An extended PCR-RFLP assay for detection of parC, parE and gyrA mutations in fluoroquinolone-resistant Streptococcus pneumoniae

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Sir,

In Gram-positive cocci, fluoroquinolone resistance is associated with mutational alterations in DNA gyrase and/or topoisomerase IV or with active efflux of the drugs.

In Streptococcus pneumoniae, fluoroquinolone resistance is mainly due to substitutions in ParC (Ser-79→Phe or Tyr, Asp-83→Asn), ParE (Asp-435→Asn) or GyrA (Ser-81→Phe or Tyr, Glu-85→Lys or Gly).1,2 Therefore, detection of these substitutions is important for the understanding of the mechanisms of resistance to fluoroquinolones and for epidemiological studies of transmission and spread of resistant strains.

A PCR-RFLP assay has been developed by Pan et al.1 for detection of point mutations within the parC (Ser-79 codon) and gyrA (Ser-81 codon) genes of S. pneumoniae. Detection included amplification of the quinolone resistance-determining regions (QRDR) of the corresponding gene and digestion of the PCR product by restriction enzymes. In this study, we report an extension of this assay to detect mutations within the Ser-79 and Asp-83 codons of parC, the Asp-435 codon of parE and the Ser-81 and Glu-85 codons of gyrA genes in S. pneumoniae which lead to decreased susceptibility to fluoroquinolones.

S. pneumoniae CP1000 and 25 in vitro or in vivo-generated resistant mutant derivatives, which were studied for mutations in the QRDR of the parC, parE and gyrA genes by sequencing of the PCR products, were used to validate the assay.

A 366 bp parC and a 290 bp parE region encompassing the QRDR were amplified with already described primers.2 Similarly, a 183 bp gyrA region containing the QRDR was amplified using primers PnGyrA-F′-TTCACCGTGCCATTCTCTACGGA-3′ and PnGyrA-R′-CATCTACAAGCATGTAACGGTAGCTCCACCATTGAGCATACGGACCATGTC-3′. Primer PnGyrA-R′ was adjacent to the Glu-85 codon and differed by one base (underlined) from the gene sequence to generate a MboII recognition site. Mutations within parC were detected using HinII (Ser-79 codon) and Lwel (Asp-83 codon), those within gyrA by HinII (Ser-81 codon) and MboII (Glu-85 codon) and that (Asp-435 codon) in parE with HinII. Restricted DNA was analysed by electrophoresis on a 3% agarose gel.

The 366 bp parC product from susceptible CP1000 contained two HinII and one Lwel recognition sites generating fragments of 183, 127 and 56 bp, and of 224 and 142 bp, respectively. Loss of a HinII site in resistant isolates following mutations at the Ser-79 codon generated two 183 bp fragments that ran as a doublet. Mutations at the Asp-83 codon suppressed the Lwel site and a 366 bp fragment was observed.

The 290 bp parE product from the susceptible strain contained two HinII sites generating 166, 87 and 37 bp fragments. Loss of one of the HinII sites in the resistant isolates following mutations at the Asp-435 codon generated fragments of 203 and 87 bp.

The 183 bp gyrA product from parental CP1000 contained a natural HinII site (Ser-81 codon) and an artificially created MboII site generating fragments of 113 and 70 bp, and 141 and 42 bp, respectively. Loss of the HinII site in resistant isolates due to mutations at the Ser-81 codon led to a 183 bp fragment. Mutations at the Glu-85 codon were associated with the loss of the MboII site leading also to a 183 bp fragment.

Mutations resulting in amino acid changes at Ser-81 and/or Glu-85 in GyrA are often associated with decreased susceptibility to quinolones in S. pneumoniae.1 The mutated site in the Ser-81 codon is part of a naturally occurring HinII restriction site; thus mutations are detected when HinII fails to digest the PCR product, as analysed by electrophoresis in agarose gel.1 However, mutations within the Glu-85 codon do not generate any restriction site. To detect mutations in the corresponding codon, we introduced a base substitution near the mutated locus to create an artificial MboII cleavage site using the primer-specified restriction site modification method.3 The DNA fragment amplified from the wild-type gyrA gene had two naturally occurring HinII restriction sites in the Ser-81 codon and an artificially created MboII cleavage site in the Glu-85 codon. Mutations in the Glu-85 codon can thus be detected by digesting the amplified DNA by MboII. This modified PCR-RFLP method has been used successfully to screen for mutations in the gyrA gene of Neisseria gonorrhoeae4 and Escherichia coli.5

The extended PCR-RFLP assay described here is simple, rapid and can be carried out in a diagnostic laboratory as a routine assay. However, this method has several limitations including the inability to assess the type of nucleotide substitution or to screen for mutations occurring at other positions in the gene. It has been shown that there is little correlation between specific mutations in type II topoisomerase genes and phenotypic susceptibility of the host to the most frequently used fluoroquinolones.6 Nevertheless, the present study provides data suggesting that this assay could be a useful screening tool for mutations and to facilitate epidemiological studies of decreased susceptibility to fluoroquinolones in clinical isolates of S. pneumoniae.

