EVALUATION OF THE RANDOM AMPLIFIED POLYMORPHIC DNA TECHNIQUE FOR THE EPIDEMIOLOGICAL INVESTIGATION OF *STREPTOCOCCUS PNEUMONIAE* OUTBREAKS

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A dissertation submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Medicine.

Johannesburg, 1994.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Hillel David Friedland

July 27, 1994

DEDICATION

I dedicate this dissertation to my wife, Elaine, and to my children, Daniel and Steven.

ACKNOWLEDGEMENT

I would like to extend grateful thanks to the following people for their assistance with this project.

Mr A Smith who taught me PCR and who did not begrudge me invading his space.

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PRESENTATIONS ARISING FROM THIS DISSERTATION

1. Oral presentation. Intercontinental spread of *Streptococcus pneumoniae* studied by arbitrarily-primed polymerase chain reaction. Federation of South African Societies of Pathology 34th Annual Congress, Cape Town, June 1994.

2. Oral presentation. Molecular typing of *Streptococcus pneumoniae* using arbitrarily-primed polymerase chain reaction. 34th Interscience Conference on Antimicrobial Agents and Chemotherapy, Orlando, Florida, USA, October 1994.

ABSTRACT

The emergence of strains of *S. pneumoniae* resistant to penicillin and to other antibiotics, and the spread of that resistance over the world, have become major concerns and increase the need for epidemiological surveillance. The following typing methods have been used to detect strain variability in pneumococci: Serotyping, antibiotic susceptibility profiles, multilocus enzyme electrophoresis (MLEE), penicillin-binding protein (PBP) profiles, pulse-field gel electrophoresis (PFGE), and ribotyping. Serotyping, antibiograms, and MLEE only detect phenotypic variability. PBP gene profiles, PFGE, and ribotyping detect genotypic differences but these techniques are labour intensive and time consuming.

Random amplified polymorphic DNA (RAPD) is a new technique that has proved useful for typing bacteria, fungi, and parasites, but has not been studied using pneumococci. Unlike conventional polymerase chain reaction (PCR), RAPD utilizes single, short primers, usually 10 oligonucleotides in length. As the primer is short and low astringency annealing temperatures are used, there will be many complimentary sites scattered randomly throughout a bacterium's genome. When such sites occur a few hundred base pairs away from each other and on opposite DNA strands, the enclosed region can be amplified by PCR. This results in numerous discrete target fragments which can be separated by agarose-gel electrophoresis and ethidium bromide staining. RAPD requires no sequence information and it scans the whole genome rather than relying on hypervariability within one specific gene.

The aims of this study were: to determine strain variability using RAPD, to determine the reproducibility of RAPD, and to demonstrate intercontinental spread of a multiresistant pneumococcal strain.

The following strains were evaluated: a) 10 strains from a day-care centre (DCC), the index case being a 3 year old girl with otitis media. An aunt from Spain had recently been staying with the family. The other strains were isolated from class mates and siblings of the index case.; b) 18 clinical isolates from Seoul, Korea.; and c) 11 epidemiologically unrelated isolates from South Africa, including the reference strain, R6.

Two DNA extraction methods were used. The first involving lysis with sodium-dodecyl-sulphate and proteinase K. Proteins were removed with phenol-chloroform, and the DNA precipitated with ethanol. The second method involved incubating the cells at 95 °C for 15 minutes, followed by centrifugation. 2 microlitres of the supernatant was then used for each PCR reaction. Three primers were evaluated.

After optimisation of the RAPD reaction for pneumococci, the final PCR mixtures per 50 µl was: primer (4 µM), template (0.5 ng), nucleotides (300 µM each), magnesium (4 mM), and *Taq* polymerase (2 U). 35 cycles were used with an annealing temperature of 35 °C.

Both DNA extraction methods gave reproducible results but were not comparable to each other. All 10 strains from the DCC gave the same banding pattern as the Spanish clone for all 3 primers. 7 of the Korean strains gave the same banding pattern as the Spanish clone using the first two primers, however one strain showed an additional band using the third primer. Of the remaining 22 strains, 21 different banding patterns were obtained.

This study has shown that RAPD is a simple and rapid technique that can distinguish strain variation among pneumococci. The reproducibility is excellent within the same laboratory. Finally using RAPD, this study identified a Spanish multiresistant 23F clone in South Africa and Korea.

LIST OF ABBREVIATIONS

AP-PCR	-	Arbitrarily-primed polymerase chain reaction
CTAB	-	Hexadecyltrimethyl ammonium bromide
DCC	-	Day-care centre
DNA	-	Deoxynucleic acid
EDTA	-	Ethylenediaminetetra-acetic acid
EPT	-	Electrophoretic protein typing
EtBr	-	Ethidium Bromide
GLC	-	Gas-liquid chromatography
LPS	-	Lipopolysaccharide
MIC	-	Minimum inhibitory concentration
MLEE	-	Multilocus enzyme electrophoresis
NCCLS	-	National Committee for Clinical Laboratory Standards
PBP	-	Penicillin-binding protein
PCR	-	Polymerase chain reaction
PFGE	-	Pulse-field gel electrophoresis
PRPs	-	Penicillin-resistant pneumococci
RAPD	-	Random amplified polymorphic DNA
RFLP	-	Restriction fragment length polymorphism
SDS-PAGE	-	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SGTs	-	Serogroups and serotypes
TE	-	Tris-EDTA
USA	-	United States of America

CONTENTS

DECLARATION ii
DEDICATION iii
ACKNOWLEDGEMENT iv
PRESENTATIONS ARISING FROM THIS DISSERTATION v
ABSTRACT vi
LIST OF ABBREVIATIONS viii
LIST OF FIGURES xii
LIST OF TABLES xiii
1. INTRODUCTION 1
1.1 Epidemiology of *Streptococcus pneumoniae* infections 1
1.2 The importance of bacterial typing 2
1.3 General bacterial typing methods 3
1.3.1 Phenotypic typing methods 3
1.3.2 Genotypic typing methods 6
1.4 Typing methods described for *S. pneumoniae* 9
1.4.1 Serotyping 9
1.4.2 Antibiotic susceptibility profile 10
1.4.3 Multilocus enzyme electrophoresis 12
1.4.4 PBP profiles 14
1.4.5 PBP gene profiles 15
1.4.6 Pulse-field gel electrophoresis 15
1.4.7 Southern blotting and ribotyping 20
1.5 Random amplified polymorphic DNA 23
1.6 Aims of the study 25

2. MATERIALS AND METHODS 26
2.1 Bacterial Strains 26
2.2 Susceptibility testing. 27
2.3 Serogroup and/or serotype determination 28
2.4 Isolation of chromosomal DNA 28
2.5 Primers 30
2.6 PCR 31
3. RESULTS 33
3.1 Optimisation of RAPD 33
3.1.1 Primer concentration 33
3.1.2 Template concentration 35
3.1.3 Magnesium concentration. 35
3.1.4 Nucleotide concentration 35
3.1.5 *Taq* polymerase concentration 38
3.2 Day-care centre isolates 41
3.3 Korean isolates 48
4. DISCUSSION 53
5. APPENDIX. 60
6. REFERENCES 61

LIST OF FIGURES

Figure 1 Concept of pulsed field electrophoresis. 17
Figure 2 Types of PFGE 19
Figure 3 Schematic illustration of RFLP and ribotyping 22
Figure 4 Influence of primer concentration on PCR patterns. 34
Figure 5 Influence of magnesium concentration on PCR patterns . . . 36
Figure 6 Influence of nucleotide concentration on PCR patterns. . . . 37
Figure 7 Comparison of 3 different *Taq* polymerases 39
Figure 8 Influence of *Taq* polymerase and template concentration on PCR patterns 40
Figure 9 DCC isolates using primer 6 42
Figure 10 DCC isolates using primer 1 43
Figure 11 DCC isolates using primer 8 44
Figure 12 Epidemiologically unrelated isolates 45
Figure 13 Korean isolates using primer 6 49
Figure 14 Korean multiresistant 23F isolates using primer 1 50
Figure 15 Korean multiresistant 23F isolates using primer 8 51

LIST OF TABLES

Table 1 South African isolates typing profiles. 47

Table 2 Korean isolates typing profiles 52

Table 3 Variability of PCR mixtures reported by different authors. . 56

Table 4 RAPD patterns of epidemiologically unrelated isolates from South Africa and Korea 58

1. INTRODUCTION

1.1 Epidemiology of *Streptococcus pneumoniae* infections

Infection due to *S. pneumoniae* (pneumococcus) results in significant human morbidity and mortality. The pneumococcus is the most common cause of community-acquired pneumonia. It is also an important cause of meningitis (most common cause in adults and second commonest in children), otitis media, and bacteraemia (Berman, 1991; Musher, 1992). Other infrequent diseases due to the pneumococcus include empyema, endocarditis, septic arthritis, osteomyelitis, and peritonitis.

Serious pneumococcal infection is most prevalent at the extreme ages of life. The incidence of pneumococcal bacteraemia in infants, less than 2 years of age, is high. The incidence is low in teenage children and young adults and increases in patients of middle age, and reaches a high level in adults older than 70 years. In the United States of America and Finland, case fatality rates of bacteraemic infections are about 20-25 % in pneumonia, and 30 % in meningitis (Kuikka et al, 1992; MMWR, 1981).

S. pneumoniae is commonly isolated from the upper respiratory tract. The rate of asymptomatic carriage varies with age, environment, serogroup and/or serotype (SGT), and the presence of upper respiratory tract infections (Hendley et al, 1975; Smith et al, 1993). Pneumococci may be carried from the first day of life, and > 95 % of children are colonized at

some time in the first 2 years of life. SGTs 6, 14, 19, 23 are acquired frequently and are carried for long periods, whereas SGTs 3, 12, 29, 31 are acquired infrequently and eliminated from the pharynx quickly (Smith et al, 1993b).

First acquisition, which is associated with an increased risk of clinical disease, is earlier in large families. Children in large families tend to carry more strains (Gray et al, 1980). Spread of the organism within a family is influenced by crowding, the season (autumn and winter), and the presence of upper respiratory tract infections or invasive pneumococcal disease such as pneumonia (Gwaltney et al, 1975).

Epidemics of pneumococcal pneumonia, even in closed populations, are uncommon. During epidemic periods person-to-person transmission occurs via droplets. Epidemics may be associated with either multiple SGTs in large susceptible populations or rarely with one type in small closed populations (Mufson, 1990).

1.2 The importance of bacterial typing

Nosocomial infections are an important cause of morbidity and mortality among hospitalised patients. Bacterial typing assays play an integral part in the investigation and control of such infections, by identifying the likely reservoir or carrier and the mode of transmission. Steps can then be taken to eradicate the outbreak.

In public health bacteriology, subtyping can determine whether previously recognized virulent clones are present in a set of strains, for recognizing outbreaks, for identifying the source of infection in outbreak and sporadic disease settings, and for ensuring that immunization programs lead to eradication of the target pathogen instead of replacement with a variant subtype.

Bacterial typing methods are not used solely for epidemiological investigations, but can aid in the identification of an organism, for example, detection of Enterohaemorrhagic *Escherichia coli* by serotyping with O157 antiserum. Typing assays can differentiate species that thrive under identical environmental conditions. Once a species is adequately recognized and typed, studies on its ecological behaviour and spread, including colonisation and cross-infection, can be initiated.

1.3 General bacterial typing methods

Typing methods that detect strain variation are based either on the phenotypic or genotypic characteristics of an organism.

1.3.1 Phenotypic typing methods

Until recently, epidemiological typing was based on phenotypic traits mentioned below. These techniques provide valuable information but are hampered by several difficulties. They vary according to the organism studied and require a wide range of methods, some involving specialized

and complicated technologies. Also, despite optimization of the methods, many strains cannot be typed (Struelens et al, 1992).

Because of the limitation in the number of phenotypes discernable in many instances, differentiation between strains is poor and results may vary with culture conditions (Mazurek, 1993).

General phenotypic typing methods include:

i) Biotyping; based on biochemical reactions of the organism. This technique has only limited ability to distinguish distinct strains within a given species (Pfaller, 1991).

ii) Bacteriocin typing; based on susceptibility of the test organism to toxins produced by other bacteria (Pfaller, 1991).

iii) Bacteriophage typing; based on the susceptibility of an organism to different bacteriophages. Phage typing used to be the mainstay of strain discrimination for *Staphylococcus aureus* and *Salmonella* spp (Blair and Williams, 1961).

iv) Antibiotic susceptibility pattern; based on the susceptibility and resistance of an organism to different antibiotics (Pfaller, 1991).

v) Serotyping; usually based on the antigenic structure of the cell wall or capsule of an organism or immunoblotting of antigenic components of bacterial cells (Pfaller, 1991).

vi) Lipopolysaccharide (LPS) analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); a relatively simple method for evaluating heterogeneity among strains within species of gram-negative bacteria (Swaminathan and Matar, 1993).

vii) Gas-liquid chromatography (GLC); based on analysis of cellular fatty acids. This method is generally inadequate for subtyping (Swaminathan and Matar, 1993).

viii) Electrophoretic protein typing (EPT) and immunoblotting. EPT is performed by isolating whole-cell or cell-surface proteins and separating them by SDS-PAGE. Immunoblotting is performed by transferring the separated bacterial products to a nitrocellulose membrane and then detecting them with use of antisera to specific type strains or with pooled human sera as a source of broadly reactive antibodies (Mulligan et al, 1988).

ix) Penicillin-binding-protein (PBP) profiles; involves identification of PBP with radiolabelled benzylpenicillin (Spratt, 1977).

x) Multilocus enzyme electrophoresis (MLEE); where isolates are characterized by the relative electrophoretic mobilities of a large number of water-soluble cellular enzymes (Selander et al, 1986).

