Combinal Detection of Autolysin and Penicillin-Binding Protein 2B Genes of *Streptococcus pneumoniae* by PCR

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PCR was used to identify penicillin resistance in 1,062 clinical isolates of *Streptococcus pneumoniae*. Three sets of primers were designed to amplify (i) a 240-bp fragment of the penicillin-binding protein (PBP) 2B gene (*pbp2b*) of penicillin-susceptible *S. pneumoniae* (PSSP), (ii) a 215-bp fragment of the class A mutations of the *pbp2b* gene present in penicillin-resistant *S. pneumoniae*, and (iii) a 286-bp fragment of the class B mutation. In addition, a set of primers that amplify 273 bp of the autolysin (*lytA*) gene was applied in combination with the above to identify *S. pneumoniae*. Of 621 isolates for which MICs of penicillin were ≤0.06 μg/ml, 614 (98.9%) were ascertained as having DNA fragments amplified by the PSSP primers. Of 441 isolates for which MICs of penicillin were ≥0.125 μg/ml, a class A mutation was detected in only 8 (1.8%), a class B mutation was detected in 310 (70.3%), and neither class A nor class B mutations were found in the remaining 123 (27.9%). However, when analysis was limited to isolates for which MICs of penicillin were ≥1.0 μg/ml, 247 isolates (89.8%) of 275 were found to possess a class B mutation. When PBPs were analyzed in 12 isolates with unclear mutations of the *pbp2b* gene by using ^[3]H]benzylpenicillin, low affinity to PBP 2B was observed in them all. These findings suggest that a *pbp2b* mutation other than class A or class B is present in these isolates. These results also indicate that it may be possible to identify PSSP and penicillin-resistant *S. pneumoniae* by applying PCR using a combination of primers to detect the susceptible *pbp2b* gene, resistant *pbp2b* gene mutations, and the *lytA* gene.

The increase in penicillin-resistant *Streptococcus pneumoniae* (PRSP) is causing serious clinical problems worldwide (1), and Japan is no exception. Following an investigation of 2,500 clinical isolates of *S. pneumoniae* collected throughout Japan, we found that PRSP accounted for 40% of cases (12). Otitis media in children and meningitis in both children and adults caused by PRSP are especially common. Cases in which changing from penicillin or cefotaxime to carbapenem antibiotics (2) or vancomycin (26) was clinically effective have been reported. With severe infections, it is most important to determine as rapidly as possible if the detected *S. pneumoniae* is PRSP, to assist in decisions regarding antibiotic treatment.

According to the susceptibility criteria for *S. pneumoniae* recommended by the National Committee for Clinical Laboratory Standards (19), isolates for which the MIC of penicillin is ≥2.0 μg/ml are resistant (PRSP), those for which the MIC is between 0.1 and 1.0 μg/ml have intermediate resistance, and those for which the MIC is ≤0.06 μg/ml are considered susceptible (PSSP). However, at least 2 days are required for MIC determinations in most laboratories.

In PRSP, there are reduced affinities for penicillin in penicillin-binding proteins (PBPs) 1A (11, 15), 2A (10), and 2B (10, 11, 15) and for cefotaxime in PBP 2X (10, 17). Among the altered PBPs, an important role in penicillin resistance is played by PBP 2B, which is encoded by a mosaic *pbp2b* gene (3). This gene has been previously sequenced in both PSSP (5) and PRSP (3, 4, 22). The *pbp2b* mutations of PRSP are highly divergent, particularly in the transpeptidase region (3, 22). The most common of these are specific mutations termed class A and class B (4).

In this study, we attempted to use PCR as a means of rapidly determining whether a *S. pneumoniae* isolate is PRSP. We describe the results of PCR performed with 1,062 isolates of *S. pneumoniae* using four sets of primers designed for amplification of (i) *pbp2b* genes found in PSSP, (ii) the class A and (iii) class B mutations of the *pbp2b* gene, and (iv) the *lytA* gene.

**MATERIALS AND METHODS**

**Strains.** Clinical isolates (n = 1,062) obtained from specimens originating at 36 institutions participating in the Working Group for Penicillin-Resistant *S. pneumoniae* were studied. These strains were collected between October 1993 and March 1994. Optochin sensitivity, inulin fermentation, and bile solubility were used to identify *S. pneumoniae*. Bacteria were routinely propagated on 5% sheep blood agar (Kyokuto Co., Ltd., Tokyo, Japan) in a humidified atmosphere supplemented with 5% CO₂. Sterilized 10% skim milk was used for the storage of bacteria at −80°C.

