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THE EMERGENCE OF PENICILLIN RESISTANCE IN NASOPHARYNGEAL AND ORAL ISOLATES OF STREPTOCOCCUS PNEUMONIAE AND VIRIDANS STREPTOCOCCI FOLLOWING BETA-LACTAM ANTIBIOTIC EXPOSURE

*

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Submitted in fulfilment of the
conditions for the degree of Master of
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University of the Witwatersrand, Johannesburg.
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To My Family
and Andrew

PREFACE

The work for this thesis was carried out under the co-supervision of Professor HJ Koornhof and Dr KP Klugman in the Department of Medical Microbiology, University of the Witwatersrand, Johannesburg, from January 1988 to May 1989.

It was my intention, in this study, to demonstrate the influence hospitalization and the use of ß-lactam antibiotics have on the development of penicillin resistance in South African strains of S. pneumoniae and the viridans streptococci, both of which have demonstrated a marked increase in resistance over the last ten years. I also wished to investigate the mechanisms of resistance of both the pneumococci and the viridans streptococci as these have been the subject of much interest in recent years.

DECLARATION

I declare that this dissertation is my own unaided work. Where use was made of the work of others, it has been duly acknowledged in the text. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

INGRID SUSAN VAN DEN BERG
Second day of June, 1990.



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ABSTRACT

THE EMERGENCE OF PENICILLIN RESISTANCE IN NASOPHARYNGEAL AND ORAL ISOLATES OF *STREPTOCOCCUS PNEUMONIAE* AND VIRIDANS STREPTOCOCCI FOLLOWING BETA-LACTAM ANTIBIOTIC EXPOSURE

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Increasing levels of penicillin-resistant *Streptococcus pneumoniae* and viridans streptococci are becoming a major cause for concern, particularly in South Africa. To investigate this problem 100 paediatric patients, admitted to Baragwanath Hospital during the period April to July 1988, were swabbed nasopharyngeally and orally.

On admission 24 out of 53 (45%) pneumococcal carriers carried penicillin-resistant strains, while on discharge 33 strains out of 45 (73%) were penicillin-resistant ($p = 0.003$). The corresponding figures for high level penicillin-resistant strains were 7 out of 53 (13%) on admission to 20 out of 45 (44%) on discharge ($p = 0.001$). Treatment with ß-lactam antibiotics reduced the pneumococcal carriage rates by eliminating penicillin-susceptible strains while many children who were not carriers on admission became carriers of penicillin-resistant strains during hospitalisation. In the case of penicillin-resistant viridans streptococci in the oral cavity the use of ß-lactam antibiotics was accompanied by significant increases in both penicillin-resistant and high level resistant strains.

An examination of the API 20 Strep system found it to be a reliable means of identifying *S. mitis* strains, but unreliable in the identification of *S. sanguis* (when compared with extensive conventional biochemical tests and a Facklam-based screening method devised by Ruoff and Kunz [63]).

Penicillin-binding protein (PBP) studies were also conducted. Amongst the API-identified *S. mitis* and *S. sanguis* strains, PBPs in the 66 to 62-Kd and 69 to 54-Kd ranges respectively, underwent a decrease in penicillin affinity. Amongst the pneumococcal strains, PBP 2b exhibited both a decrease in penicillin affinity and an increase in molecular weight. A new PBP was demonstrated in one of the moderately susceptible pneumococcal isolates.

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Chapter 1
INTRODUCTION

1.1 PENICILLIN RESISTANCE IN STREPTOCOCCUS PNEUMONIAE

1.1.1 History

The pneumococcus is the leading cause of bacterial pneumonia and an important cause of meningitis, bacteraemia and otitis media [1]. When penicillin was first introduced as a therapeutic agent in the 1940's, it led to a marked reduction in both the morbidity and mortality associated with *Streptococcus pneumoniae*. Prior to 1967 all strains of *S. pneumoniae* were thought to be highly sensitive to penicillin [2]. However, in 1967 Hansman and Bullen first described the isolation of a resistant pneumococcus from a patient in Sydney, Australia [3]. Since that year, although most pneumococcal isolates are still exquisitely sensitive to penicillin, sporadic reports of the isolation of resistant pneumococci have continued from centres all over the world [4 - 14], revealing both an increase in the number of resistant pneumococci and an increase in the degree of resistance. In a review article in 1987 [1] Appelbaum pointed out that a high prevalence of penicillin-resistant pneumococci has emerged in various countries in the last ten years, with particularly high levels being noted in South Africa (62%) [14], Spain (51%) [12], Israel (28%) [10], New Guinea (22%) [13], Oklahoma, U.S.A. (15.5%) [7] and New Mexico (14.5%) [5].

Antibiotic-resistant pneumococci were first described in South Africa in 1977 [14, 15]. Koornhof *et al*, in 1978 [16], reported a high prevalence rate of multiply-resistant pneumococci in paediatric patients in Johannesburg hospitals. Van den Ende and Rotter [17], in a multi-centre study on the aetiology of bacteraemia and meningitis in South Africa, reported prevalence rates of penicillin-resistant *S. pneumoniae* blood culture isolates in Johannesburg, of 4,1% in 1983, rising to 5,5% in 1984. The reason why more resistant isolates were not reported from the majority of other centres in this study, may have been a true reflection of the regional distribution of such strains in South Africa, but more likely reflected problems in the recognition of penicillin-resistant strains at laboratory level [18]. Klugman and Koornhof [19] analysed strains of *S. pneumoniae* isolated from cultures of blood and cerebrospinal fluid, from 1979 to 1986. During this period, strains resistant to one or more of penicillin, tetracycline, erythromycin, clindamycin, rifampicin and chloramphenicol increased from 3.8% to 14.1% among isolates from blood, and from 6.8% to 14.1% among cerebrospinal fluid isolates.

1.1.2 The Effect of Antibiotics on the Development of Resistance

Although Saah *et al* [7] in a small study with few cases of documented invasive pneumococcal disease did not find disease caused by penicillin-resistant pneumococci, most of the more recent studies [6, 20 - 25] have correlated prior antibiotic exposure with pneumococcal disease. Robins-Browne and Kharsany [20] have shown that individuals exposed to antibiotics are more likely to carry resistant pneumococci than individuals not receiving antibiotics. This is particularly the case in infants and young children, who not only have a higher carriage rate of relatively-resistant pneumococci [23], but are also more likely to carry the serogroups commonly associated with resistance, namely serogroups 6, 14, 19 and 23 [25].

1.1.3 The Hazards of Hospitalisation

In South Africa, penicillin-resistant pneumococci are most likely to be implicated in patients who have been in hospital or who have a hospital-acquired infection [2, 6, 14]. Robins-Browne and Kharsany [20] showed that the carrier rate of penicillin-resistant pneumococci was three times higher in patients hospitalised for more than 24 hours than in those who had been admitted more recently, thus bearing out the suggestion that resistant bacteria are often acquired in hospital. An earlier study which gave similar results, was carried out by Ward *et al* [26] in which he found 20% of patients at six black hospitals in Johannesburg carrying resistant pneumococci, while only 2% of persons in the community carried resistant strains. They also found a carriage rate of resistant pneumococci of only 0,5% in other hospitals (for whites, Asians and coloureds), thereby implying that this problem is particularly rife in black hospitalised patients. Overcrowded hospitals provide an ideal environment for the spread of nosocomial infections and resistant streptococci [2], as the lack of space and other considerations, such as continuous surveillance of carriers, make it very difficult for infected patients and known carriers to be isolated.

1.1.4 The Emergence of Multiple Antibiotic Resistance in Streptococcus pneumoniae

One of the major concerns associated with penicillin resistance in pneumococci is that it is increasingly being accompanied by resistance to other antimicrobial drugs as well, including chloramphenicol, erythromycin, lincomycin and cotrimoxazole [2]. Appelbaum *et al* [27], found that in their study pneumococci insensitive to two or more drugs (including penicillin) belonged to capsular groups 6 and 19 (in other words, those most commonly found in infants) and this may well be related to the frequent administration of antibacterial drugs for the treatment of infections early in childhood. In a study carried out in Johannesburg in 1977 [14], Jacobs and his co-workers found that 29% of the patients and 2% of the hospital staff members tested

in their study carried penicillin-resistant strains with MICs of between 0.12 and 4 μg/ml. Multiply resistant pneumococci (resistant to beta-lactam antibiotics, erythromycin, clindamycin, tetracycline and chloramphenicol) were isolated from 128 carriers amongst the 977 tested. Isolates from 40 other carriers were resistant to penicillin alone, or to penicillin and chloramphenicol or to penicillin, chloramphenicol and tetracycline. In a study of adults with pneumonia by Pallares *et al* [22] in Spain, 79% of penicillin-resistant pneumococci showed multiple antibiotic resistance. In South Africa this development has been shown to be associated with antibiotic use and nosocomial spread among children within hospitals [2, 20, 26].

1.2 PENICILLIN RESISTANCE IN VIRIDANS STREPTOCOCCI

1.2.1 History

The viridans streptococci are the most important group of bacteria causing subacute bacterial endocarditis. In a 1973 survey of streptococcal bacteraemia, the viridans streptococci were identified in 54% of cases [28], while in other studies, *Streptococcus sanguis* strains have been isolated from 30 - 40% of diagnosed cases [29, 30].

Because the viridans streptococci have long been shown to be highly susceptible to penicillin, this agent has been drug of choice for viridans streptococcal endocarditis for 40 years [31]. However, increased penicillin resistance has also been noted in the viridans group of streptococci. One study [32] reported that 27% of patients with endocarditis had infections with relatively penicillin-resistant viridans streptococci (MIC > 0,1 μg/ml). Six South African strains of nasopharyngeal viridans streptococci having MICs of penicillin ranging from 1 - 32 μg/ml have been

recorded [31] and in another study of 15 South African strains [33], *S. sanguis* isolates having a mean penicillin MIC value of 7 μg/ml (range 0.5 - 16 μg/ml) and *Streptococcus mitis* isolates with a mean MIC of 12 μg/ml (range 4 - 16 μg/ml) were demonstrated.

1.2.2 Antibiotics and Hospitalisation - Causes of Resistance

Colonization of the oropharynx by penicillin-resistant streptococci (other than pneumococci) was already described in 1976 in patients receiving prolonged penicillin therapy [34]. Viridans streptococci most importantly cause infective endocarditis, but are also capable of causing infections in other systems [35]. Spread of such resistant streptococci could occur particularly easily in overcrowded paediatric wards which could, in turn, serve as a reservoir of resistant streptococci for gradual dissemination to the community. Penicillin-resistant viridans streptococci are not only isolated from those who have received prior antibiotic therapy. In fact, these organisms have been isolated from the oral flora of patients, regardless of whether or not they have recently received penicillin [36, 37]. In a study by Hess *et al* [38], penicillin-resistant strains of viridans streptococci with MICs of > 0.8 units/ml were demonstrated in 39% of children. However, this high percentage was not due to previous use of penicillin, which had not been administered to the children in the two months prior to the sampling.

1.2.3 Resistance to Other Beta-lactam Agents

As with the pneumococci, penicillin-resistant viridans streptococci, too, show beta-lactam antibiotic cross-resistance, as well as high level resistance to streptomycin [31, 33]. Bourgault *et al* tested the antimicrobial susceptibilities of 78 viridans streptococcal strains and found that most of the ß-lactam antibiotics

tested showed varying degrees of reduced activity against these organisms [39]. In general, the strains of *S. mitis* and *S. sanguis* II required higher concentrations of antibiotics for inhibition than did the other species of viridans streptococci tested.

1.2.4 Carriage of Resistant Oral Streptococci and the Initiation of Bacteraemia

Hess *et al* [38] investigated the possible relationship between the presence of penicillin-resistant organisms in the oral flora and the incidence of post-extraction bacteraemia in children receiving prophylactic parenteral penicillin. Bacteraemia due to viridans streptococci occurred in 12% of children, but no relationship could be demonstrated between the presence of penicillin-resistant viridans streptococci in the oral cavity and the occurrence of post-extraction bacteraemia.

1.2.5 Proposed Study on Antibiotic Resistance in α-haemolytic Streptococci at Baragwanath Hospital

It is clear that penicillin resistance in *S. pneumoniae* and viridans streptococci has become an increasing problem, particularly in overcrowded hospitals where patients are prone to nosocomial infections caused by resistant streptococci, and are liable to become carriers of these organisms. One of the aims in this study, therefore, was to assess carriage rates and penicillin resistance levels of nasopharyngeal and oral isolates of *S. pneumoniae* and viridans streptococci taken from paediatric patients at Baragwanath Hospital for the duration of their stay in hospital. The extent of multiple antibiotic resistance in hospital isolates was also determined. In addition, the possible relationship between the carriage of resistant streptococci and the subsequent initiation of bacteraemia was investigated, and the serogroup distribution of the penicillin-resistant strains of pneumococci was determined in order to ascertain the predominant serogroups associated with penicillin resistance.

1.3 MECHANISMS OF RESISTANCE

1.3.1 S. pneumoniae

The mechanisms by which organisms develop resistance to antibiotics are of great scientific interest. In the case of penicillin-resistant laboratory mutants of pneumococci which have been well-studied [40, 41], resistance appears to be chromosomally mediated in a stepwise fashion [42]. In fact, one reason for the long delay in the emergence of penicillin resistance in pneumococci may be that high levels of resistance in this organism result from the cumulative effect of multiple mutations, which are associated with alterations in its penicillin-binding proteins (PBP's) [43, 44]. These PBP's are membrane-bound enzymes that catalyse the terminal steps in the assembly of the bacterial cell wall and are the targets of ß-lactam antibiotics [45]. Several PBP's have been described in *S. pneumoniae* based on their molecular size and assigned to three major groups 1, 2 and 3. The PBP groups 1 and 2 have been further divided into subgroups 1a, 1b and 1c, and 2a', 2a and 2b [44]. Recently PBP's of high-level penicillin-resistant strains (MIC $\geq$ 2μg/ml) isolated from different geographical sites were compared [46]. This study also supports the view that altered penicillin-binding affinities of different PBP's are necessary for penicillin resistance. Certain studies [40, 41, 47, 48] have claimed that PBP 1a of susceptible pneumococci is not detectable in resistant bacteria; instead, resistant strains exhibit PBP 1c (which is immunologically related to PBP 1a). These studies propose that the appearance of PBP 1c and simultaneous disappearance of PBP 1a (which occur at a distinct level of penicillin resistance) involves the gradual accumulation of point mutations within the gene for PBP 1a, lowering the capacity of the protein for antibiotic binding [48]. At some point, this process may result in the generation of a processing signal, causing the proteolytic splitting of PBP 1a to 1c [48].

PBP 2b has also been shown to have greatly diminished penicillin affinity in strains of higher resistance levels [40 - 42]. Immunological assays of the membranes of penicillin-resistant strains indicate that these bacteria continue to produce PBP 2b but that the greatly decreased penicillin affinity of this protein no longer allows its detection by the [^{3}H] penicillin-binding assay [47].

Handwerger and Tomasz [48] have named four PBP's involved in the acquisition of penicillin resistance: 1a; 1b; 2a; and 2b. During selection for penicillin-resistant transformants of gradually increasing resistance levels, the order in which the individual PBP's of the susceptible laboratory R6 strain are changed towards lower affinity may reflect the order of relative penicillin affinities of the pneumococcal PBP's, in other words, 1a > 2a > 1b >2b.

Chalkley and Koornhof [49] have compared the PBP profiles of a limited number of wild type South African strains (showing penicillin susceptibility, moderate susceptibility and resistance) and, in contrast to the above-mentioned studies, found PBP 2b to be the only PBP showing molecular weight alterations corresponding to increasing resistance. In this study, PBP's 1a, 1b and 1c were present in both susceptible and resistant clinical isolates. In other words, the mutual exclusiveness of PBP 1c versus 1a and 1b for penicillin resistance, as proposed by Tomasz *et al* [50] in transformation experiments in the laboratory, was not observed when wild type resistant strains were examined.

Therefore, in order to clarify further the role of PBP's 1a, 1b, 2a and 2b in the development of resistance in South African isolates of *S. pneumoniae*, further South African strains, isolated in 1988, were investigated in this study.

1.3.2 Viridans Streptococci

According to Farber *et al* [31, 33] it would seem most likely that the viridans streptococci share a common mechanism of resistance to that of the pneumococci. Several studies have suggested that major alterations in the PBP's of penicillin-resistant viridans streptococci are associated with increasing MICs of penicillin. In one such study [33], the PBP's of two penicillin-resistant South African strains of *S. mitis* differed markedly from those of two penicillin-susceptible strains, but were identical to those seen in a penicillin-resistant strain isolated in Boston. Quinn *et al* [51] investigated the mechanism of resistance to penicillin in two penicillin-resistant clinical isolates of viridans streptococci (a strain of *Streptococcus intermedius* and a strain of *S. mitis*). They found that the mechanism of resistance in both strains was associated with diminished affinity for penicillin of their penicillin-binding proteins, as compared with those of penicillin-susceptible control strains. It would, therefore, be of interest to investigate more closely the PBP patterns of penicillin-susceptible and penicillin-resistant viridans streptococci , and note any differences that exist between the two groups. It would be necessary to look at the different species of viridans streptococci individually in order to determine whether different PBP patterns exist from one species to the next.

1.4 IDENTIFICATION OF THE VIRIDANS STREPTOCOCCI

In order to conduct the PBP pattern studies on the various viridans streptococcal species, it will be necessary to ensure that the designations of our isolates, especially those studied for changes in PBP's, are in accordance with recognised classification systems. Conventional tests employed routinely in our laboratory to identify the viridans streptococcal species (namely, growth on bile-aesculin, production of H_2O_2, hydrolysis of aesculin and/or hydrolysis of arginine) were based on the Colman and Williams [52] classification and used accordingly. However, too few

tests were being used and designated species showed variable reactions with these tests resulting in inconsistent identifications. This is a common problem experienced in the identification of the viridans streptococci, and so, although the characteristics of viridans streptococcal species have been described by a number of authors [52 - 55], this heterogeneous group of organisms remain very poorly defined. The problem is further compounded by several classifications which bear very little resemblance to each other. A number of researchers [56 - 61] have attempted to establish a correlation between the physiological and serological characteristics of the viridans streptococci, but until recently they have not been completely successful in this regard. As a result, identification schemes requiring physiological tests alone have been extensively investigated by a number of authors [52 - 55, 62 - 65]. These were, unfortunately, often based on different classifications of this group of organisms.

The classification scheme proposed by Facklam [55] is widely regarded as the most authoratative of all the classifications that have been devised. Apart from a few differences, his scheme resembled closely that of Deibel and Seeley as set out in the 8th edition of the authoratative Bergey's manual of determinative Bacteriology [66]. Facklam recognised ten species, namely *Streptococcus uberis*, *Streptococcus mutans*, *S. sanguis* I, *Streptococcus salivarius*, *Streptococcus MG-intermedius* (now referred to as *S. intermedius* [67]), *S. sanguis* II, *S. mitis*, *Streptococcus anginosus-constellatus* (now referred to as *S. constellatus* [67]), *Streptococcus morbillorum* and *Streptococcus acidominimus*. On comparing his scheme with those of others, Facklam [55, 67] concluded that only his scheme and that of Parker and Ball [35] were of any use in differentiating the viridans streptococci. Facklam found agreement between his *S. mitis* and *S. sanguis* II and Parker and Ball's *S. mitior* and dextran-positive *S. mitior* respectively. They also both agreed on the identification of *S. mutans* and *S. salivarius*.

Ruoff and Kunz [63] sought to determine whether a shortened scheme based on that of Facklam [55] could be used successfully to identify viridans streptococci isolated in routine cultures of clinical specimens. Their method involved only ten

tests, compared to Facklam's twenty-nine, and included the use of miniaturized methods (Minitek) for four of the tests. They found that although the media and tests employed were somewhat different from those of Facklam, all of Facklam's species could be identified with their modified key. Coogan [68] is another author who based her classification scheme largely on that of Facklam [55]. The only discrepancy between their two schemes, was that Facklam did not recognise *Streptococcus milleri* as a single species.

Colman and Williams [52] devised a scheme which was simpler than that of Facklam's [55] in that it did not include *S. MG-intermedius*, *S. anginosus-constellatus*, *S. morbillorum*, *S. acidominimus* and *S. uberis*. They also recognised *S. mitior* in contrast to Facklam's *S. mitis*. Other schemes which recognised *S. mitior* rather than *S. mitis* include those of Hardie and Bowden (1976) [65], Jones (1978) [69], and Parker (1983) [70].

A number of schemes [35, 52, 65, 69 - 71] only recognised *S. sanguis*, the group, as a species, while Facklam [55] recognised *S. sanguis* I and *S. sanguis* II as individual species. Colman and Williams [52] did not accept *S. sanguis* II or *S. mitis* as described by Facklam but placed organisms resembling these species in the *S. mitior* group. Hardie and Bowden [65] and Jones [69] also incorporated *S. sanguis* II in the *S. mitior* species, whereas Parker and Ball [35] and Parker [70] defined it as a dextran-producing strain of the species *S. mitior*.

All schemes recognised the *S. salivarius* species and (with the exception of Deibel and Seeley [66]) the *S. mutans* species. The identification of *S. milleri* (as described by Colman and Williams [52], Coogan [68], Hardie and Bowden [65], Parker and Ball [35], Jones [69] and Parker [70]), however, is another contentious issue. According to Facklam [55] most strains of *S. milleri* were accommodated in his *S. anginosus-constellatus* and *S. MG-intermedius* species while Deibel and Seeley [66] also recognised *S. anginosus-constellatus* instead of *S. milleri*.

More recently, articles have been written on the subject of viridans streptococcal taxonomy by Kilian *et al* [72] and Hardie (in the 9th edition of Bergey's manual of systematic Bacteriology) [73]. Although Hardie looked at a wide range of differentiative techniques (including biochemical tests, serology and DNA hybridization studies) he was unable to provide a definitive system of classification as, firstly, many of the biochemical tests he prescribed as a means of differentiating the various species produced variable results and, therefore, could not be regarded as a reliable means of identification. Secondly, serology was not sufficiently species-specific to be of any major use. Hardie has, nevertheless, highlighted the potential benefits of utilizing DNA hybridization studies as an additional differentiative measure in the classification of viridans streptococci. Kilian *et al*, on the other hand, have provided a comprehensive classification based on the results of extensive biochemical and serological tests which revealed clearly defined taxa. Kilian *et al* [72] described the following species: *S. sanguis*, *Streptococcus gordonii* sp. nov. (both of which correlate with Colman and Williams' [52] *S. sanguis* and Facklam's [55] *S. sanguis* I), *Streptococcus oralis* (which correlates with Colman and Williams' *S. mitior* and Facklam's *S. sanguis* II), *S. mitis* (which correlates with Colman and Williams' *S. mitior* and Facklam's *S. mitis*), *S. salivarius*, *S. anginosus* and *S. mutans*. Kilian *et al* [72] were also able to show high degrees of intraspecies genetic relatedness when the results of genetic analysis were applied to their classification.

Despite recent studies, however, there still remains little agreement on how the viridans streptococci should be classified. Several commercial systems have been devised in an attempt to collate those physiological tests required for viridans streptococcal species differentiation into a simple, rapid identification system. They include the Minitek miniaturised system, the Rapid Strep system and the API 20 Strep system. All three systems have been evaluated [62, 74 - 76] and have shown satisfactory correlation with conventional tests with which they were compared. However, as the various systems have all been based on different physiological classifications, it is difficult to compare their relative merits.

Because of the problems associated with the classification and identification of viridans streptococci, repeat identifications of all those viridans streptococci being incorporated in the PBP studies were performed. As Facklam was able to find agreement between his *S. mitis* species [55] and Parker and Ball's *S. mitior* species [35], Colman and Ball [77] (who recognized *S. mitior* as a species) were able to assess the value of the API 20 Strep system (which recognized *S. mitis*). They concluded that, if supplemented with additional tests, the API 20 Strep system was a satisfactory substitute for biochemical tests in the identification of viridans streptococci. Another study which examined the API 20 Strep system was recently carried out by French *et al* [78]. They demonstrated that most of the viridans streptococci they examined could be classified intuitively into the six species defined by Colman and Williams [52] by means of the biochemical test results obtained using the API 20 Strep system. They constructed their classifications intuitively by giving special weight to certain test results and then performing cluster analysis of the results. The species that were thus defined were: *S. mutans*, *S. bovis*, *S. mitior*, *S. sanguis*, *S. salivarius* and *S. milleri*. French *et al*, however, do stipulate that although they would recommend using the API 20 Strep system for performing physiological tests on viridans streptococci (as they may be more reliable than conventional tests and are probably reproducible [78]), the information regarding identification and nomenclature provided by the manufacturers is in need of revision.

As the initial screening method used in this study was based on the Colman and Williams' classification scheme and the API 20 Strep system has been shown to be satisfactorily reliable [77, 78], the repeat identifications were carried out using the API 20 Strep system. These new results made it possible to compare the performance of the routine conventional tests used initially in our studies with that of the API 20 Strep system. As an additional comparison, these two identification methods were also compared with a screening procedure recommended by Ruoff and Kunz [63] which was based on Facklam's classification [55].

1.5 AIMS AND OBJECTIVES

The aims of this study were to:

1a determine the carriage rates and penicillin resistance prevalence rates of nasopharyngeal and oral isolates of *S. pneumoniae* and the viridans streptococci, with or without exposure to ß-lactam antibiotics;

b determine antibiotic resistance patterns of oral streptococcal isolates;

c determine the serogroup distribution of the penicillin-resistant isolates of *S. pneumoniae*;

d assess a possible correlation between the carriage of resistant streptococci and the initiation of streptococcal bacteraemia;

2a determine the differences in PBP patterns of penicillin-sensitive and penicillin-resistant pneumococci;

b evaluate the various techniques used in determining PBP patterns, including an assessment of both the effectiveness of the various solubilizing agents available and the value of whole cell preparations versus membrane preparations: by doing so, it was hoped that a preliminary picture of the gross changes that occur in viridans streptococcal PBP's, as they pass from penicillin susceptibility to resistance, would be provided;

3 compare the API 20 Strep system of identifying the viridans streptococci with a screening method devised in our laboratory, and with a screening procedure recommended by Ruoff and Kunz [63] which was based on Facklam's classification.

Chapter 2
MATERIALS AND METHODS

2.1 EPIDEMIOLOGICAL STUDY

2.1.1 Patient Groups

The study covered a three-month period, from May to July 1988. During this time a search for nasopharyngeal and oral carriers of pneumococci and viridans streptococci respectively, was conducted amongst 100 new paediatric admissions to Baragwanath Hospital in Soweto. The mean age of the subjects was 2 years (the range being 2 days to 9 years).

TABLE 2.1 Age, Sex and Underlying Condition Distribution of Admission Groups

	AGE		SEX		UNDERLYING CONDITION								
	0-3	4-9	M	F	M/K[1]	D/G[2]	Br[3]	Me[4]	N/S[5]	On[6]	Se[7]	Ca[8]	He[9]
Children not on antibiotics on admission (n=74)	59	15	36	38	13	6	22	10	3	4	5	8	3
Children on antibiotics on admission (n=26)	21	5	14	12	4	2	10	3	1	2	1	2	1

1 M/K = Malnutrition/Kwashiorkor
2 D/G = Diarrhoea/Gastroenteritis
3 Br = Bronchopneumoniae
4 Me = Meningitis
5 N/S = Nephrotic Syndrome
6 On = Oncology
7 Se = Septicaemia
8 Ca = Cardiac conditions
9 He = Hepatitis

A careful history was obtained on each subject about any recent evidence (< 3 months prior to initial swabbing) of beta-lactam antibiotic administration or hospitalization. Children who received antimicrobial therapy other than beta-lactam

antibiotics were not included in the study. Further information gathered on each child included age and the medical reason for his/her admission. The children were divided, according to whether or not they were receiving antibiotics, into a beta-lactam treated group and a control group respectively. The two groups were shown to be statistically comparable ($p<0.4$) with regard to age, sex and underlying condition (Table 2.1).

2.1.2 Specimen collection and processing

Each child was swabbed nasopharyngeally and orally on admission and, subsequently, on a weekly basis for the duration of his/her stay in hospital. All patients were in hospital for at least one week. The oral swab was taken across the tongue, as this is one of the optimal sites for viridans streptococcal isolation (MM Coogan, personal communication), while the nasopharyngeal swabs were taken pernasally. Calcium alginate swabs (Calgiswabs type 1, Spectrum, U.S.A.) were used to collect the specimens. The nasopharyngeal swabs were immediately plated onto blood agar plates (Oxoid Columbia base with 5% horse blood) and onto 5% horse blood agar plates containing 5μg/ml gentamicin, for the specific isolation of *Streptococcus pneumoniae*. The oral swabs were plated immediately onto 5% horse blood agar plates which were used for the isolation of viridans streptococci. The plates were then transported to the laboratory (within three hours of collection) where they were incubated overnight at 37°C in 5% CO_2.

After 24 hours incubation at 37°C, plates were examined for colonies resembling pneumococci or viridans streptococci and a single colony of a pneumococcus and/or viridans streptococcus was picked off and plated onto 5% horse blood agar. Plates with no apparent growth of streptococci were reincubated in 5% CO_2 at 37°C for an additional 24 hours and re-examined.

