Epidemiology of penicillin resistance in *Streptococcus pneumoniae* isolates in Kayseri, Turkey

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Objective To determine the penicillin resistance and serotype distribution of *Streptococcus pneumoniae* strains and to identify clonal relationships of isolates resistant to penicillin by means of pulsed-field gel electrophoresis (PFGE).

Methods In total, 193 *S. pneumoniae* strains were isolated from clinical specimens between November 1997 and January 2000. Susceptibility testing was carried out by E test, and serotyping by the Quellung reaction. Clonal relationship was analyzed by using PFGE with *sma*I endonuclease.

Results Of the *S. pneumoniae* isolates, 23% were intermediately resistant to penicillin. There were no high-level resistant pneumococci. The majority of isolates intermediately resistant to penicillin were of serogroups/serotypes 19, 23, 14 and 1, in descending order of frequency. There were eight major clones in strains intermediately resistant to penicillin. It was seen that serogroups in the 23-valent polysaccharide vaccine, 7-valent, 9-valent, and 11-valent vaccine formulations caused 92%, 75%, 78% and 87% of pneumococcal diseases in our region, respectively.

Conclusion Penicillin resistance in *S. pneumoniae* is relatively uncommon in Kayseri. All vaccine formulations can prevent the majority of pneumococcal diseases, and there is genetic heterogeneity in intermediately penicillin-resistant pneumococci in this region.

Keywords *Streptococcus pneumoniae*, penicillin resistance, serotyping, PFGE

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INTRODUCTION

Streptococcus pneumoniae is the leading bacterial cause of community-acquired pneumonia as well as acute otitis media, sinusitis, and meningitis. Despite the advent of penicillin and effective antimicrobial agents, and the availability of vaccine, pneumococcal infections are still associated with high morbidity and mortality rates [1,2]. Since the first description of a strain with diminished susceptibility in 1967, resistance among *Streptococcus pneumoniae* isolates has rapidly spread throughout the world [3,4].

Clonal spread and horizontal gene transfer are the plausible explanations for the rapid emergence of penicillin-resistant *Streptococcus pneumoniae* [5]. Clonal spread is the cause of spread of resistant strains between different countries and continents [6,7].

Prevention of pneumococcal disease with vaccine is very important, particularly for individuals in high-risk groups, to decrease morbidity and mortality. The currently available pneumococcal polysaccharide vaccine is not protective in children under 2 years old and in immunosuppressed patients [8]. To improve the current pneumococcal polysaccharide vaccines, new conjugate vaccines in which polysaccharides are covalently linked to carrier proteins have been developed [9].

It is very important to know the serotype distribution and antibiotic resistance in a particular area in order to estimate the probable efficacy of the vaccines in that region [10,11].

The aims of this study were to determine the serotype distribution and penicillin resistance of *Streptococcus pneumoniae* strains in Kayseri and to identify clonal relationships of penicillin-resistant strains by means of pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Bacterial strains

The pneumococcal strains were isolated from 193 patients in the Department of Microbiology of Erciyes University Hospital in Kayseri, a city in central Turkey with a population of about 800 000. All of the isolates were collected from patients with community-acquired infection. The study was performed

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over a period of 26 months (November 1997 to January 2000). The strains were isolated from different specimen sources: 49 from sputum, 34 from cerebrospinal fluid, 33 from ear discharge, 26 from blood infections, 22 from ocular samples, 10 from pleural fluid, eight from soft tissue, seven from nasotracheal aspirate, two from bronchoalveolar lavage, and one from peritoneal fluid.

Identification was carried out by colony morphology on blood agar, Gram stain, susceptibility to optochin, and bile solubility [12]. Strains were stored in microbanks at −70 °C until analyzed.

Streptococcus pneumoniae ATCC 49619 was used as the quality control strain for susceptibility testing, and *Staphylococcus aureus* NCTC 8325 to ensure the comparability of the gels during PFGE.

