Application of a multiplex \( pbp2b \) and \( pbp2x \) PCR for prediction of penicillin resistance in \( S. pneumoniae \)

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Sir,

In \( S. pneumoniae \), penicillin binding proteins (PBPs) 2b and 2x are the primary targets for penicillin. Hence, they are the first PBPs to be altered following mutations that lead to the development of penicillin resistance. Within the transpeptidase-encoding regions of the \( pbp2b \) and \( pbp2x \) genes, a few specific nucleotide changes have been shown to be common to penicillin-resistant pneumococci from many parts of the world. Polymerase chain reaction (PCR) primers have been developed to specifically amplify these mutant alleles. The primers are designed to be complementary to the mutant sequence, such that a PCR product is only produced if the mutation is present in the gene.\(^2\),\(^3\) However, these previously developed PCR assays are limited to only detecting mutations in one of the \( pbp \) genes, and/or they require two or more separate PCR reactions to accommodate tests for all the resistance alleles.

In this study, we report the evaluation of a one-tube, multiplex PCR reaction for detection of \( pbp2b \) and \( pbp2x \) mutations associated with penicillin resistance in \( S. pneumoniae \). The primers used in the reaction are R1 to R4, P5 and P6, specific for \( pbp2b \), and Pn-RX1 and Pn-RX2 for \( pbp2x \). The sequences of these primers have been reported previously.\(^2\),\(^3\) Possible PCR products have sizes (in bp) of 183 (primer pair Pn-RX1 and Pn-RX2), 214 (primer pair R4 and P6), 328 (primer pair R2 and P6), 331 (primer pair R1 and P6), 334 (primer pair R3 and P6) and 682 (primer pair P5 and P6—used as a PCR control).

To test the multiplex PCR assay, a collection of 383 nasopharyngeal isolates of \( S. pneumoniae \) obtained from among 1978 children aged less than 6 years attending 79 day care centres and kindergartens in all areas of Hong Kong was tested. The penicillin MICs of the isolates were determined by the Etest method. The 40 µL optimal PCR reaction comprised 2.2 mM MgCl\(_2\), dNTP at 200 µM, 1 U of AmpliTaq Gold in 1× PCR buffer (Applied Biosystems, Foster City, CA, USA), primers (P5 and P6 at 1 µM; R1 to R4 at 0.1 µM; PnRx1 and PnRx2 at 0.2 µM) and 4 µL of DNA template (generated by rapid alkaline lysis of cells). PCR was carried out using the following conditions: an initial activation at 95°C for 5 min, then 10 cycles of touchdown PCR (denaturation at 95°C for 1 min, annealing for 1 min starting at 65°C in the first cycle and decreasing by 1°C for each of the subsequent nine cycles, followed by extension for 1 min at 72°C) followed by 20 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Pneumococcal isolates R6 (no mutation), ATCC 700669 Spain\(^{2-3}\) (mutations known to result in 183 and 334 bp bands), ATCC 700670 Spain\(^{2-3}\) (mutations known to result in 183 and 328 bp bands) and a clinical isolate G111 (mutations known to result in 214 and 328 bp bands) (mutations in the strains were confirmed by sequencing in our laboratory, R. C. W. Wong & P. L. Ho unpublished data) were used as controls in the PCR.

