Phenotypic and Genotypic Characterization of Invasive *Streptococcus pneumoniae* Clinical Isolates

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ABSTRACT

Background: The emergence of infection caused by invasive penicillin-nonsusceptible (PNS) and multidrug-resistant strains of *Streptococcus pneumoniae* has become a worldwide concern, necessitating the epidemiologic surveillance of such strains.

Objectives: One aim of this study was to identify clones of invasive PNS *S pneumoniae* among isolates in Riyadh, Saudi Arabia. The second aim was to compare these clones with international clones to track their spread in Saudi Arabia.

Methods: The phenotypes of invasive isolates characterized as *S pneumoniae* were determined using susceptibility testing and serotyping (capsular test and E-test). The genotypes of PNS isolates were determined using random amplified polymorphic DNA analysis. The genetic relatedness of these local strains to the international widespread clones was investigated.

Results: Of 296 *S pneumoniae* isolates identified using biochemical and culture characteristics, 89 (30.1%) were invasive. Susceptibility testing using the E-test revealed that 17 of the 89 invasive isolates (19.1%) were PNS. Most of the 89 isolates (89.9%) were resistant to sulfamethoxazole-trimethoprim; 32.6% and 23.6% of isolates were resistant to chloramphenicol and tetracycline, respectively. All of the isolates (100.0%) were fully susceptible to ceftriaxone and vancomycin. Capsular serotyping of the 89 isolates showed that 19A (18.0%), 6B (14.6%), 23F (13.5%), 9V (11.2%), 14 (6.7%), 19F (5.6%), and 18C (4.5%) were the most predominant serogroups/serotypes. The 17 PNS strains were confirmed on polymerase chain reaction to have penicillin resistance genes. Of these 17 strains, international clone 19A-a was the most predominant (41.2%), followed by 6B-a (17.6%), and 23F-a and 9V-a (each, 11.8%).

Conclusions: The present study identified the spread of the 4 most common PNS *S pneumoniae* isolates (clones)—19A, 6B, 23F, and 9V—to Riyadh, but identified no new clones among patients having invasive infection with *S pneumoniae* in Riyadh. This study emphasizes that international PNS clones have

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contributed to the prevalence and spread of PNS pneumococci among the clinical isolates in Saudi Arabia. (*Curr Ther Res Clin Exp.* 2004;65:423–432) Copyright © 2004 Excerpta Medica, Inc.

Key words: *Streptococcus pneumoniae*, susceptibility, serotyping, random amplified polymorphic DNA analysis.

INTRODUCTION

Streptococcus pneumoniae is a leading cause of invasive and community-acquired bacterial pneumonia, meningitis, sepsis, bacteremia,¹ noninvasive upper respiratory tract infection, and otitis media worldwide.² *S pneumoniae* isolates have become increasingly resistant to penicillin, which was long considered the antibiotic of choice for the treatment of pneumococcal infections.³ However, the emergence of high-level resistance to penicillin, particularly in combination with other resistance determinants, is a serious threat to current treatment strategies.^{4,5} In some countries, the emergence of high-level penicillin resistance has reached 70%,⁶ making adequate antimicrobial therapy difficult. In addition, the excessive use of antimicrobial agents results in selection of strains harboring resistance genes,⁷ leading to treatment failure. In fact, despite Ministry of Health regulations stipulating that patients cannot obtain antibiotics without a prescription, antibiotics are available over the counter in Saudi Arabia. This had contributed to excessive antibiotic use and the emergence of resistant strains there.⁸