Sensitivity to penicillin G

Susceptibility testing was performed by E test (AB Biodisk, Solna, Sweden) on Mueller–Hinton agar with 5% sheep blood and incubation in the presence of 5% CO_2. The E test was performed according to the manufacturer's instructions. Strains with MIC ≤ 0.06 mg/L were considered to be susceptible, those with MICs between 0.1 and 1.0 mg/L intermediate, and those with MIC ≥ 2.0 mg/L highly resistant, according to the NCCLS protocol [13].

Serotyping

Serotyping was performed by the Quellung reaction with Pneumotest (Statens Serum Institute, Copenhagen, Denmark).

Pulsed-field gel electrophoresis

This part of the study was performed in Hacettepe University Faculty of Medicine, Department of Infectious Diseases, Ankara, Turkey. PFGE was carried out on intermediately resistant isolates ($n = 44$). *Streptococcus pneumoniae* DNA embedded in agarose blocks was prepared as described by Soares [6]. For digestion, restriction endonuclease *sma*I (Promega, Madison, WI, USA) was used. The DNA macrorestriction fragments were separated in 1.1% agarose gel (Genaxis, Spechbach, Germany) by PFGE (General Navigator, Pharmacia, Uppsala, Sweden). λ-Ladder (Sigma, Deisenhofen, Germany) was used as a molecular weight marker.

Analysis of PFGE profiles

Visual comparison of macrorestriction patterns of the chromosomal DNAs was done using the 'Alpha Imager Documentation and Analysis System' (USA), and distinct patterns were assigned an arbitrary PFGE designation. Assuming that a single mutational event in the chromosomal DNA could introduce maximally a three-fragment difference in the restriction pattern, strains showing more than three-fragment variations were assumed to represent major patterns (assignment of capital letters), while one- to three-fragment differences were considered to represent subtypes (capital letters with numerical subcode) [14].

RESULTS

Intermediate-level penicillin resistance was observed in 44 (23%) of the 193 strains, while there was no strain highly resistant to penicillin. MIC range, MIC_{50} and MIC_{90} (mg/L) of the strains for penicillin were 0.004–1, 0.01, and 0.25, respectively.

The serogroup/serotype distribution of 193 strains is shown in Figure 1. Fifteen strains (8%) could not be serotyped with Pneumotest. It was seen that the majority of isolates were of

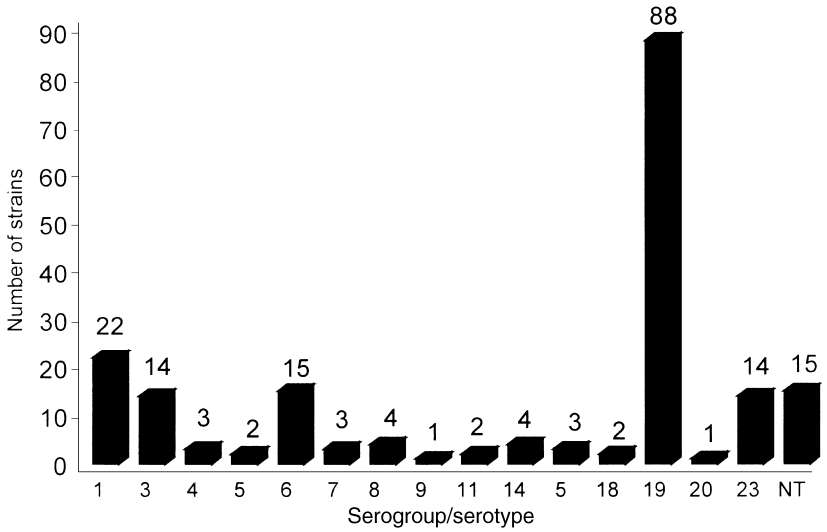

Figure 1 Serogroup/serotype distribution of 193 *Streptococcus pneumoniae* strains.

Figure 2 Representative patterns of the predominant PFGE types. M, molecular weight marker (kb). **Staphylococcus aureus* NCTC 8325; ***Streptococcus pneumoniae* ATCC 49619.

serogroups/serotypes 19, 1, 6, 23 and 3, in descending order of frequency. Among the 44 intermediately resistant strains, the most predominant serogroups/serotypes were found to be 19 ($n = 22$), 23 ($n = 11$), 14 ($n = 3$), and 1 ($n = 3$).

