Application of pbp1A PCR in Identification of Penicillin-Resistant Streptococcus pneumoniae

MIGNON DU PLESSIS,* ANTHONY M. SMITH, AND KEITH P. KLUGMAN

Pneumococcal Diseases Research Unit of MRC, SAIMR, WITS, Department of Clinical Microbiology and Infectious Diseases, South African Institute for Medical Research, Johannesburg 2000, South Africa

Received 20 August 1998/Returned for modification 13 October 1998/Accepted 12 November 1998

A seminested PCR assay, based on the amplification of the pneumococcal pbp1A gene, was developed for the detection of penicillin resistance in clinical isolates of Streptococcus pneumoniae. The assay was able to differentiate between intermediate (MICs = 0.25 to 0.5 μg/ml) and higher-level (MICs = ≥1 μg/ml) resistance. Two species-specific primers, 1A-1 and 1A-2, which amplified a 1,043-bp region of the pbp1A penicillin-binding region, were used for pneumococcal detection. Two resistance primers, 1A-R1 and 1A-R2, were designed to bind to altered areas of the pbp1A gene which, together with the downstream primer 1A-2, amplify DNA from isolates with penicillin MICs of ≥0.25 and ≥1 μg/ml, respectively. A total of 183 clinical isolates were tested with the pbp1A assay. For 98.3% (180 of 183) of these isolates, the PCR results obtained were in agreement with the MIC data. The positive and negative predictive values of the assay were 100 and 91%, respectively, for detecting strains for which the MICs were ≥0.25 μg/ml and were both 100% for strains for which the MICs were ≥1 μg/ml.

The targets for β-lactam antibiotics are cell wall-synthesizing enzymes known as penicillin-binding proteins (PBPs). β-Lactam resistance in clinical isolates of Streptococcus pneumoniae is due to extensive alterations in their PBPs that lead to decreased affinities for these drugs. Pneumococci produce five high-molecular-weight PBPs (1A, 1B, 2A, 2B, and 2X) and the low-molecular-weight PBP 3 (5). Resistance to penicillin has been shown to involve four of the five high-molecular-weight PBPs, namely, 1A, 2A, 2B, and 2X (5, 9, 10, 12). Studies have shown that alterations in PBP 2X result in low-level penicillin resistance, whereas high-level penicillin resistance requires alterations within the area of the Ser-370–Thr–Met–Lys and Ser-428–Arg–Asn motifs of pbp1A.

Due to the high morbidity and mortality associated with meningitis, early implementation of appropriate therapy requires prompt identification of the pathogen and, more importantly, its antimicrobial susceptibility pattern. Presently, susceptibility testing can only be carried out once an organism has been cultured, and this requires an additional 24 h before a result is available. Empirical combination therapy of a cephalosporin plus vancomycin is often the only choice that many clinicians have and yet one would like to avoid the extensive and sometimes inappropriate use of drugs such as vancomycin (7). Due to the development of molecular techniques, it is now possible to detect pathogens in clinical specimens by using PCR (6, 11, 20). The PCR is a rapid, specific, and sensitive method, and since it does not depend on the presence of viable organisms, it may be applicable in cases of prior antibiotic treatment. In our previous study we used a seminested PCR strategy, one based on the amplification of the pneumococcal pbp2B gene, to detect penicillin-resistant pneumococci (MICs of ≥0.125 μg/ml) in cerebrospinal fluid specimens (6). Our present study describes an assay, based on

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Position in pbp1A gene</th>
<th>Product length (bp) after amplification with downstream primer 1A-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A-R1</td>
<td>AAGAACACTGGTTATGTA</td>
<td>2662–2679</td>
<td>224</td>
</tr>
<tr>
<td>1A-R2</td>
<td>AGCATGCATTATGCAAAC</td>
<td>2317–2334</td>
<td>569</td>
</tr>
<tr>
<td>1A-1</td>
<td>ACAAAATGTAGACCAAGAAGCTCAA</td>
<td>1843–1866</td>
<td>1,043</td>
</tr>
<tr>
<td>1A-2</td>
<td>TACGAATTCATCTCTGTAGAG</td>
<td>2863–2886</td>
<td></td>
</tr>
</tbody>
</table>

* Corresponding author. Mailing address: Pneumococcal Diseases Research Unit, SAIMR, P.O. Box 1038, Johannesburg 2000, South Africa. Phone: 27-11-4899335. Fax: 27-11-4899332. E-mail: mignondp@hotmail.com.
amplification of the \( \text{pbp1A} \) gene, that is able to differentiate between isolates with intermediate resistance (MICs of 0.25 to 0.5 \( \mu g/ml \)) and those with higher-level penicillin resistance (MICs of \( \geq 1 \mu g/ml \)) by using a similar PCR strategy. Two species-specific primers were designed to bind to and amplify the pneumococcal \( \text{pbp1A} \) gene. Two additional internal primers were designed to bind to altered areas of the \( \text{pbp1A} \) gene, as identified in penicillin-resistant pneumococci isolated worldwide (1, 12, 16, 22). These altered areas occur internal to the species-specific primer binding sites. Together with the downstream primer, the upstream resistance primers amplify resistance products.

