A HOSPITAL OUTBREAK

OF MULTIRESISTANT

HAEMOPHILUS INFLUENZAE TYPE B

by

KALAWATHIE SATTAAR

Submitted in fulfilment of the requirements for the degree of:

MASTER OF MEDICAL SCIENCE

In the
Department of Medical Microbiology
University of Natal
Durban

1996
Following an outbreak of multi-resistant *Haemophilus influenzae* type b (Hib) infections in a tuberculosis hospital, this study was undertaken to determine carriage of Hib in 2 paediatric wards; to characterise all isolates of Hib, determine their antimicrobial susceptibility profile and the antibody response of the children to a conjugate vaccine.

Prior to and one month after immunisation, oro- and nasopharyngeal swab specimens as well as venous blood were collected from each child. Isolates were tested for β-lactamase and chloramphenicol acetyltransferase (CAT) production, their MIC’s determined by the agar dilution method and characterisation of Hib isolates was performed by biotyping and analysis of outer membrane protein (OMP) profiles. An ELISA was also developed to determine serum antibody levels to polyribosyl-ribitol-phosphate (PRP), the capsular polysaccharide of Hib.

The study population comprised a total of 135 children who had been hospitalised for treatment for tuberculosis. The patients were aged 4 months to 14 years with a median of 37.5 months. During the study period, none of the children developed invasive Hib disease.

The overall carriage rate of Hib increased from 38% (51/135) before immunisation to 62% (84/135) after immunisation (P < 0.01). Oropharyngeal sampling was superior to nasopharyngeal for detecting carriers of Hib. Biotyping of isolates revealed that 33% (18/51) were biotype II, 25% (12/51) biotype III, 18% (9/51) biotype V and 16% (8/51) biotype I. Isolates of invasive Hib disease which occurred prior to the study were biotype II. OMP analysis revealed greater diversity of subtypes after immunisation. Multi-antibiotic resistant strains from cases of invasive disease were subtype 1H as were two multi-resistant carriage strains.
The production of \(\beta\)-lactamase and CAT was detected in 27% and 4% of pre- and post-immunisation isolates respectively. Resistance to rifampicin increased from 92% to 100% after immunisation. All isolates were uniformly susceptible to azithromycin, cefotaxime, ceftriaxone and aztreonam and there were no differences in \(\text{MIC}_{90}\) values for \(\beta\)-lactamase producing and non-producing strains.

Prior to immunisation, only 12% (16) of the children had the expected antibody level of \(\geq 0.15\ \mu g/ml\). After immunisation, 34% (45) of patients increased their antibody levels to \(\geq 1.0\ \mu g/ml\). There was no statistical difference between the mean antibody concentrations of patients who were colonised by Hib and those who were not \((p = 0.58)\).

The vaccine did not reduce carriage of Hib in this study population of children being treated for tuberculosis and the immune response to the vaccine was not optimal. Production of \(\beta\)-lactamase and the prevalence of rifampicin resistance has implications for treatment and chemoprophylaxis in this population. OMP analysis showed a diversity of types. Multi-resistant strains causing invasive disease had the same OMP type as some multi-resistant strains which colonised the children.
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 dissertation was carried out in: the Department of Medical Microbiology, Faculty of Medicine, University of Natal under the supervision of Dr. AA Hoosen and Prof. AW Sturm; The Natal Blood Transfusion Services under the supervision of Dr. M. Bubb; the Department of Microbiology, University of Amsterdam under the supervision of Dr. L van Alphen.

KALAWATHIE SATTAR

1996
ACKNOWLEDGEMENTS

I would like to extend my sincere thanks and gratitude to:

Professor AW Sturm from the Department of Medical Microbiology for his expert advice, constructive criticism and guidance; and my supervisor, Dr. Anwar Hoosen from the Department of Medical Microbiology for his constant encouragement, constructive criticism, expert advice and in particular, for his generosity of spirit and guidance and teaching during the preparation of this dissertation.

Dr Vos (Superintendent) and Dr Sheila Bamber of King George V Hospital for assisting with access to patients.

Ms Jackie Utterson and Mr Abdul Naeem Sattar from King George V Hospital for their assistance with the preliminary Microbiology culture work.

Dr Martin Bubb and Mr Raymond Parkinson of the Natal Blood Transfusion Services for their assistance with the development of the ELISA.

Dr Loek van Alphen and Mr Paul Eijk of the Department of Microbiology, University of Amsterdam for their assistance and supply of reagents and methodology for outer membrane protein subtyping of Hib strains.

Mr Robert Vaughan-Evans from the Department of Medical Sciences, M.L. Sultan Technikon, for his invaluable moral support and advice.

Mr Logan Pillay from the Department of Medical Microbiology for his assistance with the preparation of media.
Mrs Barthi Odhav and Mrs Dimes Naidoo for their encouragement, friendship and support.

Dr Porter Anderson of Rochester University, New York, for the supply of the HbO-HA antigen as well as control and reference sera.

The Foundation for Research and Development as well as the M L Sultan Technikon for making funds available.