**Antimicrobial susceptibility tests.** Susceptibility testing of β-lactams for *S. pneumoniae* was performed using cation-adjusted Mueller-Hinton agar (Eiken Co. Ltd., Tokyo, Japan) supplemented with 10% defibrinated sheep blood by the agar dilution method. The inocula and culture conditions were based on National Committee for Clinical Laboratory Standards recommendations (18). Penicillin G and oxacillin were provided by Banyu Pharmaceutical Co. Ltd., and ampicillin and cephalotaxine were provided by Meiji Seika Kaisha Ltd. and Fujisawa Pharmaceutical Co. Ltd., respectively.

**PCR primers.** The sequences of the primers used in PCR are listed in Table 1. Primers ALY1 and ALY2, which correspond to the *lytA* gene of *S. pneumoniae*, were derived from the sequence published by Garcia et al. (18). Three sets of primers were derived from the *pbp2b* gene on the basis of the sequence reported by Dowson et al. (4, 5). Primer mixture A contained the primers for detecting the *lytA* gene and the class A mutation in the *pbp2b* gene, and primer mixture B contained primers for detecting the susceptible *pbp2b* gene of PSSP and the class B mutation in the *pbp2b* gene.

**PCR.** Two microcentrifuge tubes marked A and B were prepared for each isolate. One colony of *S. pneumoniae* grown on blood agar medium was picked and suspended in each tube containing 20 μl of lysis solution, which was constituted of 2 μl of 1 M Tris-HCl (pH 8.9), 4 μg of proteasine K, 0.225% Tween 20,
TABLE 1. Sequences of oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Position</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autolysin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALY1</td>
<td>TGAAGCCGATTATACCTG GCC</td>
<td>694–713</td>
<td>273</td>
</tr>
<tr>
<td>ALY2</td>
<td>GCTAAACCTCCCTGTATACA GG</td>
<td>945–966</td>
<td></td>
</tr>
<tr>
<td>PBP 2B (susceptible)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2B3</td>
<td>ATCAATTTGGTATACTCAGG</td>
<td>1490–1511</td>
<td>240</td>
</tr>
<tr>
<td>P2B4</td>
<td>AGTAGATTCACTTGTTAGGTC</td>
<td>1709–1729</td>
<td></td>
</tr>
<tr>
<td>PBP 2B (class A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2BR4</td>
<td>CTAGGCAATGCCGATTACG</td>
<td>1515–1534</td>
<td>215</td>
</tr>
<tr>
<td>P2B4</td>
<td>AGTAGATTCACTTGTTAGGTC</td>
<td>1709–1729</td>
<td></td>
</tr>
<tr>
<td>PBP 2B (class B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2BR3</td>
<td>CCAAACCTAACAGACTAGC</td>
<td>1444–1463</td>
<td>286</td>
</tr>
<tr>
<td>P2B4</td>
<td>AGTAGATTCACTTGTTAGGTC</td>
<td>1709–1729</td>
<td></td>
</tr>
</tbody>
</table>

0.225% Nonidet P-40, 2 μl of 10× Tth polymerase buffer (Toyobo Co. Ltd., Osaka, Japan), and sterilized distilled water to a final volume of 20 μl (23). These were incubated at 60°C for 20 min and then at 90°C for 10 min to lyse the cells with a thermal cycler (Gene Amp PCR System 9600-R; Perkin-Elmer Cetus). Afterward, 5 μl of primer mixtures A and B, which consisted of 25 ng of each primer, 8.25 mM (each) deoxynucleoside triphosphates, 1.25 U of Tth DNA polymerase (Toyobo), and 1/10 volume of 10× Tth polymerase buffer, was added to tubes A and B, respectively.

The PCR cycling conditions were as follows: 30 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 30 s. Following PCR, each sample underwent electrophoresis on a 3% agarose gel. Detection of the hytA and pbp26 genes was possible when bacterial cells were present at ≥1.2 × 10^9 CFU/ml.

Detection of PBP. A 20-ml aliquot of cells that had been incubated overnight at 37°C was inoculated into 200 ml of Todd-Hewitt broth (Difco). With gentle shaking at 37°C, the cells were made to achieve logarithmic growth. Subsequently, the cells were harvested by centrifugation at 7,000 × g for 10 min at 4°C and then washed once in 50 ml of 50 mM sodium phosphate buffer (pH 7.0). The cells were disrupted with an Ultrasonicator (model 200M, 9 kHz; Kubota Co. Ltd., Tokyo, Japan) for 20 min at 0°C. Unbroken cells were removed by centrifugation at 7,000 × g for 10 min at 4°C, and supernatant was centrifuged at 100,000 × g for 60 min at 4°C. The pellet (membrane fraction) was suspended at 4 mg of protein per ml in the same buffer and stored at −80°C until use.

