An outbreak of penicillin resistant *Streptococcus pneumoniae* investigated by a polymerase chain reaction based genotyping method

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Abstract

Aims—To characterise the genotypes of penicillin resistant *Streptococcus pneumoniae* infecting patients in a care of the elderly ward and to study its transmission in a hospital environment.

Methods—Isolates of *S pneumoniae* were cultured from specimens obtained from patients who had been admitted to a care of the elderly ward where an outbreak had occurred. Penicillin resistant *S pneumoniae* were also obtained from a series of surveillance throat swabs taken from patients in the same ward. In addition, all penicillin resistant *S pneumoniae* isolated from specimens submitted for culture at the time of the outbreak were included. Four sensitive strains isolated from a routine microbiology laboratory were included as controls. A simple polymerase chain reaction (PCR) based genotyping method for the penicillin binding protein (PBP) genes 1a, 2x, and 2b was used to characterise the genotypes.

Results—Nine patients were infected with serotype 9 *S pneumoniae*. Four of these patients died; two deaths were directly attributable to the infection. Tested against a battery of haemolytic streptococci and other organisms found in the respiratory tract, only two false positive reactions for PBP 2x were found among *S mitis*. The method demonstrated that the outbreak strain had altered PBP 1a, 2b, and 2x genes, a pattern clearly distinguishable from other penicillin resistant strains isolated at the same time.

Conclusions—This method is simple to perform and would enable many laboratories to characterise the genotype of penicillin resistant *S pneumoniae* and investigate transmission in their hospitals.

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Keywords: polymerase chain reaction; *Streptococcus pneumoniae*; penicillin resistance; penicillin binding proteins

Streptococcus pneumoniae is an important cause of morbidity and mortality in all age groups worldwide.¹ Shortly after the introduction of penicillin, laboratory mutants resistant to this agent were identified but resistant strains isolated in a clinical environment did not emerge for a further 20 years.² Resistance as a serious and common clinical problem was first described in Papua New Guinea and South

Africa.³⁻⁴ In the latter this was associated with a resistance to many other agents. Resistant isolates are now found throughout the world with a prevalence reaching up to 50% in some European countries.⁵⁻⁶ The molecular mechanisms leading to the development of penicillin resistance are now beginning to be understood. Recombination events between *S pneumoniae* penicillin binding protein (PBP) genes and short segments of homologous genes in organisms that share the same ecological niche occur at low frequency.⁵⁻⁷ These genetic modifications produce a new gene product with a reduced affinity for penicillin. The PBPs usually implicated are PBP 1a, 2b, and 2x.⁵ The origin of some of this DNA has been traced to *Streptococcus oralis* and *Streptococcus mitis*,⁸ two species closely related to *S pneumoniae*.⁹

The standard method of typing pneumococci is serotyping and this has been used to follow the spread of penicillin resistant *S pneumoniae*. The value of this approach is limited because many resistant isolates fall into a small number of serotypes.⁵⁻¹⁰ Alternative methods for typing *S pneumoniae* include multilocus enzyme electrophoresis, multilocus enzyme electrophoresis ribotyping, and field inversion gel electrophoresis,¹⁰⁻¹² but these are only available at specialist centres. To investigate an outbreak, a method to identify resistance and type the outbreak strains must be readily available. We report a simplified polymerase chain reaction (PCR) based PBP genotyping method that makes it possible to study the transmission of penicillin resistant *S pneumoniae* in a hospital environment.