It has been proved that infections with isolates intermediately resistant to penicillin, especially infections with invasive pneumococci, could be treated with ceftriaxone,⁹ a third-generation cephalosporin. Thus, vancomycin therapy is restricted for penicillin-nonsusceptible (PNS) *S pneumoniae* isolates. These results were based on the fact that beta-lactam antibiotics reach serum concentrations sufficient to overcome intermediate resistance.⁹ Beta-lactam antibiotics inhibit the growth of pneumococci by reacting to penicillin-binding proteins (PBPs), which inhibits cell-wall synthesis and leads to lysis.¹⁰ Resistance to beta-lactams is caused by a complex production of altered PBPs with resultant decreased antibiotic affinities. Streptococcal resistance genes coding for altered PBPs are acquired from the environment by natural processes of transformation and are then recombined into the genome of pneumococci.¹⁰

The high incidence of pneumococcal infections and the increasing emergence of drug-resistant isolates have become worldwide concerns and are the primary reasons for epidemiologic surveillance. Various methods of determining phenotype and genotype have been developed to assist in epidemiologic investigations to reveal the relatedness between pneumococcal isolates.⁶

Antibiotic susceptibility testing and serotyping (eg, phenotypic assays) are the most common epidemiologic tools for typing pneumococci. However, they have relatively limited discriminatory power.² In contrast, genotyping methods

(eg, ribotyping; pulsed-field gel electrophoresis; and a polymerase chain reaction [PCR]-based, molecular typing method called *random amplified polymorphic DNA* [RAPD] analysis) have shown greater discriminatory power for isolates.^{2,11} RAPD analysis allows the recognition of clusters of strains genetically similar or identical to international widespread clones, facilitating more accurate epidemiologic investigation. In addition, RAPD analysis allows genetically different patterns to be identified in strains having the same serotype.

Because phenotyping and serotyping rely on gene expression and, consequently, may be unstable, DNA-based characterization is now widely accepted.¹² Molecular epidemiology allows the differentiation of isolates that appear identical by conventional methods (ie, susceptibility testing, serotyping).¹³ Moreover, it allows the identification of the international widespread clones of penicillin-resistant pneumococci.¹⁴

One aim of this study was to identify clones of invasive PNS *S pneumoniae* among isolates in Riyadh, Saudi Arabia. A second aim was to compare these clones with international clones to track their spread in Saudi Arabia.

MATERIALS AND METHODS

Bacterial Isolates

Between October 2001 and July 2002, invasive bacterial isolates were collected from blood and cerebrospinal fluid (CSF) specimens from patients with community-acquired or nosocomial infections. The specimens were provided by laboratories at several hospitals in Riyadh. The isolates were grown on Mueller-Hinton agar (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with 5% sheep's blood and incubated at 35°C in 5% carbon dioxide overnight. Alpha-hemolytic colonies exhibiting morphologic characteristics suggestive of *S pneumoniae* were isolated. Identification of these isolates as *S pneumoniae* was confirmed using Gram staining, inhibition with optochin disks, and bile solubility testing.^{14,15}

Susceptibility Testing

Antibiotic susceptibility of the pneumococcal isolates was determined according to the guideline of the National Committee for Clinical Laboratory Standards,¹⁶ which states that the minimum inhibitory concentrations (MICs) of parenteral cepheims depend on the source of the isolate (eg, the break point concentrations used for isolates from patients having meningitis were half those used for isolates from patients not having meningitis¹⁶). The MICs of antibiotics used against *S pneumoniae* isolates were assessed on Mueller-Hinton agar (Oxoid Ltd.) supplemented with 5% sheep's blood, using E-test strips (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. Plates were incubated overnight at 35°C in 5% carbon dioxide.⁵ The standard strain, *S pneumoniae* ATCC 49619 (American Type Culture Collection,

Manassas, Virginia), was included in this study. The E-test antibiotic strips used in this study were penicillin, amoxicillin–clavulanic acid, cefprozil, cefuroxime, ceftriaxone, chloramphenicol, clindamycin, erythromycin, ofloxacin, sulfamethoxazole–trimethoprim (SXT), tetracycline, and vancomycin. High resistance to penicillin was defined as MIC ≥ 2 $\mu\text{g/mL}$; PNS was defined as MIC > 0.12 $\mu\text{g/mL}$. Multidrug resistance (MDR) was defined as resistance to ≥ 3 antibiotic drug classes.¹⁴

Serotyping

Serotyping was performed based on capsular swelling (quellung) reaction with antisera¹⁷ (Statens Serum Institute, Copenhagen, Denmark).