In PBP assays, a 3-μl volume of 37 MBq of [3H]benzylpenicillin (specific activity, 407 GBq/mmol; Amersham, Buckinghamshire, England) per ml was added to 30 μl of membrane fraction and incubated for 10 min at 30°C. Then these reactions were terminated by adding unlabeled penicillin G to give a final concentration of 2 mg/ml, and mixtures were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis by a previously described method (25). A running gel was modified to 10% (wt/vol) acrylamide and 0.065% (wt/vol) N,N'-methylenebisacrylamide.

Serotyping. Serotyping of S. pneumoniae was performed by the capsular swelling technique with antisera obtained commercially from the Statens Seruminstitut (Copenhagen, Denmark).

RESULTS

Amplified DNA profile. Figure 1 shows the results of the PCR-amplified DNA profile obtained from the PSSP UB034 isolate and from the PRSP UB023 and UB008 isolates. The two DNA bands in columns A and B from the PSSP UB034 isolate (for which the penicillin MIC was 0.016 μg/ml and the oxacillin MIC was 0.031 μg/ml) correspond to the 273-bp DNA fragment of the hytA gene and to the 240-bp DNA fragment of the susceptible pbp2b gene of PSSP, respectively. Isolates that had these DNA profiles and for which MICs of penicillin were ≤0.06 μg/ml were designated susceptible. Isolates for which the same bands were detected in the DNA profile as for the isolates in the susceptible category but for which MICs of penicillin were ≥0.125 μg/ml were distinguished from the susceptible category as unknown, since it was considered possible that they possess a mutation outside the regions that were amplified.

For isolate UB023 (Fig. 1), the MIC of penicillin was 0.125 μg/ml and the MIC of oxacillin was 1.0 μg/ml. Two DNA bands were detected in column A, corresponding to DNA fragments of the hytA gene and a 215-bp fragment of the class A mutation in the pbp2b gene. Such isolates were designated class A.

For isolate UB008 (Fig. 1), the MIC of penicillin was 4.0 μg/ml and the MIC of oxacillin was 8.0 μg/ml. This isolate showed a DNA band corresponding to the hytA gene and a band in column B corresponding to a 286-bp DNA fragment of the class B mutation of the pbp2b gene. Isolates showing this DNA profile were designated class B.

Penicillin MIC and pbp2b gene mutation. The correlations between PCR results and the MIC of penicillin for 1,062 clinical isolates of S. pneumoniae are shown in Fig. 2. From the 621 isolates for which the MIC of penicillin was ≤0.06 μg/ml, a DNA fragment corresponding to the susceptible pbp2b gene was identified in 614 (98.9%). The remaining seven isolates (1.1%) required penicillin MICs of 0.06 μg/ml but were class B.

FIG. 1. Agarose gel electrophoresis of PCR-amplified DNA fragments of the hytA and pbp2b genes in S. pneumoniae. Strain UB034 is PSSP, and strains UB023 and UB008 are PRSP. DNA fragments in lanes A, 273 bp from the hytA gene and 215 bp from class A mutation of the pbp2b gene; DNA fragments in lanes B, 240 bp from susceptible pbp2b gene and 286-bp from class B mutation of the pbp2b gene.
The existence of these few isolates for which the PCR and MIC results were not in agreement is considered to lie within the range of error of biological measurement methods.

Among 441 isolates for which the MICs of penicillin were \(\geq 0.125\, \mu g/ml\), 310 (70.3\%) were identified as class B. When analysis was limited to isolates for which MICs were \(\leq 1.0\, \mu g/ml\), almost all isolates (247 of 275 = 89.9\%) were class B. Only 8 (1.8\%) were class A, with MICs of penicillin ranging from 0.125 to 1.0 \(\mu g/ml\). There were 123 (27.9\%) isolates in the unknown category, for which the \(pbp2b\) mutation could not be identified with the primers used in the present study, despite requiring MICs of \(\geq 0.125\, \mu g/ml\). As will be described later, the affinity of PBP 2B for penicillin was apparently reduced in 38 unknown-category strains tested.

**Ceftizoxime MIC and \(pbp2b\) gene mutations.** Figure 3 shows the correlations between the MIC of ceftizoxime, a broad-spectrum cephalosporin antibiotic, and PCR results for 594 isolates. The breakpoint between susceptibility and resistance to this antibiotic is 1.0 \(\mu g/ml\). Of the 419 isolates for which MICs were \(\leq 0.5\, \mu g/ml\), 393 (93.8\%) were identified as susceptible, with the remaining 26 showing disagreement between their MIC and PCR classifications. Among 175 isolates for which MICs were \(\geq 1.0\, \mu g/ml\), 4 were classified class A (2.3\%) and 117 (66.9\%) were classified class B. The \(pbp2b\)
gene mutation status of the remaining 54 isolates (30.9%) was unclear.

