutations in gyrA and parC in a single run in 20-μl capillaries of a LightCycler (version 2.0) instrument. The parC-specific primers were optimized at unequal concentrations to increase the fluorescence and allow better discrimination of the peak $T_m$s (2).

All S. pneumoniae strains had clearly distinguishable melting curves for both gyrA and parC (Fig. 1). Wild-type parC had a $T_m$ of 65.7°C, and mutants with single Ser79 or Asp83 mutations had $T_m$s in the range of 62.1 to 63.0°C; the mutant with the Asp83Gly mutation, however, had a $T_m$ of 64.8°C, which was still significantly lower than the $T_m$s of the wild-type genes. The $T_m$s were highly reproducible. The mutant with the double Ser79-Asp83 parC mutation had a $T_m$ of 47.1°C. Wild-type gyrA had $T_m$s greater than 67°C, and the $T_m$s of all mutants were less than 65.5°C. The mean $T_m$ for wild-type gyrA was 68.2°C, that for the mutant with a single Ser81 gyrA mutation was 62.0°C, and that for the mutant with a single Glu85 gyrA mutation was 64.2°C. The mutant with a double Ser81-Glu85 gyrA mutation had an intermediate $T_m$ of 63.3°C. The results real-time PCR with FRET probes obtained for the clinical...
isolates were in agreement with the sequencing data: all strains tested except strain H548 were wild type. Strain H548 was a mutant with a single Ser81Phe mutation (9) (Table 1).

The rapid detection of gyrA and parC mutations in a single run represents a faster and more reliable approach than the current phenotypic method (13), which is unable to detect S. pneumoniae strains that have a greater potential than wild-type strains to evolve toward resistance.

The design of the set of parC probes followed the current recommendations for sensor and anchor probe design, which states that there should be a minimum difference between $T_m$ of 3 to 5°C. Use of the same recommendations for the design

### TABLE 1. Susceptibilities of S. pneumoniae strains to quinolonesa

| Strain          | QRDR mutation | Inhibition zone diam (mm) | MIC (µg/ml) |
|-----------------|---------------|---------------------------|-------------|
|                 |               | NAL | CIP | NOR | PEF | SPX | LVX | MXF | NAL | CIP | NOR | PEF | SPX | LVX | MXF |
| CIP104485       | None          | 6  | 25 | 19 | 18 | 24 | 22 | 26 | 4.0 | 8.0 | 1.0 | 0.25 | 1.0 | 0.125 |
| R6              | None          | 6  | 21 | 19 | 17 | 23 | 21 | 26 | 4.0 | 8.0 | 1.0 | 0.25 | 1.0 | 0.125 |

*Abbreviations: Nalidixic acid; norfloxacin; pefloxacin; Pefloxacin; LVX; levofloxacin; MXF, moxifloxacin; NAL, nalidixic acid; NOR, norfloxacin; PEF, pefloxacin; SPX, sparfloxacin; ND, not done.

### TABLE 2. Primers, probes, and reaction conditions for detection of QRDR mutationsa

| QRDR and oligonucleotide | Sequence (5’ to 3’) | Position | Wild-type—mutant amino acid, nucleotide (nucleotide nos.)b | Oligonucleotide concn (µM) |
|--------------------------|---------------------|----------|--------------------------------------------------------|---------------------------|
| gyrA QRDR                |                     |          |                                                       |                           |
| gyrA-F (forward primer)  | GTTCAACGTCGATCTCT   | 124–140  |                                                       | 0.5                       |
| gyrA-R (reverse primer)  | CCCATGACCATCTCAAGC  | 315–297  |                                                       |                           |
| gyrA-P1 (sensor or anchor probe) | TATCACCACACGCGGTCTCTCTTACAT- | 223–248 | Ser81→Asp, TCC→TTC (241-242-243) |                           |
| gyrA-P2 (sensor or anchor probe) | LC Red 640-ATGAAGCCATGCTGCTATGCCG-phosphate | 251–272 | Glu85→Lys, GAA→AAA (253-254-255) | 0.2                       |
| parC QRDR                |                     |          |                                                       |                           |
| parC-F (forward primer)  | CTTTATCTATGAATAAGGATAGAACTACT | 115–144 | Ser79→Tyr, TCT→TAT (217-218-219) | 1                          |
| parC-R (reverse primer)  | GCAGAGGAGATCCGTC     | 307–323  | Asp83→Ala, GAT→GCT (229-230-231) | 0.5                       |
| parC-P1 (anchor probe)   | CGATTTTTCTCAGTCGTAGACATAGAC  | 235–266 | Ser79→Tyr, TCT→TAT (217-218-219) | 0.1                       |
| parC-P2 (sensor probe)   | LC Red 705-CATCATAGATAGAAGAATCCC GTTGCG-phosphate | 205–232 | Ser79→Tyr, TCT→TAT (217-218-219) | 0.1                       |