Methods

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These organisms had been speciated by DNA-DNA hybridisation and phenotypic testing as described previously.⁹⁻¹³⁻¹⁴ The strains studied were: *S mutans* (KPSK2, 161, B48), *S sobrinus* (B 542, OMZ 176, SL-1T, OMZ 65, TH 21, TH 62), *S salivarius* (NCTC 8618, A385, NCTC 8606, H53), *S vestibularis* (JW 3, LV 71, MM1T), *S sanguis* (NCTC 7863T KPE 2), *S parasanguis* (85-81, UC 4989, MGH 143, SS 897, SS 895, FW 213), *S gordonii* (NCTC 7868, HF 9017, M 5), *S crista* (CR 311, CR 3, AK 1), *S oralis* (NCTC 7864, LGV 1 PC 1467, OPA 1), and *S mitis* (K 208, NCTC 10712). In addition, isolates of the following species were also studied: *Haemophilus influenzae* (six isolates), *H parainfluenzae* (two isolates),

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Figure 1 PCR amplified PBP 1a DNA digested with Hinf I. Lane 1, pGem marker; Lanes 2–4, sensitive *S. pneumoniae*; Lane 5, strain AD*; Lane 6, strain EA*; Lane 7, strain P; Lane 8, pGem marker. *Outbreak strains.

Moraxella catarrhalis (eight isolates), *Staphylococcus epidermidis* (three strains), *Staphylococcus aureus* (methicillin sensitive, six isolates; methicillin resistant, three isolates).

PATIENTS AND ISOLATES

Isolates of *S. pneumoniae* were cultured from specimens obtained from patients who had been admitted to one of the care of the elderly wards of the Whittington Hospital in London. Penicillin resistant *S. pneumoniae* were also obtained from a series of surveillance throat swabs taken from patients in this ward. In addition, all penicillin resistant *S. pneumoniae* isolated from specimens submitted for culture at the time of the outbreak were included. Four sensitive strains isolated from the routine microbiology laboratory of the Royal Free Hospital were included as controls. The serotypes were determined by the Streptococcal Reference Laboratory of the Public Health Laboratory Service using standard techniques. The minimal inhibitory concentration (MIC)

of penicillin, tetracycline, erythromycin, and chloramphenicol was determined by the NCCLS method.¹⁵

FINGERPRINTING PBP 1a, 2b, AND 2x GENES

All chemicals were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated. Polymerase chain reaction (PCR) methods were used to amplify *S. pneumoniae* DNA as follows. Colonies isolated on blood agar were used as a source of genomic DNA. The colony picks were placed in 50 µl sterile distilled water and heated to 95°C for five minutes. An aliquot of 10 µl was used in the PCR reaction. The entire PBP 1a gene was amplified as a 2.4 kb fragment using the following oligonucleotide primers: 5'-CGGCATTCGATTTGATT-3' and 5'-GATGTCTTCTCAGGCTTTTG-3'. The PBP 2x gene was amplified as a 2.0 kb fragment using the following oligonucleotide primers: 5'-CGTGGGACTATTTATGACCGAAATG G-3' and 5'-AATTCCAGCACTGATGGA AATAACATATTA-3'. The PBP 2b gene was amplified with the following oligonucleotide primers: 5'-GATCCTCTAAATGATTCTCA GGTGG-3' and 5'-CAATTAGCTTAGC AATAGGTGTTGG-3'. For each PCR reaction the master mix consisted of 10 µl 100 mM (NH₄)₂SO₄, 670 mM Tris-HCl (pH 8.8), 0.1% vol/vol Tween 20, 3 µl dNTP (5 mM), 3 µl MgCl₂ (50 mM), 4 µl primers (0.1 mM), 0.2 µl Taq polymerase (1 unit) (Bioline, London, UK), and 68.2 µl sterile distilled water. The thermocycling protocol was as follows: 95°C for five minutes, followed by 94°C for one minute, 55°C for two minutes, and 72°C for three minutes for 30 cycles, and a final incubation at 72°C for seven minutes using a Hybaid Omnigene II thermocycler. The amplified DNA was purified using the Wizard (Promega, Southampton, UK) PCR preparation kit according to the manufacturer's instructions. Penicillin binding protein 1a DNA was digested by Hinf I (Promega); between 10 and 50 µl of DNA sample derived from the PCR reaction was mixed with 1–5 µl ×10 buffer (10 mM Tris-HCl pH 7.5, 60 mM NaCl, 7 mM MgCl₂, 0.1 mg/ml bovine serum albumin (BSA)) and 1 µl enzyme (10 units) and incubated for 16

