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Interspecies Recombination Occurs Frequently in Quinolone Resistance-Determining Regions of Clinical Isolates of *Streptococcus pyogenes*▽

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Fluoroquinolone resistance in *Streptococcus pyogenes* has been reported only anecdotally, but a recent Belgian surveillance study found a rate of nonsusceptibility of 5.4%. From an analysis of these isolates, we show that interspecies horizontal gene transfer within the *parC* quinolone resistance-determining region is a frequent phenomenon that might contribute to fluoroquinolone resistance.

Streptococcus pyogenes, also known as the group A streptococcus (GAS), is a major human pathogen that causes a variety of respiratory tract and skin infections, ranging from asymptomatic colonization to invasive disease and postinfection sequelae. According to estimates, about 600 million cases of pharyngitis, 1.8 million new cases of severe disease, and 500,000 deaths occur each year worldwide (2).

The rates of resistance to fluoroquinolones are still low (1), but fluoroquinolones are commonly used to treat respiratory tract infections and the extensive prescription of these drugs may increase resistance rates (3). A recent Belgian surveillance study found a surprisingly high prevalence of nonsusceptibility of 5.4% (ciprofloxacin MIC ≥ 2 μg/ml) among nearly 2,800 isolates from patients with tonsillopharyngitis (6).

The fluoroquinolones inhibit the enzymes DNA gyrase and topoisomerase IV, which are essential for bacterial replication. The two enzymes are heterotetramers consisting of GyrA-GyrB and ParC-ParE subunits, respectively. Fluoroquinolone resistance is mediated mainly by point mutations in the target enzymes, especially in GyrA and ParC, whereas mutations in GyrB and ParE seem to be less relevant. These mutations tend to cluster in the so-called quinolone resistance-determining region (QRDR) and can arise spontaneously or can be acquired by horizontal gene transfer from the same species (intraspecies recombination) or from a different species (interspecies recombination).

Stanhope and colleagues have shown that both types of horizontal gene transfer occur frequently in *Streptococcus pneumoniae* and are involved in the spread of fluoroquinolone resistance (8). In contrast, the contribution of horizontal gene transfer to fluoroquinolone resistance in GAS is uncertain. A recent work detected horizontal gene transfer from *Streptococcus dysgalactiae* subsp. *dysgalactiae* within the *parC* QRDR of an individual fluoroquinolone-resistant GAS isolate (7).

The objectives of the present study were to reveal the frequency of horizontal gene transfer between *Streptococcus dysgalactiae* subsp. *dysgalactiae* and GAS and to estimate its contribution to the fluoroquinolone resistance emerging in GAS.

The study. Sixty-six isolates previously characterized for resistance mechanisms during the aforementioned Belgian surveillance study were included in this analysis. Forty-six isolates (69.7%) were resistant to fluoroquinolones (defined by a ciprofloxacin MIC of ≥2 μg/ml), and 20 isolates (30.3%) were susceptible to fluoroquinolones.

The ParC sequences of 43 strains (30 resistant and 13 susceptible strains) exhibiting a D91N amino acid substitution were analyzed for signs of interspecies recombination. This amino acid substitution was used as a surrogate marker of horizontal gene transfer for three reasons: (i) it was observed to be a feature of *Streptococcus dysgalactiae* subsp. *dysgalactiae* in the case described by Pletz et al. (7), (ii) it is most likely not involved in fluoroquinolone resistance because it can be found in both susceptible and resistant isolates, and (iii) the vast majority of GAS *parC* sequences found in the GenBank database do not exhibit this substitution.

Twenty-three isolates (16 resistant and 7 susceptible isolates) from the same study without the D91N amino acid substitution were included for comparison. The *parC* QRDR sequences were analyzed by previously described methods (7).

First, a search of the sequences in the GenBank database was done with the BLAST program and yielded more than 36 close matches for every query sequence. The first 30 hits with a high bit score and a low E value were selected. Among these were sequences of the type strains *Streptococcus dysgalactiae* subsp. *dysgalactiae* GTC 431 (GenBank accession number GI 37999015), *Streptococcus dysgalactiae* subsp. *equisimilis* GTC 842 (GenBank accession number GI 37999017), and *Streptococcus canis* GTC 423 (GenBank accession number GI 37999019).