My husband Naeem, and children Shakira, Shamira and Tasmira for love encouragement, patience and tolerance; my family and friends for their support; God Almighty for making it possible.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>i</td>
</tr>
<tr>
<td>PREFACE</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF PLATES</td>
<td>xiii</td>
</tr>
</tbody>
</table>

## CHAPTER 1

1.0 INTRODUCTION 1

## CHAPTER 2

2.0 LITERATURE REVIEW 4

2.1 EPIDEMIOLOGY 4

2.1.1 Spectrum of disease 4

2.1.1.1 In developed countries 5

2.1.1.2 In developing countries 7

2.1.2 Risk factors 10

2.1.2.1 Factors related to increased exposure 11

2.1.2.2 Factors increasing susceptibility to invasive disease 17

2.2 CARRIAGE 20

2.2.1 Factors affecting colonisation 21

2.2.1.1 Host factors 21

2.2.1.2 Bacterial factors 24

2.2.2 Oropharyngeal vs nasopharyngeal carriage 25

2.3 IDENTIFICATION AND CHARACTERISATION 26

2.3.1 General Microbiology 26

2.3.2 Serotyping 27

2.3.3 Biotyping 30
2.3.4 Outer membrane proteins (OMP) 32
2.3.4.1 Worldwide distribution 35
2.3.4.2 OMP subtypes as disease markers 37
2.4 ANTIMICROBIAL SUSCEPTIBILITY 39
2.4.1 Susceptibility testing 39
2.4.2 Antibiotic resistance 41
2.4.2.1 Ampicillin resistance 42
2.4.2.2 Chloramphenicol resistance 43
2.4.2.3 Tetracycline, Trimethoprim, sulphamethoxazole and rifampicin resistance 45
2.4.2.4 Multiple antibiotic resistance 46
2.4.3 Susceptibility to other antibiotics 48
2.4.3.1 Cephalosporins 48
2.4.3.2 Quinolones 49
2.4.3.3 Macrolides 49
2.5 IMMUNISATION 49
2.5.1 Vaccine development 49
2.5.1.1 Polysaccharide vaccine 49
2.5.1.2 Conjugate vaccines 51
2.5.2 Protective efficacy of conjugate vaccines 52
2.5.3 Effect of immunisation on incidence of disease 55
2.5.4 Antibody response in compromised hosts 56
2.5.5 Vaccine efficacy in developing countries 57
2.5.6 Effect of conjugate vaccines on carriage 58

CHAPTER 3
3.0 MATERIALS AND METHODS 61
3.1 ISOLATION AND IDENTIFICATION OF STRAINS 61
3.1.1 Sampling and immunisation 61
3.1.2 Identification of isolates 62
3.2 CHARACTERISATION OF ISOLATES 62
3.2.1 Serotyping of isolates 62
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.2</td>
<td>Biotyping</td>
<td>62</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Outer Membrane Protein (OMP) analysis</td>
<td>63</td>
</tr>
<tr>
<td>3.3</td>
<td>ANTIMICROBIAL SUSCEPTIBILITY TESTING</td>
<td>65</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Enzyme tests</td>
<td>65</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Disc susceptibility tests</td>
<td>65</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Test for Minimum Inhibitory Concentration (MIC)</td>
<td>65</td>
</tr>
<tr>
<td>3.3.3.1</td>
<td>Bacterial strains</td>
<td>65</td>
</tr>
<tr>
<td>3.3.3.2</td>
<td>Antimicrobial agents</td>
<td>66</td>
</tr>
<tr>
<td>3.3.3.3</td>
<td>Culture media</td>
<td>66</td>
</tr>
<tr>
<td>3.3.3.4</td>
<td>Inoculum preparation</td>
<td>66</td>
</tr>
<tr>
<td>3.3.3.5</td>
<td>MIC determination</td>
<td>67</td>
</tr>
<tr>
<td>3.4</td>
<td>ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)</td>
<td>68</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Preparation of antigen and reference sera</td>
<td>68</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Determination of antigen concentration for coating of plates</td>
<td>68</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Determination of dilution required for control serum and patients' sera</td>
<td>69</td>
</tr>
<tr>
<td>3.4.4</td>
<td>Determination of concentration of secondary standard</td>
<td>70</td>
</tr>
<tr>
<td>3.4.5</td>
<td>Determination of total antibody concentration of all patients' sera</td>
<td>70</td>
</tr>
</tbody>
</table>

CHAPTER 4

4.0 RESULTS | 71 |
4.1 DEMOGRAPHIC DATA | 71 |
4.2 CLINICAL DATA | 71 |
4.3 ORO- AND NASOPHARYNGEAL CARRIAGE | 72 |
4.4 CHARACTERISATION OF ISOLATES | 75 |
4.4.1 Serotype distribution | 75 |
4.4.2 Biotypes | 75 |
4.5 CHARACTERISATION OF ISOLATES FROM PATIENTS WITH INVASIVE DISEASE | 80 |
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6</td>
<td><strong>ANTIMICROBIAL SUSCEPTIBILITY TESTS</strong></td>
<td>80</td>
</tr>
<tr>
<td>4.6.1</td>
<td>Tests for enzyme production</td>
<td>80</td>
</tr>
<tr>
<td>4.6.2</td>
<td>Disc susceptibility tests (Kirby-Bauer method)</td>
<td>83</td>
</tr>
<tr>
<td>4.6.3</td>
<td>Minimum inhibitory concentration (MIC)</td>
<td>83</td>
</tr>
<tr>
<td>4.7</td>
<td><strong>OUTER MEMBRANE PROTEIN (OMP) ANALYSIS</strong></td>
<td>94</td>
</tr>
<tr>
<td>4.8</td>
<td><strong>ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)</strong></td>
<td>100</td>
</tr>
<tr>
<td>4.8.1</td>
<td>Concentration of antigen for coating of plates</td>
<td>100</td>
</tr>
<tr>
<td>4.8.2</td>
<td>Dilution of control serum and patients’ serum</td>
<td>100</td>
</tr>
<tr>
<td>4.8.3</td>
<td>Concentration of internal standard</td>
<td>100</td>
</tr>
<tr>
<td>4.8.4</td>
<td>Concentration of antibody in patients’ serum samples</td>
<td>100</td>
</tr>
<tr>
<td>4.9</td>
<td><strong>CORRELATION BETWEEN CARRIAGE AND ANTIBODY</strong></td>
<td>104</td>
</tr>
</tbody>
</table>

