

Phenotypic and genotypic discrepancy of *Streptococcus pneumoniae* strains isolated from Asian countries

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Abstract

Non-typeable isolates of *Streptococcus pneumoniae* collected from Asian countries were characterized by optochin susceptibility test, bile solubility test, multilocus sequence typing of housekeeping genes, amplification of virulence-related genes, 16S rDNA-*RsaI* digestion, and 16S rDNA sequencing. Six of 54 non-typeable pneumococcal isolates showed divergence of gene sequences of *recP* and *xpt* from typical pneumococcal strains. Of these six atypical pneumococcal strains, two showed different results in optochin susceptibility or bile solubility test from typical pneumococcal strains. All six isolates showed high sequence dissimilarities of multilocus sequence typing, 16S rDNA sequences, and *lytA* sequences from typical *S. pneumoniae* strains. Data from this study suggest that classic tests such as optochin susceptibility and bile solubility tests may lead to incorrect identification of *S. pneumoniae*. These atypical strains may belong to different bacterial species from *S. pneumoniae*.

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1. Introduction

Streptococcus pneumoniae is an important pathogen of community-acquired pneumonia, bacterial meningitis, otitis media, and sinusitis. During the past decades, antimicrobial resistance in *S. pneumoniae* has become a serious concern worldwide. Given the clinical and epidemiological importance of *S. pneumoniae*, accurate identification is essential for correct diagnosis and appropriate selection of antimicrobial agents. In the clinical microbiology laboratory, several tests are used to identify *S. pneumoniae*: colony morphology on blood

agar plates, optochin susceptibility test, bile or deoxycholate solubility test, and immunological reaction with type-specific antisera [1–5]. The optochin susceptibility test is highly sensitive (90–100%) and specific (99–100%) and bile solubility test is also highly sensitive (>98%) and very specific (100%) [1]. Besides these conventional methods, several molecular methods for identification of *S. pneumoniae* including commercial AccuProbe test targeting 16S rDNA gene, amplification of *S. pneumoniae*-specific genes such as pneumolysin (*ply*), pneumococcal surface antigen A (*psaA*), and autolysin (*lytA*) have been proposed [6]. However, these molecular methods showed variable sensitivity and specificity for identification of *S. pneumoniae*. [5–12]. Even 16S rDNA gene sequence, which is thought to be a ‘gold standard’ for description of bacterial species, do not

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discriminate the pneumococci from some other viridans streptococci such as *Streptococcus mitis* and *Streptococcus oralis* as they showed sequence similarity of >99% [13].

Based on the optochin susceptibility and bile solubility tests, previous reports documented the presence of atypical strains of *S. pneumoniae*, which were optochin-resistant or bile-insoluble and could be identified as viridans group streptococci in the clinical microbiology laboratory [3,6,8,14–19]. Recently, genotypic characteristics of these atypical strains were reported based on the sequences of several housekeeping genes [5]. These atypical strains showed sequence divergences of *recP* and *xpt* genes from typical strains of *S. pneumoniae*. It was also noted that typical strains of pneumococci show sequence dissimilarity less than 2% with other pneumococcal strains in six gene loci by multilocus sequence typing (MLST) (<http://spneumoniae.mlst.net>).

In the present study, we report the presence of atypical strains collected from Asian countries, which showed strong similarities with *S. pneumoniae*. These strains were characterized by phenotypic analysis such as optochin susceptibility and bile solubility tests and by genotypic analysis such as MLST, amplification of virulence-related genes, 16S rDNA-*RsaI* digestion, and 16S rDNA sequences.

2. Materials and methods

2.1. Bacterial strains

A total of 54 non-typeable isolates of *S. pneumoniae* were selected for the study (13 from Hong Kong, 8 Vietnam, 7 Taiwan, 6 China, 6 Thailand, 4 India, 3 Korea, 3 Philippines, 2 Sri Lanka, 1 Singapore, and 1 Malaysia, respectively). These isolates had been collected for surveillance study on pneumococcal resistance in Asian countries by the Asian Network for Surveillance of Resistant Pathogens (ANSORP) [20]. These strains were initially identified as *S. pneumoniae* by conventional microbiologic methods at each center.