2.1.3 Specimen identification

All streptococci isolated were identified with specific reference as to whether they were either *S. pneumoniae* or viridans streptococci. This was done by means of their morphological appearances, their susceptibility to an optochin disc containing 5 μg of ethyl hydrocupreine hydrochloride and their inability to grow on bile-aesculin.

2.1.3.1 Initial screening method

In order to compare various prescribed classification schemes of the viridans streptococci, all of the viridans streptococcal strains were divided into species according to a screening method devised in our laboratory. This method was based on the classification proposed by Colman and Williams [52] and included the tests for hydrolysis of aesculin and arginine. An additional test for H_2O_2 production [79] was also performed.

Forty-two of the above strains were then selected (on the basis of their penicillin MICs) for differentiation by means of the API 20 Strep system and by means of a screening procedure devised by Ruoff and Kunz [63], which was based on Facklam's classification [55].

2.1.3.2 API 20 Strep system

Forty-two organisms were selected to represent penicillin-susceptible, moderately susceptible and resistant strains of viridans streptococci. Isolates were obtained from the nasopharynx and oral cavity.

The API 20 Strep system (API System S.A., Montalieu-Vercieu, France) is composed of 20 strips, each of which comprises 20 cupules containing dehydrated substrates used for the determination of the following physiological characteristics: acetoin production; hydrolysis of hippurate and aesculin; production of the enzymes pyrrolidonylarylamidase, alpha-galactosidase, beta-glucuronidase, beta-

galactosidase, alkaline phosphatase, leucine arylamidase and arginine dihydrolase; and fermentation of ribose, L-arabinose, mannitol, sorbitol, lactose, trehalose, inulin, raffinose, starch and glycogen.

The manufacturer's instructions were followed for the API 20 Strep tests, except that horse (rather than sheep) blood agar was prepared with an Oxoid Columbia Blood Agar Base. The growth on a blood agar plate, incubated in 5% CO_2 at 37°C, was harvested and suspended in distilled water to give a density equal to or greater than a McFarland number four turbidity standard. The strips were then inoculated, incubated and interpreted according to the instructions of the manufacturer. The reactions were read after four hours incubation and also after 24 hours incubation (for the fermentation tests, and arginine and aesculin hydrolysis). A seven-digit profile number was generated in the case of each strain and these profile numbers were then compared with those provided in a code book. When organisms yielded codes which did not appear in the book, the firm was contacted by telephone and profiles were referred to their extended database.

2.1.3.3 Ruoff and Kunz screening method

This method for identification employed various physiological tests, including: fermentation of mannitol, lactose, raffinose and inulin; and hydrolysis of aesculin, arginine and hippurate. This scheme also employed the reduction of litmus milk as a further test, but as all species (bar the rarely encountered *Streptococcus morbillorum*) can accomplish this, the test was not included in this study. As all of the abovementioned tests were covered by the API 20 Strep system, the results obtained using the API 20 Strep identification kit were applied to the Ruoff and Kunz screening method. The strains were then identified according to the table of percentage positive reactions obtained from the Ruoff and Kunz study [63].

2.1.4 Suceptibility testing

Susceptibility tests using a modified Kirby-Bauer technique [14] were performed on all isolates by means of a disc diffusion method (Mast Laboratories, Liverpool). Discs used for pneumococcal isolates comprised oxacillin (1 μg), rifampicin (5 μg), erythromycin (15 μg), clindamycin (2 μg), choramphenicol (30 μg) and tetracycline (30 μg) while discs used for all other streptococcal isolates contained erythromycin (15 μg), clindamycin (2 μg), oxacillin (1 μg), vancomycin (30 μg), cefazolin (30 μg), penicillin (10 units), choramphenicol (30 μg), fucidic acid (10 μg), sulphamethoxazole (50 μg), trimethoprim (5 μg), ampicillin (10 μg) and gentamicin (10 μg). All the susceptibility tests were performed on 5% horse blood agar plates and inoculation of the plates was done using sterile cotton swabs. The plates were incubated aerobically overnight and after a 24 hour incubation period zone sizes were read with calipers. The following breakpoints (as prescribed by NCCLS [80]) were applied: oxacillin, ≤19mm (resistant) and ≥20mm (susceptible); rifampicin, ≤16mm (resistant) and ≥20mm (susceptible); erythromycin, ≤13mm (resistant) and ≥23mm (susceptible); clindamycin, ≤14mm (resistant) and ≥21mm (susceptible); chloramphenicol, ≤12mm (resistant) and ≥18mm (susceptible); tetracycline, ≤14mm (resistant) and ≥19mm (susceptible); vancomycin, ≤9mm (resistant) and ≥12mm (susceptible); cefazolin, ≤14mm (resistant) and ≥18mm (susceptible); penicillin, ≤19mm (resistant) and ≥28mm (susceptible); fucidic acid, ≤14mm (resistant) and ≥22mm (susceptible); sulphamethoxazole (the breakpoints of which were devised in our laboratory), ≤10mm (resistant) and ≥17mm (susceptible); trimethoprim, ≤10mm (resistant) and ≥16mm (susceptible); ampicillin, ≤21mm (resistant) and ≥30mm (susceptible); gentamicin, ≤12mm (resistant) and ≥15mm (susceptible).

2.1.5 MIC Determination

All pneumococcal and viridans streptococcal strains were subjected to penicillin minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determinations using a microtitre serum broth dilution method, which proceeded as follows:

The microtitre well plate (Dynatech 2000) comprised 8 rows of wells (12 wells per row). Wells 1 - 10 were the experimental wells, well 11 contained the sterile control (penicillin in serum broth, but no organisms present) and well 12 contained the growth control (organisms in serum broth, but no penicillin present).

Five colonies of the strain to be tested were inoculated into 3 ml of serum broth and incubated in a water-bath for 3 hours at 37°C.

Wells 1 - 12 of the microtitre plate were filled with 100 μl of 10% horse serum broth. As a control measure a *Staphylococcus aureus* control (ATCC 29213), of known MIC, was included in one of the rows of wells and these wells were filled with 100μl Mueller Hinton broth. A penicillin concentration of 16 μg/ml was prepared and 100 μl of penicillin was then added to the first well and mixed well with the serum broth, or Mueller Hinton broth in the case of the *S. aureus* control. 100 μl of the mixture was then withdrawn and transferred to the second well where it was, once again, mixed well. This process was continued from well to well until the 11th well had received its dilution of penicillin. The last 100 μl withdrawn from well 11 was discarded.

The serum broth bottles were then removed from the water-bath (once the 3 hours had elapsed) and 10 μl of suspension from each serum broth bottle was added to a second bottle of serum broth. One drop (from a Pasteur pipette) of the new inoculum mixture was then deposited into wells 1 - 10 and 12. With this procedure an inoculum of approximately 10^5 per ml was obtained.

The microtitre plate was then incubated overnight in 5% CO_2 at 37°C.

Purity plates were prepared, as were penicillin sensitivity plates for MIC/zone size comparisons. This involved adding inoculum from the original serum broth bottle to the second serum broth bottle until the desired turbidity (matched against the MacFarland standard) was attained. This inoculum was then plated out onto plain blood agar and a 10 μg penicillin disc was placed onto the plated-out inoculum. Penicillin susceptibility was indicated by a zone size of $\geq$28mm around the disc. This step was included as a further check on the susceptibility of the test organisms to penicillin.

After overnight incubation in CO_2, these plates (as well as the microtitre plate) were examined for bacterial growth. The MIC value was taken to be the minimal penicillin concentration that inhibited visible growth. To determine the MBC, the contents of each well (including those with no visible growth) were plated out onto plain blood agar and incubated overnight in CO_2. Growth on the plates was then recorded (with less than 5 colonies regarded as zero growth) and the MBC value was taken as the highest penicillin dilution showing no growth.

The NCCLS Minimum Inhibitory Concentration Breakpoints (μg/ml) for penicillin [80] were applied to the pneumococcal and the viridans streptococcal strains. For the pneumococcal strains, moderate resistance was defined as an MIC value from 0.1 μg/ml to 1.0 μg/ml and fully resistant strains were those having an MIC >1 μg/ml. For the viridans streptococcal strains the standards were as follows; susceptible, $\leq$0.12 μg/ml; moderately resistant, 0.25 - 2.0 μg/ml; and resistant, $\geq$4.0 μg/ml.

Gentamicin and streptomycin MICs on 30 penicillin-resistant and 10 penicillin-susceptible *S. pneumoniae* strains as well as 23 penicillin-resistant and 17 penicillin-sensitive viridans streptococcal strains of varying penicillin susceptibility were determined using the microtitre serum broth method described above.

2.1.6 Serogroup determination

Finally, the serogroups of all the penicillin-resistant pneumococcal isolates were determined by the Quellung test with the use of anti-pneumococcal sera (Statens Seruminstituut, Copenhagen).

2.2 PENICILLIN-BINDING PROTEIN STUDIES

2.2.1 Bacteria

Viridans streptococci: Twelve isolates of *S. mitis* and ten isolates of *S. sanguis* identified by the API 20 Strep system are fully described in Tables 3.23 and 3.24. This selection criterion was combined with that of optochin resistance and absence of growth on bile-aesculin. With two exceptions, only those strains with an 80% and above positive identification according to the API 20 Strep system, were used in the penicillin-binding protein studies. Included in Tables 3.23 and 3.24 are the identifications obtained on the various strains using the original screening method and the screening method recommended by Ruoff and Kunz [63].

Pneumococci: Nasopharyngeal isolates were selected to represent penicillin-susceptible, moderately resistant and resistant strains and are fully described in Table 3.26.

All isolates were initially stored in rabbit blood in liquid nitrogen. These cultures were plated for isolate colonies and a single colony subcultured for MIC determination.

2.2.2 MIC Determination

Antimicrobial activity was determined by the agar dilution method [80] using Mueller-Hinton agar supplemented with 5% defibrinated horse blood. Cultures of *S. pneumoniae* and viridans streptococci were grown in Todd Hewitt broth containing 1% (w/v) yeast extract. To 1ml of culture grown for MIC determination glycerol was added to give a final concentration of 10% (v/v) and the cultures stored at -70°C for PBP studies. All isolates were tested for optochin susceptibility.

2.2.3 PBP Labelling of Membranes

The procedure of Handwerger and Tomasz [42] was followed. Four strains representing susceptible and resistant isolates of *S. mitis* and *S. sanguis* were selected. 100 ml of Todd Hewitt broth (Oxoid Ltd., England) + yeast extract was inoculated and the cultures incubated overnight at 37°C. 1 ml samples of cells of each strain (representing 10^8 CFU/ml and 80 - 100 μg/ml of total protein) were harvested and washed in quarter-strength Ringer's solution and then suspended in 100 ml of a sonication buffer (0.12g [0.01M] Tris. HCl, containing 0.133g [5mM] $MgCl_2$, pH 7.4) to which 0.025ml (50 μg/ml) RNase (Boehringer Mannheim, West Germany)/5ml cells had been added. The cells were then disrupted by sonication (10 x 5 minutes). DNase (Boehringer Mannheim) (1mg/ml) was added (0.3ml for 6ml sonication buffer) and the disrupted cells centrifuged at 3500g for twenty minutes at 4°C in order to remove cell debris. To recover the membranes, the supernatant was centrifuged at 55000g for one hour at 4°C and the pellet washed in 1mM Tris/1mM $MgCl_2$, pH 7.8 at 55000g for one hour at 4°C. The pellet was then resuspended in phosphate buffer and a protein assay (Bio-Rad, Richmond, California) performed to estimate the protein content of the membrane preparation. Seventy nanograms of [^{3}H] benzylpenicillin (Amersham International PLC, Amersham, Bucks., U.K.) was added to 20μl of membrane suspension containing 100 μg of protein and incubated at 37°C for 20 min. 16μg of nonradioactive benzylpenicillin was then added. Membranes were solubilized at 37°C for 20 min in a solubilizing buffer comprising 0.5M Tris.HCl pH 6.8 + H_2O + either 20% Sarkosyl

(Sigma Chemical Co., St. Louis), 20% sodium dodecyl sulphate (SDS) (Sigma) or 20% Triton X-100. Finally, 50 μl of reducing buffer (as described by Zighelboim and Tomasz [40]) was added to the membranes which were boiled at 100°C for 5 min.

2.2.4 PBP Labelling of Whole Cells

Cultures were grown to a density of 1 - 5x10^8 CFU/ml in Todd Hewitt broth containing 1% (w/v) yeast extract. Radiolabelling of PBPs was performed according to Zighelboim & Tomasz [40]. Various concentrations of [^{3}H] benzylpenicillin (10 - 28 Ci/mmol), obtained from Amersham International, was added to 1 ml aliquots of exponentially growing organisms at 37°C for 10 minutes to give a final antibiotic concentration of 0.5 μg/ml. 5 μl (200 μg) of nonradioactive benzylpenicillin was added and the bacteria were recovered by centrifugation at 3500g for 5 minutes at 4°C. Cells were resuspended in 50 μl of 50mM phosphate buffer, pH 7, and 5 μl of 20% Sarkosyl or 20% SDS, to which 30 μg of mutanolysin (Sigma Chemical Co., St. Louis) had either been or not been added. The suspension was then incubated at 37°C for 20 minutes which resulted in the complete lysis of the bacteria. 30 μl of reducing buffer [40] was then added to the lysates which were then boiled at 95°C for 4 minutes. Samples were then placed on ice, until applied to a polyacrylamide slab gel.

Rainbow^{TM} protein molecular weight markers ^{14}C-labelled were obtained from Amersham International. 3 μl of molecular weight marker was used, to which 20 μl of reducing buffer was added.

2.2.5 Membrane Gel Electrophoresis

For electrophoresis, SDS-polyacrylamide (Bio-Rad, Richmond, California) gels were prepared. The stacking and separating gels contained 4% and 8% (2.67% C, where C is the cross-linkage between the acrylamide and bisacrylamide) acrylamide respectively. Electrophoresis was performed at constant current: initially 25mA/gel was applied for 1 hour, subsequently this was increased to 35mA/gel for 4 - 4.5 hours. The gels were then left overnight in 7% acetic acid. For fluorography, the gels were treated with Amplify (Amersham International) and dried at 60°C for 2 hours, followed by 20 - 30 minutes cooling. The gels were then exposed to preflashed Hyperfilm-MP (Amersham International) for 7 - 10 days at -

70°C. The number and intensity of bands on the autoradiographs were evaluated by visual observation.

2.3. DATA ANALYSIS

Data obtained were analysed using the Chi-square test with appropriate use of Yates' correction coefficient. Comparisons were considered significant at $p<0.05$.

Chapter 3
RESULTS

On admission, 74 of the children swabbed were not on ß-lactam antibiotics while 26 were; these were the admission groups. These 100 children were followed up subsequent to their admission. Of the 74 children who had not been on antibiotics on admission 45 were put onto subsequent ß-lactam antibiotics, while 29 were not. Carriage of α-haemolytic streptococci of both the group of 45 children who received subsequent ß-lactam antibiotics and the 29 children who did not was assessed on admission and again towards the end of their stay in hospital in order to determine the possible effects of antibiotic therapy and stay in hospital on the carriage of these organisms. Of the 26 children who had been on antibiotics on admission 7 were not put onto subsequent antibiotics, while 19 were. The results obtained from these study groups will be described and, later, discussed.

3.1 CARRIAGE RATES

The carriage rates of pneumococci in the nasopharynx and viridans streptococci in the oral cavity are given in Table 3.1.

3.1.1 Carriage Rates of Streptococcus pneumoniae

Although there was a trend towards reduced frequency of pneumococcal carriage there was no significant difference ($p=0.1$) between those who had not been on ß-lactam antibiotics on admission (43 out of 74, 58%) and those who were on antibiotics (10 out of 26, 38%).

The possible effects of ß-lactam antibiotics and patient's stay in hospital on pneumococcal carriage are recorded in Table 3.1 and Table 3.2. The administration of ß-lactam antibiotics was accompanied by a marked reduction in carriage rates. This is reflected in the 45 children who received ß-lactam antibiotics after admission but not before. In these children the number of carriers decreased from 30 (66%) to 10 (22%), p=0.00006. Similarly, when all 64 children who received ß-lactam antibiotics while in hospital were considered, only 17 out of the original 37 carriers (43%) remained carriers after having received ß-lactam antibiotics, p=0.0007. However, when one compares the reduction in the number of carriers following ß-lactam antibiotic therapy in the two groups (30 to 10 and 37 to 17 respectively) with the changes in the number of carriers in the comparable groups who were not on ß-lactam antibiotic therapy (13 to 8 and 16 to 11 respectively), the differences were not statistically significant (p=0.4 and p=0.6 respectively - see data in Table 3.2). The reason that some carriers who were not on ß-lactam antibiotics apparently lost their nasopharyngeal pneumococci while in hospital is not clear but may at least partly reflect natural loss of carriage in some children while others may have lost their pneumococci as a result of non-ß-lactam antibiotic therapy.

TABLE 3.1 Association of ß-lactam Administration with Carriage of α-haemolytic Streptococci[1]

	S. pneumoniae		Viridans Streptococci		S. mitior		S. sanguis	
A On Admission:	Carriers	Total(%)	Carriers	Total (%)	Carriers	Total(%)	Carriers	Total(%)
Not on ß-lactam therapy	43	74 (58%)	63	74 (85%)	47	74 (64%)	27	74 (36%)
On ß-lactam therapy	10	26 (38%)	22	26 (85%)	16	26 (62%)	8	26 (31%)
Statistical significance	NS[2] (p = 0.1)		NS		NS		NS	
B On ß-lactams in hospital but not before:	Carriers	Total(%)	Carriers	Total (%)	Carriers	Total(%)	Carriers	Total(%)
Before antibiotics	30	45 (66%)	36	45 (80%)	28	45 (62%)	15	45 (33%)
After antibiotics	10	45 (22%)	44	45 (98%)	37	45 (82%)	23	45 (51%)
Statistical significance	p = 0.00006		p = 0.02		NS (p = 0.06)		NS (p = 0.1)	
C Not on ß-lactams in hospital nor before:	Carriers	Total(%)	Carriers	Total (%)	Carriers	Total(%)	Carriers	Total(%)
On admission	13	29 (45%)	27	29 (93%)	19	29 (66%)	11	29 (40%)
Before discharge	8	29 (28%)	27	29 (93%)	20	29 (69%)	11	29 (40%)
Statistical significance	NS		NS		NS		NS	
D Acquisition of carriage in hospital:	Carriers	Total(%)	Carriers	Total (%)	Carriers	Total(%)	Carriers	Total(%)
After antibiotics	6	27 (22%)	13	13 (100%)	NA[3]		NA	
No antibiotics	11	20 (55%)	2	2 (100%)				

[1] For changes in carriage rates in children who were receiving antibiotics before admission, see Table 3.2 and Table 3.3
[2] NS = not significant
[3] NA = not analysed

Although 25 out of 53 (47%) carriers on admission lost their nasopharyngeal pneumococci during their stay in hospital, 17 out of 47 (36%) children who did not carry pneumococci on admission acquired *S. pneumoniae* during this period. Of the non-carriers more children (p=0.05) who did not receive ß-lactam antibiotics acquired pneumococci (11 out of 20; 22%). The nett loss of carriage of pneumococci was 8, from 53 carriers on admission to 45 towards the end of their stay in hospital (see Table 3.2). It is interesting that, irrespective of whether they received ß-lactam antibiotics or not, the carriage status of children who received ß-lactam antibiotics before admission did not change while in hospital with regard to loss of carriage, but 5 of the 16 non-carriers acquired nasopharyngeal pneumococci during this period.

TABLE 3.2 Association of ß-lactam Antibiotic Therapy and Stay in Hospital with Nasopharyngeal Carriage of Streptococcus pneumoniae

	Children	Carriers	Lost Carriage	Non-carriers	Gained Carriage
A On admission:	100	53	-	47	-
On antibiotics	26	10	-	16	-
Not on antibiotics	74	43	-	31	-
B In hospital, not on antibiotics on admission:					
a) On antibiotics:	45				
after admission		30	-	15	-
before discharge		10	20	32	3
b) Not on antibiotics:	29				
after admission		13	-	16	-
before discharge		8	5	12	9
C In hospital, on antibiotics on admission:					
a) On antibiotics:	19				
after admission		7	-	12	-
before discharge		7	0	9	3
b) Not on antibiotics:	7				
after admission		3	-	4	-
before discharge		3	0	2	2
Total:					
On admission	100	53	-	47	-
Before discharge	100	28[1]	25	55	17

[1] Final number of carriers at end of hospital stay is 28 + 17 = 45

3.1.2 Carriage Rates of Viridans Streptococci

The carriage rates of viridans streptococci are given in Table 3.1 and an analysis of the effect of antibiotics on carriage is represented in Table 3.1 and Table 3.3.

TABLE 3.3 Association of ß-lactam Antibiotic Therapy and Stay in Hospital with Oral Carriage of Viridans Streptococci

	Children	Carriers	Lost Carriage	Non-carriers	Gained Carriage
A On admission:	100	85	-	15	-
On antibiotics	26	22	-	4	-
Not on antibiotics	74	63	-	11	-
B In hospital, not on antibiotics on admission:					
a) On antibiotics:	45				
after admission		36	-	9	-
before discharge		33	3	1	11
b) Not on antibiotics:	29				
after admission		27	-	2	-
before discharge		27	0	0	2
C In hospital, on antibiotics on admission:					
a) On antibiotics:	19				
after admission		17	-	2	-
before discharge		17	0	0	2
b) Not on antibiotics:	7				
after admission		5	-	2	-
before discharge		5	0	2	0
Total:					
On admission	100	85	-	15	-
Before discharge	100	82[1]	3	3	15

[1] Final number of carriers at end of hospital stay is 82 + 15 = 97

There was no difference in viridans streptococcal carriage (Table 3.1) between those who had been on antibiotics on admission (22 out of 26, 85%) and those who had not (63 out of 74, 85%). Furthermore, of the 45 children who did not receive antibiotics before admission but were given ß-lactam antibiotics subsequently, 36 (80%) carried viridans streptococci before the administration of antibiotics while 44 (98%) subsequently carried these organisms. This increase in carriage rate was statistically significant ($p=0.02$). It is noteworthy that all 15 non-carriers, including 13 who were given ß-lactam antibiotics, subsequently became carriers.

Using the South African Institute for Medical Research screening procedure which is based on the Colman and Williams classification [52], the only species of viridans streptococci isolated were *Streptococcus mitior* and *Streptococcus sanguis*. As will be shown later (see section 3.5) the organisms constituting these species represent heterogeneous groups, while the sampling size from each child was limited (see Materials and Methods, Chapter 2). The validity of an analysis of carriage rates of these species is, therefore, somewhat questionable.

From Table 3.1 it is clear that the patterns observed amongst the *S. sanguis* and *S. mitior* strains were very similar to that of the viridans streptococci as a whole, except that the carrier rates were lower.

3.2 PENICILLIN RESISTANCE PREVALENCE RATES

3.2.1 Rate of Carriage of Penicillin-Resistant Bacteria

The carriage rate of penicillin-resistant organisms was expressed as a percentage of isolates exhibiting different degrees of resistance to pencillin based on MIC breakpoints. The breakpoints used for intermediate resistance and full resistance in pneumococci were 0.1 μg/ml to 1 μg/ml and >1 μg/ml respectively, while the respective breakpoints used for viridans streptococci were 0.2 μg/ml to 2 μg/ml and >2 μg/ml.

3.2.2 Prevalence of Penicillin Resistance in S. pneumoniae

3.2.2.1 Association with ß-lactam Antibiotic Therapy

The findings and relevant statistical analyses on the possible effects of ß-lactam antibiotic therapy on the carriage of penicillin-resistant *S. pneumoniae* are given in Table 3.4, Table 3.5 and Table 3.6.

TABLE 3.4 Relationship Between Beta-lactam Antibiotic Use in Pneumococcal Carriers and the Carriage of Penicillin-resistant S.pneumoniae

	MIC Range (μg/ml)						
	≤0.015-0.06	0.12-1.0	2.0	4.0	≥8.0	R[2](%)[1]	HLR[3](%)[1]
No prior antibiotics (n=43)	25	14	1	2	1	18 (42%)	4 (9%)
Received prior antibiotics (n=10)	4	3	1	2		6 (60%)	3 (30%)
No prior nor subsequent antibiotics (n=19)[4]	7	7		3	2	12 (63%)	5 (26%)
No prior antibiotics but received subsequent antibiotics (n=14)[4]	2	1	3	2	6	12 (86%)	11 (79%)
Received prior but no subsequent antibiotics (n=3)[4]	3						
Received prior and subsequent antibiotics (n=7)[4]	2	2	1	1	1	5 (70%)	3 (43%)

[1] (%) = % of pneumococcal carriers
[2] R = Resistance (MIC ≥ 0.12 μg/ml)
[3] HLR = High level resistance (MIC ≥ 2.0 μg/ml)
[4] Number of carriers prior to discharge from hospital

The carriage of penicillin-resistant pneumococci in children who were on ß-lactam antibiotic therapy on admission (6 out of 10) was not significantly different (p=0.3) than that of children who did not receive ß-lactam antibiotics for a period of 3 months before admission (18 out of 43; 42%). A similar trend which was also not significant (p=0.1) was noted with strains exhibiting high level resistance (HLR) to penicillin (Table 3.5).

Three approaches were used to assess the correlation between the use of ß-lactam antibiotics and carriage of penicillin-resistant pneumococci in children while in hospital. When carriers who received ß-lactam antibiotics in hospital were compared with those who did not, it was found that of 23 children who remained carriers after having received ß-lactam antibiotics 20 (87%) were penicillin-resistant while 13 out of 22 (59%) carriers who did not receive ß-lactam antibiotics harboured penicillin-resistant strains (p=0.08). However, when HLR strains were considered (15 out of 23; 65% versus 5 out of 22; 23% respectively) a statistically significant difference was demonstrated (p=0.01).

TABLE 3.5 Association Between ß-lactam Antibiotic Therapy and Carriage of Penicillin-sensitive and Penicillin-resistant Streptoccoccus pneumoniae

Cohort Groups	Carriers on Antibiotics						Carriers not on Antibiotics					
	Pen-R[1]	Pen-S[2]	Total	HLR[3]	Rest	Total	Pen-R	Pen-S	Total	HLR	Rest	Total
A On Admission: [n=53]	6	4	10	3	7	10	18	25	43	4	39	43
B In Hospital: Antibiotic treatment status & carriage before discharge												
No prior antibiotics [n=18]	9	1	10	7	3	10	5	3	8	1	7	8
Prior antibiotics [n=10]	5	2	7	3	4	7	0	3	3	0	3	3
Acquired carriage [n=17]	6	0	6	5	1	6	8	3	11	4	7	11
Total [n=45]	20	3	23	15	8	23	13	9	22	5	17	22

C Statistical Analysis:	Resistance		HLR	
	Pen-resistant	Pen-sensitive	HLR	Rest
a) Carriage on admission				
Antibiotics not given	18	25	4	39
Antibiotics given	6	4	3	7
Statistical significance	NS[4] (p^5=0.3)		NS (p=0.1)	
b) Carriage in hospital (before discharge)				
Antibiotics not given	13	9	5	17
Antibiotics given	20	3	15	8
Statistical significance	NS (p=0.08)		p = 0.01	
c) Change of carriage from admission to after treatment				
No prior antibiotics	18	25	4	39
Antibiotics given after admission	20	3	15	8
Statistical significance	p = 0.03		$p = 7x10^{-5}$	
d) Change of carriage in hospital when no antibiotics were given				
No prior antibiotics	18	25	4	39
No antibiotics given after admission	13	9	5	17
Statistical significance	NS (p=0.3)		NS (p=0.1)	

[1] Pen-R = Penicillin-resistant (MIC's ≥ 0.1 µg/ml)
[2] Pen-S = Penicillin-sensitive
[3] HLR = High-level resistance (MIC's >1.0 µg/ml)
[4] NS = Not significant
[5] P-values were determined by X^2 (Yates' modification) or Fisher's Exact Test

The second approach involved comparing penicillin-resistant carriage in carriers who had not received ß-lactam antibiotics before admission (18 out of 43; 42%) with that of children after they received ß-lactam antibiotics in hospital (20 out of 23; 87%). The difference was significant (p=0.03) and was highly significant when HLR was considered (4 out of 43; 9% versus 15 out of 23; 65% : p=0.00007).

Finally, the cohort of 30 carriers of *S. pneumoniae* who were not on ß-lactam antibiotics before admission but subsequently received such agents were followed up to ascertain how antibiotic therapy may have affected the carriage of penicillin-resistant strains. As can be seen in Table 3.6, out of 30 carriers on admission, 11 were resistant to penicillin and 3 were highly resistant. After ß-lactam antibiotic therapy 9 of these carriers still carried penicillin-resistant pneumococci and 7 carried high level resistant strains. The differences were not statistically significant.