**CHAPTER 5**

| 5.0     | DISCUSSION                                                          | 107  |

**CHAPTER 6**

| 6.0     | **CONCLUSIONS AND RECOMMENDATIONS**                                | 121  |
| 6.1     | CONCLUSIONS                                                        | 121  |
| 6.2     | RECOMMENDATIONS                                                   | 121  |
| 7.0     | REFERENCES                                                        | 122  |
| 8.0     | APPENDIX                                                          | 142  |
# LIST OF TABLES

| I: | Comparison of rates of *Haemophilus influenzae* meningitis and other invasive disease among children younger than 5 years of age (Vadheim and Ward, 1994) | 6 |
| II: | Age specific annual incidence of *Haemophilus influenzae* meningitis in developed and developing populations of the world (Bijlmer 1994) | 8 |
| III: | Case fatality (CFR) for *Haemophilus influenzae* meningitis in western countries versus developing countries. | 9 |
| IV: | Risk factors for *H.influenzae* type b colonisation, transmission and disease (Takala 1994a) | 11 |
| V: | Comparison of efficacy of oropharyngeal versus nasopharyngeal swabbing for the isolation of *H.Influenzae* type b (N = 31) (Michaels et al, 1976) | 26 |
| VI: | Carbohydrate composition of *H.influenzae* capsules (Mendelman and Smith, 1987) | 29 |
| VII: | Key to the differentiation of biotypes of *H.influenzae* | 30 |
| VIII: | Properties of the major outer membrane proteins of *H.influenzae* (van Alphen et al, 1983b) | 33 |
| IX: | OMP subtype distribution (van Alphen, 1994) expressed as % | 36 |
| XI: | Serum antibody levels in immunised Finnish children (Peltola, 1977) | 50 |
| XII: | *Haemophilus influenzae* type b vaccines in the United States (Murphy et al, 1993a; MMWR,1993) | 52 |
| XIII: | Reasons for variations in vaccine efficacy between developed and developing countries | 57 |
| XIV: | Effect of conjugate vaccines on carriage of *H.influenzae* type b (Takala, 1994b) | 60 |
XV: Antibiotics tested

XVI: Age distribution of patients (N = 135)

XVII: Isolation of *Haemophilus influenzae* from oro- and nasopharynx

XVIII: Serotype distribution of isolates

XIX: Carriage of *H. influenzae* type b by study patients before and after immunisation (N = 135)

XX: Distribution of *Haemophilus influenzae* type b by age

XXI: Biotypes of *Haemophilus influenzae* type b

XXII: β-lactamase (βL+) and chloramphenicol acetyltransferase (CAT+) production by *Haemophilus influenzae*.

XXIII: β-lactamase (βL+) and chloramphenicol acetyltransferase (CAT+) production by *Haemophilus influenzae* type b.

XXIV: Antimicrobial susceptibility profile of pre- and post-immunisation strains of *Haemophilus influenzae* type b using a disc diffusion method (Kirby-Bauer)

XXV: In-vitro susceptibilities of *Haemophilus influenzae* type b (N = 113) to 24 antibiotics

XXVI: In-vitro susceptibilities of β-lactamase and CAT+ producing strains of *H. influenzae* type b (N = 22)

XXVII: Age distribution and antibody response before and after immunisation (N = 133)

XXVIII: Antibody levels in patients before and one month after immunisation

XXIX: Correlation between carriage of Hib and antibody response to the vaccine (N = 133)
LIST OF FIGURES

FIGURE 1: Cumulative % minimum inhibitory concentrations for \textit{H. influenzae} type b to ampicillin, amoxycillin, clavulanic acid, imipenem, rifampicin and aztreonam 87

FIGURE 2: Cumulative % minimum inhibitory concentrations for \textit{H. influenzae} type b to cefazolin, cefuroxime, cefotaxime and ceftriaxone 88

FIGURE 3: Cumulative % minimum inhibitory concentrations for \textit{H. influenzae} type b to chloramphenicol, sulphamethoxazole, trimethoprim, gentamicin and amikacin 89

FIGURE 4: Cumulative % minimum inhibitory concentrations for \textit{H. influenzae} type b to ciprofloxacin, lomefloxacin, pefloxacin and ofloxacin 90

FIGURE 5: Cumulative % minimum inhibitory concentrations for \textit{H. influenzae} type b to erythromycin, azithromycin and roxithromycin 91

FIGURE 6: Cumulative % minimum inhibitory concentrations for \textit{H. influenzae} type b to tetracycline, doxycycline and minocycline 92

FIGURE 7: Antigen coating curve used to determine concentration of antigen required to coat ELISA plates 101
# LIST OF PLATES

<table>
<thead>
<tr>
<th>Plate</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Photograph of major outer membrane proteins after SDS-PAGE.</td>
<td>34</td>
</tr>
<tr>
<td>2</td>
<td>OMP subtypes on SDS-PAGE as described by Barenkamp et al.</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>Photograph of gel after SDS-PAGE showing OMP profiles of strains isolated before and after immunisation.</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>Photograph of gel after SDS-PAGE showing OMP profiles of strains colonising patients after immunisation only.</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>Photograph of gel after SDS-PAGE showing OMP profiles of invasive strains.</td>
<td>98</td>
</tr>
<tr>
<td>6</td>
<td>Photograph of a gel after SDS-PAGE (Amsterdam).</td>
<td>99</td>
</tr>
</tbody>
</table>
1.0 INTRODUCTION

*Haemophilus influenzae* is an important cause of a number of childhood infections, the more serious ones being meningitis and severe lower respiratory tract infection. *H. influenzae* type b (Hib) is the most frequently isolated serotype from clinical specimens. Reports from Europe and the USA indicate that up to one child in two hundred is likely to suffer from invasive Hib disease (Munson *et al.*, 1989; Congress proceedings - Bwibo and Peltola, 1992).

The organism was first isolated by Pfeiffer in 1892 (cited by Munson *et al.*, 1989) and was thought to be the cause of the influenza epidemic in Europe. The discovery of the influenza virus led to the realisation that *H. influenzae* was a secondary invader following viral infection. It was only from the mid 1930’s that *H. influenzae* gained recognition as an important cause of meningitis, epiglottitis, and other serious infections.

