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Mediterranean clone of penicillin-susceptible, multidrug-resistant serotype 6B *Streptococcus pneumoniae* in Greece, Italy and Israel

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Abstract

In 1996, 19 isolates of serotype 6B *Streptococcus pneumoniae* with a unique resistance pattern were found in carriers attending daycare centres in Patras, Southwestern Greece. These isolates were penicillin susceptible but resistant to chloramphenicol, tetracycline, erythromycin, clindamycin and trimethoprim-sulphamethoxazole. Subsequently, isolates with the same characteristics were found in 23 additional carriers in central and southern Greece in 1997–98 as well as in 19 carriers in central Italy in 1997, and in seven carriers in southern Israel in 1998. Carriers were all children under 6 years of age, attending daycare centres or outpatient hospital visits. The relatedness of the isolates was determined on representative isolates from the three countries by pulsed-field gel electrophoresis of *Sma*I digests of chromosomal DNA. Most Greek isolates were identical to each other, while isolates from Italy and Israel showed one to three band differences, with all isolates being closely related to each other as well as to the isolates from Greece. We have therefore documented the presence of this unique clone of *S. pneumoniae* in these three countries and have named this the 'Mediterranean' clone. While isolates appear to have a common origin, their source and direction of spread are unknown. However, isolates from Italy showed the most diversity, suggesting that this clone had been present in that country for a longer period than it had been in Greece.

Keywords: *Streptococcus pneumoniae*; Mediterranean clone; Resistance

1. Introduction

Administration of antimicrobial agents to young children results in eradication of susceptible isolates and promotes colonization of the upper respiratory tract by more resistant organisms [1–8]. When groups of children are simultaneously or sequentially exposed to antibiotics, the pool of susceptible organisms is replaced by a population of *Streptococcus pneumoniae* with decreased antibiotic susceptibility [8]. New clones introduced into daycare centres promptly colonize children, and colonization persists for longer if clones are resistant to antibiotics. Exposure to antimicrobial therapy in the previous 3 months, young age, female gender and the winter season were associated with colonization by resistant isolates in children attending a daycare centre [8].

Surveillance of pneumococcal resistance in nasopharyngeal isolates from children attending daycare centres has been found to be a practical and useful way to estimate the prevalence of resistant isolates in a community, and to be a good predictor of resistance in

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systemic isolates [6,8,9]. As many clones of antibiotic-resistant *S. pneumoniae* are resistant to multiple antimicrobial agents, use of any agent will select for resistance to all agents to which such clones are resistant. However, resistance in most multi-resistant clones includes penicillin, and clones such as the Spanish multi-resistant type 23F and type 6B clones have spread throughout the world [10,11]. An exception to this was the recent detection of penicillin-susceptible, multi-resistant type 6B isolates, resistant to chloramphenicol, tetracycline, erythromycin, clindamycin and trimethoprim-sulphamethoxazole in Southwestern Greece [12]. The clinical implications of such isolates are significant and include potential for treatment failure with non-β-lactam agents, limitation of choice of agents in treatment failures and penicillin-allergic patients, and failure to detect such isolates if susceptibility testing is performed by screening only for penicillin susceptibility status [6,8,13]. This work addresses the detection of such isolates in other Mediterranean countries and the clonal relationships of these isolates. As such isolates are very distinct from other pneumococci, the finding of isolates with these unique characteristics in three Mediterranean countries led us to hypothesize that these strains had a common origin.

2. Materials and methods

2.1. Isolate selection

Isolates with the susceptibility phenotype of susceptible to penicillin, resistant to chloramphenicol, tetracycline, erythromycin, clindamycin and trimethoprim-sulphamethoxazole were selected from culture collections of three investigators in Greece, Italy and Israel [12,14]. Sixty-one isolates met these criteria and were included in the study.

2.2. Laboratory procedures

Isolates were confirmed as *S. pneumoniae* by optochin inhibition and bile solubility. Isolates were serotyped by the capsular swelling method using antisera from Statens Seruminstitute (Copenhagen, Denmark).