TABLE 3.6 Follow-up of 43 S. pneumoniae Carriers Who did not Receive ß-lactam Antibiotics on Admission

A Cohort of 30 carriers of S. pneumoniae subsequently treated with ß-lactam antibiotics:	Total Resistance (IR[1] and HLR[2])				High Level Resistance			
	Pen-R[3]	Pen-S[4]	Lost C[5]	Total (%)	HLR	IR or Pen-S	Lost C	Total (%)
No prior antibiotics	11	19	0	30 (37%)	3	27	0	30 (10%)
After antibiotic therapy	9	1	20	30 (30%)	7	3	20	30 (23%)
Statistics:								
a) Change in carriage of resistant pneumococci	11 / 9	19 / 21			3 / 7	27 / 23		
	NS[6] (p^7=0.8)				NS (p=0.3)			
b) Change in carriage pattern of resistant pneumococci (Pen-R vs Pen-S or HLR vs IR & Pen-S)	11 / 9	19 / 1			3 / 7	27 / 3		
	p= 0.01				p=0.0006			
B Cohort of 13 carriers of S. pneumoniae subsequently not treated with ß-lactam antibiotics:	**Total Resistance (IR & HLR)**				**High Level Resistance**			
	Pen-R[3]	Pen-S[4]	Lost C[5]	Total (%)	HLR	IR or Pen-S	Lost C	Total (%)
No prior antibiotics	6	7	0	13 (46%)	1	12	0	13 (8%)
No subsequent antibiotics	5	3	5	13 (38%)	1	7	5	13 (8%)
Statistics:								
a) Change in carriage of resistant pneumococci	6 / 5	7 / 8			1 / 1	12 / 12		
	NS				NS			
b) Change in carriage pattern of resistant pneumococci (Pen-R vs Pen-S or HLR vs IR & Pen-S)	6 / 5	7 / 3			1 / 1	12 / 7		
	NS				NS			

1 IR = Intermediate resistance (0.1 µg/ml ≤ MIC ≤ 1.0 µg/ml)
2 HLR = High level resistance (MIC > 1.0 µg/ml)
3 Pen-R = Penicillin-resistant (MIC ≥ 0.1 µg/ml)
4 Pen-S = Penicillin-sensitive
5 Lost C = Lost carriage during therapy
6 NS = Not significant
7 P-values were determined by X^2 (Yates' modification) or Fisher's Exact Test

However, when the changes in carriage patterns in this cohort of 30 children were considered (11 out of 30 in the case of penicillin-resistant strains and 3 out of 30 versus 7 out of 10) the differences were significant ($p=0.01$ and $p=0.0006$ respectively). When the cohort of 13 carriers who did not receive ß-lactam antibiotics after admission was studied, however, it was clear that neither the number of resistant strains carried by these children nor the ratio of resistant to sensitive strains changed (see section B, Table 3.6).

In summary, the following trends emerged from the findings of the three above-mentioned approaches. The number of penicillin-resistant pneumococcal strains isolated from the nasopharynx of children on admission did not change significantly after they received ß-lactam antibiotics but HLR strains showed a significant increase ($p = 0.01$). There was, however, a loss of many penicillin-susceptible strains amongst the carriers who received antibiotic therapy. This resulted in a changed pattern of carriage with a marked predominance of resistant strains. Superimposed on this change was the acquisition of a preponderance of penicillin-resistant pneumococci in the children who did not receive ß-lactam antibiotics (8 out of 11) while all six strains acquired from children who received such agents were resistant to penicillin (5 being highly resistant). The trends described above were more pronounced when high level penicillin resistance was considered.

The nett increase in penicillin resistance from admission to discharge in children who did not receive antibiotics before admission but were subsequently given ß-lactam antibiotics was highly significant ($p=0.03$ for penicillin-resistant strains and $p=0.00007$ for HLR strains). When the resistance patterns of all carriers on admission were compared with those of all carriers on discharge they changed from 24 out of 53 (45%) to 33 out of 45 (73%; $p=0.003$) in the case of penicillin-resistant pneumococci and from 7 out of 53 (13%) to 20 out of 45 (44%; $p=0.001$) for HLR strains. Furthermore, despite the fact that there were also proportionally more penicillin-resistant pneumococci in the carriers who did not receive ß-lactam antibiotics in hospital, also due to loss of mainly penicillin-susceptible strains, there was still a significant difference in the carriage rate of HLR carriers ($p=0.01$) but not quite in the carriage rate of all penicillin-resistant carriers ($p=0.08$) between those who received ß-lactam antibiotics and those who did not.

In conclusion, overwhelming evidence has emerged from this study that therapy with ß-lactam antibiotics in the Baragwanath Hospital setting is accompanied by profound changes in the carriage of pneumococci resulting in a marked increase in penicillin-resistant, and especially HLR, strains.

3.2.2.2 Association of Carriage of Penicillin-resistant S. pneumoniae with Age

TABLE 3.7 Age-related Carriage of Resistant Pneumococci

A: CARRIERS				
a) All carriers on admission				
Age (years)	Penicillin-resistant	Penicillin-sensitive	Total	Significance
0-3	22	20	42	p=0.04 (Fisher's Exact Test)
4-9	2	9	11	
b) Carriers not on antibiotics when admitted				
Age (years)	Penicillin-resistant	Penicillin-sensitive	Total	Significance
0-3	17	17	34	p=0.04 (Fisher's Exact Test)
4-9	1	8	9	
c) Hospitalized carriers who received antibiotics				
Age (years)	Penicillin-resistant	Penicillin-sensitive	Total	Significance
0-3	17	3	20	p=0.7 (Fisher's Exact Test)
4-9	3	0	3	
B: CHILDREN				
a) All children on admission				
Age (years)	Penicillin-resistant	Rest	Total	Significance
0-3	22	58	80	p=0.08 (Fisher's Exact Test)
4-9	2	18	20	
b) Children not on antibiotics when admitted				
Age (years)	Penicillin-resistant	Rest	Total	Significance
0-3	17	42	59	p=0.07 (Fisher's Exact Test)
4-9	1	14	15	
c) Hospitalized children who received antibiotics				
Age (years)	Penicillin-resistant	Rest	Total	Significance
0-3	17	41	58	p=0.5 (Fisher's Exact Test)
4-9	3	10	13	

It has been shown that more children under the age of three years tend to carry penicillin-resistant pneumococci than do older individuals [14, 20, 23, 81 - 83]. All 100 children were divided into 0 - 3 year olds and 4 - 9 year olds in order to demonstrate a similar trend if it exists (Table 3.7).

Looking at all pneumococcal carriers on admission (Table 3.7), there was a barely significant difference ($p=0.04$, Fisher's Exact Test) between the 0 - 3 year olds (22 out of 42, 52%) and the 4 - 9 year olds (2 out of 11, 18%). The two age groups also differed marginally significantly ($p=0.04$, Fisher's Exact Test) when considering those carriers who were not receiving antibiotics on admission - 17 out of 34, 50% in the 0 - 3 year olds and 1 out of 9, 11% in the 4 - 9 year olds. There was, however, no significant difference ($p=0.7$, Fisher's Exact Test) between the two age groups amongst those hospitalised carriers who had received antibiotics - 17 out of 20, 85% in the 0 - 3 year olds and 3 out of 3, 100% in the 4 - 9 year olds.

With regard to age-related carriage of penicillin-resistant pneumococci in the children as a whole (Table 3.7) no significant difference ($p=0.08$, Fisher's Exact Test) was found to exist between the 0 - 3 year olds (22 out of 80, 28%) and the 4 - 9 year olds (2 out of 20, 10%) when considering all the children on admission. When comparing the 0 - 3 year olds with the 4 - 9 year olds amongst those children who were not receiving antibiotics on admission, an insignificant difference ($p=0.07$, Fisher's Exact Test) existed between the two age groups (17 out of 59, 29% and 1 out of 15, 7%, respectively). There were no markedly significant differences despite wide percentage differences, largely because the numbers were too small.

3.2.3 Serogroup Distribution of the Penicillin-resistant Pneumococcal Isolates

In those children not on antibiotics on admission (Table 3.8), resistant strains were of the groups 6 (53%), 11 (6%), 14 (6%), 19 (29%) and 23 (6%), showing a definite preponderance of groups 6 and 19. In those children who had received antibiotics prior to admission, penicillin-resistant pneumococci were of the groups 6 (33%), 14 (17%) and 19 (50%). In those hospitalised children who were never exposed to antibiotics the majority of resistant strains were of serogroup 6 (53%), the other

resistant strains being of the groups 19 (27%) and 23 (20%). In those hospitalised children who received antibiotics prior to and/or subsequent to admission, resistant strains were predominantly of the serogroups 6 (50%), 23 (27%) and 19 (18%). Overall, in this study, serogroup 6 was the most commonly isolated group (30 out of 60, 50%), followed by serogroup 19 (16 out of 60, 26%), 23 (10 out of 60, 17%), 14 (3 out of 60, 5%) and 11 (1 out of 60, 2%). An interesting finding was that serogroup 6 isolates increased from 11 on admission to 19 after patients had been in hospital for some time, while serogroup 23 isolates increased from 1 to 9 while in hospital.

TABLE 3.8 Serogroup Distribution of Penicillin-resistant Strains of S. pneumoniae

Sero-group	No prior anti-biotics (%)[1]	Received prior abs[2] (%)	No prior/ no subse-quent abs (%)	No prior but subse-quent abs (%)	Prior/ no subse-quent abs (%)	Prior/+ subse-quent abs (%)	Total strains tested (%)
6	9 (53%)	2 (33%)	8 (53%)	9 (56%)		2 (33%)	30(50%)
11	1 (6%)						1 (2%)
14	1 (6%)	1 (17%)				1 (17%)	3 (5%)
19	5 (29%)	3 (50%)	4 (27%)	1 (6%)		3 (50%)	16(26%)
23	1 (6%)		3 (20%)	6 (38%)			10(17%)
TOTAL	17	6	15	16	0	6	60

[1] (%) = % of penicillin-resistant pneumococcal strains
[2] abs = antibiotics

As the children were not hospitalised for sufficiently long periods of time (the average hospitalisation period being only 2.5 weeks), it was not possible to assess the carriage periods for the various serogroups. The longest carriage period observed for any one specific capsular group, in this study, was six weeks (for a pneumococcus group 23). A capsular group 6 pneumococcus was also still detectable five weeks after its initial isolation.

3.2.4 Prevalence of Penicillin Resistance in Viridans Streptococci

3.2.4.1 Association of Carriage of Penicillin-resistant Viridans Streptococci with ß-lactam Antibiotic Therapy

From Tables 3.9 and 3.10 it is clear that there was no noteworthy difference in prevalence rates of penicillin-resistant strains amongst the carriers of viridans streptococci between the control admission group and the ß-lactam-treated admission group (49 out of 63, 78% and 18 out of 22, 82%, respectively). The carriage of highly resistant strains, too, did not differ significantly between the same two groups of carriers (21 out of 63, 33% and 11 out of 22, 50%, respectively; p=0.3).

TABLE 3.9 Relationship Between Beta-lactam Antibiotic Use in Carriers of Viridans Streptococci and the Carriage of Penicillin-resistant Strains

	MIC range (µg/ml)					
	≤0.015-0.12	0.25-2.0	4.0	≥8.0	R[2](%)[1]	HLR[3](%)[1]
No prior antibiotics (n=63)	14	28	5	16	49 (78%)	21 (33%)
Received prior antibiotics (n=22)	4	7	5	6	18 (82%)	11 (50%)
No prior nor subsequent antibiotics (n=27)	3	6	8	10	24 (89%)	18 (67%)
No prior antibiotics but received subsequent antibiotics (n=42)	0	3	5	34	42(100%)	39 (93%)
Received prior but no subsequent antibiotics (n=6)	2	2	1	1	4 (67%)	2 (33%)
Received prior and subsequent antibiotics (n=17)		3	2	12	17(100%)	14 (82%)

[1] (%) = % of carriers of viridans streptococci
[2] R = Resistance (MIC ≥ 0.25 µg/ml)
[3] HLR = High level resistance (MIC > 2.0 µg/ml)

TABLE 3.10 Association Between ß-lactam Antibiotic Therapy and Carriage of Penicillin-sensitive and Penicillin-resistant Viridans Streptococci

Cohort Groups	Carriers on Antibiotics						Carriers not on Antibiotics					
	Pen-R[1]	Pen-S[2]	Total	HLR[3]	Rest	Total	Pen-R	Pen-S	Total	HLR	Rest	Total
A On Admission: [n=85]	18	4	22	11	11	22	49	14	63	21	42	63
B In Hospital: Antibiotic treatment status and carriage prior to discharge												
No prior antibiotics [n=60]	33	0	33	30	3	33	24	3	27	15	12	27
Prior antibiotics [n=22]	17	0	17	14	3	17	3	2	5	2	3	5
Acquired carriage [n=15]	13	0	13	13	0	13	2	0	2	2	0	2
Total [n=97]	63	0	63	57	6	63	29	5	34	19	15	34
C Statistical Analysis:	Resistance						HLR					
	Pen-resistant			Pen-sensitive			HLR			Rest		
a) Carriage on admission												
Antibiotics not given	49			14			21			42		
Antibiotics given	18			4			11			11		
Stat. significance	NS (p = 0.4)						NS (p = 0.3)					
b) Change of carriage from admission to after treatment												
No prior antibiotics	49			14			21			42		
Antibiotics given after admission	63			0			57			6		
Stat. significance	p = 0.00003						$p < 10^{-6}$					
c) Change of carriage from admission to prior to discharge												
No prior antibiotics	49			14			21			42		
Antibiotics given after admission	29			5			19			15		
Stat. significance	NS (p = 0.5)						p = 0.05					
d) Carriage at time of discharge												
No antibiotics given	29			5			19			15		
Antibiotics given	63			0			57			6		
Stat. significance	p = 0.004						p = 0.002					

1 Pen-R = Penicillin-resistant (MIC's ≥ 0.25 µg/ml)
2 Pen-S = Penicillin-sensitive
3 HLR = High-level resistance (MIC's > 2.0 µg/ml)
4 NS = Not significant
5 P-values were determined by X^2 (Yates' modification) or Fisher's Exact Test

There was a marked increase in penicillin resistance and HLR associated with the use of ß-lactam antibiotics in 63 carriers of viridans streptococci, from 49 out of 63 (78%) on admission to 63 out of 63 (100%) after the use of ß-lactam antibiotics ($p = 3 \times 10^{-5}$). The comparable figures for HLR viridans streptococci were 21 out of 63 (33%) to 57 out of 63 (90%), $p < 10^{-6}$ (Table 3.10). There was no significant difference in the carriage rate of penicillin-resistant viridans streptococci in carriers who were not on ß-lactam antibiotics on admission (49 out of 63, 78%) compared with that of carriers who were not given ß-lactam antibiotics in hospital (29 out of 34, 85%) but the comparable figures for carriers of HLR strains were 21 out of 63 (33%) and 19 out of 34 (56%) and tended towards significance ($p = 0.05$).

TABLE 3.11 Follow-up of 63 Carriers of Viridans Streptococci Who did not Receive ß-lactam Antibiotics on Admission

A Cohort of 36 carriers of Viridans Streptococci treated with subsequent ß-lactam antibiotics:	Total Resistance				HLR[1]			
	Pen-R[2]	Pen-S[3]	Lost C[4]	Total (%)	HLR	IR[5] or Pen-S	Lost C	Total (%)
No prior antibiotics	29	7	0	36(81%)	14	22	0	36(39%)
After antibiotic therapy	33	0	3	36(92%)	33	0	3	36(92%)
Statistics:								
o Increase in carriage of resistant viridans streptococci (Pen-R or HLR)	NS[5]				$p^6 = 8 \times 10^{-6}$			
o Increase in ratio of resistant viridans streptococci (Pen-R vs Pen-S or HLR vs IR + Pen-S)	$p = 0.008$				$p < 10^{-6}$			

B Cohort of 27 carriers of viridans streptococci who didn't receive subsequent ß-lactam antibiotics:	Total Resistance				HLR			
	Pen-R	Pen-S	Lost C	Total (%)	HLR	IR or Pen-S	Lost C	Total (%)
No prior antibiotics	23	4	0	27(85%)	8	19	0	27(30%)
No subsequent antibiotics	24	0	3	27(89%)	15	9	3	27(56%)
Statistics:								
o Change in carriage of resistant viridans streptococci (Pen-R or HLR)	NS				NS			
o Increase in ratio of resistant viridans streptococci (Pen-R vs Pen-S or HLR vs IR + Pen-S)	NS				NS			

[1] HLR = High level resistance (MIC > 2.0 µg/ml)
[2] Pen-R = Penicillin-resistant (MIC ≥ 0.25 µg/ml)
[3] Pen-S = Penicillin-sensitive
[4] Lost C = Lost carriage during therapy
[5] NS = Not significant
[6] P-values were determined by χ^2 (Yates' modification) or Fisher's Exact Test

When the carrier status of a cohort of 36 carriers who did not receive ß-lactam antibiotics on admission was compared with that of the same group after they received ß-lactam antibiotics, the number of carriers who harboured penicillin-resistant strains was not reduced significantly (from 29 out of 36; 81% to 33 out of 36; 92%) but the pattern reflecting the ratio between resistant and sensitive strains changed significantly ($p=0.008$, see Table 3.11). In the case of HLR viridans streptococci both the number of carriers (from 14 to 33), as well as the pattern, increased dramatically ($p=0.000008$ and $<10^{-6}$ respectively). No comparable differences were observed in the cohort of 27 carriers who did not receive ß-lactam antibiotics subsequent to admission (see Section B, Table 3.11).

Finally, when the prevalence of penicillin-resistant strains in carriers who received ß-lactam antibiotics during their stay in hospital (63 out of 63, 100%) was compared with that of carriers who did not receive such antibiotics (29 out of 34, 85%) there was a significant increase ($p=0.004$). A similar increase was noted in the carriage of HLR strains ($p=0.002$; see Table 3.10).

3.2.4.2 Association of Carriage of Penicillin-resistant S. mitior and S. sanguis with ß-lactam Antibiotic Therapy

Analyses of the possible effect of ß-lactam antibiotic therapy on the carriage of penicillin-resistant *S. mitior* are given in Table 3.12 and Table 3.13 and those on the carriage of penicillin-resistant *S. sanguis* in Table 3.14 and Table 3.15.

Beta-lactam antibiotic administration prior to admission was not associated with a significant difference in carriage rates of either penicillin-resistant or HLR strains of *S. mitior* or *S. sanguis* (Table 3.13 and Table 3.15). When the carriage of penicillin-resistant *S. mitior* and *S. sanguis* was assessed in carriers who did not receive antibiotics prior to admission and again in the same groups after ß-lactam antibiotics were given, the numbers of penicillin-resistant strains did not increase significantly yet the ratio between resistant and sensitive strains changed in the case of *S. mitior* ($p=0.01$) although not with *S. sanguis* isolates ($p=0.06$). In the case of HLR both the numbers and patterns of resistance changed with both *S. mitior* ($p=0.003$ and $p=0.00004$, respectively) and *S. sanguis* ($p=0.03$ and $p=0.0003$, respectively).

TABLE 3.12 Relationship Between Beta-lactam Antibiotic Use in Carriers of S. mitior and the Carriage of Penicillin-resistant Strains

	MIC range (µg/ml)					
	≤0.015-0.12	0.25-2.0	4.0	≥8.0	R[2](%)[1]	HLR[3](%)[1]
No prior antibiotics (n=47)	10	20	4	13	37 (79%)	17 (36%)
Received prior antibiotics (n=16)	4	5	3	4	12 (75%)	7 (44%)
No prior nor subsequent antibiotics (n=20)	3	5	3	9	17 (85%)	12 (60%)
No prior antibiotics but received subsequent antibiotics (n=37)	2	2	5	28	35 (95%)	33 (89%)
Received prior but no subsequent antibiotics (n=2)		1		1	2(100%)	1 (50%)
Received prior and subsequent antibiotics (n=14)	1	1	2	10	13 (93%)	12 (86%)

[1] (%) = % of carriers of S. mitior
[2] R = Resistance (MIC ≥ 0.25 µg/ml)
[3] HLR = High level resistance (MIC > 2.0 µg/ml)

A comparison of the groups that did not receive antibiotics before admission but received ß-lactam antibiotics subsequently, with those that did not receive subsequent antibiotics, did not reveal any differences in the overall numbers of penicillin-resistant isolates or in resistance patterns of either *S. mitior* or *S. sanguis*. In the case of HLR in *S. mitior* (but not in *S. sanguis*), however, both the number of HLR strains and the pattern of resistance changed significantly ($p=0.008$ and $p=0.01$ respectively; see Table 3.13). When all patients who did not receive ß-lactam antibiotics after admission were compared with all patients who received such agents in hospital, again no significant difference was noted with regard to overall penicillin resistance (data recorded in Table 3.12 and Table 3.14). Nevertheless, significant differences were noted in the case of HLR (13 out of 22 compared with 45 out of 51 for *S. mitior*; $p=0.007$ and 9 out of 16 compared with 27 out of 30; $p=0.05$ for *S. sanguis*).

TABLE 3.13 Association Between ß-lactam Antibiotic Therapy and Carriage of Penicillin-sensitive and Penicillin-resistant Streptoccoccus mitior

A On Admission:	Total Resistance				High Level Resistance			
	Pen-R[1]	Pen-S[2]	Total (%)	Statistics	HLR[3]	IR[4] & Pen-S	Total (%)	Statistics
No prior antibiotics	37	10	47 (79%)	NS[5]	17	30	47 (36%)	NS
Antibiotics given	12	4	16 (75%)	p = 0.5	7	9	16 (44%)	p = 0.8

B Carriers who did not receive prior ß-lactam antibiotics compared with those in that group who subsequently did:	Total Resistance				High Level Resistance			
	Pen-R	Pen-S	Lost Carriage During Therapy	Total (%)	HLR	IR or Pen-S	Lost Carriage During Therapy	Total (%)
No prior antibiotics	37	10	0	47 (79%)	17	30	0	47 (36%)
Antibiotics given	23	0	5	28 (82%)	21	2	5	28 (75%)
Statistics:								
a) Change in carriage of resistant S. mitior (Pen-R or HLR)	p > 0.9				p = 0.003			
b) Change in ratio of resistant S. mitior (Pen-R vs Pen-S or HLR vs IR + Pen-S)	p = 0.01				p = 0.00004			

C Carriers who did not receive prior ß-lactam antibiotics but subsequently did compared with those in that group who did not receive subsequent antibiotics:	Total Resistance				High Level Resistance			
	Pen-R	Pen-S	Lost Carriage During Therapy	Total (%)	HLR	IR or Pen-S	Lost Carriage During Therapy	Total (%)
Antibiotics given	23	0	5	28 (82%)	21	2	5	28 (75%)
No antibiotics given	12	0	7	19 (63%)	6	6	7	19 (32%)
Statistics:								
a) Change in carriage of resistant S. mitior (Pen-R or HLR)	NS (p = 0.1)				p = 0.008			
b) Change in ratio of resistant S. mitior (Pen-R vs Pen-S or HLR vs IR + Pen-S)	NS (p > 0.9)				p = 0.01			

D Carriers who did not receive prior ß-lactam antibiotics compared with those in that group who also subsequently did not:	Total Resistance				High Level Resistance			
	Pen-R	Pen-S	Lost Carriage During Therapy	Total (%)	HLR	IR or Pen-S	Lost Carriage During Therapy	Total (%)
No prior antibiotics	37	10	0	47 (79%)	17	30	0	47 (36%)
No subsequent antibiotics	12	0	7	19 (63%)	6	6	7	19 (32%)
Statistics:								
a) Change in carriage of resistant S. mitior (Pen-R or HLR)	NS (p = 0.2)				NS (p = 0.9)			
b) Change in ratio of resistant S. mitior (Pen-R vs Pen-S or HLR vs IR + Pen-S)	NS (p = 0.08)				NS(p = 0.3)			

[1] Pen-R = Penicillin-resistant (MIC's ≥ 0.1 µg/ml)
[2] Pen-S = Penicillin-sensitive
[3] HLR = High-level resistance (MIC's >2.0 µg/ml)
[4] NS = Not significant
[5] P-values were determined by X^2 (Yates' modification) or Fisher's Exact Test

TABLE 3.14 Relationship Between Beta-lactam Antibiotic Use in Carriers of S. sanguis and the Carriage of Penicillin-resistant Strains

	MIC range (µg/ml)					
	≤0.015-0.12	0.25-2.0	4.0	≥8.0	R[2](%)[1]	HLR[3](%)[1]
No prior antibiotics (n=27)	8	13	1	5	19 (70%)	6 (22%)
Received prior antibiotics (n=8)	1	3	1	3	7 (87%)	4 (50%)
No prior nor subsequent antibiotics (n=11)	1	3	4	3	10 (91%)	7 (64%)
No prior antibiotics but received subsequent antibiotics (n=23)	1	1	3	18	22 (96%)	21 (91%)
Received prior but no subsequent antibiotics (n=5)	2	1	1	1	3 (60%)	2 (40%)
Received prior and subsequent antibiotics (n=7)		1	2	4	7(100%)	6 (86%)

[1] (%) = % of carriers of S. sanguis
[2] R = Resistance (MIC ≥ 0.25 µg/ml)
[3] HLR = High level resistance (MIC > 2.0 µg/ml)

TABLE 3.15 Association Between ß-lactam Antibiotic Therapy and Carriage of Penicillin-sensitive and Penicillin-resistant Streptoccoccus sanguis

A On Admission:	Total Resistance				High Level Resistance			
	Pen-R[1]	Pen-S[2]	Total (%)	Statistics	HLR[3]	IR[4] & Pen-S	Total (%)	Statistics
No prior antibiotics	19	8	27 (70%)	NS[5]	6	21	27 (22%)	NS
Antibiotics given	7	1	8 (88%)	p = 0.3	4	4	8 (50%)	p = 0.1

B Carriers who did not receive prior ß-lactam antibiotics compared with those in that group who subsequently did:	Total Resistance				High Level Resistance			
	Pen-R	Pen-S	Lost Carriage During Therapy	Total (%)	HLR	IR or Pen-S	Lost Carriage During Therapy	Total (%)
No prior antibiotics	19	8	0	27 (70%)	6	21	0	27 (22%)
Antibiotics given	10	0	5	15 (67%)	9	1	5	15 (60%)
Statistics:								
a) Change in carriage of resistant S. mitior (Pen-R or HLR)	NS (p =0.5)				p = 0.03			
b) Change in ratio of resistant S. mitior Pen-R vs Pen-S or HLR vs IR + Pen-S)	NS (p =0.06)				p = 0.0003			

C Carriers who did not receive prior ß-lactam antibiotics but subsequently did compared with those in that group who did not receive subsequent antibiotics:	Total Resistance				High Level Resistance			
	Pen-R	Pen-S	Lost Carriage During Therapy	Total (%)	HLR	IR or Pen-S	Lost Carriage During Therapy	Total (%)
Antibiotics given	10	0	5	15 (67%)	9	1	5	15 (60%)
No antibiotics given	6	0	6	12 (50%)	4	2	6	12 (33%)
Statistics:								
a) Change in carriage of resistant S. mitior (Pen-R or HLR)	NS (p = 0.3)				NS (p = 0.3)			
b) Change in ratio of resistant S. mitior (Pen-R vs Pen-S or HLR vs IR + Pen-S)	NS (p > 0.9)				NS (p = 0.3)			

D Carriers who did not receive prior ß-lactam antibiotics compared with those in that group who also subsequently did not:	Total Resistance				High Level Resistance			
	Pen-R	Pen-S	Lost Carriage During Therapy	Total (%)	HLR	IR or Pen-S	Lost Carriage During Therapy	Total (%)
No prior antibiotics	19	8	0	27 (70%)	6	21	0	27 (22%)
No subsequent antibiotics	6	0	6	12 (50%)	4	2	6	12 (33%)
Statistics:								
a) Change in carriage of resistant S. mitior (Pen-R or HLR)	NS (p = 0.2)				NS (p = 0.4)			
b) Change in ratio of resistant S. mitior (Pen-R vs Pen-S or HLR vs IR + Pen-S)	NS (p = 0.2)				NS (p > 0.05)			

[1] Pen-R = Penicillin-resistant (MIC's ≥ 0.1 µg/ml)
[2] Pen-S = Penicillin-sensitive
[3] HLR = High-level resistance (MIC's >2.0 µg/ml)
[4] NS = Not significant
[5] P-values were determined by X^2 (Yates' modification) or Fisher's Exact Test

3.3 STREPTOMYCIN AND GENTAMICIN RESISTANCE IN S. PNEUMONIAE AND VIRIDANS STREPTOCOCCI

The relationship between penicillin-sensitive, intermediately penicillin-resistant and fully penicillin-resistant pneumococcal and viridans streptococcal isolates and streptomycin and gentamicin MIC's, is given in Table 3.16.