Second, alignment of the query sequences and the 30 closest hits obtained with the BLAST program was conducted with the

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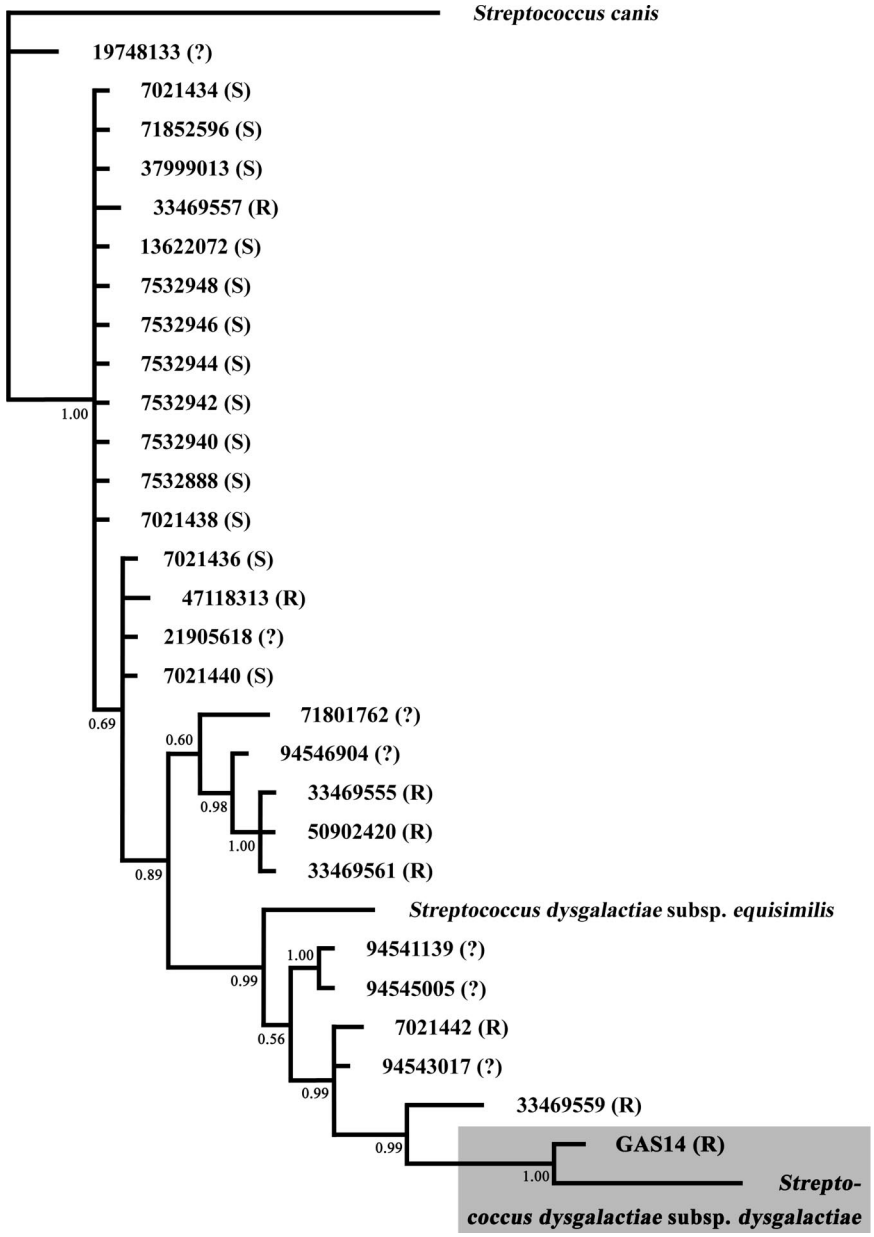

FIG. 1. The phylogenetic tree is based on the *parC* QRDR sequences, was constructed with the MrBayes program (version 3.0B4), and was rooted with *Streptococcus canis* as the outgroup. All *Streptococcus pyogenes* sequences are designated with their respective GenBank identifiers. Susceptibility to fluoroquinolones is indicated in parentheses, as follows: R, resistant; S, susceptible; ?, not found in the literature. Horizontal gene transfer between *Streptococcus pyogenes* GAS14 and *Streptococcus dysgalactiae* subsp. *dysgalactiae* is strongly suggested by the small distance and the highest possible posterior probability of the branching (posterior probability, 1.0).

