CHARACTERIZATION OF STREPTOCOCCAL INFECTIONS IN KWAZULU-NATAL DURBAN BY RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS AND DNA MACRORESTRICTION ANALYSIS

by

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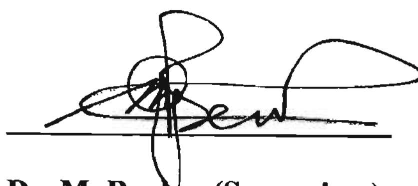
PREFACE

The experimental work described in this dissertation was carried out in the Department of Genetics, School of Molecular and Cellular Biosciences, University of KwaZulu-Natal, Pietermaritzburg, January 2003 to June 2004, under the direct supervision of Doctor M. Beukes. Part of pulsed-field gel electrophoresis was conducted at the Institute for Medical Microbiology and Immunology, University of Bonn, Germany.

I hereby declare that, unless specially indicated to the contrary in the text, this thesis is my own original work and has not been submitted to any university for a similar or any other degree.

P. Z. Madlala



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My family, Sipho, Thulisile, Bongiwe, Thembeka, Thokozani, Andile and Aphelele for the endless love, and support and for inspiring and believing in me.

DEDICATION

I would like to dedicate this thesis to my late mother Thandi Flora Madlala and late brothers, Mandla Madlala, Eric Madlala and Bongani Madlala. May their souls rest in peace.

ABSTRACT

A collection of 29 clinical streptococcal isolates obtained from the University of KwaZulu-Natal, Medical School, Durban Metro area (South Africa) were studied to establish their penicillin G susceptibility patterns often refered to as minimal inhibitory concentration (MIC) and to determine the genetic diversity among them using two genotyping methods, randomly amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (PFGE) analysis. All isolates with MIC less than or equal to 0.12 µg/ml were considered susceptible, intermediate resistant if MIC was between 0.25 µg/ml and 4 µg/ml and resistant if greater than 4 µg/ml. The percentage of isolates with resistance was relatively high (75.9%), only 10.3% of isolates showed intermediate resistance and 13.8% of the isolates were completely susceptible to penicillin G. Some of the resistant isolates were highly resistant reaching penicillin G MIC levels of 5000 µg/ml. They were speculated to contain both altered penicillin binding proteins and high level of crosslinking cell wall induced by the gene products of the *MurMN* operon. RAPD analysis was performed using three primers, MBPZ-1, MBPZ-2, and MBPZ-3, respectively. RAPD analysis allowed for the identification of 27 RAPD types with MBPZ-1 and MBPZ-3 and 26 RAPD types with MBPZ-2. Ninety-eight percent of these isolates were clustered into two groups, group I and group II, with 90% to 100% dissimilarity among them. Fifty two percent of the isolates of MBPZ-1 group I were in MBPZ-2 group I, 72% isolates of MBPZ-1 group I were in MBPZ-3 group I, and 72% of the isolates of MBPZ-2 group I were in MBPZ-3 group I. This shows the discriminatory ability of the primers used in this study. Despite clustering of isolates, relatively high diversity was seen. PFGE analysis of macrorestriction fragments obtained after digestion of chromosomal DNA by restriction enzyme, *Sma*I showed 24 PFGE patterns. The 24 PFGE patterns were divided into three groups (I, II, and III) of isolates, with an average of 85% dissimilarity (15% homology) among them. At 25% homology, four clusters, A (13 isolates), B (9 isolates), C (4 isolates), and D (4 isolates) were observed. Two pairs of isolates in group I, cluster A, showed 100% homology. This suggested that each represent the same strain. Four isolates of group I, cluster B, also exhibited 100% homology. This study showed that most of streptococcal isolates with the same penicillin G susceptibility

patterns grouped together in a phylogenetic tree by both RAPD and PFGE analysis. There was also some similarity between the results obtained by RAPD analysis and PFGE analysis. Seventeen and nine of the 29 isolates grouped into group I and group II, respectively, two pairs of isolates were indistinguishable, and two pairs of islates were closely related by both RAPD (using MBPZ-3) and PFGE analysis. Although, RAPD analysis is sensitive, specific, faster and cost effective, the ease with which PFGE analysis can be performed, high discriminatory power, reproducibility of the results, and the polymorphism seen in the patterns, suggests that PFGE method has the potential to be very useful for epidemiological evaluations of nosocomial streptococcal infections in KwaZulu-Natal.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Streptococci play a significant role in causing clinically important infections in human beings. The leading and most problematic pathogen within this genus is *Streptococcus* (*S.*) *pneumoniae*. This pathogen is the most common cause of bacterial meningitis and pneumonia that is associated with the highest case fatality rate. Several other streptococcal species play a significant role in human health. For example *S. pyogene* is a leading cause of upper respiratory tract infections, *S. mutans* is a cause of oral infections, *S. agalactiae* is a leading cause of sepsis and *S. mitis* is a cause of abdominal and poly-microbial sepsis (Casariego *et al.*, 1996; Shinzato and Saito, 1995; and Singh *et al.*, 1988).

The discovery of antibiotics such as *β*-lactams, amino glycosides, tetracycline and macrolides (Anthony *et al.*, 1969) led to predictions that bacterial diseases would soon be eradicated. However, an outbreak of bacterial isolates with resistance to commonly used anti-microbial agents requires discovery of new and novel antimicrobial agents that are safer and more effective. In addition to discovering safe and effective antibacterial agents, there is a need to determine the most effective molecular typing methods. The aim of species or strain typing studies is to provide laboratory evidence that epidemiologically related isolates collected during an outbreak of disease are also genetically related and thus represent the same strain. This information is helpful for understanding and controlling the rate of spread of infection in both hospitals and communities (Meitert and Meitert, 1978).

The first methods used for bacterial typing relied on tests based on the phenotypic properties of an individual organism. However, phenotypic-based methods have a number of limitations. For example, tests derived or designed for one group of organisms are not always useful for the other groups (Li *et al.,* 2001). Limitations of phenotypic based methods led to the development of genotypic methods for microbial typing purposes.

Many genotypic typing methods have been used for typing of bacterial species. These genotyping methods provide the information that can assist clinical microbiologists to determine the source and the rate of spread of infection and to define mechanisms of transmission and mechanism of resistance to antibiotics of epidemic strains (Tenover *et al.*, 1997; and Versalovic *et al.*, 1993). Among available techniques, pulsed-field gel electrophoresis (PFGE) has gained wide acceptance as an excellent method for typing of bacteria in terms of discriminatory power and reproducibility. Other genotypic methods such as randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphic (RFLP) and plasmid analysis have also been used (Dunne *et al.*, 2001).

The purpose of this study was to determine the genetic relatedness of streptococcus isolates obtained from human beings in KwaZulu-Natal Durban Metropolitan area, South Africa. The isolates were studied by RAPD analysis and PFGE analysis, with the objectives of this genetic characterization being (i) to establish the penicillin G minimal inhibitory concentrations (MICs) of the isolates under study (ii) to determine which of the two typing techniques is a sufficiently discriminatory method for typing streptococcus isolates and (iii) to determine to what degree these methods give concordant results.

This thesis is divided into four chapters. The first chapter is the introduction and literature review, the second chapter determines and discusses minimal inhibitory concentrations (MICs) of streptococcus isolates under study against penicillin G, the third chapter discusses the determination of genetic relatedness among the streptococcus isolates using RAPD analysis, the fourth chapter determines and discusses the genetic relatedness among isolates using PFGE analysis. Following chapter four is the discussion and conclusions of the results obtained during this study, and references

1.2 CONVENTIONAL PHENOTYPIC/ BIOLOGICAL METHODS FOR MICROBIAL TYPING

The first methods used for classification and identification of microbes relied on tests based on the phenotypic properties of individual organism. Bacterial strains were assigned to somewhat intuitive classification groups, on the basis of a small number of characteristics which could be examined easily, such as staining properties, morphology, motility, nutritional requirements, acid production, pigmentation and spore formation (Koneman *et al.,* 1997; Maslow *et al.,* 1993; and Towner and Cockayne, 1993).

Although some methods based on phenotypic properties have stood the test of time, their limitations have led to re-evaluation of the whole process of microbial identification and classification. Increasing use of molecular identification and typing methods has led to revision of some accepted classification schemes and to the recognition of new relationships among microbes. Whatever methods or criteria are used for taxonomic groupings, the basic unit for the purposes of microbial identification remains the species (Koneman *et al.*, 1997).

Identification to the species level is the primary purpose of all microbial classification schemes, but the separation and accurate recognition of subtypes (strains) within a species is assuming greater importance in all branches of microbiology, medical microbiology and particularly in epidemiology (Koneman *et al.,* 1997; and Greenwood *et al.,* 1995). There is a tendency to assume that organisms that are indistinguishable by a chosen typing technique are identical and have a common source (Greenwood *et al.,* 1995). It should always be remembered, however, that such organisms may be indistinguishable only when viewed through the typing window generated by a particular typing scheme, and may be totally different when typed by an alternative method (Towner and Cockayne, 1993).

Control of communicable diseases would not be possible without the use of typing methods to help define the source of infection, mechanism of transmission, mechanism of

resistance to antibiotics, and the rate of spread of infection in both hospitals and communities. Chapter one concerns primarily modern molecular methods for microbial typing and identification, but these methods should be considered in the comparison to older, established methods, which may eventually be superseded.

Conventional biological typing methods for microorganisms were developed originally in response to a growing awareness of different factors that influence the spread of human pathogens in various environments. As stated by Tenover *et al.* (1997) and Maslow *et al.* (1993), an ideal typing system should: (a) be able to type the vast majority of strains being studied, (b) have a good discriminatory power, with the ability to produce a clearly interpretable result for most bacterial isolates of interest, (c) show good reproducibility over a long period of time and in different centres, (d) be readily applicable to natural isolates, as opposed to laboratory collections of strains, and (e) should not be too complicated or expensive.

The main biochemical or biological typing techniques include biotyping, phage typing, serotyping and bacteriocin typing. Initially, differentiation within a newly delineated species was achieved by examining the culture and biochemical characteristics of a large collection of individual strains belonging to the species (Konman *et al.,* 1997). Such characteristics included colonial morphology, growth requirements, fermentation ability, carbon source utilization and antibiotic resistance or susceptibility (Table 1.1). Advantages, disadvantages and applications of these techniques are described in Table 1.1. From Table 1.1 it is clear that a single conventional biochemical typing method cannot give an optimal procedure for microbial species fingerprinting. One method may work for one microbial species but fail to work for other species, whereas a combination of biochemical typing methods can give the best results to many species. A combination of methods takes a longer time to perform. The limitations and disadvantages of these methods have led to establishment of molecular methods that have the ability to distinguish or study diversity in any microbial species (Mhand *et al.,* 1999; and Chetoui *et al.,* 1995).

Tablel.1 Basic principles and applications of different conventional methods for bacterial typing

Technique	Basic Principles of the Technique	Application	Disadvantages
Biotyping (Smyth *et al.*, 1997; and Kühn *et al*, 1991)	Biotyping methods reflects genotype. This technique works on the basis of agglutination tests to distinguish serotype	Bacteria, fungi and viruses	Differences in colonial morphology are often extremely subtle and therefore can be rather subjective. Biochemical reactions are more dependable and may be difficult to interpret. The relative small number of features with the potential to give differences discrimination is poor.
Phage Typing (Smyth *et al.*, 1997; Schlichting 1993; Bergan, 1979; Bergan, 1978; and Parker, 1972)	Phage typing is a bacterial strain identification method based upon sensitivity to a defined collection of bacteriophages, which have been selected to provide maximum sensitivity for differentiating strains within a particular species. Typing may be direct, based upon direct sensitivity to either unadapted or adapted phages, or indirect, based on detection or identification of phages present as prophages in bacteria.	Bacteria	Technically complex method in which many variables must be controlled. Some species may contain very few phage types, while others may contain too many within one strain. Phage types may be modified by genetic mechanisms like lysogenoic conversion, loss of phages and acquisition or loss of R plasmids. This technique cannot be applied readily to a new organism or in response to sudden emerging problem; for furthermore, for some genera, no phages have yet been isolated.
Serotyping (Smyth *et al.*., 1997; Quentin *et al.*, 1995; Schoenmaker *et al.*, 1992; and Kühn *et al*, 1991)	Typing is based on reactions with specific antisera, raised according to the antigeneric structure of microbes in each group. Serotyping can be applied to many different genera, although, in many cases, given set of reagents can be applied to a single species.	Bacteria, fungi and virus	Like phage typing, serotyping requires some time to develop a serotyping scheme for a new application, since the development of such schemes is quite a complex process, with the requirement for immunization of animals with uncreative strains, and cross-absorption experimentation. Serotyping seems to be associated with problems in antisera production and standardization of methodology. Serotyping schemes for certain genera may normally be available only in reference laboratories.
Bacteriocin Typing (Smyth *et al.*, 1997; and Munoz *et al.*, 1993)	Bacteriocin typing is performed either by testing, if the unknown strain is sensitive to the bacteriocin produced by the standard (or known) strains, or by testing if the unknown strain produces bacteriocins that are active against indicator strains	Bacteria	Although production of bacteriocins or sensitivity to them is relatively stable, it is known that these properties may be encoded by transmissible R plasmid. The second limitation with this method is that it requires more labour, especially when using cross-streaking techniques.

1.2.1 Multilocus Enzyme Electrophoresis

Multilocus enzyme electrophoresis (MLEE) detects differences in the electrophoretic mobilities of individual soluble metabolic enzymes. If the appropriate enzymes are selected, MLEE analysis can discriminate among the gene products of different alleles for a number of loci (Barrs *et al.,* 2000; and Schable *et al.*, 1991). Thus, the method can assess codominant markers in diploids for each locus, a requirement for an evolutionary biologist that is not achieved by a few of the popular DNA fingerprinting methods (Barrs *et al.*, 2000).

The MLEE method is straightforward. Cell extracts, cellular proteins of the microorganisms, are separated by starch gel electrophoresis and the individual enzymes are visualized in the gels by specific substrates (Whittam *et al.,* 1983). Variations in electrophoretic mobility typically reflect amino acid substitutions that alter the charge of the protein and thereby identify variations in the chromosomal gene encoding the enzyme. Although certain enzymes may be absent from particular isolates, the evaluation of multiple metabolic enzymes ensures that all isolates are typeable (Maslow *et al.*, 1993).

MLEE is technically demanding and, in general, only moderately discriminatory for clinical isolates (Quentin *et al.*, 1995). For example, use of MLEE to analyze or detect the genetic structure of *Staphylococcus intermedius* cultured from normal skin of dogs and those isolates from a variety of diseased conditions has shown that virulent, invasive strains are represented by a limited number of closely related genetic lineages (Barrs *et al.*, 2000; and Harvey *et al.,* 1994). Consequently, this method has had relatively limited application to epidemiological studies; but it has proved to be uniquely useful in providing qualitative data regarding the population genetics of pathogenic isolates.

1.3 GENOTYPING METHODS FOR MICROBIAL SPECIES TYPING

The problems of typeability, reproducibility, or discriminatory power associated with many phenotypic techniques have led to the development of numerous systems with the use of DNA-based (genotyping) methods. Initially, DNA-based techniques were used only in a few research laboratories, but they are widely available for use in clinical practice. In addition to the technical intricacies of these methods, there is an increasing challenge in interpreting the results obtained with these methods (Barret *et al.*, 1995).

1.3.1 Analysis of Plasmid DNA

Plasmid DNA analysis was the first molecular method to be used as a DNA based bacterial typing tool. This gives indications about the number and sizes of any plasmid present. Plasmid DNA analysis requires plasmids to be isolated and separated electrophoretically on an agarose gel. Once electrophoresis is complete, the various plasmids present can be visualized by staining with ethidium bromide (EtBr) and viewed under ultraviolet (UV) transilluminator.

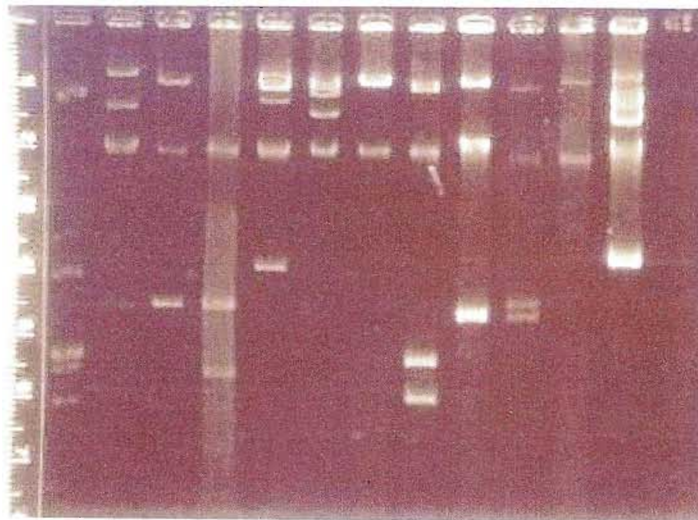

Figure 1.1. Plasmid profiles obtained by agarose gel electrophoresis. Each lane of a 0.8% gel was loaded with plasmid DNA isolated from a different strain of a bacterium.The fluorescent ruler down the left side of the image allows to the researcher measure the distance each plasmid has migrated.

A single plasmid generates at least three different bands, corresponding to the three different molecular forms of a plasmid, circular, coiled and supercoiled (Fig. 1.1) which render interpretation difficult. Therefore analysis of plasmid DNA for typing purposes requires plasmid DNA to be digested with a restriction endonuclese (section 1.3.2) to produce a number of restriction fragments with different sizes (RFLP) (Fig. 1.2). Plasmids differ considerably in the number of restriction sites that they possess for a given restriction enzyme, and a large number of restriction enzymes have been used to generate fingerprints. The generation of plasmid fingerprints is more of value for epidemiological purposes when only apparently similar plasmids of relative large size can be identified, or when similar plasmid profiles occur in strains that cannot be differentiated by other methods.

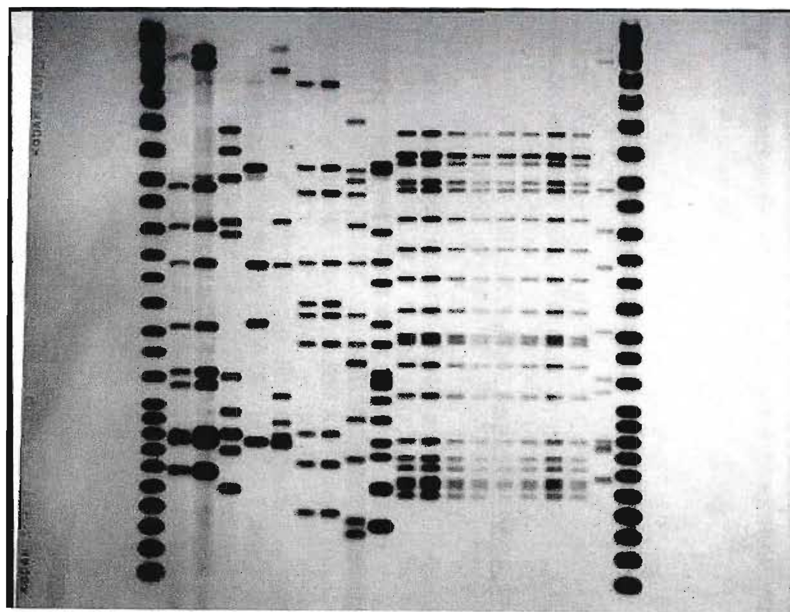

Figure 1.2. Plasmid DNA restriction fragments produced by frequent restriction enzymes. The first and the last lane containing molecular weight markers and the rest of lanes contain plasmid restriction fragments from different bacteria.

Organisms that generate totally different plasmid fingerprints certainly have totally different plasmid complements, and are epidemiologically distinct. Isolates that generate

fingerprints which differ by one or two DNA fragments, should be considered as having a possible close relationship, unless supplementary data obtained by other methods is available.

In some instances, plasmid fingerprinting may only complement other techniques, such as pulsed field gel electrophoresis analysis (section 1.4), by providing a basis for differentiating isolates that are related genotypically but are separated epidemiologically by moderate time periods, such as several months (Chatellier *et al.*, 1999; Towner and Cockyne 1993; and Tenover, 1985). A drawback of plasmid analysis technique is the lack of long-term stability in som strains. Of course, this analysis is restricted to plasmid-containing strains or species. Furthermore, plasmids may be acquired or lost in a particular environment (Maslow, 1993). Overall, plasmid analysis for typing and/or epidemiological purposes is most effective in studies that are limited both temporally and geographically (Tenover *et al.,* 1997)

1.3.2 Restriction Endonuclease Analysis (REA) of Chromosomal DNA

Each restriction endonuclease enzymatically digests DNA at a particular nucleotide recognition sequence, called restriction site. The number and size of restriction fragments generated by restriction endonuclease digestion of DNA indicates the frequency and the distribution of such restriction site for the enzyme used. In restriction enzyme analysis (REA), endonuclease with relatively frequent restriction sites are used to digest bacterial DNA, thereby generating hundreds of fragments, ranging from 5 kb to 50 kb in length (Clobots *et al.*, 1992). Such fragments can be separated by size with constant-field gel electrophoresis and the patterns detected by staining the gel with ethidium bromide and taking a photograph under ultra-violet light. Different strains of the same bacterial species have different REA because of the minute variations in their DNA sequence that alter the distribution of restriction sites. All bacterial isolates are typeable by REA, and the approach has been applied successfully to many species including streptococci (Hall, 1998).

The major limitation of this method of bacterial typing is the difficulty in interpreting the complex profile consisting of hundreds of bands that may be unresolved and overlapping (Maslow, 1993). Furthermore, REA may be compounded by the presence of plasmids, whose DNA readily contaminates genomic DNA preparation. The restriction fragments derived from the plasmids can detectably alter the REA profile and cause isolates that differ only in their plasmid content to be designated as different strains (Tenover *et al.*, 1997).

1.3.3 Southern Blot Analysis of Chromosomal DNA

The difficulty in interpreting hundreds of restriction fragments produced by REA can be solved by Southern blot analysis following agarose gel electrophoresis. Southern blot analysis was named after the investigator who described it (Southern, 1975). The separated restriction fragments can be transferred onto a nitrocellulose or nylon membrane, probed using labelled short oligonucleotide as a probe to detect restriction fragment(s) that contains sequences homologous to the probe. The variations in the number and size of these fragments are referred to as restriction fragment length polymorphism (RFLPs) and reflect variations in both the number of loci that are homologous to the probe and the location of the restriction site within or flanking those loci. All strains carrying loci homologous to the probe are typeable and the results are highly reproducible (Maslow, 1993).

The procedure requires some technical expertise and has classically used radioisotopes to label the probe. However, reliable nonradioactive systems have been developed, making Southern blot analysis more widely available. The discriminatory power of Southern blot analysis is directly related to the number and variability of the fragments detected; the most effective probes are those that detect multiple bands simultaneously. For example, an outbreak-associated strain of multidrug-resistant *Mycobacterium tuberculosis* was readily differentiated from other, sporadic multidrug-resistant strains of species by means of Southern blot analysis (Edlin *et al.*, 1992).

1.4 PULSED-FIELD GEL ELECTROPHORESIS

Conventional electrophoresis has been ineffective in influencing the size-dependent migration of large DNA. The cause of this size-independent migration of DNA is most likely related to the rod-like shape of the DNA molecules (Koneman *et al.,* 1997). Different sizes of DNA fragments differ in the lengths of their long axes, but not in the diameter of their short axes. In an electrical field, the large DNA molecules orientate themselves along their long axes as they enter a gel matrix. Since all large molecules have the same charge-to-mass ratio, and they all travel along their long axes and have identical short axes, they therefore move at the same speed in a gel.

In 1984, Schwartz and Cantour described pulsed field gel electrophoresis (PFGE), introducing a new way to separate chromosomal fragments produced by enzymatic digestion of intact bacterial genomic DNA. In contrast to conventional gel electrophoresis, PFGE is multidirectional, continually changing the location of the positive charge (Georing, 1993). The DNA molecules respond by continually reorienting their direction of migration through the agarose gel. In addition, an electric pulse of different duration favours the separation of different sizes of DNA fragments (Elliott *et al.,* 1993). With each reorientation of the electric field relative to the gel, smaller sized DNA will begin moving in the new direction more quickly than the larger DNA. Thus, large DNA lags behind, providing a separation from the smaller DNA molecules.

The size of the molecule that will separate from the others depends strongly on the length of the pulse in each direction. A fast pulse rate-short pulse time, causes smaller molecules to separate, whereas a slow pulse rate-long pulse time, causes even larger molecules to separate (Chu, 1991). If the pulse rate is increased, what is termed ramping, during the course of a run, a broader size range of molecules will separate out very distinctly (Sahm, 1996). By varying both the direction and the duration of the electric field, PFGE allows the separation or resolution of DNA molecules well over 1 000 kb in length, often referred to as mega-base sized DNA.

Other physical factors have also shown to be very important. It is known that changes in parameters such as temperature, voltage, agarose concentration and ionic strength will act inter-dependently, but non-uniformly, on the mobility of different sizes of DNA. Thus changes in one parameter might affect resolution adversely, but resolution can be restored by making an adjustment in another parameter. The agarose concentration used most widely is 1% (w/v), with the size and the shape of a gel being determined by the precise method and design of the apparatus used, but most pulsed field methods use horizontal agarose gels in a submerged mode (Towner and Kockyane, 1993). Buffers are circulated continuously and cooled, normally to a constant temperature between 10° and 15° (Chu, 1991). Electrical conditions and separation times depend on the precise method being used and the size range of the DNA molecules being separated (Chu, 1991).

After completion of the electrophoresis, gels are stained with ethidium bromide (0.5 μg/ml) for an hour, destained with distilled water as necessary, and the DNA visualized in the same manner as for conventional agarose gel electrophoresis. The effective use of PFGE requires accurate and reliable size standards for estimating the sizes of the DNA fragments being studied.

1.4.1 Types of PFGE Methods

There are different types of PFGE methods, differing only in the way the pulsed electrophoresis field is delivered to the agarose gel. Two of the most commonly cited methods are contour clamped homogeneous electric field (CHEF) (Chu *et al.*, 1986) and field inversion gel electrophoresis (FIGE) (Carle *et al.*, 1986). These two methods represent different, but complementary, ends of the PFGE spectrum.

1.4.1.1 Field Inversion Gel Electrophoresis

FIGE utilizes any conventional electrophoresis chamber box that has temperature control and periodically inverts the positive charge by 180°, hence the term *field inversion*. During electrophoresis FIGE subjects DNA molecules to an 180° re-orientation. As a result, DNA molecules spend a certain amount of time moving backward but with the net movement being forward (Fig. 1.3).

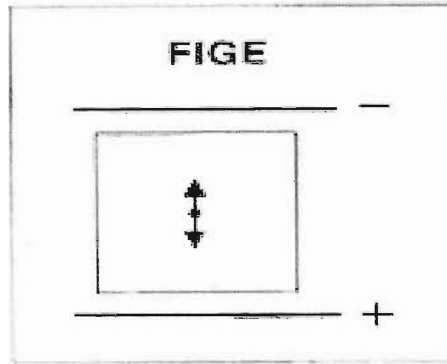

Figure 1.3. Electrode configuration of FIGE. The orientation of positive charge is indicated by the arrow in the middle of the diagram (Carle *et al.*, 1993).

FIGE has the potential for rapid electrophoretic separation, in the range of a few hours, which is somewhat preparative in nature because DNA fragments more than 300 to 400 kb tend to have a thicker, more diffuse, appearance (Carle *et al.*, 1993).

1.4.1.2 Cantour Clamped Homogeneous Gel Electrophoresis

CHEF uses a more complex electrophoresis chamber, with multiple electrodes arranged in a polygonal cantour and clamped to predetermined electric potentials to achieve a highly uniform electrophoretic field, usually reorientating the DNA molecules over 96° to 120° angle (fig. 1.4). The method applies the principles of electrostatics to gel electrophoresis (Chu *et al.,* 1986).