TABLE 3.16 Relationship between Penicillin MIC's and Resistance to Streptomycin and Gentamicin in S. pneumoniae and Viridans Streptococci

Strains	Streptomycin MIC's		Gentamicin MIC's	
	Range (μg/ml)	MIC_{50} (μg/ml)	Range (μg/ml)	MIC_{50} (μg/ml)
Pneumococci				
Penicillin-sensitive (MIC's < 0.12 μg/ml) (10 strains)	40 - 80	40	10 - 40	20
Penicillin-intermediate (MIC's 0.12 - 1 μg/ml) (24 strains)	40 - >2560	80	10 - 80	20
Penicillin-resistant (MIC's > 1 μg/ml) (6 strains)	40 - >2560	2560	20 - 40	20
Viridans Streptococci				
Penicillin-sensitive (MIC's ≤ 0.12 μg/ml) (17 strains)	8 - 128	16	0.5 - 64	16
Penicillin-intermediate (MIC's 0.25 - 2 μg/ml) (11 strains)	8 - >2560	32	0.5 - 32	16
Penicillin-resistant (MIC's ≥ 4 μg/ml) (12 strains)	4 - >2560	64	0.5 - 2560	32

It can be seen that in the case of streptomycin, fully penicillin-resistant *S. pneumoniae* strains tended to be associated with high level streptomycin resistance (MIC's > 2560 μg/ml) and there appears to be some correlation between the levels of penicillin resistance and streptomycin resistance, with the MIC_{50} concentrations increasing from 40 μg/ml in the fully penicillin-susceptible isolates to 80 μg/ml in the intermediate resistance group and 2560 μg/ml in the fully penicillin-resistant group. In the latter group five out of the six

penicillin-resistant strains had streptomycin MIC's of > 2560 μg/ml while two out of 24 strains in the intermediate penicillin resistance group had streptomycin MIC's of > 2560 μg/ml and the highest streptomycin MIC in the fully penicillin-susceptible group was 80 μg/ml.

With regard to the relationship between penicillin resistance in the viridans streptococci and streptomycin resistance, a similar pattern was observed although only 3 out of 12 fully penicillin-resistant strains had MIC's of > 2560 μg/ml, 2 out of 11 in the intermediate penicillin resistance group and nil out of 17 in the penicillin-susceptible group.

With regard to gentamicin resistance in the *S. pneumoniae* isolates there was a relatively narrow range of 10 μg/ml to 40 μg/ml resistance in all three of the penicillin-resistant/-susceptible groups, 40 μg/ml levels being found in 3 of the six fully penicillin-resistant isolates, one 80 μg/ml and one 40 μg/ml strains out of 24 strains in the intermediate penicillin resistance group and 2 out of 10 strains in the penicillin-susceptible group.

High-level gentamicin resistance with an MIC of > 2560 μg/ml was found in one viridans streptococcal strain (this strain having a penicillin MIC of 4 μg/ml and a streptomycin MIC of > 2560 μg/ml), while 2 strains had MIC's of 640 μg/ml, one with a penicillin MIC of 8 μg/ml and a streptomycin MIC of 2560 μg/ml and the other with a penicillin MIC of 16 μg/ml and a streptomycin MIC of 32 μg/ml.

3.4 ANTIMICROBIAL RESISTANCE BASED ON DISC DIFFUSION RESULTS

3.4.1 Antimicrobial Resistance Patterns of S. pneumoniae

Of the 96 strains of *S. pneumoniae* isolated, 41 (43%) were fully susceptible to penicillin and 34 strains (35%) were resistant to penicillin alone (Table 3.17). Various patterns of multiple resistance were demonstrated in the remaining 21 strains (Table 3.17). The two most common combinations were that of penicillin and

tetracycline resistance, and penicillin, tetracycline, erythromycin and clindamycin resistance (eight strains of each combination). The other resistance patterns demonstrated were: penicillin and chloramphenicol resistance (two strains); penicillin, tetracycline, chloramphenicol, erythromycin and clindamycin resistance (one strain); penicillin, tetracycline and rifampicin resistance (one strain); and penicillin and rifampicin resistance (one strain). A total of 18 strains (19%) were resistant to tetracycline, 9 strains (9%) demonstrated resistance to clindamycin, 9 strains were resistant to erythromycin, 2 strains (2%) were resistant to rifampicin, and 4 strains (4%) were resistant to chloramphenicol.

TABLE 3.17 Antimicrobial Resistance Patterns of 96 Strains of S.pneumoniae

	Number of strains (%)[1]
Susceptible to all antibiotics[2-7]	41 (43%)
P[2]	34 (36%)
P T[3]	8 (8%)
P T E[4] Cl[5]	8 (8%)
P C[6]	2 (2%)
P T C E Cl	1 (1%)
P R[7]	1 (1%)
P T R	1 (1%)

[1] (%) = % of pneumococcal strains
[2] P = penicillin G
[3] T = tetracycline
[4] E = erythromycin
[5] Cl = clindamycin
[6] C = chloramphenicol
[7] R = rifampicin

3.4.2 Antimicrobial Resistance Patterns of Viridans Streptococci

Disc susceptibility tests were performed on a total of 176 strains of viridans streptococci (Table 3.18). All of the isolates exhibited resistance to one or more of the following antimicrobial agents: penicillin G; erythromycin; clindamycin; cefazolin; fucidic acid; sulphamethoxazole; and trimethoprim. None of the isolates demonstrated penicillin resistance alone, while only five of the strains were resistant to sulphamethoxazole alone. Sulphamethoxazole resistance was demonstrated in all 176 of the strains.

Also commonly encountered was resistance to trimethoprim (in 149 (84%) of the strains). Erythromycin resistance was demonstrated in 54 (31%) of the isolates, of which 47 (87%) were also resistant to clindamycin. The 15 strains (9%) that were resistant to cefazolin were also resistant to penicillin.

TABLE 3.18 Antimicrobial Resistance Patterns of 176 Strains of Viridans Streptococci

Resistance Patterns	Number of strains (%)[1]
P[2]	21 (12%)
P Tr[3]	63 (36%)
P, F[4] Tr	22 (13%)
F[4] Tr	4 (2%)
Tr	3 (2%)
P E[5] Cz[6] F Tr	4 (2%)
P E Cz Tr	1
P E F Tr	1
P E Tr	1
P E Cl[7] F Tr	20 (11%)
P E Cl Tr	18 (10%)
P E Cl Cz Tr	8 (5%)
P Cl F Tr	2 (1%)
P E Cl	1
P Cz F Tr	1
P Cz Tr	1

[1] % = % of viridans streptococcal strains
[2] P = penicillin G
[3] Tr = trimethoprim
[4] F = fucidic acid
[5] E = erythromycin
[6] Cz = cefazolin
[7] Cl = clindamycin

The most commonly encountered pattern of multiple resistance was that of penicillin and trimethoprim (in 63 (36%) of the strains), while a combination of penicillin, fucidic acid and trimethoprim resistance was also fairly commonly demonstrated (in 22 (13%) of the strains). A combination of penicillin, erythromycin, clindamycin, fucidic acid and trimethoprim resistance was a commonly encountered pattern of resistance, being demonstrated in 20 (11%) of the isolates. 29 (16%) of the isolates were resistant to erythromycin and clindamycin (together with resistance to various other agents). None of the strains were resistant to vancomycin or chloramphenicol.

3.5 COMPARISON OF THE API 20 STREP SYSTEM WITH THE ORIGINAL SCREENING METHOD AND THE RUOFF AND KUNZ SCREENING PROCEDURE

To correlate PBP profiles with other parameters, it is necessary to ensure that the strains are pure and that the MIC value afforded each strain is accurate. Before proceeding with the PBP experiments, one colony of each isolate of *S. sanguis* and *S. mitior* (as identified by our original screening method) used in these further studies, was subcultured and an API 20 Strep identification and penicillin MIC performed.

TABLE 3.19 Analysis of Identification Findings of the Original Screening Method, the API 20 Strep System and the Ruoff and Kunz Screening Method

Original Method		API 20 Strep System		Ruoff and Kunz Method	
Species	No. strains	Species	No. strains	Species	No. strains
S. sanguis	21	S. mitis	4	S. mitis	4
		S. sanguis I	7	S. sanguis I	0
				S. sanguis II	1
				S. intermedius	5
				S. constellatus	1
		S. sanguis II	7	S. sanguis II	5
				S. intermedius	2
		S.pneumoniae	2	S.pneumoniae	2
		S. salivarius	1	S. salivarius	1
S. mitior	21	S. mitis	10	S. mitis	10
		S. sanguis II	9	S. sanguis II	7
				S. sanguis I	2
		S. morbillorum	1	S. morbillorum	1
		S. acidominimus	1	S. acidominimus	1

From Table 3.19, it can be seen that 19 of the 21 strains identified as *S. mitior* according to the original method were subsequently identified as either *S. mitis* (10) or *S. sanguis* II (9) by the API 20 Strep system.

Those strains originally identified as *S. sanguis* consisted mainly of *S. sanguis* I (7), *S. sanguis* II (7) and *S. mitis* (4) according to the API 20 Strep system, and *S. intermedius* (7), *S. sanguis* II (6) and *S. mitis* (4) according to the Ruoff and Kunz method.

Comparing the API 20 Strep system with the screening method recommended by Ruoff and Kunz (Table 3.19), complete agreement was reached between the two systems on the identification of the 14 *S. mitis* strains. The API-identified *S. sanguis* II strains also correlated fairly well with those *S. sanguis* II strains identified by the Ruoff and Kunz method. The greatest degree of disparity observed between the two systems was in the identification of the *S. sanguis* I strains; none of the original seven API-identified *S. sanguis* I strains were identified as such by the Ruoff and Kunz procedure. Five of the API-identified *S. sanguis* I strains were identified as *S. intermedius* by means of the Ruoff and Kunz scheme, the remaining two strains being identified as *S. sanguis* II and *S. anginosus-constellatus* respectively.

Looking at Tables 3.20 to 3.22 it is clear that certain discrepancies exist between the number of strains with positive reactions obtained in this study and those that should be obtained according to the API 20 Strep Identification table. Caution should, however, be exercised in stressing the percentage discrepancies, as the numbers of strains identified were small. With this reservation in mind, it can be seen that amongst the strains of *S. mitis*, the percentage positive reactions obtained for alkaline phosphatase, arginine dihydrolase, trehalose, inulin and starch, were notably lower (more than 10% so) than those prescribed in the API identification table. Amongst the *S. sanguis* I strains, the percentage positive reactions obtained for aesculin were markedly higher than those prescribed in the identification table, while those of arginine dihydrolase, inulin and starch were markedly lower. Amongst the *S. sanguis* II strains, trehalose and starch gave lower percentage positive reactions than those approved in the API identification table, while those of arginine dihydrolase and inulin were notably higher.

TABLE 3.20 Identification of Streptococcus mitis using the API 20 Strep System

Test	Percentages of Positive Reactions		
	API Reference values	Clinical Isolates API-acceptable ID[1] (n=13)	Clinical Isolates API-unacceptable ID[1] (n=1)
Voges Proskauer	0	0	100
Hippurate	0	0	0
Aesculin	1	0	0
Pyrrolidonyl-2-naphthylamide	0	0	0
6-Bromo-2-naphthyl-α-D-Galactopyranoside	1	0	0
Naphthol AS-BI-ß-D-glucuronate	0	0	0
2-Naphthyl-ß-D-galactopyranoside	47	45	0
2-Naphthylphosphate	61	31	100
L-Leucine-2-naphthylamide	100	100	100
Arginine	13	0	0
Ribose	6	15	0
L-Arabinose	0	0	0
Mannitol	0	0	0
Sorbitol	0	0	0
Lactose	95	100	100
Trehalose	16	0	0
Inulin	10	0	0
Raffinose	6	0	0
Starch	61	31	100
Glycogen	10	0	0

[1] ID = Identification

TABLE 3.21 Identification of Streptococcus sanguis I using the API 20 Strep System

Test	Percentages of Positive Reactions		
	API Reference values	Clinical Isolates API-acceptable ID[1] (n=5)	Clinical Isolates API-unacceptable ID[1] (n=2)
Voges Proskauer	0	0	0
Hippurate	0	0	0
Aesculin	64-75	100	50
Pyrrolidonyl-2-naphthylamide	0	0	0
6-Bromo-2-naphthyl-α-D-Galactopyranoside	23-96	40	100
Naphthol AS-BI-ß-D-glucuronate	0	0	0
2-Naphthyl-ß-D-galactopyranoside	1-13	0	0
2-Naphthylphosphate	7-47	40	0
L-Leucine-2-naphthylamide	100	100	100
Arginine	80-96	60	0
Ribose	3	0	0
L-Arabinose	0	0	0
Mannitol	0-1	0	0
Sorbitol	1-50	0	50
Lactose	75-99	80	100
Trehalose	82	80	100
Inulin	44-64	0	0
Raffinose	23-92	20	50
Starch	41-92	0	0
Glycogen	2	0	0

[1] ID = Identification

TABLE 3.22 Identification of Streptococcus sanguis II using the API 20 System

Test	Percentages of Positive Reactions		
	API Reference values	Clinical Isolates API-acceptable ID[1] (n=13)	Clinical Isolates API-unacceptable ID[1] (n=3)
Voges Proskauer	0	0	0
Hippurate	0	0	0
Aesculin	4	7	33
Pyrrolidonyl-2-naphthylamide	0	0	0
6-Bromo-2-naphthyl-α-D-Galactopyranoside	94	100	100
Naphthol AS-BI-ß-D-glucuronate	0	0	0
2-Naphthyl-ß-D-galactopyranoside	35	28	0
2-Naphthylphosphate	80	70	0
L-Leucine-2-naphthylamide	100	100	100
Arginine	20	43	0
Ribose	6	0	0
L-Arabinose	0	0	0
Mannitol	1	0	0
Sorbitol	1	0	0
Lactose	93	93	100
Trehalose	30	14	0
Inulin	6	14	0
Raffinose	99	100	100
Starch	86	30	0
Glycogen	2	0	0

[1] ID = Identification

Two of the strains originally classified as being of the *S. sanguis* species were API-identified as being pneumococci. They were originally identified as *S. sanguis* strains because they were both relatively optochin-resistant - one strain had an optochin zone size of 9 mm while that of the other was 11 mm. (A pneumococcal optochin zone size ranges from 16 - 20 mm, except in a few optochin-resistant variants). These same two strains were also resistant to sulphamethoxazole.

3.6 PENICILLIN-BINDING PROTEIN STUDIES

3.6.1 S. mitior and S. sanguis

In the department in which the PBP studies on clinical pneumococcal strains were carried out, there have been no problems encountered to date using a whole cell labelling procedure [40]. As problems were immediately evident with the lysis and final consistency of whole cell preparations with viridans streptococci, a membrane labelling method was tried. The comparison of the effectiveness of the three solubilizing agents (dodecyl sodium sulphate (SDS), sodium-lauryl sarcosinate (Sarkosyl) and Triton X-100) on the membranes of *S. mitis* and *S. sanguis* strains is shown in Fig. 3.1. Membranes of the *S. sanguis* and *S. mitis* strains included in this study were prepared using sonication for cell breakage (10 x 5 minutes) - the only method of breakage available in our laboratory. In order to maintain the integrity of the membrane, sonication and washing buffers contained $MgCl_2$. The Mg^{2+} present in the wash buffer may have been responsible for interfering with solubilization using Sarkosyl [84], even though the sonicated strains were ultimately resuspended in a phosphate buffer. PBP patterns were similar using Triton X-100 or SDS, however, one major PBP band of *S. sanguis* (Fig 3.1 lane 4) was more clearly demonstrated using SDS.

Using a whole cell procedure fewer PBP's were detected than when using a membrane preparation for labelling, as seen in Figure 3.2. This was very noticeable with the penicillin-resistant isolate of *S. sanguis*, strain 3 (Fig 3.2 lanes 1 & 2). This is due to differential solubilization of different PBP's during membrane preparation (Hakenbeck, personal communication) which can considerably complicate the interpretation of PBP alterations in conjunction with increasing penicillin resistance. In order to correlate membrane PBP profiles with penicillin resistance, extensive comparisons should be required but the membrane method was complex and time-consuming.

A whole cell lysis test was performed in order to determine which of the three available solubilizing agents was the most effective in solubilizing viridans streptococcal whole cells. Triton X-100 was found not to be satisfactory and, therefore, only SDS and Sarkosyl were investigated further. The comparison of the effectiveness of

Sarkosyl and SDS, with or without mutanolysin, on whole cell preparations of *S. mitior* and *S. sanguis* strains is shown in Fig. 3.3. SDS (although effective on membranes) was not effective on whole cell preparations for both lysis and loading. This can be seen by the poor demonstration of PBP's when SDS was used. When mutanolysin (which serves to break down the cell wall and thereby release the cell membrane) was added to either Sarkosyl or SDS, lysis and solubilization of membrane proteins were improved and the PBP's were demonstrated more clearly (Fig. 3.3). SDS + mutanolysin were still an inferior combination compared with that of Sarkosyl + mutanolysin. Nevertheless, although the latter combination proved effective against the majority of strains, there were still a few stubborn strains against which it was impotent.

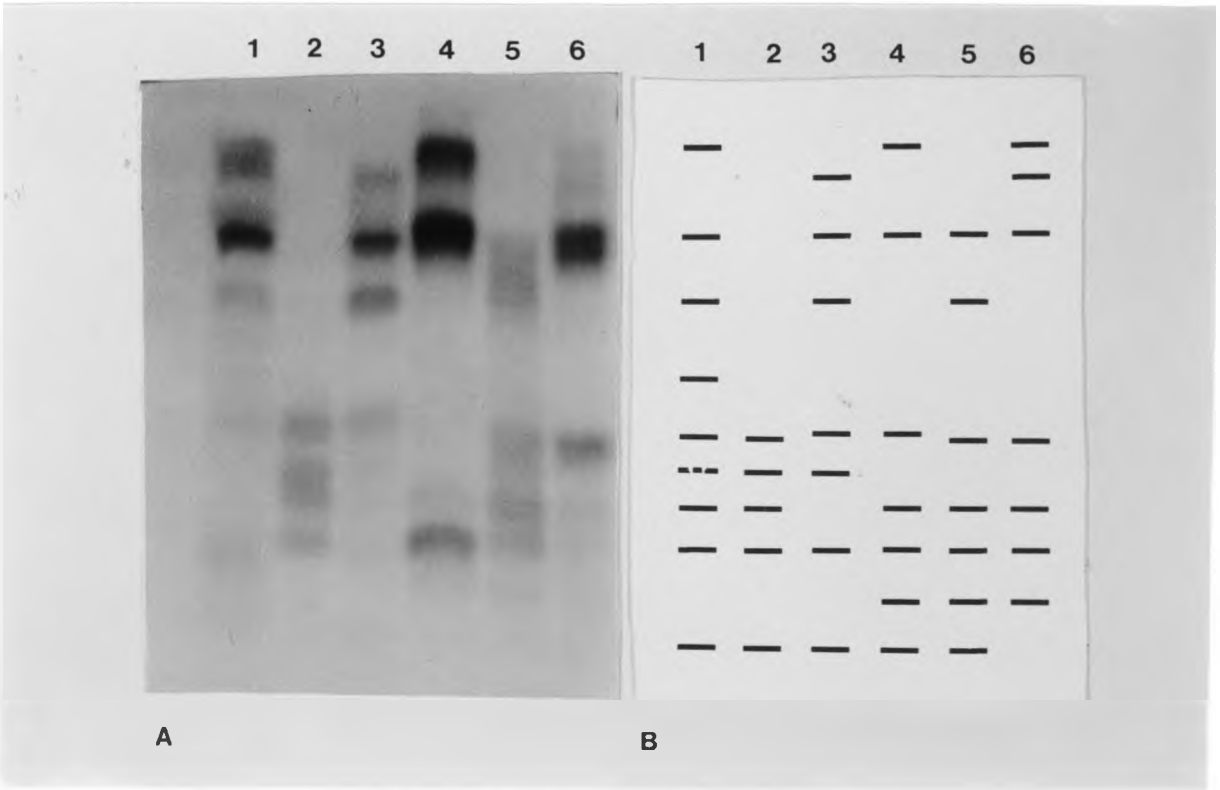

Figure 3.1 Comparison of SDS, Sarkosyl and Triton X-100 solubilization of [^{3}H] penicillin-labelled membrane preparations of *S. mitis* and *S. sanguis*. (A) Fluorograph; (B) Schematic diagram: —— indicates PBP clearly visible; -- just visible. Lanes 1, 2 and 3 strain 1; lanes 4, 5 and 6 strain 3. SDS was used in lanes 1 and 4; Sarkosyl in lanes 2 and 5; Triton X-100 in lanes 3 and 6.

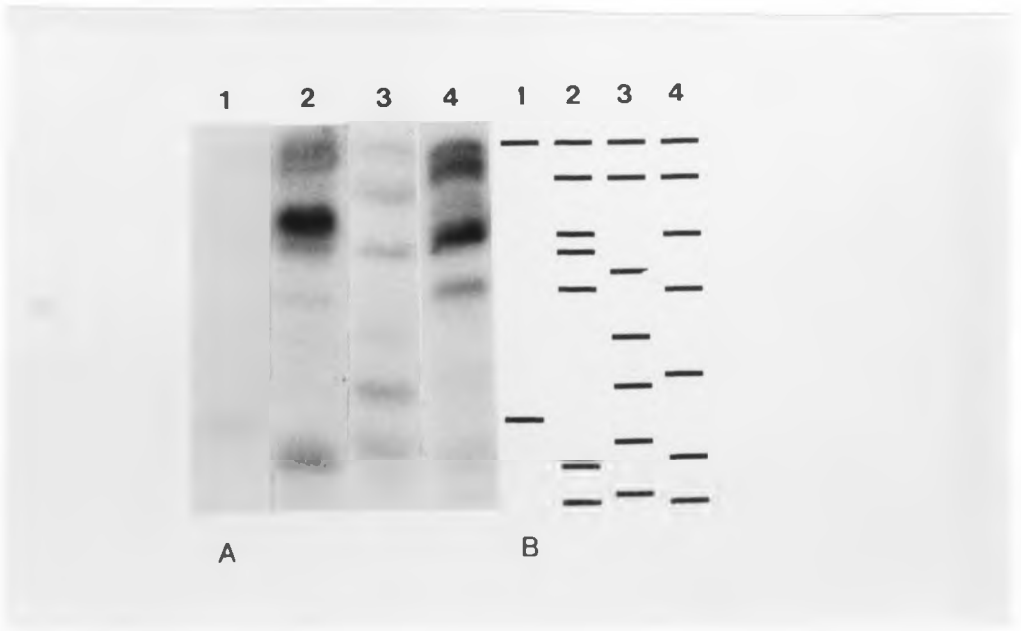

Figure 3.2 Comparison of [^{3}H] penicillin-labelled whole cell lysates and membrane preparations. (A) Fluorograph; (B) Schematic diagram: —— indicates PBP clearly visible. Lane 1 strain 3 (membrane preparation); lane 2 strain 3 (whole cell preparation); lane 3 strain 1 (whole cell preparation); lane 4 strain 1 (membrane preparation).

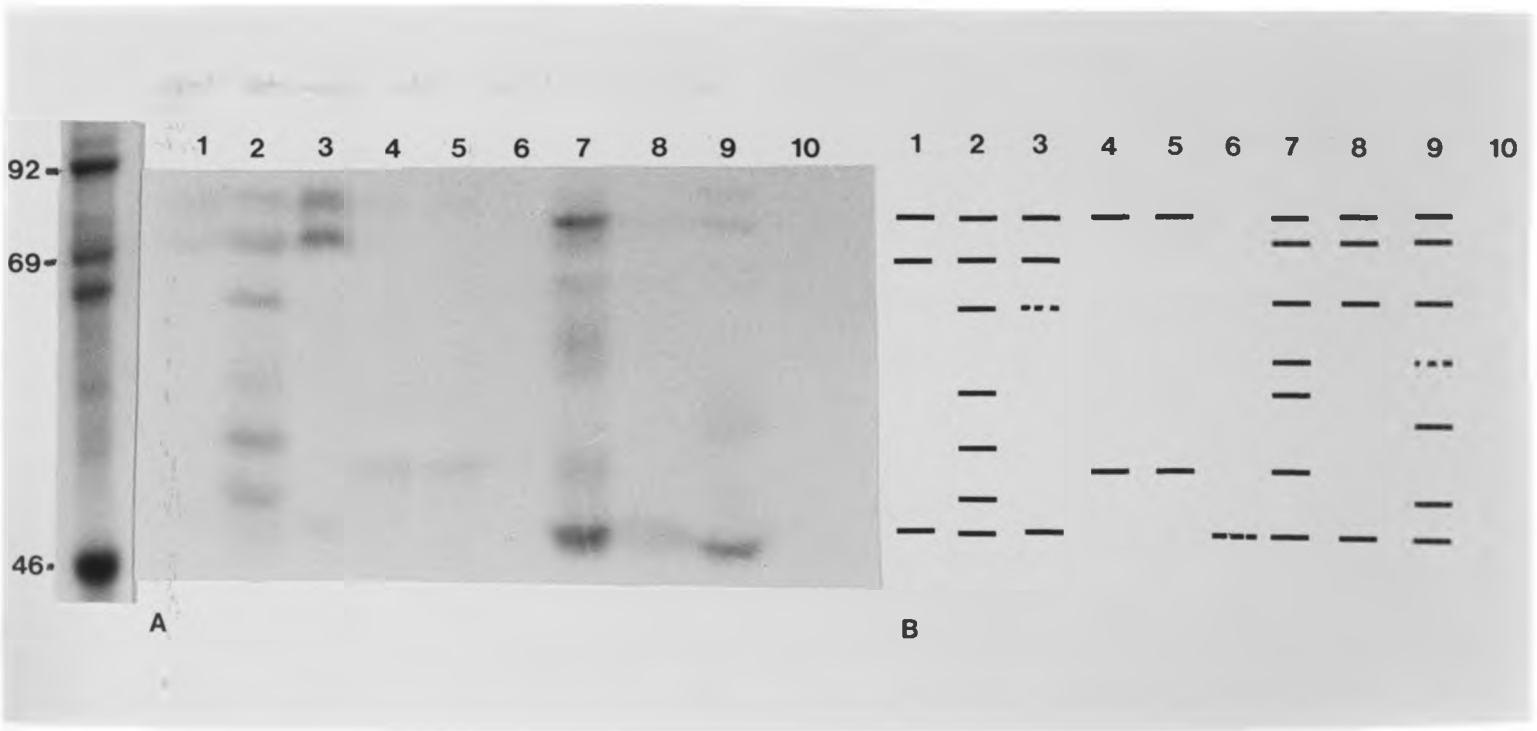

Figure 3.3 Comparison of Sarkosyl and SDS solubilization of whole cell preparations of *S. mitis* and *S. sanguis*, in the presence and absence of mutanolysin. (A) Fluorograph; (B) Schematic diagram: —— indicates PBP clearly visible; -- just visible. Lanes 1, 2 and 3 strain 1; lanes 4, 5 and 6 strain 3; lanes 7 and 8 strain 17; lanes 9 and 10 strain 20. Lane 1 Sarkosyl; lanes 2, 4, 5, 7 and 9 Sarkosyl and mutanolysin; lanes 3 and 6 SDS; lanes 8 and 10 SDS and mutanolysin.

Twelve isolates of *S. mitis* and ten isolates of *S. sanguis* (comprising both I and II), as identified by the API 20 Strep system, were selected to cover the full range of penicillin susceptibilities for the PBP studies. The MIC's and gel electrophoresis lanes of these isolates are described in Tables 3.23 and 3.24, together with the strains' identifications according to the original screening method and the screening method of Ruoff and Kunz (Tables 3.23, 3.24 and 3.25). Their penicillin-binding protein (PBP) profiles are shown in Figs. 3.4 and 3.5. The NCCLS Minimum Inhibitory Concentration (MIC) Breakpoints for penicillin [80] were applied to the viridans streptococcal strains as follows: susceptible, $\leq$0.12 μg/ml; moderately susceptible, 0.25 - 2.0 μg/ml; and resistant, $\geq$4.0 μg/ml. Some of the higher molecular weight PBP's may appear as one dense band due to overlap of bound [^{3}H] penicillin (Fig. 3.5).

In both fluorograms (Fig. 3.4 and Fig. 3.5) bands were seen below 46-kD but these were only clearly demonstrable on seven day exposure and had no visible link with resistance. Slight variations in whole cell solubilization do exist between experiments and breakdown products of major bands [85] can also complicate the picture. Therefore, PBP studies were duplicated for the majority of strains and results were reproducible.

Comparing *S. mitis* PBP profiles, they were seen to be a heterogeneous group (Fig 3.4), unlike *S. pneumoniae* (Fig 3.6). In an attempt to resolve the haphazard PBP profiles exhibited by the *S. mitis* strains, the strains were divided into groups according to their API 20 Strep profiles (Table 3.23). Comparisons were made between the PBP patterns of strains 24, 11 and 31 (lanes 2, 4 and 7), between those of strains 1, 16, 39 and 33 (lanes 3, 10-12) and between those of strains 22 and 13 (lanes 6 and 8). Regarding strains 24, 11 and 31 (lanes 2, 4 and 7) it is clear that a PBP of approximate molecular weight 75-kD is no longer detected as moderate resistance is entered. As full resistance is entered, PBP's of approximate molecular weights 88-kD and 66 - 64-kD become undetectable. Looking at strains 1, 16, 39 and 33 (lanes 3, 10-12) PBP's of approximate molecular weights 88-kD, 65-kD and 54-kD showed reduced penicillin affinity as moderate resistance is entered. As

resistance is entered a PBP of approximate molecular weight 50-kD appears to experience a decrease in penicillin binding. Finally, looking at strains 22 and 13 (lanes 6 and 8) there did not appear to be any relation between their respective PBP's even though they were both penicillin-resistant. Apart from strains 22 and 13, however, the other strains that were re-examined did demonstrate clearer PBP alterations in relation to increasing penicillin resistance than previously described.