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1.3.2 Genotypic typing methods

Molecular typing methods have revolutionized epidemiology. General genotypic typing techniques include:

i) Plasmid profiles which includes analysis of the number of plasmids present, size of plasmids, and fingerprinting of plasmids after digestion with endonucleases (Tompkins, 1992).

Plasmids are autonomous, self-replicating extrachromosomal DNA elements that are carried by many bacteria but are not essential in most species for normal bacterial growth. Plasmids are known to code for virulence factors and may carry antimicrobial resistance genes.

Plasmid analysis can be hampered by problems similar to those encountered with phenotypic markers. Absence of plasmids or presence of similar sized plasmids can prevent differentiation of strains. Also, plasmids can be lost or gained by a strain, or transferred from one strain to another (Mazurek, 1993).

Plasmid profiles have proved useful for subtyping *Escherichia coli*, *Salmonella typhi*, *Shigella*, *Campylobacter*, *Vibrio cholerae*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *N. meningitidis*, *Staphylococcus aureus*, and *Legionella* species (Holmberg and Wachsmuth, 1989; Hunter and Gaston, 1988). Plasmid profiling is not useful for subtyping *S. pneumoniae* because plasmids rarely occur in this species (Chalkley and Koornhof, 1988).

ii) Restriction fragment length polymorphism (RFLP), involves comparison of the number and the size of fragments produced following digestion of DNA with a restriction enzyme, that cuts double-stranded DNA at a constant position within a specific recognition site usually comprising 4 to 6 base pairs (Maslow et al, 1993). Because of the high specificity of restriction enzymes, complete digestion of DNA with a specific restriction enzyme provides a reproducible array of fragments. These fragments, usually ranging in size from 20000 to 1000 base pairs, can be separated by agarose gel electrophoresis and visualized by staining with ethidium bromide (EtBr).

Different series of fragments generated by a specific restriction enzyme are called restriction fragment length polymorphisms. RFLPs can result from sequence rearrangements, insertion or deletion of DNA, or base substitution within the restriction enzyme cleavage sites.

Isolation and purification of DNA are critical steps in RFLP analysis. The DNA must be sufficiently intact and should be free of impurities that can inhibit restriction enzymes and lead to incomplete cutting.

The advantages of chromosomal DNA restriction analysis are that chromosomal DNA is more stable than plasmid DNA, it is universally applicable, it is relatively easy to perform, and it is sensitive because the entire genome is evaluated for RFLP. Its disadvantage is that the restriction fragments are usually too

numerous and too closely spaced to be visualized after separation by agarose gel electrophoresis.

iii) PBP gene profiles, based on RFLP of the genes coding for PBPs (Smith et al, 1993a).

iv) Pulse-field gel electrophoresis (PFGE), involving the splicing of DNA with rare-cutting restriction enzymes to generate large fragments of chromosomal DNA, which are then separated by special electrophoretic procedures (Maslow et al, 1993).

v) Southern blot DNA fingerprints in which RFLPs on agarose gels are transferred to nitrocellulose or nylon membranes and subsequently hybridized to specific probes (Pfallen, 1991).

vi) Ribotyping, a variation of Southern blotting using probes to conserved ribosomal nucleotide sequences (Maslow et al, 1993).

vii) Random amplified polymorphic DNA (RAPD), a modification of the conventional polymerase chain reaction (PCR) (Williams et al, 1990).

viii) Mixed-linked PCR amplifying specific RFLP fragments. Genomic DNA is digested with a restriction enzyme and a double-stranded oligonucleotide linker is ligated to the ends of the restriction fragments. PCR is performed using a conventional specific primer and a second primer complementary to the linker. In one strand the

linker contains uracil in place of thymidine and specific amplification is obtained by elimination of this strand with uracil N-glycosylase. Only restriction fragments containing the specific sequence can serve as templates. The amplified RFLP pattern is analyzed by gel electrophoresis. This technique has been used to type *Mycobacterium tuberculosis* (Haas et al, 1993).

1.4 Typing methods described for *S. pneumoniae*

Neither biotyping, bacteriocin typing, bacteriophage typing, LPS analysis, EPT, immunoblotting, nor GLC are used for subtyping pneumococci. Only the following methods have been described for *S. pneumoniae*.

1.4.1 Serotyping

The most frequently used method of subtyping pneumococci is serotyping of the antigenic structure of the polysaccharide capsule. Serotyping is performed using the Quellung or capsular precipitin reaction. A suspension of the organism is air-dried on a microscopic slide and resuspended with a loopful of pneumococcal antiserum to which methylene blue has been added. After a few minutes, the slide is examined under oil immersion. If positive, a large capsule will be observed surrounding the organism. This appearance is due to both capsular swelling and greater refraction. Serotyping can also be performed by latex agglutination, counterimmunoelectrophoresis and enzyme-linked-immunosorbent assay (Pfaller et al, 1991).

There are at present 84 known SGTs. There are two systems of nomenclature, namely the American and Danish classification systems. The latter is used more extensively. The American system simply assigns serotype numbers in sequence (from 1 to 84), whereas the Danish system groups serotypes on the basis of antigenic similarities (e.g., Danish group 19 includes types 19F, 19A, 19B, and 19C, which in the American system are types 19, 57, 58, and 59, respectively) (Musher, 1992; Mufson,1990).

Cross-reactions of capsular types occur between individual pneumococcal strains as well as between *S. pneumoniae* and other bacteria. For example, types 2 and 5, types 3 and 8, types 7 and 18, and types 15 and 30 cross-react. Pneumococcal capsular polysaccharides also cross-react with polysaccharides from *Escherichia coli, Klebsiella pneumoniae, Salmonella* species, type B *Haemophilus influenzae*, and viridans streptococci (Smith et al, 1993b).

1.4.2 Antibiotic susceptibility profile

Antimicrobial resistance patterns, although based on phenotypic traits, have been highly standardized and are useful for subtyping many bacterial isolates, particularly those involved in nosocomial outbreaks.

Since its discovery, penicillin has remained the drug of choice for penicillin-susceptible *S. pneumoniae* infections. Hansman and Bullen (1967) were the first to report clinical isolates of penicillin-resistant *S. pneumoniae*.

Pneumococcal resistance to penicillin is defined according to MICs:

intermediate resistance: MICs from 0.12 to 1.0 mg/l;

high level resistance: MICs ≥ 2 mg/l (Klugman, 1990).

S. pneumoniae resistant to penicillin and other antibiotics is increasingly being reported worldwide (Klugman, 1990; Geslin et al, 1992). Other antibiotics to which resistance occurs include erythromycin, trimethoprim-sulphamethoxazole, tetracycline, chloramphenicol, rifampicin, and most recently, the extended spectrum cephalosporins (Klugman and Saunders, 1993). Multi-resistant *S. pneumoniae* isolates, defined as resistant to at least 3 different classes of antibiotics, are a particular problem in Spain, South Africa, and the USA (Klugman, 1990; and McDougal et al, 1992). Multiresistant pneumococci of several different SGTs have been reported, although the overwhelming majority of resistant strains belong to serogroups 6, 19, and 23, and serotype 14 (Klein, 1981; and Klugman, 1990).

The most active β-lactam agents against penicillin-resistant pneumococci are cefotaxime, ceftriaxone, and imipenem (Klugman, 1990; Chandy, 1994). Recently, pneumococci resistant to the third generation cephalosporins, cefotaxime and ceftriaxone, have been reported (Chandy, 1994). The National Committee for Clinical Laboratory Standards (NCCLS) have recently revised their definition of resistance to these cephalosporins. Strains with MICs of 0.5 to 1 μg/ml are classified as intermediately resistant and those with MICs ≥ 2 μg/ml as highly resistant.

1.4.3 Multilocus enzyme electrophoresis (MLEE)

MLEE analysis detects protein charge differences which can be directly linked to mutations in a variety of genes encoding metabolic enzymes. These mutations may not alter protein function but will cause an electrophoretically detectable change in the protein's charge (Selander et al, 1986).

MLEE requires a high concentration of organisms, at least 10^5 organisms/ml. Bacteria are lysed without denaturing their proteins. This can be achieved by freezing, sonification, or addition of Triton X-100. After lysis and centrifugation, aliquots of lysate are used for horizontal starch-gel electrophoresis. Following electrophoresis, three or four horizontal slices are cut from the gel with a thin wire and incubated individually at 37 ^{0}C in various enzyme staining solutions. The gels are rinsed and fixed with acetic acid and methanol. The enzymes appear on the gels as narrow, sharply defined bands.

The mobilities of enzymes from different isolates are compared on the same gel slice. For each enzyme, distinctive electromorphs (mobility variants) are numbered in order of decreasing anodal migration. Each isolate is characterized by its combination of electromorphs over the number of enzymes assayed. Distinctive profiles of electromorphs are designated electrophoretic types, which are equivalent to allele profiles.

The net charge of a protein and hence the rate of migration during electrophoresis, is determined by its amino acid sequence. Therefore, mobility variants of an enzyme can be directly equated with alleles of the

corresponding structural gene. Gel electrophoresis has the ability to detect up to 90% of amino acid substitutions (Selander et al, 1986). A proportion of these amino acid substitutions may be due to post-translational modification and not genetic variation, thus biasing estimates of genetic variation derived from the electrophoresis of proteins.

On average, between 15 and 25 enzymes are assayed with MLEE. When many polymorphic enzymes, each represented by several alleles, are examined, the number of possible genotypic combinations is enormous. However, no more than a few hundred genotypes have been encountered in any species, because in general bacteria are clonal and only a fraction of all possible genotypes are commonly represented in bacterial populations (Selander et al, 1986).

MLEE has become a standard technique in evolutionary eukaryotic biology. Early studies of enzyme polymorphism in bacteria were not particularly enlightening, although more recent studies have shown MLEE to differentiate reliably strains of *Escherichia coli* (Selander and Levin, 1980), and *Haemophilus influenzae* (Musser et al, 1986).

Muñoz et al (1991) performed MLEE on different isolates of *S. pneumoniae* by assaying 14 different enzymes. They showed that a serotype 23F, penicillin-resistant pneumococcal strain, cultured from children living in Cleveland, Ohio suffering from chronic otitis media, had the same electrophoretic type as isolates from Spain where penicillin resistance has been a problem for many years.

Data from a study by McDougal et al (1992), using MLEE for 20 enzymes, indicate widespread dissemination of the same multiresistant strain in the United States and South Africa.

1.4.4 PBP profiles

PBPs are enzymes that catalyse the terminal stages of murein synthesis and are inhibited by covalent bonding with penicillin at their active site (Tomasz, 1987). There are 6 different PBPs in most penicillin-sensitive pneumococci, i.e. PBP 1a, 1b, 2x, 2a, 2b, and 3. Penicillin resistance occurs as a result of alteration in the PBPs resulting in decreased affinity for the antibiotic (Muñoz et al, 1991).

Profiles of the PBP are performed by harvesting and lysing the bacterial cells. The cell membranes are recovered by ultracentrifugation, labelled with radioactive benzylpenicillin and separated by SDS-PAGE (Spratt, 1977). Immunoblots can also be performed using rabbit anti-PBP 1a and mouse anti-PBP 2b (Hakenbeck et al, 1986).

Studies have shown that strains of pneumococci with the same penicillin MICs not only have different PBP antigenic patterns but different penicillin affinity profiles as well (Jabes et al, 1989; Hakenbeck et al, 1991a; Hackenbeck et al, 1991b; Muñoz et al, 1991; and McDougal et al, 1992). These data indicate that PBP profiles can be an important tool for characterizing changes in proteins involved in penicillin resistance.

1.4.5 PBP gene profiles

Pneumococci are harvested and lysed with SDS and proteinase K. Chromosomal DNA is precipitated out by chloroform. The genes encoding the PBP are amplified using the polymerase chain reaction (PCR) and are purified from agarose. RFLP is then performed on the purified PCR products. The fragments are end-labelled and separated on polyacrylamide gels and are visualised using autoradiography (Smith et al, 1993a).

Based on the comparison of the DNA sequences encoding PBP 2b and PBP 2x, Muñoz et al (1991) suggested that the penicillin resistance phenotype occurs in association with multiple chromosomal lineages. Smith et al (1993a) identified 19 PBP 2b gene variants and 26 PBP 2x gene variants among serogroup 6 and 19 pneumococci. Discrete profiles to both genes were found only within narrow bands of the penicillin MIC, so that the gene pattern predicted the MIC.

