Relationship between the Original Multiply Resistant South African Isolates of *Streptococcus pneumoniae* from 1977 to 1978 and Contemporary International Resistant Clones

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High-level penicillin G-resistant as well as multidrug-resistant *Streptococcus pneumoniae* isolates were first described in South Africa in 1977. The relationship between these original multidrug-resistant South African isolates and other resistant clones was investigated. Twenty-six representative isolates isolated from initial outbreaks in South Africa from 1977 to 1978 were characterized by multilocus sequence typing and pulsed-field gel electrophoresis. Twenty-one isolates were penicillin resistant and five were penicillin intermediate, with variable susceptibilities to macrolides, clindamycin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole. Fourteen isolates were serotype 19A, 11 were serotype 6A, and one was serotype 14. Penicillin-resistant serotype 19A isolates belonged to three closely related sequence types (STs), ST 41 (n = 3), and ST 1656 (n = 1). Penicillin-resistant serotype 6A isolates belonged to two closely related STs, ST 1094 (n = 10) and ST 1607 (n = 1), and were not closely related to other international clones. The serotype 14 penicillin-intermediate isolate was not closely related to the other isolates from South Africa but was a predicted founder of a clonal group with 41 different STs. Five new STs, ST 1605, ST 1607, ST 1608, ST 1610, and ST 1656, are described for the first time in this study. New molecular methods have characterized the original multiply resistant South African pneumococcal isolates from 1977 to 1978 and have shown the relationships of these clones to major pneumococcal clones.

*Streptococcus pneumoniae* continues to be a significant cause of morbidity and mortality in humans, causing pneumonia, acute exacerbations of chronic bronchitis, sinusitis, otitis media, bacteremia, meningitis, and other diseases (17). While pneumococci were quite susceptible to penicillin G when this agent was first introduced in the 1940s, with MICs of 0.008 to 0.015 μg/ml, laboratory mutants with higher MICs were first described 20 years later, when investigators in Boston, Mass., noted penicillin G MICs of 0.1 to 0.2 μg/ml for 2 of 200 isolates (13). Hansman and Bullen were the first researchers to realize the significance of higher penicillin G MICs in *S. pneumoniae* when they isolated a strain exhibiting a penicillin G MIC of 0.6 μg/ml in Australia from the sputum of a patient with hypogammaglobulinemia (11).

Little attention was paid to antimicrobial resistance in pneumococci until 1977, when pneumococci with considerably higher levels of penicillin G resistance (2 to 8 μg/ml) were described for patients in Durban, South Africa, with meningitis, bacteremia, pneumonia, and empyema (1). In the same year, Jacobs and coworkers isolated isolates with similar penicillin G and chloramphenicol resistance levels in Johannesburg, South Africa; these isolates were also highly resistant to macrolides, lincosamides, tetracycline, and trimethoprim-sulfamethoxazole (12).

Since then, resistant pneumococci have been identified globally in steadily increasing numbers, especially since the late 1980s. Rates of penicillin G resistance among pneumococci are as high as 60% in some parts of Latin America and as high as 80% in some countries in Asia (2), and multiply resistant pneumococcal isolates are now seen worldwide (20).

In addition, resistance to macrolides is increasing worldwide (20). The most prevalent mechanisms of macrolide resistance in *S. pneumoniae* are mediated by mef(A), a gene encoding an efflux pump, and erm(B), a 23S rRNA methylase that methylates adenine in position 2058, resulting in constitutive macrolide-lincosamide-streptogramin B (cMLSₐ) resistance. In addition, mutations in the 23S rRNA gene and ribosomal proteins L4 and L22 have recently been shown to cause resistance in pneumococci (19). The main epidemiologic marker used to distinguish between pneumococcal isolates is the capsular serotype, with 90 serotypes currently being recognized. More sophisticated epidemiologic markers have subsequently become available, and multilocus sequence typing (MLST) is a recently developed technique that produces unambiguous molecular typing data that can be transmitted electronically via the Internet (http://www.mlst.net) (5, 15).

