Molecular characterization of penicillin non-susceptible \textit{Streptococcus pneumoniae} in Christchurch, New Zealand

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\textbf{Methods}: One hundred and ninety-seven pneumococcal isolates were examined by macrorestriction profile analysis of \textit{SmaI}-digested genomic DNA separated by PFGE and restriction fragment length polymorphism analysis of penicillin binding protein genes.

\textbf{Results}: Four major clonal lineages were identified, the largest and most homogenous containing 95 (48.2\%) of the isolates, the bulk of which (93.7\%), had identical macrorestriction patterns. Members of this clonal group were multidrug-resistant and exhibited high resistance to third-generation cephalosporins, with MICs $\geq$ 8.0 mg/L not uncommon (23.1\%). Two of the clonal groups, each containing 24 (12.2\%) isolates, appeared indistinguishable from the globally widespread Spain\textsuperscript{23F}-1 and France\textsuperscript{9V}-3 strains, respectively. The fourth (12.7\% of isolates) multidrug-resistant clone possessed intermediate penicillin susceptibility (MIC 0.12 mg/L).

\textbf{Conclusions}: This study shows that several distinct penicillin-resistant pneumococcal clones are present in the Christchurch community, most of which appear to have been imported into New Zealand.

Keywords: pneumococci, PFGE, cephalosporins, epidemiology

\textbf{Introduction}

\textit{Streptococcus pneumoniae} is an important cause of community-acquired respiratory infections, including sinusitis, otitis media and pneumonia as well as serious invasive infections such as septicaemia and meningitis. The ability to effectively treat pneumococcal infection has been compromised in recent years due to the acquisition of antibiotic resistance, particularly to \textbeta-lactam drugs.\textsuperscript{1} Pneumococcal resistance to \textbeta-lactams has been attributed to alterations of the penicillin-binding proteins (PBPs) which reduce their affinity.\textsuperscript{2} The first pneumococcal isolate resistant to penicillin was reported in 1967 from a patient in Australia,\textsuperscript{3} and resistant pneumococci have subsequently increased in prevalence worldwide. In some countries, extremely high rates of penicillin-resistant pneumococci (MIC $\geq$ 0.12 mg/L) have recently been reported, for example 79.7\% in Korea,\textsuperscript{4} 65.6\% in Spain,\textsuperscript{5} and 72\% in Taiwan.\textsuperscript{6}

During the 1990s, New Zealand also reported an increase in antibiotic resistance among \textit{S. pneumoniae}.\textsuperscript{7} Reduced susceptibility to penicillin (MIC $\geq$ 0.12 mg/L) increased from 0.2\% in 1992 to 24\% in 1996 among community isolates from Auckland\textsuperscript{8} and from 1.9\% in 1995 to 9.9\% in 1997 among invasive isolates nationwide.\textsuperscript{9} A study of community pneumococcal isolates from four centres during 1997 found the prevalence of high-level penicillin resistance (MIC $\geq$ 2 mg/L) to be 10\% in Christchurch.\textsuperscript{10} By comparison, the reported incidence in the other major New Zealand centres was 6\%, 5\% and 0\% in Auckland, Hamilton and Wellington, respectively, suggesting Christchurch has among the highest prevalence in New Zealand.

Through routine surveillance we noted that pneumococci with reduced susceptibility to penicillin had steadily increased in frequency in the Christchurch community, from 3\% in late 1994 to 30\% in early 1999. This rapid increase in prevalence of resistant pneumococci mirrored the situation seen in some other countries.
such as Iceland, where resistant pneumococci, which had not been observed before 1988, reached a prevalence of 17% by 1992. Likewise in France, resistant pneumococci were rare (≤1%) until 1987, but increased steadily thereafter to reach 25% prevalence in 1993. A similar observation was made in Hong Kong where pneumococci with reduced penicillin susceptibility were first reported in 1993, and by 1995 they had increased in prevalence to 28.9%.14