### MATERIALS AND METHODS

#### Bacterial strains

Clinical isolates were obtained from the South African Institute for Medical Research, a reference center for pneumococci in South Africa. A total of 159 South African \( \text{S. pneumoniae} \) strains were used in the study, together with R6 (an unencapsulated laboratory strain), \( \text{S. pneumoniae ATCC 49619} \), and 24 \( \text{S. pneumoniae} \) strains from France, Hungary, China, and The United States. Penicillin MICs were determined by the agar dilution method in Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) supplemented with 3% lysed horse blood (17). Organisms were routinely cultured at 37°C in 5% CO2

#### Preparation of genomic DNA

Pure genomic DNA was extracted from pneumococcal strains by previously described methods (21). For nonpneumococcal organisms, a swab of cells from a plate of growth was resuspended in 50 \( \mu l \) of \( H_2O \) and boiled for 10 min, and after centrifugation a supernatant containing a crude preparation of DNA was obtained.

#### PCR conditions for \( \text{S. pneumoniae} \)

A seminested PCR strategy was used. Each assay required two reactions containing primers 1A-1, 1A-2, and 1A-R1 and 1A-R2, respectively. All PCR amplifications were carried out with a Hybaid Omniprobe Thermal Cycler (Middlesex, United Kingdom). The 50-\( \mu l \) reaction mixture consisted of 50 ng of genomic DNA, 2 mM MgCl2, 200 \( \mu M \) deoxynucleotide triphosphates (Boehringer Mannheim GmbH, Germany), 50 mM KCl, 10 mM Tris-HCl (pH 8.0), a 1.0 \( \mu M \) concentration of each primer, and 2.5 \( U \) of Taq DNA polymerase (Promega Corp., Madison, Wis.). The PCR process included an initial 3-min incubation at 95°C, followed by 30 cycles of 93°C for 1 min, 55°C (when primer 1A-R1 was included) or 55°C (when primer 1A-R2 was included) for 1 min, and 72°C for 1 min. A 5-min extension at 72°C was included at the end of the final cycle. Amplified DNA fragments were analyzed by gel electrophoresis with 2% agarose.

#### PCR conditions for nonpneumococcal organisms

Conditions were exactly as described above except that 3 \( \mu l \) of boiled cells was used per PCR as opposed to genomic DNA. \( \text{S. pneumoniae ATCC 49619} \) and R6 were used as positive controls. These organisms were further tested with previously described universal 16S rRNA primers (8) to ensure that there were no false-negative results.

#### DNA sequencing

Pneumococcal \( \text{pbp1A} \) and \( \text{pbp2B} \) genes were amplified by PCR, with the forward primer biotinylated at its 5’ end. Amplified PCR products were cleaned by using a 0.6 volume of 20% polyethylene glycol–2.5 M NaCl as previously described (18). The biotylated and nonbiotylated strands were separated with streptavidin-coated paramagnetic beads according to the manufacturer’s instructions (Boehringer Mannheim). The DNA strands were sequenced by using the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemicals, Cleveland, Ohio) according to the manufacturer’s instructions.

### RESULTS AND DISCUSSION

The design of the resistance primers used in the present \( \text{pbp1A} \) seminested PCR assay is based on the published sequence data of Smith and Klugman (22). They showed that in the pneumococcal \( \text{pbp1A} \) gene, nucleotide alterations resulting in four amino acid substitutions (Thr-574→Asn, Ser-575→Thr, Glh-576→Gly, and Phe-577→Tyr) are common to all penicillin-resistant isolates for which the MICs are \( \geq 0.25 \mu g/ml \). The design of resistance primer 1A-R1 (Table 1) is based on these four consecutive mutations. In principle, this primer will anneal to the genomic DNA and result in the synthesis of an amplification product only for resistant isolates for which the MICs are \( \geq 0.25 \mu g/ml \). Resistance primer 1A-R2 (Table 1) is designed to bind to an area slightly downstream of the Ser-428→Arg→Asn motif. Mutations in this area of the \( \text{pbp1A} \) gene, resulting in the amino acid substitutions Ile-459→Met and Ser-462→Ala, only occur in isolates for which the MICs are \( \geq 1 \mu g/ml \) (22); therefore, amplification with this primer should