PBP pattern analysis, however, will not detect genetic variations responsible for resistance to non-β-lactam antibiotics. Most other genotypic methods and to a lesser extent MLEE, characterize variations in the entire chromosome and are not limited to changes in only specific genes.

1.4.6 Pulse-field gel electrophoresis (PFGE)

DNA fragments larger than 40 kb cannot be separated effectively by conventional agarose gel electrophoresis because the sieving action of the gel is lost and fragments migrate at approximately the same rate resulting in

a broad unresolved band. In contrast, PFGE is capable of separating fragments exceeding 6000 kb is size (Gardiner, 1991).

PFGE exposes DNA to electric fields that regularly change direction throughout the gel run. A schematic diagram of the general concept employed in pulsed field systems is shown in **Figure 1.**

Intact DNA is required for PFGE; therefore conventional DNA isolation methods that cause shearing of DNA are not suitable. Bacteria are incorporated into agarose plugs prior to cell lysis by enzyme and detergent. The large chromosomal DNA remains entrapped in the agarose gel matrix, while other contaminants are removed by extensive washing or dialysis. Restriction of the DNA is performed with enzymes chosen to yield a relatively small number of large restriction fragments. These enzymes recognise specific 8-base sequences (e.g., *Not*I, *Sfi*I) or selected 6-base sequences (e.g., *Sma*I, *Apa*I). The agarose plugs containing the restricted DNA are subjected to PFGE.

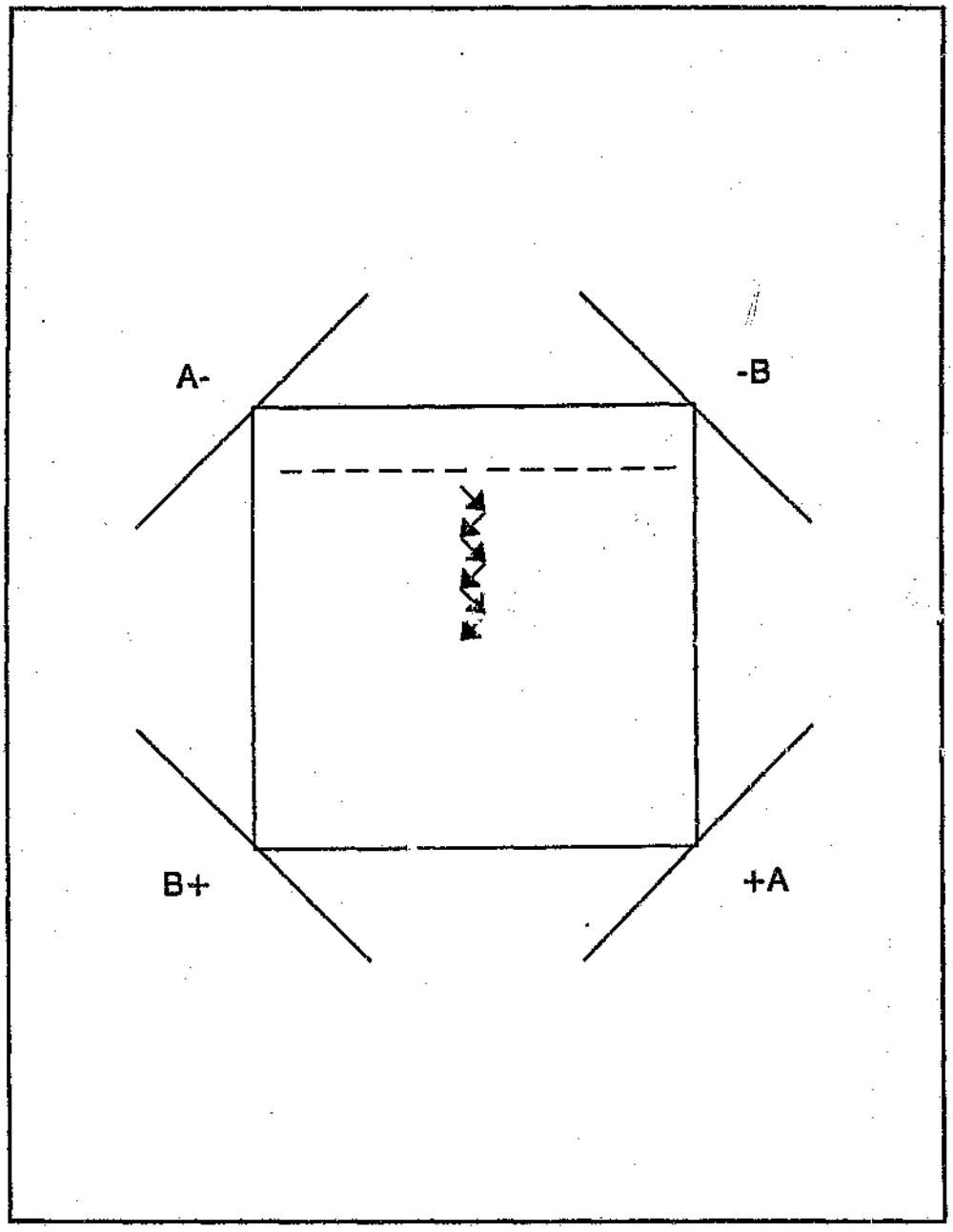

Figure 1 Concept of pulsed field electrophoresis. The box represented the agarose gel; the short horizontal lines indicate the wells, where the DNA is loaded. A and B are 2 sets of electrodes. When the electrodes A are activated, the DNA is driven downward and to the right, as indicated by the first arrow. When the A electrodes are turned off, the B electrodes are immediately activated. The DNA now moves downward and to the left. The path of the DNA in the center lane with continued alternation of field direction is shown by the arrows.

The 2 most widely used PFGE systems are (see **Figure 2**):

i) Field inversion - has the simplest design, using a single pair of electrodes and a standard submarine agarose gel electrophoresis box. The electric field regularly inverts, first driving DNA out of the wells and then back toward the wells. This system is useful for fragment sizes < 600 kb.

ii) Contour clamped homogeneous electric field (CHEF) device - uses a hexagonal array of point electrodes and produces homogeneous fields orientated at 120 °. This results in uniform fields across all lanes of the gel.

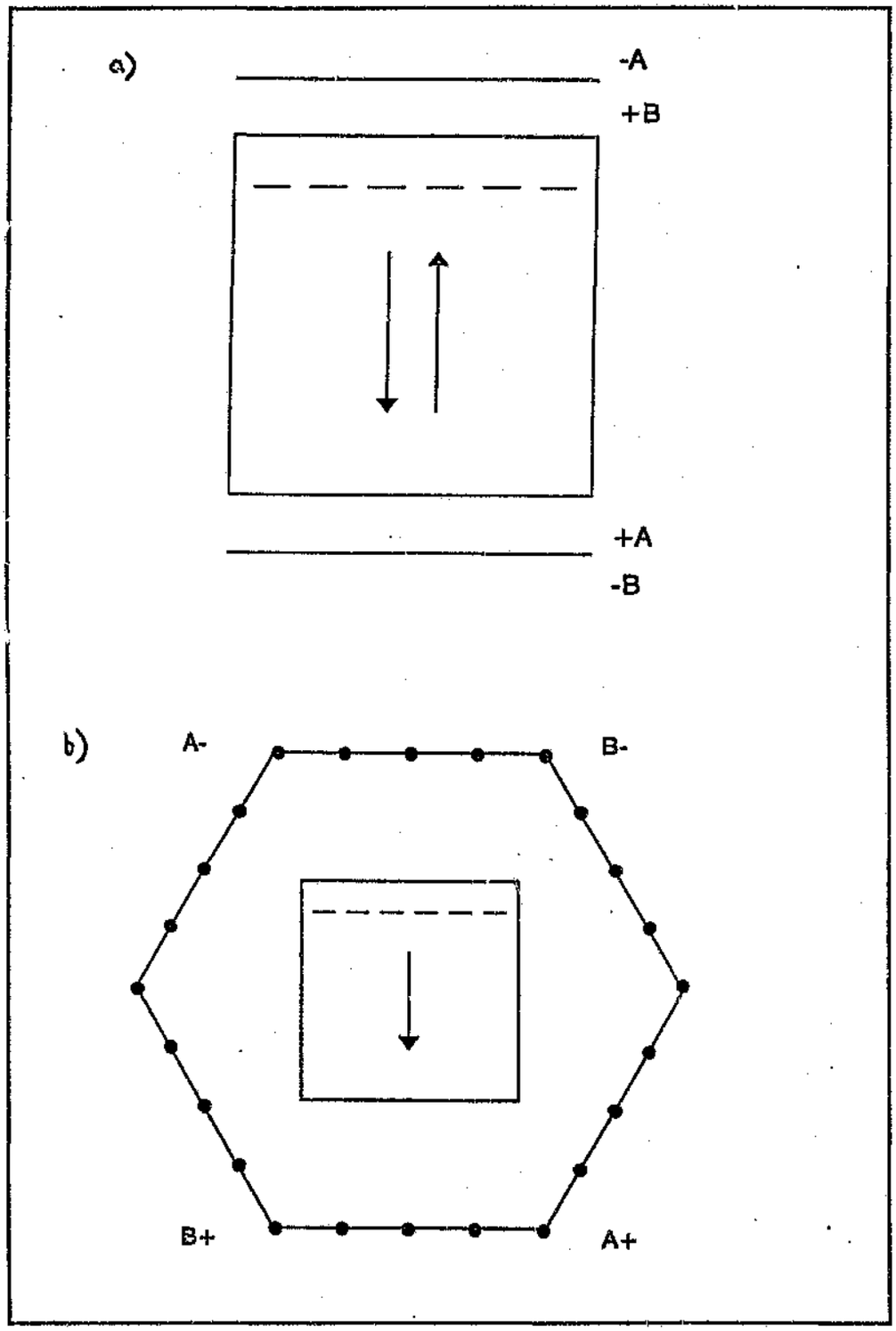

Figure 2 Types of PFGE. (a) Field inversion. A single pair of electrodes is activated alternately in the A orientation (DNA moves downward) and the B orientation (DNA moves upward). (b) CHEF. A hexagonal array of point electrodes in a resistor circuit provides control of the electric field at all points around the gel. Arrow indicates net migration.

PFGE has been useful in subtyping several gram-positive and gram-negative bacteria, including *N. meningitidis* (Bygraves and Maiden, 1992), *Enterococcus faecium* (Miranda et al, 1991), shigellae, staphylococci, *Klebsiella pneumoniae, Serratia marcescens*, and mycobacteria (Swaminathan and Matar, 1993)

Lefevre et al (1993) demonstrated that field inversion gel electrophoresis was more discriminatory than MLEE or serotyping in determining pneumococcal strain variation.

1.4.7 Southern blotting and ribotyping

With Southern blotting, restriction fragments separated by agarose gel electrophoresis for RFLP analysis are transferred to nitrocellulose or nylon membranes by placing the agarose gel between several sheets of buffer-soaked filter paper and a nitrocellulose or nylon filter. Paper towels are placed on top of the nylon or nitrocellulose. As the buffer is drawn through the agarose gel, it carries the DNA to the filter. The filter is removed after several hours and baked in a vacuum oven to permanently attach the DNA to the filter. The DNA fragments are then available for hybridization with labelled probes.

Only the restriction fragments that hybridize with the probes are visible, thus simplifying the fingerprint. Probes are either randomly cloned, based on virulence factors, or derived from insertion sequences.

Ribotyping (see **Figure 3**) describes the fingerprinting of DNA restriction fragments that contain the genes coding for the 16S and 23S rRNA. Ribotyping is similar to probing restriction fragments of chromosomal DNA with cloned probes. However, ribotyping has two advantages: first, because the genes coding for rRNA are highly conserved, a single probe can be used to subtype all eubacteria; second, because most bacteria contain multiple ribosomal operons, a reasonable number of fragments is obtained after probing to allow interspecies and intraspecies discrimination (Stull et al, 1988).

The disadvantage of ribotyping is that the multiple steps that are required (DNA isolation, restriction, electrophoresis, Southern blotting) are time-consuming and labour intensive. Also, the appropriate restriction enzyme(s) must be determined for each bacterial species.

Ribotyping has been useful for subtyping diverse bacteria including species of the family *Enterobacteriaeceae* (Hinojosa-Ahumada et al, 1991), *Campylobacter* spp (Kiehlbauch et al, 1991), *Pseudomonas cepacia* (LiPuma et al, 1991), *Haemophilus* spp, and staphylococci (Stull et al, 1988). McDougal et al (1992) successfully applied ribotyping to differenciate pneumococcal strains.

