MICROBIOLOGY AND MOLECULAR EPIDEMIOLOGY

OF MULTIRESISTANT *HAEMOPHILUS INFLUENZAE*

TYPE B IN DURBAN, SOUTH AFRICA

BY

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ABSTRACT

Microbiological and molecular epidemiological studies were conducted on 36 multi-resistant Haemophilus influenzae strains, isolated from paediatric patients, over a 26 month period (April 1986 to May 1988). The majority of strains (80,5%) had been isolated from blood and cerebrospinal fluid. More than 80% of isolates tested belonged to biotype II and 90% were of serotype B. Minimal inhibitory concentrations against 6 antibiotics (ampicillin, chloramphenicol, tetracycline, rifampicin, streptomycin and cefotaxime) confirmed the presence of multi-resistant strains. Resistance to rifampicin was confirmed in 6 (16,7%) strains. All strains were susceptible to cefotaxime.

Ten transconjugants analysed with respect to their plasmid content were shown to harbour an identical 41 MDa plasmid. Restriction endonuclease digests of these plasmids with Eco R1 and Sst1 revealed almost identical restriction patterns.

Outer membrane protein profiles of 19 strains revealed the predominance of one particular subtype.

By combining the microbiological and molecular epidemiological findings, it is concluded that one strain of H. influenzae type b is responsible for the nosocomial acquisition of infections amongst paediatric patients. The implications of these findings are discussed.

PREFACE

This study represents original work by the author and has not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

The research described in this project was carried out in the Department of Medical Microbiology, University of Natal; the Department of Paediatric Infectious Diseases, University of Washington and the Department of Paediatric Infectious Diseases, University College of Los Angeles, under the supervision of Prof J van den Ende, Prof A L Smith and Dr J I Ward respectively.

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CHAPTER 1

1.0 REVIEW OF LITERATURE

1.1 INTRODUCTION

The genus Haemophilus currently classified under the family Pasteurellaceae (Kilian and Biberstein, 1984), consists of small, non-motile, Gram-negative bacilli that require growth factors present in blood. The term Haemophilus is derived from the Greek words meaning "blood-loving".

Haemophilus-like organisms, were first described by Koch in 1883 (cited by Turk and May, 1967). In 1892, Pfeiffer isolated Haemophilus influenzae from the sputum and lung tissue of individuals who died during the influenza pandemic of the time and implicated it as the cause of influenza (cited by Konemann et al, 1988). It was only after the discovery of the influenza virus in 1933 (cited by Konemann et al, 1988) that the role of H. influenzae as a secondary invader following influenza was realised. Over the next 50 years, the role of H. influenzae in various diseases, both in adults and in children, became more widely appreciated. These infections include meningitis, acute pharyngitis, epiglottitis, bronchitis, acute sinusitis, pneumonia, conjunctivitis and septic arthritis. In the United States, an estimated 8000 to 15000 cases of H. influenzae meningitis are reported annually (Sell SH, 1987) and this organism is the commonest cause of bacterial meningitis in children between 1 month and 2 years of age. The importance of H. influenzae in developing countries is indicated by a Joint WHO/UNICEF statement that "...available evidence indicates that

Streptococcus pneumoniae and Haemophilus influenzae are the most prevalent bacterial agents of community-acquired pneumonia in children in developing countries" (A joint WHO/UNICEF Statement, 1986).

1.2 HAEMOPHILUS INFLUENZAE

1.2.1 General Microbiology

In clinical specimens, the stained organisms may vary from the coccobacillary to filamentous pleomorphic forms. In vitro, growth of H. influenzae requires both X and V factors. X factor is a group of heat-stable compounds that are provided by several iron-containing pigments such as hemin and hematin (Koneman et al, 1988). V factor (nicotinamide adenine dinucleotide (NAD)) is produced in large amounts by several micro-organisms (eg Staphylococcus aureus) and on blood agar the growth of H. influenzae is enhanced around S. aureus colonies, a phenomenon termed "satellitism" (Konemann et al, 1988). Both X and V factors are found within blood cells (eg sheep or horse erythrocytes). However, the blood may also contain enzymes that can inactivate V factors. In addition, because V factor is not released from intact erythrocytes, H. influenzae will not grow on blood agar in which the erythrocytes are intact. Gentle heating of blood as in the preparation of chocolate agar results in lysis of erythrocytes, liberation of both X and V factors, and inactivation of enzymes that could inactivate V factor (Koneman et al, 1988).

In the diagnostic setting, it is recommended that clinical specimens be plated on blood agar (with a streak of a staphylococcal strain), and chocolate agar, and incubated in an atmosphere of 5-10% carbon-dioxide at 35 to 37°C. H. influenzae colonies on chocolate agar are greyish and semi-opaque and usually visible after 18 to 24 hours of incubation. Although often grown in the presence of carbon-dioxide, this is not an essential prerequisite for growth since H. influenzae is a facultative anaerobe (Mendelman and Smith, 1987).

Further identification of putative H. influenzae colonies is by testing for X and V factor requirement by using discs impregnated with these factors on a basal medium lacking X and V factors. Further confirmation of species is obtained by the porphyrin test described below (Kilian, 1985). H. influenzae lacks the enzyme necessary for the conversion of delta-aminolaevulinic acid (δ-ALA) to porphyrins which are intermediates in the hemin biosynthetic pathway. Thus in a reagent containing δ-ALA inoculated with a Haemophilus strain, no porphyrins are detected in the case of H. influenzae (ie a negative reaction), while Haemophilus parainfluenzae serves as a positive control. Methods of detecting porphyrins include the use of a Wood's lamp to detect fluorescence or the addition of Kovac's reagent which results in a red colour in the lower aqueous phase.

1.2.2 Serotyping

Strains of H. influenzae may be either capsulated or unencapsulated. In 1931, Pitman (cited by Turk, 1982) established that the encapsulated strains may be divided into six serotypes designated a to f. Pitman's description of the association of serotype b with severe disease (cited by Turk, 1982) was a major step forward towards understanding the pathogenesis of H. influenzae infections. The use of serotyping for distinguishing between clinical H. influenzae isolates is now a routine procedure in most diagnostic microbiology laboratories.

In an effort to explain the invasiveness of the encapsulated (particularly serotype b) strains, studies on the chemical composition of the capsular substance revealed a new class of immunologically active compounds, the phosphopolysaccharides (Zamenhof et al, 1953 - cited by Omland, 1978). Using chemical and nuclear magnetic resonance spectroscopic techniques, Egar, Tsui and Zon (1982) determined the repeating units of the six H. influenzae serotype polysaccharides (Table 1.1). Only type b strains contain pentoses, all others having a hexose or a hexose derivative. Types d and e differ from the other capsular polysaccharides in lacking phosphodiester linkages.

TABLE 1.1 : Carbohydrate Composition of H. influenzae Capsules

(from Mendelman and Smith, 1987)

Type	Sugar	N-Acetyl	Phosphate
a	glucose	-	+
b	ribose, ribitol	-	+
c	galactose	-	+
d	hexose	-	+
e	hexosamine	+	-
f	galactosamine	+	+

Although serotyping as a tool for characterising H. influenzae strains has been universally accepted, the techniques used for serotyping have been the subject of recent discussion (Wallace et al, 1981; Ingram et al, 1979). The commonly used serotyping techniques are therefore briefly reviewed.

1.2.2.1 TECHNIQUES FOR SEROTYPING

1.2.2.1.1 Immunoprecipitation (Double Diffusion)

In this technique, wells punched into agar (on slides) are filled with antigen and reference antisera and allowed to diffuse towards each other. Precipitation lines usually become apparent after 24 hours and are evident as lines of identity. This method, although widely used in the 1940's and 1950's (Omland, 1978), is less widely used today principally because of the delay in obtaining results.

1.2.2.1.2 Countercurrent-Immunoelectrophoresis (CIE)

In this procedure also performed on agarose slides, the application of a low voltage current speeds up the movement of antigen and antibody towards each other, giving visible immunoprecipitation lines within an hour (Myhre, 1974). Although highly sensitive, supernatant from overnight both cultures is usually recommended as the source of antigen, thus delaying the serotyping result by about 18 hours. This method is most suitable for screening large numbers of isolates in view of the small concentrations of reagents required.