The present study uses modern molecular methods, including this technique, to analyze resistant *S. pneumoniae* isolates from South Africa (1977 to 1978), which were available in the isolate collections of authors who originally described these isolates.
isolates, and to compare results from these isolates with those of other isolates of *S. pneumoniae* encountered worldwide.

**MATERIALS AND METHODS**

**Bacterial isolates.** Twenty-six pneumococcal isolates, representative of the serotypes and resistance patterns of the original South African isolates and which had been isolated from patients or carriers in Johannesburg and Durban from 1977 to 1978, had been stored at −70°C since their initial isolation and were available for the current study. The identity of isolates as *S. pneumoniae* was confirmed by optochin sensitivity and bile solubility testing, and the capsular serotypes and resistance patterns of the original South African isolates and which were reconstituted with cation-adjusted Mueller-Hinton broth (Oxoid, Wesel, Germany) at 105 CFU/ml.

**Susceptibility testing.** MIC testing was performed using the broth microdilution method recommended by the Clinical Laboratory Standards Institute (CLSI) (4). Dried microtiter plates (Sensititre Susceptibility Plates; TREK Diagnostics Ltd., East Grinstead, West Sussex, England) containing penicillin G, ceftoxime, amoxicillin, clarithromycin, clindamycin, tetracycline, teftothromycin, levofloxacin, trimethoprim-sulfamethoxazole, and chloramphenicol were reconstituted with cation-adjusted Mueller-Hinton broth (Oxoid, Wesel, Germany) plus 5% lysed horse blood (Oxoid) with inocula of 5 × 10^5 CFU/ml. MICs were determined following incubation at 35°C for 20 to 24 h in ambient air and interpreted according to current CLSI (formerly NCCLS) interpretive criteria. For penicillin G, breakpoints of 0.1 to 1 µg/ml (intermediate) and ≥2 µg/ml (resistant) were used (4). *S. pneumoniae* ATCC 49619 was included as a control strain.

**Determination of resistance genotypes and phenotypes.** Primers described previously by Tait-Kamradt et al. and Tricu-Cuot et al. were chosen for detection of *erm* (B) and *mef* genes in macrolide-resistant isolates (24, 26). Preparation of DNA and real-time PCR were performed as described previously (21). Macrolide resistance phenotypes were determined as described previously (16). **Serotyping.** Isolates were serotyped by Neufeld’s capsular swelling reaction on September 7, 2017 by guest http://jcm.asm.org/ Downloaded from using type and factor sera obtained from the Statens Serum Institut, Copenhagen, Denmark (18).

**PFGE.** Genetic relatedness of the isolates was investigated by pulsed-field gel electrophoresis (PFGE) using SmaI digests of chromosomal DNA. Restriction fragments were separated in 1% agarose gels and electrophoresed for 20 h according to the instructions of the manufacturer (GenePath strain typing system; Bio-Rad, Hercules, CA). PFGE patterns were analyzed visually and compared according to standard criteria (25).

**MLST.** MLST was carried out as described previously (5). Briefly, internal fragments of the *aroE*, *gdh*, *gki*, *recF*, *spi*, *spt*, and *ddl* genes were amplified by PCR from chromosomal DNA with the primer pairs described previously by Enright and Spratt (5). The alleles at each of the seven loci provided the allelic profile of each isolate and also defined their sequence types (STs). The allelic profiles of the South African isolates were compared with each other as well as with other isolates in the pneumococcal MLST database using software available at the MLST website (http://www.mlst.net).

**Phylogenetic analysis.** MLSTs were analyzed using the program eBURST. This program is able to display the relationships between closely related isolates of a bacterial species or population. eBURST, unlike cluster diagrams, trees, or dendrograms, uses a simple but appropriate model of bacterial evolution in which an ancestral (or founding) genotype increases in frequency in the population and while doing so begins to diversify to produce a cluster of closely related genotypes that are all descended from the founding genotype. This cluster of related genotypes is referred to as a “clonal complex” (http://eburst.mlst.net) (6). Nine STs (ST 41, ST 124, ST 172, ST 1094, ST 1605, ST 1607, ST 1608, ST 1610, and ST 1656) were compared with all the isolates available in the MLST database. Analysis was done for single-locus variants (SLVs) and double-locus variants.