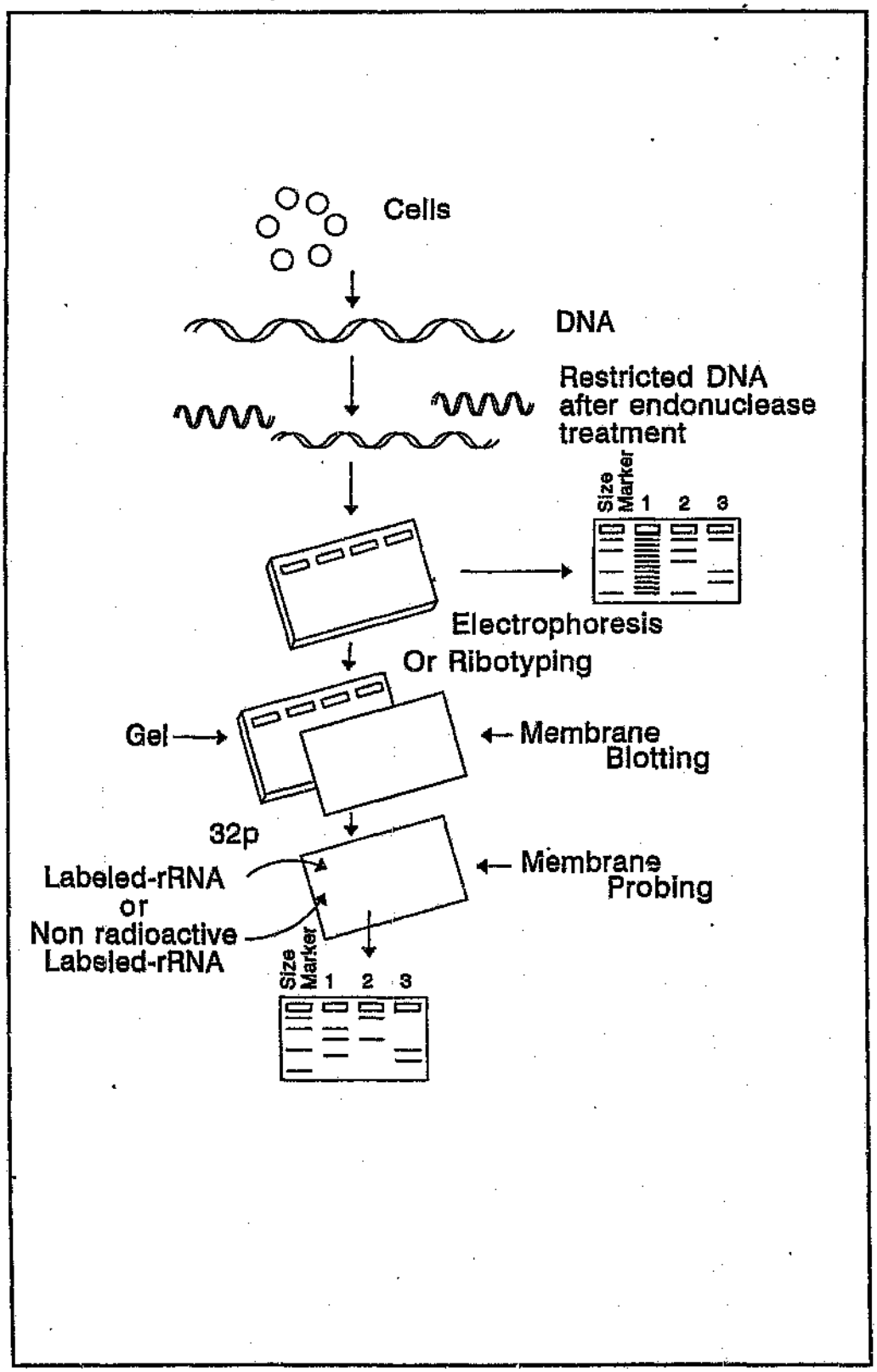

Figure 3 Schematic illustration of RFLP and ribotyping

1.5 Random amplified polymorphic DNA (RAPD)

The conventional polymerase chain reaction (PCR) is based on the annealing and extension of 2 oligonucleotide primers that flank the target region in double-stranded DNA. After denaturation of DNA, these primers hybridize or anneal to opposite strands of the target sequence and are oriented so that DNA synthesis (extension) by the DNA polymerase proceeds across the region between the primers. These 3 steps (denaturation, annealing and extension) represent a single PCR cycle. Each step is carried out at a discrete temperature: denaturation at 94 ^{0}C, annealing at 37 to 50 ^{0}C, and extension at 72 ^{0}C (Cherfas, 1990).

The extension products themselves serve as primer binding sites and templates for subsequent PCR cycles, resulting in exponential accumulation of the specific target fragment.

Unlike conventional PCR, which requires knowledge of the flanking sequences of the target DNA, RAPD utilizes random short oligonucleotides as primers to amplify discrete fragments of the target DNA (Williams et al, 1990). This process is also known as arbitrarily primed PCR (AP-PCR) (Welsh, 1990).

Because the primer is a short oligonucleotide (about 10 nucleotides long), many sites complementary to it are randomly dispersed throughout a genome. When such sequences occur a few hundred base pairs away from each other and on opposite strands, the enclosed region can be amplified by PCR. Absence of that amplified DNA product in another strain can be due to loss of a priming site either by mutation, deletion or insertion. RAPD

requires no sequence information and it scans the whole genome rather than relying on hypervariability within one specific gene.

RAPD has potential application in epidemiology and bacterial strain identification, as well as pedigree analysis, examining interspecific hybridization and the study of genetic variation in natural populations.

A major problem with RAPD is poor reproducibility among different laboratories. Penner et al (1993) found that the type of thermocycler used appeared to be the main source of variation, manifested by different amplified product lengths. In addition, certain primers gave more reproducible results than others.

Other workers noted that *Taq* polymerase concentrations of 2.5 U per 50 µl reaction, resulted in nonspecific DNA amplification patterns and found concentrations of 0.8 U per 50 µl reaction, to be optimal (Schierwater, 1993).

In a study by Ellsworth et al (1993) variation in banding patterns could be attributed to differences in:

i) the primer to template concentration ratio,

ii) the annealing temperature during the amplification reaction, and

iii) the magnesium ion concentration.

Small fragments (less than 500 bp) are generated at high primer concentrations whereas additional large fragments amplify readily at lower primer concentrations. Progressively fewer products appear with increasing template concentration. It is suggested that for efficient and consistent amplifications, primer concentrations between 1.6 and 6.4 μM with approximately 0.3 to 1.0 μg of template DNA should be used.

Variation in banding patterns occurs with differing magnesium concentrations below 2 mM. Concentrations of magnesium above 2 mM have no effect on the resulting banding pattern.

Wang et al (1993) showed that RAPD is more sensitive than MLEE for distinguishing related *Escherichia coli* strains. RAPD has also been useful in subtyping *Haemophilus influenzae* (Jordens et al, 1993) and *Acinetobacter baumannii* (Graser et al, 1993).

1.6 Aims of the study

i) To determine the degree of strain variation demonstrated by the random amplified polymorphic DNA technique (RAPD).

ii) To determine the reproducibility of RAPD.

iii) To prove intercontinental spread of a multi-resistant *Streptococcus pneumoniae* strain.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

The genetic and phenotypic relatedness of the following strains were compared.

- 10 strains previously obtained from a epidemiological study (Klugman et al, 1994). The index case was a 3-year-old child with otitis media. Other strains were isolated from the nasopharynx of her 1-year old sister, her mother and 2 children (4 and 6 years old) from the same day-care centre (DCC). Further investigation revealed isolates from the siblings of the 2 classmates (1 and 7 years old). Following nasal mupirocin application, in an attempt to eliminate nasal carriage, *S. pneumoniae* was again isolated from 3 children.

- 18 clinical isolates of *S. pneumoniae* from Seoul, Korea. These strains were isolated from nasal swabs, sputum and blood cultures.

- The penicillin-sensitive reference strain R6, which is a derivative of the unencapsulated Rockefeller University strain *S. pneumoniae* R36A (derivative of a capsular type 2 strain, D39; American Type Culture Collection, Rockville, Md.).

- 10 epidemiologically unrelated clinical isolates from different parts of South Africa.

2.2 Susceptibility testing

Antimicrobial susceptibility was determined by the Kirby-Bauer disk-diffusion method using 5% horse-blood agar plates (Mueller-Hinton base, Oxoid). An inoculum of approximately 10^6 organisms was obtained by incubating five colonies in serum broth to a desired turbidity comparable with a barium sulphate standard (NCCLS, 1993a). Plates were inoculated with a sterile cotton swab on a wooden applicator and incubated aerobically at 37 ^{0}C for 18 hours.

A multidisk ring (Mastring-S, Mast Laboratories, UK) was used, which included the following: chloramphenicol, 30 µg; tetracycline, 30 µg; erythromycin, 15 µg; clindamycin, 2 µg; rifampicin, 5 µg.

Inhibition zone diameters around 1 µg oxacillin disks were used to assess susceptibility of the pneumococci to penicillin as zone diameters around these disks correlate more accurately with penicillin G MICs than the use of 6 µg penicillin disks (Gartner, 1979; Swenson et al, 1986).

Antimicrobial susceptibility was confirmed with MIC determination by microtiter serum broth dilution (NCCLS, 1993b)

2.3 Serogroup and/or serotype determination

Serogrouping and serotyping was performed using the Quellung reaction as described in section 1.4.1. The pneumococcal antiserum was obtained from the Statens Seruminstitut, Copenhagen, Denmark.

2.4 Isolation of chromosomal DNA

For the RAPD technique a previously described method was used (Smith et al, 1993a). From a single colony, pneumococci were grown overnight (18 hours) on blood agar plates (Appendix A) at 37 ^{0}C under enhanced CO_2 conditions. Organisms covering the full plate were harvested and suspended in 700 µl of distilled water. The suspension was centrifuged at 12000 r.p.m. for 2 minutes to pellet the bacterial cells. Cells were resuspended in 470 µl of Tris-EDTA (TE) buffer (Appendix A). The cells were lysed using 30 µl of 10% sodium dodecyl sulphate (SDS) (Sigma Chemical Co., St. Louis, Mo.) and 5 µl of 10 mg/ml proteinase-K (Sigma Chem. Co.) for 1 hour at 37 ^{0}C. Proteins were precipitated out by incubating with 83 µl of 5 M NaCl (BDH Chem. Ltd, Poole, England) and 66 µl of 10% hexadecyltrimethyl ammonium bromide (CTAB) (Appendix A) for 10 minutes at 65 ^{0}C. The lysate was then mixed with an equal volume of chloroform (BDH Chem. Ltd.) and centrifuged at 12000 r.p.m. for 5 minutes. The clear aqueous phase was transferred to a new Eppendorf tube and the chromosomal DNA was precipitated out by the addition of 1 ml of 100% ice-cold ethanol (BDH Chem. Ltd.). The DNA precipitate was collected by centrifugation at 12000 r.p.m. (4 ^{0}C) for 10 minutes. The DNA pellet was washed with 50% ice-

cold ethanol (BDH Chem. Ltd.). After further centrifugation at 12000 r.p.m. the pellet was dried at 45 °C

The pellet was resuspended in 50 µl of TE buffer and the DNA concentration was estimated by electrophoresis of a 3 µl sample through a 1% high strength analytical grade agarose gel (Bio-Rad, Richmond, CA., USA) at 90 V for 30 minutes. The 3 µl sample was mixed with 1 µl loading dye (Appendix A). The 1% gel (30 ml) was stained with 2 µl of a 10 mg/ml stock of ethidium bromide (EtBr) and a 1 X TAE running buffer (Appendix A) was used. The DNA concentration was corrected to ± 1 µg/µl and then stored at -20 °C. All centrifugation steps were performed using a Hägar microfuge model HM- 2 (Hägar designs, Pinelands, S. A.).

A second method of DNA extraction modified from Graser (1993) and Jordens (1993) was compared to the above method. Overnight cultures were obtained from a single colony of a particular isolate as above. From the overnight culture, 10 colonies were suspended in 100 µl of distilled water. The suspension was then incubated at 95 °C for 15 minutes and then briefly vortexed. After brief centrifugation at 12000 r.p.m., 2 µl of the lysate was removed and added to the RAPD PCR mixture. The remaining lysate was stored at -20 °C.

2.5 Primers

7 random single primers of 10 oligonucleotides and 1 of 20 oligonucleotides in length with the following sequences were tested:

Primer 1: 5'- CGGTGGCGAA -3' (GC content 70%)
Primer 2: 5'- TGGTCACTGA -3' (GC content 50%)
Primer 3: 5'- ACGGTACACT -3' (GC content 50%)
Primer 4: 5'- TCACGATGCA -3' (GC content 50%)
Primer 5: 5'- AAACCAATGA -3' (GC content 30%)
Primer 6: 5'- TTGACAACTG -3' (GC content 30%)
Primer 7: 5'- ATTTCTACTT -3' (GC content 20%).
Primer 8 5'- CCTGCGAGCGTAGGCGTCGG -3'

Primers 1 to 4 and 8 have been described previously (MacPherson et al, 1993; Williams et al, 1990; Bingen et al, 1993b; Shawar et al, 1993). Primers 5 to 7 were selected arbitrarily with a GC content less than 50%, because the GC content of the pneumococcal genome varies from 33 to 42 % (Mufson, 1990).