1.2.2.1.3 Capsular Swelling ("Quellung") Reaction

A broth culture of the organism to be serotyped is mixed with specific antiserum on a slide and observed for capsular swelling under a microscope (Neufeld, 1902 - cited by Omland, 1978). Although highly sensitive and rapid, the reaction may not always be easy to interpret, particularly with small organisms like H. influenzae.

1.2.2.1.4 Slide Agglutination

This is now the most widely used, simplest and most rapid serotyping method in use in most diagnostic laboratories. Specific antiserum is added to a loopful of well emulsified, unknown strain and the slide rotated for two minutes. Agglutination is easily

visible. It is important that appropriate controls be included to exclude false positive reactions due to autoagglutination.

1.2.2.1.5 Other Techniques

Other techniques which have been used for serotyping include latex agglutination, immunofluorescence (Wallace, 1981; Ingram et al, 1979) and radioimmunological techniques (cited by Omland, 1978). Latex agglutination was reported by Ingram et al (1979) to be difficult to read due to weak reactions; cross-reactions with nasopharyngeal isolates were also observed with this procedure. These workers reported that H. influenzae serotyped by the slide agglutination test, CIE, the Quellung reaction and latex agglutination gave essentially comparable results. Wallace and colleagues (1981) on the other hand, reported frequent discrepancies between results of CIE, immunofluorescence and slide agglutination. In examining H. influenzae isolates obtained from adults with meningitis or septicemia, they noted that serotyping of non-typeable strains as typeable, was the commonest error encountered. Seventy percent of 45 strains in their study were grouped as serotypes b by slide agglutination, but only 29% of these strains tested positive for type b by CIE.

Serotyping is important because since its introduction encapsulated strains (particularly serotype b) have been shown consistently to be the most pathogenic amongst H. influenzae strains. This in turn has led to the development of vaccines containing the type b capsular material (Robbins and Schneerson, 1978). The major

limitation of serotyping is that most clinical H. influenzae isolates, particularly from non-invasive disease, are non-capsulated and hence non-typable. These strains are being increasingly recognised as important human pathogenesis (reviewed by Murphy and Apicella, 1987), both in adults and in children. Typing these strains can be expected to assume increasing importance as their role in disease becomes more widely recognised.

In addition to the limitations of serotyping outlined above, the fact that most invasive strains, (particularly in children), are identified as serotype b limits the value of serotyping for epidemiological studies.

In order to expand the discriminatory potential of typing H. influenzae for epidemiological purposes, several other methods have recently been proposed. These include biotyping (Kilian, 1976, cited by Kilian, 1985), outer membrane protein typing (Loeb and Smith, 1980; Barenkamp, Munson and Granoff, 1981) and multilocus enzyme electrophoresis (Selander et al, 1986). Some of these techniques and their applications to the epidemiology of H. influenzae are discussed further.

1.2.3 Biotyping

In the scheme proposed by Kilian in 1976 (cited by Kilian, 1985), Haemophilus species could be divided into several biotypes based on indole production, urease activity and ornithine decarboxylase activity. To date, 8 biotypes of H. influenzae (Table 1.2) have

been identified. Specific biotypes have been associated with different

TABLE 1.2 : Key to the Differentiation of H. influenzae biotypes (From Doern and Chapin, 1987)

Biotype	Indole Production	Urease Activity	Ornithine Decarboxylase Activity
I	+	+	+
II	+	+	-
III	-	+	-
IV	-	+	+
V	+	-	+
VI	-	-	+
VII	+	-	-
VIII	-	-	-

types of infections, sources of isolation and antimicrobial susceptibility patterns. In a Danish study 94% of 16 H. influenzae isolates from patients with meningitis were of biotype I (Braun and Frus-Moller, 1976). A similar percentage (93% of 130 strains) of H. influenzae isolates causing meningitis studied by Kilian, Sorensen and Frederiksen (1979) were also biotype I. However, although a study of over 500 H. influenzae isolates in Canada by Albritton et al, 1978, found that 93% of the blood isolates were either biotype I

or II, these authors found no correlation between biotypes and any specific clinical syndrome. Granato, Jurek and Winer (1983), in a later study found that all of 10 H. influenzae isolates from cerebrospinal fluid were biotype I. The association between biotype III and conjunctivitis was illustrated in the study by Controni et al (1981), who found that 70% of the 60 isolates from the eye were biotype III. Biotype IV on the other hand, has been associated with obstetric and gynaecological infections and in neonatal infections (Wallace, Baker and Quinones, 1983 - cited by Konemann et al, 1988). These strains are usually non-typeable by serological techniques.

The association of biotype with antimicrobial susceptibility appears to be less clear-cut than initially proposed. Albritton et al (1978) claimed a significant association between biotypes I and II and antibiotic resistance. However, other studies of isolates from several body sites indicate that B-lactamase mediated ampicillin resistance maybe more generally distributed among the different H. influenzae biotypes (Konemann et al, 1988).

Although biotyping is reproducible and easy to perform it fails to distinguish between the majority of invasive type b strains (89% are biotype I), thus limiting its usefulness in epidemiological studies.

1.2.4 Outer Membrane Proteins (OMPs)

The cell envelope of H. influenzae is typical of that found in

gram-negative bacteria, consisting of a cytoplasmic or inner membrane, a peptidoglycan layer and an outer membrane. The composition of the outer membrane has also been shown to be typical of that found in other gram-negative bacteria (Loeb and Smith, 1982).

Although more than 30 outer membrane proteins have been identified on sodium dodecyl sulfate polycrylamide gels, seven proteins account for 80% of the total outer membrane protein (Loeb and Smith, 1982a). These major outer membrane proteins have approximate molecular weights of 16 000, 27 000, 30 000, 35 000, 40 000 and 49 000 respectively (Barenkamp, Munson and Granoff, 1981). On the basis of the major outer membrane proteins, 21 distinct sub-types of H. influenzae have been described (Granoff, Barenkamp and Munson, 1982). Repeated in vivo and in vitro passage of H. influenzae strains investigated have shown that OMP markers were stable (Barenkamp, Munson and Granoff, 1981; Loeb and Smith, 1980).

These varying outer membrane protein patterns have been successfully used to obtain useful epidemiological data which could not be obtained by serotyping or biotyping alone or in combination (Campos et al, 1987; van Alphen et al, 1987; Barenkamp, Munson and Granoff, 1981; Granoff, Barenkamp and Munson, 1981). Barenkamp, Munson and Granoff (1981) found complete agreement in the sub-types of H. influenzae isolated from 10 index cases and their household contacts. In addition, in cases of recurrent H. influenzae disease, the same outer membrane protein sub-types

were isolated from seven children, ie the sub-type responsible for primary disease was the same as that isolated during the recurrent attack (Granoff, Barenkamp and Munson, 1982). These investigations also showed that in 6 day-care centres, with secondary cases of Haemophilus disease, sub-type IH organisms were responsible for most cases of disease. Overall, in a study of 256 type b isolates from various regions of the United States, three sub-types (IH, 2L and 3L) accounted for nearly three-quarters of the isolates, with sub-type IH being the commonest cause of endemic disease in the United States. These findings support the earlier findings of Loeb and Smith (1980) in which outer membrane protein sub-typing was used to demonstrate several features of H. influenzae type b (Hib) disease such as :

i Siblings who acquired Hib disease within a short time had similar outer membrane protein sub-types;

ii Blood and cerebrospinal fluid isolates were identical and that Hib infections were due to a single strain and not to mixtures of strains;

iii Reinfection of an individual was always due to a similar outer membrane sub-type as the one causing the primary infection.

In an epidemic of Hib meningitis involving 9 patients, outer membrane protein analysis was used to show that a single strain was not responsible for the epidemic (Loeb and Smith, 1980).

Most outer membrane protein analyses of H. influenzae have been conducted in the United States. However, in Europe, investigations undertaken by van Alphen and colleagues (van Alphen et al, 1987), indicate that only one sub-type of Hib predominates in Western Europe and these strains differ from those most frequently encountered in the United States. In a recent study, Takala et al (1987) have suggested that a particular outer membrane sub-type (Ic by van Alphen's classification) is significantly associated with meningitis.

In addition to their use as tools in sub-typing isolates of H. influenzae, the role of outer membrane protein components in pathogenesis of H. influenzae disease and their role as potential vaccine candidates is receiving increasing attention (Loeb and Smith, 1980).