2.2. Multilocus sequence typing (MLST)

The template DNA for PCR was prepared from a few colonies on blood agar plate. The DNA was extracted using the simple boiling-lysis method. Briefly, colonies were suspended in lysis buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, and 1% Triton X-100), and were incubated at 70 °C for 10 min. Then, the mixture was centrifuged for a moment and aqueous phase was used as a template for PCR. For amplification of *recP* and *xpt* gene fragments in 54 non-typeable isolates, we used two primer sets as described previously [21]. Pneu-

mococcal strains, which showed significant sequence divergence of *recP* and *xpt* genes from typical *S. pneumoniae*, were subjected to the sequence typing of other housekeeping genes (*aroE*, *gdh*, *gki*, and *spi*) in MLST except *ddl* gene because it is closely placed with penicillin-binding protein gene (*pbp2b*) and often contains highly diverged sequences [22]. Amplification of housekeeping genes of *S. pneumoniae* was also performed as described previously [21]. Six of seven MLST loci in six pneumococcal strains were concatenated using program at the MLST website (<http://spneumoniae.mlst.net/sql/concatenate>). DNA sequences of 47 sequence types (STs), which had been obtained from 110 erythromycin-resistant pneumococcal isolates from Asian countries in the previous study [23], were also concatenated and compared. Fifty-four strains analyzed for sequence types of *recP* and *xpt* genes and 53 concatenated sequences were phylogenetically analyzed using neighbor-joining method. Branch supporting values were evaluated with 1000 bootstrap replications [24].

2.3. Phenotypic assays

The optochin susceptibility test and bile solubility test were performed with six strains which showed significant sequence divergence in *recP* and *xpt* genes from typical pneumococci. For the optochin susceptibility test, a 5 µg-optochin disk (Mast Diagnostics) was placed on the agar plate which was incubated at 37 °C overnight. Isolates with zone diameter of >14 mm were considered susceptible. Bile solubility was determined by the tube method [25]. Colonies were suspended in 1 ml of 0.85% NaCl to a 1.0 McFarland turbidity standard and were divided equally into two small glass tubes. Three to four drops of 10% sodium deoxycholate were added to one tube (test), and 3–4 drops of 0.85% NaCl were added to the other tube (control). After incubation for up to 2 h at 35 °C, turbidity in the tube was compared with the 0.85% NaCl control. Complete lysis was interpreted as ‘bile-soluble’.

2.4. Amplification of virulence-related genes

To detect the genes related to virulence of *S. pneumoniae*, amplifications of *ply* (pneumolysin), *psaA* (pneumococcal surface antigen), and *lytA* (autolysin) gene fragments were performed as described previously [6]. Amplified fragments of *ply*, *psaA*, and *lytA* genes were visualized as 209, 838, and 101 bp, respectively.

2.5. 16S rDNA-*RsaI* digestion and 16S rDNA tree

Amplification of the 16S rDNA and subsequent digestion with *RsaI* was performed as described previously [6,26]. Briefly, amplified products of 16S rDNA were digested with *RsaI* in 37 °C for more 4 h. Digested

fragments were visualized in 3% agarose gel. Since 16S rDNA sequences of *S. pneumoniae* and *Streptococcus sanguinis* lack an *RsaI* restriction site (GTAC) at *Escherichia coli* position 635, *S. pneumoniae* shows restriction fragments of 114, 146, 335, and 887 bp [6]. In contrast, *RsaI* restriction site is present in 16S rDNA sequences of *S. mitis*, *S. oralis*, and *S. parasanguinis* that produce more fragments of 114, 146, 262, 355, and 625 bp. 16S rDNA sequences of six pneumococcal strains were also obtained and compared with those of typical isolates of *S. pneumoniae*, *S. mitis*, *S. oralis*, *S. gordonii*, *S. sanguinis*, and *S. parasanguinis* retrieved from GenBank. 16S rDNA tree was generated by the neighbor-joining method and bootstrap values were evaluated with 1000 replications.

2.6. Nucleotide sequence accession numbers

Sequences of six strains (Kor 145, HK P47, HK P55, HK P116, SI P25, and VN O27) determined in this study have been deposited in GenBank under Accession Nos. AY525788 to AY525842.