In particular, the electric field vector is confined to two dimensions and has two components, Ex(x, y) and Ey(x, y). To simplify the problem, the electric field may be expressed as the negative gradient of a single function, the scalar potential field $\phi(x, y)$ (Chu *et al.,* 1986).

$$Ex(x, y) = -\partial\phi(x, y)/\partial x \qquad (1a)$$

$$Ey(x, y) = -\partial\phi(x, y)/\partial x \qquad (1b)$$

A homogeneous electric field is generated by two parallel, infinitely long electrodes in such a way that if one electrode is located along the x axis (y = 0) and the other is separated by a fixed distance (y = a), the potential field between the electrodes is,

$$\phi(x, y) = \phi_\theta y/a \qquad (2)$$

where ϕ_θ is the voltage applied across the electrodes (Chu *et al.,* 1986). Substitution of equation 2 into equation 1 shows that the corresponding electric field is homogeneous and oriented perpendicular to the electrode,

$$Ex(x, y) = 0 \qquad (3)$$

$$Ey(x, y) = \phi_\theta \qquad (3b).$$

It is impractical to use infinitely long electrodes, but it is possible to produce a homogeneous electric field with a finite system. A solution is to use multiple electrodes arranged along a polygonal contour, in which two faces of the polygon coincide with the positions of the infinite electrodes (Chu *et al.,* 1991 and 1986). The electrodes along y = 0 and x = a are clamped to the potentials 0 and ϕ_θ, respectively, whereas the remaining electrodes located at intermediate positions are clamped to intermediate potentials, as determined by equation two (Chu *et al.,* 1986).

Thus positions along the cantour are clamped to potentials equal to those generated by two infinite electrodes. It follows that everywhere inside the contour the potential field will be equal to that generated by two infinite electrodes.

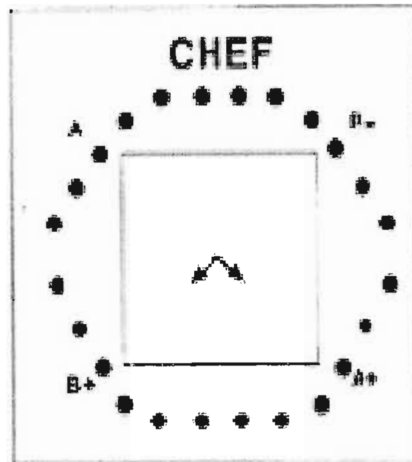

Figure 1.4. Electrode configuration of CHEF. The orientation of positive charge is indicated by the arrows in the middle of the diagram.

In a hexagonal contour, the two opposing sides of the polygon are used to define the electric field orientation. Alternation in the orientation is achieved by electronic switching. The re-orientation of DNA molecules at smaller oblique angles, generally between 96° and 120°, causes DNA molecules to always move forward in a zigzag pattern down the gel (Clark *et al.,* 1990). CHEF separations are slower, in the range of 20 to 26 hours, but appears more analytical, as the practical upper limit of DNA fragment resolution is greater for CHEF than FIGE, 10 mb versus 700 to 800kb, respectively, and larger DNA fragments retain a more compact appearance.

Other methods of PFGE analysis include zero integrated field electrophoresis (ZIFE) (Turmel *et al.*, 1990), transverse alternating field electrophoresis (Gardine *et al.*, 1986) and programmable autonomously controlled electrophoresis (PACE). For more detailed review of PFGE technology, refer to Birren and Lia, 1993.

1.5 DNA PREPARATION AND DIGESTION USING RESTRICTION ENZYME FOR PFGE ANALYSIS

Isolation of intact chromosomal DNA is a prerequisite for the reproducible generation of restriction fragments by rare-cutting enzymes. Chromosome-size DNA is easily sheared during the normal DNA preparation processes (Towner and Cockayne, 1993). Thus, for DNA analysis by PFGE, chromosomal DNA is commonly prepared by *in situ* lysis of cells embedded in agarose (Schwartz and Cantor, 1984). This provides an environment protected from molecular shearing forces that allow the reproducible generation of mega-base sized restriction fragments.

Bacterial culture is grown to an OD_{600} of 0.8 to 1.0 at the appropriate temperature. It is then treated with chloramphenicol that synchronizes ongoing rounds of chromosomal replication while inhibiting further rounds of replication. Agarose plugs are then prepared by mixing the cell suspension with the low melting agarose (held briefly at 50°C) and the mixture transferred into plug molds (Mastushek *et al.*, 1996; Tenover *et al.*, 1995; and Hermans *et al.*, 1995). When set, the plugs containing intact cells are treated with different enzymes: lysozyme; proteinase K, and RNase, and detergents, *N-lauroyl sarcosine* or *Brij*, in order to lyse the cells *in situ* and degrade the cell wall, RNA and proteins.

Table1.2 Restriction endonucleases suitable for use in conjunction with pulsed-field gel electrophoresis

Restriction Endonuclease (Enzyme)	Recognition site sequence
ApaI	GGGCC/C
ClaI	AT/CGAT
NotI	GC/GGCCGC
PvuI	CGAT/CG
SalI	G/TCGAC
SmaI	CCC/GGG
XhoI	C/TCGAG

Portions of DNA-containing agarose plugs can be loaded directly into wells of 1% agarose gel, where they are sealed in place with 1% agarose gel before PFGE. Smith *et al.,* 1988 gives a detailed review of protocols for the preparation of chromosome-sized DNA from different sources.

The choice of an appropriate enzyme (Table 1.2) for typing purposes depends on the frequency of the enzyme's restriction sites in the DNA molecule to be digested. For example, *Sma*I will be rare cuting in an AT-rich organism but a frequent cutter in GC-rich (mycoplasms). Agarose plugs containing free DNA must first be treated with phenylmethylsulphonyl fluoride (PMSF) in order to inhibit any remaining serine proteases (proteinase K) that could degrade the restriction endonuclease (Smith *et al.*, 1998). In turn, the PMSF must be removed by successive washing with detergents and EDTA. Small pieces of agarose plug can then be incubated overnight in microfuge tubes containing digestion buffer and an appropriate restriction endonuclease. The endonuclease diffuses into the agarose and digestion occurs *in situ*.

1.6 APPLICATIONS OF PFGE ANALYSIS FOR TYPING PURPOSES

The epidemiological analysis of nosocomial isolates is most commonly associated with multiple isolations of a given organism, either from the same patient (a question of therapeutic efficacy) or a series of different patients (a question of infection control) (Maslow, 1993). In either case, chromosomal analysis by PFGE provides a fundamental comparison of the organisms in question, thus allowing an assessment of whether a therapeutic or infection control problem exists.

PFGE analysis has been applied to the genetic and/or epidemiological analysis of pathogens or pathogen groups including *Acinetobacter baumanni* (Gouby *et al.*, 1992); *Acinetobacter calcoaceticus* (Allardet-Servent *et al.*, 1989); *Enterobacter cloaca* (Haertl *et al.*, 1993); *Enterococcus faecalis* and *Enterococcus faecium* (Murray *et al.*, 1990; and Georing *et al.*, 1990); *Escherichia coli* (Arbeit *et al.*, 1990); mycobacteria including *Mycobacterium avium*; *Mycobacterium intracellulae* and *Mycobacterium tuberculosis*

(Arbeit *et al.*, 1993; Coffin *et al.*, 1992; Varnerot *et al.*, 1992; and Levy-Frebault *et al.*, 1989); *Staphylococcus aureus* and a variety of coagulase-negative staphylococci (Inglis *et al.*, 1993; Ichiyama *et al.*, 1991; and Georing *et al.*, 1990); group A streptococci, group B streptococci, group C streptococci and group D streptococci (Benson *et al.*, 2002; Benson *et* al., 2001; Bartie *et* al., 2000; Davis *et al.*, 1999; Chatellier *et al.*, 1996; Coffey *et al.*, 1995 and Elliot *et al.*, 1993).

Although PFGE is one of the most reproducible and highly discriminatory typing techniques available and currently the typing method of choice for many species, a few caveats are, however, necessary. Firstly, major difficulties associated with PFGE relate to the technical demands of the procedure and the initial cost of the equipment. Secondly, initial extraction of high molecular weight chromosomal DNA requires two to four days, depending on the organism tested. Thirdly, PFGE is relatively less sensitive than PCR-based typing for detecting small differences between strains (Tenover *et al.,* 1997). Thus identical PFGE patterns obtained with a particular enzyme do not necessarily mean that two strains are identical. Differences are conclusive, but similarities are not.

Finally, it is important to note that PFGE tends to reflect the gross clonal structure of a collection of strains, rather than the fine architecture that may be of more interest to the epidemiologist. Despite the caveats, PFGE is one of the most reproducible techniques.

1.7 TYPING METHODS USING POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) is a technique that has been used for a number of years for direct detection of many types of infectious agents in clinical samples (Persing, 1993). It was further adapted as a typing tool (van Belkum, 1994; Welsh and McClelland, 1990; and Williams *et al.*, 1990). PCR has the ability to produce literally millions of copies of a particular DNA sequence within three to four hours. PCR procedure requires template DNA, or RNA if a reverse transcriptase step is used initially, which may be present in minute quantities; two oligonucleotide primers, single-stranded DNA molecules, which

flank a specific sequence on the template DNA, to be amplified (thus defining the starting point for DNA polymerase activity); deoxynucleotide triphosphates (dNTPs); and a heat-stable DNA polymerase (Mullis, 1985).

Efficient amplification is accomplished readily for templates with less than 2 000 base pairs, although templates as large as 35 kb have been amplified (Tenover *et al.*, 1997). A typical PCR assay requires approximately three hours to complete 30 cycles, where each cycle consists of a heat denaturation step, in which double-stranded DNA molecules are separated into single strands; an annealing step, in which primers bind to the target DNA sequences on the single strand; and an extension step, in which DNA synthesis proceed from the primers along each strand of the template DNA, thereby generating two new double-stranded copies of the original template (Fig. 1.5). After 30 cycles, a single initial copy of template DNA theoretically can be amplified to millions of copies (Black, 1993; and Mullis, 1985). These strands exist at this stage as double-stranded DNA molecules (fig. 1.6).

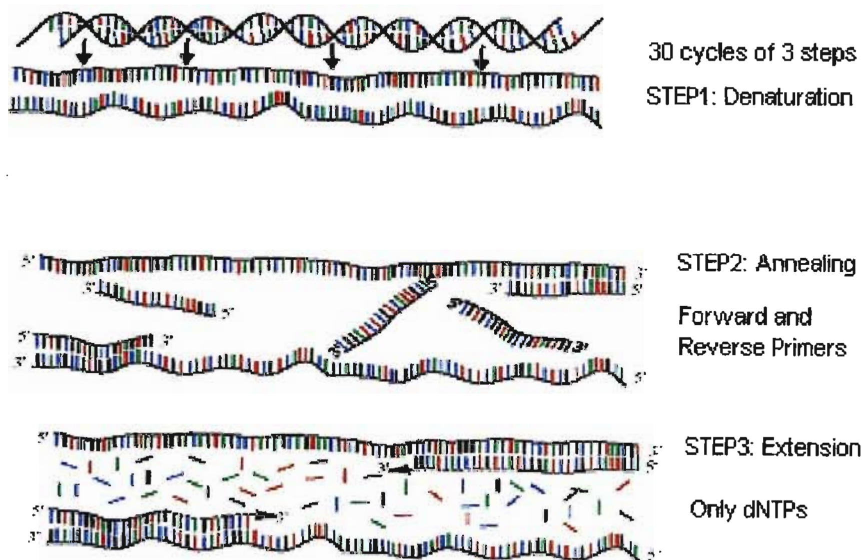

Figure 1.5. Schematic diagram of polymerase chain reaction showing the three major steps involved in PCR.

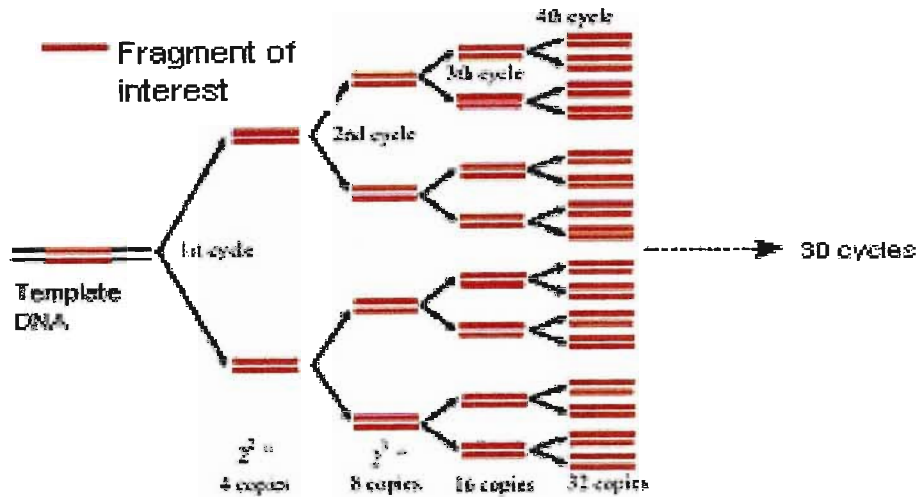

Figure 1.6. Shows the schematic diagram of repeated cycles for strand synthesis by PCR.

The bases complementary to the template are coupled to the primer on the 3' end, the polymerase adds dNTPs from 5' to 3', reading the template from 3' to 5' side. Bases are added that are complementary to the template (fig 1.5).

Strand synthesis can be repeated by heat denaturation of double stranded-DNA, annealing of primers by cooling the mixture and primer extension by DNA polymerase at a temperature suitable for the enzyme reaction. Each repetition of strand synthesis comprises a cycle of amplification. Each new DNA strand synthesized becomes a template for any further cycle of amplification and so the amplified target DNA sequence is selectively amplified, cycle after cycle (fig 1.6).

1.8 RANDOMLY AMPLIFIED DNA POLYMORPHISM (RAPD) ANALYSIS

Randomly amplified DNA polymorphism (RAPD) analysis is a variation of the PCR technique, employing a single short, 10 bases pairs long, primer that is not targeted to amplify any specific DNA sequence (Williams *et al.*, 1991; and Welsh and Maclelland, 1990). Rather, at low annealing temperatures, the primer will hybridize at multiple random chromosomal locations and initiate DNA synthesis. Low melting temperatures

allow the primer to anneal to random regions of the genome, which may be fully complementary to the primer (Black, 1993). In order for amplification to occur, the primer must anneal on complementary strands of the template DNA and the 3' ends of the annealed primers must face each other. Furthermore, the annealing sites must be separated at a distance of no longer than 300 bp, as this is the maximum size that can be amplified with routine PCR (Black, 1993). The requirement that the 3' ends of the annealed primers face each other suggests that annealing sites are exact or similar inverted repeats. The observation that single substitutions, especially in the 3' end of the primer, can change amplified banding patterns (Williams *et al.*, 1991) implies that annealing in RAPD-PCR must be precise.

The products of RAPD-PCR reaction from a species genome are typically a series of fragments that vary in intensity and in size, from 200 to 2000 bp (Black, 1993). Differences in size (polymorphism) occur as the presence or absence of a specific fragment among species. Absence of a fragment presumably occurs because amplification cannot proceed on DNA strands from either of the homologous chromosomes in a species. This can occur through point mutations at one or both primer annealing sites on a DNA strand, inversions surrounding a site or insertions that separate the annealing sites at a greater distance than can be amplified. Polymorphism among species can be analyzed by gel electrophoresis and visualized by staining with ethidium bromide.

The advantages of RAPD-PCR is that it does not require cloning or DNA sequence information for primer design, it does not use radioactive markers, and genetic polymorphisms can be visualized within 24 h from extraction of genomic DNA (van Belkum, 1994). RAPD-PCR is appreciably faster than other typing systems, such as PFGE and RFLP. Often 20 to 30 organisms can be completed in a single day.

RAPD-PCR is, however, not without drawbacks. The number, sizes and intensities of amplified fragments are extremely sensitive to small changes in PCR buffers, the condition and concentration of template DNA and amplification parameters (Martinez *et*

al., 2000; Mahenthiralingam *et al.*, 1996; Render *et al.*, 1996; and van Belkum, 1994). All studies to date have noted that the primer, dNTP and magnesium concentrations affect the numbers and the intensities of bands. In addition, the amount of template DNA and the manner in which it was extracted also affects banding patterns (Black *et al.*, 1992).

While one laboratory can standardize reagent concentrations and settle on optimal template isolation procedures, two laboratories, especially those employing different thermal cyclers, may not be able to obtain similar patterns. This implies that great care should be taken in optimising RAPD-PCR parameters within and among laboratories if they are to share and compare results.

There are a wide variety of other potential problems to consider. Extraction of chromosomal DNA from other organisms (e.g. bacteria) with DNA of the target taxon is inevitable and it is likely that some amplified bands will arise from the contaminating template. If contaminating species occur sporadically throughout the taxon, or if the contaminants are themselves polymorphic, then amplified bands can be mistaken for polymorphisms in the genome of the target taxon (Black, 1993).

Finally, it should be emphasized that, as with all PCR, it is extremely important to process a negative control as a test for contamination. This should be a tube containing all of the components of the reaction except template DNA and handled at the same time and in an identical manner to tubes containing the template (Williams *et al.*, 1991).

1.9 THE INTERPRETIVE CRITERIA

To interpret the DNA fragment/band patterns generated by PFGE or RAPD analysis and translate them into useful epidemiological information, the microbiologist must learn how to compare PFGE patterns or RAPD patterns and how random genetic events can

alter the patterns. Ideally, Both PFGE and RAPD patterns of isolates representing the outbreak strain would be indistinguishable from each other and distinctly different from those of epidemiologically unrelated strains (Tenover *et al.,* 1997).

The results obtained by either PFGE or RAPD analysis are interpretated and translated the same. For the purposes of this study PFGE analysis will be used to discuss the interpretation and translation of RAPD and PFGE analysis.

Table 1.3 Microbiological interpretation of DNA profiles from PFGE analysis

Microbiological Interpretation based on typing results	No. of genetic differences compared with out break strain	Typical no. of fragment differences compared to outbreak pattern	Epidemiological correlation
Indistinguishable	0	0	Isolate is part of the outbreak
Closely related	1	2-3	Isolate probably is part of the outbreak
Possibly related	2	4-6	Isolate possibly is part of the outbreak
Different	3	≥ 7	Isolate is not part of the outbreak

More commonly, random genetic events, including point mutations and insertions and deletions of DNA, alter PFGE patterns during the course of an outbreak. While this makes, interpretation of the patterns a little more challenging. Knowledge of how such genetic events affects the PFGE patterns enables the microbiologist to correctly assign the patterns to each isolate to one of four categories (Tenover *et al.,* 1997): indistinguishable from the outbreak pattern, closely related to the outbreak pattern, possibly related to the outbreak pattern, or unrelated to the outbreak pattern (Table 1.3 and Fig. 1.7).

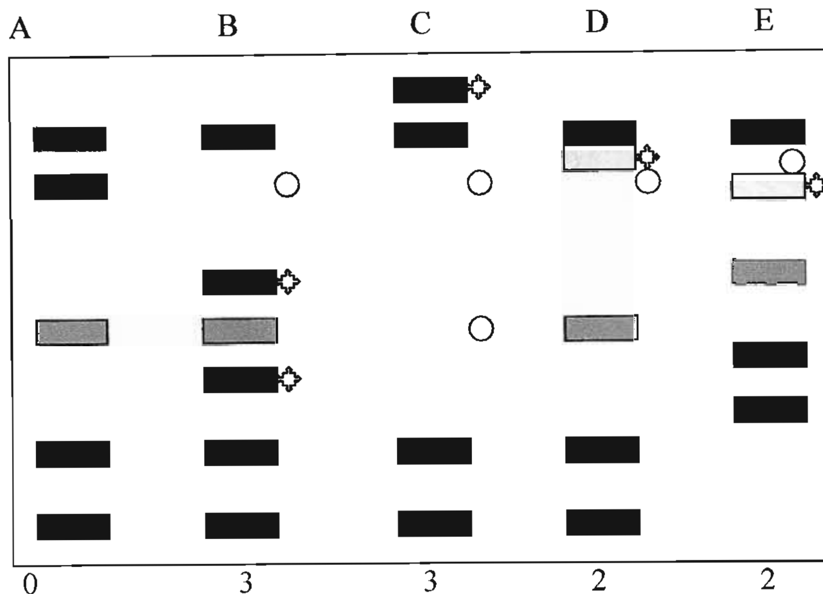

Figure 1.7. Schematic diagram showing the changes in the PFGE patterns of an isolate as a result of various genetic events. Lane A, outbreak pattern; lane B, gain of restriction site; lane C, loss of restriction site; lane D, insertion of DNA in an existing fragment; lane E, deletion of DNA from an existing fragment. The open circles (○) indicate fragments present in the outbreak pattern and missing from the test isolate after a genetic event; asterisks (✥) indicate fragments present after genetic event but absent from the outbreak pattern (Tenover et al., 1997).

The criteria proposed herein are reliable if PFGE resolves at least 10 distinct fragments. When fewer bands are detected, the robustness and discriminatory ability of the criteria are unknown. It is believed that the comparison of restriction patterns remains, in part, a subject process, that cannot totally be reduced to rigid algorithm. However, the process becomes easier and more consistent with experience (Tenover *et al.,* 1997).

CHAPTER TWO

MINIMAL INHIBITORY CONCENTRATIONS (MICS) OF STREPTOCOCCUS AGAINST PENICILLIN

ABSTRACT

A collection of 29 clinical streptococcal isolates obtained from the University of KwaZulu-Natal, Medical School, Durban, and *Escherichia coli* were tested for susceptibility to penicillin G. All isolates with penicillin minimal inhibitory concentration (MIC) less than or equal to 0.12 μg/ml were considered susceptible, intermediate resistant if penicillin G MIC was between 0.25 μg/ml and 4 μg/ml and resistant if greater than 4 μg/ml. The percentage of isolates with resistance was relatively high (73%), only 10% of isolates with intermediate resistance and 16.7% of the isolates were completely susceptible to penicillin G. Some of the resistant isolates were highly resistant, reaching penicillin G MIC levels of 5000 μg/ml and are speculated to contain both altered penicillin binding proteins and a high level of crosslinking cell wall induced by the gene products of the *Mur*MN operon.

2.1 INTRODUCTION

During the three decades since their first detection in clinical specimens in the late 1960s, penicillin-resistant and multi-drug-resistant species or strains of streptococci have achieved a global spread and have become a major public health concern (Filepe and Tomasz, 2002). The molecular mechanism of penicillin resistance in these pathogens was shown to involve remodelling of β-lactam target enzyme: penicillin-binding proteins (PBPs), in such a way that their affinity is greatly reduced towards the antibiotic molecule (Zighelboim and Tomasz, 1980; and Paredes *et al.*, 1979).

The physiological function of PBPs is in the terminal stages of bacterial cell wall peptidoglycan assembly. It was suggested that the reduced affinity of PBPs may affect their catalytic performance (Garcias-Bustos and Tomasz, 1988) with their natural substrates, precursor muropeptides, the D-alanyl-alanine carboxyl terminal, which has close structural analogy to the β-lactam ring (Filipe and Tomasz, 2000 and Fontana *et al.*, 1992). This proposal was based on the intriguing observation that penicillin-resistant clones of streptococci were often found to produce cell wall peptidoglycans of grossly abnormal muropeptide composition (Garcia-Bustos and Tomasz, 1988; and Garcia-Bustos *et al.*, 1988).

A common feature of this structural abnormality is the replacement of linear structural muropeptides, typical of the peptidoglycan of penicillin-susceptible strains, with branched structured muropeptides carrying short alanyl-alanine or seryl-alanine substituents on the epsilon amino group of the lysine residues. Such branched components are rare in the cell wall of penicillin-susceptible strains (Strunden *et al.*, 1997; and Severin *et al.*, 1996). The frequent occurrence of such a distorted cell wall composition among penicillin-resistant strains suggested some association between the mechanism of penicillin-resistance and the chemical abnormality of cell walls.

In 2000, Filipe and Tomasz identified the genetic determinants for MurM and MurN proteins, which are involved with the biosynthesis of branched cell wall peptides. They

reported that the inactivation of *mur*MN operon in penicillin-resistant strains resulted in the correction of cell wall abnormalities and virtually a complete loss of penicillin resistance, indicating that the functioning of these two non-PBP genes is an integral component of the penicillin-resistance mechanism in streptococci.

When characterizing streptococcal infections, it is therefore crucial to determine the antibiotic susceptibilities of the isolates under investigation (Smith *et al.,* 1993). Streptococcal strains are categorized into susceptible, if their minimal inhibitory concentration, MIC, is less than 0.12 μg/ml, intermediate if their MIC is between 0.25 and 2.0 μg/ml and resistant if they have an MIC greater than 4.0 μg/ml (Tracy *et al.*, 2001).

2.2 MATERIALS AND METHODS

2.2.1 Bacterial Species and Media

Streptococcal isolates (Table 2.1) used in this study were obtained from the University of KwaZulu-Natal, Medical School, Durban. The streptococcal isolates were first streaked onto Tryptone Soya Agar (TSA) (Merck) containing 5% sheep blood. Colonies qualifying phenotypically as streptococci were isolated and gram stained, with *Escherichia coli* (XL 1-Blue) used as a negative control. The cultures were incubated at 37°C in 5% CO_2 atmosphere. Working stocks were maintained on TSA plates, subcultured every four weeks and stored at 4°C. Stock cultures were stored at –70°C in Tryptone Soya Broth (TSB) (Merck) containing 30% glycerol.

The organisms tested were originally isolated from clinical specimens. A total of 29 isolates (Table 2.1) and one control were processed. These were identified by routine microbiological methods and their identies were confirmed by the Medical Microbiology Laboratory of the Medical school, Univerity of KwaZulu-Natal.

Table 2.1 Isolates of streptococcus used in this study

Name of Isolate	Lab. Number	Identification
KE 1	P4448	*Streptococcus pyogenes*
KE 2	S2425	*Streptococcus dys. equisimilis*
KE 3	S2429	*Streptococcus pyogenes*
KE 4	B2436	*Streptococcus agalactiae*
KE 5	B2479	*Streptococcus pyogenes*
KE 6	PAB101	*Streptococcus agalactiae*
KE 7	PAB103	*Streptococcus agalactiae*
KE 8	PAB111	*Streptococcus agalactiae*
KE 9	PAB32	*Streptococcus agalactiae*
KE 10	PAB132	*Streptococcus agalactiae*
KE 11	S 2112	*Streptococcus pyogenes*
KE 12	S 2125	*Streptococcus agalactiae*
KE 13	S 2317	*Streptococcus dys. equisimilis*
KE 14	4734	*Streptococcus pyogenes*
KE 15	M 4431	*Streptococcus pyogenes*
KE 16	P 4348	*Enterococcus faecalis*
KE 17	B 2478	*Streptococcus agalactiae*
KE 18	4161	*Streptococcus agalactiae*
KE 19	S 2319	*Streptococcus pyogenes*
KE 20	3999	*Streptococcus agalactiae*
KE 21	4178	*Streptococcus pyogenes*
KE 22	4484	*Streptococcus pyogenes*
KE 23	4121	*Streptococcus constellatus*
KE 24	4480	*Streptococcus pyogenes*
KE 25	4219	*Enterococcus faecalis*
KE 26	2109	*Streptococcus agalactiae*
KE 27	2013	*Streptococcus agalactiae*
KE 28	PAB 36	*Streptococcus agalactiae*
KE 29	4755	*Streptococcus constellatus*
XL 1-Blue		*Escherichia coli*

2.2.2 Penicillin Susceptibility Testing

Micro-titre plate dilution assay. Bacterial cultures were grown at 37°C in 5% CO_2 atmosphere to the exponential phase, optical density of 0.6 at 600nm (OD_{600} of 0.6) in a final volume of 5 ml TSB. Antibiotic susceptibility test was carried out by the micro-broth dilution method (NCCLS, 2001), using cation-adjusted Mueller-Hinton broth containing different concentrations of the antibiotic. The concentration range of penicillin G, obtained by serial dilution, was from 0 μg/ml to 5000 μg/ml. Each micro-titre plate containing varied levels of penicillin was inoculated with 1% culture in a final volume of

200 μl. The wells corresponding to the 11th and the 12th column of the micro-titre plate did not contain antibiotic. The 12th column was inoculated with 2μl of the culture and served as a positive control whereas the 11th column was not inoculated and served as a negative control. This was incubated at 37°C in 5% CO_2 atmosphere for 18h. The concentration in which an isolate could not grow was taken as the MIC of that isolate.