Isolates were tested for susceptibility to chloramphenicol, tetracycline, erythromycin, clindamycin and trimethoprim-sulphamethoxazole by disk diffusion following the guidelines of the National Committee for Clinical Laboratories Standards [15]. Minimum inhibitory concentrations (MICs) of penicillin were determined using the E-test method (AB Biodisk Solna, Sweden) [16].

2.3. Pulsed-field gel electrophoresis

Bacteria from single colonies were inoculated into 5 ml BHI broth supplemented with 10% horse serum (Difco Laboratories, Detroit, MI) and incubated in a 37°C shaking waterbath for 4 h or until turbidity equalled a 2 McFarland standard. Cell pellets were collected by centrifugation of 500 μl culture for 2 minutes at 12 000 × *g*. Cell pellets were resuspended in 100 μl cold cell suspension buffer (Bio-Rad, Hercules, CA) and placed in a dry-bath at 50°C. An equal volume of 2% clean cut agarose (Bio-Rad) and 2 μl lysozyme (Bio-Rad) were then added to the warmed cells, and 100 μl of the cell–agarose mixture was distributed into plug moulds and allowed to solidify. Plugs were placed in 500 μl lysis buffer (10% sodium desoxycholate, 1% disodium phosphate (Bio-Rad)) and incubated for 1 h at 37°C. After the plugs were washed with 1 × wash buffer (10mM Tris–HCl (pH 7.4), 1 mM ethylenediamine tetraacetic acid (EDTA) (Bio-Rad)), a solution of proteinase k reaction buffer and proteinase k (Bio-Rad) was added into each tube and the plugs were incubated overnight in a dry-bath at 50°C. The solution was then decanted and the plugs were washed three times with 1 ml of 0.1 × wash buffer (10 mM Tris–HCl (pH 7.4), 0.1 mM EDTA (Bio-Rad)), and then stored at 4°C.

Approximately 2 mm of each plug was then digested to completion by the addition of 25 U *Sma*I (Bio-Rad) and incubated at room temperature overnight. Pulsed-field gel electrophoresis (PFGE) was performed using a Gene Path System (Bio-Rad). Digested chromosomal DNA was separated using 100 ml of 1% Pulsed-Field Certified Agarose (Bio-Rad) in 1 × TBE (130 mM Tris, 45 mM boric acid, 2.5 mM EDTA). PFGE running conditions were as follows: 18.5 h, 5–20 s ramp time, 6.6 V/cm, 14°C. *Sma*I-digested *Staphylococcus aureus* isolate 8325 (Bio-Rad) was used as a size standard. DNA was visualized by staining with ethidium bromide and gels documented using Gel Doc 1000 gel documentation system (Bio-Rad). The interpretation of the PFGE patterns was based on the criteria of Tenover et al. [17]. These were modified slightly as they are based on application to small (< 30 isolates), single institution, temporally related outbreaks, unlike this study. Isolates with no fragment differences were considered indistinguishable; those with one to three differences, closely related; those with four to six differences, probably related; and those with greater than six differences, unrelated.

2.4. Mef and erm gene detection by polymerase chain reaction

Isolated isolates derived from single colonies were placed in 1 ml saline and total DNA extracted as