3. Results

3.1. Sequence analysis of *recP* and *xpt* genes

Six (Kor 145, HK P47, HK P55, HK P116, SI P25, and VN O27) of 54 non-typeable isolates of *S. pneumoniae* showed sequence divergence of *recP* and *xpt* genes compared with typical pneumococci. These six strains were obtained from patients with pneumonia (five strains) or otitis media (one) (Table 1). Gene trees of 54 non-typeable strains were developed based on *recP* (448 bp) and *xpt* (486 bp) sequences, using neighbor-joining method, respectively (Fig. 1). In both gene trees, five strains (HK P47, HK P55, HK P116, SI P25, and VN O27) showed sequence divergences with each other and with other pneumococcal isolates. Although one Korean strain, Kor 145, belonged to a

group of typical *S. pneumoniae* in *recP* tree (Fig. 1(a)), it showed a significant divergence in *xpt* tree like other atypical strains (Fig. 1(b)). Nucleotide divergence of *recP* and *xpt* genes of six atypical strains ranged from 1.1% to 5.3% and from 3.2% to 8.4% compared with other typical strains, respectively, while sequence dissimilarity was less than 1% between typical pneumococcal isolates.

3.2. Concatenated sequence analysis of MLST

Four other housekeeping genes (*aroE*, *gdh*, *gki*, and *spi*) were additionally amplified and sequenced in six pneumococcal strains, which showed sequence divergence in *recP* and *xpt* genes. However, *spi* gene fragment of Kor 145 could not be amplified possibly due to mutation in primer sites, and thus *spi* sequences of Kor 145 were coded as missing. Concatenated sequences of six atypical strains showed nucleotide dissimilarities ranging from 1.6–2.5% (Kor 145) to 4.4–6.3% (other isolates) compared with those of STs of typical *S. pneumoniae* (data not shown). Phylogenetic analysis indicated that 47 STs from 110 erythromycin-resistant pneumococcal isolates formed a group with bootstrap value of 99% and showed short branch lengths within a group. On the other hand, six atypical strains were placed outside a cluster of typical *S. pneumoniae* and were highly divergent among them (Fig. 2). A strain Kor 145 was the closest to a cluster of typical *S. pneumoniae*.

3.3. Optochin susceptibility and bile solubility tests

In the optochin susceptibility test, five strains (Kor 145, HK 47, HK P55, HK P116, and SI P25) were susceptible to optochin, which is generally indicative of *S. pneumoniae*. Another (VN O27) was shown to be resistant to optochin (Table 1). In the bile solubility test, only five strains (Kor 145, HK P47, HK P116, SI P25, and VN O27) were bile-soluble (Table 1). In summary, four strains (Kor 145, HK P47, HK P116, and SI P25) were optochin-susceptible and bile-soluble, which could

Table 1
Phenotypic and genotypic characteristics of six strains examined in this study

| Strain ^a | Isolated year | Specimen source | Category of infection | Optochin susceptibility ^b | Bile solubility | PCR result ^c | | | 16S rDNA- <i>RsaI</i> digestion pattern ^d |
|---------------------|---------------|-----------------|-----------------------|--------------------------------------|-----------------|-------------------------|-------------|-------------|--|
| | | | | | | <i>ply</i> | <i>psaA</i> | <i>lytA</i> | |
| Kor 145 | 1998 | Sputum | Pneumonia | S | + | + | + | + | M |
| HK P47 | 2000 | Sputum | Pneumonia | S | + | + | – | + | P |
| HK P55 | 2001 | Sputum | Pneumonia | S | – | + | – | + | M |
| HK P116 | 2001 | Sputum | Pneumonia | S | + | + | + | + | P |
| SI P25 | 2000 | Sputum | Pneumonia | S | + | + | – | + | M |
| VN O27 | 2001 | Middle ear | Otitis media | R | + | + | – | – | M |

^a Kor, Korea; HK, Hong Kong; SI, Singapore; VN, Vietnam.

^b S, susceptible; R, resistant.

^c *ply*: pneumolysin gene; *psaA*: pneumococcal surface antigen A gene; *lytA*: autolysin gene.