When such dramatic increases in resistance rates occur during a relatively short time period, detailed molecular analysis of the pneumococcal isolates often reveals that a significant proportion of the resistant population are clonally related. In Iceland, 57 serotype 6B isolates were shown to be indistinguishable from a Spanish 6B clone by both Smal macrorestriction profile analysis (MRP-Smal) determined by PFGE and multilocus enzyme electrophoresis (MLEE).15 In France, 65% of resistant isolates were found to belong to serotype 23F, and were genetically related as assessed by MRP-Smal.14 In Hong Kong, molecular analysis by MRP-Smal of 105 resistant isolates showed that 74% comprised a major clonal group that was indistinguishable from the globally widespread Spanish serotype 23F clone. However, in Hong Kong, this clonal lineage was found to express capsaicin specific transporters (C. trust). Without the ability to discern an index case, types are considered to belong to a given group if they differ by no more than six modifications. Tenover et al. suggested that patterns differing in up to six bands from an index pattern can be considered highly related, and those differing in up to six bands considered possibly related. As the current investigation was spread temporally over 5 years, it was decided that all ‘possibly related’ profiles were to be considered as belonging to the same macro-restriction group. Each group was numbered arbitrarily as new profiles were observed. Within each group, unique profiles were considered distinct subgroupings, and distinguished with an uppercase letter, e.g. 1A, 1B, 1C, etc.

Based on these overseas observations, we hypothesized that the recent rapid increase in pneumococcal penicillin resistance in Christchurch was due to the dissemination of an existing resistant clone. To address this hypothesis, we examined the population structure of penicillin non-susceptible pneumococci collected in Christchurch between 1997 and 2001.

Materials and methods

Bacterial strains and growth conditions

Clinical pneumococcal isolates used in this study were obtained from Medlab South, a community medical laboratory that provides routine diagnostic testing for medical practitioners in Christchurch (population 400 000). This laboratory is used by about half the general practitioners in the region. Isolates were routinely cultivated on tryptic soya agar (TSA) supplemented with 5% defibrinated sheep’s blood (Life Technologies, Auckland, New Zealand) at 37°C in 5% CO₂. Pneumococci were isolated from clinical specimens and identified using standard techniques including characteristic colony morphology, α-haemolysis on TSA 5% sheep’s blood agar, optochin sensitivity and bile solubility. Repeat isolates from the same patient were excluded.

Antibiotic susceptibility testing

Isolates were tested for penicillin non-susceptibility with a 1 μg oxacillin disc. Isolates with reduced susceptibility were tested against five other antibiotics by the disc diffusion method; erythromycin (15 μg), tetracycline (30 μg), co-trimoxazole (25 μg), chloramphenicol (30 μg) and vancomycin (30 μg) (Oxoid, Basingstoke, UK). Tests were carried out and interpreted according to the recommendations of the NCCLS. S. pneumoniae ATCC 49619 was used as a susceptible control strain. Penicillin and cefotaxime MICs were determined using Etest strips (AB-Biodisk, Solna, Sweden). All pneumococcal isolates with reduced susceptibility to penicillin (MIC ≥0.12 mg/L) were collected during two sampling periods: August 1997 through to May 1999 (163 isolates) and March 2001 through to July 2001 (34 isolates).

Pulsed-field gel electrophoresis

PFGE was carried out as described by Hall et al.13 with minor modifications. Overnight growth was suspended in 3 mL of PETT IV (10 mM Tris–HCl pH 7.6, 1 M NaCl) buffer to give turbidity equivalent to a 3.0 McFarland Standard. The cells were collected by centrifugation, resuspended in 125 μL of PETT IV buffer and mixed with an equal volume of 1.6% pulsed-field certified agarose (Bio-Rad, Hercules, CA, USA). The molten agarose was injected into plug moulds, and once solidified, incubated overnight in 2 mL of ESP buffer (0.5 M EDTA pH 8.0, 0.5% N-lauroyl sarcosine) containing proteinase K (final concentration 0.5 mg/mL) at 50°C. Following cell lysis, plugs were transferred to sterile 50 mL Falcon tubes and were washed three times in TE buffer (10 mM Tris–HCl pH 7.6, 1 mM EDTA), overnight at 4°C. Embedded DNA was digested using either of the restriction enzymes Smal or Apal (Roche, Mannheim, Germany) in 150 mL volumes using 20 U of enzyme in accordance with the manufacturer’s instructions. PFGE was carried out in a 1% agarose gel (pulsed-field certified agarose; Bio-Rad, Hercules, CA, USA) in 0.5 × TBE buffer (50 mM Tris–HCl, 50 mM boric acid, 0.1 mM EDTA). Concatamers of lambda DNA (PFG marker; New England Biolabs, Beverly, MA, USA) were used as a size standard. Electrophoresis was carried out on a CHEF DRIII system (Bio-Rad). Electrophoretic conditions were: running temperature 14°C, gradient 6.0 V/cm, run time 22 h, initial switch time 5 s, final switch time 35 s and a linear ramping factor. Gels were stained with ethidium bromide (0.5 mg/L) post-electrophoresis and visualized on a UV trans-illuminator.