The oligonucleotide primers were synthesized by the Department of Biochemistry, University of Cape Town, using a Auto-Gene 6500 DNA Synthesizer, Genetic Design.

2.6 PCR

PCR was performed using varying concentrations of primer, template DNA, *Taq* DNA polymerase, magnesium chloride, and deoxynucleosides (Boehringer Mannheim, GmbH, Mannheim) to find the most reproducible combination. The final volume of the RAPD PCR mixture was 50 µl overlaid with 1 drop of mineral oil (Sigma Chem. Co.).

Three different *Taq* DNA polymerases were used, viz. Promega Corp., Madison, USA; Advanced Biotechnology, London, UK; and DynaZyme, Finnzymes Oy, Finland. The final mixture also contained 1 X *Taq* DNA polymerase buffer (Promega Corp.: 50 mM KCl, 10 mM Tris-HCl pH 8, 0.01% gelatin, 0.1% triton X-100; Advanced Biotechnology: 20 mM ammonium sulphate, 75 mM Tris-HCl pH 9, 0.01% Tween; DynaZyme: 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100).

PCR was performed in a Perkin Elmer Cetus DNA thermal cycler model 480 (Perkin Elmer Cetus, Norwalk, CT., USA) with the following cycle program: initial denaturation at 93 ^{0}C for 5 minutes, then 35 cycles of denaturation at 93 ^{0}C for 1 minute, primer annealing at 35 ^{0}C for 1 minute, and primer extension at 72 ^{0}C for 2 minutes, followed by a final extension step at 72 ^{0}C for 5 minutes.

A 10 µl sample of the amplified products together with 2 µl of loading dye were electrophoresed through a 2 % high strength analytical grade agarose gel (50 ml) (Bio-Rad), stained with 2 µl of 10 mg/ml EtBr, using a MINNIE submarine agarose gel unit, model HE 33 (Hoefer Scientific Instruments,

San Francisco, USA). A 1 X TAE running buffer containing 0.5 mg/ml of EtBr was used. Running conditions were 100 V for 1 hour at 4 °C.

To avoid contamination the following steps were taken:

i) sample preparation was performed in a different area to PCR mixture,

ii) solutions were autoclaved,

iii) disposable gloves were worn,

iv) reagents were divided into single use aliquots and stored in an area free of PCR-amplified products.

v) splashes were avoided, and

vi) negative controls, mixtures not containing template DNA, were used.

3. RESULTS

3.1 Optimisation of RAPD

Variations in concentration of primer, magnesium, nucleotides, template, and *Taq* polymerase were tested to obtain the optimum combination. In addition, the different *Taq* polymerases were evaluated against each other. For all RAPD experiments the same molecular size markers were used, with the following size bands (in base pairs): 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220, 154.

3.1.1 Primer concentration

All reactions were conducted using magnesium (4 mM), template (1 µg), and nucleotides (300 µM). Primer concentration ranged from 1.6 to 10 µM (**Figure 4**). The best concentration was taken where the most bands could be seen clearly.

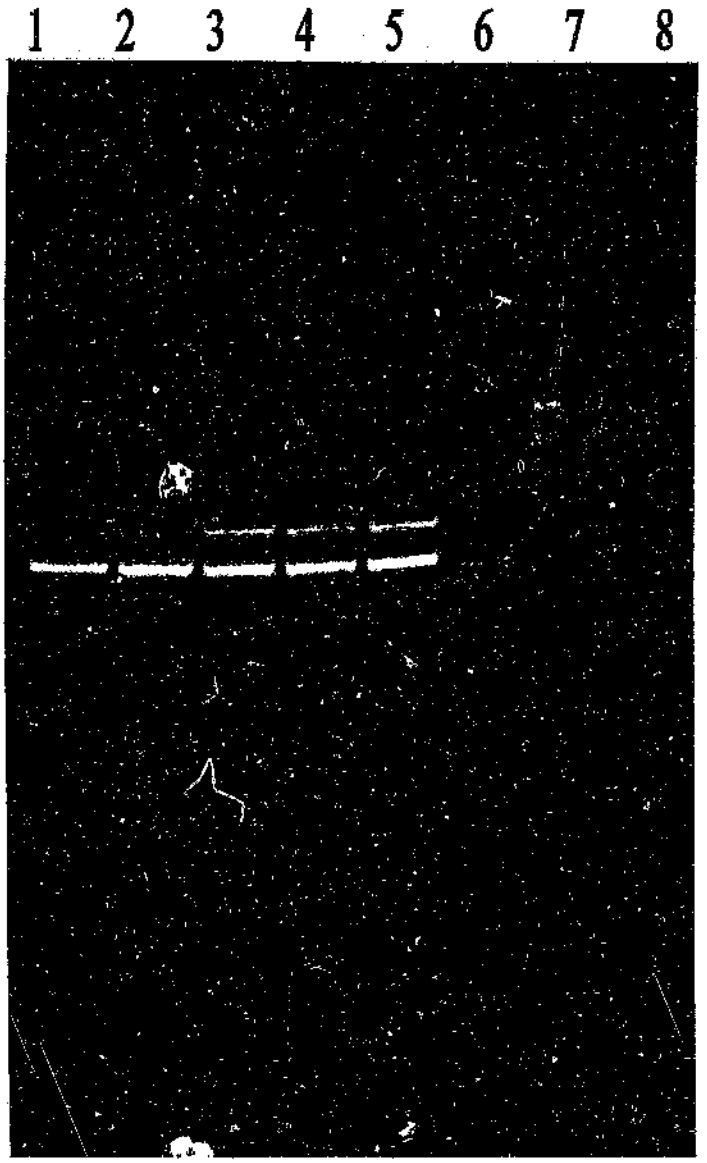

Figure 4 Influence of primer concentration on PCR patterns.
Primer concentrations: lane 1 (3.2 μM), lane 2 (4.8 μM), lane 3 (5.4 μM), lane 4 (7.0 μM), lane 5 (8.6 μM), lane 6 (10 μM). Lane 7 contained molecular size markers.

3.1.2 Template concentration

All reactions were conducted using magnesium (4 mM), primer (4 μM), and nucleotides (300 μM). Template concentration varied from 0.25 to 2 μg **(Figure 8)**.

3.1.3 Magnesium concentration

All reaction were conducted using primer (4 μM), template (1 μg), and nucleotides (300 μM). Magnesium concentrations varied from 1 to 4 mM **(Figure 5)**

3.1.4 Nucleotide concentration

All reactions were conducted with magnesium (4 mM), primer (4 μM), and template (1 μg). Nucleotide concentration ranged from 100 to 300 mM **(Figure 6)**.

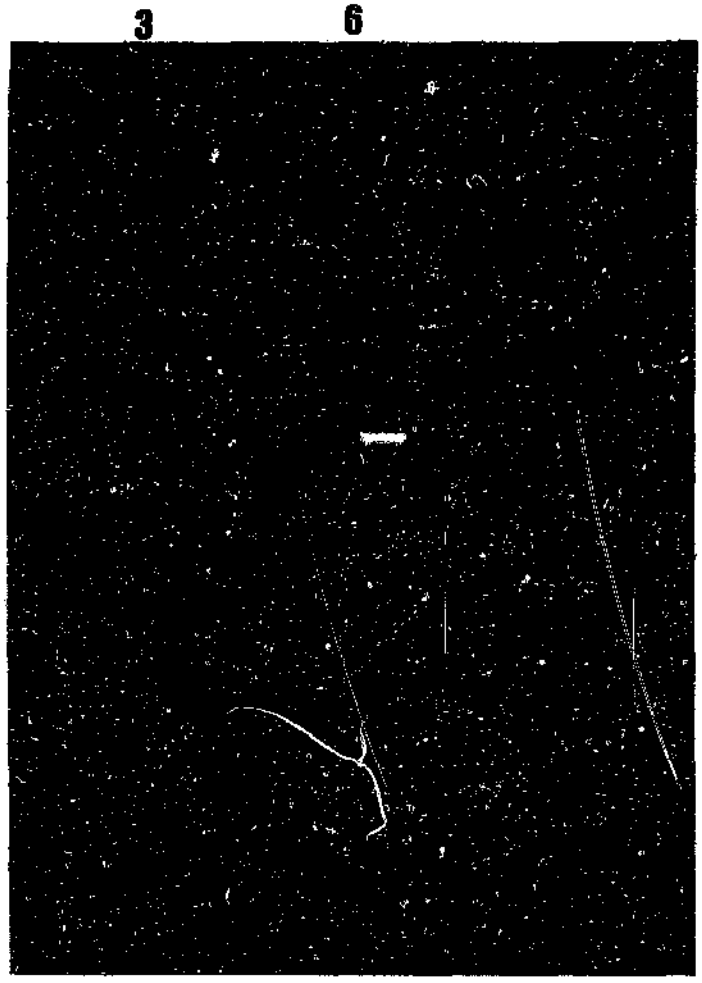

Figure 5 Influence of magnesium concentration on PCR pattern.
Magnesium concentrations: lane 1 (1 mM), lane 2 (1.5 mM), lane 3 (2 mM), lane 4 (2.5 mM), lane 5 (3 mM), lane 6 (3.5 mM), lane 7 (4mM). Lane 8 contains molecular size markers.

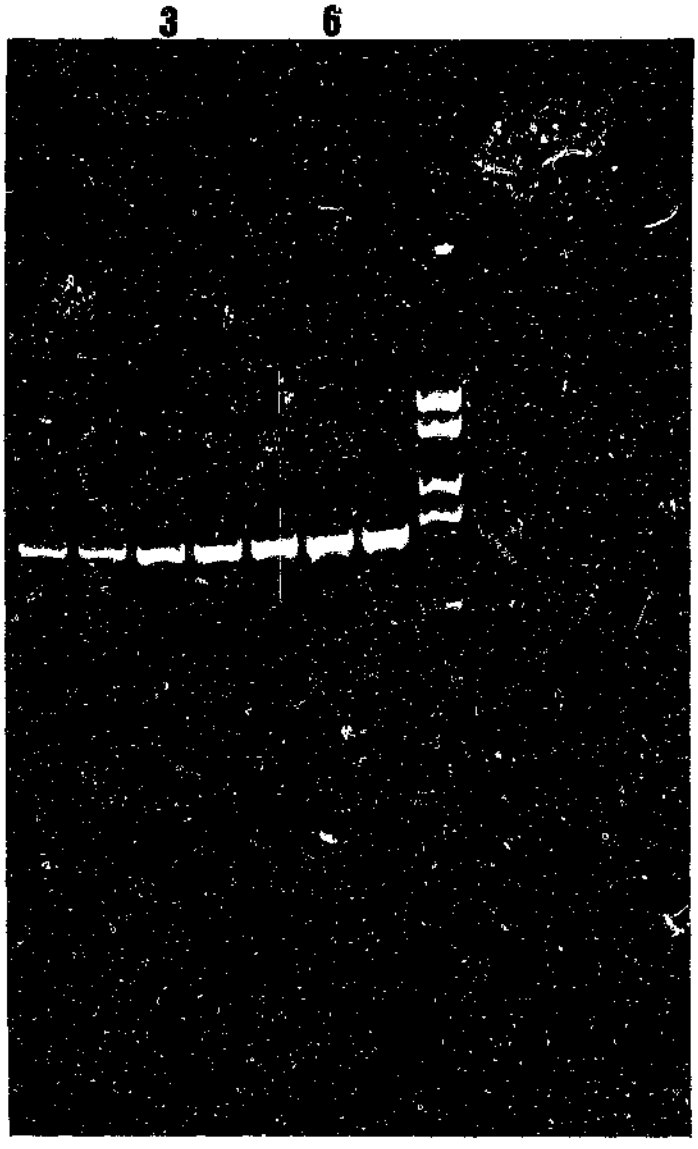

Figure 6 Influence of nucleotide concentration on PCR patterns.
Nucleotide concentrations: lane 1 (100 µM), lane 2 (150 µM), lane 3 (200 µM), lane 4 (250 µM), lane 5 (300 µM). Lane 6 contains molecular size markers.

3.1.5 *Taq* polymerase concentration

The 3 different *Taq* polymerase enzymes were compared (**Figure 7**). All 3 gave different banding patterns. The Promega *Taq* was the most expensive. The Dynazyme *Taq* gave fewer bands than the others. Advanced Biotechnology *Taq* was used for all the experiments as it was the cheapest and its buffer foamed the least during mixing.

For the optimisation of the *Taq* concentration, reactions were conducted with magnesium (4 mM), primer (4 µM), nucleotide (250 mM), and template (1 µg). *Taq* polymerase concentrations varied from 0.8 to 2 U per 50ml reaction mix (**Figure 8**).