Genotyping

DNA Extraction and Amplification

Chromosomal DNA was extracted from bacterial cells, and the streptococcal resistance gene(s) coding for altered PBPs was amplified using PCR.¹⁰ Genomic DNA of each isolate was prepared using the isolation kit and protocols (provided by Amersham Biosciences AB, Uppsala, Sweden). Briefly, bacterial cells were harvested using centrifugation at 5000 rpm for 3 to 4 minutes at room temperature. The cell lysate, obtained by cell lysis solution, was extracted 3 times with saturated phenol and once with a mixture of phenol–chloroform (1:1). Cellular RNA was eliminated using an RNase. Total DNA was then collected using ethanol precipitation.¹² Penicillin resistance genes were detected using amplification reaction. Briefly, the reaction mixtures contained 2.5 μL PCR buffer, 0.5 μL dNTP (10 mmol/L), 10 ng (1 μL) template DNA, 1.6 ng (1 μL) of each of the 2 reverse primers of resistance gene (5' $\rightarrow$ 3' sequence, 5'GCGCAAGCTTAATATGTATCCGCTCATG3' and 5' $\rightarrow$ 3' sequence, 5'GCGCGAATTCCTATCTCACCAGTCTGTC3'), and 4 U Taq polymerase. The amplification was performed in a DNA thermocycler (model 480, Perkin-Elmer, Inc., Norwalk, Connecticut) programmed for one 5-minute denaturation cycle at 94°C, one 45-second denaturation cycle at 90°C, one 1-minute cycle of annealing at 48°C, forty 1-minute cycles of extending at 72°C, and 10 minutes of holding at 72°C.

The thermocycler amplification products were subjected to electrophoresis on agarose gel (1.2% W/W) in 1 $\times$ tris–borate–ethylenediaminetetraacetic acid buffer stained with ethidium bromide and photographed using a gel documentation system (Gel Doc 2000, Bio-Rad Laboratories, Inc., Hercules, California).

Random Amplified Polymorphic DNA Analysis

Using RAPD analysis, isolates with penicillin resistance genes (as identified on PCR) were analyzed to identify clones. We conducted preliminary experiments with 6 oligonucleotide primers to determine their capacity to amplify

purified DNA of a standard *S pneumoniae* strain (ATCC 49619). This method allowed the selection of 1 working primer.

The 25- μ L reaction mixture, containing 25 pmol RAPD primer (5' $\rightarrow$ d[GCTGCGGGAA]-3'), 50 ng purified template DNA, and distilled water up to 25 μ L, was added to a tube containing the RAPD analysis bead according to the manufacturer's instructions (Ready-To-Go RAPD Analysis Beads, Amersham Biosciences AB). The contents of the tube were mixed using gentle vortexing and were centrifuged in a microfuge briefly (at 14,000 rpm for 30 seconds at 4°C) so the contents would accumulate at the bottom of the tube and could be collected. The reaction mixture was overlaid with 50 μ L sterile mineral oil. The samples were placed in the thermocycler using the following cycle profile: one 5-minute cycle at 95°C, forty-five 1-minute cycles at 95°C, 1 minute at 36°C, and one 2-minute cycle at 72°C.¹¹ A negative control without DNA was included in each RAPD PCR run. The amplified DNA fragments (amplicons) obtained were compared after electrophoresis on 1.5% agarose gel (SeaKem, FMC BioProducts, Rockland, Maine) in triacetate buffer, stained with ethidium bromide, and visualized and photographed using the gel documentation system. A similarity matrix was produced and genetic relatedness was determined and analyzed using the Austin Bio-Diversity Project Database. Clones were labeled with their identified serotype and the letter *a* to signify a clone.