MRP-Smal analysis

Band patterns were compared visually and were interpreted as described by Tenover et al. with modifications. Tenover et al. suggested that patterns differing in up to three bands from an index pattern can be considered highly related, and those differing in up to six bands considered possibly related. As the current investigation was spread temporally over 5 years, it was decided that all ‘possibly related’ profiles were to be considered as belonging to the same macro-restriction group. Each group was numbered arbitrarily as new profiles were observed. Within each group, unique profiles were considered distinct subgroups, and distinguished with an uppercase letter, e.g. 1A, 1B, 1C, etc.

PCR amplification of pbp genes

PCR amplification of the pbp genes was carried out using the previously described primers; Pn2Bup 5’-GAT CCT CTA AAT GAT TCT CAG GTG G-3’ and Pn2Bdown 5’-CAA TTA GCT TAG CAA TAG GTG TGT G-3’ for pbp2b,22 and pbp2x-up 5’-CGT GGG ACT ATT TAT GAC CGA AAT GG-3’, pbp2x-down 5’-ATT TCC AGC ACT GAT GGA AAT AAA CAT ATT A-3’ for pbp2x. Total cellular DNA extraction was carried out using the rapid guanidinium thiocyanate procedure described by Pitcher.
et al. Amplification was carried out in 50 mM volumes, each reaction consisted of 1 μg of template DNA; the appropriate primers at a final concentration of 2 μM per reaction; each dNTP at a final concentration of 200 μM; MgCl2 at a concentration of 1.5 mM; and 2.5 U of Taq DNA polymerase (Roche) in the reaction buffer provided. Reaction mixtures were overlaid with two drops of mineral oil and amplification was carried out in a Corbett Research FTS 320 thermal cycler. Amplification consisted of an initial denaturation of 1 min at 94°C, 35 cycles of the following three-step cycle; template denaturation for 15 s at 94°C, primer annealing for 45 s at 55°C and template elongation for 1 min at 72°C. A final extension step of 5 min at 72°C was included.

Restriction fragment length polymorphism (RFLP) analysis of pbp genes

PCR-amplified pbp genes were subjected to restriction endonuclease digestion using either DdeI or HinfI (Roche). Restriction digests were carried out in final volume of 15 μL and incubated at 37°C for 3 h. RFLP patterns were visualized after electrophoresis through 3.0% agarose gels (ultraPURE, Life Technologies, Auckland, New Zealand) at 6 V/cm for 2 h.

Serotyping

Serotyping was carried out by the New Zealand Reference Laboratory (Porirua, Wellington) using the capsular reaction test (Neufeld test) and the Danish system of nomenclature. Serotype data were not available for all isolates.

Results

The age of the patients from whom the isolates were recovered ranged from 1 week to 91 years. Ninety-one isolates (46.1%) were recovered from patients 2 years or younger, and 33 (16.8%) were recovered from patients 60 years or older. Isolates were recovered from 101 (51.2%) females, 94 (47.7%) males, with the sex of two patients unknown. The most prominent sites of isolation were ears (29.4%), eyes (28.4%) and sputa (20.3%), with the sex of two patients unknown. The most prominent sites consisted of an initial denaturation of 1 min at 94°C, 35 cycles of the following three-step cycle; template denaturation for 15 s at 94°C, primer annealing for 45 s at 55°C and template elongation for 1 min at 72°C. A final extension step of 5 min at 72°C was included.