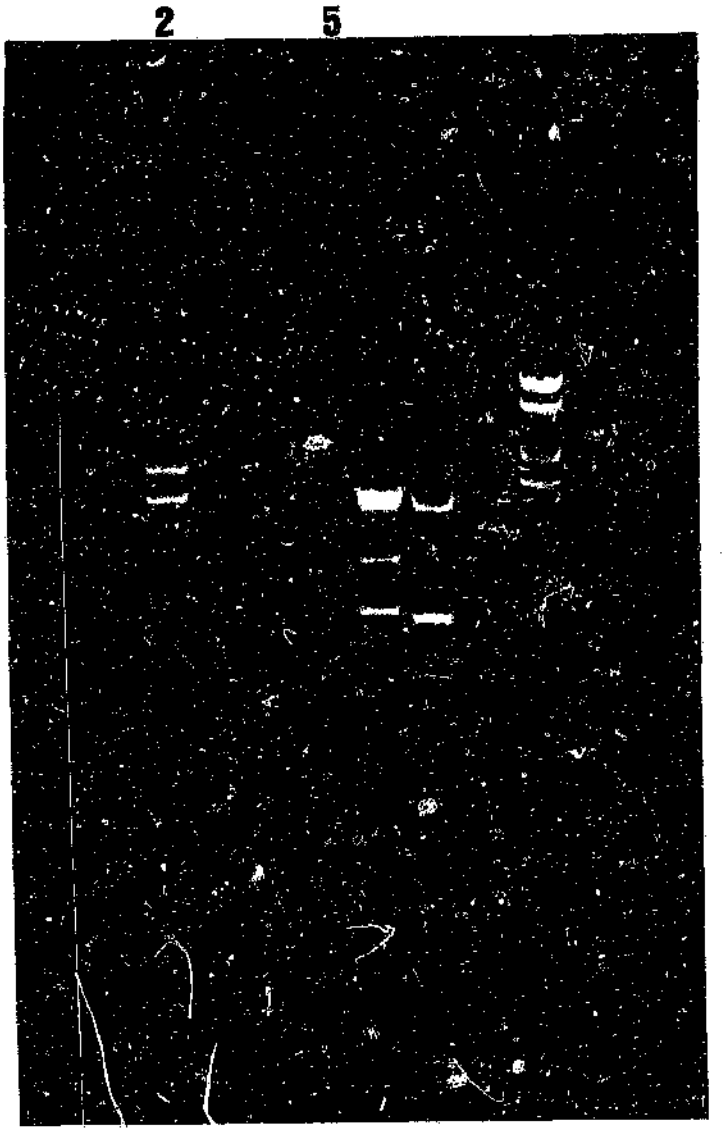

Figure 7 Comparison of 3 different *Taq* polymerases. Dymazyme in lanes 4 and 8 Advanced Biotechnology in lanes 2 and 6. Promega in lanes 3 and 7. Lanes 1 to 3 contain strain 23 and lanes 5 to 7 contain strain 24. Lane 8 contains the molecular size markers and lane 4 is empty.

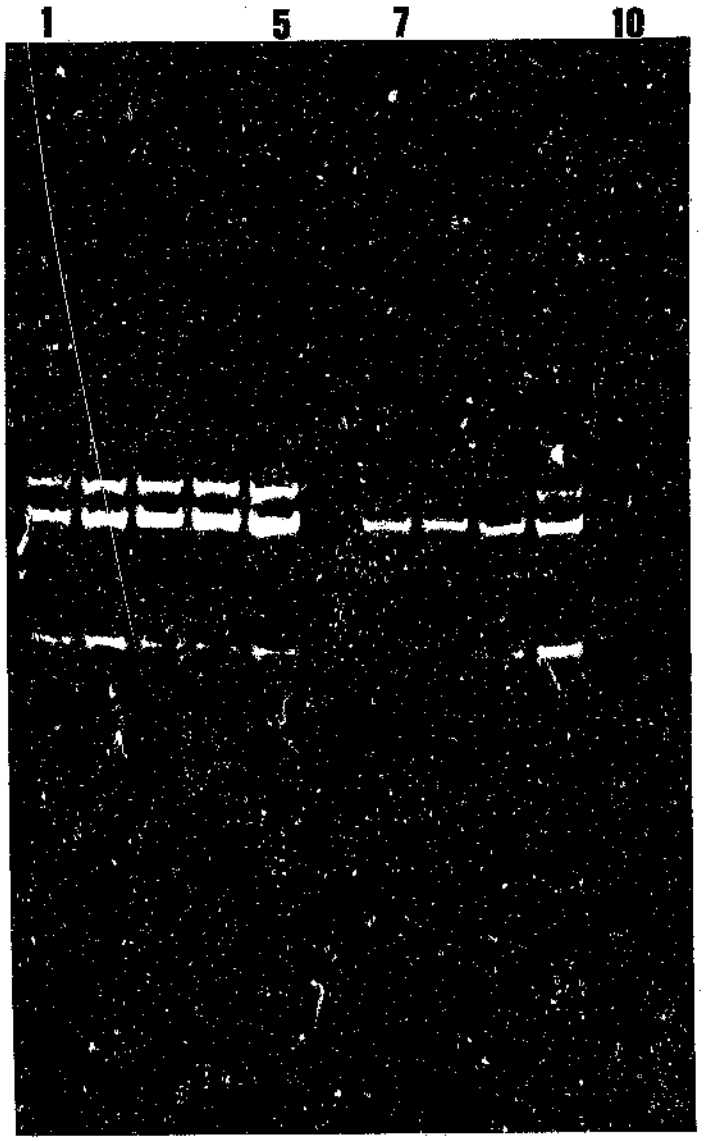

Figure 8 Influence of *Taq* polymerase and template concentration on PCR patterns. Template concentration per 50 µl PCR mix: lane 1 (0.25 µg), lane 2 (0.5 µg), lane 3 (1 µg), lane 4 (1.5 µg), lane 5 (2 µg). *Taq* polymerase concentrations per 50µl PCR mix: lane 7 (0.8 U), lane 8 (1.2 U), lane 9 (1.6 U), lane 10 (2 U). Lane 11 contains molecular size markers. Lane 6 is empty.

3.2 Day-care centre isolates

The 10 isolates from the DCC were compared to a multi-resistant 23F Spanish strain using primer 6 (**Figure 9**), primer 1 (**Figure 10**), and primer 8 (**Figure 11**).

All 11 isolates showed identical banding patterns for each primer.

The DCC isolates were further compared to a penicillin-sensitive reference strain (R6), and 10 epidemiologically unrelated isolates from different parts of South Africa using primer 6 (**Figure 12**). All 11 isolates gave different banding patterns.

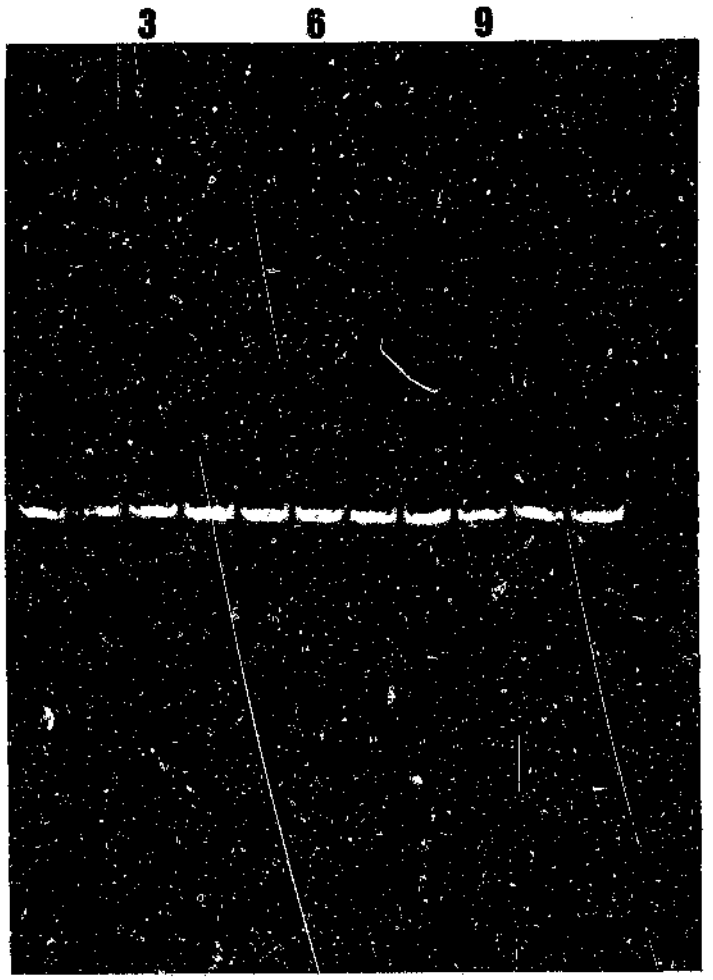

Figure 9 Day-care centre isolates using primer 6. Lane 1 contains the Spanish strain. Lanes 2 to 11 contain the different day-care centre isolates. Lane 12 contains the molecular size markers.

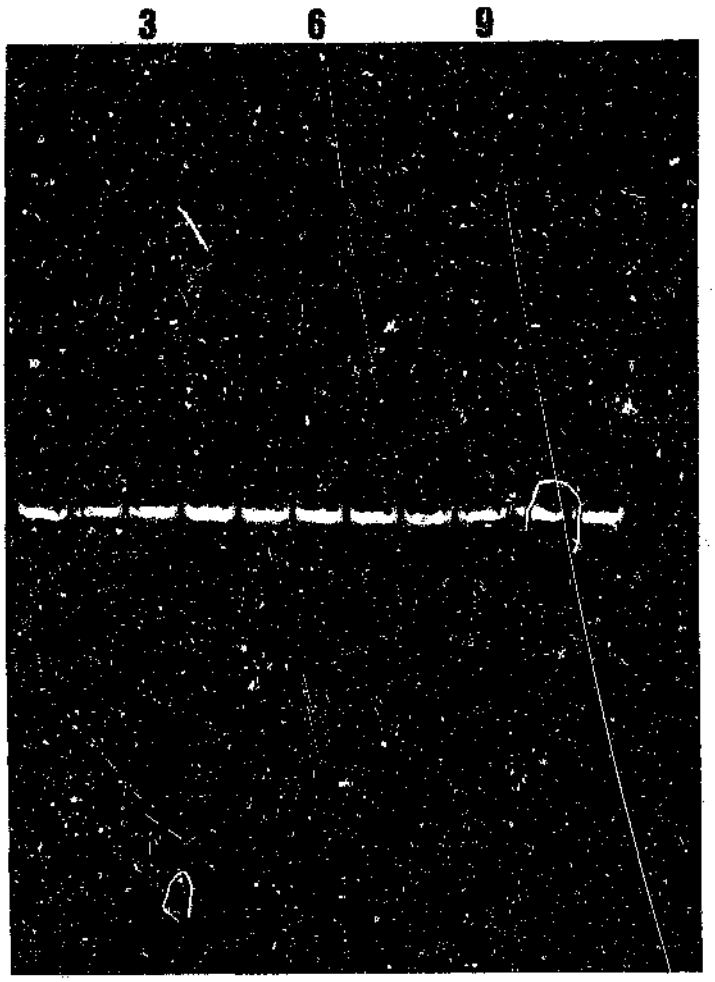

Figure 9 Day-care centre isolates using primer 6. Lane 1 contains the Spanish strain. Lanes 2 to 11 contain the different day-care centre isolates. Lane 12 contains the molecular size markers.

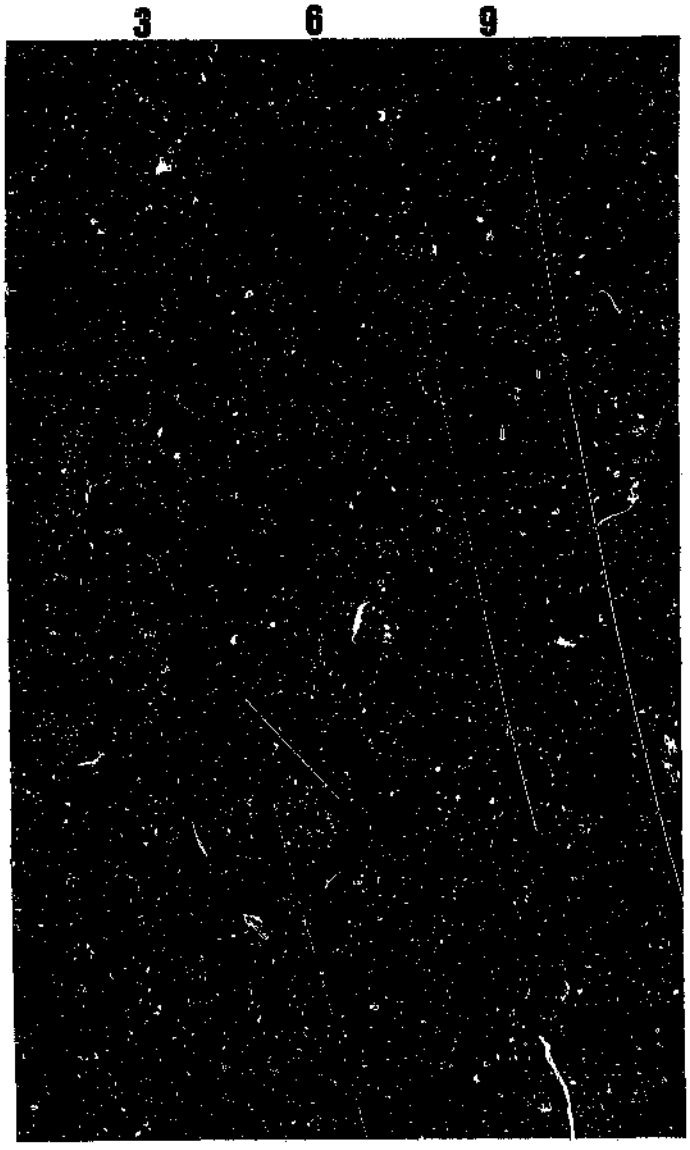

Figure 10 Day-care centre isolates using primer 1. Lane 1 contains the Spanish strain. Lanes 2 to 11 contain the different day-care centre isolates. Lane 12 contains the molecular size markers.