follows. Samples were centrifuged at 10 000 × *g* at room temperature for 4 min, and the pellet was then resuspended in 200 μl of a 5% Chelex resin (Perkin Elmer Applied Biosystems, UK) and heated at 60°C for 30 min. After vortexing, samples were heated at 100°C for 10 min and finally centrifuged for 4 min at 10 000 × *g*. Ten micrometers of this supernatant were then used for each amplification. Previously described primer sets for amplification of the *erm* and *mefE* genes were used [18,19]. An initial denaturing step at 94°C for 6 min and a final elongation step at 72°C for 12 min were performed for each polymerase chain reaction (PCR) run. The *erm* gene was amplified under the following conditions: 40 cycles of three steps: 94°C for 75 s, 40°C for 90 s and 72°C for 60 s. PCR products for *erm* genes were of 530 bp. Amplifications for the *mefE* gene were carried out in 40 cycles of three steps: 94°C for 90 s, 52°C for 60 s and 72°C for 60 s. Typical PCR products for *mefE* were sized 348 bp. A negative control with no DNA was included in each PCR run. Amplifications were carried out in a final volume of 50 μl covered with mineral oil. PCR reaction buffers, AmpliTaq Gold DNA polymerase and oligonucleotide primers were obtained from Perkin Elmer Applied Biosystems. The final PCR mixture contained: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 200 μM of each of the deoxynucleotides dATP, dCTP, dGTP and dTTP, 30–50 pmol of each primer set, 1.25 U AmpliTaq Gold. PCR for *erm* gene sequences were performed in 1.5 mM $MgCl_2$, whereas amplifications for *mef* were run in 4.0 mM $MgCl_2$. Fifteen-microlitre samples of each PCR reaction were then electrophoresed on 1.7% agarose gels (Boehringer Mannheim, Germany) containing 0.5 μg/ml ethidium bromide in 0.5 × TBE buffer. PCR products were visualized under UV illumination. Their size was estimated using standard DNA molecular weight markers (φ*X*174 RF DNA cut with *Hae*I, Boehringer Mannheim).

3. Results

The 61 isolates included in this study were selected based on city and country of origin, and date of isolation, and were made up of 35 isolates from Greece, 19 from Italy and seven from Israel. All 61 isolates were confirmed to be type 6B, penicillin susceptible, with modal penicillin MIC value of 0.016 mg/l (range, 0.08–0.03 mg/l), and resistant to chloramphenicol, tetracycline, erythromycin, clindamycin and trimethoprim-sulphamethoxazole by disk diffusion. All isolates were also positive for *erm* gene products and none were positive for *mef* gene products.

Thirty-five isolates had been recovered from paediatric carriers in southwest and central Greece: 28 from Patras, one from Amaliada, one from Pyrgos, one from Agrinio, two from Larissa and two from Trikala between January 1996 and June 1998 (Table 1). Of the 19 isolates isolated from carriers in central Italy in 1997, seven were from Rome, eight from Cassino and four from L'Aquila. Seven isolates were isolated from children attending daycare centres in Beer Sheva, Israel in 1998. Isolates from Greece and Italy were isolated from children attending daycare centres or outpatient clinics. In Italy, isolates from Rome were isolated from carriers attending outpatient clinics of the Department of Paediatrics, University of Rome 'La Sapienza' and from children in daycare centres in two other cities in central Italy (Cassino and L'Aquila).

PFGE analysis of the 61 isolates yielded ten patterns (Table 2 and Fig. 1). The Greek isolates had one predominant pattern, A1, with 33 isolates and two closely related PFGE patterns, A2 and A3, each represented by a single isolate differing by three bands from pattern A1 isolates. The two Greek isolates with different PFGE patterns were isolated from outpatients in 1997 (one from Patras and the other from Pyrgos). Isolates from Amaliada, Agrinio, Larissa and Trikala were identical to the predominant clone in Patras.

In contrast, the Italian isolates had six different but closely related PFGE patterns, with two or three band differences. There were seven, six and three isolates with patterns A4, A5 and A6, respectively, and single isolates with patterns A7–A9. Isolates with PFGE patterns A4, A6, A7 and A9 came from patients in Rome with two, three, one and one isolates in each pattern. Isolates from L'Aquila all belonged to pattern A4.