Antimicrobial susceptibilities

The criterion for inclusion in this study was a penicillin MIC of 0.12 mg/L or greater. The highest penicillin MIC observed was 8.0 mg/L, and the MIC50 and MIC90 were 2 and 4 mg/L, respectively. Cefotaxime MICs ranged from 0.06 to 16 mg/L, with MIC50 and MIC90 values of 1.0 and 8.0 mg/L, respectively. In contrast, MICs of penicillin and cefotaxime of 0.5–4.0 mg/L and 0.25–2.0 mg/L, respectively. All isolates of this group were susceptible to chloramphenicol. Fourteen of the isolates belonging to this group were serogrouped and determined to belong to serogroup 19, four of the 14 were typed further as 19F.

Macrorestriction group 2 comprised 24 (12.2%) isolates. The group was more genetically heterogeneous than MRP group 1, evident by the identification of a total of 13 different subtypes, each containing from one to eight isolates. Subtypes arose due to slight but distinct changes in mobility of individual bands around 200–250 kb. Isolates within MRP group 2 had a range of penicillin and cefotaxime MICs of 0.5–4.0 mg/L and 0.25–2.0 mg/L, respectively. All isolates were multi-resistant, typically exhibiting resistance to co-trimoxazole, erythromycin and chloramphenicol, with some isolates also resistant to tetracycline. Ten representative isolates from MRP group 2 were serotyped, and expressed capsular type 23F. MRP group 2 isolates were related to the PMEN-defined Spain23F-1 clone (ATCC 700669), being most similar to profile 2E (Figure 1b).

Macrorestriction group 3 consisted of 24 isolates (12.2%) and five subtypes. Each subtype contained between four and six isolates. Penicillin and cefotaxime MICs for this group were in the range 0.5–4.0 mg/L and 0.5–2.0 mg/L, respectively. This group was typically not multidrug-resistant, although 18 isolates (75%) were resistant to co-trimoxazole as well as penicillin. Only two members of this group were serotyped, and were determined to be one of each serotype 9 and 14. MRP group 3 isolates were related to a representative of the France9-3 clone (ATCC 700671), the most similar profile being 3C (Figure 1c).

Macrorestriction group 4 contained 25 isolates (12.7%) distributed among 10 subtypes (Figure 1d). Each subtype contained between one and eight isolates. A degree of heterogeneity was exhibited between MRPs, however, most of the diversity observed could be attributed to a single Smal fragment, which
varied in size between 100 and 150 kb. MRP group 4 isolates were all intermediately resistant to penicillin (MICs 0.12–0.25 mg/L), but all were multidrug-resistant. All MRP group 4 isolates were resistant to co-trimoxazole and erythromycin. Most (96%) were also resistant to tetracycline. Only two group 4 isolates were serotyped, and both of these were found to be non-typeable.

Macrorestriction profiles 5–25 contained the remaining 29 isolates and represented unique profiles that differed significantly (six bands or greater) from each of the major groups, and from each other. Of these 21 MRP groups, most (81%) contained only one isolate representative of that profile (Table 2). Four MRP groups (5, 9, 10 and 12) could be differentiated into two subgroups, the largest of which (9A) contained three isolates. Many of the isolates were also resistant to antibiotics besides penicillin; resistance to co-trimoxazole, tetracycline, erythromycin and chloramphenicol was noted in 20, 17, 16 and seven isolates (69%, 59%, 55%, 24%), respectively. A notable multidrug-resistant cluster, MRP group 10, contained four isolates in three subgroups and each of these four isolates was serotyped as 19F. These isolates had penicillin and cefotaxime MICs of 2–4 mg/L and 1–4 mg/L, respectively. They also expressed resistance to all of the non-\(\beta\)-lactam drugs tested, except vancomycin.

### Analysis of \(pbp\) RFLP patterns

Twelve different RFLP profiles of the \(pbp2b\) gene were observed (Figure 2a). The three most frequently occurring profiles...
(A, B and H) were associated with 98 (49.7%), 55 (28.0%) and 26 (13.2%) isolates, respectively. Twelve different RFLP profiles of the \( \text{pbp2x} \) gene were observed (Figure 2b). The three most frequently occurring profiles (A, B and F), were associated with 86 (43.7%), 67 (34.0%) and 27 (13.7%) isolates, respectively.