ClustalX program (version 1.8) (9). On the basis of this alignment (369 bp, codons 35 to 157), a phylogenetic analysis was carried out for each isolate by using the Bayesian method implemented by use of the program MrBayes (version 3.0B4) (4). The resulting trees were rooted by using the sequence with the lowest similarity (*Streptococcus canis*) as the outgroup.

The posterior probability of a phylogenetic tree is its probability given the observed data. It cannot be calculated exactly, but it can be estimated by sampling millions of trees created through Markov chain Monte Carlo algorithms. This process starts with a random tree and proposes changes that are accepted or rejected until an equilibrium distribution of the sampled posterior probabilities is reached.

Horizontal gene transfer can be inferred from a phylogenetic gene tree when the sequences of different species appear to be more closely related than expected with regard to species similarity because recombination makes them share genes. If the GAS query sequence was grouped with another species and the posterior probability of this branching was greater than 0.9, horizontal gene transfer was assumed.

Subsequently, for those isolates, the assumed interspecies recombination was verified by a statistical test (the maximum

chi-square test included in the START package, version 1.0.8 [5]) on the basis of the aligned parental sequences (426 bp, codons 16 to 158). To improve reproducibility and comparability, a majority consensus of all GAS *parC* reference sequences found in GenBank (accession numbers GI 94993396, GI 94991497, GI 94989509, GI 94987631, GI 71902667, GI 21909536, GI 19745201, GI 15674250, and GI 71909814) was calculated with the MegAlign program (version 5.00) and was used as the parental GAS sequence for all isolates.

Results and conclusions. The phylogenetic analysis revealed that 15 GAS isolates (7 isolates resistant to fluoroquinolones and 8 isolates susceptible to fluoroquinolones) were grouped with *Streptococcus dysgalactiae* subsp. *dysgalactiae*. An example is shown in Fig. 1. In all cases, the high posterior probability of 1.00 indicated that the real probability of this branching given the sequences is 100% and therefore strongly suggested interspecies recombination. The maximum chi-square test confirmed this hypothesis at a statistically significant ($P < 0.05$) or highly significant ($P < 0.001$) level.

Accordingly, in our sample the frequency of interspecies horizontal gene transfer within the *parC* QRDR of *Streptococcus pyogenes* was 22.7%, and in all cases the putative donor was *Streptococcus dysgalactiae* subsp. *dysgalactiae*. The GAS isolates involved in horizontal gene transfer belong to different *emm* types and pulsed-field gel electrophoresis clusters. These findings exclude mere clonal expansion as an explanation for the unexpectedly high frequency.

The ParC D91N amino acid substitution could be validated as a surrogate marker of interspecies horizontal gene transfer in *Streptococcus pyogenes*, since none of the 23 isolates lacking the mutation presented horizontal gene transfer, whereas 15 of 43 isolates (34.9%) featuring the mutation presented horizontal gene transfer. A closer look at the sequences revealed that, besides silent mutations (mostly at the third codon position), another amino acid substitution indicated horizontal gene transfer from *Streptococcus dysgalactiae* subsp. *dysgalactiae*. The ParC S140P amino acid substitution was present in all sequences with proven horizontal gene transfer, irrespective of the fluoroquinolone resistance or susceptibility of the isolate. The latter finding suggests that this substitution does not confer fluoroquinolone resistance.

The frequency of horizontal gene transfer and its role in the emergence of fluoroquinolone resistance in the entire GAS population are difficult to quantify, since for that purpose a more representative sample and additional sequence data from GenBank are needed. Nevertheless, we could generate evidence that interspecies horizontal gene transfer occurs frequently in the *parC* QRDR of *Streptococcus pyogenes* and is consequently able to contribute to fluoroquinolone resistance.

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