Table 1
Origins of isolates used in the study

City, country	Number of isolates	Year of isolation	Source
Patras, Greece	12	1996	Daycare centres
Patras, Greece	16	1997–98	Outpatients
Amaliada, Greece	1	1997	Outpatients
Agrinio, Greece	1	1997	Outpatients
Larissa, Greece	2	1998	Outpatients
Pyrgos, Greece	1	1997	Outpatients
Trikala, Greece	2	1998	Outpatients
Rome, Italy	7	1997	Outpatients
Cassino, Italy	8	1997	Daycare centres
L'Aquila, Italy	4	1997	Daycare centres
Beer-Sheva, Israel	7	1998	Daycare centres

Table 2
PFGE patterns of isolates from Greece, Israel and Italy

Country	PFGE pattern										
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	Total
Greece	33	1	1	0	0	0	0	0	0	0	35
Italy	0	0	0	7	6	3	1	1	1	0	19
Israel	0	0	0	0	0	0	0	0	0	7	7

Pattern A5 was only found in isolates from Cassino, with single isolates of patterns A4 and A8 also being from Cassino. All seven Israeli isolates had the same PFGE pattern (A10), which was closely related to the other PFGE patterns found in isolates from Greece and Italy, and which differed by one fragment from the predominant Greek pattern (A1) and by a different fragment from the predominant Italian pattern (A4).

Comparison of the PFGE patterns between isolates from the three countries showed all isolates to be closely related, with one to four fragment differences between isolates. However, none of the isolates from any country was identical to isolates from the other countries. Italian patterns A5 and A8 differed from the predominant Italian pattern (A4) by the absence of one band around 210 kb in size, and the addition of one extra band around 150 kb in size in pattern A5 and two in A8. The A6 PFGE pattern lacked two fragments, one around 360 kb and the other around 210 kb in size, and had one extra fragment >360 kb in size. Pattern A7 had a similar >360 kb fragment to pattern A6, but was missing a fragment around 80 kb. Pattern A9 was similar to A4 except for absence of a 210 kb band and the addition of a 250 kb band.

The A6 Italian and A1 Greek patterns differed by the presence of a >360 kb band in the A6 pattern that was not present in the A1 pattern, and by the presence of 360 and 250 kb bands in the A6 but not the A1 pattern. Comparison of the A1 Greek pattern and the A4 Italian pattern showed the absence of a 250 kb band in A4 as in the other Italian patterns, and an additional band around 210 kb in size in A4, which was not present in any of the Greek isolates. The other two Greek patterns were different from the A1 pattern by the presence of three extra bands around 30, 80 and 170 kb in size in A2, and two extra bands around 80 and 200 kb in size in A3. The major difference between the Israeli PFGE pattern (A10) and the predominant Greek pattern (A1) was the absence of the 250 kb band present in the Greek isolates. While five band differences were found between some isolates, the progression of patterns found is likely to be due to the widespread temporal and geographic distribution of the isolates studied.

4. Discussion

While isolates of *S. pneumoniae* were initially susceptible to many antimicrobial agents, resistant isolates have been described since the introduction of antimicrobial agents [11]. Examples include sulphonamide resistance in the USA in 1943, tetracycline resistance in Britain in 1963, macrolide resistance in Canada and the USA in 1967, and chloramphenicol resistance in Poland in 1970 and in Japan in 1977 [10,11,20]. However, resistant isolates did not become widely disseminated, and most isolates continued to be susceptible worldwide except for isolated reports of low-level penicillin resistance from Papua-New Guinea, Australia, Canada and the USA between 1965 and 1976 [21–23]. This situation changed dramatically in 1977 with the description in South Africa of isolates resistant to penicillin (high-level), chloramphenicol and trimethoprim-sulphamethoxazole in Durban, and isolates additionally resistant to tetracycline, erythromycin and clindamycin in the Johannesburg area [20,24].

Multi-resistant isolates have subsequently been described throughout the world, and are common in