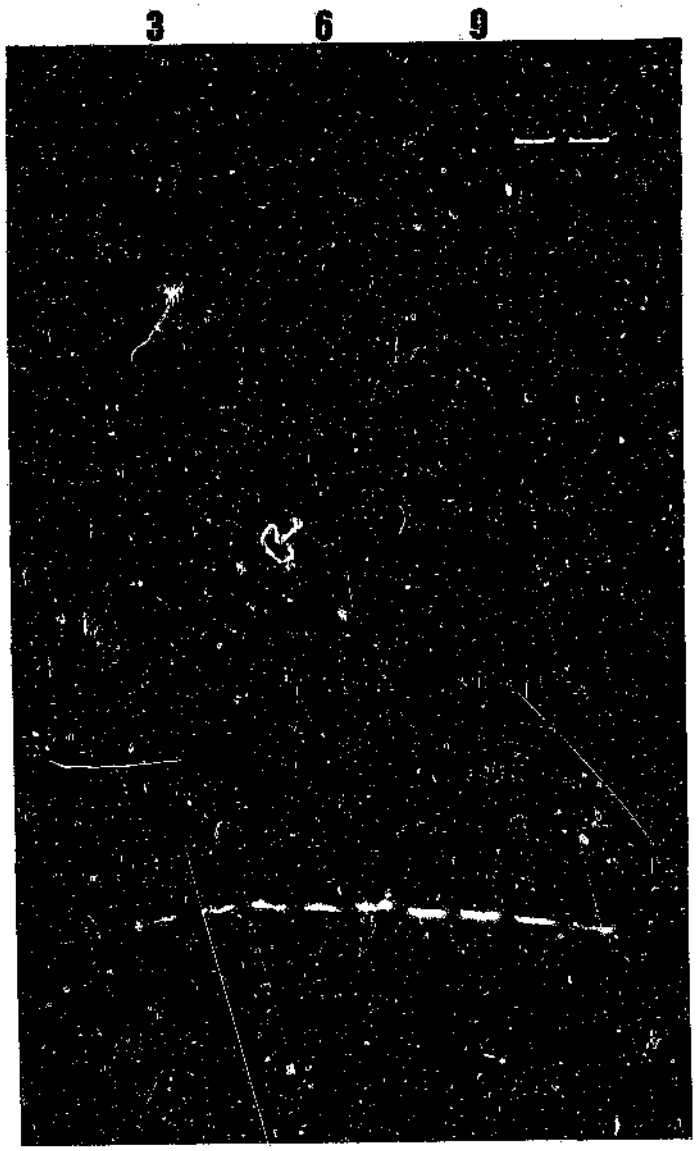

Figure 11 Day-care centre isolates using primer 8. Lane 1 contains the Spanish strain. Lanes 2 to 11 contain the different day-care centre isolates. Lane 12 contains the molecular size markers.

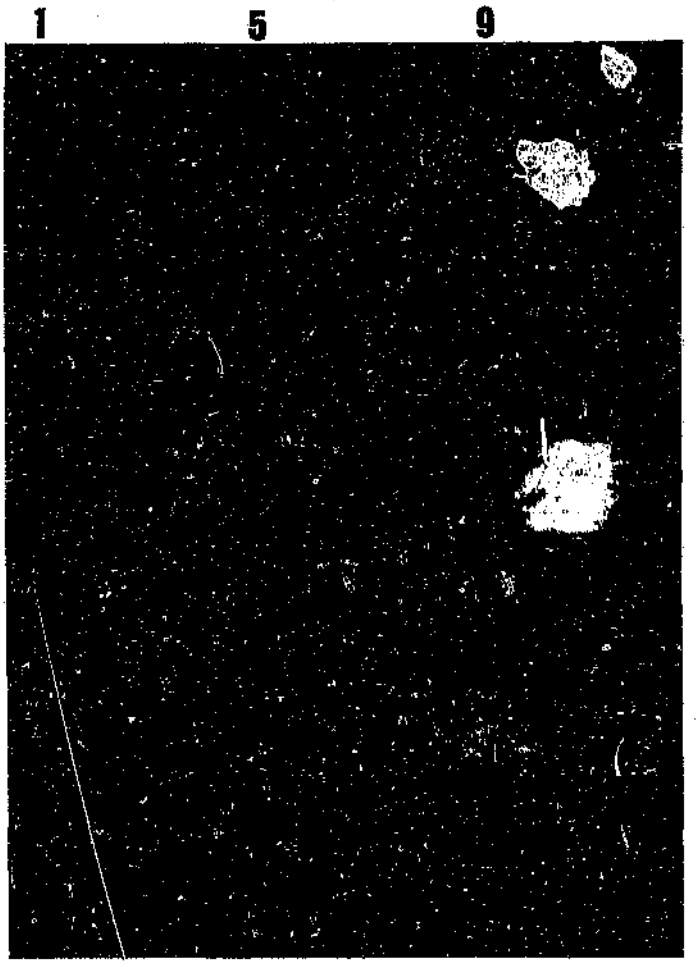

Figure 12 Epidemiologically unrelated isolates. Lanes 1 to 11 contain the following isolates respectively; R6, 33, 34, 37, 38, 39, 40, 41,42, 43, and 47. Lane 12 contains the molecular size markers

Results of serotyping, antibiotic profiles, and RAPD of the above 22 strains is summarised in **Table 1.**

Table 1 South African isolates typing profiles. DCC:- Day-care centre. Antibiogram: 1 - resistant to penicillin, chloramphenicol, and tetracycline; 2 - resistant to penicillin, chloramphenicol, tetracycline, and erythromycin; 3 - fully sensitive.

Isolate	Serotype	Antibiogram	RAPD profile
Spanish strain	23F	1	a
DCC1	23F	2	a
DCC2	23F	2	a
DCC3	23F	2	a
DCC4	23F	2	a
DCC5	23F	2	a
DCC6	23F	2	a
DCC7	23F	2	a
DCC8	23F	2	a
DCC9	23F	2	a
DCC10	23F	2	a
R6	2	3	b
33	6B	3	c
34	23F	2	d
37	23F	1	e
38	6B	3	f
39	1	3	g
40	23F	1	h
41	19	1	i
42	19	1	j
43	19	1	k
47	6B	1	l

3.3 Korean isolates

Clinical isolates of 18 penicillin-resistant and multi-resistant pneumococci were evaluated for their relatedness to each other and to the Spanish clone using primer 6 (**Figure 13**).

7 of the multiresistant serotype 23F pneumococcal isolates had the same RAPD profile as the Spanish strain. These 7 isolates were further tested using primer 1 (**Figure 14**) and primer 8 (**Figure 15**).

Results of serotyping, antibiotic profiles, and RAPD of the Korean isolates is summarised in **Table 2**.

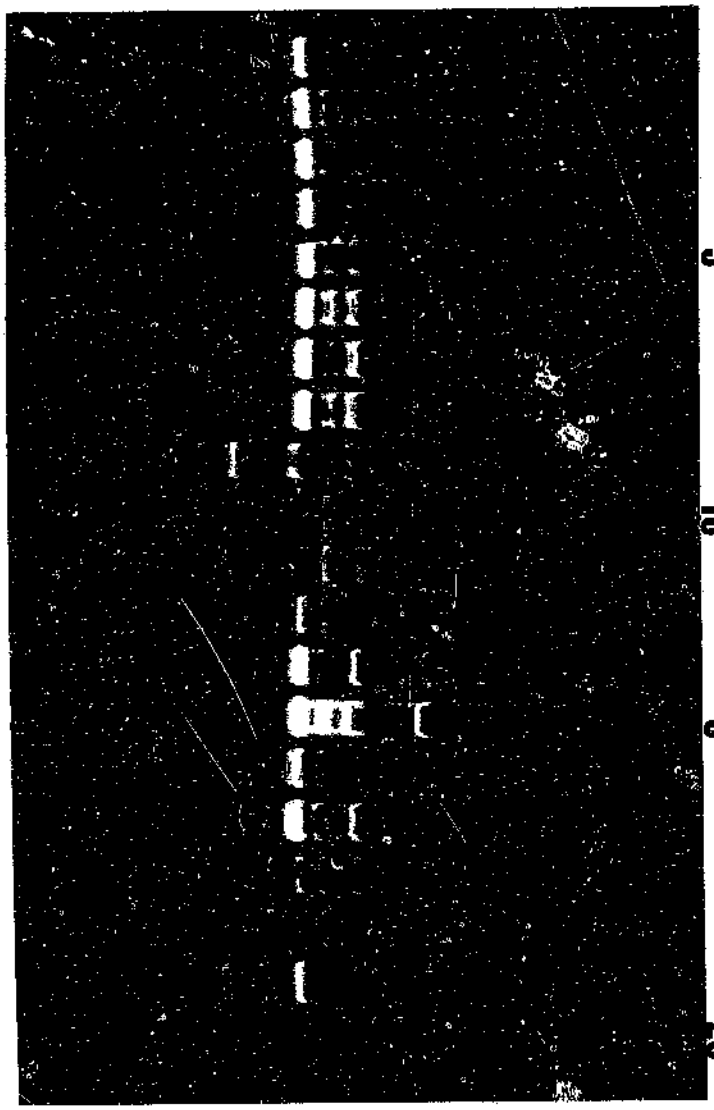

Figure 13 Korean isolates. Lane 1 contains the Spanish strain. Lanes 2 to 19 contain the following isolates respectively; 11, 12, 13, 24, 25, 28, 29, 30, 20, 19, 21, 27, 31, 22, 23, 18, 26, 32. Lane 20 contains the molecular size markers. All reactions with primer 1.

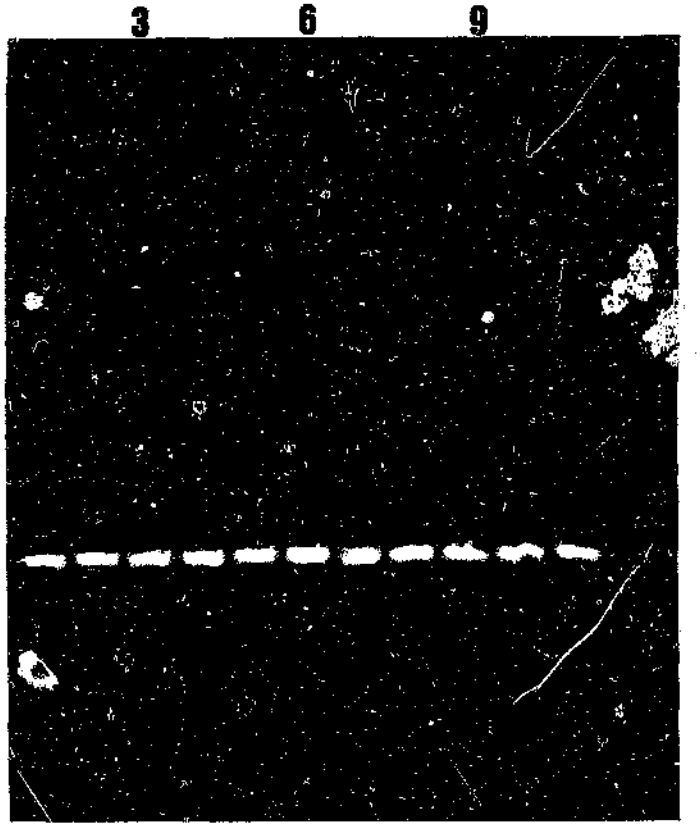

Figure 14 Korean multiresistant 23F isolates using primer 1. Lanes 1, 2 and 11 contain the Spanish strain. Lanes 3 and 4 contain isolate 11. Lanes 5 to 10 contain isolates 12, 13, 24, 25, 28, and 29 respectively. Lane 12 contains the molecular size markers.