1.2.5 Antimicrobial Susceptibility of H. influenzae

Early confusion concerning the role of penicillin in the therapy of H. influenzae meningitis (Smith, 1976), appeared to be resolved by the introduction of chloramphenicol in the 1950's. Subsequently, ampicillin, introduced in 1961 was shown to have good in vitro activity against H. influenzae (Rolinson and Stevens, 1961). Its efficacy in the therapy of H. influenzae meningitis (Thrupp, Leedom and Ivler, 1964 - cited by Smith 1976), combined with its greater clinical safety (in comparison to that of chloramphenicol) soon led to the widespread use of this antibiotic in the therapy of H. influenzae disease. The few reports of therapeutic failure in

serious infections were attributed to reasons other than ampicillin resistance (Needham, 1988).

1.2.5.1 Ampicillin Resistance

The first report of H. influenzae isolates resistant to ampicillin appeared in 1972 (Mathies, 1972). In this paper, five H. influenzae isolates obtained from blood or cerebrospinal fluid were shown to have minimum bactericidal concentrations for ampicillin of 100mg/ℓ. Over the next 2 years numerous reports of ampicillin resistance in H. influenzae appeared worldwide (Schiffer et al, 1974; Tomeh et al, 1974; Price and Boswell, 1974; Turk, 1974; Thomas et al, 1974; Khan et al, 1974; Morbidity and Mortality Weekly Report (MMWR) 1974a; MMWR, 1974b).

In South Africa, a study from Johannesburg indicated that during a 17 month study period between August 1978 and December 1979, three of 10 invasive H. influenzae isolates were beta-lactamase producing (Black, Arntzen and Gardner, 1980). In an analysis of blood culture isolates from seven South African teaching hospital centres, van den Ende and Rotter (1985), reported an overall 10% prevalence of beta-lactamase-producing H. influenzae.

The prevalence of ampicillin-resistant H. influenzae has continued to increase wherever such trends have been followed. In the United Kingdom, countrywide surveys involving up to 25 laboratories have shown an increase in ampicillin resistance from 1,6% in 1977 to 7,8% in 1986 (Powell et al, 1987; Philpott-Howard and

Williams, 1982) amongst clinical H. influenzae isolates. The prevalence of resistant type b isolates increased from 14% to 18%. In Finland, Jokipii and Jokipii (1980) found that whereas no ampicillin-resistant H. influenzae isolates were apparent in 1975, 13% of H. influenzae strains isolated between 1977 and 1978 were ampicillin-resistant. The majority of strains were non-invasive (only one resistant isolate was obtained from blood) and no information on serotypes was provided in this study. In a recent survey of clinical H. influenzae isolates in the United States, Doern et al (1988) showed that ampicillin resistance had increased from 15,2% in 1984 to 20% in 1986; with resistance among type b strains showing an increase from 21% to 31,7% during the same period. In this study, the highest rates of resistance were amongst isolates from children less than 2 years of age.

The increase in ampicillin resistance both in the United States and the United Kingdom, has prompted the recommendation of chloramphenicol as first line empirical therapy for invasive H. influenzae disease (Powell et al, 1987; Doern et al, 1988).

1.2.5.2 Mechanisms of Ampicillin Resistance

First reports on the mechanism of resistance to ampicillin established the involvement of a constitutive beta-lactamase (Williams, Kattan and Cavanagh, 1974; Khan et al, 1974), which resembled the beta-lactamases produced by other gram-negative bacilli (Farrar and O'Dell, 1974). Medeiros and O'Brien (1975) established that H. influenzae possessed much less of a permeability barrier to

ampicillin than Eschericha coli. They postulated that this was responsible for the lower levels of resistance of H. influenzae possessing the TEM type beta-lactamase, compared to E. coli strains producing a similar enzyme.

Although ampicillin-resistance in H. influenzae is most commonly associated with production of a Tem-1 beta-lactamase, a second beta-lactamase termed "Rob" has been recently described (Rubin et al, 1981). Interestingly, this enzyme tested falsely negative with the chromogenic cephalosporin test.

In addition to beta-lactamase production, non-beta-lactamase-producing strains of H. influenzae have also been reported (Bell and Plowman, 1980). In one study, 9 of 115 ampicillin-resistant strains were found to exhibit this type of resistance (Philpott-Howard and Williams, 1982). More recent studies seem to indicate an increase in this type of resistance (Doern et al, 1988; Jorgenssen et al, 1988). The mechanism for this form of resistance is presumed to be due to chromosomally mediated alterations in penicillin-binding proteins (PBPs) (Reid et al, 1987; Mendeman, 1986). PBPs are enzymes involved in cell wall synthesis and are the targets of beta-lactam antibiotics. Alterations in PBPs may result in B-lactam antibiotics binding less strongly to the target PBPs. Such chromosomally mediated non-beta-lactamase-resistance has so far been restricted mainly to non-type b respiratory isolates. The significance of this mechanism of resistance is that it also results in decreased susceptibility to other beta-lactam antibiotics such as the second and third generation cephlasporins

(Needham, 1988). Minimum inhibitory concentrations (MICs) for ampicillin for these organisms generally range from between 4 to 8mg/mℓ (Woolfrey, Lally and Ederer, 1987), although MICs of greater than or equal to 32mg/mℓ have been reported (Mendelman et al, 1984).

1.2.5.3 Chloramphenicol Resistance

As early as 1972, Barrett et al, in a review of 127 H. influenzae strains from patients with meningitis reported one strain to be chloramphenicol resistant. In 1978, Kinmouth, Storrs and Mitchell reported the isolation of a H. influenzae type b strain from cerebrospinal fluid which was resistant to chloramphenicol. The overall prevalence of chloramphenicol resistance amongst H. influenzae strains, is appreciably lower than that reported for ampicillin. In the most recent National Collaborative Study conducted in the United States (Doern et al, 1988), only 0,5% of 28 clinical isolates were reported as resistant to chloramphenicol. Only two of these 14 strains were type b. In a countrywide British study (Powell et al, 1987), 1,7% of over 2 400 strains of H. influenzae were reported to be chloramphenicol resistant, compared to a prevalence rate of 1,03% 5 years earlier (Philpott-Howard and Williams, 1982). This increase in chloramphenicol resistance was considered not to be significant and the authors concluded that chloramphenicol could still be deemed suitable for initial treatment of invasive H. influenzae disease.

1.2.5.4 Mechanisms of Chloramphenicol Resistance

Roberts et al (1980) studied 9 chloramphenicol resistant isolates (including 4 serotype b strains) and concluded that resistance was due mainly to the production of chloramphenicol acetyltransferase (CAT), a finding which has subsequently been confirmed (Doern et al, 1988; Powell et al, 1987). However, a permeability barrier resulting from lack of one of the outer membrane proteins has also been postulated as mediating chloramphenicol resistance (Burns and Smith 1987, Burns et al, 1985).

1.2.5.5 Tetracycline, Trimethoprim, Sulphamethoxazole and Rifampicin Resistance

Most data on the susceptibility of H. influenzae to these antibiotics comes from the countrywide studies conducted in the United States and Britain (Doern et al, 1988; Powell et al, 1987), and are briefly reviewed.

Tetracycline resistance amongst United Kingdom isolates of H. influenzae decreased slightly from 3,1% in 1982 to 2,7% in 1987 (Powell et al, 1987) whilst trimethoprim resistance increased significantly from 1,4% in 1981 to 4,2% in 1986 (p 0,001). The number of type b strains resistant to these antibiotics totalled only three. The prevalence of sulphamethoxazole resistant strains stood at 3,5%. Resistance to rifampicin and erythromycin were not evaluated in this study.

The comparative prevalence of resistance in the United States (Doern et al, 1988) was as follows:

tetracycline 2,3% (20 type b strains), trimethoprim-sulphamethoxazole 0,9% (5 type b isolates). In this study, only 0,1% of strains were susceptible to erythromycin while 0,7% of strains were considered resistant to rifampicin (MIC 4mg/mℓ). The mechanisms of resistance to these antibiotics remain mostly unknown, but is assumed to be similar to that found in other species of bacteria.