Table 1 Demographic and outbreak details

Isolate	Age	Ward area	Source of isolate	Infection	Outcome	Minimum inhibitory concentration (mg/l)				Sero-type
						Pen	Ery	Chlor	Tet	
CG	92	CoE	Blood	LRTI	Died, related	0.5	0.06	2	0.125	9
AY	86	CoE	Sputum	LRTI	Recovered	1.0	0.06	2	0.125	9
JF	93	CoE	Blood	LRTI	Died, related	1.0	0.03	2	0.125	9
LM	82	CoE	Pharynx	Carriage	Died, unrelated	2.0	0.06	4	0.25	9
AD	92	CoE	Sputum	LRTI	Recovered	1.0	0.06	2	0.125	9
WS	86	CoE	Sputum	LRTI	Recovered	1.0	0.03	1	0.125	9
PS	73	CoE	Sputum	LRTI	Recovered	0.125	0.06	4	0.25	19
BB	85	CoE	Pharynx	Carriage	Well	1.0	0.06	2	0.125	9
EA	75	CoE	Pharynx	Carriage	Died, unrelated	1.0	0.015	2	0.06	9
E	70	W	Pharynx	Carriage	Well	1.0	0.06	4	0.25	9
N	82	Com	Pharynx	Carriage	Well	1.0	0.03	2	0.125	9
P	65	W	Pharynx	Carriage	Well	0.03	0.06	2	0.125	5
Sq	71	Com	Pharynx	Carriage	Well	0.125	0.015	16	8	6
W	71	W	Pharynx	Carriage	Well	1.0	0.03	2	0.125	9
X	52	Com	Sputum	LRTI	Recovered	1.0	0.06	2	0.25	9

CoE, care of the elderly ward; W, another ward area of the Whittington Hospital; Com, community; LRTI, lower respiratory tract infection; Pen, penicillin; Ery, erythromycin; Chlor, chlorhexidine; Tet, tetracycline.

Figure 2 PCR amplified PBP 2b DNA digested with *Hinf* I. Lane 1, pGem marker; Lane 2, sensitive *S pneumoniae*; Lane 3, strain AY*; Lane 4, strain BB*; Lane 5, strain AD*; Lane 6, strain PS; Lane 7, strain P; Lane 8, strain Sq; Lane 9, pGem marker. *Outbreak strains.

Figure 3 PCR amplified PBP 2x DNA digested with *Hinf* I. Lane 1, pGem marker; Lanes 2 and 3, sensitive strains; Lane 4, strain PS; Lane 5, strain EA*; Lane 6, strain AD*; Lane 7, strain P; Lane 8, strain Sq; Lane 9, pGem marker. *Outbreak strains.

hours at 37°C. Penicillin binding protein 2b DNA was digested with *Dde* I (Promega) or *Hinf* I; between 10 and 50 µl of DNA sample derived from the PCR reaction was mixed with 1–5 µl ×10 buffer (*Dde* I: 10 mM Tris-HCl pH 8.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol; *Hinf* I: 10 mM Tris-HCl pH 7.5, 60 mM NaCl, 7 mM MgCl₂, 0.1 mg/ml BSA) and 1 µl enzyme (*Dde* I, 12 units; *Hinf* I, 10 units) and incubated for 16 hours at 37°C. Penicillin binding protein 2x DNA was digested with *Hinf* I or *Dde* I (Promega); between 10 and 50 µl DNA derived from the PCR reaction was mixed with 1–5 µl of the relevant buffer and 1 µl enzyme (*Hinf* I, 10 units; *Dde* I, 6 mM Tris-HCl pH 7.9, 150 mM NaCl, 1 mM dithiothreitol) the tubes were incubated at 37°C for 16 hours. The products of each of the reactions were run on a 1.8% agarose gel and visualised by incorporating 0.02% vol/vol ethidium bromide into

the gel and examining under UV illumination (UV Products, Cambridge, UK).