2.3 RESULTS AND DISCUSSION

2.3.1 Antibiotic Susceptibility Testing

As determined by penicillin MIC breakpoints, recently established by the National Committee for Clinical Laboratory Standards (NCCLS) (Kaneko *et al.*, 2000) for application to *Streptococcus* species, it was found that 13.7% (4 isolates) of the isolates obtained from the Durban Medical School can be considered susceptible (MIC, ≤ 0.12 μg/ml) and 10.3% (3 isolates) intermediate (MIC, 0.25 μg/ml < MIC ≤ 2.0 μg/ml) to penicillin G, while 75.9% (22 isolates) of the isolates were resistant to penicillin G (Table 2.2).

In a study in 2000, the rate of intermediate-resistant strains was reported to be between 1.4% and 12.5% (Kaneko *et al.*, 2000). In our study three isolates with intermediate resistance to penicillin G demonstrated MIC levels between 0.25-2.0μg/ml when compared with susceptible isolates and 22 isolates with MIC levels from 4.0-5000μg/ml (Table 2.2 and Fig 2.1).

Other studies (Smith *et al.*, 1993; and Zighelboim and Tomsz, 1980) reported that the molecular mechanism of penicillin resistance in streptococci involve the remodelling of PBP in such a way that their affinity is greatly reduced towards the antibiotic molecule. In 2002, Fillipe and Tomasz described the identification of the genetic factors, *MurM* and *MurN*. These factors are considered different from PBPs and confer branched muropeptides phenotypes of the cell wall.

This finding suggests that the underlining mechanism for resistance to the antibiotic, penicillin G, is due to both altered PBP's and the phenotypic expression of the *murMN* operon. Isolates KE4, KE11, KE25, KE26 and KE27, demonstrated high levels of resistance to penicillin G (Fig. 2.1), which could indicate that these isolates contain both altered PBPs and *MurM* and *MurN* genetic determinants in their genome.

The other resistant isolates (MIC between 4 μg/ml and 200 μg/ml) can be speculated to contain either PBP genes that have been altered or *MurM* and *MurN* genetic determinants. Isolates KE1, KE2, KE3, KE5 and KE29 including XL1-Blue were susceptible to penicillin G.

Table 2.2 *In vitro* activities of penicillin G against streptococcal isolates

Antibiotic	MIC (μg/ml)									Antibiotic relation
	≤ 0.1	0.5	1.0	1.5	2.0	2.5	3.0	3.5	≥ 4.0	
Penicillin G	KE1									Susceptible
Penicillin G		KE2								Intermediate
Penicillin G	KE3									Susceptible
Penicillin G									KE4	Resistant
Penicillin G	KE5									Susceptible
Penicillin G									KE6	Resistant
Penicillin G									KE7	Resistant
Penicillin G									KE8	Resistant
Penicillin G									KE9	Resistant
Penicillin G									KE10	Resistant
Penicillin G									KE11	Resistant
Penicillin G									KE12	Resistant
Penicillin G									KE13	Resistant
Penicillin G									KE14	Resistant
Penicillin G									KE15	Resistant
Penicillin G									KE16	Resistant
Penicillin G									KE17	Resistant
Penicillin G									KE18	Resistant
Penicillin G									KE19	Resistant
Penicillin G									KE20	Resistant
Penicillin G						KE21				Intermediate
Penicillin G									KE22	Resistant
Penicillin G									KE23	Resistant
Penicillin G						KE24				Intermediate
Penicillin G									KE25	Resistant
Penicillin G									KE26	Resistant
Penicillin G									KE27	Resistant
Penicillin G									KE28	Resistant
Penicillin G	KE29									Susceptible
Penicillin G	Xl-1 Blue									Susceptible

Minimal Inhibitory Concentration (MIC; μg/ml)

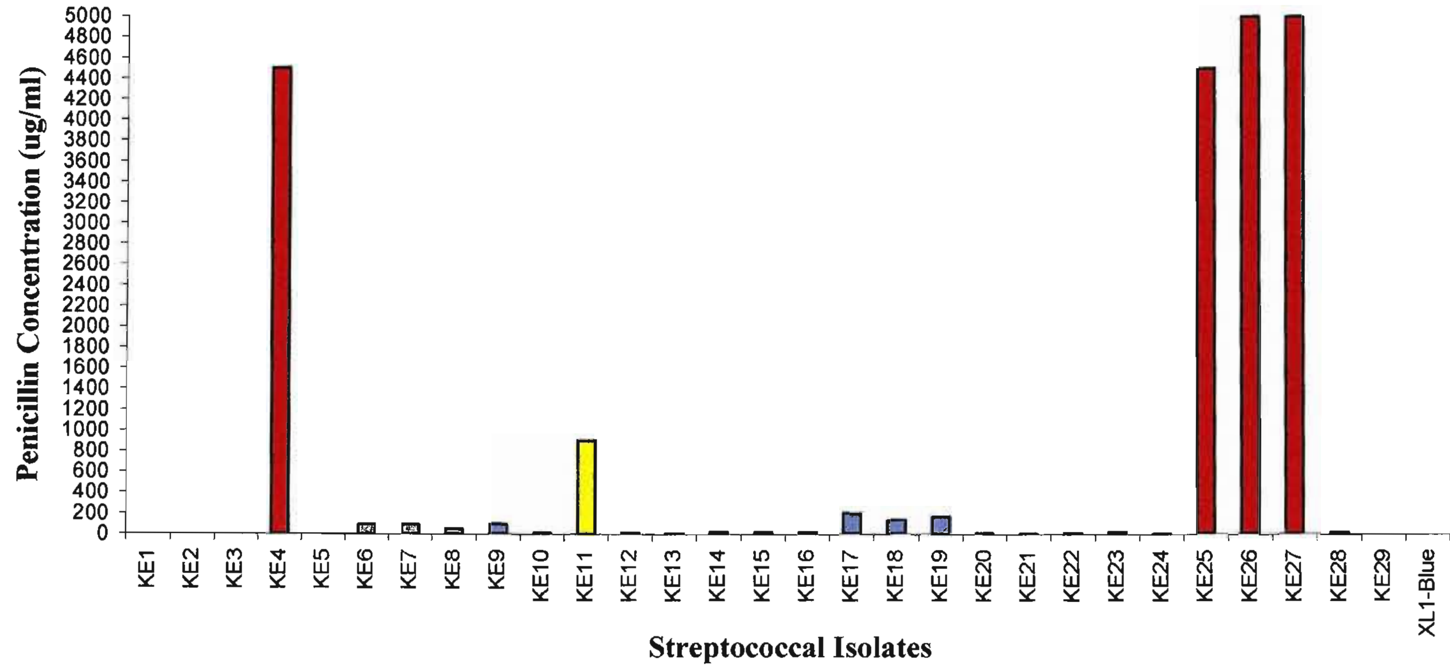

Figure 2.1. Minimal Inhibitory Concentration of Penicillin G against streptococcal isolates

In conclusion, high level of penicillin G resistance was common among the isolates of *S. agalactiae*, this is evident from the fact that all of *S. agalactiae* isolates were resistant to penicillin G (Table 2.1 and Table 2.2). *S. constellatus* isolates were also resistant to penicillin G. From penicillin susceptibility results, it is expected to find most if not all *S. agalactiae* isolates clustered together when analyzed by UPGMA neighbor-joining computer progaramme, software. Although *S. constellatus* isolates showed the same antibioc patterns as *S. agalactiae*, they are not expected to be in one cluster with *S. agalactiaei* because they are different species. *S. pyogenes* isolates were fully represented in the three categories of penicillin G susceptibility (susceptible, intermediate resistant and resistant) (Table 2.2). *S. pyogenes* with the same antibiotic relation are expected to cluster together or show very few differences when analyzed by RAPD and PFGE analysis, chapeter 3 and chapte 4 respectively. Other isolates, *S. dys. equisimilis* and *Enterococcus* faecalis, were also found to represent different antibiotic relations within a species (Table 2.2). Penicillin G MIC levels determined in the present study will help in the analysis of genetic relatedness of the streptococcal isolates established by RAPD (Chapter 3) and PFGE (Chapter 4).

CHAPTER THREE

GENOTYPING OF SELECTED CLINICAL STREPTOCOCCAL ISOLATES BY RANDOMLY AMPLIFIED POLYMORPHIC DNA -POLYMERASE CHAIN REACTION (RAPD-PCR) ANALYSIS

ABSTRACT

The genetic diversity of 29 streptococcal isolates was examined by randomly amplified polymorphic DNA (RAPD) analysis with three primers named MBPZ-1, MBPZ-2 and MBPZ-3. The strains included 29 isolates of human origin and one *Escherichia coli* strain called XL-1 Blue. This investigation allowed for the identification of 27 RAPD types with MBPZ-1 and MBPZ-3 and 26 RAPD types with MBPZ-2. Ninety-eight percent of these isolates were clustered into two groups, group I and group II, with 90% to 100% dissimilarity among them. Fifty-two percent of the isolates of MBPZ-1 group I were in MBPZ-2 group I, 72% isolates of MBPZ-1 group I were in MBPZ-3 group I, and 72% of the isolates of MBPZ-2 group I were in MBPZ-3 group I. This shows the discriminatory ability of the primers used in this study. Despite clustering of isolates, relatively high diversity was seen.

3.1 INTRODUCTION

Although a variety of PCR based methods have been developed for fingerprinting purposes, the RAPD analysis has evolved as the most popular method for DNA fingerprinting of infectious pathogens (van Belkum, 1994; and Caetano-Anolles, 1993). RAPD analysis utilizes random primers of approximately 10 bases long that is not targeted to amplify any specific bacterial DNA sequence (Williams *et al.*, 1990). At low annealing temperatures, the primer will hybridize at multiple random chromosomal locations and initiate DNA amplification (Tenover, *et al.*, 1995; and Lefevre *et al.*, 1993). The resulting RAPD-PCR products represent a number of different-sized DNA amplicons that are resolved by staining with ethidium bromide following agarose gel electrophoresis.

In the first reaction on each strand, a sequence is replicated by *Taq* polymerase, beginning at the site of hybridization and extending beyond the point of the cognate sequence on the opposite strand (Dunne *et al.,* 2001; Maslow *et al.*, 1993). However, in the second reaction, the primer finds the homologous site within the first amplified strand and the replication reaction extends the second strand to the terminus of the sequence, which is the homologous sequence to the opposite strand. This second reaction produces the first amplified sequences equal in length to the targeted amplicon. In the amplification reaction that follows, primers continue to promote exclusively the synthesis of fragments of the amplicon sequence (Tenover *et al.*, 1995).

In the development of a RAPD DNA fingerprinting system for a particular species, a number of oligonucleotide primers must be tested and those that provide the best variability among independent isolates are selected (Pujol *et al.*, 1997). Although a single primer can generate a relatively complex pattern that varies among isolates, in most cases a single primer provides one to three intense bands that may differ among isolates (Tenover *et al.*, 1995). Therefore one must select more than one primer, run each independently for each test isolate and combine the information (Pujol *et al.*, 1997).

The RAPD method of DNA fingerprinting has become popular for most infectious bacteria and has been successfully applied to *Streptococcus agalactiae* (Martinez *et al.*, 2000; Chatellier *et al.*, 1997; and Williams *et al.*, 1990), *Streptococcus suis* (Chatellier *et al.*, 1998), *Streptococcus pneumoniae* (Gerardo *et al.*, 1999; Hermans *et al.*, 1995; and van Belkum, 1994), *Streptococcus dysgalactiae* (Oliver *et al.*, 1998) and *Enterococcus faecalis* (Barbier *et al.*, 1996).

Several disadvantages of the procedure must, however, be kept in mind. First, there is a problem of reproducibility not only among laboratories, but also within a laboratory over time. This single problem, although not insurmountable, makes the development of a common database difficult (Tenover *et al.*, 1997).

Virtually every methodological aspect of PCR can affect reproducibility. Artificial variation can occur as a result of small differences in the primer-to-template concentration ratio, the temperatures during the amplification reaction and the concentration of magnesium in the reaction mixture (Ellsworth *et al.*, 1993). Changes in these parameters affect notably the presence of low-intensity bands but can also affect the position and intensity of high-intensity bands.

3.2 MATERIALS AND METHODS

3.2.1 Chromosomal DNA Purification

Chromosomal DNA was isolated from all bacteria listed in Table 2.1. The DNA manipulations or purification were performed generally as described by the DNaesy Tissue Handbook (Qiagen), with minor modifications. The chromosomal DNA was extracted by harvesting a maximum of 2 x 10^9 bacterial cells (OD_{600} value of 0. 5 to 1.0) in a 1.5 ml microcentrifuge tube by centrifugation at 7500 rpm (5000 x g) and this pellet was then resuspended in 180 μl of lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1.2% Triton® X- 100) containing 1 mg/ml of lysozyme; 1 mg/ml of mutanolysin; and 0.05 μg/ml of RNase A (Roche Diagnostics). The lysis was completed after 30 minutes of incubation at 37°C. Deproteinization of the extract was done by the addition of 10

mg/ml of proteinase K and 200 μl of buffer AL (Qiagen), and incubated at 70°C for thirty minutes. The DNA was precipitated with absolute ethanol (96 – 100%) and washed through a DNaesy spin column by centrifugation using washing buffer AW1 (Qiagen) and then buffer AW2 (Qiagen). After washing with AW2 (Qiagen) the DNA was eluted twice into two separate sterile microcentrifuge tubes, using 50 μl of elution buffer, AE (Qiagen), by centrifugation at 8000 rpm for 1 min. Ten μl of the eluted DNA was used for analysis (see section 3.2.2) and the rest was stored at –20°C for further use.

3.2.2 Analysis of DNA Extracts by Agarose Gel Electrophoresis

DNA extracts were analysed by agarose gel electrophoresis on a 0.8% agarose gel, prepared in 1X TAE buffer (4.846g of Tris, 0.41 g of anhydrous sodium acetate, and 0.372 g EDTA, pH 7.8) and stained with ethidium bromide (0.5μg/ml). Molecular weight marker (MWM) III (Roche) was used as the standard markers. Electrophoresis parameters were: (i) maximum current; (ii) 72 volts; and (iii) 1 hour and 30 minutes running time. The results were visualized by ultraviolet (UV) transilluninator following staining with ethidium bromide and then photographed by a gel documenting system (UVP's GDS 5000).

3.2.3 Quantification of DNA

The quantity of extracted DNA was measured using a GenQuant spectrophotometer (E-52) (Pharmacia). DNA samples were first diluted 100 times (5 μl of DNA sample to 495 μl of AE buffer (Qiagen) in AE buffer (Qiagen). AE buffer (Qiagen)) was also used as a blank. The absorbance readings were taken at wavelengths of 260nm and 280nm. The readings at 260 nm allows for the calculation of nucleic acids concentration. An absorbance of 1 corresponds to approximately 50 μg/ml of double stranded DNA (Brown, 1993). The ratio between the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of the nucleic acids.

3.2.4 RAPD-PCR Optimization

RAPD-PCR analysis was performed as described by Martinez *et al.*, 2000, with modifications. Optimization was done by magnesium titration where all other reagents

(Table 3.1) were kept constant. Initially four primers (Table 3.2) were used to determine if they would all give the most discriminative results.

Table 3.1 Parameters used during $MgCl_2$ profiling for optimization of RAPD-PCR assay

Reagent	Initial Concentration	Required Concentration	Volume (μl) to take out of reagent	X 6
SdH_2O			A	A x 6
10X reaction buffer without $MgCl_2$	10X	1X	2.5	15.0
$MgCl_2$	25mM	B	C	C x 6
dNTP	10mM	0.2mM	0.5	3.0
Primer	100μM	2μM	0.5	3.0
DNA Template	10μg/ml	100ng	10	
Taq (Hot Start)	5U/μl	1U	0.2	

A - volume of sterile distilled water to add to the reaction tube
B - Concentration of $MgCl_2$ required
C – The volume of stock $MgCl_2$ to add to the reaction tube

The concentration of $MgCl_2$ used ranged from 1.0 mM to 3.5 mM. Each concentration was tested with five species (KE1, KE2, KE3, KE4, and KE5). Amplification was performed in a 25 μl reaction mixture (Table 3.1) with a single primer being used in each reaction. Amplification was done using a GeneAmp® PCR Systems model 9700 thermocycler.

Each sample was subjected to the first cycle of amplification of four minutes of denaturation at 94°C, one minute of annealing at 36°C and two minutes of extension at 72°C. Each of the 44 subsequent cycles consisted of the denaturaion at 94°C for one

minute, annealing at 36°C for one minute and extension at 72°C for two minutes and the last cycle consisted of an extension at 72°C for ten minutes.

3.2.5 Analysis of RAPD-PCR Product by Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed as previously described (section 3.2.2), with minor modifications. A 1.5% agarose gel was prepared in 0.5 X TBE buffer (5.4 g Tris, 2.5 g boric acid, 0.45 g EDTA).

The electrophoresis parameters were: 100 V and 50 mA constant current for 6.5 hours. Molecular weight marker III (Roche) was used as molecular standards, negative control consisting of the same reaction mixture, but with sterile distilled water, instead of template DNA and a positive control containing the same reaction mixture with template DNA, from *E. coli* (XL-1 Blue) was also included for analysis.

3.2.6. Computer Analysis of Banding Pattern Data

Data obtained by RAPD analysis contains discrete bands that in most cases can be automatically identified and digitized by computer-assisted systems (Abbott *et al.* 1995; Avise, 1994; and Rohlf, 1963). The object of pattern comparison is to obtain a measure of commonness or difference between the gel patterns of two isolates. Many different measures of genetic distance, or similarity coefficients, exist. For the purposes of the present study, Euclidean formula for genetic distance measure, which takes into consideration the presence and the absence of a band, was used to analyze patterns produced by RAPD analysis (Abbott *et al.* 1985).

The data for banding patterns was synopsised by binary values 0 and 1, where zero indicates no band at a position and 1 indicate a band at that position (Avise, 1994; Clifford and Stephenson, 1975; Sneath and Sokal, 1973; and Sneath, 1957). Euclidean distance measure discard negative matches between pairs of isolates and provides a more accurate picture of relatedness (Abbott *et al.* 1995). Euclidean distance measure is described by, $Di_{AB} = \sqrt{(a_A - a_B)^2 + (b_A - b_B)^2}$, where Di_{AB} is the shortest distance between species A and B, a_A is a band that exists in species A, a_B is a band that exists in species B,

b_A is a band that exists in species A and b_B is a band that exist in species B. The phylogenetic trees were constructed using unweighted-pair group arithmetic mean (UPGMA) (Saitou, and Nei, 1987; and Sneath, 1957).

3.3 RESULTS AND DISCUSSION

3.3.1 Identification of Informative Primers

To identify primers that generate informative and discriminatory arrays of RAPD products, four different 10-mer primers (Table 3.2) were chosen at random to obtain RAPD profiles from five isolates of streptococci that were selected from the entire panel of isolates. These four oligonucleotides were selected according to the following criteria: (i) 10 nucleotides in length, (ii) between 40 and 77% G + C in composition, (iii) containing no palindromic sequence. Performance data for each primer in PCR with the five DNA samples are presented in Table 3.2. Three out of the four primers exhibited different degrees of discrimination among the isolates and therefore they were selected for subsequent studies with the full panel of thirty isolates. Three primers named MBPZ-1 (5'-AGGGGGTTCC-3'), MBPZ-2 (5'- AACGCGCAAC-3'), and MBPZ-3 (5'-GCATACAATC-3') were selected, as they showed polymorphism among individual isolates and gave reproducible patterns comprising fragments with a large size range and a small number of low intensity bands (Fig. 3.1).

Table 3.2 Short random oligonucleotide primers tested by RAPD analysis of streptococci

Name	Sequence (5'→3')	%GC	Fragments Size Range (kb)	Fragments No.	No. of Patterns
MBPZ-1	AGGGGGTTCC	70	0.6 – 3	6 – 12	27
MBPZ-2	AACGCGCAAC	60	0.6 – 4	3 – 14	27
MBPZ-3	GCATACAATC	40	0.5 – 1.5	4 – 16	28
MBPZ-4	AGTCGGGTGG	70	0.1 – 4.5	3 - 11	8

The reproducibility of the RAPD patterns obtained with these primers were verified by repeating the experiments under the same conditions, using DNA preparation prepared at different times from the five isolates tested (Fig. 3.1).

The most informative and reproducible fingerprint patterns were obtained with 3.5 mM $MgCl_2$. More $MgCl_2$ resulted in a higher background, without additional prominent bands. The number of amplified DNA fragments observed by agarose gel electrophoresis ranged from five to seventeen bands (Fig. 3.1).

Primer MBPZ-4 with GC contents of 70% resulted in less than six different pattern of bands, or no amplification product at all. This primer also gave a large number of low intensity bands. The intensities of the minor fragments varied in repeated tests (Table 3.1 and figure 3.2D), so this primer was not tested further with any of the other species.

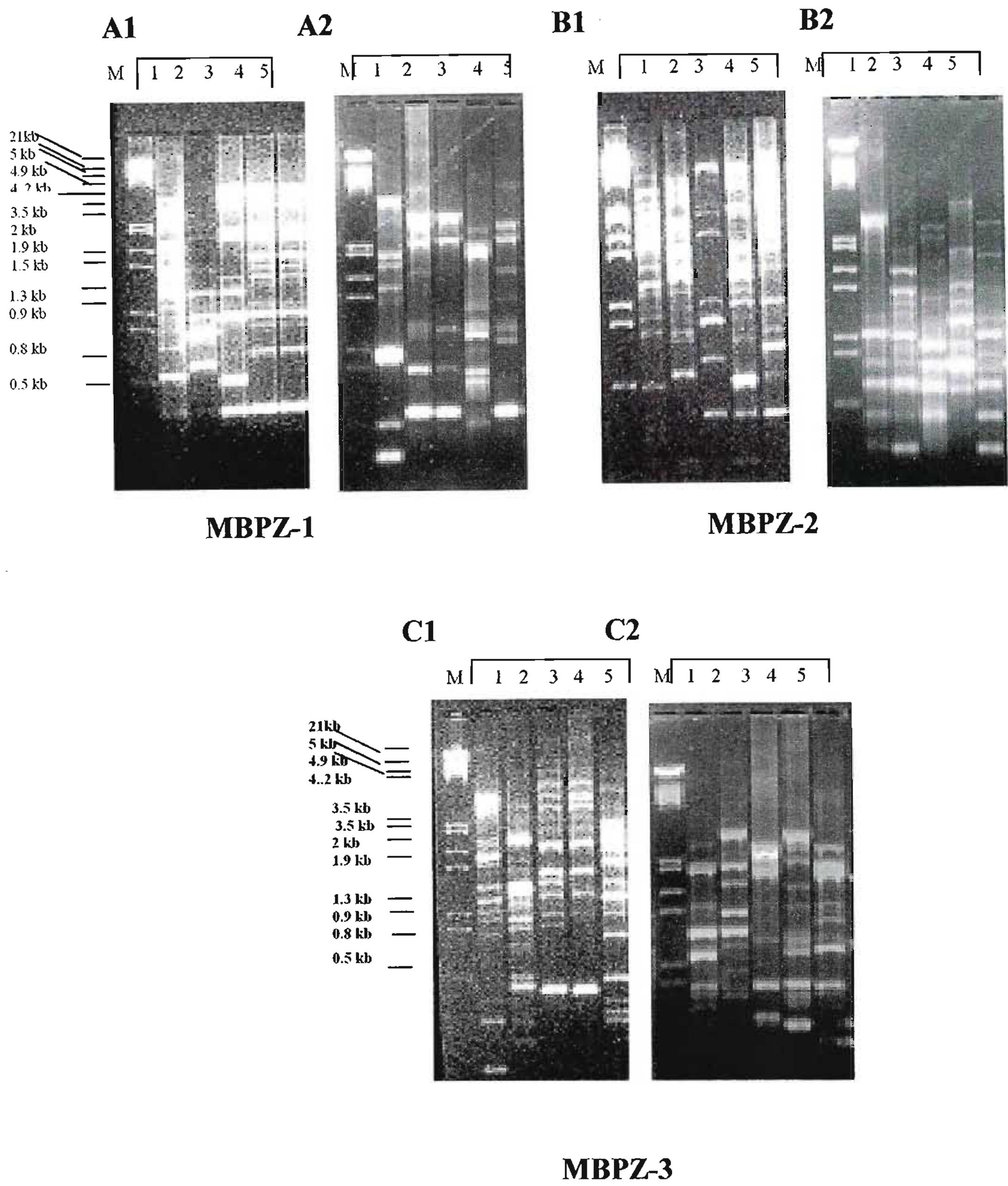

Figure 3.1. Reproducibility of the RAPD patterns obtained with three primers. A1 and A2 represent RAPD patterns obtained with MBPZ-1 using the same DNA but prepared at different times. B1 and B2 represent RAPD patterns obtained with MBPZ-2 using the same DNA but prepared at different times. C1 and C2 represent RAPD patterns obtained with MBPZ-3 using the same DNA but prepared at different times. Lane: M, Molecular Weight Marker III (Roche); 1, KE1; 2, KE2; 3, KE3, 4, KE4; 5, KE5.

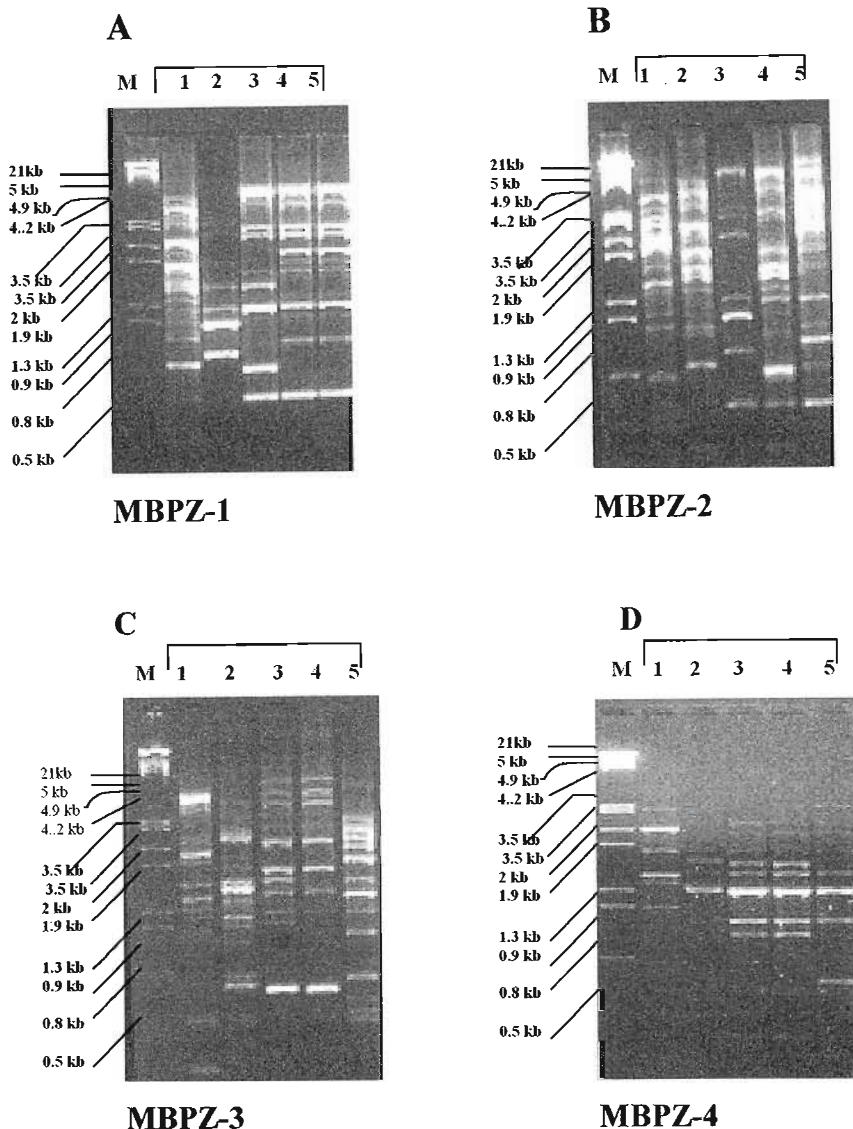

Figure 3.2. RAPD patterns generated with primers MBPZ-1 (A1 to A5), MBPZ-2 (B1 to B5), MBPZ-3 (C1 to C5), and MBPZ-4 (D1 to D5), which includes only isolates of streptococci. Lane: M, Molecular Weight Marker III (Roche); 1, KE1; 2, KE2; 3, KE3, 4, KE4; 5, KE5.