The association of \( \text{pbp} \) RFLP types with MRPs and their associated \( \beta \)-lactam MICs is shown in Tables 1 and 2. MRP group 1 was almost exclusively associated with the \( \text{pbp2b/2x} \) RFLP profile A/A, and this combination was associated with resistance to both penicillin (MIC \( \leq 4 \) mg/L; range 1.0–8.0 mg/L) and cefotaxime (MIC \( \leq 2 \) mg/L; range 0.5–16 mg/L). MRP groups 2 and 3 were associated with the \( \text{pbp2b/2x} \) RFLP profile B/B. It has been noted previously that the \( \text{Spain}^{23F} \)-1 and \( \text{France}^{9V} \)-3 clones (MRP groups 2 and 3, respectively) have identical RFLP profiles for \( \text{pbp1a}, \text{pbp2b} \) and \( \text{pbp2x} \) genes, suggesting a common origin of resistance within these two clones. The \( \text{pbp2b/2x} \) RFLP profile B/B was noted in 52 isolates (including MRP groups 8, 10 and 20), and was associated with penicillin resistance (MIC \( \leq 2 \) mg/L; range 0.5–4.0 mg/L) and intermediate cefotaxime susceptibility (MIC \( \leq 2 \) mg/L; range 0.5–4.0 mg/L). The MRP group 4 produced \( \text{pbp} \) RFLP profile H/F, which was consistently associated with intermediate penicillin susceptibility (MIC \( \leq 2 \) mg/L) and susceptibility to cefotaxime. Among the 21 non-clonal MRP groups, considerable variation was observed within the \( \text{pbp2b/2x} \) RFLP patterns indicating further the genetic heterogeneity among these isolates.

**Discussion**

The isolation frequency of penicillin non-susceptible pneumococci has increased significantly in many parts of the world during the 1990s. The application of molecular techniques (such as MRP) has introduced a means of effectively discriminating between clonally related isolates, and genetically unrelated isolates sharing a common phenotype (e.g. serotype). When applied to rapidly emerging resistant pneumococcal populations, clonal lineages are often found to comprise a large proportion of the population.

One of the most successful multi-resistant pneumococcal clones throughout the world was first identified in Spain during the early 1980s and subsequently designated the \( \text{Spain}^{23F} \)-1 clone according to PMEN nomenclature. Given the global distribution of the \( \text{Spain}^{23F} \)-1 clone, it was unsurprising to detect this clone in New Zealand. However, the Christchurch variants of the \( \text{Spain}^{23F} \)-1 clone differ slightly from its previously described antibiotic susceptibility profile. The more common profile observed included resistance to erythromycin as well as co-trimoxazole and...
chloramphenicol, with or without resistance to tetracycline (48% and 44%, respectively). This resistance pattern has been associated with the Spain 23F-1 clone previously; in Hong Kong, 76 of 105 isolates were shown to be related to the Spain 23F-1 clone by MRP-Smal/ApaI, yet 93% were resistant to erythromycin. This illustrates the further evolution of this highly successful clone in response to macrolide usage, to which very high rates of resistance have been reported in some parts of the world.

Table 2. Distribution of Smal MRP subgroups, and their associated serotypes and antibiotic resistance profiles, among the 21 minor MRP groups of penicillin-resistant pneumococci in Christchurch

<table>
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<tr>
<th>MRP group</th>
<th>MRP subgroup</th>
<th>n</th>
<th>Serotype</th>
<th>pbp RFLP</th>
<th>MIC range (mg/L)</th>
<th>Resistance profile</th>
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*Number of isolates serotyped shown in parentheses; ND, not determined; NT, non-typeable.