The PBP's demonstrated in Fig. 3.4 lanes 5, 10 and 11 were all from strains having the same MIC value. The PBP patterns of these strains did not resemble each other, however, and may represent different biotypes. Similarly, strains 13 and 33 (lanes 8 and 12) - also of the same MIC value - differed slightly in that strain 13 exhibited an extra PBP band (molecular weight of approximately 88-kD). A general trend of reduced binding can be seen with increasing penicillin resistance. PBP's of molecular weight 66 - 62-kD were only weakly demonstrated in isolates with MIC's $\geq$ 0.5mg/l. Decreased affinity of major bands 70 - 90-kD was also evident.

Comparing *S. sanguis* I/II strains (as identified by the API system) there was a clear PBP pattern difference in Fig 3.5 lanes 3, 5, 6, 7 and 10. According to Table 3.23 these strains were all arginine positive and, therefore, *S. sanguis*. The remaining strains, on the other hand, were lending towards *S. mitis*. Amongst the *S. sanguis* strains, overlapping of PBP bands was due to incomplete solubilization and poor resolution. There was reduced binding of [^{3}H] penicillin to PBP's of approximate molecular weight 84-kD as seen in Fig 3.5 lane 10. There was also reduced binding of [^{3}H] penicillin to major PBP's 70 - 90-kD in resistant isolates of *S. mitis* (Fig 3.5 lanes 8 and 9).

PBP and penicillin susceptibility comparisons in viridans streptococci proved difficult and will require more extensive identification procedures, better solubilization of PBP's (especially *S. sanguis*) and affinity studies.

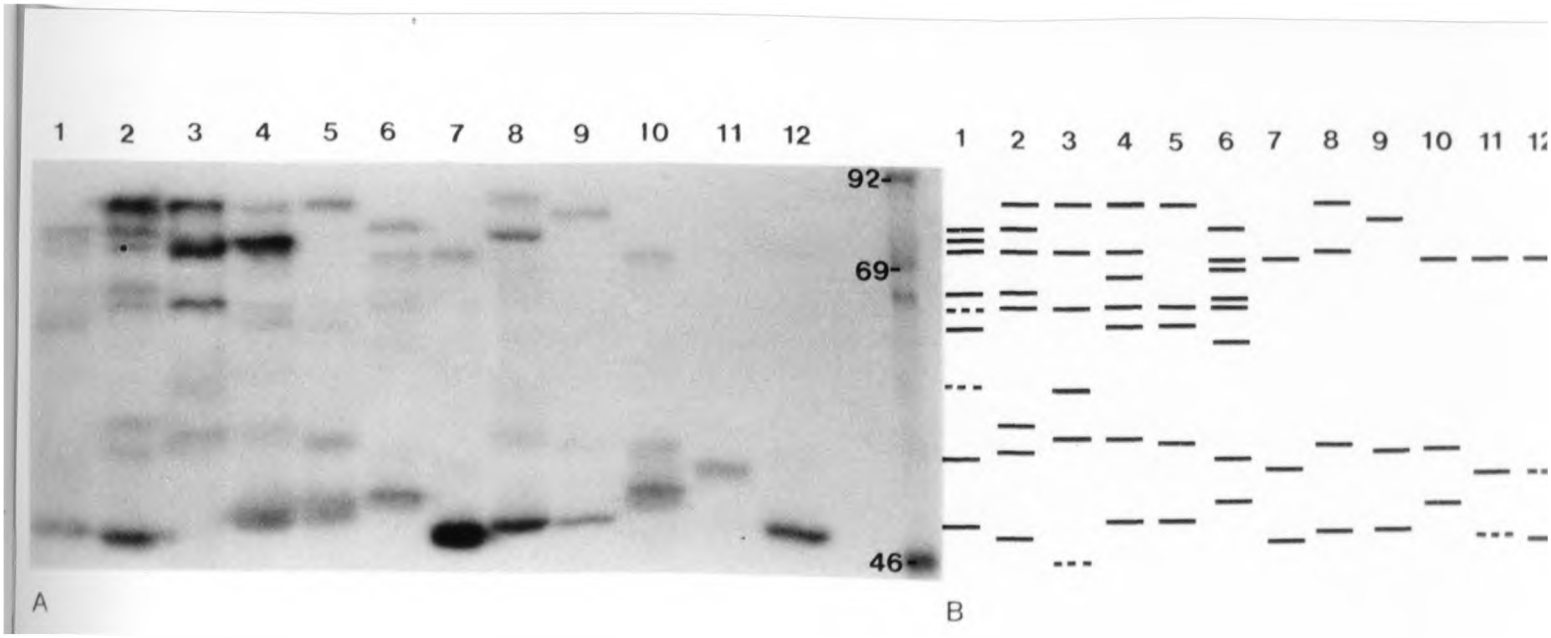

Figure 3.4 PBP profiles of *S. mitis* strains. (A) Fluorograph; (B) Schematic diagram: —— indicates PBP clearly visible; -- just visible. Lanes 1, 2, 3 and 4 susceptible; lanes 5, 6 and 7 moderately susceptible; lanes 8 and 9 resistant; lanes 10 and 11 moderately susceptible - questionable identities; lane 12 resistant - questionable identity. Detailed MIC's of the isolates are listed in Table 3.21.

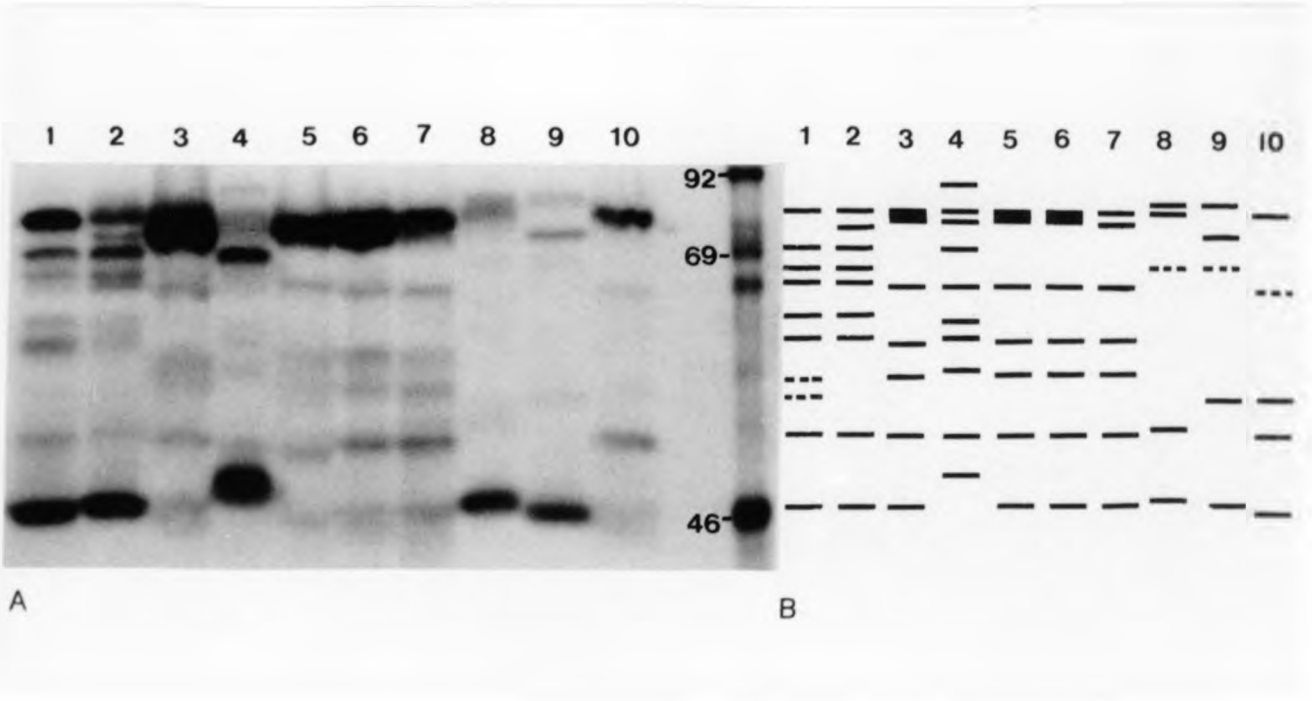

Figure 3.5 PBP profiles of *S. sanguis* I/II strains. (A) Fluorograph; (B) Schematic diagram: —— indicates PBP clearly visible; -- just visible; ▬ possible combination of PBP's. Lanes 1, 2 and 3 susceptible; lanes 4, 5, 6 and 7 moderately susceptible; lanes 8, 9 and 10 resistant. Detailed MIC's of the isolates are listed in Table 3.22.

TABLE 3.23 Details of Clinical Isolates of S. mitis (based on the API 20 Strep Identification System)

Strain	Penicillin MIC (μg/ml)	Fig. 3.4 PBP Lane	Original Screening Method[1]	Ruoff and Kunz Screening Method[2]
17	≤0.015	1	S. mitior	S. mitis
24	0.03	2	S. mitior	S. mitis
1	0.06	3	S. sanguis	S. mitis
11	0.12	4	S. sanguis	S. mitis
14	0.5	5	S. mitior	S. mitis
22	1.0	6	S. mitior	S. mitis
31	2.0	7	S. mitior	S. mitis
13	8.0	8	S. mitior	S. mitis
42	32.0	9	S. sanguis	S. mitis
16	0.5	10	S. mitior	S. mitis
39	0.5	11	S. sanguis	S. mitis
33	8.0	12	S. mitior	S. mitis

[1]Screening method based on Colman and Williams's classification [52]
[2]Screening method based on Facklam's classification [55]

TABLE 3.24 Details of Clinical Isolates of S. sanguis I/II (based on the API 20 Strep Identification System)

Strain	Penicillin MIC (μg/ml)	Fig. 3.5 PBP Lane	API 20 Strep System	Original Screening Method[1]	Ruoff and Kunz Screening Method[2]
35	≤0.015	1	S. sanguis I	S. sanguis	S. intermedius[3]
25	0.06	2	S. sanguis II	S. mitior	S. sanguis II
9	0.12	3	S. sanguis II	S. sanguis	S. intermedius[3]
19	0.25	4	S. sanguis II	S. mitior	S. sanguis II
40	0.5	5	S. sanguis I	S. sanguis	S. intermedius[3]
7	1.0	6	S. sanguis II	S. sanguis	S. sanguis II
5	2.0	7	S. sanguis II	S. sanguis	S. sanguis II
21	4.0	8	S. sanguis II	S. mitior	S. sanguis I
20	8.0	9	S. sanguis II	S. mitior	S. sanguis II
3	16.0	10	S. sanguis II	S. sanguis	S. sanguis II

[1]Screening method based on Colman and Williams's classification [52]
[2]Screening method based on Facklam's classification [55]

[3]S. intermedius = S. MG-intermedius

TABLE 3.25 Details of Viridans Streptococcal Isolates

Strain	API Identification APICODE	Species	(%)	Variable Reactions E A	A B P	R'T I R S	Penicillin MIC (μg/ml)
35	4242410	S. sanguis I/1	87	+ -	+ - -	+ + - - -	<0.015
25	0260440	S. sanguis II	99	- -	+ - +	- - - + -	0.06
9	4261441	S. sanguis II	58	+ +	+ - +	- - - + +	0.125
19	0240441	S. sanguis II	98	- -	+ - -	- - - + +	0.25
40	4261450	S. sanguis I/1	82	+ +	+ - +	- + - + -	0.5
7	0271450	S. sanguis II	92	- +	+ + +	- + - + -	1.0
5	0261440	S. sanguis II	97	- +	+ - +	- - - + -	2.0
21	0270460	S. sanguis II	99	- -	+ + +	- - + + -	4.0
20	0270441	S. sanguis II	99	- -	+ + +	- - - + +	8.0
3	0261450	S. sanguis II	74	- +	+ - +	- + - + -	16.0
17	0050401	S. mitis	99	- -	- + -	- - - - +	<0.015
24	0050400	S. mitis	94	- -	- + -	- - - - -	0.03
1	0040400	S. mitis	87	- -	- - -	- - - - -	0.06
11	0050400	S. mitis	94	- -	- + -	- - - - -	0.125
14	0070401	S. mitis	99	- -	- + +	- - - - +	0.5
22	0062401	S. mitis	99	- -	- - +	- - - - +	1.0
31	0050400	S. mitis	98	- -	- + -	- - - - -	2.0
13	0060401	S. mitis	99	- -	- - +	- - - - +	8.0
42	0070400	S. mitis	99	- -	- + +	- - - - -	32.0
16	0040400	S. mitis	87	- -	- - -	- - - - -	0.5
39	0040400	S. mitis	87	- -	- - -	- - - - -	0.5
33	0040400	S. mitis	87	- -	- - -	- - - - -	8.0

D = Doubtful
E A = esculin, arginine
A B P = αGAL, ßGAL, PAL
R'T I R S = RIB, TRE, INU, RAF AMD
All VP HIP PYRA BGUR ARA MAN SOR GLYG - negative
All LAP LAC - positive

3.6.2 S. pneumoniae

Twenty-one isolates of *S. pneumoniae* were selected to cover the full range of penicillin susceptibility for the PBP studies. The MIC's and gel electrophoresis lanes of these isolates are described in Table 3.26. The penicillin-binding protein (PBP) profiles of these pneumococcal isolates are shown in Figs. 3.6 and 3.7. As with the viridans streptococci, the NCCLS MIC Breakpoints for penicillin [80] were also applied to the pneumococcal strains as follows: susceptible, ≤0.06 μg/ml; moderately resistant, 0.12 - 1.0 μg/ml; and resistant, ≥2.0 μg/ml.

PBP's 1a and 1b may have appeared as one dense band due to overlap of bound [^{3}H] penicillin (Figs. 3.6 and 3.7). The slightly higher molecular weight PBP 3 (52 - kD) (Fig. 3.6) has been described before and has been found not to be associated with reduced susceptibility to penicillin [48, 50]. PBP 2b could be seen to alter to an apparently higher molecular weight as the MIC of the isolates reached 0.125 - 0.25 μg/ml (Figs. 3.6 and 3.7). This slightly higher PBP 2b then appeared to decrease its binding affinity for penicillin through moderately susceptible isolates and was only just visible or not detected in isolates with MICs $\geq$ 1.0 μg/ml. Faint PBP 2b bands could be detected in the resistant isolates (Fig. 3.6) which may have been residual production of the PBP 2b which still bound penicillin. Saturation studies are required to confirm reduced affinity of PBP 2b and the PBP's of Group 1. Two susceptible strains had a slightly lower molecular weight PBP 2a (Fig. 3.6 lanes 2 and 3). There was a preponderance of detectable PBP 2a' (Figs. 3.6 and 3.7) throughout the full range of penicillin susceptibilities selected. A new PBP was demonstrated in one of the moderately susceptible isolates (Fig. 3.6, lane 7), its molecular weight being approximately 60 - kD.

TABLE 3.26 Details of Clinical Isolates of S.pneumoniae

Strain	Penicillin MIC (μg/ml)	Fig. 3.6 PBP Lane
11	0.03	1
721	0.06	2
1002	0.06	3
781	0.125	4
621	0.25	5
21	0.25	6
132	0.25	7
1022	0.5	8
1042	2.0	9
1152	4.0	10
1212	2.0	11

Strain	Penicillin MIC (μg/ml)	Fig. 3.7 PBP Lane
1971	$\leq$0.015	1
1161	0.06	2
10221	0.25	3
1053	0.25	4
111	0.25	5
1052	2.0	6
1602	4.0	7
11522	4.0	8
1452	4.0	9
602	0.06	10

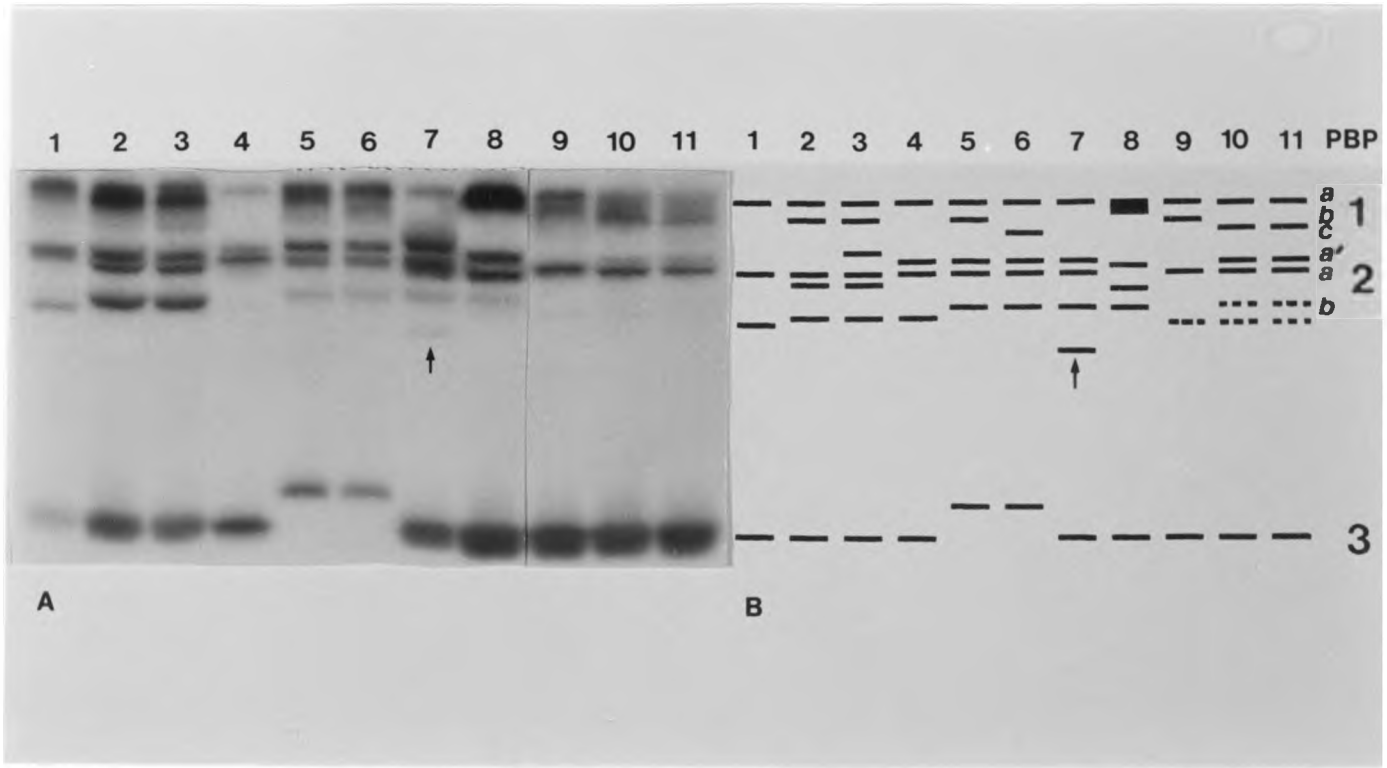

Figure 3.6 PBP profiles of *S.pneumoniae* strains. (A) Fluorograph; (B) Schematic diagram: —— indicates PBP clearly visible; -- just visible; ▬ possible combination of PBP's 1a and 1b; ↑ location of new PBP. Lanes 1, 2 and 3 susceptible; lanes 4, 5, 6, 7 and 8 moderately susceptible; lanes 9, 10 and 11 resistant. Detailed MIC's of the isolates are listed in Table 3.24.

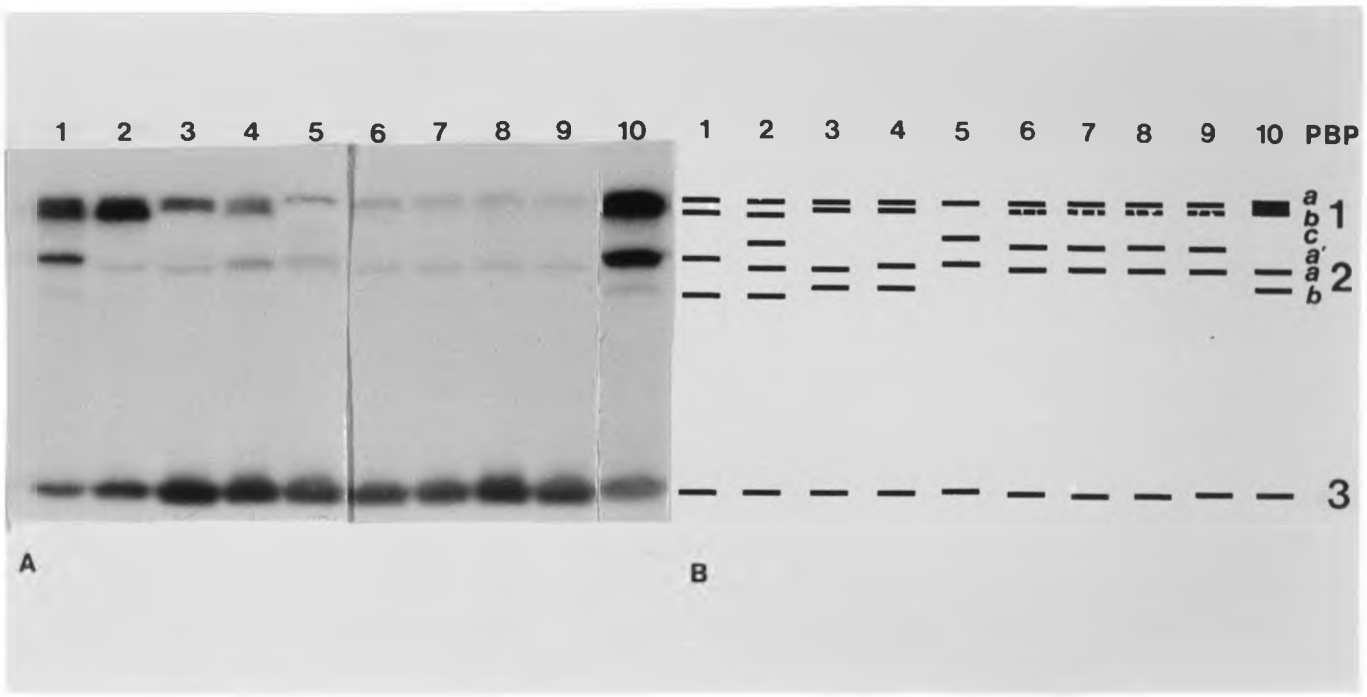

Figure 3.7 PBP profiles of *S.pneumoniae* strains. (A) Fluorograph; (B) Schematic diagram: —— indicates PBP clearly visible; -- just visible; ▬ possible combination of PBP's 1a and 1b. Lanes 1, 2 and 10 susceptible; lanes 3, 4 and 5 moderately susceptible; lanes 6, 7, 8 and 9 resistant. Detailed MIC's of the isolates are listed in Table 3.24.

Chapter 4
DISCUSSION

4.1 PENICILLIN-RESISTANT PNEUMOCOCCI

4.1.1 Incidence of Penicillin-resistant Pneumococci

The first reported isolation of a clinically relevant pneumococcus with decreased susceptibility to penicillin, occurred in Sydney, Australia in 1967 [3]. Although sporadic reports of pneumococcal isolates showing increased resistance to penicillin appeared subsequently, concern with such strains being a cause of potential therapeutic problems did not emerge until early in the 1970's [86, 87].

In recent years there has been a significant increase in South Africa and elsewhere in the prevalence of penicillin-resistant pneumococci in the hospital enviroment. This increase is particularly marked in Spain [11, 12] and in South Africa [88]. Studies have shown that patients, particularly children, who have received antibiotics while in hospital, are more likely to carry penicillin-resistant pneumococci than other individuals [14, 23, 25, 26]. The results in this study are in accordance with this.

An important feature of the present study is that it describes an attempt to monitor directly the carriage of penicillin-resistant pneumococci in hospitalized children. It was clearly shown that ß-lactam antibiotic therapy markedly reduced the frequency of carriage of *S. pneumoniae* from 37 to 17 in the carriers who received such antibiotics. The carriage of pneumococci in children who did not receive ß-lactam antibiotics was also somewhat reduced (from 16 to 11). Thus a total of 25 children lost their pneumococci, while 17 out of the 46 children who were not carriers on admission acquired nasopharyngeal pneumococci (6 of these children were on ß-

lactam antibiotics and 11 were not). This study thus showed a considerable turn-over in the carriage of pneumococci, some (mainly those on antibiotics) losing their strains while others who were previously not carriers became colonized with new strains. Strains belonging to serogroups 6 and 23 were mainly responsible for colonizing non-carriers in hospital. Although there was a nett loss of 8 carriers (15%) during hospitalisation (from 53 to 45), the prevalence of penicillin-resistant pneumococcal carriers increased from 23 out of 53 (43%) to 33 out of 45 (73%; $p = 0.003$) in the case of penicillin-resistant pneumococci and from 7 out of 53 (13%) to 20 out of 45 (44%; $p = 0.001$) in the case of high level resistance (HLR) strains. On closer examination it became clear that children who were treated with ß-lactam antibiotics showed a strong trend to lose their penicillin-susceptible strains: from 25 susceptible strains out of 43 carriers (58%) to 3 out of 20 (15%) in relation to penicillin-resistant strains ($p = 0.03$) and from 39 relatively susceptible strains out of 43 carriers (91%) to 8 out of 23 (35%) when susceptible strains and strains of lesser resistance were compared with HLR strains ($p = 0.00007$). Conversely, there were corresponding increases in resistant strains (18 out of 43 to 20 out of 23) and in HLR strains (from 4 out of 43 to 15 out of 23) (see Table 3.5 C). Similar but insignificant trends were observed when carriers who were not on ß-lactam antibiotics were followed up (the respective p values being 0.3 and 0.1). These trends were accompanied by the acquisition of mainly penicillin-resistant strains in children who did not previously carry pneumococci. Of the 17 non-carriers who subsequently became carriers, all 6 strains from the 6 carriers who were on ß-lactam antibiotics were resistant to penicillin (and 5 of these were HLR strains), while the majority of strains acquired in the 11 children who were not on ß-lactam antibiotics were also resistant to penicillin (8 were penicillin-resistant and 4 of these were HLR). The nett result was the emergence of a carrier population with a markedly altered penicillin resistance pattern featuring a significant increase in the number of carriers of penicillin-resistant pneumococci. This increase was most noticeable in the case of HLR strains. The two main factors involved in these changes appear to be the selection of resistant pneumococci by ß-lactam antibiotics, largely through the elimination of susceptible strains from carriers, and

the acquisition of penicillin-resistant strains by hospital cross-infection in children who did not carry pneumococci on admission.

Although no significant difference in carriage of resistant pneumococci existed between those carriers who had been on ß-lactam antibiotics on admission and those who had not (6 out of 10, 60% and 18 out of 43, 42%; p=0.25, Fisher's Exact Test) this could be due to the fact that the ß-lactam-treated admission group carriers would only have been on antibiotics for a few days prior to their admission - a far shorter period of time than those hospitalised carriers who received antibiotics would have been exposed to these agents.

It is, unfortunately, not feasible to isolate carriers of resistant pneumococci from other non-carriers in an over-crowded hospital like Baragwanath Hospital. The wards, therefore, become a potential haven for cross-infection. Even those children not receiving antibiotics and, therefore, not exposed to this important risk factor in the acquisition of resistant pneumococci, can easily become carriers of these organisms while in hospital. In this study, even those hospitalised carriers never exposed to antibiotics while in hospital were found to carry a high rate of resistant pneumococci at the end of their hospital stay (13 out of 22; 59% and 5 out of 22; 23% for HLR strains). The corresponding figures for the carriers who were on ß-lactam antibiotics were 20 out of 23 (87%; p = 0.08) for all resistant strains and 15 out of 23 (65%; p = 0.01) for HLR strains (see Table 3.5 B).

Another study which stresses the hazards of overcrowding is one that was carried out by Ward in Johannesburg hospitals [2]. At two of the hospitals involved in the study, resistant pneumococci acquisition rates for newly admitted patients were 90% and 15% respectively during the first week; in the latter hospital less overcrowding occurred, thus showing that overcrowding does encourage the spread of pneumococci.

Another major problem facing hospitals like Baragwanath, where a marked percentage of children already carry moderately resistant pneumococci, is the possibility that the acquisition of higher level penicillin resistance is more frequent in pneumococci already possessing the lower levels of resistance, as suggested by in vitro studies [42, 48]. Thus, the latter population may provide a reservoir of pneumococci from which strains with dangerously high levels of penicillin resistance may emerge. This could well explain the dramatic difference ($p=0.00007$) in the proportions of highly resistant to sensitive pneumococci (MIC $\geq$ 2 μg/ml) between those who were not on antibiotics on admission (4 out of 43, 9%) and those hospitalized children who received antibiotics prior to admission and/or while in hospital (15 out of 23, 65%).