The remaining three primers (MBPZ-1, MBPZ-2, and MBPZ-3) with G + C contents of 40 to 70 % were selected, as they gave more or less reproducible patterns comprising fragments with large size range and a small number of low intensity bands (Fig. 3.1 A to C; and Fig. 3.2 A to C). Although the selected primers did not give 100% reproducibility of RAPD patterns of the five isolates selected from the panel, they gave good

differentiation of unrelated strains/species. The reproducibility of the RAPD patterns obtained with these three primers were verified by repeating experiments under the same conditions. Each isolate was tested at least twice.

3.3.2 Genetic Diversity of Isolates as Defined by RAPD Fingerprinting.

For the whole panel of 29 isolates, 27 RAPD patterns, each composed of four to fourteen bands with sizes between 0.2 and 4.0 kb, were obtained with primer MBPZ-1 (Fig. 3.3); 27 RAPD patterns, each characterized by three to ten bands in a 0.1 to 3.5 kb size range resulted with MBPZ-2 (Fig. 3.5) and 26 RAPD patterns, each with six to eighteen bands in a 0.1 to 5.0 kb size range, were observed with MBPZ-3 (Fig. 3.7).

Genetic relationships, generated by UPGMA–neighbour joining computer software (section 3.2.6), among the 27 RAPD patterns obtained with MBPZ-1 are represented in the dendogram shown in Fig. 3.4. A total of 98% of the isolates was clustered by MBPZ-1 into two groups (I and II), with 75% to 80% dissimilarity among them. Two clusters, A (17 isolates) and B (8 isolates), 69% to 76% dissimilarity, were identified within group I. Only one cluster (8 isolates), with 54 to 85% dissimilarity, was identified within group II.

All thirteen isolates of *S. agalactiae* were grouped together in group I. Twelve of the thirteen *S.agalactiae* isolates were clustered in cluster A of group I and the other isolate, KE 12 was in cluster B of group I. Although *S. agalactiae* isolates exhibit different percentages of similarity among themselves, it is evedent from the phylogenetic tree that they are isolates of the same species since they grouped together in one group (fig. 3.4 group I, cluster A). Four *S. agalactiae* isolates (KE 9 and KE 10, and KE 6 and KE 7) of group I, cluster A, showed 0% dissimilarity, i.e. 100% homology. This suggest that RAPD analysis using MBPZ-1 exhibits KE 9 and KE 10, representing the same strain of *S. agalactiae,* and KE 6 and KE 7 representing another strain of *S.agalactiae*. Table 3.5 shows 0% dissimilarity between KE 26 and KE 27. However, this 100% homology is not shown on a dendogram, but they appear as the closest relatives (Fig. 3.3). These results also suggested that primer MBPZ-1 is a good primer to discriminate for *S. agalactiae*.

Three isolates (KE 3, KE 5 and KE 19) of *S. pyogens* and two *S. sys. equisimilis* isolates (KE 2 and KE 13) were also found in cluster A of group I (Fig. 3.4). Even though the three isolates of *S. pyogens* and two isolate of *S. dys. equisimilis* were clustered with *S . agalactiae* in one group, primer MBPZ-1 managed to exhibit the two *S. sys. equisimilis* isolates as related (50% similar) isolates as well as the three *S. pyogenes* isolates, as related (60% similar) isolates (Fig. 3.4 group I, cluster A). Cluster B of group I contained only *S. pyogenes* isolates (KE 1, KE 11, KE 14, KE 15, KE 21, KE 22 and KE 24) except one *S. agalactiae* isolate (KE 12). Only five isolates were grouped into group II, two isolates of this group were *S. contellatus* (KE 23 and KE 29), two of them were *Enterococcus feacalis* (KE 16 and KE 25) and the last one was *E.coli*. KE 29 and *E. coli* of group II showed 100% homology but *E. coli* was expected not to cluster with any other isolate because it is a different genus.

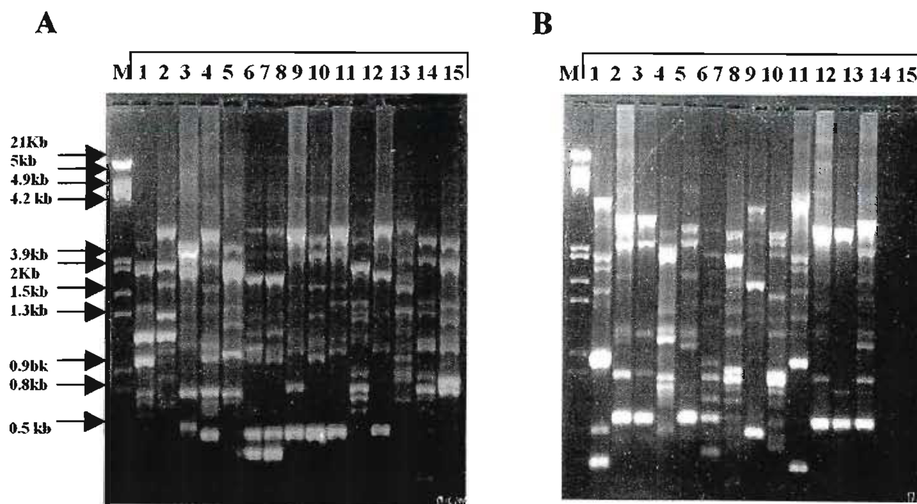

Figure 3.3. RAPD patterns of chromosomal DNA obtained from MBPZ-1 primer and resolved on a 1.5% agarose gel in 0.5 X TBE buffer. **A:** Lane M: MWMII; lane 1 to lane 15: KE 1; KE 2; KE 3; KE 4; KE 5; KE 6; KE 7; KE 8; KE 9; KE 10; KE 11; KE 12; KE 13; KE 14; KE 15. **B:** LaneM: MWMII; lane 1: KE 16; lane 2: KE 17; lane 3: KE 18; lane 4: KE 19; lane 5: KE 20; lane 6: KE 21; lane 7 to 15: KE 22; KE 23; KE 24; KE 25; KE 26; KE 27; KE 28; KE 29; *E. coli* (XL 1-Blue).

Table 3.3. Scores of the bands generated with primer MBPZ-1

Strain	Band Letter																				
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
KE1	0	0	0	1	1	0	0	1	0	0	1	1	0	1	0	1	1	0	1	0	1	1
KE2	0	0	0	0	0	1	1	1	1	0	1	0	0	1	1	1	0	0	1	0	1	1
KE3	0	0	0	1	0	1	0	1	0	0	1	1	0	1	0	1	1	0	1	0	1	1
KE4	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1	1	1
KE5	0	0	1	1	0	0	1	1	0	1	1	0	0	1	0	1	1	0	0	0	1	1
KE6	0	0	0	1	0	1	0	1	0	0	0	0	1	1	1	1	1	0	0	1	1	1
KE7	0	0	0	1	0	1	0	1	0	0	0	0	1	1	1	1	1	0	0	1	1	1
KE8	0	0	0	1	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	1	0	1
KE9	0	0	0	1	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	1	1	1
KE10	0	0	0	1	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	1	1	1
KE11	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	1	0	0	1	1
KE12	0	0	0	1	0	1	1	1	0	0	0	0	0	1	1	1	1	0	0	1	0	1
KE13	0	0	0	0	0	0	1	1	0	1	1	0	0	1	1	1	0	0	1	1	0	1
KE14	0	0	0	1	1	0	1	1	0	0	0	1	1	0	1	1	0	1	1	1	1	1
KE15	0	0	0	1	1	1	1	0	1	0	0	1	1	1	1	1	0	1	0	1	0	1
KE16	0	1	0	1	1	0	0	0	0	0	0	1	1	1	1	1	0	0	1	1	0	0
KE17	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1	0
KE18	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1	0
KE19	0	0	0	1	0	1	0	1	0	1	0	0	1	1	1	0	0	0	0	0	1	1
KE20	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1	0
KE21	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0	1	0	0	1	0
KE22	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1	0	1	1	0	0	1	1
KE23	0	0	0	1	0	0	0	0	0	1	1	0	1	1	0	0	0	1	0	0	1	0
KE24	0	0	0	1	0	0	0	1	0	0	0	1	1	1	1	0	1	1	0	1	0	1
KE25	0	0	0	1	0	1	1	1	0	0	0	0	1	1	0	1	1	1	0	1	1	1
KE26	1	0	1	1	1	1	1	1	0	0	0	0	0	1	0	0	1	0	0	1	1	1
KE27	1	0	1	1	1	1	1	1	0	0	0	0	0	1	0	0	1	0	0	1	1	1
KE28	1	0	1	1	1	0	1	1	0	0	0	0	0	1	0	0	1	0	0	1	1	1
KE29	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0
E. coli	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1	0

Table 3.4. Distance matrix of streptococcal isolates generated with primer MBPZ-1

	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	
E1	0																														KE1
E2	5	0																													KE2
E3	5	8	0																												KE3
E4	9	6	6	0																											KE4
E5	3	6	6	8	0																										KE5
E6	9	8	6	4	6	0																									KE6
E7	10	9	7	5	7	1	0																								KE7
E8	8	7	9	5	7	3	2	0																							KE8
E9	9	8	10	8	6	4	3	3	0																						KE9
E10	8	7	9	7	9	7	6	4	5	0																					KE1
E11	3	8	6	10	6	10	11	11	10	11	0																				KE1
E12	8	7	9	7	9	5	4	2	5	2	11	0																			KE1
E13	5	4	6	6	6	6	7	7	10	9	6	7	0																		KE1
E14	3	6	4	8	6	8	9	9	12	11	4	9	2	0																	KE1
E15	3	6	4	8	6	8	9	9	12	11	4	9	2	0	0																KE1
E16	7	8	12	8	6	6	5	3	2	5	8	5	8	10	10	0															KE1
E17	8	7	9	5	7	3	2	0	3	4	11	2	7	9	9	3	0														KE1
E18	4	7	9	9	7	7	6	4	5	4	7	4	7	7	7	3	4	0													KE1
E19	2	3	7	7	3	7	8	6	7	6	5	6	3	5	5	5	6	4	0												KE1
E20	7	6	10	8	8	6	5	3	2	3	8	3	8	10	10	2	3	3	5	0											KE2
E21	1	6	6	8	4	8	9	7	8	7	4	7	6	4	4	6	7	3	3	6	0										KE2
E22	5	6	6	8	6	6	7	9	8	9	6	9	4	4	4	8	9	5	5	8	4	0									KE2
E23	9	8	8	6	10	8	7	7	8	7	8	7	4	6	6	6	7	7	7	6	8	6	0								KE2
E24	5	8	4	6	8	6	7	7	10	9	4	7	6	4	4	10	7	7	7	8	4	6	8	0							KE2
E25	9	6	10	8	10	8	7	7	8	5	8	5	6	8	8	8	7	7	7	6	8	6	6	6	0						KE2
E26	9	8	8	4	8	6	5	3	6	3	12	3	8	10	10	6	3	7	7	6	8	10	6	8	6	0					KE2
E27	9	8	8	4	8	6	5	3	6	3	12	3	8	10	10	6	3	7	7	6	8	10	6	8	6	0	0				KE2
E28	7	6	6	4	6	4	3	3	6	7	8	5	4	6	6	6	3	7	5	6	8	8	6	6	6	4	4	0			KE2
E29	5	8	6	8	8	6	5	5	8	7	6	5	4	4	4	6	5	3	5	6	6	6	6	6	8	8	8	4	0		KE2
.coli	6	9	11	11	9	11	10	8	7	8	7	8	9	9	9	5	8	4	6	5	5	7	7	9	9	9	9	9	7	0	*E.cc*
	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	

Table 3.5. Euclidean distance between RAPD band patterns for streptococci generated with primer MBPZ-1

	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	
KE1	0																														KE1
KE2	53	0																													KE2
KE3	17	43	0																												KE3
KE4	50	60	38	0																											KE4
KE5	43	53	43	50	0																										KE5
KE6	53	53	43	38	53	0																									KE6
KE7	53	53	43	38	53	0	0																								KE7
KE8	64	64	54	36	64	27	27	0																							KE8
KE9	57	57	46	27	57	18	18	11	0																						KE9
KE10	57	57	46	27	57	18	18	11	0	0																					KE1
KE11	62	50	62	58	62	62	62	75	67	67	0																				KE1
KE12	60	50	50	46	50	25	25	20	27	27	69	0																			KE1
KE13	60	38	60	67	50	60	60	62	64	64	58	46	0																		KE1
KE14	50	59	59	65	67	50	50	69	63	63	57	56	56	0																	KE1
KE15	67	59	67	65	74	50	50	60	63	63	75	47	65	38	0																KE1
KE16	60	76	69	82	83	60	60	71	73	73	87	67	67	47	47	0															KE1
KE17	54	73	64	71	73	54	54	77	69	69	85	71	71	50	60	36	0														KE1
KE18	54	73	64	71	73	54	54	77	69	69	85	71	71	50	60	36	0	0													KE1
KE19	67	57	57	54	57	33	33	45	36	36	67	54	64	63	63	73	69	69	0												KE1
KE20	75	75	75	83	75	64	64	91	82	82	78	83	83	79	79	73	50	50	70	0											KE2
KE21	73	64	73	71	64	64	64	86	79	79	50	80	62	69	69	71	67	67	58	50	0										KE2
KE22	43	63	53	38	43	53	53	54	46	46	50	60	69	59	67	76	73	73	57	85	64	0									KE2
KE23	71	80	71	58	62	71	71	85	77	77	73	87	79	75	75	79	64	64	55	63	33	62	0								KE2
KE24	60	76	60	46	69	38	38	36	42	42	69	46	67	47	47	57	71	71	54	83	71	50	69	0							KE2
KE25	56	56	47	31	47	23	23	46	38	38	64	31	63	44	44	71	57	57	50	67	67	56	64	43	0						KE2
KE26	56	65	56	43	47	47	47	46	38	38	81	43	71	61	61	78	67	67	60	86	89	47	81	63	40	0					KE2
KE27	56	65	56	43	47	47	47	46	38	38	81	43	71	61	61	78	67	67	60	86	89	47	81	63	40	0	0				KE2
KE28	53	71	63	50	43	53	53	54	46	46	80	50	69	59	67	76	64	64	67	85	88	43	80	60	47	8	8	0			KE2
KE29	83	83	83	92	92	83	83	90	91	91	100	92	82	86	86	70	63	63	80	60	78	92	75	82	85	93	93	92	0		KE2
E.coli	75	75	75	83	75	64	64	91	82	82	78	83	83	79	79	73	50	50	70	0	50	85	63	83	67	86	86	85	60	0	*E.cc*
	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	

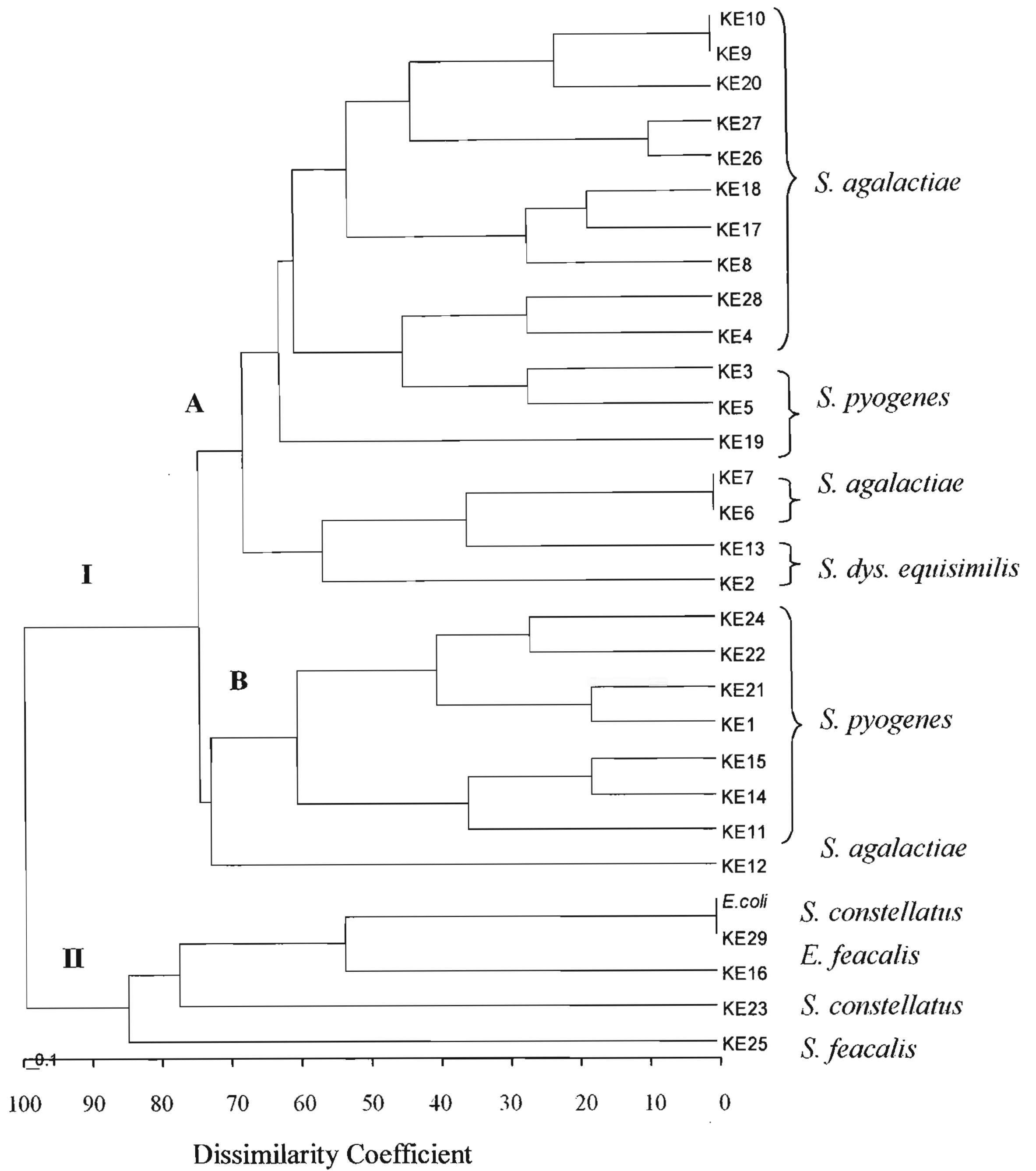

Figure 3.4. Genetic relationships among 29 streptococcal isolates and one *Escherichia coli* strain, as estimated by cluster analysis of RAPD patterns obtained with primer MBPZ-1. The dendogram was generated by the unweighted pair group method with arithmetic means.

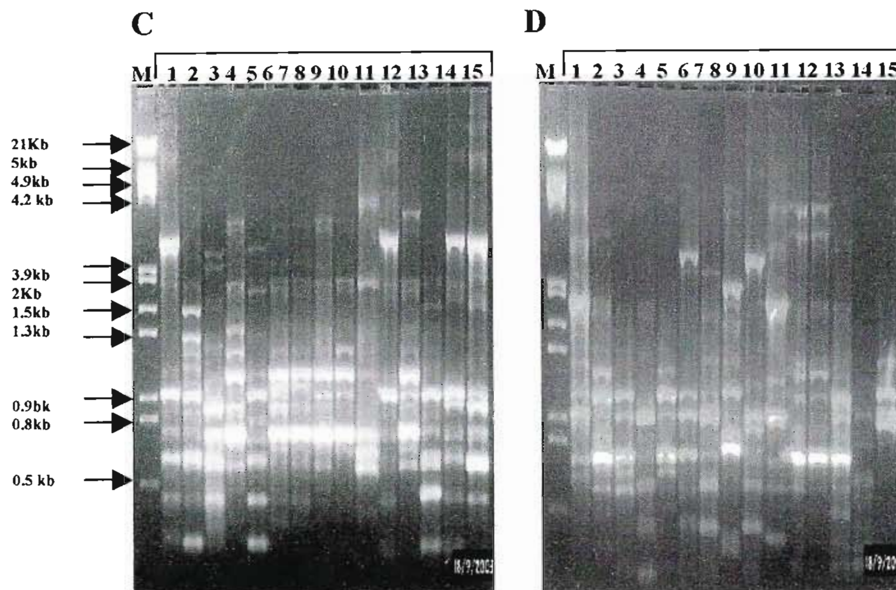

Figure 3.5. RAPD patterns of chromosomal DNA fragments obtained with MBPZ-2 primer and resolved on a 1.5% agarose gel in 0.5 X TBE buffer. **C**: Lane M: MWMII; lane 1 to 15: KE 1; KE 2; KE 3; KE 4; KE 5; KE 6; KE 7; KE 8; KE 9; KE 10; KE 11; KE 12; KE 13; KE 14; KE 15. **D**: Lane M: MWMII; lane1 to 15: KE 16; KE 17; KE 18; KE 19; KE 20; KE 21; KE 22; KE 23; KE 24; KE 25; KE 26; KE 27; KE 28; KE 29; E. coli (XL 1-Blue).

Table 3.6. Scores of the bands generated with primer MBPZ-2

Strain	Band Letter																				
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**
KE1	0	0	1	0	0	0	0	0	0	1	1	0	1	0	1	1	0	1	0	0	0	0
KE2	0	0	0	0	1	1	1	0	1	1	1	0	1	0	1	1	0	1	0	0	0	0
KE3	0	0	1	1	0	0	0	0	1	1	0	1	1	0	1	1	1	1	0	0	0	0
KE4	0	1	0	0	1	0	1	0	1	1	0	1	1	0	1	1	1	0	0	0	0	0
KE5	0	1	1	1	1	0	0	0	0	1	1	0	1	0	1	1	0	1	0	0	0	0
KE6	0	1	0	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0
KE7	0	1	0	1	1	0	0	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0
KE8	0	1	0	0	1	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0
KE9	0	1	0	1	1	0	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0
KE10	1	0	0	0	1	0	0	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0
KE11	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	1	1	1	0	0	0	0
KE12	1	0	0	0	1	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0
KE13	0	0	0	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0
KE14	0	0	1	0	0	1	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0
KE15	0	0	1	0	0	1	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0
KE16	0	1	0	0	1	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0
KE17	0	1	0	0	1	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0
KE18	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	0	0	0	0	0
KE19	0	0	0	0	1	0	0	0	0	1	1	0	1	0	1	1	0	1	0	0	0	0
KE20	0	0	0	0	1	0	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0
KE21	0	0	1	0	0	0	0	0	0	1	1	0	1	0	1	1	0	0	0	0	0	0
KE22	0	0	0	1	0	1	0	0	0	1	1	0	1	0	1	1	1	0	0	0	0	0
KE23	0	0	0	0	1	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0
KE24	0	0	1	0	0	0	0	0	1	0	1	1	1	0	1	1	1	0	0	0	0	0
KE25	1	0	0	0	1	1	0	0	1	0	1	0	1	0	1	0	1	0	0	0	0	0
KE26	1	1	0	0	1	0	0	0	1	1	0	1	1	0	1	0	0	0	0	0	0	0
KE27	1	1	0	0	1	0	0	0	1	1	0	1	1	0	1	0	0	0	0	0	0	0
KE28	0	1	0	0	1	0	0	0	1	1	1	1	1	0	1	0	1	1	0	0	0	0
KE29	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	0	0	0
E. coli	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0

Table 3.7. Distance matrix of streptococcal isolates generated with primer MBPZ-2

	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	
KE1	0																														KE1
KE2	5	0																													KE2
KE3	5	8	0																												KE3
KE4	9	6	6	0																											KE4
KE5	3	6	6	8	0																										KE5
KE6	9	8	6	4	6	0																									KE6
KE7	10	9	7	5	7	1	0																								KE7
KE8	8	7	9	5	7	3	2	0																							KE8
KE9	9	8	10	8	6	4	3	3	0																						KE9
KE10	8	7	9	7	9	7	6	4	5	0																					KE1
KE11	3	8	6	10	6	10	11	11	10	11	0																				KE1
KE12	8	7	9	7	9	5	4	2	5	2	11	0																			KE1
KE13	5	4	6	6	6	6	7	7	10	9	6	7	0																		KE1
KE14	3	6	4	8	6	8	9	9	12	11	4	9	2	0																	KE1
KE15	3	6	4	8	6	8	9	9	12	11	4	9	2	0	0																KE1
KE16	7	8	12	8	6	6	5	3	2	5	8	5	8	10	10	0															KE1
KE17	8	7	9	5	7	3	2	0	3	4	11	2	7	9	9	3	0														KE1
KE18	4	7	9	9	7	7	6	4	5	4	7	4	7	7	7	3	4	0													KE1
KE19	2	3	7	7	3	7	8	6	7	6	5	6	3	5	5	5	6	4	0												KE1
KE20	7	6	10	8	8	6	5	3	2	3	8	3	8	10	10	2	3	3	5	0											KE2
KE21	1	6	6	8	4	8	9	7	8	7	4	7	6	4	4	6	7	3	3	6	0										KE2
KE22	5	6	6	8	6	6	7	9	8	9	6	9	4	4	4	8	9	5	5	8	4	0									KE2
KE23	9	8	8	6	10	8	7	7	8	7	8	7	4	6	6	6	7	7	7	6	8	6	0								KE2
KE24	5	8	4	6	8	6	7	7	10	9	4	7	6	4	4	10	7	7	7	8	4	6	8	0							KE2
KE25	9	6	10	8	10	8	7	7	8	5	8	5	6	8	8	8	7	7	7	6	8	6	6	6	0						KE2
KE26	9	8	8	4	8	6	5	3	6	3	12	3	8	10	10	6	3	7	7	6	8	10	6	8	6	0					KE2
KE27	9	8	8	4	8	6	5	3	6	3	12	3	8	10	10	6	3	7	7	6	8	10	6	8	6	0	0				KE2
KE28	7	6	6	4	6	4	3	3	6	7	8	5	4	6	6	6	3	7	5	6	8	8	6	6	6	4	4	0			KE2
KE29	5	8	6	8	8	6	5	5	8	7	6	5	4	4	4	6	5	3	5	6	6	6	6	6	8	8	8	4	0		KE2
E.coli	6	9	11	11	9	11	10	8	7	8	7	8	9	9	9	5	8	4	6	5	5	7	7	9	9	9	9	9	7	0	*E.cc*
	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	

Table 3.8. Euclidean distance between RAPD banding patterns for streptococci generated with primer MBPZ-2

	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	
E1	0																														KE1
E2	45	0																													KE2
E3	45	57	0																												KE3
E4	69	46	46	0																											KE4
E5	30	46	46	57	0																										KE5
E6	64	53	43	31	43	0																									KE6
E7	71	60	50	38	50	8	0																								KE7
E8	67	54	64	42	54	25	18	0																							KE8
E9	75	62	71	62	50	33	27	30	0																						KE9
E10	73	58	69	58	69	54	50	40	50	0																					KE1
E11	38	67	55	77	55	71	79	85	83	92	0																				KE1
E12	67	54	64	54	64	38	33	20	45	22	85	0																			KE1
E13	45	33	46	46	46	43	50	54	71	69	55	54	0																		KE1
E14	30	46	33	57	46	53	60	64	80	79	40	64	18	0																	KE1
E15	30	46	33	57	46	53	60	64	80	79	40	64	18	0	0																KE1
E16	70	67	86	67	55	50	45	33	25	56	80	50	67	77	77	0															KE1
E17	67	54	64	42	54	25	18	0	30	40	85	20	54	64	64	33	0														KE1
E18	50	64	75	75	64	58	55	44	56	50	78	44	64	64	64	43	44	0													KE1
E19	25	30	58	58	30	54	62	55	64	60	56	55	30	45	45	56	55	50	0												KE1
E20	70	55	77	67	67	50	45	33	25	38	80	33	67	77	77	29	33	43	56	0											KE2
E21	14	55	55	67	40	62	69	64	73	70	50	64	55	40	40	67	64	43	38	67	0										KE2
E22	50	50	50	62	50	46	54	69	67	75	60	69	36	36	36	73	69	56	50	73	44	0									KE2
E23	82	67	67	55	77	62	58	64	73	70	80	64	40	55	55	67	64	78	70	67	80	60	0								KE2
E24	50	62	36	50	62	46	54	58	77	75	44	58	50	36	36	83	58	70	64	73	44	55	73	0							KE2
E25	75	50	71	62	71	57	54	58	67	50	73	45	50	62	62	73	58	70	64	60	73	55	60	55	0						KE2
E26	75	62	62	36	62	46	42	30	55	33	92	30	62	71	71	60	30	70	64	60	73	77	60	67	55	0					KE2
E27	75	62	62	36	62	46	42	30	55	33	92	30	62	71	71	60	30	70	64	60	73	77	60	67	55	0	0				KE2
E28	58	46	46	33	46	31	25	27	50	58	67	42	33	46	46	55	27	64	45	55	67	62	55	50	50	36	36	0			KE2
E29	50	62	50	62	62	46	42	45	67	64	60	45	36	36	36	60	45	38	50	60	60	55	60	55	67	67	67	36	0		KE2
.coli	75	82	92	92	82	85	83	80	78	89	88	80	82	82	82	71	80	67	75	71	71	78	88	90	90	90	90	82	78	0	*E.cc*
	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	