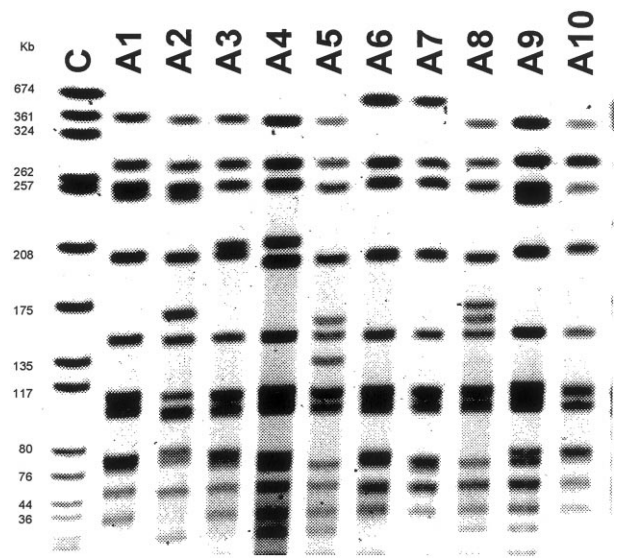

Fig. 1. PFGE patterns of *Sma*I-digested isolates representative of patterns A1–A10. Size markers are shown using *Staphylococcus aureus* isolate 8325 as a size standard (lane marked C, with band sizes (kb) on left).

many countries [3,5,25–32]. A notable feature of most multi-resistant isolates is that resistance usually includes penicillin, and penicillin-susceptible, multi-resistant isolates have rarely been reported [11,23]. One report, however, documented 21 pneumococcal isolates that were penicillin and chloramphenicol susceptible but resistant to tetracycline, erythromycin, clindamycin, and trimethoprim-sulphamethoxazole [9]. These isolates were recovered from nasopharyngeal specimens collected in Johannesburg, South Africa from 1978 to 1985, and included multiple resistance patterns and serotypes 6B, 14, and 19F.

However, a unique group of type 6B *S. pneumoniae* was recently found in southwestern Greece by Syrogiannopoulos et al. in 1996: these isolates were resistant to chloramphenicol, tetracycline, erythromycin, clindamycin and trimethoprim-sulphamethoxazole, but were fully susceptible to penicillin [12]. Detection of phenotypically similar penicillin-susceptible, multi-resistant isolates in Italy [14] and Israel, and their clonal relationships with Greek isolates, therefore led to the findings presented in this paper. In the Italian study, 49 of the 64 (76.6%) multiply resistant strains were penicillin susceptible, 28 serogroup 6 strains also being resistant to the other antibiotics tested [14]. Such strains had not previously been reported from Italy but have the same features as strains recently found in childhood carriers in Greece [12]. The multi-resistant type 6B isolates from Israel also represented a unique phenotype that had not previously been noted in Israel. More recently, a high prevalence of multiply resistant strains was found in childhood carriers in daycare centres in Athens, Greece. More than one-half of the multidrug-resistant strains were susceptible to penicillin, and most belonged to serogroup 6 [33].

The PGFE data showed that all isolates were closely related, with only minor differences between isolates from each country and between the countries, and support our hypothesis that the Italian, Greek and Israeli isolates have a common ancestry. The fact that the isolates from Italy showed the most diversity indicates that isolates may have been present in that country for a longer time period than in Greece. In contrast, isolates from Greece were much more closely related, with 33 of 35 isolates belonging to a single clone, including isolates recovered from carriers in several cities and over a 30-month time period. Although all isolates from Israel were identical, the time period over which these isolates were collected and the limited geographic area sampled limits drawing any further conclusions about these isolates.

The finding of penicillin-susceptible, multi-resistant isolates has important clinical implications. Treatment of infections caused by these isolates is limited to β-lactams, quinolones and vancomycin, and choice of suitable oral agents for paediatric use is particularly limited, especially in penicillin-allergic patients. This problem is compounded by empiric treatment of most infections where *S. pneumoniae* is the predominant pathogen, such as acute otitis media and acute sinusitis. Options for oral treatment of penicillin-allergic patients or in treatment failures in areas where these isolates are prevalent are particularly limited. The presence of such isolates should be specifically investigated when pneumococci are isolated by testing several classes of agents, such as penicillins, macrolides, clindamycin and trimethoprim-sulphamethoxazole, rather than initially screening isolates for penicillin resistance only as is often done.

In conclusion, this work documents the finding of a unique, recently described, clone of penicillin-susceptible, multi-resistant type 6B *S. pneumoniae* in three Mediterranean countries: Greece, Italy and Israel. Further spread of this and other resistant clones will further compromise antimicrobial therapy of respiratory tract infections.

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