^d M, *S. mitis*-specific pattern; P, *S. pneumoniae*-specific pattern.

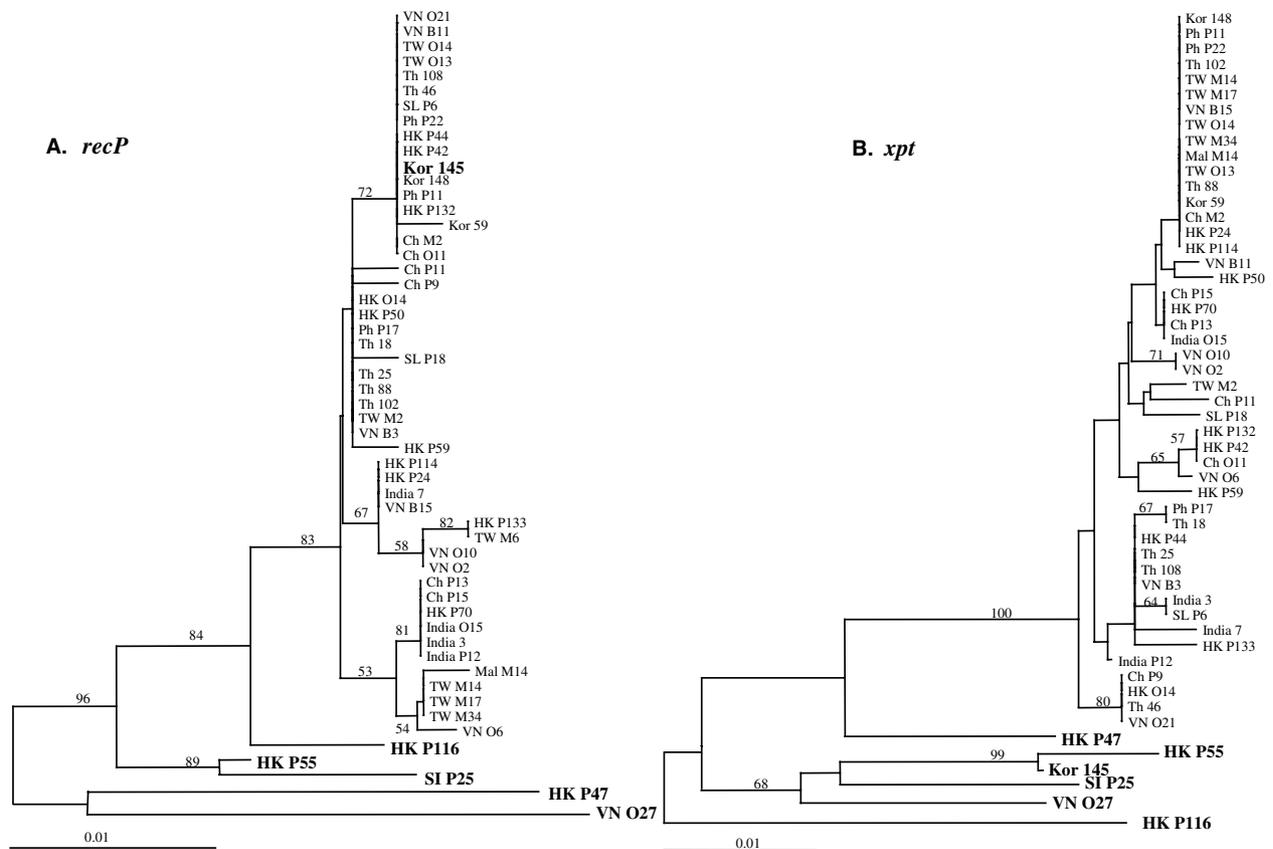


Fig. 1. Phylogenetic trees of 54 non-typeable pneumococcal strains deduced from *recP* (448 bp) and *xpt* (486 bp) gene sequences. These trees were inferred with the method of neighbor-joining and branch support values were evaluated by using bootstrap analysis of 1000 replications. Only bootstrap values higher than 50% in the bootstrap analysis are indicated at the corresponding branches. Six strains showing diverged sequences are represented as bold types.

be identified as *S. pneumoniae*. The other two strains (HK P55 and VN O27) showed incongruent results (Table 1).