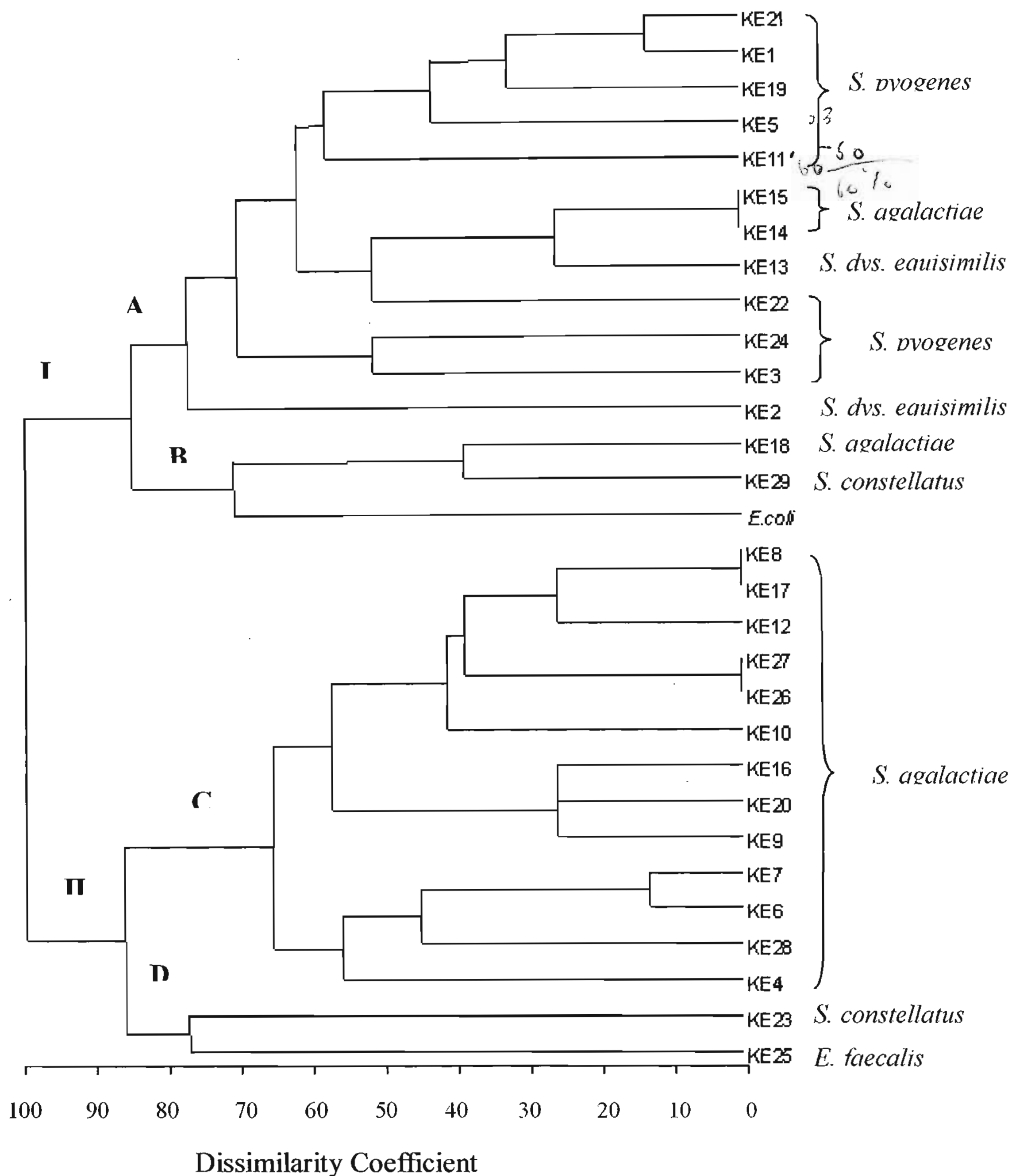

Figure 3.6. Genetic relationships among 29 streptococcal isolates and one *Escherichia coli* strain as estimated by cluster analysis of RAPD patterns obtained with primer MBPZ-2. The dendogram was generated by the unweighted pair group method, with arithmetic means.

Genetic relationships, generated by UPGMA–neighbour joining computer software (section 3.2.6), among the 27 RAPD patterns obtained with MBPZ-2 are represented in Fig. 3.6. The 27 RAPD patterns clustered into two groups (group I and group II [Fig. 3.6]) of isolates, with an average of 86% dissimilarity among them. Two clusters, A (12 isolates) and B (3 isolates), with 78% to 85% dissimilarity, were identified within group I. Another two clusters, C (13 isolates), and D (3 isolates), with 65% to 78% dissimilarity, were identified within group II. Out of twelve isoaltes of group I cluster A, 10 isolates were isolates of *S. pyogenes* and the other two isolates isolates of *S. dys. equisimilis*. One isolates of *S. agalactiae*, isolates of *S. constellatus* and the other species was *E. coli.* Two isolates (KE 14 and KE 15) of group I, cluster A, showed 100% homology. This means that RAPD analysis using MBPZ-2 showed that KE 14 and KE 15 are the same strain of *S. pyogenes.*

Although all isolates of *S. pyogenes* were clustered in cluster A of group I which exhibit primer MBPZ-2 as a good primer to discriminate for isolates of *S. pyogenes*, *S. dys. equisimilis* isolates were found among isolates of *S. pyogenes*. *E. coli* was found to be among the isolates of streptocci, which was not expected to happen (Fig. 3.6 group I cluster A). Cluster C of group II contained only isolates of *S. agalactiae* (Fig. 3.6 group II cluster C). Fig. 3.6 suggests that primer MBPZ-2 is a good primer to discriminate for isolates of *S. agalactiae.* Four isolates (KE8 and KE17, and KE 26 and KE 27) of group II cluster C, showed 100% homology. This means that RAPD analysis using MBPZ-2 exhibit KE 8 and KE 17 represents the same strain of *S. agalactiae* and KE 26 and KE 27 another strain of *S. agalactiae*. Cluster D of group II contained only two isolates, *S. constellatus* and *Enterococcus faecalis*, were exhibited not related (80% dissimilarity) by primer MBPZ-2. This was, of course expected since they are different species.

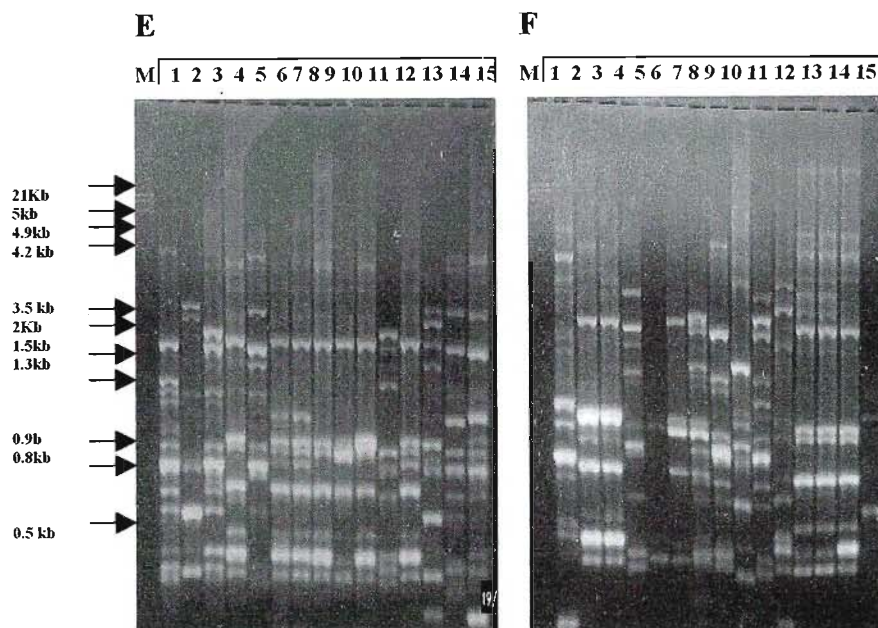

Figure 3.7. RAPD patterns of chromosomal DNA RAPD-PCR products obtained from MBPZ-3 primer and resolved on 1.5% agarose gel in a 0.5 X TBE buffer. **E:** Lane M: MWMII; lane 1 - 15: KE 1; KE 2; KE 3; KE 4; KE 5; KE 6; KE 7; KE 8; KE 9; KE 10; KE 11; KE 12; KE 13; KE1 4; KE 15. **F:** Lane M: MWMII; lane 2 - 15: KE 16; KE 17; KE 18; KE 19; *E. coli* (XL 1-Blue); KE 20; KE 21; KE 22; KE 23; KE 24; KE2 5; KE 26; KE 27; KE 28; KE 29.

Table 3.9. Scores of the bands generated with primer MBPZ-3

Strain	Band Letter																				
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**
KE1	0	0	0	1	1	0	0	1	0	0	1	1	0	1	0	1	1	0	1	0	1	1
KE2	0	0	0	0	0	1	1	1	1	0	1	0	0	1	1	1	0	0	1	0	1	1
KE3	0	0	0	1	0	1	0	1	0	0	1	1	0	1	0	1	1	0	1	0	1	1
KE4	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1	1	1
KE5	0	0	1	1	0	0	1	1	0	1	1	0	0	1	0	1	1	0	0	0	1	1
KE6	0	0	0	1	0	1	0	1	0	0	0	0	1	1	1	1	1	0	0	1	1	1
KE7	0	0	0	1	0	1	0	1	0	0	0	0	1	1	1	1	1	0	0	1	1	1
KE8	0	0	0	1	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	1	0	1
KE9	0	0	0	1	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	1	1	1
KE10	0	0	0	1	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	1	1	1
KE11	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	1	0	0	1	1
KE12	0	0	0	1	0	1	1	1	0	0	0	0	0	1	1	1	1	0	0	1	0	1
KE13	0	0	0	0	0	0	1	1	0	1	1	0	0	1	1	1	0	0	1	1	0	1
KE14	0	0	0	1	1	0	1	1	0	0	0	1	1	0	1	1	0	1	1	1	1	1
KE15	0	0	0	1	1	1	1	0	1	0	0	1	1	1	1	1	0	1	0	1	0	1
KE16	0	1	0	1	1	0	0	0	0	0	0	1	1	1	1	1	0	0	1	1	0	0
KE17	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1	0
KE18	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1	0
KE19	0	0	0	1	0	1	0	1	0	1	0	0	1	1	1	0	0	0	0	0	1	1
KE20	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1	0
KE21	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0	1	0	0	1	0
KE22	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1	0	1	1	0	0	1	1
KE23	0	0	0	1	0	0	0	0	0	1	1	0	1	1	0	0	0	1	0	0	1	0
KE24	0	0	0	1	0	0	0	1	0	0	0	1	1	1	1	0	1	1	0	1	0	1
KE25	0	0	0	1	0	1	1	1	0	0	0	0	1	1	0	1	1	1	0	1	1	1
KE26	1	0	1	1	1	1	1	1	0	0	0	0	0	1	0	0	1	0	0	1	1	1
KE27	1	0	1	1	1	1	1	1	0	0	0	0	0	1	0	0	1	0	0	1	1	1
KE28	1	0	1	1	1	0	1	1	0	0	0	0	0	1	0	0	1	0	0	1	1	1
KE29	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0
E. coli	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1	0

Table 3.10. Distance matrix of streptococcal isolates generated with primer MBPZ-3

	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	
1	0																														KE1
2	9	0																													KE2
3	4	7	0																												KE3
4	8	9	4	0																											KE4
5	8	5	4	4	0																										KE5
6	6	11	6	4	6	0																									KE6
7	6	11	6	4	6	0	0																								KE7
8	8	11	6	2	6	2	2	0																							KE8
9	8	11	6	2	6	2	2	0	0																						KE9
10	8	11	6	2	6	2	2	0	0	0																					KE1
11	9	4	7	7	7	7	7	7	7	7	0																				KE1
12	7	10	5	3	5	1	1	1	1	1	6	0																			KE1
13	9	4	7	9	5	11	11	11	11	11	6	10	0																		KE1
14	7	8	9	13	9	11	11	11	11	11	8	10	8	0																	KE1
15	7	10	9	13	9	11	11	11	11	11	10	10	6	2	0																KE1
16	6	11	10	10	10	6	6	8	8	8	7	7	11	7	7	0															KE1
17	8	7	10	10	6	10	10	10	10	10	9	9	7	9	9	10	0														KE1
18	9	8	9	9	5	9	9	9	9	9	10	8	8	10	10	11	1	0													KE1
19	7	4	7	7	5	11	11	9	9	9	6	10	4	8	8	11	5	6	0												KE1
20	9	6	11	11	9	11	11	11	11	11	6	10	8	8	10	9	3	4	6	0											KE2
21	8	7	8	10	8	10	10	10	10	10	7	9	7	9	9	12	4	5	5	5	0										KE2
22	6	11	8	8	10	8	8	6	6	6	9	7	11	7	7	10	8	9	7	9	6	0									KE2
23	7	10	9	13	11	11	11	13	13	13	8	12	8	10	8	7	7	8	8	8	5	9	0								KE2
24	2	7	6	8	8	6	6	8	8	8	7	7	7	9	9	8	6	7	5	7	6	6	7	0							KE2
25	9	8	11	9	9	11	11	9	9	9	12	10	8	12	10	11	5	6	6	8	7	7	10	7	0						KE2
26	8	11	10	8	10	10	10	8	8	8	13	9	11	9	9	10	10	11	9	11	12	10	15	10	7	0					KE2
27	8	11	10	8	10	10	10	8	8	8	13	9	11	9	9	10	10	11	9	11	12	10	15	10	7	0	0				KE2
28	7	12	9	7	9	9	9	7	7	7	12	8	10	8	8	9	9	10	8	10	11	9	14	9	8	1	1	0			KE2
29	6	7	8	8	10	10	10	8	8	8	7	9	9	9	11	10	6	7	5	5	6	6	9	4	7	10	10	9	0		KE2
oli	9	10	11	9	13	11	11	9	9	9	8	10	12	12	14	11	7	8	8	4	7	7	10	7	8	11	11	10	3	0	E.co
	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	

Table 3.11. Euclidean distance between RAPD band patterns for streptococci generated with primer MBPZ-3

	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	
1	0																														KE1
2	69	0																													KE2
3	33	58	0																												KE3
4	62	75	36	0																											KE4
5	57	45	33	36	0																										KE5
6	46	79	46	36	46	0																									KE6
7	46	79	46	36	46	0	0																								KE7
8	62	85	50	22	50	20	20	0																							KE8
9	62	85	50	22	50	20	20	0	0																						KE9
10	62	85	50	22	50	20	20	0	0	0																					KE1
11	75	50	64	70	64	64	64	70	70	70	0																				KE1
12	54	77	42	30	42	10	10	11	11	11	60	0																			KE1
13	64	40	54	69	42	73	73	79	79	79	60	71	0																		KE1
14	50	62	60	81	60	69	69	73	73	73	67	67	57	0																	KE1
15	47	67	56	76	56	65	65	69	69	69	71	63	43	15	0																KE1
16	46	79	67	71	67	46	46	62	62	62	64	54	73	50	47	0															KE1
17	62	64	71	77	50	71	71	77	77	77	82	69	58	64	60	71	0														KE1
18	64	67	64	69	42	64	64	69	69	69	83	62	62	67	63	73	11	0													KE1
19	58	44	58	64	45	79	79	75	75	75	67	77	40	62	57	79	50	55	0												KE1
20	75	67	85	92	75	85	85	92	92	92	75	83	73	67	71	75	38	44	67	0											KE2
21	57	58	57	71	57	67	67	71	71	71	64	64	54	60	56	75	36	42	45	50	0										KE2
22	46	79	57	62	67	57	57	50	50	50	75	54	73	50	47	67	62	64	58	75	46	0									KE2
23	50	71	60	81	69	69	69	81	81	81	67	75	57	63	50	50	54	57	62	67	38	60	0								KE2
24	20	64	50	67	62	50	50	67	67	67	70	58	58	64	60	62	55	58	50	70	50	50	54	0							KE2
25	60	62	69	64	60	69	69	64	64	64	86	67	57	71	59	69	42	46	50	67	50	50	63	54	0						KE2
26	53	73	63	57	63	63	63	57	57	57	87	60	69	56	53	63	67	69	64	79	71	63	79	67	47	0					KE2
27	53	73	63	57	63	63	63	57	57	57	87	60	69	56	53	63	67	69	64	79	71	63	79	67	47	0	0				KE2
28	50	80	60	54	60	60	60	54	54	54	86	57	67	53	50	60	64	67	62	77	69	60	78	64	53	8	8	0			KE2
29	60	78	73	80	83	83	83	80	80	80	88	82	82	75	79	83	67	70	63	71	60	60	75	50	64	77	77	75	0		KE2
oli	82	100	92	90	100	92	92	90	90	90	100	91	100	92	93	92	78	80	89	67	70	70	83	78	73	85	85	83	60	0	*E.co*
	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	*E. coli*	

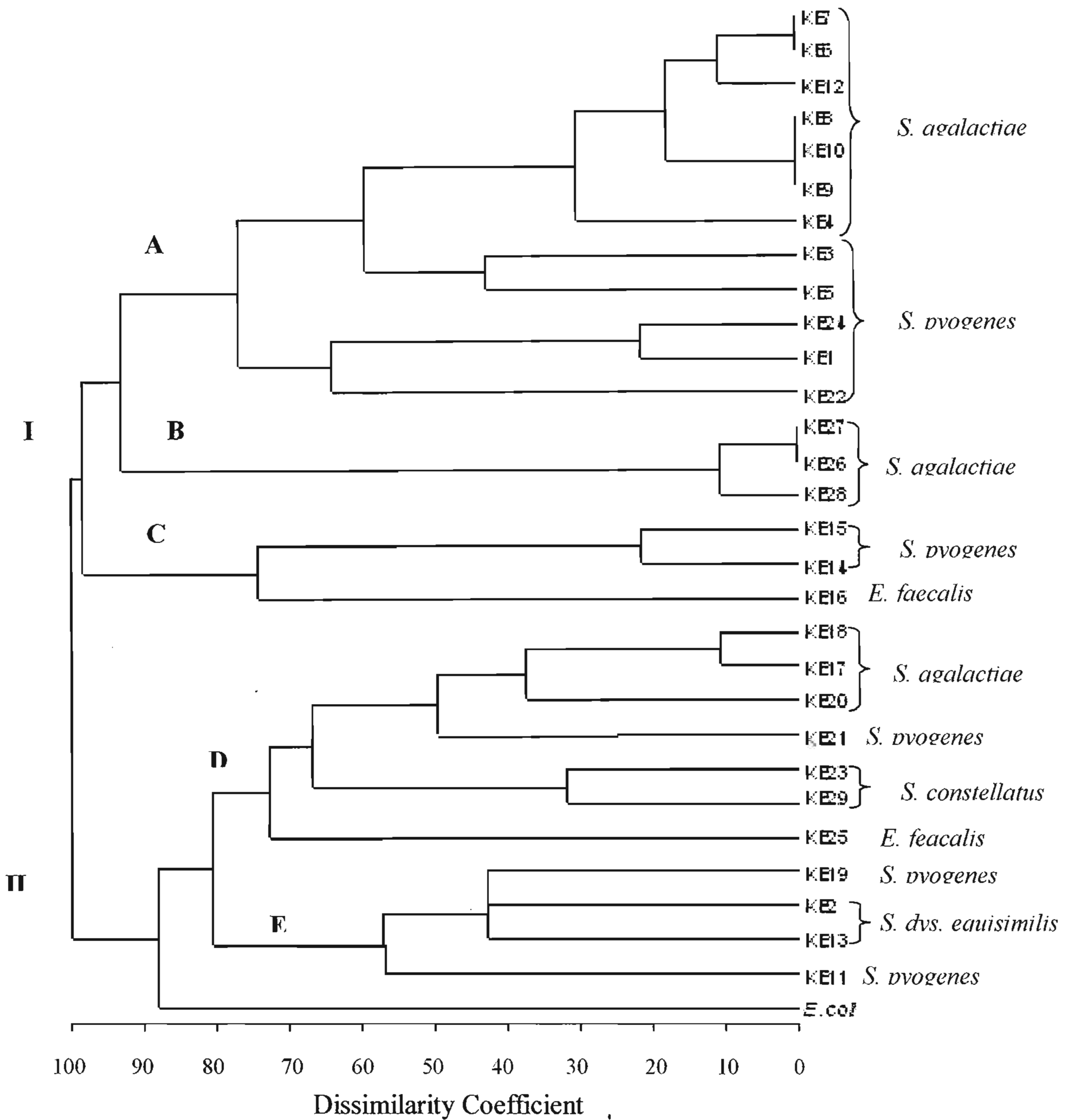

Figure 3.8. Genetic relationship between 29 streptococcal isolates and one *Escherichia coli* strain as established by cluster analysis of RAPD patterns obtained with primer MBPZ-3. The dendogram was generated by the unweighted pair group method with arithmetic means.

Genetic relationships, generated by UPGMA–neighbour joining computer software (section 3.2.6), among the 26 RAPD patterns obtained with MBPZ-3 are represented in the dendogram shown in Fig. 3.8. The 26 RAPD patterns clustered into two groups, group I and group II (Fig. 3.8) of isolates with 88% to 98% dissimilarity among them. Three clusters, A (12 isolates), B (3 isolates) and C (3 isolates), with 10 % to 75% dissimilarity, were identified within group I. Another two clusters, D (7 isolates), E (4 isolates), with 73% to 88% dissimilarity, were identified within group II. Cluster A contained seven isolates of *S. agalactiae* and five isolates of *S. pyogenes* (fig. 3.8 group I cluster A). Cluster B of group I contained isolates of *S. agalactiae* only but not all them and cluster C of the same group contained three isolates, three of which *S. pyogenes* and the othe one was *Enterococcus faecalis* (fig 3.8 group I cluster C). The two isolates of *S. pyogenes* found in clusre C of group I are closely related (25% dissimilar) where as *E. faecalis* is not since it only 20% similar (80% dissimilarity) to *S. pyogenes* in cluster C (fig. 3.8 group I cluster C). KE 6 and KE 7, KE 8, KE 9, and KE 10 of group I cluster A showed 100% homology. KE 26 and KE 27 of group I, cluster B, showed 100% homology. This means that RAPD analysis using MBPZ-3 exhibits KE 6 and KE 7, representing the same strain of *Streptococcus agalactiae*e, KE 8, KE 9, and KE 10 representing another strain of *Streptococcus agalactiae* and KE 26 and KE 27 representing yet another strain of *Streptococcus agalactiae*.

Three isolates of group II, cluster D were isolates of *S. agalactiae*, two were isolates of *S. constellatus* (67% similarity), one was *E. enterococcus* and the last one was *S. pyogenes* (fig. 3.8 group II clusre D). Two isolates of group II, cluster E were isolates of *S. pyogenes* and the other two were isolates of *S. dys. equisimilis* (fig. 3.8 group II clusre E). One of the *S. pyogenes* isolates (KE 19) apperared to be more related to *S. dys. equisimilis* (55% similarity) that to the other isolate (KE 11) of *S. pyogenes* (45% similarity). This was not expected to happen.

Escherichia coli could not be grouped in any of the two groups (I and II). MBPZ-3 exhibits *Escherichia coli* as an "out group", meaning that *Escherichia coli* is a different genus.

RAPD analysis obtained with all three primers showed no differences between KE 26 and KE 27 (Table 3.5, 3.8, and 3.11 and Fig. 3.4, 3.6, and 3.8). There were no differences obtained between KE 6 and KE 7, with MBPZ–1 exhibiting 100% homology and MBPZ-3 (Table 3.5 and 3.11 and Fig. 3.4 and 3.8). MBPZ-2 exhibited a high percentage of similarity, 92% between these isolates (Table 3.8 and Fig. 3.6), suggesting that KE 6 and KE 7 did not represent the strain of *Streptococcus agalactiae* since only differences of any RAPD analysis that can be concluded (Tenover *et al.,* 1997). This diversity is caused by a third smallest band that exists in KE 6, but not in KE 7 (Fig. 3.4).

The results obtained with primer MBPZ-3 showed 100% similarity between KE 8, KE 9 and KE 10. Primer MBPZ-1 showed 100% similarity between KE 9 and KE 10 only, but 82% similarity between KE 8 and KE 9, and KE 8 and KE 10 likewise, 60% to 70% similarity was obtained with primer MBPZ-2. This suggests that KE 9 and KE 10 belong to the same strains of *Streptococcus agalactiae*, and KE 8 is a closely related strain to KE 9 and KE 10. These results suggest that isolates that have the same RAPD patterns are actually the same species obtained from different individuals. The isolates that share the same RAPD cluster may have originated from a common ancestor (Martinez *et al.,* 2000; and Clifford and Stephenson, 1975).

Each of the primers divided the isolates into two groups, group I and group II. However, some isolates that clustered in group I with one primer did not necessarily cluster in the group I when analyzed with a different primer (Figures 3.4, 3.6, and 3.8). Thirteen of 25 (52%) isolates of MBPZ-1 group I were in MBPZ-2 group I, 18 of 25 (72%) isolates of MBPZ-1 group I were in MBPZ-3 group I, and 13 of 18 isolates (72%) of MBPZ-2 group I were in MBPZ-3 group I. This suggests that not all isolates were differentiated from each other.

Most of the isolates of the same species that had the same antibiotic relations (Table 2.3) were grouped or clustered together by all three primers. Isolates of *S. agalactiae* which were all resistant to penicillin G (Fig. 2.1 and Table 2.2) clustered together in one cluster (Fig. 3.6 group II cluster C) when analyzed by primer MBPZ-2, they also clustered together in one cluster except one isolate KE 12, which appeared in a different cluster but of the same group (fig. 3.4 group I clusters A and B). Two of the three suceptable isolates of *S. pyogenes* (Table 2.2) were also clustered in one cluster (fig. 3.4 group I cluster A and fig. 3.8 group I cluster A). Both MIC results and RAPD analysis of streptococcal isolates and entirococci suggests that all three primers used in this study are both discriminative and informative. This is evident from the fact that isolates of the same species and antibiotic relation were mostly grouped or clustered together.

The present work used RAPD analysis to study 29 of human streptococcal isolates from South Africa, Durban Metro area, KwaZulu-Natal. In general, high genetic diversity was found. A possible explanation for this diversity is that different isolates originated from different humans, although unlikely, it is possible that these specimens were mislabelled in the past, or that that there was a contamination or laboratory error.

Analysis by RAPD-PCR demonstrated good discriminatory power, despite some lack of reproducibility of faint bands.

CHAPTER FOUR

PULSED-FIELD GEL ELECTROPHORESIS (PFGE) ANALYSIS OF STREPTOCOCCAL ISOLATES

ABSTRACT

The genetic diversity of 29 streptococcal isolates in KwaZulu-Natal, Durban Metro area, South Africa, was evaluated by pulsed-field gel electrophoresis (PFGE) of genomic DNA restricted with *Sma*I. The method was highly discriminative, results were reproducible and the PFGE patterns were easy to interpret. Among all the streptococcal isolates, 24 different PFGE patterns were observed. The 24 PFGE patterns were divided into three groups (I, II and III) of isolates with an average of 85% dissimilarity (15% homology) among them. At 25% homology, four clusters, A (13 isolates), B (9 isolates), C (4 isolates) and D (4 isolates) were obseved. Two pairs of isolates (KE 6 and KE 7, and KE 14 and KE 15) of group I, cluster A, showed 100% homology. This suggested that KE 6 and KE 7 represent the same strain of *Streptococcus agalactiae* and KE 14 and KE 15 represent the same strain of *Streptococcus pyogenes*. Four isolates (KE19, KE25, KE26, and KE27) of group I, cluster B, also exhibited 100% homology. The ease with which this analysis can be performed, together with the clarity and the polymorphism seen in the patterns, suggests that this technique will be very useful for epidemiological evaluations of nosocomial streptococcal infections.