RESULTS

Of 296 *S pneumoniae* isolates identified using biochemical and culture characteristics, 89 (30.1%) were invasive (75 [84.3%] from blood and 14 [15.7%] from CSF). Of these, 17 isolates (19.1%) were PNS. Fifteen of 17 isolates (88.2%) were highly resistant to penicillin.

Phenotype

Antibiotic Susceptibility

Susceptibility testing revealed that 80 of the 89 invasive isolates (89.9%) were resistant to SXT, 29 (32.6%) to chloramphenicol, 21 (23.6%) to tetracycline, 15 (16.9%) to erythromycin, 9 (10.1%) to clindamycin, 6 (6.7%) to cefprozil, 4 (4.5%) to cefuroxime, 3 (3.4%) to amoxicillin-clavulanic acid, and 3 (3.4%) to ofloxacin. All of the isolates (100.0%) were fully susceptible to ceftriaxone and vancomycin.

Serotype

Serotyping of isolates revealed that the most predominant antigenic groups were 19A (16 [18.0%]), 6B (13 [14.6%]), 23F (12 [13.5%]), 9V (10 [11.2%]), 14 (6 [6.7%]), 19F (5 [5.6%]), and 18C (4 [4.5%]). The prevalence of other serotypes (22F, 2, 3, 4, 7F, 8, 10A, 11A, 12F, 20, and nonvaccine serotype from pool sera D and E) ranged from 1 (1.1%) to 3 (3.4%). Six strains (6.7%) were nontypeable.

Genotype

Of the 89 isolates, PCR showed 17 strains (19.1%) had penicillin resistance genes. In these 17 PNS *S pneumoniae* strains, RAPD analysis revealed that international clone 19A-a was the most predominant (7 [41.2%]), followed by 6B-a (3 [17.6%]); 23F-a (2 [11.8%]); 9V-a (2 [11.8%]); and 19F-a, 14-a, and 18C-a (each, 1 [5.9%]) (Table I and Figure).

The epidemiologic parameters of these 17 PNS *S pneumoniae* strains are presented in Table II. Invasive PNS strains were confined to 7 serogroups/serotypes: 19A (7 [41.2%]); 6B (3 [17.6%]); 23F and 9V (each, 2 [11.8%]); and 14, 18C, and 19F (each, 1 [5.9%]). Fourteen of these PNS strains (82.4%) were MDR. PNS strains were most commonly resistant to penicillin and SXT (16 [94.1%]), chloramphenicol (13 [76.5%]), and tetracycline (10 [58.8%]).

DISCUSSION

S pneumoniae species is an important cause of morbidity and mortality, and this pathogen is a leading cause of meningitis and sepsis.⁶ The treatment of infection with penicillin-resistant *S pneumoniae* has become challenging, and reports of treatment failure, especially in infections with invasive MDR *S pneumoniae* species, have increased.¹⁸

The present study agrees with others showing that the increased resistance to antibiotics (other than penicillin) often used for treating pneumococcal infections was in parallel with the spread of penicillin resistance.^{3,18,19}

Table I. Genetic relatedness between the 17 invasive penicillin-nonsusceptible *Streptococcus pneumoniae* (local) strains and international widespread clones.

Local Strain No.	International Clone (Highest % of DNA Relatedness to Local Strains)
1	19A-a (17.1)
3	14-a (6.4)
4	19F-a (100.0)
5	18C-a (56.5)
10	19A-a (59.5)
24	19A-a (67.5)
29	6B-a (60.5)
30	23F-a (69.7)
32	9V-a (46.8)
34	6B-a (25.2)
36	6B-a (33.2)
37	19A-a (88.6)
41	19A-a (30.8)
42	9V-a (9.9)
59	19A-a (77.7)
70	19A-a (11.7)
72	23F-a (34.1)

Figure. Random amplified polymorphic DNA patterns of 9 international widespread penicillin-nonsusceptible (PNS) *Streptococcus pneumoniae* clones and 8 (A) and 9 (B) of the 17 invasive PNS *S. pneumoniae* local strains.