The elucidation of the presence of capsulate and non-capsulate strains by Pittman in 1931 (cited by Turk, 1963) and further typing of capsulate strains into six serotypes served to highlight the clinical significance of type b strains. Strains of Hib can be characterised further by biotyping, outer membrane protein profile, multilocus enzyme electrophoresis and analysis of plasmid profiles (van Alphen, 1994).

Adherence of an organism to the mucosa is the first step in the pathogenesis of a number of invasive diseases (Beachey, 1981). With reference to Hib, adherence to the mucosal epithelium of the upper respiratory tract would precede colonisation and this could, by unknown mechanisms, be followed by invasive disease.

In closed populations, carriage of Hib tends to persist for longer periods of time and secondary cases of Hib illness have been recognised among close contacts.
of children with Hib infections viz. day-care centre contacts and long-term care hospital contacts (Glode et al., 1976; Granoff and Daum, 1980).

For the treatment of established Hib infections, the drugs of choice have been ampicillin and chloramphenicol. Reports of strains resistant to ampicillin emerged from 1972 onwards and chloramphenicol resistance was reported in 1976. This led to the use of other antimicrobial agents, notably the cephalosporins, for therapy.

From 1971 onwards, interest in the development of a vaccine against Hib gained momentum. The early vaccines comprised purified polysaccharide of type b strains viz. polyribosylribitol-phosphate (PRP). Efficacy of a PRP- Hib vaccine was investigated in Finland in 1977 and based on the results of this trial, the vaccine was registered in the USA in 1985. However, the PRP vaccine was not effective in children less than 18 months old. This led to investigations into the use of vaccines comprising PRP conjugated to diphtheria toxoid. Subsequently, other conjugate vaccines were developed which resulted in protective immunity in infants who were 2 months old.

An outbreak of multi-antibiotic resistant *Haemophilus influenzae* type b infections occurred in an institution where children with tuberculosis were being treated ie. King George V Hospital, Durban. These strains were resistant to ampicillin, chloramphenicol, erythromycin, tetracycline and rifampicin. During the 6 month period January to June 1993, 44 cases of Hib infection were recorded from the paediatric wards. Of these, 25 isolates (57%) demonstrated multiple antibiotic resistance. During this period, serious infections such as septicaemia and meningitis were recorded in 6 children in the paediatric wards that were studied subsequently.

In an attempt to intervene in the outbreak, a decision was taken to immunise children with the newly registered HibTITRE vaccine (Lederle). At the time, this particular conjugate vaccine was the only one registered for use in South Africa
The aim of this study was to:

a. determine whether immunisation eradicated or reduced mucosal carriage of *Haemophilus influenzae* type b by children in the paediatric wards of King George V TB Hospital, Durban.

b. characterise *Haemophilus influenzae* isolates by biotyping, serotyping and resistance profiles to ampicillin (for β-lactamase production) and chloramphenicol (chloramphenicol acetyltransferase production)

c. determine whether the same Outer Membrane Protein (OMP) types of Hib were prevalent before and after immunisation.

d. determine whether antibiotic resistant strains of *Haemophilus influenzae* type b causing invasive disease were of the same OMP type as those which colonised the mucosa of the children.

e. evaluate the *in vitro* activity of various antimicrobial agents against *Haemophilus influenzae* type b.

f. determine the antibody response of the immunised children to the vaccine by developing an in-house ELISA.
CHAPTER 2

2.0 LITERATURE REVIEW

2.1 EPIDEMIOLOGY

2.1.1 Spectrum of disease

*Haemophilus influenzae* has the ability to cause a wide range of clinical syndromes. Apart from colonisation, this microorganism has proved to be very versatile in overcoming the defence barriers of its host (Bijlmer, 1992).

*Haemophilus influenzae* type b (Hib) causes diverse invasive diseases in children, such as meningitis, epiglottitis, pneumonia, arthritis, osteomyelitis and pericarditis (Todd and Bruhn, 1975; Dajani et al, 1979; Malik, 1995; Fogarty et al, 1995).

Dajani et al (1979) conducted a study over a four year period during which 292 patients with systemic Hib disease were seen at their hospital. Their subsequent report presents a composite picture of systemic Hib disease in Michigan, USA also incorporating an analysis of the less emphasised disease entities. They noted concomitant disease entities in 24 (8.2%) patients viz. meningitis and pneumonia occurred in 10 children, meningitis and arthritis in 2, epiglottitis and pneumonia in 8, arthritis and adjacent osteomyelitis in 3 and cellulitis and adjacent osteomyelitis in 1. One disease entity was detected in the remaining patients. The relative frequencies of different clinical entities due to *H. influenzae* type b infection were meningitis 51.0%, epiglottitis 17.4%, pneumonia 14.6%, arthritis 7.6%, cellulitis 6.0%, bacteraemia 1.9% and osteomyelitis 1.6%. Excluding meningitis, pneumonia was the most common manifestation of Hib bacteraemia.

In a 12 month survey carried out by Todd and Bruhn (1975), they reported the following percentage frequencies for different disease entities; meningitis 54%, pneumonia 14%, bacteraemia 11%, cellulitis 10%, epiglottitis 10%, periorbital
cellulitis 1%, arthritis 1% and thyroglossal duct abscess 1%. Again, pneumonia was the most common manifestation excluding meningitis.

2.1.1.1 In developed countries

In developed countries, prior to the advent of Hib vaccines, Hib disease occurred endemically. Meningitis accounted for more than 50% of all cases of invasive Hib disease. (Todd and Bruhn, 1975; Dajani et al, 1979; Granoff and Basden, 1980). Incidence rates in children less than 5 years of age have decreased dramatically in several populations since 1990, probably due to increased vaccination rates (Vadheim and Ward, 1994). It is not known whether higher rates of disease reported in the United States since the mid 1970s represent a true increase in disease incidence due to changes in risk factors, such as increased use of day-care facilities, or are due to improvements in diagnosis and surveillance.