The combination of the selective pressure of ß-lactam antibiotic usage and the facility of hospital cross-infection of pneumococci could paint a very bleak future for overcrowded hospitals like Baragwanath. These conditions will also promote the emergence of highly resistant pneumococci in carriers of moderately susceptible organisms. The hospital thus becomes a major source of resistant pneumococci which will inevitably spread to the community [2, 20, 24] (see Section 4.1.3).

4.1.2 Relationship between Age and the Carriage of Resistant Pneumococci

Another important factor which usually influences the carriage of penicillin-resistant pneumococci, is the age of the child. It has been shown, on a number of occasions [14, 20, 23], that infants are more predisposed to acquiring penicillin-resistant pneumococci than older children. This may be due to a poorly developed immune response to pneumococcal polysaccharides 6 and 19 (which are two of the serotypes most commonly associated with penicillin resistance in children under two years of age [82]). Older children previously exposed to penicillin-resistant serogroups 6 and 19 (which are the most prevalent serogroups in young children) would have preformed antibodies to these serogroups and may thus resist colonization.

On dividing the children in this study into their respective age groups, according to whether they received ß-lactam antibiotics or not (Table 3.7), it can be seen that amongst all the carriers on admission, the carriage rate of resistant pneumococci was marginally higher ($p=0.04$) in the 0 - 3 year olds (22 out of 42 individuals, 52%) than in the 4 - 9 year olds (2 out of 11 individuals, 18%). One could assume, therefore, that within the community infants (0 - 3 years old) will tend to carry higher rates of penicillin-resistant pneumococci than older children (4 - 9 years old) would. A significant difference ($p=0.04$) was also evident between the 0 - 3 year olds and 4 - 9 year olds amongst those carriers not on antibiotics on admission (17 out of 34, 50% and 1 out of 9, 11%, respectively). This would suggest that without the effects of antibiotic exposure and hospital-related factors, resistant pneumococci will be more prevalent in infants than in older children. The fact, however, that amongst those hospitalised carriers who received antibiotics (both prior to and/or subsequent to admission) there was an insignificant difference ($p=0.5$, Fisher's Exact Test) between the 0 - 3 year olds and 4 - 9 year olds in their respective carriage rates of resistant pneumococci - 16 out of 21, 76% and 3 out of 3, 100% - would imply that the combination of antibiotic pressure and hospitalisation, with a high risk of cross-infection involving resistant pneumococci, have an over-riding effect on the carriage of resistant pneumococci in children, irrespective of age. The insignificant difference ($p=0.5$, Fisher's Exact Test) that existed between the two age groups amongst all those hospitalised children who were exposed to antibiotics (16 out of 58, 28% in the 0 - 3 year olds and 3 out of 13, 23% in the 4 - 9 year olds) supports this finding.

No significant difference in the children's pneumococcal carriage rates was noted (Table 3.1 and Table 3.2) between the period before their admission and the period covering their stay in hospital (the combined rates of carriage being 53 out of 100, 53% and 45 out of 100, 45%, respectively). The profound effect of antibiotics on the carriage of pneumococci by eliminating susceptible strains from the nasopharynx and also the acquisition of pneumococci in children in hospital who

previously did not carry these organisms have already been alluded to earlier. Ward *et al* [26] showed the carriage of pneumococci by the community to be 50% and that of hospitalised patients to be only 20%, thereby suggesting that hospitalization may lower the carrier rates of pneumococci. However, in the Ward *et al* study, the community sample comprised mainly children (who tend to have higher carriage rates of pneumococci than older individuals) while the hospital sample included all ages. Another plausible explanation for the marked difference between the present study and the one described by Ward *et al*, may be that the interplay between prolonged antibiotic administration in hospital, which could have lowered pneumococcal carrier rates, and the acquisition of new carriers in hospital may have differed in the two studies. It is noteworthy that, compared with the non-ß-lactam-treated admission group (who exhibited a carrier rate of 43 out of 74; 58%), the hospitalised group that did not receive antibiotics in the present study exhibited a pneumococcal carriage rate of 11 out of 36 (31%) and the hospitalised ß-lactam-treated children a slightly lower rate of 17 out of 64 (27%). This shows that children do lose their nasopharyngeal pneumococci during hospitalization. Many children who were not carriers, however, acquired pneumococcal strains in hospital, thus raising the overall carriage rate. Loss of carriage during antibiotic therapy correlates well with another study [90] that was conducted among children with sickle cell anaemia, receiving prophylactic penicillin. The rate of pneumococcal carriage was shown to be significantly reduced in those receiving the drug when compared with an untreated control group. The inverse relationship demonstrated between pneumococcal carriage and the incidence of penicillin resistance, may be partly due to the fact that the administration of penicillin therapy will destroy most of the penicillin-sensitive pneumococci, thereby allowing subsequent proliferation of resistant strains.

Also worth noting is the fact that in all of the study groups, there were no significant differences in pneumococcal carriage rates between the 0 - 3 year olds and 4 - 9 year olds: in those carriers who were not on antibiotics on admission the rates were 34 out of 59 (58%) and 9 out of 15 (60%), respectively; in those carriers who

were receiving antibiotics on admission the rates were 8 out of 21 (38%) and 2 out of 5 (40%), respectively; in those hospitalised carriers never exposed to antibiotics the rates were 15 out of 22 (68%) and 4 out of 7 (57%), respectively; $p=0.5$, Fisher's Exact Test; and in those hospitalised carriers who received antibiotics the rates were 21 out of 58 (36%) and 3 out of 13 (23%), respectively; $p=0.3$, Fisher's Exact Test. On the whole, however, the pneumococcal carriage rates of the two age groups were either equivalent (as in the two admsission groups) or lower in the 4 - 9 year olds (as in the hospitalised carriers). Perhaps if larger sample sizes had been used, the lower rates observed in the 4 - 9 year olds could have been shown to differ significantly from those rates exhibited by the 0 - 3 year olds. An overall trend towards lower pneumococcal carriage rates in the older children may also have been observed. In other studies [26, 89, 91] carriage of pneumococci has been shown to be significantly higher in infants less than 5 years of age than in older individuals. This is due, in part, to their duration of carriage being longer, presumably because their immune response to capsular antigens is poor [92, 93]. According to Gray *et al* [89] acquisition of the first type of pneumococcal strain occurs (on average) at six months, and the duration of carriage decreases with successive types carried. In their study, Gray *et al* found that duration of pneumococcal carriage ranged from one to 17 months. In general, the younger the infant was at the time of acquisition, the longer a strain was likely to be carried. As a group, types 6, 14, 19 and 23 were found to be carried significantly longer (mean, 4.2 months) than other types (mean, 2.7 months).

4.1.3 Pneumococcal Resistance in the Community

The above discussion has tended to focus on the markedly high levels of resistant pneumococci observed in the hospital environment. Yet, one cannot disregard the disturbingly high proportions of resistant to sensitive pneumococci isolated from those children who had not been on antibiotics on admission (42%), particularly if one considers that until recently, infections due to pneumococci, as well as the

asymptomatic carrier state, were almost exclusively nosocomially acquired [94]. Apparently, community-acquired bacteraemia due to penicillin-resistant pneumococci is occurring with increasing frequency in black children in Johannesburg [19, 24]. In a study performed in 1978 [14] the rate of acquisition of the carrier state among 152 healthy household contacts of children known to be colonized with resistant pneumococci, was 4.6%. Robins-Browne and Kharsany [20] found 6 (2%) carriers of resistant pneumococci in a study population of 305 children, in a survey conducted in Durban. In another study of urban children [88], Klugman and Koornhof showed that as many as 14.2% of black children carried penicillin-insensitive pneumococci. It is clear, therefore, that control measures also need to be applied to the prescribing of antibiotics in the community.

4.1.4 Penicillin-Resistant Pneumococcal Serogroup Distribution

Information about serogroups of penicillin-resistant pneumococcal strains is needed in order to design an epidemiological policy for the control of pneumococcal disease caused by these serogroups. For the polyvalent pneumococcal vaccine to be effective, the serogroups of penicillin-resistant pneumococcal strains should be identified so that the appropriate serogroups can be included in the vaccine. In this study, it was also important that the resistant pneumococci be serogrouped in order to, firstly, establish whether there was any difference between the serogroups isolated from the non-ß-lactam-treated groups and those isolated from the ß-lactam-treated groups, and secondly, determine whether there was a relationship between any particular serogroup(s) and the incidence of bacteraemia.

4.1.4.1 Effect of Antibiotics on Serogroup Carriage

The carriage of pneumococci is widespread in normal individuals, especially in infants. According to Gray *et al* [89], the number of types carried per infant varies from zero to six, with most children carrying one to three types. Although carriage

(with the exception of infants) is not usually associated with disease, the serotypes most frequently carried are also those most often associated with pneumococcal disease in susceptible infants, on acquisition of new strains [89]. Serogroups 6, 14 and 19 are those groups usually associated with nasopharyngeal carriage and with bacteraemia in children in most parts of the world. Together they account for 28% to 59% of pneumococcal bacteraemia in children in the United States [95 - 98]. Dowling *et al* [99], however, found type 3 accountable for almost two-thirds of the strains in the first year of life, decreasing markedly during the following three years, when type 19 became dominant.

Based on these facts and the fact that children with pneumococcal disease will almost certainly receive antibiotics (particularly penicillin or one of its derivatives), it is logical that those serogroups most commonly associated with infection (namely 6, 14, 19 and 23) would also be those most often associated with penicillin resistance. In fact, in South Africa, resistant pneumococci were initially only of two serotypes, 6A and 19A [1]. It is, therefore, not surprising that serogroups 6 and 19 (together with groups 14 and 23) predominated in this study. This correlates well with past studies which have not only found that these four types are most often associated with penicillin resistance [1, 10, 12], but have also shown them to be the ones most commonly isolated from infants [89]. The emergence of these four groups as resistant serogroups and as the serogroups most commonly isolated from infants, may well be related to the frequent administration of antibacterial drugs for the treatment of infections early in childhood [100]. Besides groups 6, 14, 19 and 23, many different capsular types have shown resistance [2, 19], and in a report from New Mexico [5] eleven different serogroups were found to be resistant to 0.1 μg/ml or more of penicillin.

4.1.4.2 Carriage of Resistant Serogroups and Initiation of Bacteraemia

One cannot comment on whether there is a connection between any particular serogroup(s) and the initiation of bacteraemia, as no penicillin-resistant pneumococci were isolated from the clinical specimens of any of the patients dur

ing their hospital stay. Nevertheless, in one of the patients a penicillin-susceptible pneumococcus was isolated from a blood specimen, while a resistant strain was isolated from the nasopharynx. In another patient, a penicillin-susceptible *S. pneumoniae* strain was isolated from both the nasopharynx and the blood, implying a possible endogenous invasion by the susceptible strain. To confirm this, the penicillin-sensitive pneumococci would have to be serotyped in order to establish whether it was, indeed, the same serotype (and, therefore, probably the same pneumococcal strain) that was isolated from both the nasopharynx and blood. For the same reason, it would also have been necessary to have serotyped all resistant pneumococcal isolates to have enabled one to determine whether the isolation of an invasive resistant pneumococcus was preceded by the isolation of a resistant strain of the same capsular type from the nasopharynx.

Another reason why it would be of interest to serotype all penicillin-susceptible and -resistant pneumococcal isolates from the nasopharynx in future studies assigned to elucidate the invasiveness of resistant strains, would be that one could then see whether, in any one individual, the isolation of a resistant pneumococcus was preceded by the isolation of a sensitive strain of the same capsular group, where the patient had been receiving ß-lactam antibiotics in the interim. Although one would not be able to establish with certainty that the two isolates represented the same pneumococcal strain, it would, nevertheless, be strongly indicative of this possibility and would, therefore, emphasize the strong link between antibiotic administration and the emergence of resistance.

Although more data would be needed to make a more concrete comment, on the whole, there did not appear to be any correlation between the nasopharyngeal carriage of resistant streptococci and the development of bacteraemia. Differences in the ability of nasopharyngeal epithelial cells of different individuals to adsorb pneumococci, and differences in the ability of strains of pneumococci of the same or of different capsular types to adhere *in vitro* to the epithelial cells of a single individual, have been reported [100, 101, 102]. However, although there is evidence

that the adhesiveness of a particular pneumococcal strain may have been associated with otitis media, more data will be needed to evaluate fully the importance of adherence preceding invasion. One could study children who have a bacteraemic infection, and swab them nasopharyngeally in order to determine the possibility of a strain from the nasopharynx being responsible for the initiation of the bacteraemia. This would require serotyping of the pneumococcal strains involved.

4.1.5 Progress in the Control and Treatment of Resistant Pneumococcal Infections

4.1.5.1 The Pneumococcal Vaccine

The slowly increasing occurrence of drug-resistant pneumococcal strains provides added impetus to the importance of developing a vaccine which can be used for active immunization against infection with those pneumococcal types most frequently responsible for infection in man. A number of studies have focussed on the benefits of immunizing children with such a polyvalent vaccine, the aim of which is to prevent the development of systemic pneumococcal disease and reduce transmission of penicillin-resistant strains. MacLeod *et al* [103] and Sivonen [104] have suggested that antibodies developed after immunization with vaccines of bacterial capsular polysaccharides may reduce the acquisition of homotypic organisms by previously uncolonized adults. According to Andersson *et al* [102], the antigen-antibody complexes produced after immunization may interfere with the interaction of the pneumococcal 'adhesin' (responsible for the adherence of pneumococci to nasopharyngeal epithelial cells) with receptors on respiratory epithelial cells. Austrian [100] pointed out that, while vaccination with a type-specific capsular antigen had no effect on carriage of the homotypic organism if colonization had occurred prior to immunization, it reduced by approximately half, the chance of acquiring the homotypic organism in the previously uncolonized individual.

Nevertheless, the issue of a polyvalent vaccine is a controversial one and a number of authors have disputed the effectiveness of such a vaccine. In one such study [105], no consistent effect on nasal carriage rates of *S. pneumoniae* belonging to the serotypes included in the vaccine was observed in children aged 6 - 54 months. Another study [106] also showed that the serum antibody response to the vaccine is age-dependent - the principal paediatric types are 6, 14, 19 and 23, and the humoral response in young children to these polysaccharides is much less than that for young adults. In fact, Riley *et al* [107] claimed that even if the serotype of the resistant isolate were included in the vaccine and the patients were old enough to have an immunologic response to the vaccine, it was still doubtful that transmission via pharyngeal colonization would be completely eliminated.

In a study by Simberkoff *et al* [108], all of the ten resistant pneumococcal strains isolated were found to belong to serotype 19A. As the 14-valent vaccine does not contain serotype 19A antigen, the authors prescribed the use of the newer 23-valent vaccine instead. The 23-valent vaccine is in current use in the U.S.A. [27] and, although it does not include serotype 19A, it does include the capsular polysaccharides of serotypes 6A and 19F. There is a noteworthy degree of immunologic cross-reactivity between serotypes 19F and 19A, and there is a strong likelihood that antibodies to the former would provide partial, if not complete, protection against the latter [14]. However, here, too, the probable effectiveness of the vaccine is limited by the relative inability of children under two years of age to mount an effective immune response, and the lesser degree of immunogenicity of polysaccharides generally (and of types 19F and 6A specifically) in this age group [2].

Faced with the continuing problem of pneumococcal infection, therefore, Davies and Kumaratne [109], in 1988, made various recommendations concerning the improvement of current immunotherapeutic practices. For instance, conjugation of capsular polysaccharides to protein carriers such as tetanus toxoid could overcome the physiological inability of young children to produce antibodies directed

against the polysaccharide moiety. This approach (although it requires further investigation) has shown results which may point the way towards the development of second generation pneumococcal vaccines. Another approach is the possible development of a vaccine aimed at the cell wall components involved in the pathogenesis of pneumococcal infection, as this could also be effective in reducing the morbidity associated with this illness. The final idea proposed by Davies and Kumaratne concerns the possible addition of serotherapy to antibiotic regimens. As some commercial immunoglobulin preparations have a high titre of anti-capsular antibody directed against the commonly pathogenic pneumococcal serotypes, doing this may be of benefit in pneumococcal infections.

The persistently high mortality of severe pneumococcal infection in the post-penicillin era should be a spur for research into optimal therapy for this condition. Tuomanen [110] recently indicated that four modalities of drugs that could augment the effect of antibiotics in the treatment of bacterial meningitis ("partner drugs") are being researched, and show great promise in reducing the current unacceptable mortality rate associated with pneumococcal meningitis. All four of the modalities involve reducing the inflammation associated with bacterial meningitis. They are: the design of nonlytic ß-lactam antibiotics, such as imipenem, which lyse bacteria in a manner that releases less inflammatory debris from the bacterial cell wall; to capture inflammatory cell wall components to render them inert (for instance, the use of anti-cell wall antibodies); to decrease leucocyte-associated damage by developing antibodies that block leucocyte adhesion to endothelia; and to inhibit the activation of host mediators of inflammation by the use of steroids and nonsteroidal anti-inflammatory drugs.

4.1.5.2 Control Measures

Unfortunately, no effective control programme has been instituted on a wide enough scale to combat the growing problem of penicillin resistance in both pneumococci and viridans streptococci. Such a programme is especially neces-

sary because of the difficulty involved in developing another antimicrobial agent that is as effective, safe and convenient as penicillin, and is comparable in price. The extensive research being carried out in this regard will hopefully, in time, result in the development of a suitable antibiotic (or antibiotics) that will not only satisfy all the aforementioned requisites, but will also be effective against the growing numbers of multiply resistant streptococci.

Certain control measures have been suggested as a means of countering the problems associated with increasing resistance in *S. pneumoniae*. Appelbaum *et al* [27] recommended the following: upon recovery of a penicillin-resistant pneumococcus from a patient, surveillance studies should be performed to determine the prevalence of such organisms in the hospital population; in sporadic cases restriction of antibiotic use in the immediate environment, isolation of the patient (if possible) and enhanced surveillance (including nasopharyngeal and oral culture of family members and hospital personnel); if resistant pneumococci are identified in a limited population group at increased risk of developing systemic infection (for instance, in a paediatric medical ward), consideration should be given to enforced isolation procedures, separating susceptible patients (where possible) from those likely to harbour resistant pneumococci. Ward and Koornhof [94] have recommended that: antibiotics should be restricted to treatment of proven or suspected bacterial infections; where possible, crowding in hospital wards should be alleviated, especially for children under six years of age; effectiveness of the pneumococcal vaccine containing (in particular) type 19A and 6A antigens be evaluated; erythromycin be considered drug of choice for pneumococcal infections other than meningitis in areas where a large proportion of cases of severe pneumococcal infections are resistant to penicillin; and consideration be given to the possible effectiveness of high doses of penicillin in the initial treatment of patients with pneumococcal bacteraemia.

Although extensive measures have been taken in South Africa to control the spread of multiply resistant pneumococci (with relative success), the problem is still present throughout the country. The carrying-out of susceptibility tests on all isolates

from problem patients and periodic susceptibility surveys in reference laboratories would help detect development of resistance at an early stage, allowing for prevention of the spread of resistant organisms.

4.1.5.3 Alternative Treatment Regimens

Whether penicillin resistance should influence the treatment of pneumococcal infections depends largely on the degree of resistance and the site of infection. For instance, meningitis caused by pneumococci with any degree of penicillin resistance is associated with a poor response to treatment and a poor prognosis, even with a high dose penicillin regimen [2, 15].

Alternative therapies for meningitis caused by these resistant strains have been investigated and the most suitable would seem to be chloramphenicol, if susceptible, vancomycin and the third generation cephalosporins, cefotaxime and ceftriaxone. Imipenem (N-formimidoyl thienamycin) could also be added to the list [111], but is at present not recommended for the treatment of meningitis because of its increasing tendency to cause seizures in the presence of a disturbed blood-brain barrier. These considerations do not constitute firm recommendation for the treatment of penicillin-resistant pneumococcal meningitis, but rather suggest alternatives to penicillin in this commonly lethal disease.

In the case of bacteraemia or pneumonia, the relevance of pneumococcal resistance is less obvious as one is able to attain peak serum levels of penicillin well above the MIC of even highly resistant pneumococci. Nevertheless, whichever antibiotic is chosen, it is vital that therapy for pneumococcal pneumonia be started immediately. In areas where resistance is commonplace or where the patient is desperately ill, vancomycin should be administered from the start. Elsewhere, however, the use of penicillin as routine initial therapy for community-acquired pneumonia is an acceptable approach [22, 120].

Pallares *et al* [22] recommended the use of erythromycin, clindamycin and rifampicin for the treatment of penicillin-resistant pneumococcal pneumonia, but Klugman and Koornhof [113] have recently emphasized resistance to both erythromycin and clindamycin in pneumococcal isolates from blood and cerebrospinal fluid, of both adults and children in South Africa [114, 115]. They also make mention of the fact that in countries like South Africa, where tuberculosis is endemic, rifampicin resistance is increasing due to its extensive use in hospitals and in the community. In fact, from the period 1979 - 1982 to the period 1983 - 1986, rifampicin resistance doubled [113].

Chloramphenicol has also been recommended as an alternative to penicillin for the treatment of severe penicillin-resistant pneumococcal infections [116]. However, Istre *et al* [117] found that one of the seven relatively penicillin-resistant pneumococcal strains that they isolated was also resistant to chloramphenicol, and Garau *et al* [118] described a patient with penicillin-resistant pneumococcal meningitis that did not respond to chloramphenicol therapy. Chloramphenicol resistance, although relatively uncommon, occurs in South Africa [14, 15, 24, 119] and this should be kept in mind when initial therapy for meningitis is instituted in centres where chloramphenicol resistance occurs.

It would appear, therefore, that the best procedure to follow for the treatment of penicillin-resistant pneumococcal meningitis in populations where multiple antibiotic resistance occurs, would be that recommended by Koornhof [120]. He suggests that in pneumococcal meningitis due to intermediately resistant strains, high doses of cefotaxime or ceftriaxione could be considered, while vancomycin would be appropriate treatment. Rifampicin could also be considered as an adjunct in the treatment of penicillin-resistant pneumococcal meningitis, but only if the organism is susceptible to it and it is used together with another appropriate antibiotic. However, in infections caused by high-level resistant strains, only vancomycin can be regarded as a drug likely to give satisfactory results.

4.2 PENICILLIN-RESISTANT VIRIDANS STREPTOCOCCI

4.2.1 Incidence of Penicillin-Resistant Viridans Streptococci

High carriage rates of viridans streptococci were demonstrated in this study in both ß-lactam antibiotic treatment and in control groups. This good isolation rate is no doubt due to the fact that the oral cavity was the site selected for the isolation of viridans streptococci. The oral cavity (rather than the nasopharynx) is known to be the site for optimal carriage of these organisms. In a previous study done [81] the nasopharynx was the site selected for isolation of the viridans streptococci. The carriage rates of these organisms were found to be as low as 16% in patients on penicillin prophylaxis and 37% in normal children.

The fact that in this study those hospitalised children who received ß-lactam antibiotics (prior to and/or subsequent to admission) demonstrated higher carriage rates of viridans streptococci than the ß-lactam-treated admission group, suggests that the use of antibiotics in hospital probably exerted a selective effect by reducing the number of carriers with penicillin-sensitive strains, but that these were rapidly replaced with resistant strains. It is also possible that reduction in the number of other oral bacteria may have facilitated colonization with viridans streptococci in hospital.

It is clear from the results in this study that penicillin resistance amongst the viridans streptococci is reaching alarming proportions. In all of the study groups, the proportion of resistant organisms to sensitive organisms was greater than 70%. We demonstrated clearly that antibiotic administration had an effect on the carrier rate of penicillin-resistant strains as there were no significant differences in the carriage of resistant organisms, between the ß-lactam-treated groups and the control groups (Table 3.10 and Table 3.11).

As the carriage rates of penicillin-resistant viridans streptococcal isolates increased in both the ß-lactam antibiotic-treated group of hospitalised patients and in those not treated with ß-lactam antibiotics, it would appear that the hospital environment, in addition to antibiotic administration, was responsible for the acquisition of resistant organisms by hospital cross-infection. What is also of great interest, is the highly significant increase in the carriage of highly resistant (MIC $\geq$ 4 μg/ml) oral streptococci, from 33% in the control admission group to 90% in those hospitalised children who received antibiotics subsequent to admission (see Table 3.10 C(b)). This implies that, in addition to the likely effect of antibiotics on increasing the rate of resistance amongst clinical isolates of oral streptococci, they may also have a positive influence on increasing the degree of resistance in the isolates.

Using our routine screening procedure, the majority of viridans streptococci isolated, in all of the study groups, were *S. mitior* (the mean isolation rate being 68%). This correlates with a previous study [81], in which 63% of the viridans streptococcal isolates were of the *S. mitior* species. In the same study, the second most commonly isolated species of viridans streptococci was *S. sanguis* (as in this study).

Both *S. mitior* and *S. sanguis* isolates correlated with the viridans streptococcal group as a whole, in that those hospitalised children who received antibiotics prior to and/or subsequent to admission had higher carriage rates of penicillin-resistant isolates than the ß-lactam-treated admission groups.

Finally, regardless of whether antibiotic administration or hospitalization is the more influential agent, what is important to note from these results, is the alarming prevalence of highly resistant viridans streptococcal isolates (90%) amongst those children who received antibiotics while in hospital.

4.2.2 Progress in Treatment of Resistant Viridans Streptococcal Infections

As with the pneumococci, extensive research has gone into the development of alternative antibiotic regimens for the treatment of penicillin-resistant viridans streptococci. In vitro studies have indicated that viridans streptococcal strains which are not killed by penicillin alone are killed by penicillin + gentamicin or penicillin + streptomycin [121 - 123]. Yee *et al* [124] found that the mechanism of increased uptake of aminoglycoside, when added to penicillin, may not be dependent on the cell wall disruptive effect of penicillin, but rather on the direct effect penicillin has on the membrane potential leading to stimulation of aminoglycoside uptake. These findings suggested that the mechanism of penicillin stimulation of aminoglycoside uptake was direct stimulation of a transport process.

Miller *et al* [125], although they showed increased uptake of streptomycin in the presence of penicillin in enterococci, could not demonstrate such a synergistic effect in viridans streptococci over a 2 hour period. However, Miller's group performed their streptomycin uptake studies only over a period of 2 hours while Yee *et al* [124] assessed uptake for 24 hours. The latter investigators showed that the increased streptomycin uptake increased substantially after 120 minutes. It is also interesting that Yee and colleagues showed a pronounced synergistic effect in viridans streptococci over a period of 24 hours but minimal synergy after 4 hours.

The finding of high-level gentamicin resistance in viridans streptococci in our studies has important implications for the treatment of viridans streptococcal endocarditis. Although Farber *et al* [31] could still show synergy between penicillin and gentamicin in a South African *S. mitis* isolate that exhibited high-level streptomycin resistance, the gentamicin MIC of this strain was only 4 μg/ml. Although this still has to be confirmed, it is doubtful whether meaningful synergy will occur in strains with a high level of resistance to gentamicin. It would be interesting to know whether our viridans streptococci with high-level gentamicin resistance are also resistant to tobramycin, netilmicin and amikacin. If these strains also exhibit high-

level resistance to these aminoglycoside antibiotics, endocarditis due to high-level gentamicin resistance may well have to be treated with a combination of penicillin and vancomycin, or even rifampicin plus vancomycin, especially when strains are, in addition, resistant to penicillin. Obviously appropriate synergy studies will have to be performed before such alternative treatment schedules are recommended.

Shanson *et al* [126] compared netilmicin (which may be less nephrotoxic and ototoxic than gentamicin) with gentamicin and streptomycin, alone and in synergy with penicillin, against penicillin-tolerant viridans streptococci. In combination with penicillin, netilmicin appeared to show greater bactericidal activity than gentamicin, however, it is not known whether this difference has any clinical significance for the treatment of streptococcal endocarditis. They also found streptomycin to be less effective as a synergistic agent than either netilmicin or gentamicin. The situation is aggravated by the emergence of high level streptomycin resistance amongst South African isolates of viridans streptococci which have been shown to lack penicillin-streptomycin synergy [31].

4.3 MULTIPLE ANTIBIOTIC RESISTANCE

4.3.1 Multiple Antibiotic Resistance Patterns amongst S. pneumoniae

Although there had been previous reports of pneumococcal resistance to antibiotics other than penicillin (namely chloramphenicol, erythromycin, lincomycin and cotrimoxazole) [94], it was only when strains insensitive to nearly all the then known potentially useful therapeutic agents for pneumococcal infection started emerging, that any major interest in the problem of multiple antibiotic resistance in pneumococci developed. In 1978, Jacobs *et al* [14] reported the isolation of pneumococci insensitive to penicillin and a number of ß-lactam antibiotics, macro-

lides, tetracycline, chloramphenicol, cotrimoxazole and aminoglycosides, from both infected children and carriers in South Africa. An examination by Robins-Browne and Kharsany [20] in 1981, of the prevalence of antibiotic-resistant pneumococci in hospitalized black children under 12 years of age in Durban, identified 21 strains that were either resistant to penicillin alone or to multiple antibiotics among isolates recovered from 178 (31%) of 573 subjects.