![FIG. 1. Primer binding sites in the \( \text{S. pneumoniae} \) \( \text{pbp1A} \) gene. 1A-1 and 1A-2 represent pneumococcal specific primers. 1A-R1 and 1A-R2 represent resistance primers which amplify DNA from isolates with penicillin MICs of \( \geq 0.25 \) and \( \geq 1 \mu g/ml \), respectively.](http://jcm.asm.org/Downloaded from http://jcm.asm.org)
only occur for higher-level resistant isolates (MICs of ≥1 μg/ml). The positions of primer binding to the \(pbp1A\) gene are indicated in Fig. 1. A universal reverse primer 1A-2 amplifies, together with the forward primers 1A-R1 and 1A-R2, to generate 224- and 569-bp resistance products, respectively. The forward primer 1A-1 and the universal reverse primer 1A-2 are pneumococcus specific and generate a 1,043-bp product. To determine the effectiveness of this \(pbp1A\) assay in identifying penicillin-resistant pneumococci, 183 pneumococcal isolates, with penicillin MICs ranging from 0.03 to 16 μg/ml, were analyzed. The results are summarized in Tables 2 and 3. An excellent correlation was found between PCR products and the MIC data. For 98.3% (180 of 183) of the isolates tested, the results obtained were in agreement with the MIC data. For comparative purposes, the 183 isolates were also analyzed with our previously described \(pbp2B\) assay (6). According to this \(pbp2B\) assay, 96.7% (177 of 183) of the PCR results were in agreement with the MIC data.

Table 3 shows those 6 of 183 (3.3%) isolates that exhibited discrepant PCR results when compared with their MIC data. This table shows the results obtained for the present \(pbp1A\) assay and our previously described \(pbp2B\) assay (6). For these six isolates, the penicillin-binding domains of \(pbp1A\) and \(pbp2B\) were also sequenced and compared to that of the penicillin-susceptible strain R6. Table 4 shows the amino acid substitutions present in the penicillin-binding domains of the \(pbp1A\) and \(pbp2B\) genes of these isolates. Isolates 29, 36, and 89 revealed MICs of 0.125 to 0.25 μg/ml; therefore, positive PCR results were expected for both their \(pbp1A\) and \(pbp2B\) genes. However, only the \(pbp1A\) assay gave resistance amplification products. The negative \(pbp2B\) assay was supported by DNA sequencing, which revealed an unaltered gene. These results were unexpected, considering that previous data have shown that the development of penicillin resistance occurs in a stepwise manner with an alteration of \(pbp2B\) occurring before an alteration of \(pbp1A\) (15, 22, 23). This uncommon situation was found at the intermediate level of resistance. At a higher level of penicillin resistance an altered \(pbp2B\) would probably be required. For isolates 129, 139, and 143 (MICs of 0.125 to 0.5 μg/ml) are considered “borderline” and 50% of the time are PCR positive for the assay. For comparative purposes, the 183 isolates were also analyzed with our previously described \(pbp2B\) assay (6). According to this \(pbp2B\) assay, 96.7% (177 of 183) of the PCR results were in agreement with the MIC data.

**FIG. 2.** Agarose gel electrophoresis of PCR amplified fragments of the \(pbp1A\) gene from *S. pneumoniae*. Lane M, molecular weight marker. Primer combinations are as follows: 1A-R1 + 1A-1 + 1A-2 (lanes a); 1A-R2 + 1A-1 + 1A-2 (lanes b). The penicillin MICs for the isolates are as follows: 0.03 μg/ml (lanes 1), 0.06 μg/ml (lanes 2), 0.125 μg/ml (lanes 3), 0.25 μg/ml (lanes 4), 0.5 μg/ml (lanes 5), 1 μg/ml (lanes 6), 2 μg/ml (lanes 7), 4 μg/ml (lanes 8), and 8 μg/ml (lanes 9). A, a 1,043-bp product arising from amplification with primers 1A-1 and 1A-2; B, a 569-bp product arising from amplification with primers 1A-R2 and 1A-2; C, a 224-bp product arising from amplification with primers 1A-R1 and 1A-2.
have the potential to transfer resistance genes to pneumococci and vice versa (3, 19). We do not expect the viridans group streptococci to cause significant misdiagnosis in the setting of meningitis.

In the PCR-based diagnosis of penicillin-resistant pneumococci, the present \textit{pbp1A} assay is an improvement on our previously described \textit{pbp2B} assay. Two resistance primers are used in the \textit{pbp1A} assay compared to the four used in the \textit{pbp2B} assay. In addition, the \textit{pbp1A} assay can also differentiate between intermediate (MICs of 0.25 to 0.5 \(\mu\text{g}/\text{ml}\)) and high-level (MICs of \(\geq 1 \mu\text{g}/\text{ml}\)) resistance. PCR-based diagnosis of penicillin resistance is complicated by the participation of multiple PBPs in the development of resistance. Further research will determine which PBPs will serve best as a target in a PCR-based diagnostic kit aimed at the identification of all pneumococci with resistance to penicillin and other \(\beta\)-lactams.

### REFERENCES