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References

Sir,

As a part of our research into the diversity of bacterial agents associated with amoebae in hospital water supplies, we have previously identified new α-Proteobacteria belonging mostly to Afipia and Bosea genera. As it has been established that Afipia is responsible for cat scratch disease and nosocomial osteitis, and in addition we have demonstrated that patients with nosocomial pneumonia hospitalized near contaminated water in a public hospital in our city had elevated antibody titres to these bacteria and that seroconversion to near contaminated water in a public hospital in our city had elevated antibody titres to these bacteria and that seroconversion to intensive care unit. As few data about the antibiotic susceptibility of this group of bacteria are available, we tested 24 antibiotics including the new compound telithromycin (HMR 3647).

Strains and antibiotics used in the study are presented in Table 1. Antibiotics were provided by the different antibiotic manufacturers. Strains were grown for 72–96 h on BCYE agar before testing. Antibiotic susceptibility testing was carried out using a micro broth dilution method in nutrient broth. For the testing of co-trimoxazole, 5% lysed horse blood was added. The final inoculum for all broth tests ranged from 1 × 10^3 to 5 × 10^3 cfu/mL. The plates were incubated at 30°C and read between 72 and 96 h. As there is no standard method to test antibiotic susceptibility of these fastidious bacteria, Escherichia coli (ATCC 25922) and Enterococcus faecalis (ATCC 29212) tested under the same conditions, were used as controls.

MICs results are summarized in Table 1. The results of MICs observed for controls are in accordance with those obtained using the NCCLS guidelines. In this study, most of the strains had high MICs to all antibiotics tested and none of the antibiotics tested was efficient against all strains. This work confirms that Afipia felis is a bacterium resistant to almost all antibiotics. In contrast, rifampicin, tobramycin and imipenem were more effective against the phylogenetically homogeneous group of Afipia, except for the three Afipia genospecies. Doxycycline, the sole antibiotic with low MICs, was effective against all Bosea species. In all cases, MICs of levofloxacin were equal to or lower than the MICs of ciprofloxacin against all the species of Afipia, except Afipia birgiae and Afipia massiliensis. However, none of the MICs observed were lower than 2 mg/L for this antibiotic. Telithromycin was effective against B. massiliensis and Afipia clevelandensis with MICs ≤0.25 mg/L, but the MICs were high for other species.

Most antibiotic regimens currently proposed for patients with hospital-acquired pneumonia would be ineffective owing to these agents having high MICs against most Bosea and Afipia species. The use of imipenem, alone or in combination with an aminoglycoside such as tobramycin would be the most appropriate regimen, except for Bosea species that should be treated with doxycycline or telithromycin in the case of B. massiliensis-related infection. Further studies are needed to evaluate the true prevalence of these bacteria in cases of hospital-acquired pneumonia as their response to current antibiotic protocols is likely to be poor.

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References


Comparison of the in vitro efficacy of telithromycin (HMR 3647) and levofloxacin with 22 antibiotic compounds against Bosea and Afipia species

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As a part of our research into the diversity of bacterial agents associated with amoebae in hospital water supplies, we have previously identified new α-Proteobacteria belonging mostly to Afipia and Bosea genera. It has been established that Afipia is responsible for cat scratch disease and nosocomial osteitis, and in addition we have demonstrated that patients with nosocomial pneumonia hospitalized near contaminated water in a public hospital in our city had elevated antibody titres to these bacteria and that seroconversion to Bosea massiliensis was significantly associated with pneumonia in the intensive care unit. As few data about the antibiotic susceptibility of this group of bacteria are available, we tested 24 antibiotics including the new compound telithromycin (HMR 3647).  

Antibiotics were provided by the different antibiotic manufacturers. Strains were grown for 72–96 h on BCYE agar before testing. Antibiotic susceptibility testing was carried out using a micro broth dilution method in nutrient broth. For the testing of co-trimoxazole, 5% lysed horse blood was added. The final inoculum for all broth tests ranged from 1 × 10^3 to 5 × 10^3 cfu/mL. The plates were incubated at 30°C and read between 72 and 96 h. As there is no standard method to test antibiotic susceptibility of these fastidious bacteria, Escherichia coli (ATCC 25922) and Enterococcus faecalis (ATCC 29212) tested under the same conditions, were used as controls.

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