One of the most common patterns of resistance in multiply resistant pneumococci has been to penicillin + tetracycline [94]. This corresponds well with the results from this study in which 8 (8%) of the 96 pneumococcal strains were resistant to both penicillin and tetracycline. In all, penicillin + tetracycline resistance (with or without resistance to various other agents) was demonstrated in 18 (19%) of the pneumococcal strains. Resistance to penicillin, tetracycline + chloramphenicol is also one of the more commonly encountered patterns of multiple resistance in pneumococci [94]. Other patterns of multiple resistance that have been reported, include: penicillin + chloramphenicol [19, 24, 119, 127]; penicillin, erythromycin + clindamycin [10, 12, 19, 100, 128]; and penicillin, tetracycline, chloramphenicol, erythromycin + clindamycin [12, 14, 19]. Linares *et al* [127] found that 45% of the isolates tested in their study exhibited chloramphenicol resistance, with MICs ranging from 16 - 64 μg/ml. The results in this study, however, demonstrated ony four strains (4%) with resistance to chloramphenicol. Linares *et al* also found only 2.5% of the 200 strains studied to be resistant to erythromycin + clindamycin (none of which were resistant to penicillin). Jacobs *et al* [14], on the other hand, demonstrated combined erythromycin/clindamycin resistance in 20% of the 168 isolates included in their study, the majority of them also being resistant to penicillin (+ a number of other agents). In this study, 9 (9%) of the strains demonstrated this resistance combination. Rifampicin resistance was demonstrated in 9 (9%) of the strains (together with penicillin, tetracycline, erythromycin +/- chloramphenicol resistance). Jacobs *et al* [14] demonstrated rifampicin resistance (in combination with resistance to various other agents) in 40 (25%) of the 168 strains used in their study. The high prevalence of rifampicin resistance was probably a result of carriers having been treated with this antibiotic.

Although multiply resistant pneumococci are usually characterized by resistance to ß-lactam antibiotics in addition to other agents [6, 14, 129], there has been a recent report of the isolation of novel multiply resistant pneumococci demonstrating susceptibility to ß-lactam antibiotics but resistance to tetracycline, clindamycin, erythromycin and cotrimoxazole [114, 115]. The presence of these organisms is not likely to pose a therapeutic problem for serious pneumococcal infections, but does, nevertheless, demand consideration of resistant organisms in the management of those less serious community-acquired diseases (such as otitis media and sinusitis) for which erythromycin and cotrimoxazole are often prescribed.

In Johannesburg, control measures were instituted in an attempt to confine the spread of multiply antibiotic-resistant pneumococci [130]. Firstly, surveillance was intensified by testing all pneumococcal isolates for antibiotic susceptibility and by surveying all paediatric wards for carriers of resistant strains. Secondly, carriers of resistant strains were isolated. Thirdly, antibiotics to which the resistant strains were susceptible, were administered in an attempt to reduce the nasopharyngeal carriage of the resistant strains. Although difficult to confirm, it would appear that these control measures were effective to a point, as, at the time of the study, only four patients in Johannesburg had developed systemic infections due to resistant strains, while in Durban (where no control measures were initially instituted) systemic infections due to resistant pneumococci continued to occur [2, 130]. Unfortunately, enforcement of these control measures on a wide scale, although desirable, would be very costly, and attempts at reducing nasopharyngeal carriage of resistant pneumococci carry the risk of selecting for more resistant strains.

4.3.2 Multiple Antibiotic Resistance Patterns amongst Viridans Streptococci

It is a remarkable fact that all of the penicillin-resistant strains of viridans streptococci demonstrated resistance to at least one other antimicrobial agent as well. This is particularly worrying in the light of the very high prevalence rates of both in-

termediately penicillin-resistant (27%) and fully resistant (59%) strains of viridans streptococci observed in this study. However, as the medical importance of the viridans streptococci relates mainly to infective endocarditis, resistance to agents other than ß-lactam and aminoglycoside antibiotics is of less concern.

High-level streptomycin resistance has been shown to occur in *S. pneumoniae* [14] and viridans streptococci [120, 121]. However, high-level resistance to gentamicin (MIC $\geq$ 2000 μg/ml) has not yet been described in viridans streptococci. The finding of one viridans streptococcal strain in this study with a gentamicin MIC of 2560 μg/ml (high-level) and two further strains with gentamicin MIC's of 640 μg/ml has implications for the treatment of endocarditis caused by such strains, especially since all three strains were also resistant to penicillin. The synergy that one expects with the combination of an aminoglycoside and penicillin in strains with low-level resistance to the aminoglycoside antibiotic [31, 121, 124] is not likely to occur in these strains. In such cases treatment with vancomycin alone or in combination is probably indicated.

Currently there are three accepted regimens, according to Bisno *et al* [131], for the treatment of endocarditis due to viridans streptococci. These include penicillin alone for 4 weeks, penicillin for 4 weeks plus streptomycin for the initial 2 weeks, and penicillin plus streptomycin for 2 weeks [32]. The use of combination therapy has been based on the repeated observations that penicillin plus streptomycin is synergistically active against strains of viridans streptococci [31, 121, 124]. Whether such combination therapy improves the clinical outcome is still unclear. In vitro studies [121 - 124] have also demonstrated the killing power of penicillin and gentamicin in combination with each other. Farber *et al* [31] and Shanson *et al* [126] found the combination of gentamicin and penicillin to be more effective than streptomycin and penicillin, in the killing of viridans streptococci.

An increasing prevalence of both gentamicin and streptomycin resistance may, therefore, pose a problem for clinicians faced with cases of infective endocarditis caused by penicillin-resistant viridans streptococci. Further research into the development of new bactericidal agents active against viridans streptococci will be needed to counter this problem.

4.4 API 20 STREP IDENTIFICATION SYSTEM

Although differentiating the viridans streptococci into species is of little importance to clinical microbiologists, one possible use for such a differential procedure is to determine the identity of strains isolated from patients with recurring endocarditis. One could then determine whether the isolates indicated treatment failure or infection by another strain [55]. Another reason for devising a differential scheme for the viridans streptococci is the alarmingly high rate of resistance observed in these organisms in this study. With such a scheme one could determine the pattern of penicillin resistance in specific species and this would provide a helpful guide for clinicians in the future management of serious conditions like endocarditis. Viridans streptococci are, of course, of great interest to dentists and in dental research where their role in caries and oral infections has been studied extensively.

Various authors [35, 52, 54, 55, 132] have researched and developed differential procedures with which to speciate the viridans streptococci, most of which are based on the physiological characteristics of the strains, although cluster analysis [78], cell wall composition [133 - 135] and antigenic structure [52, 136 - 138] have also been employed. Along with an increasing interest in identification at the species level of clinically important viridans streptococci, there have been numerous reports describing simplified identification schemes. Most of these schemes have concentrated on reducing the number of tests required for viridans species identification [74, 139 - 141] but, although they may be improvements over

classical identification schemes, they still require the use of unusual media and incubation periods of from two to five days. A number of them have also proved to be rather unsatisfactory [62, 140]. In our studies, we assumed that the API 20 Strep system (by mere virtue of its involving a greater number of tests) would be preferable to the original screening method used by us. Only 14 out of 23 (61%) of the API-identified *S. sanguis* strains correlated with the original identifications, the remaining 9 strains originally having been classified as *S. mitior*. A possible reason for this could be that, according to the API 20 Strep system, *S. sanguis* 11 strains will produce negative aesculin and arginine hydrolysis results in the majority of cases - a characteristic of *S. mitior* strains according to the original method of identification. Since most of the *S. sanguis* strains identified by means of the API 20 Strep system were *S. sanguis* 11, this could explain why a significant number of *S. sanguis* isolates were identified originally as being *S. mitior* isolates.

Table 4.1 depicts the prescribed percentages of positive reactions (PPR's) for the tests included in the API 20 Strep system and the Ruoff and Kunz screening procedure [63]. It is the way the results of these tests were interpreted, that distinguishes the API 20 Strep system from the Ruoff and Kunz procedure. The reason for the perfect correlation of the API 20 Strep system with the Ruoff and Kunz procedure in the identification of the *S. mitis* strains, is the close similarity of their prescribed PPRs for *S. mitis* (Table 4.1); similarly, this is why the two systems correlated well in the identification of the *S. sanguis* II strains. However, with the *S. sanguis* I strains, the API 20 Strep system quoted the PPR for inulin fermentation as < 50% while the Ruoff and Kunz method quoted a PPR of 100%. This would explain the low level of agreement between the API 20 Strep-identified *S. sanguis* I strains and those identified as *S. sanguis* I by the Ruoff and Kunz procedure.

The API-identified *S. salivarius* and *S. morbillorum* strains were identified as such by the Ruoff and Kunz method and this is most probably also due to the close similarity between the respective PPR's in each species' case.

TABLE 4.1 Percentages of Positive Reactions for Isolates of Different Viridans Streptococcal Species, in various tests using (i) the API 20 Strep System and (ii) the Ruoff and Kunz Screening Method

Species	% Positive by Following Physiological Test					
	Mannitol	Lactose	Raffinose	Inulin	Aesculin	Arginine
S. mitis - API 20 S[1]	0	95	6	10	1	13
- R + K[2]	0	100	0	0	0	30
S. sanguis I - API 20 S[1]	0	75	23	44	75	80
- R + K[2]	0	96	49	100	56	78
S. sanguis II - API 20 S[1]	1	93	99	6	4	20
- R + K[2]	0	100	100	0	0	21
S. salivarius - API 20 S[1]	3	84	87	60	98	0
- R + K[2]	0	89	89	100	78	0
S. morbillorum - API 20 S[1]	0	0	1	1	0	4
- R + K[2]	0	0	0	0	0	0
S. MG-intermedius - R + K[2]	0	100	16	0	100	30
S. anginosus-constellatus-R + K[2]	0	0	14	0	35	17
S. milleri I - API 20 S[1]	1	1	1	0	1	99
S. milleri II - API 20 S[1]	1	94	2	8	97	99
S. milleri III - API 20 S[1]	99	95	95	6	99	100

[1]API 20 S = API 20 Strep System
[2]R + K = Ruoff and Kunz Screening Method

The API 20 Strep system does not recognise the *S. milleri* groups, *S. MG-intermedius* and *S. anginosus-constellatus*. Instead it recognises *S. milleri* I, II and III. From Table 4.1, it would appear that *S. MG-intermedius* correlated with *S. milleri* II (except for the PPR for arginine hydrolysis) and that *S. anginosus-constellatus* correlated with *S. milleri* I (except, too, for the PPR for arginine hydrolysis). It is not possible in the present study to show these correlations, as no strains of *S. milleri* were identified by the API 20 Strep system. Instead, the majority of strains (5 out of 7) identified as *S. MG-intermedius* by the Ruoff and Kunz procedure had been API-identified as *S. sanguis* I. On examining the PPR patterns for *S. MG-intermedius* and *S. sanguis* I (which one would have expected to find very similar), however, one can see that although there was a fair degree of agreement between the two species, they nevertheless differed in their respective PPR's for arginine hydrolysis and, less notably, inulin fermentation. A possible reason for this disparity, is that the API 20 Strep system involved a wider range of tests than the Ruoff and Kunz method did and, therefore, may not have relied solely on the results derived from the tests depicted in Table 4.1 for their identification of *S. sanguis* I.

According to Tables 3.20, 3.21 and 3.22, the results in this study indicated a drift from the API 20 Strep-approved percentage positive reactions. This is evident in the following tests in particular: alkaline phosphatase, arginine dihydrolase, trehalose, inulin and starch. The reason for this shift is not immediately clear, however, the numbers investigated in this study may have been too limited for any meaningful conclusions to be drawn from these results.

Considering the discrepancies witnessed between the percentages of positive reactions obtained in this study and those prescribed by the API 20 Strep system, there may be a need to look more closely at the accuracy of some of the biochemical tests incorporated in this method. For instance, Colman and Ball [77] felt that the alpha-galactosidase and beta-galactosidase sugar fermentation tests should be replaced with the actual enzyme tests if, in time, new tests (still using 20 reactions) became available, as the enzyme tests were (according to the authors) more reliable. They also recommended the inclusion of the bile-aesculin test and suggested that the API 20 Strep system be used in conjunction with other tests, such as sensitivity to optochin and a procedure that would demonstrate possible production of an extracellular polysaccharide (for instance,levan) from sucrose.

French *et al* [78] came out in support of the API 20 Strep system by suggesting that the tests for aesculin/arginine hydrolysis were more reliable than conventional tests and also probably reproducible. Our results would appear to support this. Referring to Table 3.23 a number of those strains identified by the API 20 Strep system as *S. mitis* (and which were subsequently confirmed to be such by the Ruoff and Kunz screening method) were originally classed (by conventional tests) as *S. sanguis* because of these strains demonstrating positive aesculin and/or arginine hydrolysis reactions. According to Table 3.25, however, all the API-identified *S. mitis* strains demonstrated negative aesculin and arginine reactions which would suggest that API 20 Strep aesculin/arginine tests may, indeed, be more reliable than conventional tests.

With regard to the optochin-resistant pneumococci isolated, other similar cases have been previously described. In one such study [142], optochin resistance was found in pneumococci isolated from the blood of two patients. According to the API 20 Strep system, the one resistant variant received a presumptive identification

of *S. pneumoniae* 2 while the other suggested an identification of *S. sanguis* 11 or *S. pneumoniae* 2. The respective API 20 Strep profiles of the two resistant strains in this study were almost identical: the one strain received a presumptive identification of *S. pneumoniae* 2 and the other suggested an identification of *S. sanguis* 11 or *S. pneumoniae* 1. Sensitivity to optochin is a cornerstone in the identification of pneumococci and is often used in laboratories as the primary and sometimes the only identification method. According to Kontiainen and Sivonen [142], if optochin-resistant variants of pneumococci of the rough colony type predominated, the isolate could easily be misidentified as a viridans group streptococcus and biochemical tests may not be very helpful in differentiating between viridans streptococci and optochin-resistant rough colony type pneumococci.

In summary, therefore, it is clear that, in the differentiation of the viridans streptococcal species, many disparities were evident when comparing the original screening procedure with the API 20 Strep system and the Ruoff and Kunz screening method. It is possible that by extending the range of tests incorporated in the API 20 Strep system, one may be able to improve the reliability and reproducibility of the system. Further research could also be done into improving the reliability of the tests themselves. Kilian *et al* [72] claimed that many of the biochemical tests used to examine streptococci are not sufficiently optimized for these bacteria, and small alterations in a test may give different results. For instance, in their study the type strain of *S. sanguis*, strain ATCC 10556, was found to be clearly positive for aesculin hydrolysis in the test substrate recommended by Carlsson [64], but negative, together with several other *S. sanguis* strains, if tryptic soy broth was substituted for trypticase peptone in the medium. They also mention that discrepancies in the reported abilities of strains to ferment carbohydrates may be due to the use of different pH indicators. However, it is difficult to compare various identification systems if the authors' interpretations of the various tests included in the systems differ. Therefore, for total compatibility to be achieved between the various identification schemes, it would not only be necessary to decide upon an internationally acceptable range of physiological tests, but also to come to an agreement on how these tests' results should be interpreted. In the review article by Kilian *et al* [72] they were able to resolve a number of the taxonomic problems related to the viridans streptococci, however, further research is still necessary, particularly with regard to *S. milleri/S. anginosus-constellatus/S. intermedius*

speciation and identification.

Finally, in a further attempt to devise a means of identifying the viridans streptococci French *et al* [143] investigated the use of an isothermal method of pyrolysis-gas chromatography (Py-GC), as gas-chromatographic analysis (either by detection of specific chemical components or by "fingerprint" patterns of chromatographic peaks) can be used in the characterization of bacteria. French *et al* managed to distinguish five groups representing recognised species but, once again, *S. milleri* strains posed a problem, proving to be indistinguishable from *S. sanguis*. They concluded that while Py-GC was moderately successful for the identification of viridans streptococci, their study indicated that the technique had limited use in diagnostic medical microbiology because it was both time-consuming and lacked flexibility.

4.5 VIRIDANS STREPTOCOCCAL PENICILLIN-BINDING PROTEIN STUDIES

From the preliminary fluorogram results obtained in this study, it is plain that the PBP's of clinical strains of viridans streptococci could not as easily be divided into groups as those of *S. pneumoniae*. This made it more difficult to determine any molecular weight changes that may have occurred in the PBP's. Although clear alterations of specific PBP's were not demonstrable electrophoretically in this study, past studies, which have investigated the PBP patterns of *S. mitis* strains, have shown otherwise [33, 51]. Farber *et al* [33] demonstrated molecular weight changes fairly well in their study. The obvious changes that occurred were: a decrease in molecular weight from 44-kD in susceptible strains to 42-kD in resistant strains; an increase in molecular weight from 92-kD in susceptible strains to 86-kD in resistant strains; and a decrease in molecular weight from 109-kD in susceptible strains to 105-kD in resistant strains. They also demonstrated an extra band that appeared amongst the resistant South African strains when compared with the susceptible South African strain. It should be noted, though, that membrane preparations were used in this study and that only five strains were studied for PBP's. In the study by Quinn *et al* [51], too, only one penicillin-resistant strain of *S. mitis* was studied and compared against one penicillin-susceptible control strain and, there-

fore, the authors would not have been faced with the problem of having to define PBP groups amongst a whole range of strains, all with differing MIC values.

One of the main problems experienced in this study with the PBP profiles of the *S. mitis* and *S. sanguis* strains, was that no baseline "reference" PBP (in other words, a PBP band which has been shown not to alter penicillin binding affinity with increasing penicillin resistance) was evident. Such a standard PBP band does allow a direct comparison of initial cell density, lysis and solubilization. Therefore, without affinity studies to determine whether PBP differences observed between strains of viridans streptococci of different MIC values are directly due to reduced penicillin affinity, only tentative gross alteration comparisons can be made. However, the reduced binding of some PBP's in penicillin-resistant isolates is very obvious when compared with the susceptible isolates.

The PBP patterns observed in this study, although only preliminary, differ quite markedly from other previous studies [33, 51]. However, none of these other studies have themselves demonstrated similar patterns. For instance, Farber *et al* [33], described five PBP bands in his penicillin-resistant *S. mitis* strains, with molecular weights of 105, 100, 93, 86 and 42-kD respectively. In this present study no bands >92kD were detected. This difference, however, could be due to Farber *et al* having used cell membranes in their study as opposed to the whole cell preparations used in this study. Quinn *et al* [51], on the other hand, demonstrated four major PBP bands in his penicillin-resistant *S. mitis* strain, with molecular weights of 79 - 44-kD. Neither of the two earlier studies demonstrated changes in PBP affinity (from susceptibility through to resistance) as markedly as this study did. Farber *et al* [33] did describe one PBP band which became undetectable (in a *S. mitis* strain isolated in Boston) as the strain entered resistance, and Quinn *et al* [51] (although only having studied one strain) showed a reduction in penicillin affinity in the resistant strain, when compared with the susceptible strain.

In this study, *S. mitis* PBP's in the range 62 - 66-kD were no longer detected as the organisms entered moderate penicillin resistance. The *S. mitis* strains also possessed a PBP of approximate molecular weight 75-kD that showed an apparent reduction in binding at the same MIC as a strain in the study by Farber *et al* [33].

The apparent complexity of PBP alterations seen with *S. mitis* appear to be due to the heterogenicity of the strains. According to the API 20 Strep system (Table 3.25) certain of the API-identified *S. sanguis* strains exhibited negative aesculin and arginine hydrolysis reactions. The only factor common to all the *S. sanguis* strains (except strain 35) was their ability to ferment raffinose. French *et al* [78] have discriminated between organisms resembling *S. sanguis/S. mitior* by testing for arginine hydrolysis - a positive result in the API test is characteristic of *S. sanguis* [144] while, in contrast, *S. mitior* is API arginine-negative. However, both *S. sanguis* and *S. mitior* can hydrolyse aesculin [78]. The API-identified *S. sanguis* strains (Table 3.25) demonstrating an inability to hydrolyse arginine and aesculin (namely strains 25, 19, 21 and 20) are, therefore, classified according to French *et al* [78] and Colman and Williams as *S. mitior*. Furthermore, according to French *et al* [78] organisms resembling *S. sanguis* that are arginine-negative and aesculin-positive in the API 20 Strep system (cf. strain 35) also have a low cell-wall rhamnose content, and thus can be classified as *S. mitior*. Therefore, of those strains originally identified as *S. sanguis* by the API 20 Strep system for the purpose of the penicillin-binding protein studies (Fig 3.5), only strains 9 (lane 3), 40 (lane 5), 7 (lane 6), 5 (lane 7) and 3 (lane 10) can be confidently classified as *S. sanguis* strains.

The PBP profiles of the "confirmed" *S. sanguis* strains (Fig 3.5 lanes 3, 5 - 7 and 10) correlated well with those obtained by Zito and Daneo-Moore [85] in their study of the penicillin-binding proteins of *S. sanguis*. The one difference between the two studies was the detection of a PBP of molecular weight 86-kD in Zito and Daneo-oore's study. In the present study, the resistant *S. sanguis* (strain 3) isolate (Fig 3.5 lane 10) exhibited reduced binding in a PBP of approximate molecular weight 80 - 84-kD. Due to different gel composition and running conditions this PBP probably corresponds to the major penicillin target, PBP 4, which showed a dramatic decrease in penicillin binding in the Zito and Daneo-Moore study [85]. The solubilization problem and poor resolution of high molecular weight PBP's (Fig 3.5 lanes 3, 5 - 7 and 10) could have been due to the fact that whole cell preparations were used in this study instead of membrane preparations; however, Zito and Daneo-Moore [85] experienced a similar problem with solubilization and poor resolution of high molecular weight PBP's even though they used membrane preparations in their study.

An important point to note, though, is the fact that because the above-mentioned *S. sanguis* strains exhibited such similar PBP profiles, this could give backing to the proposed use of PBP's as a means of identifying the viridans streptococci. Williamson *et al* [145], in view of the difficulties involved in using physiological and fermentation tests for the identification of enterococci, examined the value of PBP's for the identification of these organisms. Their results showed that PBP patterns may be useful for identification purposes since some strains with unusual fermentation characteristics were assigned to species by this technique. The PBP results obtained in this study (in both the *S. mitis* and *S. sanguis* strains' PBP patterns) suggest that such a system may also be useful in the identification of viridans streptococci which have proved difficult to identify by means of conventional physiological and biochemical tests.

Considering the problems associated with the classification and identification of the viridans streptococci, probably the best approach for future viridans streptococcal PBP studies, would be to run a high number of physiological, biochemical and serological tests on the strains to be studied (possibly based on the methods used by Kilian *et al* [72] in their comprehensive review article). One could then include those strains that were the most similar/closely related (according to the results of these tests) in the same gel run, ideally pairing off each resistant strain with a closely related susceptible strain. By ensuring that the strains being compared were almost identical biochemically, one could then be more confident that the PBP changes one observed, related exclusively to increasing penicillin resistance, and not to possible species/biotype differences.

4.6 PNEUMOCOCCAL PENICILLIN-BINDING PROTEIN STUDIES

Amongst the pneumococcal isolates, PBP's 1a, 1b and 1c would appear not to undergo any molecular weight changes indicative of increasing resistance. This is in agreement with Chalkley *et al* [49]). As in this present study, other studies have shown a PBP 3 of increased molecular weight [47, 49]. However, all experimental results obtained thus far [40, 44, 47, 48] indicate that the high-affinity PBP 3 does

not undergo detectable changes along with penicillin resistance; the extra polypeptide material responsible for the larger molecular size appears to lie distant from the beta-lactam binding site [47]. PBP 2b is the only PBP which shows an alteration in molecular weight which correlates with increased resistance to penicillin. PBP 2b has been identified as a major target for penicillin activity in a previous study [49] and this has been verified by the PBP profiles demonstrated in the nasopharyngeal isolates of *S. pneumoniae* used in this study. Alterations of PBP 2b to higher molecular weights occur whether strains are isolated from blood culture, CSF or the nasopharynx [146]. Decreased binding of penicillin to other PBP's [44] may be necessary for ultimate high-level resistance (MIC $\geq$ 1 μg/ml).

A recent study [147] sought to establish whether the changes in PBP size and affinity for penicillin that accompanied the acquisition of resistance determinants in transformants, did not also affect the normal activity of these enzymes in vivo. The results indicated that the structure of the peptidoglycan portion of the cell wall in the transformant with the highest resistance level, underwent a major change. It was found that the cell wall stem peptides (that became major components of the walls of the highly resistant cells) all contained greatly increased proportions of alanine which were in the cross-bridges. The abundance of such alanine-rich peptides in the cell walls of highly resistant pneumococcal strains suggested that for resistance at this level to be successfully expressed, some mutation(s) must have occurred that would ensure adequate supplies of cross-bridge-bearing stem peptides. In other words, for high level resistance to be achieved, there must be not only an alteration of penicillin-sensitive enzymes, but also a change(s) in cell wall precursor synthesis. Nevertheless, even though the chemical nature of the cross-links changed, the PBP's remained fully functional in the resistant cells. The altered peptidoglycan structure of the highly resistant strains appeared to be fully compatible with the normal physiological function of the cells.

It was recently reported [46] that PBP 2a' has low affinity for penicillin in high-level resistant strains and, according to Zighelboim *et al* [40, 44], PBP 2a' seemed to appear as the level of resistance increased. However, in the South African nasopharyngeal isolates investigated in this study, PBP 2a' was clearly detectable throughout the full range of penicillin susceptibilities selected, being demonstrated in 68% of the isolates - nearly four times as frequently as in South African isolates

from blood culture and CSF studied to date [146]. Therefore, the presence or absence of PBP 2a' would appear to have no correlation with the development of resistance in South African isolates of *S. pneumoniae*. It is, as yet, not known whether this predominance of PBP 2a' in these nasopharyngeal isolates is of clinical significance and, therefore, remains to be investigated.

The differences that appear to exist between the pneumococcal PBP patterns observed in this study and those that have been described by other authors [40, 41, 44, 47, 48], may be due to the fact that in pneumococci, remodeling of critical PBP's in more than one way may result in comparable levels of penicillin resistance. Markiewicz and Tomasz [148], in a 1989 study of clinical isolates of pneumococci, found that although the vast majority of penicillin-susceptible pneumococcal strains appeared to share a common characteristic PBP pattern, several penicillin-susceptible strains showed deviation from this shared typical pattern. The significance and interpretation of these anomalous PBP patterns were not clear. They also detected a marked degree of variation in the number and molecular sizes of PBP's in penicillin-resistant isolates, and suggested that these anomalous-size PBP's may represent mutant derivatives of one or the other normal PBP's. These same PBP's were reproduced upon repeated cultivation and, therefore, these PBP patterns may be considered as heritable within particular resistant mutant strains. So, it would appear that more than one kind of molecular restructuring of these PBP's may be compatible with low antibiotic affinity and increased penicillin resistance, suggesting a surprising degree of plasticity in the genetic determinants of pneumococcal PBP's.

Of interest, too, is the new PBP with a molecular weight of approximately 60-kD demonstrated in this study. No such PBP has been detected previously and no function has, as yet, been assigned to it. Further investigation should determine whether this observation is owing to the limited numbers of oropharyngeal isolates from South Africa that have been investigated to date, or that a PBP change has not occurred until recently.

REFERENCES

1 Appelbaum PC. Worldwide development of antibiotic resistance in pneumococci. *Eur J Clin Microbiol* 1987; 4: 367 - 377

2 Ward JI. Antibiotic-resistant *Streptococcus pneumoniae*: clinical and epidemiological aspects. *Rev Infect Dis* 1981; 3: 254 - 266.

3 Hansman D, Bullen MM. A resistant pneumococcus. *Lancet* 1967; 2: 264.

4 Dajani AS. Antibiotic-resistant pneumococci. *Pediatr Infect Dis* 1982; 1: 143 - 144.

5 Tempest B, Carney JP, Eberle B. Distribution of sensitivities to penicillin of types of *Diplococcus pneumoniae* in an American Indian population. *J Infect Dis* 1974; 130: 67 - 69.

6 Radetsky MS, Istre GR, Johansen TL, Parmalee SW, Lauer BA, Wiesenthal AM, Glode MP. Multiply-resistant pneumococcus causing meningitis: its epidemiology with a day-care centre. *Lancet* 1981; 2: 771 -773.

7 Saah AJ, Mallonee JP, Tarpay M, Thornsberry CT, Roberts MA, Rhoades ER. Relative resistance to penicillin in the pneumococcus. *JAMA* 1980; 243: 1824 - 1827.

8 Williams EW, Watts JA, Potten MR. *Streptococcus pneumoniae* resistant to penicillin and chloramphenicol in the U.K. [letter]. *Lancet* 1981; 2: 699.

9 Modde HK. *Streptococcus pneumoniae* isolates relatively insensitive to penicillin G recovered from patients in Switzerland. *Chemotherapy* 1978; 24 :227 - 230.