Most studies from other developed countries in the pre-vaccine era suggest incidence rates substantially lower than in the United States. Vadheim and Ward (1994) summarised the incidence of *Haemophilus influenzae* disease in various developed countries and this data is presented in Table I. They also cite studies which rank Hib as the leading cause of bacterial meningitis in Sweden, Finland, the Netherlands and Canada.

High rates of invasive Hib disease have been reported for selective populations in developed countries such as Navajo Indians and Native Alaskans (Ward et al, 1982; Ward et al, 1986). While these populations reside in developed countries, the living conditions under which they reside closely approximates those found in developing regions of the world.

With the widespread use of Hib conjugate vaccines in infancy, dramatic decreases in Hib disease incidence have been reported. Table I (Vadheim and Ward, 1994) summarises data for the United States as a whole and for seven specific U.S. populations as well as for Helsinki and Finland. In each of these populations, Hib incidence decreased by 60-80% between the late 1980s and 1991. The greatest
decrease in disease incidence was in children less than 2 years of age.

**TABLE I: COMPARISON OF RATES OF HAEMOPHILUS INFLUENZAE MENINGITIS AND OTHER INVASIVE DISEASE AMONG CHILDREN YOUNGER THAN 5 YEARS OF AGE (Vadheim and Ward, 1994)**

<table>
<thead>
<tr>
<th>Geographic area</th>
<th>Study period</th>
<th>Annual incidence of H. influenzae Meningitis</th>
<th>All invasive disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior to infant immunisation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alaska (non-Natives)</td>
<td>1980 - 1982</td>
<td>69</td>
<td>129</td>
</tr>
<tr>
<td>Colorado (6 months)</td>
<td>1981 - 1982</td>
<td>68</td>
<td>112</td>
</tr>
<tr>
<td>Fresno County, California</td>
<td>1976 - 1978</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>Jefferson County</td>
<td>1981 - 1983</td>
<td>62</td>
<td>not available</td>
</tr>
<tr>
<td>Monroe County, New York</td>
<td>1982 - 1983</td>
<td>55</td>
<td>86</td>
</tr>
<tr>
<td>Dallas County, Texas</td>
<td>1982 - 1984</td>
<td>67</td>
<td>109</td>
</tr>
<tr>
<td>Minnesota</td>
<td>1982 - 1984</td>
<td>45</td>
<td>67</td>
</tr>
<tr>
<td>Atlanta, Georgia</td>
<td>1983 - 1984</td>
<td>57</td>
<td>82</td>
</tr>
<tr>
<td>Los Angeles County, California</td>
<td>1983 - 1988</td>
<td>not available</td>
<td>33 - 43</td>
</tr>
<tr>
<td>Sweden</td>
<td>1971 - 1983</td>
<td>27 - 31</td>
<td>not available</td>
</tr>
<tr>
<td>Finland</td>
<td>1976 - 1981</td>
<td>27</td>
<td>41</td>
</tr>
<tr>
<td>Geneva, Switzerland</td>
<td>1976 - 1989</td>
<td>not available</td>
<td>60</td>
</tr>
<tr>
<td>France</td>
<td>1980 - 1989</td>
<td>13</td>
<td>21</td>
</tr>
<tr>
<td>Israel</td>
<td>1988 - 1990</td>
<td>18</td>
<td>34</td>
</tr>
<tr>
<td>Manitoba, Canada</td>
<td>1981 - 1984</td>
<td>32</td>
<td>not available</td>
</tr>
<tr>
<td>The Netherlands</td>
<td>1977 - 1982</td>
<td>22</td>
<td>not available</td>
</tr>
<tr>
<td>Wales</td>
<td>1988 - 1990</td>
<td>22</td>
<td>35</td>
</tr>
<tr>
<td>After infant immunisation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Los Angeles County, California</td>
<td>1991</td>
<td>not available</td>
<td>12.9</td>
</tr>
<tr>
<td>N CA Kaiser, California</td>
<td>1991</td>
<td>not available</td>
<td>5.7</td>
</tr>
<tr>
<td>Connecticut</td>
<td>1991</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Greater Pittsburgh, PA</td>
<td>1991</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Minnesota</td>
<td>1991</td>
<td>not available</td>
<td>11 (7 - 15)</td>
</tr>
<tr>
<td>Dallas, Texas</td>
<td>1991</td>
<td>not available</td>
<td>9 (5 - 14)</td>
</tr>
<tr>
<td>United States</td>
<td>1991</td>
<td>not available</td>
<td>11</td>
</tr>
<tr>
<td>Helsinki, Finland</td>
<td>1990 - 1991</td>
<td>2.9</td>
<td>not available</td>
</tr>
</tbody>
</table>
2.1.1.2 In developing countries

In a study carried out by Bijlmer et al (1990), it was observed that the incidence of *H. influenzae* meningitis in the Gambia, West Africa, was as high as that in the USA and that it had a 10-fold more devastating outcome. Over a 2 year period, 76 children had Hib meningitis with a case-fatality rate of 37% and only fifty-four percent of the patients recovered completely.