Results

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Negative results were obtained for all species other than *S pneumoniae* and all three PBP gene PCRs with the exception of two isolates of *S mitis* which were positive in the PBP 2x PCR reaction (data not shown). All *S pneumoniae* were positive in all three tests.

PATIENTS

An outbreak of penicillin resistant *S pneumoniae* occurred in a 26 bedded acute care of the elderly ward in October and November 1992. Nine patients were infected and four died; in two, death was directly related to the infection. The demographic and outbreak details are given in table 1. All the patients were male and the outbreak was confined to three six bedded bays.

The index case was a 92 year old man (CG) who had been admitted with a cerebrovascular accident. Six weeks later he developed a nosocomial pneumonia, was treated with intravenous benzyl penicillin, but died the following day. Antemortem blood cultures grew a moderately resistant serogroup 9 strain of *S pneumoniae*. Four days later the next case occurred. The patient (AY) was in the adjacent bed to the index case. The third case (JF) occurred on the sixth day in another bay. This organism was isolated from the patient's blood and he died 17 days later from pneumonia. The ward was closed to admissions and transfers. Known cases were nursed in source isolation and patients were screened for nasopharyngeal carriage. Day 7 and 8 saw the discovery of one throat carrier (LM) and a further clinically infected case (AD), one each in the two affected bays. The remaining patients were immunised with 23 valent pneumococcal polysaccharide vaccine (Merck Sharp and Dohme, Welwyn, Hertfordshire, UK) and on day 17, following a review of infection control procedures, the ward was reopened. The review had found that some respiratory equipment, particularly the space inhalers used for the delivery of bronchodilators, was being shared. This was contrary to pre-existing guidelines and the practice was ended.

Twenty four days after the last patient had presented a further clinical case (WS) occurred, on the 32nd day of the outbreak and in a previously unaffected bay. Ten days later three further cases, one of infection and two of pharyngeal carriage were detected (PS, BB, and EA). Patient PS had been immunised more than three weeks before on a previous admission. Subsequently, the strain was shown to be serogroup 19 and this represented vaccine failure. No further cases were seen after this time.

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The strains isolated from the ward where the outbreak occurred were predominantly serogroup 9. However, isolates from moderately penicillin resistant strains of serogroup 6, 19, and 5 were found in surveillance swabs and

clinical specimens submitted to the laboratory from patients in the hospital and community; these were not thought to be part of the outbreak. These data together with the results of MIC evaluation are recorded in table 1.

PENICILLIN BINDING PROTEIN GENOTYPE

The PBP genotype of isolates from the outbreak and other penicillin moderate resistant *S. pneumoniae* collected at the same time was determined. Examples of restriction fragment length polymorphism (RFLP) patterns of restricted PBP amplimers for the PBP 1a, 2b, and 2x genes are illustrated in figs 1–3. All of the sensitive strains had a similar PBP 1a pattern as did the other non-outbreak resistant strains. This was clearly distinguishable from the outbreak type 9 strain. For PBP 2b the epidemic strain differed from the sensitive strains, strain PS (serogroup 19 with an MIC on the borderline of sensitive and moderate resistance genotype) and from strain P (serogroup 5, sensitive), which had a genotype similar to the sensitive strains. Isolate Sq (serogroup 6) had a PBP 2b genotype different from the sensitive and outbreak strains and an MIC on the borderline of sensitive and intermediate resistance.

The sensitive strains exhibited considerable heterogeneity in PBP 2x RFLP. Outbreak strains and other resistant isolates had distinguishable RFLP patterns.