**RESULTS**

Twenty-six antibiotic-resistant *S. pneumoniae* strains isolated in South Africa from 1977 to 1978 were available for the present investigation, with 25 isolates originating from Johannesburg and 1 isolate (isolate 638) originating from Durban. The antimicrobial susceptibility pattern of isolate 638 was identical to that of the five original penicillin G- and chloramphen-
The multiply resistant Johannesburg serotype 19A isolates were found to belong to ST 41 and ST 1605, with ST 1605 being closely related to ST 41. The serotype 6A strains were predominantly ST 1094, with one being ST 1607, which is closely related to ST 1094. The five penicillin G-intermediate isolates belonged to four STs, two of which were less closely related to the STs of the 21 penicillin G-resistant isolates. Five new sequence types were found (ST 1605, ST 1608, ST 1607, ST 1610, and ST 1656).

eBURST analysis showed that ST 1608 and ST 1610, ST 1094 and ST 1607, and ST 1605 and ST 41 are SLVs of each other. These STs form three groups of two isolates each; i.e., no other SLVs were found in the MLST database. ST 1656 has one SLV in the MLST database (MLST 68). MLST 68 is represented in the MLST database by only one penicillin-resistant serogroup 19 isolate, also from South Africa, which was isolated in 1977. ST 1608/ST1610 and ST1094/ST1607 have no double-locus variants in the MLST database. ST 1605/ST 41 and ST 1656/ST 68 are double-locus variants of each other, forming a group of four clones.

MLST 172 is part of a group composed of 89 sequence types, all connected by SLVs. The predicted founder of this group is MLST 176, represented by serotype 6B/serotype 6A isolates from the United Kingdom, Canada, Italy, Kuwait, and Poland (Fig. 1A). In this analysis, ST 172 is the founder of a subgroup with six SLVs (ST 361, ST 1131, ST 1150, ST 1373, ST 1447, and ST 1526). These strains were isolated in the Philippines.

### Table 2: Serotypes, resistance profiles, MLSTs, and PFGE types of 26 antibiotic-resistant *S. pneumoniae* isolates in South Africa from 1977 to 1978

<table>
<thead>
<tr>
<th>Isolate</th>
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<th>Resistance profile</th>
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<th>PFGE type</th>
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*Abbreviations: p, penicillin G intermediate; P, penicillin G resistant; M, macrolide resistant; L, lincosamide resistant; C, chloramphenicol resistant; T, tetracycline resistant; S, trimethoprim-sulfamethoxazole resistant. Breakpoints (intermediate and resistant, respectively) according to CLSI are as follows: penicillin G, 0.12 to 1 μg/ml and ≥2 μg/ml; erythromycin A (macrolide), 0.5 μg/ml and ≥1 μg/ml; clindamycin (lincosamide), 0.5 μg/ml and ≥1 μg/ml; chloramphenicol, not determined and ≥8 μg/ml; tetracycline, 4 μg/ml and ≥8 μg/ml; trimethoprim-sulfamethoxazole, 1/19 μg/ml 2/38 μg/ml, and ≥47/6 μg/ml.*
Uruguay, Portugal, the United States, South Africa, and Hungary. ST 1447 from South Africa was isolated in 2002 and shows intermediate resistance to penicillin G and resistance to macrolides.

ST 124 (serotype 14) is the predicted founder of a group of 41 different STs (Fig. 1B). ST 124 isolates were reported from Germany, The Netherlands, Scandinavia, the United Kingdom, Australia, and Canada. All isolates in the database were reported to be penicillin G and macrolide susceptible, with the exception of one macrolide-resistant penicillin G-intermediate isolate from Germany. Other members of this group are mostly penicillin and erythromycin susceptible (1 out of 41 was penicillin intermediate, and 6 out of 41 were macrolide resistant).

PFGE patterns generally correlated with ST results. The six isolates of ST 41 (serotype 19A) showed five PFGE profiles (B, C, C2, C3, and D). The isolates of ST 1094 (serotype 19A) were more closely related by PFGE and showed only four profiles (A1, E, F, and F1) (Fig. 2).