Bijlmer (1994) described a high incidence rate for *Haemophilus influenzae* meningitis in 'developing societies' living within the boundaries of 'Western' countries. These societies have a culture distinct from Western culture, a different family structure, live in less affluent areas and are genetically very distinct from Caucasians. He further states that due to a lack of data on incidence from developing countries, this parameter is almost blank as far as the Third World is concerned. However, from the little available data from the Gambia and Senegal, it can be concluded that the incidence is high compared to Western societies. The incidence of Hib meningitis in developing communities living in developed countries and in developing countries is shown in Table II. Bijlmer (1994) also states that much more is known about the case-fatality rates (CFR) in developing countries and that this was very high as illustrated in Table III.
TABLE II: AGE SPECIFIC ANNUAL INCIDENCE OF *HAEMOPHILUS INFLUENZAE* MENINGITIS IN DEVELOPED AND DEVELOPING POPULATIONS OF THE WORLD (Bijnmer 1994)

<table>
<thead>
<tr>
<th>Population Location</th>
<th>&lt; 5 years</th>
<th>&lt; 1 year</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Australia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Western, Aboriginals</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td><strong>United States</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alaska Eskimos</td>
<td>282</td>
<td>871</td>
</tr>
<tr>
<td>White Mountain Apaches</td>
<td>254</td>
<td></td>
</tr>
<tr>
<td>Navajo Indians</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td><strong>Africa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The Gambia</td>
<td>60</td>
<td>297</td>
</tr>
<tr>
<td>Senegal</td>
<td>132</td>
<td></td>
</tr>
</tbody>
</table>
TABLE III: CASE FATALITY (CFR) FOR *HAEMOPHILUS INFLUENZAE* MENINGITIS IN WESTERN COUNTRIES VERSUS DEVELOPING COUNTRIES. (Bijlmer, 1994)

<table>
<thead>
<tr>
<th>LOCATION</th>
<th>CFR(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Developed</td>
<td></td>
</tr>
<tr>
<td>The Netherlands</td>
<td>1.95</td>
</tr>
<tr>
<td>United States (Alaska)</td>
<td>3</td>
</tr>
<tr>
<td>Finland</td>
<td>3.3</td>
</tr>
<tr>
<td>United States (Navajo Indians)</td>
<td>4</td>
</tr>
<tr>
<td>United States (overall)</td>
<td>5</td>
</tr>
<tr>
<td>Israel</td>
<td>3.3</td>
</tr>
<tr>
<td>Developing</td>
<td></td>
</tr>
<tr>
<td>Ivory Coast</td>
<td>22</td>
</tr>
<tr>
<td>Nigeria (Ibadan)</td>
<td>26</td>
</tr>
<tr>
<td>Ghana (Kumasi)</td>
<td>30</td>
</tr>
<tr>
<td>Senegal (Dakar)</td>
<td>33</td>
</tr>
<tr>
<td>The Gambia</td>
<td>37</td>
</tr>
<tr>
<td>Egypt (Cairo)</td>
<td>40</td>
</tr>
</tbody>
</table>

Hussey *et al* (1994b) concur with Bijlmer that the epidemiology of *Haemophilus influenzae* in developing countries has not been well documented. The results of the Cape Town study indicate 59.9% meningitis, 24.6% pneumonia, 7.0% septicaemia, 4.9% arthritis and 2.8% sinusitis. *Haemophilus influenzae* type b accounted for 86.5% of cases in children which is less than that found in industrialised countries where virtually all cases are due to Hib (Broome, 1987; Makela *et al*, 1992). Hussey *et al* (1994b) found that the incidence rate in Black children was about two-fold greater than that in other race groups and for meningitis was similar to that reported from the Gambia and Senegal. They also found that the case-fatality rate in the South African study was similar to that of developed countries (1-5%) and much lower than that in developing countries (12-47%) (Bijlmer, 1994). They ascribed this to the availability and accessibility locally of health care facilities.
Munson et al (1989) reviewed existing data on diseases caused by *H. influenzae* in children from developing countries. Their findings are consistent with those of Bijlmer (1990) in the study done in Gambia. However, they cite a study by Gratten and co-workers in Papua New Guinea, where the incidence of *H. influenzae* meningitis was spread over a wide range of 0 - 90% (Gratten et al, 1985). They suggest that this could be due to a laboratory bias in the ability of laboratory personnel to isolate the organism as well as a variation in quality control of media preparation and optimal culture conditions.

### 2.1.2 Risk factors

Although invasive Hib disease occurring in multiple members of a household was reported in 1909 by Davis (cited by Cochi et al, 1986), only within the last decade has documented evidence become available that invasive Hib disease is contagious. The findings of Tarr and Peter (1978), Cochi et al (1986), Takala et al (1989), Munson et al (1989), and Peterson et al (1991) suggest that multiple socioeconomic factors as well as inherent racial predilection affect the occurrence of this disease in young children. In different populations the incidence of Hib disease may vary considerably with varying risk factors that are largely dependent on cultural and socioeconomic factors (Takala, 1989). This variation may result in differences in the incidence rate and age distribution of Hib disease and indicates an urgent need for further epidemiologic studies especially in developing countries.

This literature survey on risk factors is broadly divided into factors relating to increased exposure and factors that increase the susceptibility to invasive disease. However, it is important to note that factors associated with increased exposure also have a bearing on increased susceptibility, e.g. age and carriage. These risk factors should be viewed in a broad context as being interrelated (Takala, 1994a). In this regard, Takala 1994 summarises the risk factors illustrated in Table IV.
TABLE IV: RISK FACTORS FOR *H. INFLUENZAE* TYPE b
COLONISATION, TRANSMISSION AND DISEASE (Takala 1994a)

<table>
<thead>
<tr>
<th>Increased risk for colonisation and transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young age</td>
</tr>
<tr>
<td>Large, crowded households</td>
</tr>
<tr>
<td>Siblings of preschool age</td>
</tr>
<tr>
<td>Day-care attendance</td>
</tr>
<tr>
<td>Concurrent viral infection</td>
</tr>
<tr>
<td>Season</td>
</tr>
<tr>
<td>Smoking of parents and other respiratory irritants</td>
</tr>
<tr>
<td>Short duration of breast feeding</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Increased risk for disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young age</td>
</tr>
<tr>
<td>Male sex</td>
</tr>
<tr>
<td>Frequent infections in general</td>
</tr>
<tr>
<td>Concurrent viral respiratory infections</td>
</tr>
<tr>
<td>Smoking of parents</td>
</tr>
<tr>
<td>Short duration of breast feeding</td>
</tr>
<tr>
<td>Genetic factors</td>
</tr>
<tr>
<td>Selected racial/ethnic groups</td>
</tr>
<tr>
<td>Eskimos, American Indians, Blacks, Hispanics</td>
</tr>
<tr>
<td>Antibody or complement deficiencies</td>
</tr>
<tr>
<td>Sickle cell anaemia, malignancies</td>
</tr>
</tbody>
</table>