The gene coding for trimethoprim resistance in H. influenzae has recently been cloned (de Groot et al, 1988). These authors concluded that resistance to this antibiotic was mediated by the overproduction of chromosomally located dihydrofolate reductase.

1.2.5.6 Multiple Antibiotic Resistance

Although resistance to ampicillin and chloramphenicol has for reasons of convenience been discussed separately, the presence of H. influenzae strains resistant to multiple antibiotics has been evident since 1978 (Bryan, 1978). Occasional ampicillin-chloramphenicol resistant strains have been reported from various parts of the world (Overturf, Cable and Ward, 1987; Catry and Vaz Pato, 1983; Rotimi and Turk, 1981). In South Africa, a beta-lactamase-producing, chloramphenicol-resistant H. influenzae type b from the blood and cerebrospinal fluid of a patient was first recorded in Durban in 1985 (Coovadia, Coovadia and van den Ende, 1986). Subsequently, a similar case was reported from Pretoria (Krajewska

et al, 1986). A nationwide study in the United States (Jorgensen et al, 1988), identified 106 H. influenzae strains resistant to multiple antibiotics. The majority of these strains were non-type b respiratory isolates. However, included in this group were two type b strains resistant to ampicillin, chloramphenicol and tetracycline.

More disturbing, were the series of reports from Spain which concluded that "...emergence of multiply resistant strains seems to be an endemic problem" (Campos and Garcia-Tornel, 1987; Campos et al, 1986; Campos, Garcia.Tornel and Sanfeliu, 1984). Between 1981 and 1984, 57% of H. influenzae type b strains isolated from community acquired cases of meningitis were resistant to both ampicillin and chloramphenicol. In addition, a high percentage of contacts of patients with invasive H. influenzae type b disease were shown to be carriers of multiply-resistant strains.

An earlier report (Simasathien, Duangmani and Echeverria, 1980) of an outbreak of multiply-resistant H. influenzae strains causing meningitis, involving three children, came from an orphanage in Thailand.

1.2.6 Plasmids in Antibiotic-Resistant H. influenzae

Plasmids are autonomous self-replicating extrachromosomal deoxyribonucleic acid (DNA) elements; and maybe either self-transmissible (conjugative) or non-self-transmissible (non-conjugative). Conjugative plasmids can be transferred between

bacteria of the same or different genera (Elwell and Falkow, 1986). In addition to carrying genes that code for their own replication and transfer, plasmids may also carry genes coding for toxin production, antibiotic resistance, adhesin and bacteriocin production (Elwell and Falkow, 1986). Relevant to plasmid biology was the recognition that genes specifying resistance to certain antibiotics such as tetracycline and penicillin, can be carried on plasmids that have no homology with the bacterial chromosome. These genes, termed transposons or "jumping" genes, were found to be DNA sequences that code for enzymes that bring about the insertion of an identical copy of themselves into a new DNA site. Transposons can move from plasmids to the bacterial chromosome and vice versa (Watson et al, 1987).

Following the reports of beta-lactamase mediated resistance in H. influenzae, studies were soon underway to determine the genetic basis of resistance. Thorne and Farrar (1975) and Sykes, Matthew and O'Callaghan (1975) were the first to demonstrate that ampicillin resistance was transferable by conjugation, from resistant to susceptible strains. A variety of plasmids have been identified in resistant H. influenzae strains, ranging in size from 3,5 megadaltons (MDa) (de Graaff, Elwell and Falkow, 1976 - cited by Needham, 1988), to 62 MDa (Rotimi and Turk, 1981). Large conjugative plasmids between 30 to 52 MDa in size appear to be most commonly associated with ampicillin and multi-resistant strains (Campos et al, 1987; Mendelman et al, 1985; Willard, Johnson and Daum, 1982; Roberts et al, 1980; Elwell et al, 1977; van Klingeren, van Embden and Dessons-Kroon, 1977). The DNA sequence

coding for the TEM-1 beta-lactamase is a 3,0 MDa transposon (TnA) which is widely distributed amongst many different bacterial species (Heffron et al, 1975 - cited by Needham, 1988). An interesting observation with regard to the conjugative H. influenzae plasmids was made by Stuy (1980) who concluded that most of the conjugative plasmids were integrated into the H. influenzae chromosome. This finding was confirmed by Willard, Johnson and Daum (1982), who showed that the majority of ampicillin-resistant H. influenzae clinical isolates were "plasmid-free" but transferred resistance to susceptible strains. They were able to demonstrate plasmid DNA in the transconjugants. More recently, further proof of plasmid integration into the H. influenzae chromosome has been demonstrated by restriction enzyme and Southern blot analysis of DNA in plasmid-free beta lactamase-producing isolates (Murphey-Corb, Nolan-Willard and Daum, 1984). Willard, Johnson and Daum (1982) proposed an evolutionary advantage to H. influenzae carrying plasmids both in the integrated and extrachromosomal forms. Extrachromosomal plasmids are efficient in disseminating resistance genes by conjugation but are easily lost in the absence of selective pressure. On the other hand, H. influenzae with chromosomally integrated resistance, plasmids are "uniformly stable and perpetuate resistance determinants in the H. influenzae gene pool".

Between April 1986 and October 1986, multi-resistant H. influenzae were isolated from 9 children at Clairwood Hospital, Durban. These infections (bacteraemia and/or meningitis) had been acquired after admission to hospital (Hoosen, van den Ende and Kharsany, 1987).

At least 30 more nosocomial infections due to multiply-resistant H. influenzae were noted at Clairwood Hospital during 1987 and the first quarter of 1988.

The objective of this study was to investigate the microbiology and molecular epidemiology of these multi-resistant strains.

CHAPTER 2

2.1 MATERIALS AND METHODS

2.2 BACTERIAL STRAINS

2.2.1 Clinical Isolates

Antibiotic resistant clinical isolates of H. influenzae b were obtained from the diagnostic microbiology laboratories at King Edward VIII and Clairwood Hospitals. Strains isolated between June 1986 and May 1988 were included in the study. All strains had been identified as H. influenzae b by routine laboratory methods which included satellitism, the requirements for X and V factors and a negative porphyrin test (Konemann et al, 1988). In addition, isolates are routinely tested for beta-lactamase production by the chromogenic cephalasporin test (NEU, 1986). Chloramphenicol acetyl transferase is routinely tested for by the method of Slack et al (1977). All isolates were serotyped using a commercial slide agglutination kit (Wellcome).

2.2.2 Laboratory strains

The following strains were used:

H. influenzae R906, erythromycin and streptomycin resistant, non-capsulated served as the recipient strain during conjugation experiments.

H. influenzae Eagan, capsulated type b

H. influenzae S2 non-capsulated. This strain tests positive by immunoprecipitation with type b antiserum after boiling

H. influenzae R1689 ampicillin, chloramphenicol, tetracycline and streptomycin resistant

E. coli Sa carrying a 25 MDa plasmid

E. coli RP4 carrying a 34 MDa plasmid

E. coli R1 carrying a 62 MDa plasmid

All strains were obtained from Dr A L Smith, University of Washington.

2.2.3 Culture Medium

Brain heart infusion agar supplemented with 10ug of hemin-chloride/mℓ 10ug of L-histidine/mℓ and 2ug of B-NAD+/mℓ (sBHI) was used for growth. Plates were incubated at 37°C in 5% CO_2; liquid cultures (sBHI broth) were incubated at 35°C in air and shaken at 200 rpm.

2.2.4 Biotyping

H. influenzae isolates were biotyped using the API 10S strip as recommended by Mehtar and Afshar (1983). The API strips were inoculated with H. influenzae isolates suspended in serum broth.

2.2.5 Counterimmunoelectrophoresis (Myhre, 1974)

Reagents

Sodium Barbital Buffer (Sigma Cat No B6632) was used at pH 8,2.

Agarose (Sigma A-6013). A 1% solution was prepared in sodium barbital buffer and dissolved by boiling briefly.

Preparation of Slides

Ordinary microscope slides (2,5cm by 7,5cm) were overlaid with molten 1% agar and the agarose allowed to solidify at room temperature for 1 hour. Using a template, wells 3mm in diameter were punched out of the agarose. Wells were 5mm apart and 2mm from the adjacent pair (Fig 2.1).