4.1 INTRODUCTION

Molecular techniques have received increasing attention as a means of analyzing epidemiological interrelationships, thus leading to use of the term "molecular epidemiology" (George, 1993). Since chromosomal DNA is the fundamental molecule of cellular identity, there has been particular interest in assessing chromosomal similarity as a measure of epidemiological relatedness.

One attractive approach has been to digest chromosomal DNA with restriction enzymes, which recognize numerous sites within the bacterial chromosomal DNA, resulting in a series of different-sized fragments that form patterns when comparatively analyzed by agarose gel electrophoresis. In this context, differences in fragment patterns are commonly referred to as restriction fragment length polymorphism (RFLP) (Tenover *et al.*, 1997).

With such enzymes, restriction digestion of DNA from different bacterial isolates results in hundreds of fragments ranging from ~0.5kb to 50kb in length, that are too numerous to compare accurately after conventional agarose gel electrophoresis. Even though different strains of the same bacterial species have different RFLP profiles, restriction fragments produced by these enzymes would be still difficult to analyze (Tenover *et al.*, 1997; and Maslow, 1993).

Conventional agarose gel electrophoresis is unidirectional, constantly "pulling" DNA molecules (which are negatively charged) through an agarose matrix towards a fixed positive charge. Under these conditions, DNA molecules < 40 to 50 kb in size migrate through the gel in a size-dependent fashion. This is not the case with DNA molecules greater than 40 to 50 kb in size, which exhibit aberrant, size-independent, electrophoretic migration that may relate to a longitudinal orientation of molecules in the agarose matrix. This is called reptation (Georing *et al.*, 1993).

In contrast, PFGE is multidirectional, continually changing the location of the positive charge. The DNA molecules respond continually, re-orientating their direction of migration through the agarose gel matrix (Chu *et al.*, 1986; Carle *et al.*, 1986; and Schwartz and Cantor, 1984).

In addition, electrical pulses of different duration favour the re-orientation of different-sized DNA molecules. Longer pulse times favour the re-orientation of larger molecules and *vice versa* (George, 1993; Carle *et al.*, 1986; Schwartz and Cantor, 1984; and Chu *et al.*, 1986). By varying both the direction and the duration of the electric field, PFGE allows the resolution of DNA molecules well over 1000kb (mega-base pairs) in length (Maslow, 1993).

There are disadvantages associated with PFGE. These include the technical demands of the procedure and the initial cost of the equipment, as mentioned previously (section 1.4).

4.2 MATERIALS AND METHODS

Isolation of intact chromosomal DNA is a prerequisite for reproducible generation of restriction fragments by rare-cutting restriction endonucleases. Thus, for analysis by PFGE, chromosomal DNA is commonly prepared by the *in situ* lysis of cells embedded in agarose blocks or plugs. This provides an environment where molecules of chromosomal DNA are protected from molecular shearing forces and allow the reproducible generation of mega-base sized restriction fragments.

4.2.1 Preparation of Agarose Embedded Bacterial DNA

4.2.1.1 Lysis (Day 1)

Bacterial cultures (Table 2.1) were inoculated into 5ml TSB and grown to an OD_{600nm} of between 0.6 and 0.8 at 37°C in a 5% CO_2 atmosphere. When the desired OD was reached, 1 ml of the bacterial culture was harvested by centrifugation at 4°C, at 14 500 rpm for 5 minutes, using a Sigma Centrifuge, and the supernatant discarded. The pellet was resuspended in 1 ml of ice-cold PIV solution (100 mM Tris-HCl and 1 M NaCl). This

suspension was centrifuged under the same conditions, the supernatant removed and, depending on the size of the pellet, the cell suspension was resuspended in a volume between 300µl and 1000µl of PIV solution. The OD_{578} values of bacterial suspension were then determined, by diluting the suspension 100 times (10µl of bacterial suspension was mixed with 990µl PIV). The OD_{578} values were adjusted to be between 0.01 and 0.025, using PIV solution. PIV was also used as a blank.

Low Melting Point (LMP) agarose (1.2%) was prepared by dissolving LMP agarose particles in PIV solution and bringing to boil in a 100°C water bath and thereafter equilibrated at 50°C.

The agarose plugs were prepared by mixing 50µl of 1.2% LMP agarose with 50 µl of the cell suspension. The cells-agarose mixture, termed an agarose plug, was kept at 50°C and then transferred into plug molds. The agarose plug was allowed to solidify in plug molds. This step was expedited by placing the molds containing the cells-agarose mixture at –20°C for 15min and then at 4°C for a further 20 min. The agarose plugs were transferred into sterile microfuge tubes containing lysis solution (1M NaCl, 100mM EDTA, 6mM Tris-HCl, pH 8.0, 0.5mg/ml *N-laurolysarcosin*, 60µg/ml RNaseA, 10µg/ml lysozyme, and 10µg/ml mutanolysin). The microfuge tubes containing lysis buffer and agarose plugs were incubated for 24 hours at 37°C in a water-bath, to facilitate lysis.

4.2.1.2 Deproteinization (Day 2)

After 24 hours of lysis at 37°C, lysis solution was aspirated out with a pipette, autoclaved and discarded. The agarose plugs were washed twice for 30 min in 1ml of ES buffer (0.5 M EDTA, pH 9.0, 0.1 mg/ml *N-laurylsarcosin*). After the second wash, the agarose plugs were deproteinized in 1 ml of ESP solution (ES buffer containing 1 mg/ml proteinase K) by incubating at 50°C for overnight in a water bath.

4.2.2 Digestion Using Restriction Endonuclease

The choice of restriction enzyme for this purpose depends on the frequency of the enzyme recognition sites on the chromosomal DNA. As a group, gram-positive bacteria contain chromosomal DNA rich in adenine (A) and thymine (T). Restriction enzymes that recognize sequences rich in guanine (G) and cytosine should be used (section 1.4).

4.2.2.1 Digestion of Plugs Using Restriction Endonuclease *Sma*I (Day 3)

After overnight of deproteinization (incubation at 50°C) the ESP solution was removed. Agarose plugs were transferred into sterile test tubes containing 14ml of TE buffer (10mM Tris-HCl, 1mM EDTA, pH7.5). Agarose plugs were washed twice for 30 min each time in this buffer. After each wash, TE buffer was slowly poured out of each test tube into a clean petri dish in order to retain the agarose plug in the test tube. In case an agarose plug was transferred into a petri dish, the buffer was gently poured from this petri dish into another petri dish, thereby retaining a plug in the first dish. The plug was then transferred into the original tube with a glass spatula. This had to be done very carefully, because the plugs are almost transparent. After the second wash, 1 ml of the laboratory prepared *Sma*I buffer (3.3 mM Tris-acetate, 1mM magnesiun-acetate, potassium-acetate and 0.5mM Dithiothreitol (DTT), pH 7.9) was aliquated into 15 2 ml reaction tubes. The blocks were then transferred into the reaction tubes containing laboratory prepared *Sma*I buffer and equilibrated at room temperature for 30 min. The buffer was then removed by aspiration first with blue tips, then with yellow tips. Next, 60µl of restriction digestion solution (720µl sterile distilled water (sdH_2O); 80 µl of buffer A; and 25 µl of 10 U/µl *Sma*I) containing the added to each of the 2 ml microfuge tubes containing agarose plug and incubated at 25°C for 20 hours.

4.2.3 Separation of Restriction Fragments by Pulsed-Field Gel Electrophoresis

After 20 hours of digestion, 60µl of loading buffer (200mg Na2-EDTA, 20g sucrose, 15mg Bromophenol Blue in 50ml dH_2O) was added to each reaction tube containing the restriction digest.

A 1% agarose gel containing ethidium bromide was added, to reach a final concentration of 0.5µg/ml was prepared. Agarose plugs were cut quadratically, transferred to the wells of the gel, and sealed with 1% pulsed field certified agarose (Bio Rad) and allowed to polymerize for 10 to 15 minutes.

The agarose plugs were electrophoresed in the 1% agarose gel (Bio Rad) with buffer TBE (89mM Tris-HCl, 89mM borate, 0.4mM EDTA [pH 8.4]), using a cantour-clamped homogeneous electric field (CHEF DRIII, Bio-Rad Laboratories) (Chatellier *et al.,* 1999). Pulse times were ramped from 1 to 18 seconds over 18 hours at 6 V/cm and 120° included angle. PFGE patterns were detected with a UV transilluminator. *Staphylococcus aureus* 8325 NCTC obtained from the Institut für Medizinische Mikrobiologie und Immunologie, Bonn Univesität was used as a standard marker. After electrophoresis and staining in ethidium bromide, the resulting restriction endonuclease digestion profiles were recoded by scoring presence and absence of bands, denoted by 1 and 0, respectively, and analyzed by Euclidean Distance (ED) as a dissimilarity measure.

Euclidean distance measure discards negative matches between pairs of isolates and provides a more accurate picture of relatedness (Abbott *et al.*, 1995). Euclidean distance measure is described by $Di_{AB} = \sqrt{(a_A - a_B)^2 + (b_A - b_B)^2}$, where Di_{AB} is the shortest distance between species A and B, a_A is a band that exists in species A, a_B is a band that exists in species B, b_A is a band that exists in species A and b_B is a band that exists in species B.

4.3 RESULTS AND DISCUSSION

4.3.1 Genetic diversity of streptococcal isolates as defined by PFGE.

The reproducibility of the PFGE patterns was established by repeated testing of four isolates, selected from the panel of 29 isolates, on separate occasions and different gels. All such tests yielded identical PFGE patterns, suggesting suitability of the PFGE (Fig. 4.1).

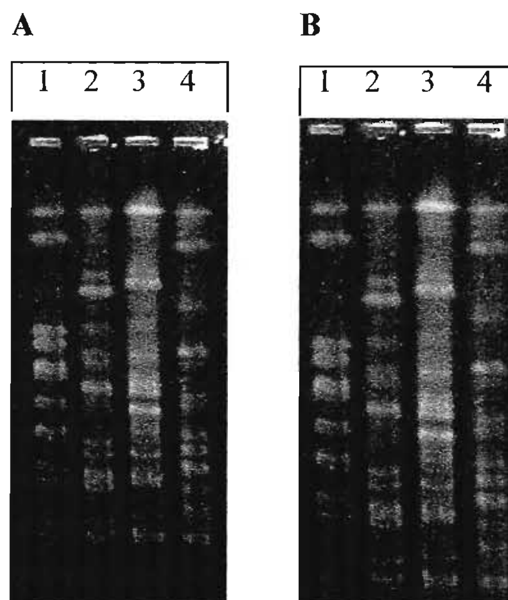

Figure 4.1. The reproducibility of the PFGE patterns obtained by digestion using restriction endonuclease *Sma*I. Lanes: 1 - 4; contain KE1 to KE 4. **A** and **B** represent two independent experiments, respectively.

4.3.2 Genetic diversity of streptococcal isolates as defined by PFGE

PFGE patterns of genomic DNA from streptococcal isolates digested with *Sma*I, were characterized by 7 to 16 restriction fragments (fig. 4.2A, lanes 2 and 13). Among the 29 streptococcal isolates, 24 PFGE patterns were identified. The genetic relationships, generated by UPGMA–neighbour joining computer software (section 3.2.6), between the 29 isolates of streptococcus are presented in the dendogram (fig. 4.3) and table (Table 4.3) and they diverged by up to 93% (7% homology). At about 15% homology, three groups, I, II, and III, were identified. A 25% homology, four clusters, A to D, were observed (fig. 4.3).

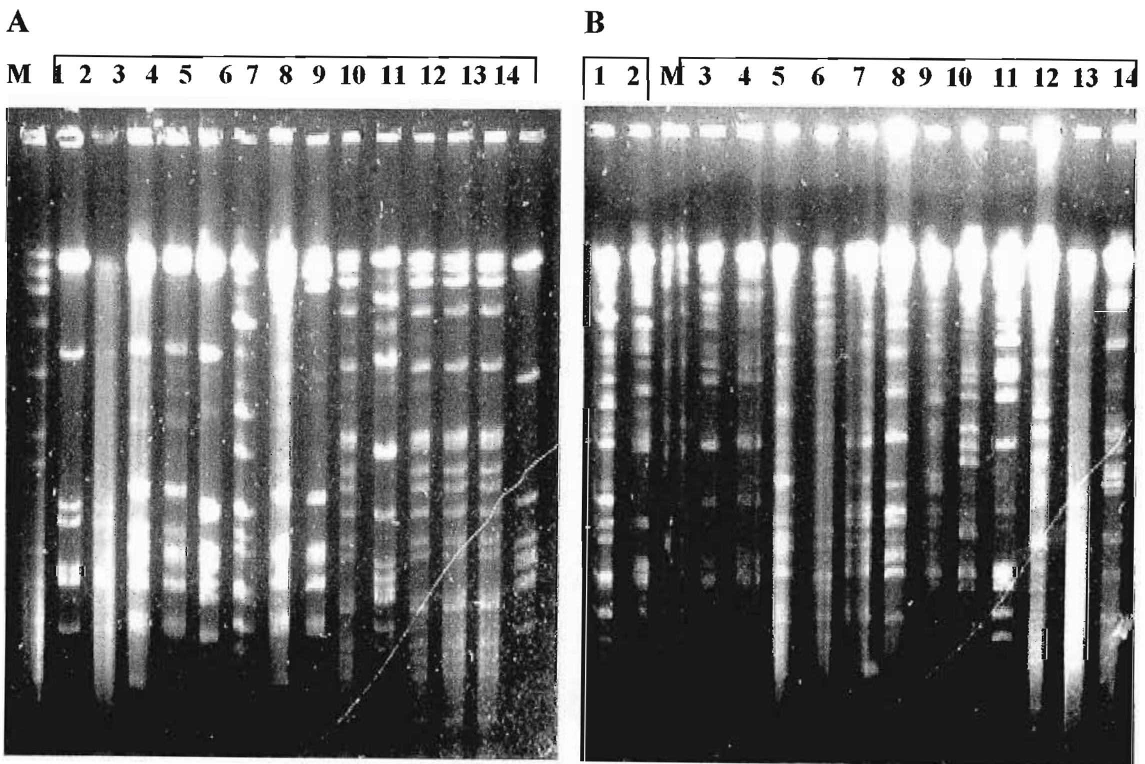

Figure 4.2. *Sma*I microrestriction patterns of streptococcal isolates analyzed by PFGE on a 1% pulsed field certified agarose stained with ethidium bromide. (A) Lane M, S. aureus; lane 1 – 14: KE; KE7; KE28; KE8; KE9; KE11; KE17; KE18; KE19; KE22; KE25; KE26; KE27; and KE24. (B) Lane 1 - 2, KE1; KE2; lane M, S. aureus; lanes 3-14, KE3; KE4; K5; KE10; KE12; KE13; KE14; KE15; KE16; KE20; KE21; and KE23.

Table 4.1. Scores of the fragments produced with restriction enzyme *Sma*I

Strain	Band Letter																	
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**
KE1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0
KE2	1	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	0
KE3	1	0	1	1	0	1	0	1	1	0	0	0	0	1	1	0	0	0
KE4	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1
KE5	1	1	1	0	0	0	1	0	1	0	1	1	1	0	1	1	1	0
KE6	1	1	0	0	0	0	1	0	0	0	1	1	0	0	1	0	1	0
KE7	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1	1	1	0
KE8	1	1	0	0	0	0	1	0	0	0	0	0	1	1	1	0	1	0
KE9	1	1	0	0	0	0	1	0	0	0	0	1	1	1	1	0	1	0
KE10	1	1	1	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0
KE11	1	0	1	1	0	1	1	0	1	0	1	1	1	1	0	0	0	1
KE12	1	1	0	0	0	0	1	0	0	0	0	1	1	1	0	1	0	0
KE13	1	1	1	0	1	1	1	0	0	1	0	1	0	1	0	1	0	0
KE14	1	1	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1
KE15	1	1	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1
KE16	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	0
KE17	1	1	1	0	0	0	0	1	1	0	0	0	1	1	1	0	1	0
KE18	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0
KE19	1	1	1	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0
KE20	1	1	1	1	1	1	0	1	0	0	1	0	1	1	0	1	1	0
KE21	1	1	1	0	0	0	1	0	0	0	1	0	1	1	0	0	1	1
KE24	1	0	0	0	0	0	0	0	1	0	1	0	1	1	1	0	1	1
KE23	1	1	0	1	0	1	0	0	0	0	0	0	1	1	0	0	1	1
KE28	1	1	0	0	0	0	1	0	0	0	1	0	1	1	1	0	1	1
KE22	1	0	1	1	0	1	1	1	0	0	0	1	1	1	1	0	1	1
KE25	1	1	1	0	1	0	0	1	0	0	0	1	1	1	0	1	0	0
KE26	1	1	1	0	1	0	0	1	0	0	0	1	1	1	0	1	0	0
KE27	1	1	1	0	1	0	0	1	0	0	0	1	1	1	0	1	0	0
KE29	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus	1	1	0	0	0	1	0	1	1	0	0	1	1	1	1	1	0	0

Table 4.2. Matrix of correlation coefficients between the Euclidean distances generated with restriction enzyme *Sma*I

	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29
KE1	0																												
KE2	6	0																											
KE3	11	7	0																										
KE4	13	9	2	0																									
KE5	6	8	11	11	0																								
KE6	7	7	12	12	3	0																							
KE7	7	7	12	12	3	0	0																						
KE8	10	10	9	9	6	5	5	0																					
KE9	9	9	10	10	5	4	4	1	0																				
KE10	10	10	9	9	6	7	7	6	7	0																			
KE11	10	8	7	7	8	11	11	1	9	12	0																		
KE12	10	8	11	11	6	5	5	4	3	6	8	0																	
KE13	9	5	10	12	9	8	8	9	8	9	9	5	0																
KE14	14	8	9	7	10	9	9	6	7	8	8	6	5	0															
KE15	14	8	9	7	10	9	9	6	7	8	8	6	5	0	0														
KE16	7	5	6	8	10	10	10	9	10	7	13	11	6	9	9	0													
KE17	10	10	5	5	6	9	9	4	5	4	10	8	11	8	8	7	0												
KE18	14	10	7	7	8	7	7	4	5	4	10	6	7	4	4	7	4	0											
KE19	10	8	9	9	8	9	9	8	7	4	10	4	5	8	8	7	6	6	0										
KE20	7	7	8	10	9	10	10	9	10	5	9	9	8	11	11	4	7	7	5	0									
KE21	12	10	11	9	6	7	7	4	5	6	6	6	9	4	4	11	6	4	8	7	0								
KE22	11	13	8	6	7	8	8	5	6	9	7	9	14	9	9	12	5	7	11	10	5	0							
KE23	11	11	8	8	11	10	10	5	6	7	7	7	10	7	7	8	7	5	9	6	5	6	0						
KE24	10	10	11	9	6	5	5	2	3	8	8	6	11	6	6	11	6	6	10	9	2	3	5	0					
KE25	9	7	6	6	9	10	10	7	6	9	5	9	10	9	9	8	7	9	9	8	7	8	6	7	0				
KE26	10	8	9	9	8	9	9	8	7	4	10	4	5	8	8	7	6	6	0	5	8	11	9	10	9	0			
KE27	10	8	9	9	8	9	9	8	7	4	10	4	5	8	8	7	6	6	0	5	8	11	9	10	9	0	0		
KE28	10	8	9	9	8	9	9	8	7	4	10	4	5	8	8	7	6	6	0	5	8	11	9	10	9	0	0	0	
KE29	14	12	7	7	10	7	7	6	7	6	10	6	9	6	6	11	8	4	8	11	8	7	7	8	11	8	8	8	0

KE1 KE2 KE3 KE4 KE5 KE6 KE7 KE8 KE9 KE10 KE11 KE12 KE13 KE14 KE15 KE16 KE17 KE18 KE19 KE20 KE21 KE22 KE23 KE24 KE25 KE26 KE27 KE28 KE29

Table 4.3. Euclidean distance between restriction fragment patterns for streptococci produced with restriction enzyme *Sma*I

	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE20	KE21	KE22	KE23	KE24	KE25	KE26	KE27	KE28	KE29	
KE1	0																													KE1
KE2	35	0																												KE2
KE3	65	50	0																											KE3
KE4	72	60	22	0																										KE4
KE5	38	50	73	73	0																									KE5
KE6	47	50	86	86	27	0																								KE6
KE7	47	50	86	86	27	0	0																							KE7
KE8	63	67	75	75	50	50	50	0																						KE8
KE9	56	60	77	77	42	40	40	13	0																					KE9
KE10	63	67	75	75	50	64	64	60	64	0																				KE10
KE11	56	50	54	54	53	73	73	71	64	80	0																			KE11
KE12	63	57	85	85	50	50	50	44	33	60	62	0																		KE12
KE13	53	36	71	80	60	62	62	69	62	69	60	45	0																	KE13
KE14	78	57	75	64	71	75	75	60	64	73	62	60	45	0																KE14
KE15	78	57	75	64	71	75	75	60	64	73	62	60	45	0	0															KE15
KE16	41	33	46	57	65	67	67	64	67	54	72	73	43	64	64	0														KE16
KE17	59	63	45	45	46	69	69	40	45	40	67	67	73	67	67	50	0													KE17
KE18	82	71	70	70	67	70	70	50	56	50	77	67	64	50	50	58	44	0												KE18
KE19	59	53	69	69	57	69	69	67	58	40	67	40	42	67	67	50	50	60	0											KE19
KE20	41	44	57	67	56	67	67	64	67	42	56	64	53	73	73	29	50	58	38	0										KE20
KE21	67	63	79	69	46	58	58	40	45	55	46	55	64	40	40	69	50	44	62	50	0									KE21
KE24	65	76	67	55	54	67	67	50	55	75	54	75	88	75	75	75	45	70	79	67	45	0								KE22
KE23	65	69	67	67	73	77	77	50	55	64	54	64	71	64	64	57	58	56	69	46	45	55	0							KE23
KE28	59	63	79	69	46	45	45	22	30	67	57	55	73	55	55	69	50	60	71	60	20	30	45	0						KE24
KE22	50	44	46	46	56	67	67	54	46	64	36	64	63	64	64	50	50	69	60	50	50	57	46	50	0					KE25
KE25	59	53	69	69	57	69	69	67	58	40	67	40	42	67	67	50	50	60	0	38	62	79	69	71	60	0				KE26
KE26	59	53	69	69	57	69	69	67	58	40	67	40	42	67	67	50	50	60	0	38	62	79	69	71	60	0	0			KE27
KE27	59	53	69	69	57	69	69	67	58	40	67	40	42	67	67	50	50	60	0	38	62	79	69	71	60	0	0	0		KE28
KE29	93	92	88	88	91	88	88	86	88	86	91	86	90	86	86	92	89	80	89	92	89	88	88	89	92	89	89	89	0	KE29
	KE1	KE2	KE3	KE4	KE5	KE6	KE7	KE8	KE9	KE10	KE11	KE12	KE13	KE14	KE15	KE16	KE17	KE18	KE19	KE 20	KE21	KE22	KE23	KE 24	KE25	KE26	KE27	KE28	KE29	

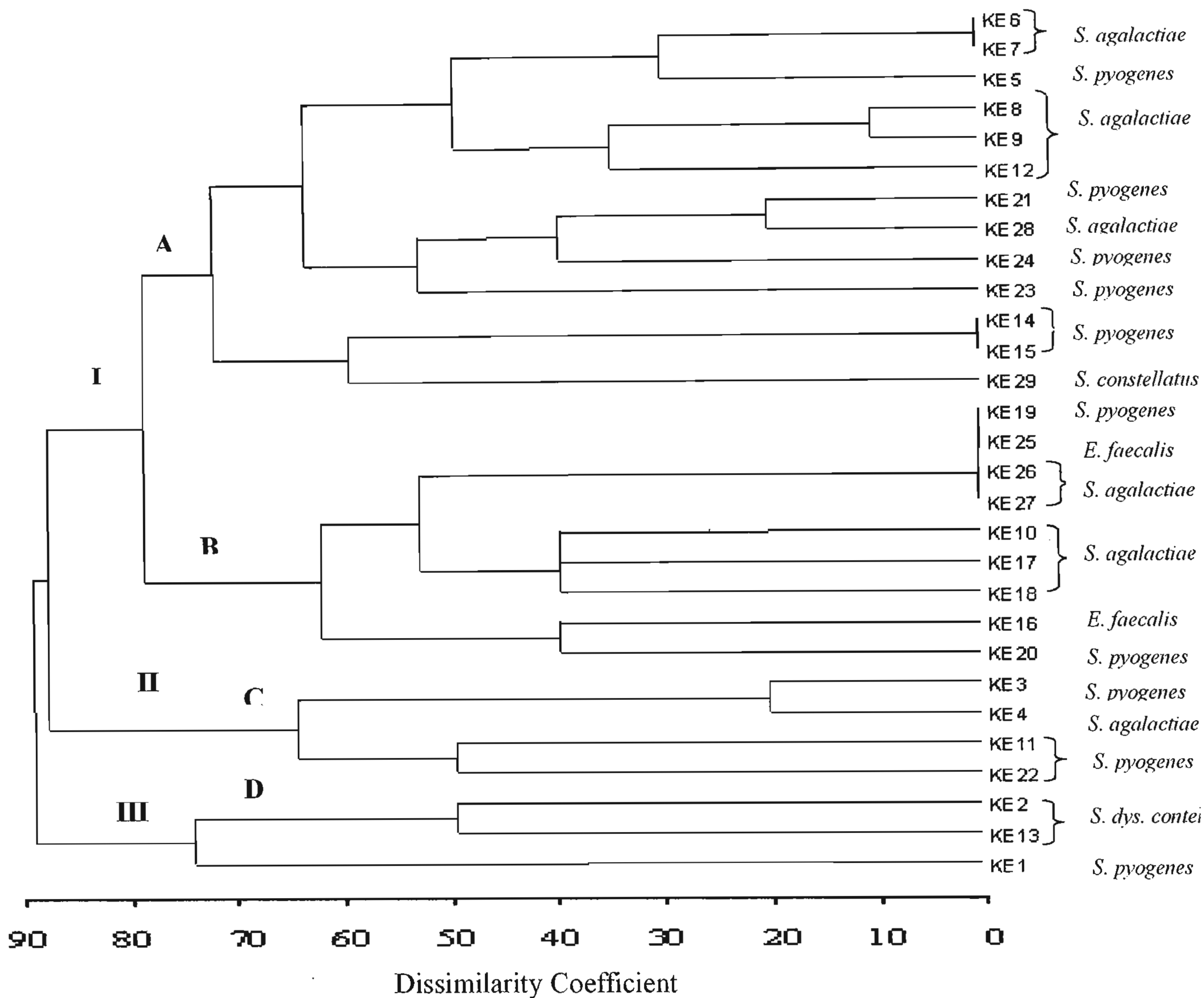

Figure 4.3. Genetic relationship between 29 streptococcal isolates, established by clustering analysis of PFGE patterns obtained with restriction enzyme SmaI. The tree was generated by the unweighted pair group method, with arithmetic means.

The present study shows that there is considerable polymorphism among PFGE patterns, which is important since it allows the inference that isolates that have the same genomic restriction pattern are likely to represent a single strain. PFGE analysis using *Sma*I as the restriction enzyme divided the 29 streptococcal isolates into three groups (I, II, II). At 80% dissimilarity, group I was further divided into four clusters, A to D (Fig. 4.3).