Discussion

Spread of penicillin resistant *S. pneumoniae* in closed communities such as prisons and hospitals is well recognised and is a growing threat worldwide; in some countries these strains make up almost 50% of all isolates.^{5–6} Guidelines to reduce the impact of penicillin resistant *S. pneumoniae* have been formulated by a working party sponsored by the Centers for Disease Control and Prevention (Atlanta, Georgia, USA) and have been published recently.¹⁶ The outbreak described here in a care of the elderly ward was brought under control readily by conventional infection control measures, but not before two deaths, directly attributable to this strain, had occurred.

Invasive *S. pneumoniae* infection has a high mortality, which has not improved as much as might have been expected after the introduction of penicillin.¹ The prognosis can only worsen as penicillin resistant *S. pneumoniae* become more common in the community. Prevention of spread in the hospital environment depends on the rapid identification of resistant strains. Many rapid diagnostic techniques for the diagnosis of *S. pneumoniae* including latex agglutination, enzyme linked immunosorbent assay, and PCR have been described.^{17–19} Rapid detection of penicillin resistant *S. pneumoniae* is more difficult: identification of penicillin resistance by conventional culture has been complicated by methodological controversy. A PCR based genotyping method is likely to be a more reproducible approach. Polymerase chain reaction based methods using autoradiography have been reported previously but these were more suitable for epidemiological studies.²⁰ In

this study we have developed PCR-RFLP methods suitable for application in the routine microbiology laboratory. Each of the PCRs uses the same basic reaction mixture and thermocycling protocol enabling these reactions to be run in parallel. Another sensitive and specific PBP 2b PCR has been described for the diagnosis of *S. pneumoniae* bacteraemia using specimens of whole blood. It provided a sensitivity of 80% from clinical specimens but did not look at the genotype by RFLP analysis.²¹ Another approach has been to use a combination of PCR targeting of known resistant genotypes together with a *lytA* PCR.²² The PBP 2b PCRs were able to detect 72.1% of the resistant isolates and the remainder were only detected by the *lytA* reaction. As only the central portion of the gene is amplified there is no possibility of determining epidemiologically relevant genotyping data.

In the context of this outbreak, the PCR-RFLP methods we report were able to distinguish the outbreak strains from other penicillin resistant *S. pneumoniae* isolated in surveillance swabs and casual clinical isolates. Although these epidemiological relations would also be inferred from the results of serotyping, it is clearly beneficial to have determined the PBP genotype of the resistant organisms, especially when community isolates are compared. The outbreak strain had alterations in all three PBP genes examined whereas the PBP 1a gene of all of the casual strains was indistinguishable from the sensitive strains.

The data generated by PCR-RFLP should be available in hospital laboratories and the technique described here makes that possible in a timely manner. The application of modern gel scanning technology and computer RFLP comparison software opens the possibility of standardised reporting between laboratories and genotypic prediction of MIC.

Serotyping remains the reference method for control of infection studies but for penicillin resistant *S. pneumoniae* subtyping is needed. Ribotyping and multilocus enzyme electrophoresis have been evaluated for this purpose and these have been shown to be useful, although they are unable to subtype clonal serotypes such as type 7 and type 14.¹² In addition, these techniques are only available at a limited number of specialist centres. PCR-RFLP techniques are becoming more widely applied in many laboratories. The technique described here is simple to perform and would enable many laboratories to characterise penicillin resistant *S. pneumoniae* and to investigate transmission within the hospital environment.

The results of the PCR reactions in species other than *S. pneumoniae* are very encouraging. The organisms were chosen to include species that are taxonomically closely related¹⁴ together with those likely to be found in specimens of sputum or saliva. Only two false positive results were obtained out of the bacteria studied. As all of the *S. pneumoniae* were positive in all three assays false positive reactions in a single PBP PCR would not cause confusion as the results from the PBP 2b and PBP 1a assays were negative.

These data open the possibility of determining the identity and susceptibility of *S pneumoniae* directly from patients' specimens. Our initial experiments with stored specimens have been successful (data not shown) and prospective studies are now underway. If these studies are successful, susceptibility and genotype data could be determined even when sputum samples are rendered falsely negative by pre-admission antimicrobial therapy.

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