Overall, 161 (42%) of 383 isolates were shown to be susceptible to penicillin, 122 (31.9%) were intermediate and 100 (26.1%) were resistant. The MICs for the isolates range from 0.016 to 4 mg/L. The multiplex PCR correctly identified penicillin susceptibility (MIC \( \leq 0.06 \) mg/L) or non-susceptibility (MIC \( \geq 0.12 \) mg/L) in 367 (95.8%) of 383 isolates evaluated (Table 1), yielding sensitivity, specificity, and positive and negative predictive values of 98.2% (218/222), 92.5% (149/161), 94.8% (218/230) and 97.4% (149/153), respectively. Discordant results included four (1%) penicillin non-susceptible isolates with negative PCR and 12 (3.1%) penicillin susceptible isolates with positive PCR. On repeated testing, these isolates had reproducible penicillin MICs at, or close to, the susceptibility breakpoint. To confirm the identification of the 16 isolates, PCR of the pneumococcal surface antigen gene (\( psa \)) was carried out as described previously,\(^4\) and all were \( psa \) positive. To resolve the reason for the most important (i.e. false negative) PCR errors, the \( pbp2b \) and \( pbp2x \) genes of the four penicillin non-susceptible isolates with negative PCR results were sequenced.\(^1\),\(^6\) This revealed nucleotide sequence divergence from the equivalent genes in \( S. pneumoniae \) R6 of between 5% and 7% and between 6% and 13% in \( pbp2b \) and \( pbp2x \), respectively. In terms of predicted amino acid sequence, several key substitutions were present at or adjacent to the known conserved motifs in these PBPs, including T445S or A (\( pbp2b \)), T338P (\( pbp2x \)), which are all recognized as being important in the development of penicillin resistance.\(^7\) However, the reason why the PCR reactions were negative was that the region to which the primers were designed to bind are either wild-type, or the nucleotide changes present are not complementary to those in the primers.

In this study (Table 1), six and 21 penicillin non-susceptible isolates, respectively, would not have been detected if only one, and not both of the \( pbp2b \) and \( pbp2x \) primer sets were used. Despite the improved test performance, discordant results were found for a high proportion of the isolates (16/40) with MICs (0.06–0.12 mg/L) close to the penicillin breakpoint. This is not unexpected as breakpoints were designed artificially for the prediction of clinical outcome, not for the detection of resistance mechanisms. Theoretically, the sensitivity of this PCR approach might be enhanced further by incorporation of a third primer set to detect mutations in \( pbp1a \). The contribution of this, however, is expected to be small as altered \( pbp1a \) is rarely found in clinical isolates in the absence of pre-existing altered \( pbp2x \) and/or \( pbp2b \).\(^8\) We conclude that this assay may well be useful for PCR-based predictive diagnosis of penicillin resistance in \( S. pneumoniae \).

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Table 1. PCR results and MICs of penicillin for 383 S. pneumoniae isolates

<table>
<thead>
<tr>
<th>Penicillin MIC (mg/L) distribution</th>
<th>0.016a</th>
<th>0.032a</th>
<th>0.06a</th>
<th>0.12b</th>
<th>0.25b</th>
<th>0.5b</th>
<th>1b</th>
<th>2b</th>
<th>4c</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pbp2b or pbp2x alteration (n = 153)</td>
<td>23</td>
<td>115</td>
<td>11</td>
<td>4</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pbp2b alteration only (n = 6)</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pbp2x alteration only (n = 21)</td>
<td>11</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Both pbp2b and pbp2x alterations (n = 203)</td>
<td>1</td>
<td>4</td>
<td>12</td>
<td>19</td>
<td>67</td>
<td>87</td>
<td>13</td>
<td></td>
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</tbody>
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References


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Fluoroquinolone resistance in environmental urease-positive campylobacters: why we don’t see it

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Sir,

Antibiotic resistance arising from the treatment of clinical and veterinary pathogens has become a widely debated topic over the last 5 years, in particular, the use of fluoroquinolones in poultry production and the emergence of ciprofloxacin-resistant thermophilic campylobacters in humans. Currently, what remains unclear is the contribution—to antibiotic resistance in human campylobacteriosis—of treating poultry with fluoroquinolones, and the contribution of clinical employment of fluoroquinolones in human medicine. Regardless of the source and routes of transmission, fluoroquinolone resistance has now emerged as a significant problem in both human and animal medicine.

We wish to report on the incidence of antibiotic resistance in environmental campylobacters, namely the urease-positive thermophilic Campylobacter (UPTC), isolated from shellfish in Northern Ireland, where no selective antibiotic pressure has been applied and where we have not been able to demonstrate antibiotic resistance, particularly among the fluoroquinolones. UPTC, a microaerophilic and Gram-negative bacterium, is an organism identified only relatively recently in England. After the original descriptions of UPTC appeared, isolates of UPTC were reported in France, Ireland and The Netherlands, and UPTC strains have also been found recently in Japan.1 The UPTC group was first described by Bolton et al.2 These strains were isolated from river water, sea water, mussels and cockles.

References