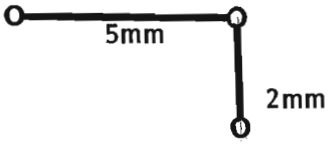

Figure 2.1 : Diagram showing placement of wells in CIE (not to scale)

Procedure

One mℓ of an overnight broth culture of H. influenzae grown in supplemented Brain heart infusion broth was centrifuged at 6 000rpm for 2 minutes. The supernatant was diluted 1:50 in phosphate buffered saline; 4uℓ of the diluted supernatant was placed in wells of the agarose (cathode side). 4 uℓ of

appropriate antiserum (Wellcome) was loaded in the anodal well. Whatman No 1 filter paper wicks soaked in sodium barbital buffer were used to complete the circuit between anode and cathode.

Electrophoresis was carried out at 35V/cm (200V maximum) for 60 minutes. Gels could be read immediately or after 24h at 4°C, using a viewing box (Precision Viewer, Hyland). Appropriate control serotypes (a-f) were included in each run. A positive result was indicated by an obvious line of precipitation which was absent for negative strains.

2.2.6 Immunoprecipitation (Omland, 1978).

Agarose slides were prepared as described above, and wells were cut using a circular template (Fig 2.2). The central well was filled with appropriate antiserum. Supernatant from overnight cultures was loaded in the surrounding wells.

Figure 2.2 : Template for Immunoprecipitation - Actual size

2.2.7 Minimum Inhibitory Concentrations (Emerson et al, 1975)

The agar plate dilution method was used.

Antibiotics

The following antibiotics were tested:

ampicillin (A) - Sigma Chemical Company

chloramphenicol (C) - Aldrich Chemical Company

tetracycline (T) - Sigma " "

streptomycin (Sm) - Sigma " "

rifampicin (R) - Sigma " "

cefotaxime (ctx) - Sigma " "

Culture Media

sBHI agar was prepared as described previously and kept molten at 55°C. Freshly prepared , appropriately diluted antibiotic solutions were added to the molten agar immediately prior to pouring the plates. All plates were stored at 4°C and were used within one week of being prepared.

Testing Procedure

All strains were grown in sBHI broth at 35°C with shaking. Upon reaching an optical density (O.D) of 0,6 at 600nm the cultures were put on ice. Cultures were centrifuged at 10 000rpm for 15 min at room temperature and the pellet resuspended in 2mℓ phosphate-buffered saline (pH 7,4) with 0,1% gelatin (PBSG). An appropriate dilution of the culture was transferred via a Steer's replicator so that 10^4-10^5 colony forming units were delivered per spot. Antibiotic-free plates were inoculated at the beginning and end of each set of antibiotic plates. Colony counts were determined on antibiotic-free medium. Ampicillin sensitive and resistant strains were included as controls. Plates were incubated for 18-24h at 37°C in 5% CO_2. The MIC was defined as the lowest concentration

of the antibiotic which inhibited visible growth of the inoculum. Strains were defined as sensitive, intermediate or resistant based on the following National Committee for Clinical Laboratory Standards (NCCLS 1986) guidelines

Ampicillin MIC	$\leqslant$2mg/ℓ	=	sensitive
	$\geqslant$4mg/ℓ	=	resistant
Chloramphenicol	$\leqslant$4mg/ℓ	=	sensitive
	$\geqslant$8mg/ℓ	=	resistant
Rifampicin	$\leqslant$1mg/ℓ	=	sensitive
	2mg/ℓ	=	intermediate
	$\geqslant$4mg/ℓ	=	resistant
Tetracycline	$\leqslant$4mg/ℓ	=	sensitive
	$\geqslant$8mg/ℓ	=	resistant
Streptomycin	$\leqslant$4mg/ℓ	=	sensitive
	$\geqslant$8mg/ℓ	=	resistant
Cefotaxime	$\leqslant$8mg/ℓ	=	sensitive
	16-32mg/ℓ	=	intermediate
	$>$32mg/ℓ	=	resistant

The guidelines for chloramphenicol and rifampicin are based on more recent NCCLS guidelines (Second Informational Supplement for Interpretation of Susceptibility Tests, M100-S2, 1987 - cited by Jorgensen et al, 1987).

2.2.8 Filter Matings (Roberts et al, 1980)

Donor and recipient strains of H. influenzae were grown in sBHI

broth to give a final count of 10^8cfu/mℓ (OD 0,6 at A600nm). Equal volumes (0,5mℓ) of the donor (10^8cfu/mℓ) and recipient strains (10^5cfu/mℓ) were filtered through an 0,22um millipore filter. The filter was removed with sterile forceps and incubated overnight on an sBHI plate and the resultant growth was then rinsed off in 0,5mℓ of sBHI broth; 0,1mℓ aliquots of ten-fold dilutions (10^{-1}-10^{-5}) of the sBHI broth were then spread on selective antibiotic containing plates (chloramphenicol 5ug/mℓ, erythromycin 20ug/mℓ and streptomycin 250ug/mℓ). Plates were incubated in CO_2 for 18-24h at 37°C. Thirty putative transconjugants were tooth-picked onto sBHI plates containing ampicillin and chloramphenicol separately. Non-type b (R906) colonies growing on these plates were considered transconjugants and investigated further for the presence of conjugative plasmids.

2.2.9 Plasmid Studies

2.2.9.1 Rapid Miniscreen (Kado and Liu, 1981)

Materials

Lysis Solution

Tris base 1,21gm (50mM)

Sodium dodecyl sulfate (SDS) 6,0gm (3%)

2N NaOH (fresh) - titrate to pH 12,4

TE 10mm Tris base, pH 8, 1mM EDTA

Phenol/chloroform (1:1, v/v)

Phenol must be distilled and tris-saturated (Maniatis, Fritsch and Sanbrook, 1982)

Chloroform is a mixture of chloroform (24) : isoamyl alcohol(1)

Tracking dye : 20% Ficoll 400 000; 0,7% bromophenol blue

Procedure

Using a sterile tooth-pick a small inoculum from an overnight sBHI plate containing 4ug/mℓ chloramphenicol was resuspended in 40uℓ TE. Addition of 100uℓ of lysis buffer was followed by incubation in a 65°C water bath for 45 minutes. 160uℓ of phenol/chloroform was added to the mixture and after gentle mixing, it was centrifuged in a microfuge at 4°C. 30uℓ of the supernatant was transferred to an Eppendorf tube and mixed with 6uℓ of tracking dye. The mixture was loaded on a 0,7% agarose (Seakem) gel and electrophoresed at 100V for 4½ hours. E. coli strains RP4, RIA and Sa were used as controls

2.2.9.2 Large Scale Plasmid Isolation

(Portnoy, Moseley and Falkow, 1981)

Solutions

TE and Lysis buffer were prepared as described previously

2M Tris base pH 7

5M NaCl (sodium chloride)

Procedure

Transconjugants were grown overnight on sBHI plates supplemented with 4ug/mℓ chloramphenicol. The growth of 2 plates was resuspended in 1mℓ of TE. To the suspension, 10mℓ of lysis buffer was added and the mixture incubated at 60°C for 20 minutes. To this was added 0,6uℓ of 2M Tris (pH 7) mixed and followed by the addition of 2,6mℓ 5M NaCl. The solution was put on ice for one

hour, then centrifuged at 20 000rpm for 20 minutes at 4°C. The supernatant was decanted to another tube and 7,5mℓ of cold isopropanol (-20°C) added and frozen (-20°C) for one hour. This was then centrifuged at 3 500rpm for 5 minutes at 4°C, the pellet dried and resuspended in 3,8 uℓ TE.

2.2.10 Centrifugation in Cesium Chloride - Ethidium Bromide Gradients (Maniatis, Fritsch and Sambrook, 1982)

Sufficient cesium chloride (CeCl) was added to the solution to yield a density of 1,58 to 1,60. The solution was transferred to a quick seal centrifuge tube and ethidium bromide added to give a final concentration of 600ug/mℓ.

Centrifugation was carried out in a fixed angle rotor at 60 000rpm for 18h. The lower band visible under long wave ultraviolet light was harvested via a hypodermic needle (18 gauge). The ethidium bromide was removed by extracting several times with NaCl saturated isopropanol.