2.1.2.1 Factors related to increased exposure

The epidemiology of invasive Hib disease varies considerably among populations. Low incidence rates are found among Caucasian populations in Australia and Europe, whereas up to 10-fold greater rates are found among certain ethnic groups, such as Native American and Australian populations (Takala, 1994a). Crowding and increased contact between young children have been related to
increased risk of invasive Hib disease. The factors related to increased exposure are (a) siblings and crowded households, (b) day-care attendance, (c) race, (d) hospitalisation and (e) other socioeconomic circumstances.

(a) Siblings and crowded households

Factors that have been associated with increased exposure to Hib in the family setting include the presence of siblings and crowding. The presence of crowded large households and especially extended family groups has been reported to be a risk factor for Hib disease among Alaskan Eskimos (Ward et al, 1986; Peterson et al, 1991) and is probably also an important risk factor among Navajo Indians, who traditionally live in extended families (Takala, 1994a). Peterson et al (1991) suggest that susceptible infants in extended family households are exposed to a greater number of caretakers or an increased number of non-sibling children than infants in nuclear families. These individuals were more likely to be Hib carriers and therefore introduce Hib into the family. Older siblings did not predispose a susceptible infant to Hib disease possibly due to the fact that the risk of exposure from other individuals in extended families was much greater than the risk from siblings.

Reports by Granoff and Daum (1980) and Granoff and Basden (1980) demonstrate that household contacts are at increased risk of developing invasive disease. Both reports indicate that the risk was greatest in contacts less than two years of age. Granoff and Basden (1980) do however cite two studies which indicate that a lesser degree of risk also exists for contacts two to four years of age.

Istre et al (1985) conclusively state that the only two significant risk factors are attendance at day-care centres and household contact. Household contact as a risk factor was associated with the presence of a school-aged child in the household. This risk factor was the most important among the youngest children with a doubling of the risk for each additional sibling (Takala et al, 1989). Cochi et al (1986), Clements et al (1992), Takala and Clements (1992) and Makela et al
(1992), also confirm that household crowding was significantly associated with Hib disease. Cochi et al (1986) further state that the magnitude of risk increased with the extent of crowding.

Takala (1994a) cites the results of Michaels et al (1976) in their study of oropharyngeal carriage rates of Hib. They found that carriage rates were highest in preschool children. Thus, the more siblings of this age a susceptible infant has, the more likely he or she is to acquire Hib and subsequently contract invasive Hib disease. However, in a study in Ireland by Fogarty et al (1995) household crowding and family membership characteristics did not emerge as risk factors. The homogeneity of the Irish population may account for the similarity in distribution of these characteristics that have been shown to be risk factors elsewhere.

(b) Day-Care attendance

Day-care attendance provides an increased exposure to many infectious agents, including Hib. Contact between infants and young children in day-care is close. Thus it is likely that infectious agents are shared, leading to increased pathogen transmission and probably to transmission of a high dose of infectious agent (Takala, 1994a). The risk for invasive Hib disease has been shown to be significantly higher during the first months of day-care attendance than the risk from contact during later months (Takala, 1989).

The magnitude of the association of day-care attendance with primary Hib disease was highly age dependent (Cochi et al, 1986). The peak odds ratio was 12.8 for children 2 to 5 months of age. Reports of spread of Haemophilus influenzae type b in day-care centres or in other institutional settings indicate that most of the associated cases are in children less than 2 years of age (Granoff and Daum, 1980; Cochi et al, 1986). During the period of the study conducted by Cochi et al (1986), 50% of all invasive Hib disease that occurred was attributable to exposure to day-care. Variations in the level of risk of Hib disease associated with day-care attendance may be due to the size and practice of the centre
(Fogarty et al, 1995). The greater the number of children per room and the longer the duration of stay, the higher the risk of disease (Istre et al, 1985; Cochi et al, 1986).

Children who did not attend a day-care centre showed a rather typical age distribution for *H. influenzae* disease, with a peak among the 6- to 11-month-old group (Istre et al, 1985). Those who did attend a day-care centre were mostly older children ( > 18 months) and the results of the study showed an increasing risk with increasing age. The association of infected children with day-care attendance and the presence of a school-aged household member remained significant even when meningitis and other cases were analyzed separately, when day-care attendance was redefined as > 4 or > 8 hours per week and when day-care attendance of other household members was considered.

(c) Race

In a retrospective study of hospital records in North Carolina between 1966 and 1970, Parke et al (1972) reported 86 cases of Hib meningitis. Three children and one adult died - all were black. The overall mortality rate for Blacks was 8%. They suggest that increased overcrowding in ghetto areas, deficient nutrition and sickle cell haemoglobin should be considered as contributing factors and that further studies were necessary in order to evaluate these factors. However, Fraser et al (1974) conducted a similar survey in New Mexico and their results suggested that much of the differences in risk between blacks and whites in some populations may be associated with differences in level of education.