3.4. Amplification of *ply*, *psaA*, and *lytA* genes

Of six atypical strains, two (Kor 145 and HK P116) possessed all three genes. However, HK P47, HK P55, and SI P25 possessed only *ply* and *lytA* genes, while VN O27 was positive for only *ply* gene (Table 1). Remaining 48 strains, which belonged to typical *S. pneumoniae* group based on *recP* and *xpt* gene sequences, possessed all three virulence-related genes.

3.5. 16S rDNA-RsaI digestion pattern

Only two (HK P47 and HK P116) of six atypical strains showed *S. pneumoniae*-specific digestion pattern (114, 146, 335, and 887 bp-fragments), while four strains showed *S. mitis* group-specific digestion pattern (114, 146, 262, 355, and 625 bp) (Table 1). Sequence analysis of 16S rDNAs of six strains was consistent with the *Rsa*

I digestion patterns. At *E. coli* position 635 nucleotide sequences are 'GTAG' in HK P47 and HK P116 strains, while 'GTAC' was noted in other four strains. Forty-eight isolates excluding six atypical strains showed *S. pneumoniae*-specific digestion patterns of 16S rDNAs (data not shown).

3.6. 16S rDNA tree

16S rDNA tree was drawn based on the sequences of six atypical strains and 23 strains of six streptococcal species retrieved from GenBank (seven of *S. pneumoniae*, five of *S. mitis*, three of *S. oralis*, three of *S. gordonii*, two of *S. sanguinis*, and two of *S. parasanguinis*) (Fig. 3). All seven strains of *S. pneumoniae* including type strain (NCTC 7465^T) belonged to one clade in spite of low bootstrap value. Six atypical strains did not cluster with pneumococcal strains and showed paraphyly. Moreover, SI P25 clustered with two strains of *S. mitis*. *S. gordonii*, *S. sanguinis*, and *S. parasanguinis* showed monophyly with robust support, respectively. Both *S. mitis* and *S. oralis* did not show monophyletic groupings.