Figure 2. Comparison of RFLP profiles of pbp genes. Molecular size marker (M) indicated in base pairs. (a) PCR amplified pbp2b gene digested with DdeI; lanes 1–10 correspond to RFLP profiles A–J, respectively; (b) PCR amplified pbp2x gene digested with Hinfl; lanes 1–11 correspond to RFLP profiles A–K, respectively.
Another successful clone, the France 9V-3 clone, has also demonstrated its capacity to disseminate globally, and establish itself in regions geographically widespread. Consequently, it was not surprising to observe the France 9V-3 clone in New Zealand in approximately equal abundance to the Spanish clone. Although only two isolates of the New Zealand France 9V-3 variant were serotyped, one was found to be serotype 14 suggesting exchange of genes for capsule biosynthesis had occurred. The acquisition of serotype 14 by the France 9V-3 clone has been shown previously in Uruguay, and was later confirmed by Coffey et al. Isolates belonging to PFGE group 1 were notably associated with high levels of resistance to cefotaxime; 26 of 95 (27.3%) of the isolates had a cefotaxime MIC $\geq 4$ mg/L. In 1997–98, a significant increase in prevalence of pneumococcal resistance to cefotaxime (MIC $\geq 2$ mg/L) was described in New Zealand. Prevalence increased from 5.4% in 1996 to 25.4% during the first 6 months of 1997. During this time, 216 cefotaxime-resistant (MICs $\geq 2$ mg/L) pneumococcal isolates were submitted to the New Zealand antibiotic resistance reference laboratory. Of these, 113 (52%) had cefotaxime MICs of $\geq 4$ mg/L (the revised non-meningitis cefotaxime resistance breakpoint in the 2002 NCCLS guidelines) and most (112/113) belonged to serotype 19F. DNA MRP analysis also suggested that these isolates were clonal. This was the first report of the epidemic multiresistant serotype 19F clone that is now prolific in New Zealand. The MRPs described in 1997 were indistinguishable from the MRP group 1 penicillin-resistant isolates from Christchurch described in this study (data not shown).

This study shows that the major New Zealand clone (MRP group 1) is homogenous as assessed by MRP analysis. Other globally widespread pneumococcal clones identified in New Zealand during this study (e.g. Spain 23F-1) have small, but significant changes in their MRP profiles due to evolutionary divergence. The high level of homogeneity observed in the New Zealand 19F clone suggests that it arrived in New Zealand recently and therefore dissemination of this clone would also have been recent. Comparison of the MRP 1 with the published macro-restriction profile of the Taiwan 19F-14 clone shows many similarities with the predominant New Zealand 19F clone. MRP group 4 was also multidrug-resistant (resistant to erythromycin, co-trimoxazole and tetracycline), yet it had only intermediate resistance to penicillin (MIC 0.12 mg/L) and was susceptible to cefotaxime (MIC range 0.06–0.12 mg/L). Of the 25 isolates belonging to this group, 23 (92%) were isolated from eye swabs. This is considerably more frequent than non-group 4 isolates of which 19% were isolated from eyes ($P \leq 0.001$, $\chi^2$ test). Two of the MRP group 4 isolates were submitted for serotyping and were found to be non-typeable. Non-typeable pneumococci have previously been implicated in outbreaks of conjugavititis and molecular analysis of such outbreaks often shows the non-typeable pneumococci to be genetically indistinguishable. Consequently, isolates of MRP group 4 are likely to be responsible for many cases of pneumococcal conjunctivitis in Christchurch.

Overall, although four clonal groups have been shown to be present in Christchurch, the most predominant appears to be a New Zealand variant of the Taiwan 19F-14 clone, which accounted for nearly half the isolates characterized in this study. Assuming this to be the case, it would seem there is an intimate link in the movement of antibiotic resistance between New Zealand and countries of South-East Asia. This is likely to be influenced by increased tourism and other movements of population in this area of the world. This observation is significant, as future surveillance strategies must therefore consider Asia as a likely candidate for the introduction of novel resistance mechanisms in New Zealand. However, it should also be noted that the New Zealand variant of the Taiwan 19F-14 clone appears to have characteristics which make them unique from their parental lineage; notably high level macrolide resistance mediated by the $erm$ (B) gene, and its high-level resistance to cephalosporins.

The surveillance of antibiotic-resistant pneumococci in New Zealand should be continued, with due attention to the mechanisms of resistance. This will enable the rapid identification of new resistant clones and novel resistance genotypes, and better allow the therapeutic consequences to be anticipated. However, for long-term extrapolation of resistance trends in New Zealand, antimicrobial resistance surveys in neighbouring countries within the Asia/Pacific region must be considered, as these are the most likely reservoirs for the importation of antibiotic-resistant pathogens into New Zealand.

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References

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