Plasmid DNA was precipitated as follows:
To 100uℓ of the CeCl DNA mixture was added 800uℓ absolute ethanol and 300uℓ double distilled water. This was put on ice for 30 minutes. The solution was centrifuged in an Eppendorf centrifuge for 30 minutes at 4°C; the ethanol poured off and the pellet washed in 70% ethanol. This was then spun for a further 15 minutes at 4°C, the pellet dried and resuspended in 10uℓ TE. The concentration of DNA was measured in a fluorometer (Hoefer

Scientific Instruments) and appropriate volumes mixed with tracking dye were electrophoresed as described previously.

2.2.11 Restriction Enzyme Digestion (Maniatis, Fritsch and Sambrook, 1982)

EcoR1, PsTI and Sst1 digests of selected plasmids were prepared as follows:
250 ng of DNA was digested with 1uℓ of enzyme in a volume of 10uℓ at 37°C for 1h. The reaction was stopped by addition of tracking dye and the mixture electrophoresed on a 0,5% agarose gel at 20V for 14-16h.

Restriction enzymes were purchased from Bethesda Research Laboratories, USA.

2.2.12 Outer Membrane Proteins (Barenkamp, Munson and Granoff, 1981)

Ten millilitres of an overnight culture was added to 200mℓ SBHI broth and incubated at 35°C to an O.D of 0,8 at 600nm. Cells were harvested by spinning at 10 000rpm at 4°C for 10 minutes, washed with 0,05M Tris (pH 7,8), and respun at 10 000rpm for 10 minutes. The pellet was resuspended in 8mℓ of 0,05M Tris-2mM $MgCl_2$ (pH 7,8) and sonicated for 3 minutes (in 15 second bursts). After centrifuging at 10 000rpm for 5 minutes at room temperature, the supernatant was spun at 50 000rpm for 60 minutes at 4°C. The pellet was resuspended in 0,5mℓ, 0,01M Hepes pH 7,4 with 18, 20

and 26 gauge syringes and mixed with 7mℓ of 0,01M Hepes (pH 7,4) - 2% Triton X-100. After 20 minutes at room temperature, the mixture was spun at 50 000rpm for 1h at 4°C. Using 18, 20 and 26 gauge needles, the pellet was resuspended in 0,5mℓ Hepes, and the triton extraction repeated. The pellet was resuspended in 0,01M Hepes (pH 7,4) and frozen (approximately 0,1-0,2mℓ).

Protein separation was by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Laemmli (1970)) and gels were stained with Coomassie brilliant blue R (Davis, Dibner and Battey, 1986).

CHAPTER 3

3.1 RESULTS

3.1.1 Clinical Isolates

A total of 36 strains were studied. These had been isolated from the following body sites : blood (26 isolates), CSF (3 isolates), sputum (2 isolates), eye and joint fluid (1 isolate each). For 3 isolates the body site was not indicated.

3.1.2 Biotyping

Of 24 H. influenzae isolates tested 20 (83,3%) belonged to biotype II. Of the remaining 4 isolates 3 (12,5%) were biotype I and 1 (4,2%) belonged to biotype III. The 3 biotype I isolates were all from patients at King Edward Hospital. Hence 95,2% (20/21) of isolates from Clairwood Hospital belonged to biotype II.

3.1.3 Serotyping

Twenty clinical isolates of H. influenzae which had been reported as type b by slide agglutination, were checked both by CIE and latex agglutination. Seventeen strains (85%) were confirmed as type b by CIE while 18 strains (90%) were positive by slide agglutination. The one additional strain that tested weakly positive by slide agglutination was subsequently shown (by OMP typing) to be a nontypeable strain. Of the 3 strains that were negative by CIE,

2 strains tested positive with the type b antisera by the immuno-precipitation technique using boiled culture supernatant. Overall, therefore, 1 strain (5%) which had been reported as type b was nontypeable. This strain had been isolated from blood.

3.1.4 Minimum Inhibitory Concentrations (MICs)

The MICs of 36 strains were determined against 6 antibiotics.

For ampicillin the MIC values ranged from 0,5mg/ℓ to 64mg/ℓ. For 33 strains (91,7%) the MIC value was 16mg/ℓ. One beta-lactamase negative strain (MIC = 0,5mg/ℓ) had been reported as resistant by the Stokes disc-diffusion method.

Thirty-three strains (91,7%) were resistant to chloramphenicol, with MICs of 8mg/ℓ. The MIC values for chloramphenicol ranged between 0,5mg/ℓ and 15mg/ℓ.

MICs for tetracycline ranged between 0,5mg/ℓ and 16mg/ℓ; 33 strains (91,7%) exhibited tetracyline MICs of 8mg/ℓ and were considered resistant.

Six of 36 strains (16,7%) were found to have MIC values of 4mg/ℓ for rifampicin (range 0,5mg/ℓ to 16mg/ℓ) and were considered to be resistant to this antibiotic.

Only 4 strains (11,1%) were sensitive to streptomycin (MIC: 8mg/ℓ), while all strains were sensitive to cefotaxime with

MICs 0,06mg/ℓ (range 0,015-0,06mg/ℓ). The antibiotic resistance patterns to the 6 antibiotics tested is summarised in Table 3.1.

TABLE 3.1

Antibiotic Resistance Patterns of 36 strains of H. influenzae to 6 Antibiotics

Resistance*	Number of Resistant strains (%)
A, C, T, Sm and R	5 (13,9)
A, C, T and Sm	24 (66,7)
A, T, Sm and R)	
A, C and T)	
A, C and Sm)	
A and T)	1 each (2,8)
A and Sm)	
A)	
C)	
TOTAL	36 (100)

* Strains resistant to ampicillin (A), chloramphenicol (C), tetracycline (T), streptomycin (Sm) and rifampicin (R).

3.1.5 Filter Matings

Filter mating done on 20 strains eventually yielded transconjugants on 10. In general, matings had to be attempted at least twice and for some strains up to 4 times before transconjugants appeared.

3.1.6 Plasmid Studies

Ten transconjugants were analysed with respect to their plasmid content. All were shown to harbour a single plasmid. The migration of intact plasmids, prepared by the rapid method (Kado and Liu, 1981), in a 0,7% agarose gel is shown in Plate 3.1. All plasmids were calculated to have a molecular weight of 41 MDa (Fig 3.1).

The restriction enzyme patterns generated by Eco R1 and Sac I are depicted in Plates 3.2 and 3.3. All 10 strains had very similar restriction patterns when digested with each restriction enzyme.

3.1.7 Outer Membrane Proteins (OMPs)

A total of 19 strains were analysed with respect to their OMP profiles. A representative SDS-PAGE (Laemmli) gel is shown in Plate 3.4. The type b strains could be categorised into two groups designated 2L and 2L′ Strains 1190 and 129S (Lanes 2 and 6) are representative of strains falling into the former category. The remaining type b strains (14) fell into the 2L′ category. These 2L′ strains differed from the 2L strain in possessing a 45K protein vs a 46 K protein for the 2L strains and having a double band in the region of the 30K protein. Three nontypeable strains (represented by strain 1345- Lane 3), exhibited OMP profiles which were distinctly different to those of the type b isolates.