DISCUSSION

The first description of pneumococci that were highly resistant to penicillin G was reported previously by Appelbaum et al. in Durban, South Africa, in 1977 (1). The patients described were five children aged between 3 and 24 months, presenting between March and May 1977, and all had received penicillin G or chloramphenicol for relatively long periods prior to the development of these infections, with three of the cases being nosocomial. The pneumococci isolated from these five patients were all serotype 19A and were resistant to penicillin G (MICs, 4 μg/ml), ampicillin (MICs, 2 μg/ml), chloramphenicol (MICs, 16 μg/ml), and trimethoprim-sulfamethoxazole (MICs, 2/38 μg/ml) (1, 27). Initial surveillance studies of nasopharyngeal carriage of resistant pneumococci among hospital contacts and outpatients in Durban did not yield any such isolates. These findings in Durban were followed by the isolation of multidrug-resistant serotype 19A pneumococci in Johannesburg in July.

FIG. 1. (A) eBURST analysis of the clonal relatedness of MLST 172. MLST 172 is part of a group composed of 89 sequence types, all connected by SLVs, with a predicted founder, MLST 176. MLST 172 is a subgroup founder of a group of six SLVs and two double-locus variants. (B) eBURST analysis of the clonal relatedness of MLST 124. ST 124 (serotype 14) is the predicted founder of a group of 41 different STs.
1977 in two hospitals as well as the isolation of isolates with other resistance patterns, some of which were serotype 6A (12). The first Johannesburg isolate was from the sputum of a 3-year-old patient with postoperative pneumonia following cardiac surgery. This isolate had levels of resistance to penicillin G, chloramphenicol, and trimethoprim-sulfamethoxazole that were similar to those found in the Durban isolates and in addition was resistant to erythromycin (MIC > 32 μg/ml), clindamycin (MIC > 32 μg/ml), and tetracycline (MIC, 32 μg/ml). In contrast to the initial experience in Durban, nasopharyngeal carriers of these resistant isolates were found in 19% of pediatric patients and 2% of hospital staff contacts in August 1977 in Johannesburg. A compilation of findings in Durban and Johannesburg from 1977 to 1978 documented penicillin G-resistant pneumococcal infections in 41 children, representing 8.5% of pneumococcal infections, and carriage in 323 hospitalized children (14). Most isolates were penicillin G resistant, with MICs of 2 to 4 μg/ml, although a few were penicillin G intermediate, with MICs of 0.12 to 0.25 μg/ml. Resistant isolates were predominantly serotype 19A, with a few serotype 6A isolates, and a variety of resistance patterns was found.

For many years, epidemiologic discrimination of pneumococcal clones has been based on capsular serotyping. PFGE and MLST were then introduced and were highly discriminative for pneumococcal isolates, and the results of the two methods were generally in agreement (22). In addition, DNA fingerprinting of penicillin-binding protein genes and arbitrarily primed PCR were used to identify predominant penicillin-resistant South African clones isolated from 1987 to 1989 (23).

Most of the clones found in South Africa in the 1970s do not appear to have spread to other countries, with the exception of ST 124 and ST 172. In a study of the clonal distribution of invasive pneumococcal isolates from children and selected adults in the United States, Gertz et al. reported that ST 124, which was demonstrated to be a founder of a clonal complex by the present study, accounted for a large percentage of penicillin-resistant type 14 isolates (10). ST 172 was also described as a serotype 19A strain isolated in the United Kingdom in 1996 from a patient with meningitis. Interestingly, this related isolate was found in a country with strong historic relationships to South Africa, suggesting that the spread of pneumococcal clones may in part parallel human migration to and from these countries. The latter finding has also been observed in other genetically more conserved species, such as Helicobacter pylori (7).

In summary, modern molecular methods have allowed us to characterize the first highly penicillin G-resistant isolate and many multidrug-resistant isolates of S. pneumoniae that had been isolated from 1977 to 1978 in South Africa and to show the relationship of these isolates to other international pneumococcal clones. The serotype 14 South African isolate from 1977 appears to be more widespread and more related to other pneumococcal clones in several countries than the serotype 6A and 19A isolates from this time period.

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REFERENCES