The clonal relationships of isolates that were intermediately resistant to penicillin were analyzed by means of PFGE. In these strains, eight major patterns (A–H) were observed. The majority of the strains (41%) belonged to PFGE type A; however, there were three different subtypes in clone A. Representative patterns of the predominant PFGE types are shown in Figure 2. No relationship was found between serotypes and PFGE types. Genetic heterogeneity was seen in strains intermediately resistant to penicillin.

DISCUSSION

Shortly after the isolation of pneumococci with moderate penicillin resistance in Australia and New Guinea [15], penicillin-resistant pneumococci emerged in many countries. Today, the prevalence of pneumococci resistant to penicillin is increasing worldwide, particularly in Spain [16,17], Hungary [18], and South Africa [19]. Antimicrobial use is generally considered to be one of the major factors in resistance [20,21]. Although antimicrobial use is relatively unrestricted, and self-administration is common in Kayseri, it is surprising that there is still no highly resistant pneumococcal strain and no increase in the prevalence of intermediate-level resistance from 1992 to 2000 in this region [22,23].

It is known that serotype distribution of strains causing invasive disease, nasopharyngeal colonisation and antibiotic resistance is related to age, geography, and socio-economic conditions. Analysis of the leading serotypes in a particular area is very important to evaluate the efficacy of vaccines [10,11].

Penicillin resistance is restricted worldwide to a few serogroups. These serogroups are 6, 9, 14, 19, and 23 [24–27]. However, there is a much wider range in serogroups of the strains intermediately resistant to penicillin [28]. In this study, we found that the leading serogroups/serotypes were 19, 1 and 6 in Kayseri. Intermediate-level resistance to penicillin was mostly observed in serogroups 19 and 23. It was observed that serogroups in the 23-valent-polysaccharide vaccine, 7-valent, 9-valent and 11-valent vaccine formulations caused 92%, 75%, 78% and 87% of pneumococcal diseases in Kayseri, respectively. Each formulation can be used and prevent most of the pneumococcal diseases in this region.

There are three hypotheses about the rapid spread of penicillin resistance:

1. The altered penicillin-binding protein (PBP) genes arise by interspecies recombinational events in which segments of native PBP genes are replaced with the corresponding segments from related streptococcal species [29,30].
2. Horizontal transfer of altered PBP genes from resistant strains to susceptible strains is the main cause of spread of resistance [31].
3. The importation and clonal spread of a small number of resistant clones are important factors in the global increase in the incidence of penicillin-resistant pneumococci [6,7].

In general, it can be proposed that resistant pneumococci that are not closely related genetically but that contain identical altered PBP genes have arisen by horizontal spread, whereas

isolates that are genetically indistinguishable are the result of clonal spread [5]. Clonal spread is the most important mechanism, and causes the spread of resistant clones to geographically distinct areas [6,7].

Spanish serotype 23F strains [32] resistant to penicillin, tetracycline, and chloramphenicol, and variably resistant to erythromycin, have been described as a distinct clone that has spread to numerous countries in Europe [6,31,33], the USA [32,34], and South Africa [35]. The same bacterial clone, but with a 19F capsule, has been found in many countries, suggesting capsular switching [31,36,37]. Serotype 23F clones unique to certain areas have been identified in Finland [38], Italy [37], Israel [39], and Bulgaria [40]. Nevertheless, in Japan and Korea, genetic heterogeneity has been reported in serotype 23F strains resistant to penicillin [41,42]. Not only the 23F clone, but also the 14 [33,40], 9V [31,37] and 19A [18,40] clones, have been dispersed throughout the world.

Although resistant strains have highly related PFGE patterns, there is genetic heterogeneity in intermediate and susceptible strains [38,43]. We found eight different major clones, of which 41% belonged to PFGE type A in our strains.

In conclusion, there are still no high-level penicillin-resistant pneumococci in Kayseri. All of the vaccine formulations are sufficient to prevent most of the pneumococcal diseases in this region. There is no relationship between PFGE type and serotype, and there is genetic heterogeneity in pneumococci intermediately resistant to penicillin.

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