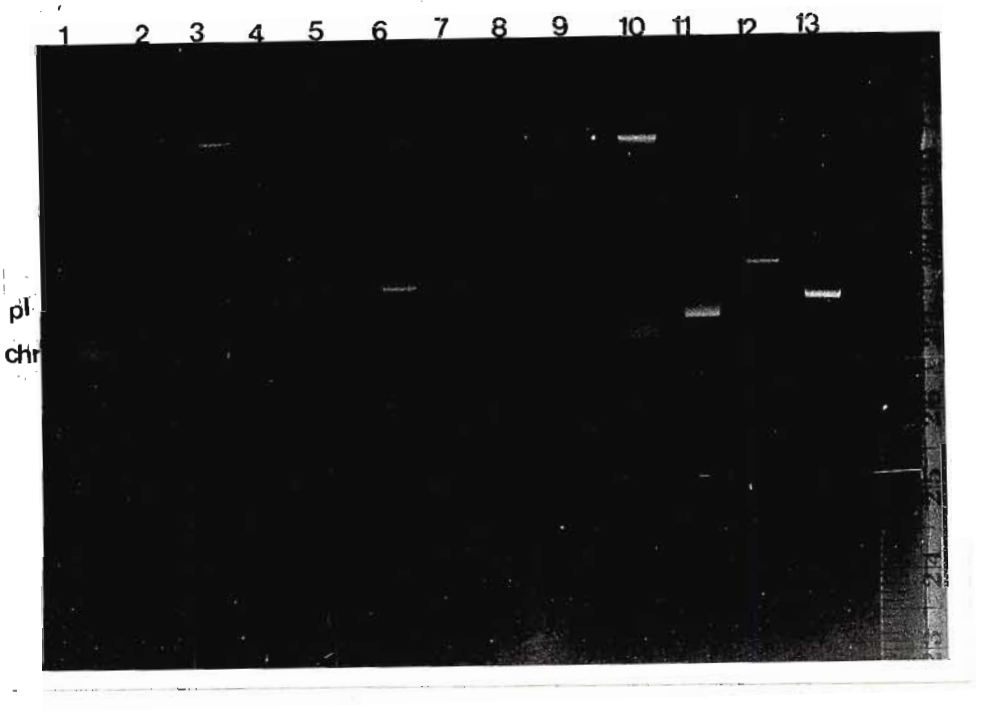

PLATE 3.1

Agarose gel electrophoresis of DNA of multiply resistant H. influenzae. Lanes 1 and 3-10 prepared from type b strains. Lane 2 was prepared from a nontypeable strain. Lanes 1-4 were from strains isolated in 1986, Lane 5 was from a strain isolated in 1987, Lanes 6-10 were isolates from 1988. Lanes 11, 12 and 13 contain E. coli plasmids of known molecular weights (25 MDa, 62 MDa and 34 MDa respectively).

(chr = chromosomal DNA, pl = plasmid DNA.

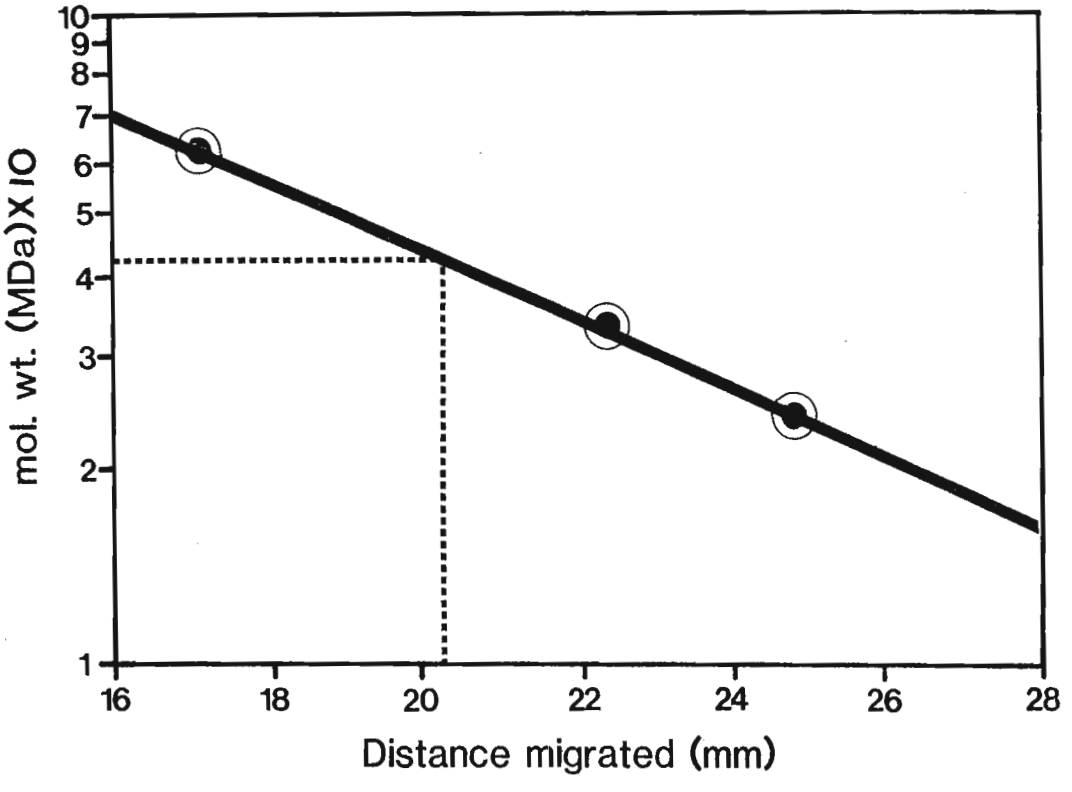

FIGURE 3.1

Semi-log graph to determine molecular weights of plasmids. Dotted line indicates molecular weight of plasmids isolated from transconjugants.

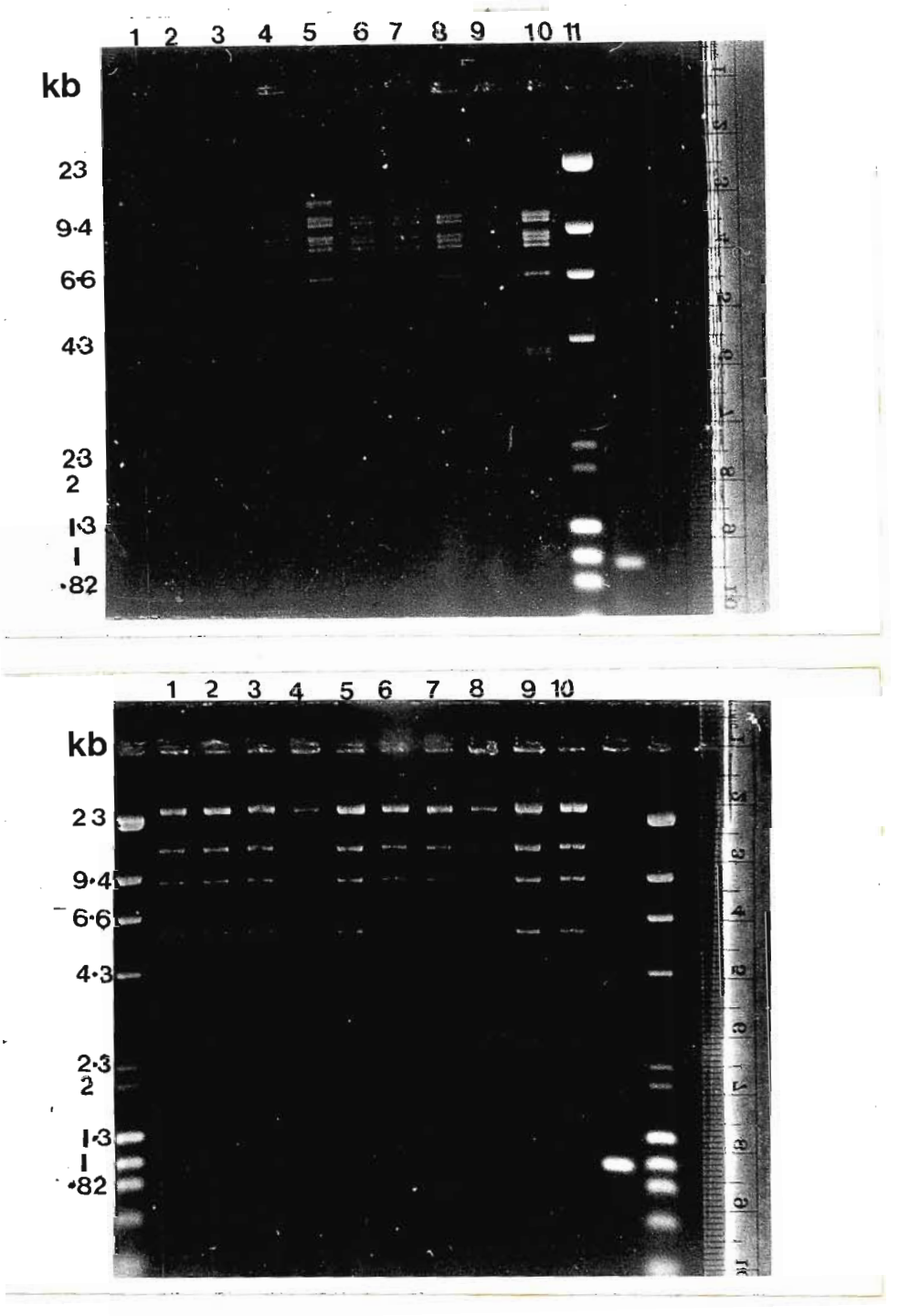

PLATE 3.2 and 3.3

Agarose gel electrophoresis of restriction endonuclease digest of 10 cesium chloride-purified plasmids : Eco R1 (top), SstI (bottom). Lane Std contains molecular weight standards of DNA digested with Hind III and ØX174 digested with Hae III. Lanes 1-10; plasmids from strains 1-10 respectively (See legend to Plate 3.1).