Two pairs (KE 6 and KE 7, and KE 14 and KE 15) of the 22 isolates of group I, belonging to cluster A, showed indistinguishable PFGE patterns (100% homology), hence KE 6 and KE 7 were interpreted as representing a single strain of *Streptococcus pyogenes* and KE 14 and KE 15 as representing a single strain of *Streptococcus agalactiae.* Four (KE 19, KE 25, KE 26, and KE 27) of the 22 isolates of group I, belonging to cluster B, showed indistinguishable PFGE patterns (100% homology) and these isolates were interpreted as representing a single strain (fig. 4.2A, lanes: 9; 11; 12; and 13, fig.4.3 and Table 4.3). This 100% homology was not expected among these four isolates as they were initially identified as different species, except KE 26 and KE 27, which were initially identified as *Streptococcus agalactiae* (Table 2.1). It is possible, though unlikely that the specimens were mislabeled in the past, or that there was contamination or laboratory error. KE 8 and KE 9 had identical PFGE patterns with 85% homology, and they differed by only two bands (fig. 4.2A, lane 5 and lane 6, Fig. 4.3 [group I, cluster A] and Table 4.3).

Since isolates with identical restriction patterns are interpreted as representing the same strain or a recent derivative (Murray *et al.*, 1990), KE 8 and KE 9 from group I cluster A represent one strain of *Streptococcus agalactiae* and KE 21 and KE 28 from group I cluster A another strain. In group II, there were only four isolates (KE 3, KE 4, KE 11 and KE 22) with KE 3 and KE 4 showing 80% similarity between them, representing a single strain. Isolates that were more than 75 % dissimilar represent a recent derivative. Group III had only three isolates (KE1, KE2, and KE13) clustered together and there is a considerable restriction fragment length polymorphism among them (Fig. 4.2B, lane 1and 2, and Fig. 4.3, group III). PFGE analysis using *Sma*I as a restrition enzyme manage to group or cluster some of the isolates belonging to the same species together. To improve or get even better results with this technique (PFGE) different rare restriction enzymes should been used.

FINAL DISCUSSION AND CONCLUSION

It is important to be able to compare isolates of a particular species. This may be helpful in epidemiological studies, in which the demonstration that different persons are infected with a single strain would suggest that an outbreak had occurred, while the presence of a different strain will point away from this (Murray *et al.*, 1990). Comparing isolates may also have clinical relevance for individual patients. For example, the demonstration that the same strain is present in a post therapy urine culture has clinical and therapeutic implications different from those of the demonstration of different strains.

In the past, bacteria have been compared by using phenotypic properties, including those relating to biochemical reactions, antibiotic resistance, phage typing, bacteriocin typing and serotyping (Table 1.1). Biotyping can be useful when multiple tests are done in a standardized manner, such as with multilocus enzyme analysis (Hall *et al.*, 1996), but routinely available kits, such as API, are not usually sufficient, especially for organisms which grow poorly or are largely non-fermentative. Some techniques may be useful for only a limited number of species, some require individualized reagents for each species or genus, some require a large number of individual assays, and some may not be applicable to all members of a species (Bartie *at al.*, 2000; Mhand *et al.*, 1999; and Hall *et al.*, 1996).

Genotyping techniques have also been used to compare bacterial strains. One type of analysis compares the total plasmids content of isolates. This technique is most useful when there is a reliable and easy lysis method for the organism being investigated and when there is a high copy number of plasmid in all isolates examined (Tang *et al.*, 2002; Skoneman *et al.*, 1997; Greenwood *et al.*, 1995; Lietra *et al.*, 1989; Walla *et al.*, 1988; and Hoimberg *et al.*, 1984). Comparison of chromosomal digestion patterns, using the same electrophoretic conditions as those used for plasmid analysis, have been performed in epidemiological studies, but results typically show a large number of fragments that are close together and may be difficult to analyse (Benson and Ferrieri, 2001). This technique has been more successful when combined with hybridisation using a gene probe to select a small number of fragments that generate more readily visible, and thus

more easily compared, patterns (Vicki *et* al., 2000; Tenover *et al.*, 1997; Maslow *et al.*, 1993).

The application of PCR-based techniques has had a revolutionary impact on the diagnosis of infectious diseases. Because these techniques have the ability to detect or allow analysis of minute amounts of microbial DNA or RNA sequences, they have emerged as a highly sensitive and specific method for identifying pathogens. The PCR-based RAPD fingerprinting technique of utilizing random oligonucleotides to prime DNA synthesis is a particularly powerful typing method (Lin *et al.*, 2001). Unlike the traditional PCR analysis, which requires a specific knowledge of DNA sequences and the application of target-specific sequences, RAPD analysis does not require any specific knowledge of the DNA sequences of the target organism (Welsh *et al.*, 1990). This makes it a tool of great power and general applicability.

The most challenging aspect of the protocol for bacterial sub-typing by RAPD analysis is the selection of suitable primers (Lin *et al.*, 1996). In the present study, five isolates of the entire panel of isolates were used to identify primers that are appropriate for streptococcus typing. Using this approach, a total of four primers was examined for suitability. Of these four primers, three proved to be very discriminatory for the RAPD analysis of streptococcus isolates. The reliability of the RAPD analysis was confirmed by the fact that most isolates of the same species were grouped together on dendograms (Fig. 3.4, 3.6 and 3.8).

PFGE has been used to resolve very large DNA fragments generated from bacterial genomic DNA using restriction endonucleases that have few recognition sites (Berthelot-Herault *et al.*, 2002; Vicki *et al.*, 2000; Rolland *et al.*, 1999; and Murray *et al.*, 1990). The choice of the restriction endonuclease depends on the G + C content of the organism being studied as well as the recognition sequence of the enzyme. *Not*I, for example, has an 8-base pair recognition sequence and cleaves *E. coli* chromosomal DNA an average of 25 times (Dunne *et al.*, 2001; and Hall *et al.*, 1996.). Restriction enzymes with GC rich sequence such as *Sma*I, whose recognition sequence is CCCGGG, cleave *E. coli*

chromosomal DNA numerous times, because *E. coli* has a GC-rich sequence. However, *Sma*I cleaves organisms with a lower G + C content, such as staphylococci, enterococci, and streptococci, less frequently. *Sma*I has been used for digestion in an epidemiological investigation of streptococci (Berthelot-Herault *et al.*, 2002; Allgaier *et al.*, 2001; Vicki *et al.*, 2000).

The present study reports penicillin G resistance patterns of streptococcal isolates and the application of the RAPD analysis, using primer MBPZ-3 and PFGE analysis (Table 5.1) to the differentiation of the same streptococcal isolates from KwaZulu-Natal, Durban Metro area, South Africa. Results obtained from RAPD analysis using primer MBPZ-3 were chosen from others, because MBPZ-3 outgrouped *E.coli* from streptococcal isolates, as was expected, since *E. coli* belongs to a different genus.

The present study shows that there is considerable randomly amplified polymorphism and restriction fragment length polymorphism among the streptococcal isolates, even among isolates of the same species. For many species, comparitive studies indicate that isolates that are indistinguishable by PFGE are unlikely to demonstrate substantial differences by other typing techniques (Tenover *et al.,* 1994; Olsen *et al.*, 1994; Gordille *et al.*, 1993; Schoenmaker *et al.*, 1992; Struelens *et al.*, 1992; and Miranda *et al.*, 1991). In the present study the argument will be based more on PFGE analysis results than RAPD analysis results.

Polymorphism is important, since it allows the inference to be made that isolates that have the same banding patterns are likely to represent a single strain (Mayo *et al.*, 2002; Quale *et al.,* 2001; Renders *et al.*, 1996; Liu *et* al., 1995; and Murray *et al.*, 1990). Since KE 6 and KE 7 exhibited 100% homology by both RAPD and PFGE analysis (Table 5.1) and have the same penicillin G profile (Table 2.2) they can be interpreted as representing a single strain of *Streptococcus agalactiae*. Also KE 26 and KE 27 exhibited 100% homology by both RAPD and PFGE analysis (Table 5.1) and have the same penicillin G profile (Table 2.2) they can be interpreted as representing a single strain of *Streptococcus agalactiae*.

When PFGE patterns of isolates differ by changes consistant with a single genetic event, i.e. changes resulting in two or three bands difference, like point mutation or insertion/deletion of DNA, it can be concluded that such isolates are closely limited, at least strains belonging to the same species (Tenover *et al.*, 1995). KE 9 and KE 10 can be interpreted as closely related, or strains of *Streptococcus agalactiae*, KE 14 and KE 15 strains of *Streptococcus pyogenes*, since they differ by two bands and they have the same antibiotic relation (Table 2.2).

KE 21 and KE 28 were initially identified as two different species, *Streptococcus pyogenes* and *Streptococcus agalactiae*. However, PFGE analysis exhibited them as strains belonging to the same species (fig. 4.2 group I cluster A), since their PFGE patterns differ only by two bands. These streptococcal isolates (KE 21 and KE 28) also have the penicillin G relation (Table 2.2). The difference in banding patterns between these isolates could be due to mutational gain of a restriction site, which split one restriction fragment into two smaller fragments. It is possible that these strains were mislabled in the past, or that a contamination occured, which resulting in them initially being identified as different strains.

RAPD analysis using each primers exhibited KE 21 and KE 28 as different species. KE 21 was clustered with *S. pyogene* and KE 28 was also always associated with *S. agalactiae* when characterized by RAPD analysis. Since only differences of species can be concluded, results obtained with RAPD analysis with regards to these two isolates are more conclusive than the one obtained by PFGE analysi.

Isolates (KE3 and KE4), whose PFGE patterns differed by changes consistent with two independent genetic events (i.e. four to six restriction bands) were considered possibly to be related species. However, since these isolates were initially identified as different species, have different penicillin G relations (Table 2.2) and also exhibited as different isolates by RAPD anlysis it means that KE 3 and KE 4 are not the strains of the same species. Isolates whose PFGE patterns differed by changes consistent with three or more

Table 5.1. Comparison of clonal relationships among 29 streptococcal isolates with RAPD with MBPZ-3 prime and by PFGE with *Sma*I

Isolates grouped into group I by both RAPD and PFGE	Isolates grouped into group II by both RAPD and PFGE	Isolate(s) grouped into group I by RAPD and into group II by PFGE	Isolates grouped into group II by RAPD and into group I by PFGE	Indistinguishable patterns by both RAPD and PFGE	Indistinguishable patterns by RAPD and related patterns by PFGE	Related patterns by both RAPD and PFGE	Related patterns by RAPD and unrelated patterns by PFGE	Unrelated patterns by RAPD and related patterns by PFGE
(KE3), (KE4), (KE5), KE6, KE7, KE8, KE9, KE10, KE12, KE14, KE15, KE16, KE22, KE24, KE26, KE27, KE28	KE2, KE13	KE1	KE11, KE17, KE18, KE19, KE20, KE21, KE23, KE25, KE29	KE6 and KE7 KE26 and E27	KE8, KE9 and 10, no band difference by RAPD and 2-band difference by PFGE	KE14 and KE15, 3- to 4-bands difference by RAPD and 2-band difference by PFGE, KE1 and KE24, 4-band difference by RAPD and 1- to 2-band difference by PFGE	KE17 and KE18, 2-band difference by RAPD and 7-band difference by PFGE	KE3 and KE4, 6-band difference by RAPD and 4- to 5-band difference by PFGE KE21 and KE28, 8- to 9-band difference by RAPD and 2- to 3-band difference by PFGE

genetic events, i.e. seven or more band differences (Table 5.1), were considered unrelated or distinct.

Interpretation of isolates that differ by only a few bands is rather difficult, because such differences arise within a single individual from inversions, deletions, or other rearrangements of the chromosome, or acquisition or loss of prophage, transposon or plasmid. On the other hand, such differences could indicate that isolates are distantly related (Allgaier *et al.,* 2001; and Murray *et al.*, 1990).

Compared with the procedure of PFGE, which requires a week to complete, RAPD analysis, once the optimal primers and reaction conditions for generation of suitable numbers of DNA bands are determined, takes only a day and prove less laborious. Although RAPD analysis offers good intralaboratory reproducibility, an interlaboratory reproducibility is less predictable (Vu-Thein *at al.*, 1999; Liu *et al.*, 1995; and Swaminathan and Matar, 1993). The differences in the number of strains obtained by each method actually reflect the difference of principle in which these methods are based It was concluded nevertheless that both RAPD and PFGE analysis are useful discriminatory DNA-based techniques for differentiation of clinical streptococcus isolates.

In conclusion, RAPD analysis is more specific, faster, and less laborious.However, results obtained by RAPD analysis are not consistent. The inconsistence of results is not only between laboratories, but even in one laboratory when the reaction is performed at different times. Although PFGE analysis takes a week to give results, provide greater discrimination and give reproducible results. The present study showed that results obtained by PFGE analysis should be interpreted with caution until further data is available to confirm of complement PFGE results. The present study indicated that RAPD should serve as a first screen for clinical isolates of streptococcus typing because of the simplicity and high speed of the technique and that the bacterial grouping results attained are similar with those of PFGE analysis

REFERENCES

1. **Abbott, L.A., F.A. Bisby, and D. J. Rojers.** 1985. Taxonomic analysis in biology. Computers, models, databases. Colombia University Press. New York.

2. **Alam, S., S. R. Bradford, R. A. Whiley, and D. Beighton.** 1999. PCR-based methods for genotyping viridins group streptoccoci. J. Clin. Microbial 37: 2772-2776.

3. **Allardet-Servent, A., N. Bouziges, and M-J. Carles-Nurit.** 1989. Use of low-frequency-cleavage restriction endonucleases for DNA analysis in epidemiological investigations of nosocomial bacterial infections. J. Clin. Microbiol. **27**: 2057-2061.

4. **Allgaier, A., R. Goethe, H. J. Wisselink, H. E. Smith, and P. Valentin-Weigang.** 2001. Relatedness of *Streptococcus sius* isolates of various serotypes and clinical backgrounds as evaluated by macrorestriction analysis and expression of potential virulence traits. J. Clin. Microbiol. **39**: 445-453.

5. **Anderson, M.T., K. Gull, and D.W. Denning.** 1996. Molecular typing by random amplification of polymorphic DNA and M13 southern hybridization of related paired isolates of *Aspergillus fumigatus*. J. Clin. Microbiol. **34**: 87-93.

6. **Anthony, B. F., E.L. Kaplan, and L.W. Wannamaker.** 1969. Attack rates of acute nephritis after type 49 streptoccocal infection of the skin and the respiratory tract. J. Clin. Invest. **48:** 1697-1704.

7. **Arbeit, R.D., A. Slutsky, T.W. Barber, J.N. Maslow, S. Niemezyk, J.O. Falkimham, III, G.T. O'Conner, and C.F. van Reyn.** 1993. Genetic diversity among strains of *Mycobacterium avium* causing monoclonal and polyclonal bacterium in patients with AIDS. J. Infect. Dis. **167**: 1384-1390.

8. **Avis, J.C.** 1994. Molecular markers, natural history and evolution. Chapman & Hall, New York, N.Y.

9. **Barbier, N., P. Saulnier, E. Chachaty, S. Dumontier, and A. Andremont.** 1996. Random amplified polymorphic DNA typing versus pulsed-field gel electrophoresis for epidemiological typing of vancomycin-resistant entirococci. J. Clin. Microbiol. **34**: 1096-1099.

10. **Barrett, T.J., H. Lier, J.H. Green, R. Khakhria, J.G. Wells, B.P. Bell, K.D. Greene, J. Lewis, and M.P. Griffin.**1995. Laboratory investigation of a multistate food-borne outbraek of *Escherichia coli* O157:H7 by using pulsed-field gel electrophoresis and phage typing. J. Clin. Microbiol. **32**: 3013-3017.

11. **Barthelot-herault, F., C. Marois, M. Cottschalk, and M. Kobisch.** 2002. Genetic diversity of *Streptococcus suis* strains isolated from pigs and humans as revealed by pulsed-field gel electrophoresis. J. Clin. Microbiol. **40**: 615-619.

12. **Bartie, K.L., M.J. Wilson, D.W. Williams, and M.A.O. Lewis.** 2000. Macrorestriction fingerprinting of *Streptococcus milleri* group bacteria by pulsed-field gel electrophoresis. J. Clin. Microbiol. **38**: 2141-2149.

13. **Benson, J.A., and P. Ferrieri.** 2001. Rapid pulsed field gel electrophoresis method for group B streptococcus isolates. J. Clin. Microbiol. **39**: 3006-3008.

14. **Benson, K.D., J.B. Luchansky, J.A. Elliot, A.J. Degnan, H.J. Wiienberg, J.M. Thornbery, and H.H. Kay.** 2002. Pulsed-field fingerprinting of vaginal group B streptococcus in pregnancy. Els. Sci. Inc. **100**: 545-551.

15. **Bergan, T.** 1978. Phage typing of *Proteus*, in Methods in Microbiology. Academic Press, London. **11**: 243-258.

16. **Bergan, T.** 1979. Bacteriophage-typing of shigella, in Methods in Microbiology. Academic Press, London. **11**: 178-286.

17. **Bingen, E., S. Bonacorsi, P. Rohrlich, M. Duval, S. Lhopital, N. Brahimi, E. Vilmer, and R.V. Georing.** 1996. Molecular epidemiology provides evidence of genotypic heterogeneity of multidrug-resistant *Pseudomonas aeruginosa* serotype O: 12 outbreak isolates from a pediatric hospital. J. Clin. Microbiol. **34**: 3226-3229.

18. **Birren, B., and E. Lai.** 1993. Pulsed-field gel electrophoresis: a practical guide. San Diego, CA. Acad. Press. Inc.

19. **Black, W.C.** 1993. PCR with arbitrary primers: approach with care. Ins. Mol. Biol. **2**: 1-6.

20. **Black, W.C., I.V. DuTeau, N.M. Puterka, G.J. Nechols, J.R.Pettorini.** 1992. Use of random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) to detect DNA polymorphisms in aphids. Bull. Ent. Res. **82**: 151-159.

21. **Caetano-Anolles, G.** 1993. Amplifying DNA with arbitrary oligonucleotide primers. Genome Res. **3**: 85-94.

22. **Carle, G. F., M. Frank, and M. V. Olson.** 1986. Electrophorestic separation of large DNA molecules by periodic inversion of the electric field. Science. **232**: 65-68.

23. **Casariego, E., A. Rodriguez, J.C. Orredoira.** 1996. Prospective study of *Streptococcus milleri* bacteremia. Eur. J. Clin. Microbiol. Infect. Dis. **15**: 194-200.

24. **Chachaty, E., P. Saulneir, A. Martin, N. Mario, and A. Andremont.** 1994. Comparison of ribotyping, pulsed-field gel electrophoresis and random amplified polymorphic DNA for typing *Clostridium difficile* strains. FEMS. Microbiol. Lett. **122**: 61-68.

25. **Chatellier, S., H. Huet, S. Kenzi, A. Rosenau, P. Geslin, and R. Quentin.** 1996. Genetic diversity of rRNA operons of unrelated *Streptococcus agalactiae* strains isolated from cerebrospinal fluid of neonates suffering from meningitis. J. Clin. Microbiol. **34**: 2741-2747.

26. **Chatellier, S., M. Gottschalk, R. Higgins, R. Brousseau, and J. Harel.** 1999. Relatedness of *Streptococcus sius* serotype 2 isolates from different geographic origins as evaluated by molecular fingerprinting and phenotyping. J. Clin. Microbiol. **37**: 362-366.

27. **Chatellier. S., C. Ramananthsoa, P. Harriau, K. Rolland, A. Rosenau, and R. Quentin.** 1997. Characterization of *Streptococcus agalactiae* strains by randomly amplified polymorphic DNA analysis. J. Clin. Microbiol. **35**: 2573-2579.

28. **Chetoui, H., E. Delhalle, P. Osterrieth, and D. Rousseaux.** 1995. Ribotyping for use in the study of molecular epidemiology of *Serratia marcescens*: comparison with biotyping. J. Clin. Microbiol. **33**: 2637-2642.

29. **Chu, G.** 1986. Bag model for DNA migration during pulsed-field electrophoresis. Proc. Natl. Acad. Sci. **88**: 11071-11075.

30. **Chu, G., D. Vollrath, and R.W. Davis.** 1986. Separation of large molecules by contour-clamped homogeneous electric fields. Science. **234**: 1582-1585.

31. **Coffey, T.J., M. Daniels, L.K. McDougal, C. G. Dowson, F.C. Tenover, and B.G. Spratt.** 1995. Genetic analysis of clinical isolates of *Streptococcus pneumoniae* with high-level resistance to expanded-spectrum cephalosporins. Antimicrob. Agents Chemother. **39**: 1306-1313.

32. **Coffin, J.W. C. Condon, and C.A. Compston.** 1992. Use of restriction length polymorphism resolved by pulsed field gel elctrophoresis for subspecies identification of mycobacteria in the *Mycobacterium avium* complex for isolationofDNA probes. J. Clin. Microbiol. **30**: 1829-1836.

33. **Collier, M.C., F. Stock, P.C. DeGrirolami, M. H. Samore, and C.P. Cartwright.** 1996. Comparison of PCR-based approaches to molecular epidemiologic analysis of *Clostridium difficile*. J. Clin. Microbiol. **34**: 1153-1157.

34. **Corbel, M.J.** 1984. Phage typing of ***Brucella.*** In Methods in Microbiology. Academic Press. London. **16**: 23-74.

35. **Corso, A., E. P. Severina, V.F. Petruk, Y.R. Mauriz, and A. Tomasz.** 1998. Molecular characterization of penicillin-resistant *Streptococcus pneumoniae* isolates causing respiratory disease in the United States. Microb. Dug Rsist. **4**: 325-337.

36. **Davis, M.A., D.D. Hancock, T.E. Besser, and D.R. Call.** 2003. Evaluation of pulsed-field gel electrophoresis as a tool for determining the degree of genetic relatedness between strains of *Escherichia coli.* O157: H7. J. Clin. Microbiol. **41**: 1842-1849.

37. **Davis, T., R.V. Goering, M. Lovegren, J.A. Talbot, M.R. Jacobs, and P.C. Appelboum.** 1999. Molecular epidemiological survey of penicillin-resistant *Streptococcus pneumoniae* from Asia, Europe, and North America. Diadn. Microbiol. Infect. Dis. **34**: 7-12.

38. **Dmitriev, A., Y.Y. Hu, A.D. Shen, A. Suvorov, and Y.H. Yang.** 2001. Chromosomal analysis of group B streptococcal clinical strains: *bac* gene-positive strains are genetically homogenous. FEMS. Microbiol. Lett. **208**: 93-95.

39. **Doern, G.V.** 1995. Susceptibility tests of fastidious bacteria. p. 1342-1343.

40. **Doern, G.V., A.B. Brueggemann, M. Blocker, M. Dunne, H.P. Holley, Jr., K.S. Kehl, J. Duval, K. Kugler, S. Putnam, A. Rauch, and A. Pfaller.** 1998. Clonal relationships among high-level penicillin-resistant *Streptococcus pneumoniae* in the United States. Clin. Infect. Dis. **27**: 270-274.

41. **Doern, G.V., M.A. Pfaller, K. Kugler, J. Freeman, and R.N. Jones.** 1998. Prevalence of antimicrobial resistance among respiratory isolates of *Streptococcus pneumoniae* in North America: 1997 results form SENTRY Antimicrobial Surveillance Program. Clin. Infect. Dis. **27**: 764-770.

42. **Dowson, C.G., A. Hutchison, J.A. Brannigan, R.C. George, D. Hansman, J. Linares, A. Tomasz, J.M. Smith, and B.G. Spratt.** 1989. Horizontal transfer of penicillin-binding protein genes in penicillin-resistant isolates of *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA. **86**: 8842-8846.

43. **Dunne, W.M., Jr., K.S. Kehl, C.A. Holland-staley, A.B. Brueggeman, M.A. Pfaller, and G.V. Doern.** 2001. Comparison of results generated by serotyping, pulsed-field restriction analysis. Ribotyping, and repetitive-sequence PCR used to characterize penicillin-resistant pneumococci from the United States. J. Clin. Microbiol. **39**: 1791-1795.

44. **Eisenstein, B. I.** 1989. New molecular techniques for microbial epidemiolgy and the diagnosis of infectous diseases. J. Infect. Dis. **161**: 595-602.

45. **Edlin, B.R., J.L. Tokars, and M.L.L. Grieco.** 1992. An outbreak of multidrug-reistant tuberculosis among hospitalized patients with acquired immunodeficiency syndrome. N. Engl. J. Med. **326**: 1514-1521.

46. **Elliot, P.M., H. Williams, and I.A.B. Brooksby.** 1993. A case of infective endocarditis in a farmer caused by *Streptococcus equines.* Eur Heart J **14**: 1291-1293.

47. **Ellsworth, D.L., K.D. Rittenhouse, and R.L. Honeycutt.** 1993. Artificial variation in random amplified polymorphic DNA banding pattern. Bio-Techniques **14**: 214-217.

48. **Enright, M.C., and B.G. Spratt.** 1999. Extensive variation in the *ddl* gene of penicillin-resistant *Streptococcus pneumoniae* results from hitchhiking effect driven by the penicillin-binding protein 2b gene. Mol. Biol. Evol. **16**: 1687-1695.

49. **Ezaki, T., R.R. Facklam, and N. Takeuchi.** 1986. Genetic relatedness between the type strain of *Streptococcus anginosus* and minute-colony-forming *beta*-hemolytic streptococci carrying different Lancefield grouping antigeris. Int J Syst Bacteriol. **36**: 345-347.

50. **Farris, J.** 1972. Estimating phylogenetic trees from distance matrices. Am. Nat. **106**: 645-668.

51. **Fasola, E., C. Livdahl, and P. Ferrieri.** 1993. Molecular analysis of multiple isolates of the major serotypes of group B streptococci. J. Clin. Microbiol. **36**:2616-2620.

52. **Filipe, S.R., and A. Tomasz.** 2000. Inhibition of the expression of the penicillin resistance in *Streptococcus pneumoniae* by inactivating of cell muropeptides branching genes. Proc. Natl. Acad. Sci. USA. **97**: 4891-4896.

53. **Filpe, S.R., E. Severina, and A. Tomasz.** 2001. Functional analysis of *Streptococcus pneumoniae* murM reveals the region responsible for its specificity in the synthesis of branched cell wall peptides. J. Biol. Chem. **276**: 39618-39628.

54. **Filpe, S.R., E. Severina, and A. Tomasz. 2002**. Distribution of the moisac structured *murM* genes among natural population of *Streptococcus pneumoniae*. J. Bacteriol.182: 6798-6805.

55. **Filpe, S.R., E. Severina, and A. Tomasz.** 2002. The *murMN* operon: A functional link between antibiotic resistance and antibiotic tolarence in *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA. **99**: 1550-1555.

56. **Filpe, S.R., M.G. Pinho, and A. Tomasz.** 2002. Characterization of *murMN* operon involved in the synthesis of branched peptidoglycan peptides in *Streptococcus pneumoniae*. J. Biol. Chem. **276**: 27768-27774.

57. **Finney, M.** 1993. Pulsed-field gel electrophoresis, p. 2.2.9-2.517. *In* F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (ed), Current protocols in molecular biology, vol. 1. Current protocols, Greene-Wiley, New York.

58. **Fitch, W.M., and E. Margoliash.** 1967. Construction of phylogenetic trees. Sci. **155**: 279-284.

59. **Fitzgerald J.R., D.E. Sturdevant, S.M. Mackie, S.R. Gill, and J.M. Musser.** 2001. Evolutionary genome of *Staphylococcus aureus*: Insight into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. Proc. Natl. Acad. Sci. USA. **98**: 8821-8826.

60. **Fontana, R., G. Amalfitano, and L. Rossi.** 1992. Mechanism of resistance to growth inhibition and killing by *beta*-lactam antibiotics in enterococci. Clin Infect Dis **15**: 486-489.

61. **Fox, K., J. Turner, A. Fox.** 1993. Role of *beta*-hemolytic group C streptococci in pharyngitis: incidence and biochemical characteristics of *Streptococcus equisimilis* and *Streptococcus anginosus* in patients and healthy controls. J. Clin Microbiol. **31**: 804-807.

62. **Garcia-Bustos, J., and A. Tomasz.** 1988. A biological price of antibiotic resistance: major changes in peptidoglycan structure of penicillin-resistant pneumococci. Proc. Natl. Acad. Sci. USA **87**: 5415-5419.

63. **Garcia-Bustos, J., B.T. Chait, and A. Tomasz.** 1988. Altered peptidoglycan structure in a pneumococcal transformant resistant to penicillin. J. Bacteriol. **170**: 21432147.