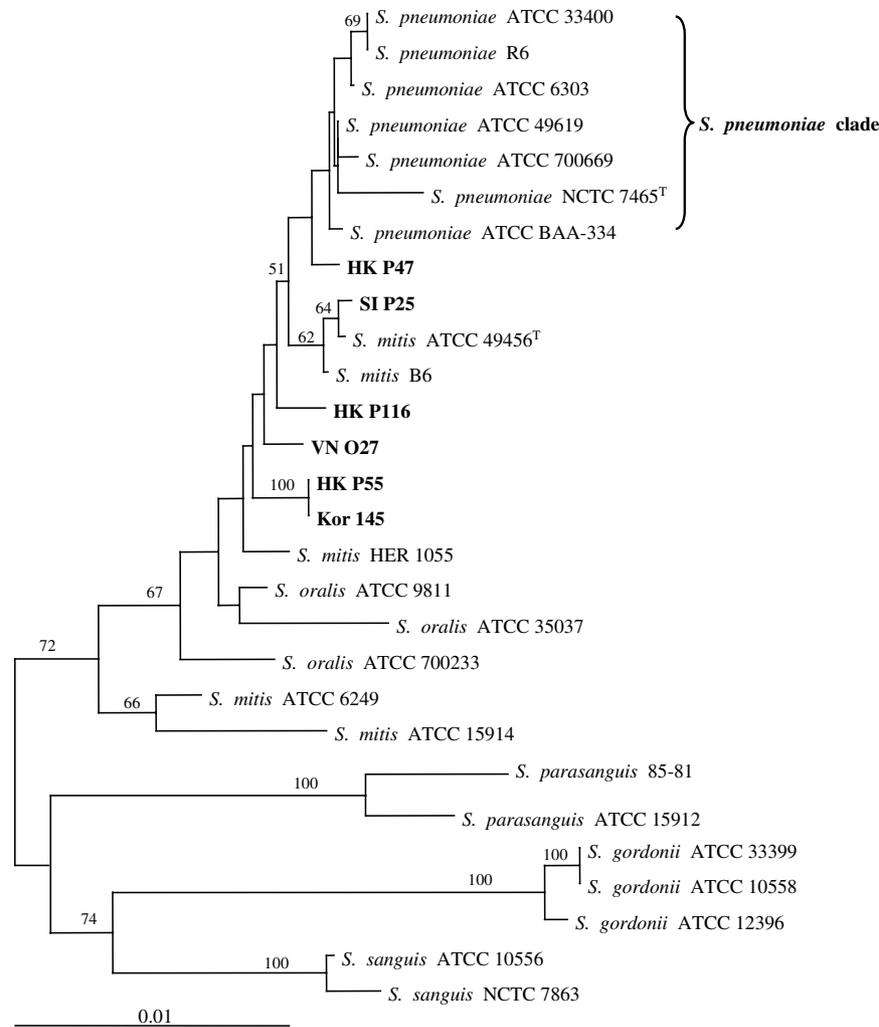


Fig. 3. 16S rDNA tree of six strains used in this study and strains of other *Streptococcus* species. 16S rDNA sequences of six strains were determined in this study, and those of other strains were retrieved from GenBank database. Sequences were aligned by using the CLUSTAL X program. The tree was also reconstructed using the same method of Fig. 1.

showed sequence dissimilarities of at least 4.4% compared with typical *S. pneumoniae* strains in concatenated sequences combining six housekeeping genes. Since intra-species variation of sequence similarities is less than 2% in *S. pneumoniae* by multilocus sequence typing, these strains could be considered different species from *S. pneumoniae*. Although Kor 145 showed sequence divergences of less than 2% from *S. pneumoniae* in *aroE*, *gdh*, *gki*, and *recP* genes, it showed 5.2 to 6.0% sequence divergence in *xpt* gene and its *spi* gene could not be amplified.

Molecular detection of virulence genes of *S. pneumoniae* such as *ply* (pneumolysin), *psaA* (pneumococcal surface antigen A), and *lytA* (autolysin) may not be highly specific for identification of *S. pneumoniae* [6,8,12,27,28]. Our data also indicated that presence of *ply*, *psaA*, and *lytA* could not confirm the identification of *S. pneumoniae* because all or some genes exist in six pneumococci-like atypical strains used in this study.

Therefore, our data suggested that not a single test either phenotypic or genotypic method could not confirm the identification of *S. pneumoniae*.

Obergón et al. [7] reported that atypical pneumococcal strains insoluble to bile contain highly divergent *lytA* genes comparing with typical *S. pneumoniae* and have deletion of two amino acids in the carboxy-terminal domain. In this study, *lytA* gene sequences of Kor 145, HK P47, HK P55, HK P116, and SI P25, were similar to those strains reported by Obergón and others (sequence divergence 2.3–5.8%), while it was highly diverged from typical pneumococci (sequence divergence 19.7–22.3%). These data support the presence of potentially pathogenic bacterial group distinct from *S. pneumoniae*.

Phylogenetic analysis showed that six atypical isolates in this study did not match with *S. pneumoniae*, *S. mitis* or *S. oralis* in 16S rDNA tree, which suggest that they may belong to a new bacterial species. Recently, Arbique et al. [27] proposed *Streptococcus*

pseudopneumoniae, a novel species that resembles *S. pneumoniae* but is included in the *S. oralis*–*S. mitis* group of viridans streptococci based on DNA–DNA hybridization analysis. Although we did not perform DNA–DNA hybridization analysis, six isolates analyzed in this study may belong to *S. pseudopneumoniae* because they share nearly identical 16S rRNA gene sequences.

In summary, we found six isolates among 54 non-typeable *S. pneumoniae* strains from Asian countries that showed sequence divergence of *recP* and *xpt* genes from typical pneumococcal strains. Further genotypic analyses suggested that these six strains were distinct from typical *S. pneumoniae* strains with regard to the sequence divergences of housekeeping genes, analysis of 16S rDNA, and the presence of virulence-related genes. Our data suggest the presence of new, potentially pathogenic bacterial species distinct from *S. pneumoniae*. In addition, conventional microbiologic tests such as optochin susceptibility and bile solubility tests may result in incorrect identification of *S. pneumoniae*.

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