**Affinity of PBP 2B.** The PBP affinities of 12 of 38 investigated unknown-category strains are shown in Fig. 4. These strains could not be assigned to class A or class B, even though the MICs of penicillin for them were $\geq 0.125 \mu g/ml$.

All these strains showed a marked reduction in the affinities for penicillin of PBP 1A and 2B in comparison with the PSSP S223 strain in lane 1. These results suggest that most isolates classified as unknown possess $pbp2b$ mutations distinct from those in class A and class B isolates.

**Relation between PBP 2B gene ($pbp2b$) mutation and serotypes.** Table 2 shows the relationship between the serotypes of the 1,062 isolates and the $pbp2b$ mutations found by PCR. The isolates were classified into four groups: susceptible, class A, class B, and unknown. The most common serotype in the 614 susceptible strains was serotype 6, followed by serotypes 23 and 14. Many other serotypes also were found.

Among the 8 class A strains and 317 class B strains, two serotypes, 19 and 23, accounted for 84.6%, followed by serotype 6, with 8.0%. Of the 123 unknown-category strains, 32.5% were serotype 6, 30.1% were serotype 23, and 27.6% were serotype 19.

**DISCUSSION**

Low affinity of PBPs 1A (11, 15), 2A (10), 2B (10, 11, 15), and 2X (10, 17) is involved in the penicillin or cefotaxime resistance of *S. pneumoniae*. The genes encoding PBPs 1A (16), 2B (3, 4, 22), and 2X (13, 14) have been sequenced in both PSSP and PRSP, and it has been shown that highly divergent genotypes are present within PRSP. In addition, these regions of divergence are partially shared by *Streptococcus oralis* (6), *Streptococcus sanguis* (6), and *Streptococcus mitis* (3).

Mixing of DNA from PRSP may transform a susceptible strain into one with a full level of penicillin or cefotaxime resistance, and reciprocal transfer also has been confirmed (7, 17, 20). Therefore, the genes encoding PBPs 1A, 2B, and 2X are thought to be mosaic genes formed from different species.

We have analyzed the PBPs of PRSP isolated in Japan and detected low affinity of PBPs 1A, 2A, and 2B (24). These results suggest that the main cause of penicillin resistance is a $pbp2b$ mutation. Clinically, the isolation rate of PRSP from cases of meningitis, sepsis, and otitis media has significantly increased, making it necessary to rapidly determine if the detected *S. pneumoniae* is PRSP.

Attempts to detect *S. pneumoniae* directly by PCR have already been made using primers of the $lytA$ gene or $pbp2b$ gene from blood (21, 28), otitis media (27), and sputum (9). We further included the $pbp2b$ gene of PRSP for combinational detection to characterize penicillin resistance and evaluated a possible method for rapid identification of PRSP by PCR.

On the basis of results from the present study, it appears that class B mutations are present at a high rate in isolates for which the MIC of penicillin is $\geq 1.0 \mu g/ml$ in Japan. There were very few class A mutations in this resistance level. It is noteworthy that serotypes 19, 23, and 6 accounted for 92% of all PRSP, and these serotypes were also isolated most frequently in other regions of the world, suggesting that selected PRSP strains are widely distributed.

Among PRSP strains for which MICs are $\geq 1.0 \mu g/ml$, 10.1% of the strains had a mutation(s) other than class A or class B. The $pbp2b$ gene in seven of these strains has been partially sequenced, and a specific mutation consisting of a 9-nucleotide insertion has been located between the Ser-X-X-Lys region and the Ser-X-Asn region of the conserved amino acid sequences in three strains (23a). Another PRSP strain also included similar mutations near the conserved amino acid sequences described by Smith and Klugman (22). Work is currently under way to apply PCR for the direct detection of PRSP from specimens that include mutations other than class A or class B.

<table>
<thead>
<tr>
<th>Classification of $pbp2b$ gene</th>
<th>No. (%) of strains of serotype:</th>
<th>Total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Susceptible</td>
<td>8 (1.3)</td>
<td>130 (21.2)</td>
</tr>
<tr>
<td>Class A</td>
<td>2</td>
<td>24 (7.6)</td>
</tr>
<tr>
<td>Class B</td>
<td>1 (0.3)</td>
<td>1 (0.8)</td>
</tr>
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</table>
ACKNOWLEDGMENTS

We thank the members of the Working Group for collecting isolates of PRSP.

REFERENCES