10 Michel J, Dickman D, Greenberg Z, Bergner-Rabinowitz S. Serotype distribution of penicillin-resistant pneumococci and their susceptibilities to seven antimicrobial agents. *Antimicrob Agents Chemother* 1983; 23: 397 - 401.

11 Perez JL, Linares J, Bosch J, Lopez de Goicoechea MJ, Martin R. Antibiotic resistance of *Streptococcus pneumoniae* in childhood carriers. *Eur J Clin Microbiol* 1986; 6: 278 - 280.

12 Latorre C, Juncosa T, Sanfeliu I. Antibiotic resistance and serotypes of 100 *Streptococcus pneumoniae* strains isolated in a children's hospital in Barcelona, Spain. *Antimicrob Agents Chemother* 1985; 28: 357 - 359.

13 Hansman D, Devitt L, Miles H, Riley I. Pneumococci relatively insensitive to penicillin in Australia and New Guinea. *Med J Aust* 1974; 2: 353 - 356.

14 Jacobs MR, Koornhof HJ, Robins-Browne RM, Stevenson CM, Vermaak ZA, Freiman I, Miller GB, Witcomb MA, Isaacson M, Ward JI, Austrian R. Emergence of multiply resistant pneumococci. *N Engl J Med* 1978; 299: 735 - 740.

15 Appelbaum PC, Bhamjee A, Scragg NJ, Hallett AF, Bowen AJ, Cooper RC. *Streptococcus pneumoniae* resistant to penicillin and chloramphenicol. *Lancet* 1977; ii: 995 - 997.

16 Koornhof HJ, Jacobs M, Isaacson M, et al. Follow-up on multiple-antibiotic-resistant pneumococci - South Africa. *MMWR* 1978; 27: 1 - 7.

17 Van den Ende J, Rotter ML. An analysis of blood culture isolates from 7 South African teaching hospital centres. *SAMJ* 1986; 69: 89 - 93.

18 Centers for Disease Control. Multiply resistant pneumococcus - Colorado. *MMWR* 1981; 30: 197 - 198.

19 Klugman KP, Koornhof HJ. Drug resistance patterns and serogroups or serotypes of pneumococcal isolates from cerebrospinal fluid or blood, 1979 - 1986. *J Infect Dis* 1988; 158: 956 - 964.

20 Robins - Browne RM, Kharsany ABM. Antibiotic-resistant pneumococci in hospitalized children. *J Hyg Camb* 1984; 93: 9 - 16.

21 Jackson MA, Shelton S, Nelson JD, McCracken GH. Relatively penicillin-resistant pneumococcal infections in pediatric patients. *Pediatr Infect Dis* 1984; 3: 129 - 132.

22 Pallares R, Gudiol F, Linares J, Ariza J, Rufi G, Murguil, Dorca J, Viladrich PF. Risk factors and response to antibiotic therapy in adults with bacteremic pneumonia caused by penicillin-resistant pneumococci. *N Engl J Med* 1987; 317: 18 - 22.

23 Amitai Y, Rotenberg M, Wirtschafter D, Haas H, Michel J. Increasing frequency of penicillin-resistant pneumococci: epidemiological aspects and case-control study. *Isr J Med Sci* 1985; 21: 340 - 345.

24 Oppenheim B, Koornhof HJ, Austrian R. Antibiotic-resistant pneumococcal disease in children at Baragwanath Hospital, Johannesburg. *Pediatr Infect Dis* 1986; 5: 520 - 524.

25 Willet LD, Dillon HC Jr, Gray BM. Penicillin-intermediate pneumococci in a children's hospital. *Am J Dis Child* 1985; 139: 1054 - 1057.

26 Ward JI, Fraser DW, Bennet JV. Multiple antibiotic-resistant pneumococci, South Africa. Limited distribution paper. Public Health Service - CDC - Atlanta EPI 77-93-2 (not for publication) 1978.

27 Appelbaum PC, Koornhof HJ, Jacobs M, *et al.* Multiple antibiotic resistance of pneumococci - South Africa. *Center for Disease Control MMWR* 1977; 26: 285 - 286.

28 Horstmeier C, Washington JA II. Microbiological study of streptococcal bacteremia. *Appl Microbiol* 1973; 26: 589 - 591.

29 Bayliss R, Clarke C, Oakley CM, Somerville W, Whitfield AGW, Young SEJ. The microbiology and pathogenesis of infective endocarditis. *Br Heart J* 1983; 50: 513 - 519.

30 Roberts RB, Krieger AG, Schiller NL, Gross KC. Viridans streptococcal endocarditis: the role of various species, including pyridoxal-dependent streptococci. *Rev Infect Dis* 1979; 1: 955 - 965.

31 Farber BF, Eliopoulos GM, Ward JI, Ruoff KL, Moellering RC Jr. Resistance to penicillin-streptomycin synergy among clinical isolates of viridans streptococci. *Antimicrob Agents Chemother* 1983; 24: 871 - 875.

32 Wilson WR, Geraci GE, Wilkowske CJ, Washington JA. Short-term intramuscular therapy with procaine penicillin plus streptomycin for infective endocarditis due to viridans streptococci. *Circulation* 1978; 57: 1158 - 1162.

33 Farber BF, Eliopoulos GM, Ward JI, *et al.* Multiply resistant viridans streptococci: susceptibility to ß-lactam antibiotics and comparison of penicillin-binding protein patterns. *Antimicrob Agents Chemother* 1983; 24: 702 - 705.

34 Phillips I, Warren C, Harrison JM, Sharples P, Ball LC, Parker MT. Antibiotic susceptibilities of streptococci from the mouth and blood of patients treated with penicillin or lincomycin and clindamycin. *J Med Microbiol* 1976; 9: 393 - 403.

35 Parker MT, Ball LC. Streptococci and aerococci associated with systemic infection in man. *J Med Microbiol* 1976; 9: 275 - 302.

36 Sprunt K, Redman W, Leidy G. Penicillin-resistant alpha streptococci in pharynx of patients given oral penicillin. *Pediatrics* 1968; 42: 957 - 968.

37 Drucker DB, Jolly M. Sensitivity of oral micro-organisms to antibiotics. *Br Dent J* 1971; 131: 442 - 449.

38 Hess J, Holloway Y, Dankert J. Penicillin prophylaxis in children with cardiac disease: postextraction bacteremia and penicillin-resistant strains of viridans streptococci. *J Infect Dis* 1983; 147: 133 - 136.

39 Bourgault A, Wilson WR, Washington JA II. Antimicrobial susceptibilities of species of viridans streptococci. *J Infect Dis* 1979; 140: 316 - 321.

40 Zighelboim S, Tomasz A. Penicillin-binding proteins of multiply antibiotic-resistant South African strains of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1980; 17: 434 - 442.

41 Hakenbeck R, Tarpay M, Tomasz A. Multiple changes of penicillin-binding proteins in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1980; 17: 364 - 371.

42 Handwerger S, Tomasz A. Alterations in penicillin-binding proteins of clinical and laboratory isolates of pathogenic *Streptococcus pneumoniae* with low levels of penicillin resistance. *J Infect Dis* 1986; 153: 83 - 89.

43 Shockley TE, Hotchkiss RD. Stepwise introduction of transformable penicillin resistance in pneumococci. *Genetics* 1970; 64: 397 - 408.

44 Zighelboim S, Tomasz A. Multiple antibiotic resistance in South African strains of *Streptococcus pneumoniae*: mechanism of resistance to ß-lactam antibiotics. *Rev Infect Dis* 1981; 3: 267 - 276.

45 Frere JM, Joris B. Penicillin-sensitive enzymes in peptidoglycan biosynthesis. *CRC Crit Rev Microbiol* 1985; 11: 299 - 396.

46 McDougal LK, Thornsberry C. Structurally diverse penicillin-binding proteins of high-level penicillin resistant *S. pneumoniae* from the United States, South Africa and Czechoslovakia. Abstr, Interscience Conference, *Antimicrob Agents Chemother* 1988: 153.

47 Hakenbeck R, Ellerbrok H, Briese T, et al. Penicillin-binding proteins of penicillin-susceptible and -resistant pneumococci: immunological relatedness of altered proteins and changes in peptides carrying the beta-lactam binding site. *Antimicrob Agents Chemother* 1986; 30: 553 - 558.

48 Handwerger S, Tomasz A. Alterations in kinetic properties of penicillin-binding proteins of penicillin-resistant *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1986; 30: 57 - 63.

49 Chalkley LJ, Koornhof HJ. Penicillin-binding proteins of *Streptococcus pneumoniae*. *J Antimicrob Chemother* 1988; 22: 791 - 800.

50 Tomasz A, Zighelboim-Daum S, Handwerger S, Liu H, Qian H. Physiology and genetics of intrinsic beta-lactam resistance in pneumococci. In: Lieve L and Schlessinger D (eds). *Microbiology*. American Society for Microbiology, Washington D.C., 1984, 393 - 397.

51 Quinn JP, DiVincenzo CA, Lucks DA, Luskin RL, Shatzer KL, Lerner SA. Serious infections due to penicillin-resistant strains of 'viridans' streptococci with altered penicillin-binding proteins. *J Infect Dis* 1988; 157: 764 - 769.

52 Colman G, Williams REO. Taxonomy of some human viridans streptococci. In: Wannamaker LW and Matsen JM (eds). *Streptococci and streptococcal diseases*. Academic Press Inc., New York, 1972, 282 - 299.

53 Bouvet A, Villeroy F, Cheng F, Lamesch C, Williamson R, Gutmann L. Characterization of nutritionally variant streptococci by biochemical tests and penicillin-binding proteins. *J Clin Microbiol* 1985; 22: 1030 - 1034.

54 Carlsson J. A numerical taxonomic study of human oral streptococci. *Odontol Revy* 1968; 19: 137 - 160.

55 Facklam RR. Physiological differentiation of viridans streptococci. *J Clin Microbiol* 1977; 5: 184 - 201.

56 Soloway M. A serological classification of viridans streptococci with special reference to those isolated from subacute bacterial endocarditis. *J Exp Med* 1942; 76: 109 - 126.

57 Selbie RR, Simon ED, Robinson RGM. Serological classification of viridans streptococci from subacute bacterial endocarditis, teeth, and throats. *Br Med J* 1949; ii: 667 - 672.

58 Williamson CK. Serological classification of viridans streptococci from the respiratory tract of man. In: Leone CA (ed). *Taxonomic biochemistry and serology*. Ronald Press, New York, 1964, 607 - 622.

59 Austrian R, Buettiger C, Dole M. Problems in the classification and pathogenic role of alpha and non-hemolytic streptococci of the human respiratory tract. In: Wannamaker LW and Matsen JM (eds). *Streptococci and streptococcal diseases.* Academic Press Inc., New York, 1972, 355 - 370.

60 Karakawa WW, Kane JA, Austrian R. Filamentous capsulated streptococci from the human respiratory tract. II. Antigenic structure of provisional capsular types 89 and 83/89. *Infect Immun* 1973; 8: 962 - 968.

61 Karakawa WW, Kane JA, Austrian R. Filamentous capsulated streptococci from the human respiratory tract. III. Immunochemical studies of the cross-reactivity between cell wall antigens of a filamentous streptococcus and of pneumococcus. *Infect Immun* 1973; 8: 969 - 976.

62 Waitkins SA, Ball LC, Fraser CAM. A shortened scheme for the identification of indifferent streptococci. *J Clin Pathol* 1980; 33: 47 - 52.

63 Ruoff KL, Kunz LJ. Identification of viridans streptococci isolated from clinical specimens. *J Clin Microbiol* 1982; 15: 920 - 925.

64 Carlsson J. Presence of various types of non-haemolytic streptococci in dental plaque and in other sites of the oral cavity in man. *Odontol Revy* 1967; 18: 55 - 74.

65 Hardie JM, Bowden GH. Physiological classification of oral viridans streptococci. *J Dent Res* 1976; 55: A166 - A176.

66 Deibel RH, Seeley HW. Streptococceae. In: Buchanan RE, Gibbons NE (eds). *Bergey's Manual of Determinative Bacteriology* (8th ed). The Williams and Wilkins Company, Baltimore, 1974.

67 Facklam RR. The major differences in the American and British streptococcus taxonomy schemes with special reference to *Streptococcus milleri*. *Eur J Clin Microbiol* 1984; 3: 91 - 93.

68 Coogan MM. A code for the identification of the 'viridans' streptococci. *Journal of the Dental Association of South Africa* 1986; 41: 129 - 133.

69 Jones D. Composition and differentiation of the genus Streptococcus. *Society for Applied Bacteriology Symposium series* 1978; 7: 1 - 49.

70 Parker MT. Streptococcus and lactobacillus. In: Wilson G, Miles A, Parker MT (eds). *Topley and Wilsons Principles of Bacteriology, Virology and Immunity* (6th ed). Edward Arnold, London, 1983, Vol 3, Ch 29.

71 Cowan ST, Steel KJ. Characters of gram positive bacteria. In: *Manual for the Identification of Medical Bacteria* (2nd ed). University Press, Cambridge, 1974.

72 Kilian M, Mikkelsen L, Henrichsen J. Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp. nov. and emended descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrewes and Horder 1906). *Int J Syst Bacteriol* 1989; 39: 471 - 484.

73 Hardie JM. Oral streptococci. In: Sneath PHA *et al* (eds). *Bergey's Manual of Systematic Bacteriology* (9th ed). Williams and Wilkins, Baltimore, 1986, Vol 2, 1054 - 1063.

74 Holloway Y, Schaareman M, Bankert J. Identification of viridans streptococci on the Minitek miniaturised differentiation system. *J Clin Pathol* 1979; 32: 1168 - 1173.

75 Ruoff KL, Kunz LJ. Use of the Rapid STREP system of identification of viridans streptococcal species. *J Clin Microbiol* 1983; 18: 1138 - 1140.

76 Waitkins SA, Anderson DR, Todd FK. An evaluation of the API-STREP identification system. *Med Lab Sci* 1981; 38: 35 - 39.

77 Colman G, Ball LC. Identification of streptococci in a medical laboratory. *J Appl Bacteriol* 1984; 57: 1 - 14.

78 French GL, Talsania H, Charlton JRH, Phillips I. A physiological classification of viridans streptococci by use of the API-20 Strep system. *J Med Microbiol* 1989; 28: 275 - 286.

79 Whittenbury R. Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. *J Gen Microbiol* 1964; 35: 13 - 26.

80 National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. *NCCLS, Villanova Pa*, 1985; 5: 583 - 587.

81 Van den Berg IS. Penicillin susceptibility of nasopharyngeal streptococcal isolates from rheumatic fever patients receiving penicillin prophylaxis. BSc (Lab Med). Dissertation, University of the Witwatersrand, Johannesburg, 1987.

82 Gray BM, Converse GM III, Huhta N, Johnston RB Jr, Pichichero ME, Shiffman G, Dillon HC Jr. Epidemiologic studies of *Streptococcus pneumoniae* in infants: antibody response to nasopharyngeal carriage of type 3, 19 and 23. *J Infect Dis* 1981; 144: 312 - 318.

83 Cowan MJ, Almmann AJ, Wara DW, Howie VM, Schultz L, Doyle N, Kaplan M. Pneumococcal polysaccharide immunization in infants and children. *Pediatrics* 1978; 62: 721 - 727.

84 Filip C, Fletcher G, Wulff JL, Earhart CF. Solubilization of the cytoplasmic membrane of *Escherischia coli* by the ionic detergent Sodium-lauryl sarcosinate. *J Bacteriol* 1973; 115: 717 - 722.

85 Zito ET, Daneo-Moore L. Transformation of *Streptococcus sanguis* to intrinsic penicillin resistance. J Gen Microbiol 1988; 134: 1237 - 1249.

86 Hansman D, Glasgow H, Sturt J, *et al*. Increased resistance to penicillin of pneumococci isolated from man. *N Engl J Med* 1971; 284: 175 - 177.

87 Finland M. Increased resistance in the pneumococcus. *N Engl J Med* 1971; 284: 212 - 214.

88 Klugman KP, Koornhof HJ. Carriage of penicillin-resistant pneumococci. *Arch Dis Child* 1986; 61: 377 -381.

89 Gray BM, Converse GM III, Dillon HC Jr. Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis* 1980; 142: 923 - 933.

90 Anglin DA, Siegel JD, Pacini DL, Smith SJ, Adams G, Buchanan GR. Effect of penicillin prophylaxis on nasopharyngeal colonization with *Streptococcus pneumoniae* in children with sickle cell anaemia. *J Pediatr* 1984; 104: 18 - 22.

91 Hendley JO, Sande MA, Stewart PM, Gwaltney JM Jr. Spread of *Streptococcus pneumoniae* in families. I. Carriage rates and distribution of types. *J Infect Dis* 1975; 132: 55 - 61.

92 Gwaltney JM Jr, Sande MA, Austrian R, *et al*. Spread of *Streptococcus pneumoniae* in families. II. Relation of transfer of *Streptococcus pneumoniae* to incidence of colds and serum antibody. *J Infect Dis* 1975; 132: 62 - 68.

93 Masters PL, Brumfitt W, Mendez RL, *et al*. Bacterial flora of the upper respiratory tract in Paddington families, 1952 - 4. *Brit Med J* 1958; 1: 1200 - 1205.

94 Ward JI, Koornhof HJ. Antibiotic-resistant pneumococci. In: Remington JS and Swartz MN (eds). *Current Clinical Topics in Infectious Diseases*. McGraw-Hill International Book Co., New York, 1980, 265 - 287.

95 Jacobs NM, Lepdkachornsuk S, Metzger WI. Pneumococcal bacteraemia in infants and children: a ten - year experience at the Cook County Hospital with special reference to the pneumococcal serotypes isolated. *Pediatrics* 1979; 64: 296 - 300.

96 Siegel JD, Poziviak CS, Michaels RH. Serotypically defined pneumococcal infections in children. *J Pediatr* 1979; 93: 249 - 250.

97 Gray BM, Converse GM III, Dillon HC Jr. Serotypes of *S. pneumoniae* causing disease. *J Infect Dis* 1979; 140: 979 - 983.

98 Bratton L, Teele DW, Klein JO. Outcome of unsuspected pneumococcemia in children not initially admitted to the hospital. *J Pediatr* 1977; 90: 703 - 706.

99 Dowling JN, Sheehe PR, Feldman HA. Pharyngeal pneumococcal acquisitions in "normal" families: a longitudinal study. *J Infect Dis* 1971; 124: 9 - 17.

100 Austrian R. Some aspects of the pneumococcal carrier state. *J Antimicrob Chemother* 1986; 18: 35 - 45.

101 Andersson B, Dahmén J, Frejd T, Leffler H, Magnusson G, Noori G, Eden CS. Identification of an active disaccharide unit of a glycoconjugate receptor for pneumococci attaching to human epithelial cells. *J Exp Med* 1983; 158: 559 - 570.

102 Andersson B, Fogh A, Jorgensen F, Larsson S, Leffler H, Magnusson G, Nylen O, Siidow CV, Svanborg Edén C. Attachment of *Streptococcus pneumoniae* to human pharyngeal epithelial cells in vitro - mechanism of binding. *Otolaryngology - Head and Neck Surgery* 1984; 92: 266 - 269.

103 MacLeod CM, Hodges RG, Heidelberger M, Bernhard WG. Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. *J Exp Med* 1945; 82: 445 - 465.

104 Sivonen A. Effect of *Neisseria meningitidis* group A polysaccharide vaccine on nasopharyngeal carrier rates. *J Infection* 1981; 3: 266 - 272.

105 Douglas RM, Hansman D, Miles HB, *et al*. Pneumococcal carriage and type-specific antibody. *AJDC* 1986; 140: 1183 - 1185.

106 Douglas RM, Paton JC, Duncan S, *et al*. Antibody response to pneumococcal vaccination in children younger than 5 years of age. *J Infect Dis* 1983; 148: 131 - 177.

107 Riley ID, Tarr PI, Andrews M, Pfeiffer M, Howard R, Challands P, Jennison G, Douglas RM. Immunization with a polyvalent pneumococcal vaccine. *Lancet* 1977; 1: 1338 - 1341.

108 Simberkoff MS, Lukaszewski M, Cross A, Al-Ibrahim M, Baltch AL, Smith RP, Geiseler PJ, Nadler J, Richmond AS. Antibiotic-resistant isolates of *Streptococcus pneumoniae* from clinical specimens: a cluster of serotype 19A organisms in Brooklyn, New York. *J Infect Dis* 1986; 153: 78 - 82.

109 Davies AJ, Kumaratne DS. The continuing problem of pneumococcal infection. *J Antimicrob Chemother* 1988; 21: 387 - 393.

110 Tuomanen E (Editorial). Partner drugs: a new outlook for bacterial meningitis. *Ann Intern Med* 1988; 109: 690 - 692.

111 Tweardy DJ, Jacobs MR, Speck WT. Susceptibility of penicillin-resistant pneumococci to eighteen antimicrobials: implications for treatment of meningitis. *J Antimicrob Chemother* 1983; 12: 133 - 139.

112 Sacho H, Klugman KP, Koornhof HJ, Ruff P. Community-acquired pneumonia in an adult due to a multiply-resistant pneumococcus. *J Infection* 1987; 14: 188 - 189.

113 Klugman KP, Koornhof HJ. Bacteremic pneumonia caused by penicillin-resistant pneumococci. *N Engl J Med* [Lett] 1988; 318: 123 - 124.

114 Klugman KP, Koornhof HJ, Kuhnle V, Miller SD, Ginsburg PJ, Mauff AC. Meningitis and pneumonia due to novel multiply-resistant pneumococci. *Br Med J* 1986; 292: 730.

115 Klugman KP, Koornhof HJ, Kuhnle V. Clinical and nasopharyngeal isolates of unusual multiply-resistant pneumococci. *Am J Dis Child* 1986; 140: 1186 - 1190.

116 Ward JI, Moellering RC Jr. Susceptibility of pneumococci to 14 beta-lactam agents: comparison of strains resistant, intermediate-resistant, and susceptible to penicillin. *Antimicrob Agents Chemother* 1981; 20: 204 - 207.

117 Istre GR, Humphreys JT, Albrecht KD, Thornsberry C, Swenson JM, Hopkins RS. Chloramphenicol and penicillin resistance in pneumococci isolated from blood and cerebrospinal fluid: a prevalence study in Metropolitan Denver. *J Clin Microbiol* 1983; 17: 472 - 475.

118 Garau J, Linares J, Dominguez C. Chloramphenicol-resistant pneumococci. *Lancet* 1981; ii: 147 - 148.

119 Jacobs MR, Mithal Y, Robins-Browne RM, Gaspar MN, Koornhof HJ. Antimicrobial susceptibility testing of pneumococci: determination of Kirby-Bauer breakpoints for penicillin G, erythromycin, clindamycin, tetracycline, chloramphenicol, and rifampin. *Antimicrob Agents Chemother* 1979; 16: 190 - 197.

120 Koornhof HJ. The emergence of antimicrobial resistance in *Streptococcus pneumoniae*. In: Berkarda B and Kuemmerle HP (eds). *Progress in antimicrobial and anticancer chemotherapy.* Proceedings of the 15th International Congress of Chemotherapy, Landsberg, 1987, 91 - 93.

121 Sande MA, Irvin RG. Penicillin-aminoglycoside synergy in experimental Streptococcus viridans endocarditis. *J Infect Dis* 1974; 129: 572 - 579.

122 Shanson DC, Namnyak S. Viridans streptococci with reduced bactericidal susceptibility to penicillin from patients with endocarditis; susceptibilities to erythromycin, rifampin, vancomycin and aminoglycosides. In: Periti P and Grassi CG (eds). *Current Chemotherapy and Immunotherapy.* Proceedings of the 12th International Congress of Chemotherapy, Florence, Italy, 1981, 311 - 313. American Society for Microbiology, Washington, DC.

123 Wilson WR, Zak O, Sande MA. Penicillin therapy for treatment of experimental endocarditis caused by viridans streptococci in animals. *J Infect Dis* 1985; 152: 1028 - 1033.

124 Yee Y, Farber B, Mates S. Mechanism of penicillin-streptomycin synergy for clinical isolates of viridans streptococci. *J Infect Dis* 1986; 154: 531 - 534.

125 Miller MH, El-Sokkary MA, Feinstein SA, Lowy FD. Penicillin-induced effects on streptomycin uptake and early bactericidal activity in viridans group and enterococcal streptococci. *Antimicrob Agents Chemother* 1986; 30: 763 - 768.

126 Shanson DC, Tadayon M, Bakhtiar M. Bactericidal activity of netilmicin compared with gentamicin and streptomycin, alone and in combination with penicillin, against penicillin tolerant viridans streptococci and enterococci. *J Antimicrob Chemother* 1986; 18: 479 - 490.

127 Linares J, Garau J, Dominguez C, Perez JL. Antibiotic resistance and serotypes of *Streptococcus pneumoniae* from patients with community-acquired pneumococcal disease. *Antimicrob Agents Chemother* 1983; 23: 545 - 547.

128 Champion LAA, Wald ER, Luddy RE, Schwartz AD. *Streptococcus pneumoniae* resistant to erythromycin and clindamycin. *J Pediatr* 1978; 92: 505 - 506.

129 Simberkoff MS, Richmond A, Lukaszewski M, *et al*. Isolation of multiply antibiotic-resistant pneumococci - New York. *MMWR* 1985; 34: 545 - 546.

130 Koornhof HJ, Jacobs MR, Ward JI, Appelbaum PL, Hallet FA. Therapy and control measures of antibiotic resistant pneumococcal disease. In: Schlesinger D (ed). *Microbiology - 1979*. American Society of Microbiology, Washington DC, 1979, 286 - 289.

131 Bisno AL, Dismukes WE, Durack DT, Kaplan EL, Karchmer AW, Kaye DK, Sande MD, Sanford JP, Wilson WR. Treatment of infective endocarditis due to viridans streptococci. American Heart Association Report. *Circulation* 1981; 63: 730A - 733A.

132 Lennette EH, Balows A, Hausler WJ Jr, Shadomy HJ (eds). *Manual of Clinical Microbiology* (4th ed). A.S.M., Washington DC, U.S.A., 1985.

133 Colman G, Williams REO. The cell walls of streptococci. *J Gen Microbiol* 1965; 41: 375 - 387.

134 Mizuno J, Torii M, Yamamoto T, Hamada S. Detection of amphipathic antigen unique to biotype B *Streptococcus sanguis* [letter]. *FEMS Microbiol* 1983; 16: 209 - 212.

135 Rosan B. Absence of glycerol teichoic acids in certain oral streptococci. *Science* 1978; 201: 918 - 920.

136 Cole RM, Calandra GB, Huff E, Nugent KM. Attributes of potential utility in differentiating among "group H" streptococci or *Streptococcus sanguis*. *J Dent Res* 1976; 55 (Special Issue A): A142 - A153.

137 Hamada S, Torii M, Tsuchitani Y, Kotani S. Isolation and immunobiological classification of *Streptococcus sanguis* from human tooth surfaces. *J Clin Microbiol* 1980; 12: 809 - 817.

138 Rosan B. Antigens of *Streptococcus sanguis*. *Infect Immun* 1983; 7: 205 - 211.

139 Appelbaum PC, Jacobs MR, Heald JI, Palko WM, Duffett A, Crist R, Naugle PA. Comparative evaluation of the API 20S system and Auto-Microbic system Gram-Positive Identification Card for species identification of streptococci. *J Clin Microbiol* 1984; 19: 164 - 168.

140 Ruoff KL, Ferraro MJ, Jerz ME, Kissling J. Automated identification of gram-positive bacteria. *J Clin Microbiol* 1982; 16: 1091 - 1095.

141 Appelbaum PC, Chaurushiya PS, Jacobs MR, Duffett A. Evaluation of the Rapid Strep system for species identification of streptococci. *J Clin Microbiol* 1984; 19: 588 - 591.

142 Kontiainen S, Sivonen A. Optochin resistance in *Streptococcus pneumoniae* strains isolated from blood and middle ear fluid. *Eur J Clin Microbiol* 1987; 6: 422 - 424.

143 French GL, Talsania H, Phillips I. Identification of viridans streptococci by pyrolysis-gas chromatography. *J Med Microbiol* 1989; 29: 19 - 27.

144 Price T, French GL, Talsania H, Phillips I. Differentiation of *Streptococcus sanguis* and *S. mitior* by whole-cell rhamnose content and possession of arginine dihydrolase. *J Med Microbiol* 1986; 21: 189 - 197.

145 Williamson R, Gutmann L, Horaud T, Delbos F, Acar J. Use of penicillin-binding proteins for the identification of enterococci. *J Gen Microbiol* 1986; 132: 1929 - 1937.

146 Chalkley LJ, van den Berg IS, Koornhof HJ. Penicillin-binding proteins of clinical isolates of *Streptococcus pneumoniae* from South Africa. *SAJS* 1989; 85: 263 - 266.

147 Garcia-Bustos JF, Chait BT, Tomasz A. Altered peptidoglycan structure in a pneumococcal transformant resistant to penicillin. *J Bacteriol* 1988; 170: 2143 - 2147.

148 Markiewicz Z, Tomasz A. Variation in penicillin-binding protein patterns of penicillin-resistant clinical isolates of pneumococci. *J Clin Microbiol* 1989; 27: 405 - 410.