64. **Garnier, F., G. Gerbaud, P. Courvalin, and M. Galimand.** 1997. Identification of clinically relevant viridans group streptococci to the species level by PCR. J. Clin. Microbiol. **35**: 2337-2341.

65. **Gremmill, R.M.** 1991. Pulsed-filed gel electrophoresis. In advances of electrophoresis. Vat Weinhein Germany. **4**: 1-48.

66. **Georing, R.V.** 1993. Molecular epidemiology of nosocomial infection: analysis of chromosomal restriction fragment patterns by pulsed-field gel electrophoresis. Infect. Contr. Hosp. Epidemiol. **14**: 595-600.

67. **Georing, R.V. and T. D. Duensing.** 1990. Rapid field inversion gel electrophoresis in combination with an rRNA gene probe in the epidemiological evaluation of staphylococci. J. Clin. Microbiol. **28**: 426-429.

68. **Gerardo, S. H., D. M. Citron, and E. J. C. Goldstein.** 1999. PCR fingerprinting analysis for differentiation of *Streptococcus pneumoniae* reinfection versus relapse. Diag. Microbiol. Infect. Dis. **36**: 275-278.

69. **Geslin, P., G. Sissia, J. Jelinkova, A. Fremaux, and J. Motlova.** 1992. Serotype distribution of group B streptococci isolated from human sources in France over a 10-year period (1980-1989). Zentbl. Bakteriol. Suppl. **22**: 484-485.

70. **Gordille, M.E., K.V. Singh, C.J. Baker, B.E. Murray.** 1993. Typing of group B streptococci: comparison of pulsed-field gel electrophoresis and conventional electrophoresis. J. Clin. Microbiol. **31**: 1430-1434.

71. **Gouby, A., M-J. Carles-Nurit, and N. Bouziges.** 1992. Use of pulsed-field gel electrophoresis for investigatuion of hospital outbreaks of *Acinetobacter baumannii*. J. Clin. Microbiol. **30**:1588-1591.

72. **Greenwood, D., R. Slack, and J. Peutherer,** 1995. Medical Microbiology. 4th edition. Churchill Livingstone. Hong Kong. 211-229.

73. **Hall, L.M.C.** 1998. Application of molecular typing to the epidemiology of *Streptococcus pneumoniae*. J. Clin. Pathol. **51**: 270-274.

74. **Hall, L.M.C., R.A. Whiley, B. Duke, R.C. George, and A. Efstratiou.** 1996. Genetic relatedness within and between serotypes of *Streptococcus pneumoniae* from the United Kingdom: analysis of multilocus enzyme electrophoresis, pulsed-field gel electrophoresis and antimicrobial resistance patterns. J. Clin. Microbiol. **34**: 853-859.

75. **Hawkey, P.M.** 1989. Laboratory investigation of nosocomial infection, in medical bacteriology: A Practical Appraoch (eds P.M. Hawkey, and S.A. Lewis), IRL Press, Oxford. 247-269.

76. **Haertl, R., and G. Bandlow.** 1993. Epidemiological fingerprinting of *Enterobacter cloacae* by small-fragment restriction endonuclease analysis and pulsed-field gel electrophoresis of genomic restriction Fragments. **31**: J. Clin. Microbiol. 128-133.

77. **Hermans, P. W. M., M. Sluijter, T. Hopgenboezem, H. Heersma, A. van Belkum, and R. de Groot.** 1995. Comparitive study of five different DNA fingerprinting techniques for molecular typing of *Streptococcus pneumoniae* strains. J. Clin. Microbiol. **33**: 1606-1612.

78. **Hookey, J.V., V. Edwards, B.D. Cookson, and J.F. Richardson.** 1998. Molecular typing of *Staphylococcus aureus* based on PCR-restriction fragment length polymorphism and DNA sequence analysis of the coagulase gene. J. Clin. Microbiol. **36**: 1083-1089.

79. **Hookey, J.V., V. Edwards, B.D. Cookson, and J.F. Richardson.** 1999. PCR-RFLP analysis of the coagulase gene of *Staphylococcus aureus*: application to differentiation of epidenic and sporadic methicillin-resistant strains. J. Hospit. Infect. **142**: 205-212.

80. **Howard, P.J., K.D. Harsono, and J.B. Luchansky.** 1992. Differentiation of *Listeria monocytogenes*, *Listeria ivanovii*, and *Listeria* by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. **58**: 709-712.

81. **Ichiyama, S., M. Ohta, K. Shimokata, N. Kato, and J. Takeuchi.** 1991. Genomic DNA fingerprinting by pulsed-field gel electrophoresis as an epidemiological marker for study of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **29**: 2690-2695.

82. **Inglis, B., W. El.Adhami, and P.R. Stewart.** 1993. Methicillin-resistant and-resistant homologues of *Staphylococcus aureus* occur together among clinical isolates. J. Infect. Dis. **167**: 323-328.

83. **Jacobs, J.A., and E. Stobberingh.** 1996. In-vitro antimicrobial susceptibility of the "*Streptococcus milleri*" group (*Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*). J. Antimicrib. Chemother. **37**: 371-375.

84. **Jedrzejas, M.J.** 2001. Pneumococcal virulence factors: structure and function. Microbiol. Molec. Biol. Rev. **65**: 187-188.

85. **Jones, R.N., M.A. Pfaller, P.R. Rhomberg, and D.H. Walter.** 2002. Tiamulin activity against fastidious and nonfastidious veterinary and human bacterial isolates: intial development of *in vivo* susceptibility test methods. J. Clin. Microbiol. **40**: 461-465.

86. **Kaneko, A., J. Sasaki, M. Shimadzu, A. Kanayama, T. Saika, and I. Kobayashi.** 2000. Comparison of *gyrA* and *parC* mutations and resistance levels among fluoroquinolene-resistant isolates and laboratory-derived mutants of oral streptococci. J. Antimicrib. Chemother. **45**: 771-775.

87. **Kato, H., N. Kato, K. Watanabe, K. Ueno, H. Ushijama, S. Hashira, and T. Abe.** 1994. Application of typing by pulsed field gel electrophoresis to the study of *Clostridium difficile* in neonatal intensive care unit. J. Clin. Microbiol. **32**: 2067-2070.

88. **Kell, C.M., J.Z. Jordens, M. Daniels, T.J. Coffey, J. Bates, J. Paul, C. Gilks, and B.G. Spratt.n** 1993. Molecular epidemiology of penicillin-resistant pneumococci isolates in Nirobi, Kenya. Infect. Immun. **61**: 4382-4391.

89. **Kikuchi, K., T. Enari, K. Totsuka, and K. Shimizu.** 1995. Comparison of phenotypic characteristics, DNA-DNA hybridisation results, and results with a commercial rapid biochemical and enzymatic reaction system for identification of viridins group streptococci. J. Clin. Microbiol. **33**: 1215-1222.

90. **Koneman, E. W., S.D. Allen, W.M. Janda, P.C. Schreckenberger, Jr., and W. C Winn.** 1997. Diagnostic Microbiology. Fifth edition. Lippincott. New York. 578-618.

91. **Kreiswirth, B., J. Kornblum, R.D. Arbeit, W. Eisner, J.N. Maslow, A. McGreer, D.E. Low, and R.P. Novick.** 1993. Evidence for a clonal origion of methicillin-resistance in *Staphylococcus aureus*. Science. **259**:227-230.

92. **Kühn, I., K. Tullus, and L.G. Burman.** 1991. The use of the biochemical fingerprinting system in epidemiological studies of faecal *Enterbacter cloacae* strains from infants in Swedish neonatal wards. Epidemiology and Infection. **107**: 311-319.

93. **Lefevre, J.C., G. Faucon, A.M. Sicard, and A.M. Gasc.** 1993. DNA fingerprinting of *Streptococcus pneumoniae* strains by pulsed-field gel electrophoresis. J. Clin. Microbiol. **31**: 2724-2728.

94. **Lèvy-Frèbault, V.V., M-F. Thorel, A. Vamerot, and B. Gicquel.** 1989. DNA polymorphism in *Mycobacterium paratuberculosis*, "wood pegion mycobacteria," and related mycobacteria analyzed by pulsed-field inversion gel electrophoresis. J. Clin. Microbiol. 2823-2826.

95. **Li, W.-H., and D. Grauer.** 1991. Fundamentals of molecular evolution. Sinauer Associates, Sunderland, Mass.

96. **Lin, A. W., M. A. Usera, T. J. Barret, and R.A. Goldsby.** 2001. Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella enteritidis*. J. Clin. Microbiol. **34**: 870-876.

97. **Liu, P.Y.-F., Y.-J. Lau, B,-S. Hu, J.-M. Shi, W.-S. Tsai, Y.-H. Lin, and C.-Y. Tseng.** 1995. Analysis of clonal relationships among isolates of *Shigella sonnei* by different molecular typing methods. J. Clin. Microbiol. **33**: 1779-1783.

98. **Liu, P.Y.-F., Y.-J. Lau, B,-S. Hu, J.-M. Shi, W.-S. Tsai, Y.-H. Lin, and C.-Y. Tseng.** 1995. Comparison of different PCR approaches for characterization of *Burkholderia* (*Pseudomonas*) *sepacia* isolates. J. Clin. Microbiol. **33**: 3304-3307.

99. **Luna, V.A., and M.C. Roberts.** 1998. The presence of the tet *O* gene in a variety of tetracycline resistant *Streptococcus pneumoniae* serotypes from Washington state. J. Antimicrobial. Chemother. **42**: 613-619.

100. **Luna, V.A., D.B. Jernigan, A. Tice, J.D. Kellner, and M.C. Roberts.** 2002. A novel multiresistant *Streptococcus pneumoniae* serogroup 19 clone from Washington State identified by pulsed-field gel electrophoresis and restriction fragment length patterns. J. Clin. Microbiol. **38**: 1575-1580.

101. **Malathum, K., K.V. Singh, G.M. Weinstock, and B.E. Murray.** 1998. Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. J. Clin. Microbiol. **36**: 211-215.

102. **Malienthiralingam, E., M. E. Campell, J. Foster, J. S. Lam, and D. P. Speert.** 1996. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. J. Clin. Microbiol. **34**: 1129-1135.

103. **Martinez, G., J. Harel, R. Higgins, S. Lacouture, D. Daignault, and M. Gottschalk.** 2000. Characterization of *Streptococcus agalactiae* isolates of bovine and human origin by randomly amplified polymorphic DNA analysis. J. Clin. Microbiol. **38**: 71-78.

104. **Maslow, J.N., A.M. Slutsky, and R.D. Arbeit.** 1993. The application of pulsed-field gel electrophoresis to molecular epidemiology. In: Persing, D.H., T.F. Smith, F.C. Tenover, J.J. White, eds. Diagnostic Molecular Microbiology: Principles and Applications. Washington, DC: American Society of Microbiology. Pp 563-572.

105. **Maslow, J.N., M.E. Mulligan, and R.D. Arbeit.** 1993. Molecular epidemiology: application of contemporary techniques to typing of microorgarnisms. Clin. Infect. Dis. **17**: 153-164.

106. **Matushek, M.G., M.J. Bonten, and M.K. Hayden.** 1996. Rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. J. Clin. Microbiol. **34**: 2598-2600.

107. **Mayo, S.R., J.A. Maeland, and K. Bergh.** 2002. Typing of human isolates of *Streptococcus agalactiae* (group B streptococcus, GBS) strains from Zimbabwe. J. Med. Microbiol. **51**: 595-600.

108. **Meitert, T., and E. Meitert.** 1978. Usefulness, applications and limitations of epidemiology typing methods to evaluate nosocomial infections and the spread of

communicable diseases. In Methods in Microbiology. Academic Press. London. **10**: 1-37.

109. **Mhand, R. A., N. Brahimi, N. Moustaoui, N. E. Mdaghri, H. Amarouch, F. Grimont, E. Bingen, and M. Benbachir.** 1999. Characterization of extended-spectrum β-lactamase-producing *Salmonella typhimurium* by phenotypic and genotypic typing methods. J. Clin. Microbiol. **37**: 3769-3773.

110. **Milla`n, R.S., G. Quindos, J. Garaizar, R. Salesa, J. Guarro, and J. Ponton.** 1997. Characterization of *Scedosporium prolificans* clinical isolates by randomly amplified polymorphic DNA analysis. J. Clin. Microbiol. **35**: 2270-2274.

111. **Miranda, A.G., K.V. Singh, and B.E. Murray.** 1991. DNA fingerprinting of *Enterococcus faecium* by pulsed-field gel electrophoresis may be a useful epidemiologic tool. J. Clin. Microbiol. **29**: 2752-2757.

112. **Moissenet, D., M. Tabone, J. Girardet, G. Leverger, A. Garbarg-Chenon, and H. Vu-Thein.** 1996. Nosocomial CDC group IV c-2 bacteremia: epidemiological Investigation by randomly amplified polymorphic DNA analysis. **34**: 1264-1266.

113. **Montesinos, I., E. Salido, T. Delgado, M. Cuervo, and A. Sierra.** 2002. Epidemiological genotyping of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis at the university hospital and comparison with antibiotyping and protein A and coagulase gene polymorphisms. J. Clin. Microbiol. **40**: 2119-2125.

114. **Munoz, P., T. Coque, M.R. Creixems.** 1993. Purification and characteristics of streptolysin O secreted by *Streptococcus equisimilis* (group c). Infect Immun. **61**:2727-2731.

115. **Munoz, R., J.M. Musser, M. Crain, D.E. Briles, A. Marton, A.J. Parkinson, U. Sorensen, and A. Tomasz.** 1992. Geographic distributionof penicillin-resistant clones of *Streptococcus pneumoniae*: characterization by penicillin-binding protein profile, surface protein A typing, and multilocus enzyme analysis. Clin. Infect. Dis. **15**: 112-118.

116. **Murray, B.E., K.V. Singh, J.D. Heath, B.R. Sharma, and G.M. Weinstock.** 1990. Comparison of genomic DNA of different enterococcal isolates using restriction endonucleases with infrequent restriction site. J. Clin. Microbiol. **28**: 2059-2063.

117. **Murray, R.G.E., D.J. Brenner, and R.R. Colwell.** 1990. Report of the ad hoc committee on approaches to taxonomy within the Proteobacteria. Internat. J. Syst. Bacteriol. **40**: 213-215.

118. **Musser, J.M., S.J. Mattingly, R. Quentin, A. Goudeau, and R.K. Slander.** 1989. Identification of high-virulence clone type III *Streptococcus agalactiae* (Group B streptococcus) causing invasive neonatal disease. Pro. Natl. Acad. Sci. USA **86**: 4731-4735.

119. National Committee for Clinical Laboratory standards. 2000. Method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically-approved standard, 5th ed, M7-A5. Villanova, P.A: NCCLS.

120. **Oliver, S. P., B. E Gillespie, and B. M. Jayaroa.** 1998. Detection of new and persistant *Streptococcus urberis* and *Streptococcus dysgalactiae* intramammary infections by polymerase chain reaction-based DNA fingerprinting. FEMS. Microbiol. Lett. **160**: 69-73.

121. **Olsen, J.E., M.N. Skov, E.J. Threlfall, and D.J. Brown.** 1994. Clonal lines of *Salmonella entirica* serotype *enteritidis* documented by IS200-, ribo-, pulsed-field gel electrophoresis and RLFP typing. J. Med. Microbiol. **40**: 15-22.

122. **Onasanya, A., H.D. Mignouna, and G. Thottappilly.** 2003. Genetic fingerprinting and phylogenetic diversity of *Staphylococcus aureus* isolated from Nigera. Afric. J. Biotech. **2**: 246-250.

123. **Paredes, A., Wong, P., and Mason, E.O.** 1979. Failer of peinicillin to eradicate group B streptococcal colonization in the pregnant woman. Am J. Obstet Gynecol. **135**: 1062-1065.

124. **Parker, M.T.** 1972. Phage typing of *Staphylococcus aureus*. In Methods in Microbiology. Academic Press, London/. 7B: 1-128.

125. **Pujol, C., S. Joly, S. Lockhart, S. Noel, M. Tibayrene, and D.R. Soll.** 1997. Parity of MLEE, RAPD and Ca3 hybridization as fingerprinting methods for *Candila albicans*. J. Clin. Microbiol. **35**: 2348-2358.

126. **Quale, J.M., D. Landman, C. Flores, and J. Ravishankar.** 2001. Comparison of automated ribotyping to pulsed-field gel electrophoresis for genetic fingerprinting of *Streptococcus pneumoniae*. J. Clin. Microbiol. **39**: 4175-4177.

127. **Quentin, R., H. Huet, F. Wang, P. Geslin, A. Goudeau, and R.K. Selander.** 1995. Characterization of *Streptococcus agalactiae* strains by multilocus enzyme genotype and serotype: identification of multiple virulent clone families that cause invasive neonatal disease. J. Clin. Microbiol. **33**: 2576-2581.

128. **Renders, N., U. Romling, H. Verburgh, and A. van Belkum.** 1996. Comparative typing of *Psuedomonas aeruginosa* by random aplification of polymorphic DNA or pulsed-field gel electrophoresis of DNA macrorestriction fragments. J. Clin. Microbiol. **34**: 3190-3195.

129. **Rieseberg, L. H.** 1996. Homology among RAPD frragments in interspecific comparison. Mol. Ecol. **5**: 99-105.

130. **Rohlf, F.J.** 1963. Classification of *aedes* by numerical taxonomic methods (Diptera: Culcidae). Ann. Entomol. Soc. Am. **56**: 798-804.

131. **Rolland, K., C. Morois, V. Siquier, B. Chatteir, and R. Quentin.** 1999. Genetic features of *Streptococcus agalactiae* strains causing severe neonatal infections, as revealed by pulsed-filed gel electrophoresis and *hylB* gene analysis. J. Clin. Microbiol. **37**: 1892-1898.

132. **Rudolph, K.R., A. Parkinson, and M.C. Roberts.** 1998. Molecular analysis by pulsed-field gel electrophoresis and antibiogram of *Streptococcus pneumoniae* serotype 6B isolates from selected areas within United States. J. Clin. Microbiol. **36**: 2703-2707.

133. **Sahm, D. F.** 1996. Molecular typing of bacteria using pulsed-field gel electrophoresis (PFGE). Lab. Med. New. **4**: No. 2.

134. **Saitou, N., and M. Nei,** 1987. The neighbor-joining method: a new method for constructiong phylogenetic trees. Mol. Biol. Evol. **4**: 406-425.

135. **Schable, B., M.E. Villarino, M.S. Favero, J.M. Miller.** 1991. Application of multilocus enzyme electrophoresis to epidemiologic investigation of *Xanthomonas maltophilia.* Infect. Contr. Hosp. Epidemiol. **12**: 163-167.

136. **Schlichting, C., C. Branger, J.M. Fournier, W. Witte, A. Boutonnier, C. Wolz, P. Goullet, and G. Doring.** 1993. Typing of *Staphyloccus aureus* by pulsed-field gel electrophoresis, zymogram, capsular typing, and phage typing: resolution of clonal relationships. J. Clin. Microbiol. **31**: 227-232.

137. **Schoenmaker, D., T. Heimberger, and G. Birkbird.** 1992. Comparison of ribotyping and restriction enzyme analysis using pulsed-field gel electrophoresis for distinguishing *Legionella pnemophila* isolates obtained during nosocomial outbreak. J. Clin. Microbiol. **30**: 1491-1498.

138. **Severin, A., A.M.S. Figueiredo, and A. Tomasz.** 1996. Separationof abnormal cell wall composition from penicillin resistance through genetic transformation of *Streptococcus pneumoniae*. J. Bacteriol. **178**: 1788-1792.

139. **Severin, A., and A. Tomasz.** 1996. Naturally Occurring peptidoglycan variants of *Streptococcus pneumoniae*. J. Bacteriol. **178**: 186-174.

140. **Shinzato, T., and A. Saito.** 1995. The *Streptococcus milleri* group as cause of pulmonary infections. Clin. Infect. Dis. **21**: 238-243.

141. **Singh, K.P., A. Morris, and S.D. Lang.** 1988. Clinically significant *Streptococcus anginosus* (*Streptococcus milleri*) infections: a review of 186 cases. N.Z. Med. J. **101**: 813-816.

142. **Smith, A.M., K.P. Klugman, T.J. Coffey, and B.G. Sratt.** 1993. Gentic diversity of penicillin-binding protein 2B and 2X genes from *Streptococcus pneumoniae* in South Africa. Antimocrob. Agents Chemother. **37**: 1938-1944.

143. **Smyth, C.J., H. Metthews, M.K. Halpenny.** 1997. Biotyping, serotyping and phage typing of *Streptococcus faecalis* isolated from dental plaque in human mouth. J. Med. Microbiol. **23**: 845-854.

144. **Sneath, P.H.A.** 1957. Some thoughts on bacterial classification. J. Gen. Microbiol. **17**: 184-200.

145. **Sneath, P.H.A., and R.R. Sokel.** 1973. Numerical taxonomy. W.H. Freeman & Co., San Fransico, Calf.

146. **Souther, E.M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503-517.

147. **Struelens, M.J. 1996.** Members of the European study group on epidemiological markers. Consensus guidelines for appropriate use and evaluation microbial epidemiology typing systems. Clin. Microbiol. Infect. **2**: 2-11.

148. **Struelens, M.J., A. Deplano, C. Gordard, N. Maes, and E. Serruys.** 1992. Epidemiologi typing and delineating of genetic relatedness of methicillin-resistant *Staphylococcus aureus* by macrorestriction analysis of genomic DNA by pulsed-field gel electrophoresis. J. Clin. Microbiol. **30**: 2599-2605.

149. **Strunden, A.M., K. Ehlert, H. Labischinski, and B. Berger-baĕhi.** 1997. Cell wall monoglycin cross-bridges and methicillin hypersusceptibility in *femAB* null mutant of methicillin-resistant *Staphylococcus aureus*. J. Bacteriol. **179**: 9-16.

150. **Swaminathan, B., G.M. Matar.**1993. Molecular typing methods: definitions, applications, and advantages. In. Persing D.H., T.F. Smith, F.C. Tenover, J.J. White. Eds. Diagnostic Molecular Microbiology: Principles and Applications. Washington, DC: American Society for Microbiology. Pp. 26-50.

151. **Tang, Y., M.G. Waddington, D.H Smith, J.M. Manahan, P.C. Kohner, L.M. Highsmith, H. Li, F.R. Cockerill III, R.L. Thomson, S.O. Montagomery, and D.H. Persing.** 2002. Comparison of protein A gene sequencing with pulsed-field gel electrophoresis and epidemiologic data for molecular typing of methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **38**: 1347-1351.

152. **Tarcy, M., Wanahita, A., Y. Shuhatovich, E.A.Goldsmith, J.E. Clarridge III, and D.M. Musher.** 2001.Antibiotic susceptibility of genetically characterized *Streptococcus milleri* Group Strains. J. Clinic. Microbiol. **45:** 1511-1514.

153. **Tenover, F.C.** 1985. Plasmid fingerprinting. A tool for bacterial strain identification and surveillance of nosocomial and community-acquired infections. Clin. Lab. Med. **5**: 413-436.

154. **Tenover, F.C., R. Arbeit, G. Archer, J. Biddle, S. Byrne, R. Georing, G. Hancock, G.A. Hebert, B. Hill, R. Hollis, W.R. Jarvis, B. Krueiswirth, W. Eisner, J. Maslow, L.K. McDougal, J.M. Miller, M. Mulligan, and M.A. Pfaller.** 1994. Comparison of traditional and molecular methods of typing isolates *Staphylococcus aureus*. J. Clin. Microbiol. **32**: 407-415.

155. **Tenover, F.C., R.D. Arbeit, R.V. Georing, P.A. Mickelen, B.E. Murray, D.H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33**: 2233-2239.

156. **Tenover, F.C., R.D. Arbeit, R.V. Goering.** 1997. How to select and interpret molecular strain typing methods for epidemiological studies bacterial infections: a review for healthcare epidemiologists. Infect. Contr. Hosp. Epidemiol. **18**: 426-439.

157. **Towner, K.J., and A. Cockayne.** 1993. Molecular methods for microbial identification and typing. Chapman and Hall, New York, N.Y.

158. **Van Belkum, A.** 1994. DNA fingerprinting of medically important microorganisms by use of PCR. Clin. Microbiol. Rev. **7**: 174-184.

159. **Varnerot, A., E. Clèment, M. Gheorghiu, and V. Vincent- Lèvy-Frèbault.** 1992. Pulsed-filed gel elctrophoresis of representatives of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCg strains. FEMS. Microbiol. Lett. **98**: 155-160.

160. **Versalovic, J., V. Kapur, E.O. Mason, Jr., U. Shah, T. Koeuth, J.R. Lupski, and J.M. Musser.** 1993. Penicillin-resistant *Streptococcus pneumoniae* strains recovered in Houston: identification and molecular characterization of multiple clones. J. Infect. Dis. **167**: 850-856.

161. **Vicki, A. L., D. B. Jernigan, A. Tice, J. D. Keliner, and M.C. Roberts.** 2000. A novel multiresistant *Streptococcus pneumoniae* serogroup 19 clone from Washington state identified by pulsed-field gel electrophoresis and restriction fragment length patterns. J. Clin. Microbiol. 38: 1575-1580.

162. **Vu-Thein, H., C. Dulot, D. Moissenet, B. Fauroux, and A. Garbarg-Chenon.** 1999. Comparison of randomly amplified polymorphic DNA analysis and pulsed-field gel electrophoresis for typing of *Moraxella catarrhalis* strains. J. Clin. Microbiol. **37**: 450-452.

163. **Welsh, J., and M. McClelland.** 1990. Fingerprinting genonmes using PCR with arbitrary primers. Nucl. Acids Res. **18**: 7213-7219.

164. **Whatmore, A. M., A. Efstratiou, A. P. Pickerill, K. Broughton, G. Wooddard, D. Sturgeon, R. George, and C. G. Dowson.** 2000. Genetic relationships between clinical isolates of *Streptococcus pneumoniae, Streptococcus oralis,* and *Streptococcus mitis:* characterizatrion of "typical" pneumococci and organisms allied to *S. mitis* harbouring *S. pneumoniae* and encoding genes. Infect. Immun. **68**: 1374 –1382.

165. **Whiley, R.A., and D.Beighton.** 1991. Emended description and recognition of *Streptococcus constillatus, Streptococcus intermedius, and Streptococcus anginosus* as distinct species. Int. J. Syst. Bacteriol. **41**: 1-5.

166. **Whiley, R.A., H. Fraser, J.M. Hardie, and D. Beighton.** 1990. Phenotypic differentiation of *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* strains within the "*Streptococcus milleri* group". J. Chin. Microbiol. **28**: 1497-1501.

167. **Whiley, R.A., L.M.C. Hall, J.M. Hardie, and D. Beighton.** 1997. A study of small colony, β-haemolytic, Lancefield group C streptococcus within the anginosus group: description of *Streptococcus constellatus* subsp. *pharynges* subsp. nov., associated with human throat and pharyngitis. Int. J. Syt. Bacteriol. **49**: 1443-1449.

168. **Whiley, R.A., L.M.C. Hall, J.M. Hardie, and D. Beighton.** 1999. Genotypic and Phenotypic diversity within *Streptococcus anginosus*. Int. J. Bacteriol. **47**: 645-650.

169. **Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey.** 1991. DNA polymorphisms amplified by arbitrary primers are useful genetic markers. Nucl Acids Res. **18**: 6531-6535.

170. **Whittam, T.S., H. Ochman, and R.K. Selander.** 1983. Multilocus genetic structure in natural population of *Escherichia coli*. Proc. Natl. Acad. Sci USA. **80**: 1751-1755.

171. **Yano, H., M. Suetake, A. Kuga, K. Irinoda, R. Okamoto, T. Kobayashi, and M. Inoue**. 1999. Pulsed-field gel electrophoresis analysis of nasopharyngeal flora in children attending a day-care centre. J. Clin. Microbiol. 38: 625-629.

172. **Yao, J.D.C., J.M. Cnly, and M. Krajden.**1995. Molecular typing of *Xanthomonas maltophilia* by DNA macrorestriction analysis and randomly amplified DNA polymorphic DNA analysis. J. Clin. Microbiol. 33: 2195-2198.

173. **Zighelboin, S., and A. Tomasz.** 1980. Penicillin-binding proteins of multiply antibiotic-resistance South Africa strains of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. 17: 434-442.