*For the reactions with both the gyrA and parC QRDRs, 3 mM Mg2+ was used and the reaction mixtures were run for 40 cycles of 95°C for 8 s, 60°C for 5 s, and 72°C for 9 s. The melting conditions were 95°C for 5 s and 55°C for 15 s, with a ramp to 95°C at 0.2°C s−1.
bBoldface indicates the mutated nucleotide.
of the gyrA probes, however, did not yield satisfactorily distinguishable melting curves between mutant and wild-type strains. We therefore designed the probes for gyrA with identical $T_m$s so that each probe covered one mutation (Ser81 or Glu85) and behaved as an anchor for the other; thus, only the wild-type strain had a perfect complementary match. To the best of our knowledge, this is the first report of the use of such a strategy to successfully detect mutations lying in close proximity. Since a single probe pair is required for the detection of two putative mutations, this avoids the risk that sets of probes will overlap or that dimerization, which would require the use of two capillaries, will occur.

The limitations of the assay, as is the case with all sequence-specific techniques, are that mutations outside the sequence covered by the sensor probe escape detection and that resistance resulting from a mutation in another gene, e.g., parE or gyrB, or other mechanisms, such as efflux, also remains undetected. However, although these mechanisms tend to confer resistance, they are less well described for high-level resistance than for the resistance conferred by double parC and gyrA mutations.

The assay could be useful as an alternative to DNA sequencing for the screening of resistance in epidemiological surveys and is more sensitive for estimation of the prevalence of resistance than phenotypic screening. We have confirmed that nine isolates considered levofloxacin susceptible according to the CLSI breakpoint (4 μg/ml) by the phenotypic test (4) and five isolates not detected by the nonmolecular test for the detection of low-level resistance to fluoroquinolones by Varon et al. (13) possessed single QRDR mutations. Although our assay is not as accurate as DNA sequencing for the determination of the precise genotype of the mutant strains, it is cheap, fast, and accurate for the detection of the most common QRDR mutations and allows rapid testing if a clinical isolate has a wild type or a "non-wild-type" gyrA and parC QRDR genotype.

Recently, Fukushima et al. (6) also reported the use of melting curve analysis for the detection of QRDR mutations in S. pneumoniae. They used two pairs of probes for the detection of the Ser79 and Asp83 mutations in ParC and two pairs of probes for the detection of the Ser81 and Glu85 mutations in GyrA. The sensitivity and specificity of our assay allow the direct detection of mutations in clinical isolates, rendering the assay useful for the establishment of timely therapeutic decisions. Moreover, the design of our hybridization probes for the detection of the two mutations in GyrA with a single probe pair indicates that in a single run and with a single capillary, all types of gyrA mutants (mutants with a single Ser81 mutation, a single Glu85 mutation, and double ser81 and Glu85 mutations) can be detected.

Decousser et al. (5) described an assay that allows the clear identification of Ser79 or Asp83 parC mutations in S. pneumoniae with a single capillary and two locked nucleic acid probes (TaqMan format), with each probe matching a mutated sequence; mutated strains are identified on the basis of the absence of fluorescence with the corresponding probe. However, due to the absence of an internal control, mutants with double Ser79 and Asp83 mutations are characterized on the basis of the lack of fluorescence, similar to what would be observed with a negative control or PCR inhibition. By using another FRET technology with hybridization probes (Hyb-Probe format), our assay yields detectable amplification whatever the genotype of the strain studied is. Additionally, our assay also allows genotyping of gyrA, which is the other major gene implicated in quinolone resistance in S. pneumoniae.

In conclusion, our assay is a two-capillary, single-run, easy and rapid technique that detects the major gyrA and parC QRDR mutations associated with quinolone resistance in S. pneumoniae and can be used for both surveillance and treatment regimen decision-making purposes.

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