Serotyping of *S. pneumoniae* strains revealed that 7 serogroups/serotypes were represented among invasive PNS *S. pneumoniae* isolates, with 19A, 6B, 23F, and 9V being the most predominant. These 4 serotypes have been shown in previous studies to be the most common causes of invasive *S. pneumoniae* infections and are most often associated with antibacterial drug resistance.^{3,20,21} The present study confirmed the spread of international clones 19A, 6B, 23F, and 9V worldwide,^{5,22} including to Riyadh.

The conjugate pneumococcal vaccines 7, 9, and 11 do not include the 19A serotype. However, the 23 valent vaccine covers all serogroups/serotypes found in the present study. Reduced colonization with the serogroups/serotypes in the vaccine will minimize the spread of these resistant strains.³

In Saudi Arabia, only the 23 polyvalent vaccines are available, and these are given only to children aged >2 years in certain cases based on the patient's clinical background and risk factors (eg, sickle cell anemia, immunocompromise, organ transplantation). To decide which conjugate pneumococcal vaccine to implement in Saudi Arabia's vaccination program, the Ministry of Health is conducting a program for *S. pneumoniae* serotyping at its hospitals (personal communication, Atef Shibl, 2004).

Table II. Epidemiologic parameters of the 17 invasive penicillin-nonsusceptible *Streptococcus pneumoniae* (local) strains.

Local Strain No.	Serotype	Clone Type	Penicillin MIC, µg/mL	Resistance Profile
1	19A	19A-a	4	PEN, SXT, CHL, TET
3	14	14-a	4	PEN, SXT, CHL, ERY
4	19F	19F-a	8	PEN, SXT, CHL, TET, ERY
5	18C	18C-a	1	PEN, SXT
10	19A	19A-a	8	PEN, SXT, CHL, TET, ERY, OFL
24	19A	19A-a	4	PEN, SXT
29	6B	6B-a	4	PEN, SXT, CHL, TET
30	23F	23F-a	2	PEN, SXT, CHL
32	9V	9V-a	4	PEN, SXT, CHL, TET
34	6B	6B-a	1	PEN, SXT
36	6B	6B-a	4	PEN, SXT, CHL, TET, CLI
37	19A	19A-a	8	PEN, SXT, CHL, TET, ERY, Amoxiclav, CEF, CEFP, CLI
41	19A	19A-a	8	PEN, SXT, CHL, TET, ERY
42	9V	9V-a	4	PEN, CHL, ERY
59	19A	19A-a	4	PEN, SXT, CHL, TET, ERY
70	19A	19A-a	4	PEN, SXT, CHL, ERY
72	23F	23F-a	4	PEN, SXT, CHL, TET, ERY

MIC = minimum inhibitory concentration; PEN = penicillin; SXT = sulfamethoxazole-trimethoprim; CHL = chloramphenicol; TET = tetracycline; ERY = erythromycin; OFL = ofloxacin; CLI = clindamycin; Amoxiclav = amoxicillin-clavulanic acid; CEF = cefuroxime; CEFP = cefprozil.

In the present study, RAPD analysis showed that isolates of clones sharing serotypes also shared similar antibiotic susceptibility patterns. These data are consistent with those from previous investigations.^{3,14,15,20,21,23} Future studies should focus on the hazards caused by resistant clones, especially 19A, 6B, 23F, and 9V.

CONCLUSIONS

The present study identified the spread of the 4 most common PNS *S pneumoniae* isolates (clones)—19A, 6B, 23F, and 9V—to Riyadh, Saudi Arabia, but identified no new clones among patients having invasive infection with *S pneumoniae* in Riyadh. This study emphasizes that international PNS clones have contributed to the prevalence and spread of PNS pneumococci among the clinical isolates in Saudi Arabia.

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