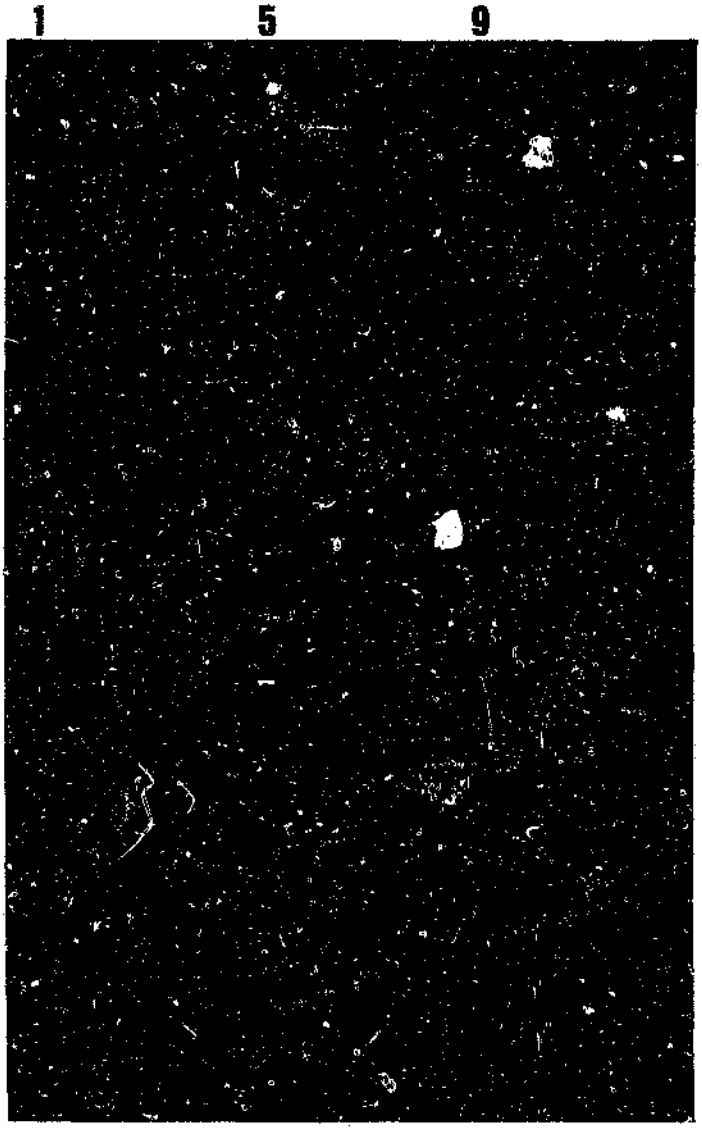

Figure 15 Korean multiresistant 23F isolates using primer 8. Lanes 1, 2 and 9 contain the Spanish strain. Lanes 3 to 8 contain isolates 11, 12, 13, 24, 25, 28, and 29 respectively. Lane 10 contains the molecular size markers.

Table 2 Korean isolates typing profiles. Antibiogram: 1 - resistant to penicillin, chloramphenicol, and tetracycline; 2 - resistant to penicillin, chloramphenicol, tetracycline, and erythromycin; 3 - resistant to penicillin alone.

Isolate	Serotype	Antibiogram	RAPD profile
11	23F	1	a
12	23F	1	a
13	23F	1	a
24	23F	1	a
25	23F	1	a
28	23F	1	a
29	23F	1	a
30	23F	2	b
20	23F	1	c
19	23F	2	d
21	23F	2	e
27	23F	3	f
31	23F	3	g
22	6B	2	h
23	6B	2	f
18	23F	2	d
26	19F	1	i
32	19F	1	j

4. DISCUSSION

The emergence of strains of *S. pneumoniae* resistant to penicillin and to other antibiotics, and the spread of that resistance over the world have become major concerns and increase the need for epidemiological surveillance (Klugman, 1990). Phenotypic and genotypic techniques have been used in epidemiological investigations. These include antibiograms, serotyping, PBP patterns, MLEE, PBP gene profiles, RFLP, ribotyping and PFGE (Lefevre et al, 1993).

Serotyping is associated with variable expression of the polysaccharide capsule and some isolates are not typeable (Lund and Hendrichsen, 1978). PBP pattern analysis is limited to changes in genes encoding penicillin resistance proteins. RFLP using conventional electrophoresis is difficult to interpret because of the large number of bands. MLEE analysis detects protein charge differences which can be directly linked to mutations in a variety of genes, encoding for metabolic enzymes, throughout the pneumococcal genome. Lefevre et al (1993) demonstrated that PFGE was more discriminatory than MLEE or serotyping. Ribotyping is simpler to interpret than RFLPs, but there are limited data on the use of this technique in pneumococci (McDougal et al, 1992). MLEE, ribotyping, and PFGE are time consuming and labour intensive.

Multiresistant serotype 23F pneumococci have been prevalent in Spain for at least 16 years (Fenoll et al, 1990), whereas most multiresistant strains in

South Africa prior to 1978 belonged to SGTs 6, 14, and 19 (McDougal et al, 1992). Subsequently, an increase in the isolation of multiresistant 23F pneumococci have been documented in South Africa (Koornhof et al, 1992). Muñoz et al (1991) showed a clonal relationship between a Spanish strain and other multiresistant 23F pneumococci, isolated in Cleveland, Ohio, by MLEE and RFLP of the PBP 2X and 2B genes. McDougal et al (1992), using PBP profiles, MLEE and ribotyping, showed clonality between multiresistant 23F pneumococci from Spain, South Africa, and America. They also concluded that MLEE and ribotyping provided greater sensitivity in differentiating strain variation than did PBP profiles. Furthermore Lefevre et al (1993), using PFGE, showed genetic relatedness between the Spanish 23F multiresistant strain and strains from England, Finland, Germany, South Africa and the United States. Versalovic et al (1993) using MLEE and repetitive extragenic palindromic-polymerase chain reaction genomic profiling showed clonality between penicillin-resistant 6B pneumococci isolated in Houston, Spain, Alaska, and Iceland.

In this study RAFD was used for the epidemiological evaluation of pneumococcal strains. A major disadvantage of the classical PCR is contamination. RAPD, however, is less vulnerable to contamination than is diagnostic PCR, since larger amounts of pure template DNA are used (Van Belkum, 1994). All PCR-products derived from previous assays, however, can act as contaminating templates in later experiments. Van Belkum (1994) showed that by keeping the PCR-products physically separated from template DNA, reliable fingerprints with no contamination could be achieved. The present study confirmed this, as all negative controls remained bandless.

RAPD requires no sequence information and it scans the whole genome rather than relying on hypervariability within one specific gene. Each primer gives a different pattern of PCR products, each with the potential to detect polymorphisms between strains. Therefore, discrimination between isolates can be maximised by combining the RAPD results obtained with several primers. Care must be taken in selecting primers because some primers may not generate polymorphic patterns with a particular organism and some generate a conserved PCR profile that is species-specific (Matthews, 1993). An alternative to repeating RAPD with different primers each time, is to use two short arbitrary primers in combination for each RAPD (Kaemmer et al, 1992).

RAPD, unlike MLEE, ribotyping and PFGE, is a simple and rapid technique and is well suited for studies involving a large number of isolates. A reported disadvantage of RAPD has been the lack of reproducibility between laboratories. The reproducibility in this study, which was conducted in only one laboratory, was however excellent. The same strain investigated numerous times during one PCR experiment gave the same banding pattern. In addition, the same strain investigated at different times consistently gave the same banding pattern.

Studies using RAPD for subtyping *Pseudomonas cepacia* (Bingen et al, 1993), *Actinobacillus actinomycetemcomitans* (Preus et al, 1993), *Proteus mirabilis* (Bingen et al, 1993), *Escherichia coli* (Alos et al, 1993; Bingen et al, 1992), *Porphyromonas gingivalis* (Steenbergen et al, 1993), *Acinetobacter baumannii* (Graser et al, 1993), *Haemophilus influenzae* (Jordens et al), *Haemophilus somnus* (Myers et al, 1993), and *Staphylococcus aureus* (Matthews et al, 1994) have used different

concentrations of magnesium, primer, template, *Taq* polymerase, and nucleotides. Even the number of cycles and temperature of denaturation, annealing and extension differed (**Table 3**).

As RAPD has not been described for *S. pneumoniae*, differing concentrations of the above variables were assessed, in order to find the best combination.

Table 3 Variability of PCR mixture reported by different authors. n/s - not stated

Reference	Primer (µM)	Template (µg)	*Taq* U	dNTP (µM)	Mg^{++} (mM)	Cycles	Annealing T °C
Bingen, 1992	3	0.05	3	400	4	35	35
Preus, 1993	0.2	0.05	1	100	5	32	42
Graser, 1993	25	2.5	3	200	4.5	27	50
Jordens, 1993	0.3	1	2	250	2.5	44	32
Myers, 1993	0.4	0.01	2	200	3	45	40
Matthews, 1994	2.5	0.02	4	100	n/a	35	30

From initial experiments in this study, the optimal concentration of PCR components was found to be:

primer (4 µM),
template (0.5 µg),
nucleotides (300 mM),
magnesium (4 mM), and
Taq polymerase (2 U).

Of the 8 primers tested only primer 1,6 and 8 gave optimal banding patterns at an annealing temperature of 35 °C. Primer 2, 3, and 4 gave banding patterns only at an annealing temperature of 50 °C, whereas primers 5 and 7 gave no bands at any temperature. All the banding patterns were different.

The comparison of the 2 DNA extraction methods gave interesting results. The more classical method of DNA extraction as described by Smith et al (1993a), gave larger fragment sizes than the boiling method. This could be due to the greater shearing of DNA by vortexing after the boiling step. Thus before the PCR is started the template DNA comprises smaller fragments than in the PCR mixtures from the classical DNA extraction method. As long as the DNA extraction method was kept constant, however, both methodologies gave good and reproducible results. In a recent study, Ménard et al (1992) showed that RNA, proteins and other contaminants in the PCR mixtures did not affect the final RAPD pattern. Therefore for RAPD a simple and rapid DNA extraction method can be used.

When the DCC isolates were compared with the Spanish clone using primer 6, the same banding pattern was found. This observation was confirmed with primer 1 and primer 8. These same strains compared previously, by MLEE and fingerprinting of the PBP genes (Klugman et al, 1994), were found to be identical, confirming the RAPD results.

When epidemiologically unrelated multiresistant 23F pneumococci, as well as other resistant and sensitive serotypes, from both South Africa and Korea were compared, 21 different RAPD patterns were obtained (**Table 4**), confirming the excellent discriminatory ability of the RAPD technique.

Table 4 RAPD patterns of epidemiologically unrelated isolates from South Africa and Korea

Serotype	No. of Isolates	No. of different RAPD profiles
2	2	2
6B	5	5
19F	5	5
23F	10	9

As multiresistant 23 F pneumococci seem to have originated in Spain (Klugman, 1990) and as there was a possible link between the index case from the DCC and Spain (through the child's aunt), it is postulated that spread of this multiresistant 23F strain from Spain to South Africa has occurred.

With the use of primer 6, 7 of the 14 multiresistant 23F pneumococcal isolates from Korea, were found to have RAPD patterns identical to the Spain clone. Using primer 1, these isolates also had the same RAPD pattern, which was different from the pattern obtained using primer 6. When primer 8 was used however, one isolate showed a different RAPD pattern. Therefore after using 3 different primers, 6 of the Korean multiresistant 23F isolates were shown to have the same RAPD pattern to the Spanish clone. These data indicate that the Spanish clone and 6 of the Korean isolates originated from the same source. It is interesting to

speculate that spread could possibly have occurred during the Seoul Olympic Games in 1988.

In conclusion, after optimization of the RAPD reaction, reproducibility of the banding patterns was maintained. Reproducibility between laboratories or thermocyclers however, was not tested.

In the RAPD technique, a rapid and simple DNA extraction technique can be used which gives equivalent results to the more classical and expensive method. Allowing 2 hours for DNA extraction and PCR preparation, 4 hours for the PCR procedure, and 2 hours for electrophoresis, RAPD can give results the same day.

This study has demonstrated that RAPD has excellent discriminatory value in the detection of strain variation among *Streptococcus pneumoniae* isolates. The 10 South African DCC isolates and 6 of the Korean isolates showed identical banding patterns to a Spanish multiresistant 23F strain, suggesting intercontinental spread of this Spanish strain.

MLEE, ribotyping, and PFGE have proven useful for subtyping pneumococci, but they are labour intensive, expensive, time consuming and require a high level of expertise. RAPD is a useful additional technique for typing pneumococci either alone or in conjunction with other typing techniques.

5. APPENDIX A

Blood Agar: Columbia Agar Base (Oxoid, Basingstoke, England)
5% horse blood.

Loading dye: 40% Sucrose
0.025% Bromophenol blue

Tris (1M): 121,1 g Tris
800 ml distilled water
Buffered to pH 8 with concentrated hydrochloric acid

EDTA (0.5M): 186,1 g EDTA
800 ml distillied water
Buffered to pH 8 with 1M sodium hydroxide

TE buffer: 10 mM Tris
1 mM EDTA

CTAB/NaCl: 4.1 g NaCl in 80 ml distilled water
10 g CTAB
Add distilled water to 100 ml
Final concentration - 10 % CTAB, 0.7 M NaCl

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