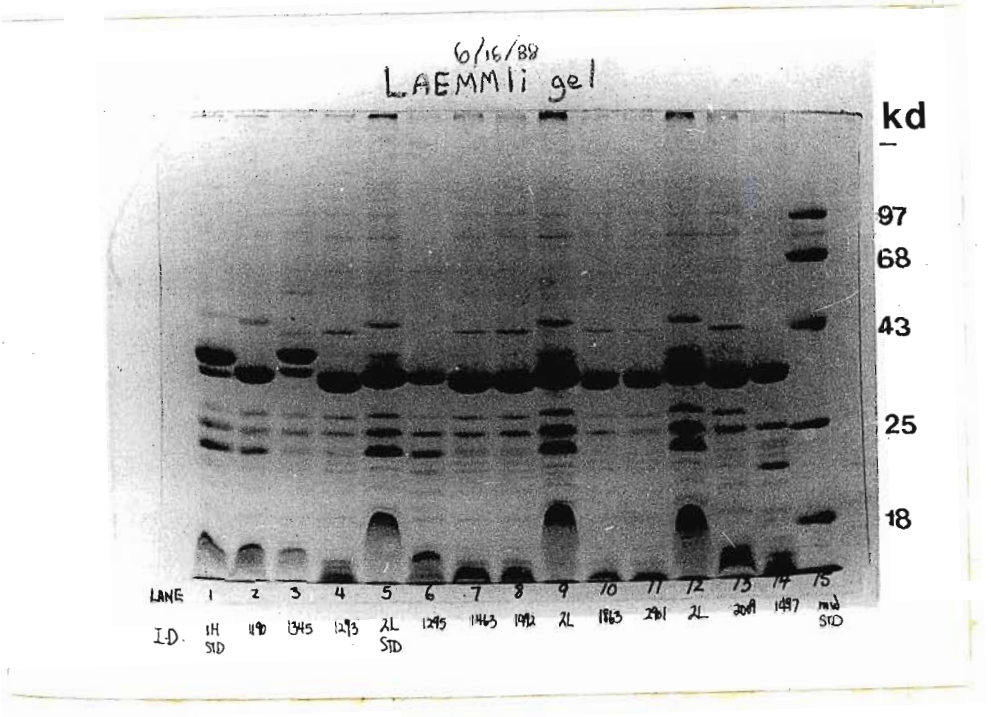

PLATE 3.4

SDS-PAGE of OMP preparations of 10 representative strains of resistant H. influenzae. Strains 1190 and 1295 appear identical to the control strain 2L; strain 1345 is a nontypeable strain, the rest of the isolates fall into the category designated 2L'. Lanes labelled 2' and IH (STD) are control strains while lanes labelled MW STD contain proteins of known molecular mass.

The 2L strains (1190 and 1295) were isolated early in 1986. All clinical isolates from mid-1986 to mid-1988 were of the 2L′ OMP profile.

CHAPTER 4

4.1 DISCUSSION

Beginning in April 1986, multi-resistant H. influenzae causing invasive disease have been increasingly isolated from the paediatric wards of a convalescent hospital (Clairwood Hospital) in Durban. Epidemiological studies done in 1986 (Hoosen, Kharsany and van den Ende, 1987) and 1987 (Peer et al), suggested the nosocomial acquisition of infection in these patients. Typing the multiresistant H. influenzae both by conventional microbiological methods (sero-typing, biotyping and antimicrobial susceptibility patterns) and the newer techniques of OMP profile analysis and plasmid isolation and fingerprinting are logical approaches to determining similarities or differences between multiresistant disease causing isolates obtained during this period (April 1986 up to and including May 1988).

Both by capsular typing and biotyping all the blood and CSF isolates appeared to be identical. This finding is not unexpected and the limitations of these phenotypes as epidemiological tools was discussed in Chapter 1.

As far as antimicrobial susceptibility patterns are concerned, the majority of isolates appeared to be similar both with regard to the MIC values for individual antibiotics as well as the susceptibility pattern (Table 3.1). The only other centre in the world where large numbers of multiresistant H. influenzae type b have been reported is Barcelona, Spain (Campos and Garcia-Tornel, 1987;

Campos et al, 1986, Campos, Garcia-Tornel and Sanfeliu, 1984). Comparing the MICs and antibiotic susceptibility patterns of local strains with the Spanish isolates reveals very little apparent differences between these two groups. The only striking difference is the higher prevalence of rifampicin-resistant strains present locally. Whereas only 1 of 83 (1,2%) ampicillin-chloramphenicol-resistant H. influenzae isolates from Spain were resistant to rifampicin; 16,7% (6/36) of local isolates were rifampicin-resistant. It is possible that this is linked to selective pressure imposed by the increase use of rifampicin locally in the treatment of pulmonary tuberculosis in South Africa.

The epidemiological applications of OMP profiles has been alluded to in Chapter 1. In the study on the multi-resistant H. influenzae from Spain, 3 different OMP profiles were found and when used together with plasmid restriction patterns, provided useful epidemiological data (Campos et al, 1987). Amongst local strains, 14 of 16 (87,5%) H. influenzae type b isolates studied fell into the category designated 2L'. Interestingly, the remaining 2 isolates which were designated 2L were isolated early in 1986 when only one case of multi-resistant H. influenzae had been reported, and one of these strains was resistant to ampicillin alone. This suggests that OMP strains, other than 2L` are probably present in the community, but that the 2L' OMP strains is endemic at Clairwood Hospital.

The applications of plasmid analysis to the study of disease outbreaks and the transmission of antibiotic resistance have been

mostly utilised for studying resistance plasmids in the Enterobacteriaceae (Wachsmuth 1986, John and Twitly, 1986). The application of molecular epidemiology to the study of large numbers of H. influenzae has been limited to 2 studies (Campos et al, 1987; Mendelman et al, 1985). The study by Mendelman et al was confined to H. influenzae type b strains resistant to ampicillin only. These isolates had been isolated from different regions of Alaska. Among 29 invasive isolates examined, 55% had a 40 MDa plasmid, 14% had a 3 MDa plasmid and 31% had no plasmid. Restriction enzyme digestion with Pst I and Sac I revealed 5 distinct plasmid patterns. The authors concluded that despite variable OMP profiles the plasmids showed significant DNA homology.

Campos et al (1987), studied the epidemiology of carriage and infection with multiresistant H. influenzae type b in day care centres. Plasmids with a mass of either 45 MDa or 52 MDa were reported. Restriction endonuclease analysis of 11 plasmids revealed 3 different patterns, which nevertheless were markedly similar.

In the present study, the plasmids from different isolates were all the same size. The majority of plasmids had identical restriction enzyme patterns; with only minor differences being apparent in one plasmid.

By combining OMP profiles and plasmid restriction endonuclease patterns, Campos et al (1987), were able to demonstrate the presence of more than one strain and plasmid type. In the present study however the multiresistant strains isolated from Clairwood

Hospital over a 26 month period appear to be similar by multiple criteria including serotyping, biotyping OMP profile and plasmid analysis.

In conclusion, several unique features regarding H. influenzae disease in Durban need to be stressed. Firstly, this would be the largest number of nosocomial infections due to H. influenzae type b reported to date. Secondly, no report of such a prolonged duration of nosocomial H. influenzae infections (26 months) has appeared in the literature. Thirdly, the frightening reality of endemic multiresistant H. influenzae type b infections has now materialised in Durban and is likely to spread to other centres in South Africa. Lastly, we can expect more cases of community-acquired invasive infections due to multiresistant H. influenzae type b to present at hospital out-patient departments. The microbiology laboratory should be alert to identifying such strains as soon as possible and the paediatrician will, in the near future, need to reconsider his choice of empiric antimicrobial therapy, particularly in the febrile child with a possible diagnosis of meningitis.

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