Tarr and Peter (1978) reviewed data from 1970 to 1974 in Rhode Island. They found that the disease incidence among black children under five was significantly higher than that among white children. The estimated risk to a black child of contracting *H. influenzae* meningitis in Rhode Island was one in 193. Their findings confirm the increased incidence of *H. influenzae* in blacks and indicate that socioeconomic factors do not affect the incidence of the disease in white children.
The results of Santosham et al (1979) concur with the findings of Tarr and Peter (1978). However, their study revealed a twofold increase in incidence in black children aged 13-60 months compared to white children of the same age. There was no difference in incidence between the two groups from birth to 12 months. Subsequent studies by Granoff and Basden (1980), also demonstrate an increased incidence among blacks and Hispanics - 3.3 times and 1.6 times greater than for whites respectively. Istre et al (1985) did not find race to be a significant factor. This could be attributed to the fact that the large proportion of whites in Colorado made it difficult to evaluate the effect of race.

Further investigations by Cochi et al (1986) and Makela et al (1992) suggest that whilst the incidence of Hib disease was often higher among blacks, this trend was due to other socioeconomic factors. When such factors were controlled for, race was not a determinant. This supports the findings of Fraser et al (1974). The results of the study by Cochi et al (1986) do, however, demonstrate that excess risk in blacks may be restricted to children >1 year of age. They suggest that this may be due to genetically determined differences in host susceptibility to disease which affects age distribution of disease.

(d) Hospitalisation

Many of the factors that may contribute to the risk of nosocomial infections with Hib are poorly defined (Gilsdorf and Herring, 1987), including the role of environmental Hib contamination and the survival of the organisms on fomites. Experiments by Gilsdorf and Herring (1987) demonstrated a decreasing density of Hib over a 2-hour period suggesting that the organisms were not actively replicating on the environmental surfaces.

Secondary cases of Hib illness have been recognised among close contacts of children with Hib infections, such as household and day-care centre contacts (Granoff and Daum, 1980) and long-term care hospital contacts (Glode et al, 1976). Glode et al (1976), report five Hib meningitis cases occurring within six months in an enclosed population of approximately 30 chronically ill children.
Subsequently, two additional children, who were non-carriers of Hib, acquired Hib meningitis. A carriage rate of 20% was observed in this closed population. This finding supports the observation of higher carriage rates in enclosed populations by Turk (1963) and Mpairwe (1970) who also suggest that a single carrier may infect a large group over a prolonged period of time.

In a report by Bachrach (1988), an outbreak of *H.influenzae* bacteremia involving four out of 11 patients in the infant unit of an intermediate care hospital is described. Consequently, they suggest that there can be a significant risk to hospital patients from a patient with Hib disease. The attack rate was 36% and was unusually high especially since it occurred in a hospital population already compromised by chronic disease and tracheostomies. The results of epidemiological studies carried out in Durban in 1986 (Hoosen *et al*) and 1987 (Peer *et al* - conference proceedings) also suggest nosocomial acquisition of infection by paediatric patients.

In the study by Peter and Tarr (1978) a high hospitalisation rate was not found to be associated with an increased risk of Hib disease. Takala *et al* (1989) found that previous hospitalisation and a history of otitis media were associated with increased risk of Hib disease. However, this risk was only moderately increased and it was possible that some socioeconomic factors associated with the probability of the child being hospitalised may have remained unrecognised in the study. A child's history of infectious disease as evidenced by otitis media was strongly associated with the risk of Hib disease. Otitis media has been shown to be 15 times more common among populations with the highest incidence of invasive Hib disease viz. native Americans and Alaskan natives (Takala, 1992).

**(e) Other socioeconomic factors**

In a study by Fraser *et al* (1974), the association of meningitis risk with average income and education was examined in Bernalillo County, New Mexico and was compared with three other American populations. Incidence rates of *H.influenzae* were significantly higher in parts of the county with lower than average education
levels but appeared to be independent of income in high education areas. They concluded that average education level was the only contributing risk factor and suggest that it may relate to benefits derived from early utilisation of medical treatment for "minor" respiratory illnesses caused by potentially pathogenic organisms. In contrast to this, Cochi et al (1986) found no significant association for level of income and education after controlling for confounding variables in their study of risk factors for Hib disease. Similar results were obtained by Tarr and Peter (1978).

2.1.2.2 Factors increasing susceptibility to invasive disease

(a) Age

Of all risk factors for invasive Hib disease, young age is the most important (Peltola et al, 1977). At least 95% of cases in all carefully studied populations occur in children less than 5 years of age. However, within this age range, there are large differences between populations. In Scandinavian countries approximately 10% of Hib meningitis occurs in infants less than 6 months old, while the corresponding figure among Alaskan natives is 34% (Cochi et al, 1986). The results of studies on Alaskan Eskimos by Peterson et al (1991) indicate that 95% of cases were under 18 months of age with a mean age of 8.7 months. Ward et al (1986) found that the peak disease incidence for Native Alaskans was 6 to 11 months of age. Studies on Apache Indians (Losonsky et al, 1984) demonstrate similar results as do studies by Istre et al (1984) and Hitchcock et al (1994).

In a field study in the Gambia, 84% of all recorded cases of H.influenzae meningitis occurred before the age of 1 year and 44% of patients were younger than 6 months old (Bijlmer et al, 1990). Cochi et al (1986) found that there was a correlation between day-care attendance and age, with 2- to 5- month old infants having the highest risk. Similarly, Fraser et al (1974), found that the persons at highest risk were in the age group 2 to 9 months with the maximum risk in the 6 to 7 month age group.
A study in the Republic of Ireland (Fogarty et al., 1995) also reports a strong relationship between age and risk of Hib disease for day-care attendance with the highest risk experienced by children in the second year of life. In North West England (Quigley et al., 1993), the mean age for 87 cases of Hib disease was one year 10 months. Of these, 64% were under the age of two years and 7% were under the age of 6 months.

(b) Gender

Although studies indicate a predominance of invasive Hib disease among males, there is no overwhelming evidence to suggest that males are more susceptible than females. The distribution of cases by sex was shown to be 48% male in Minnesota and 51% in Dallas County (Murphy et al., 1992). Similar observations were made by Santosham et al. (1979), Bijlmer (1990) and Istre et al. (1984).

(c) Underlying disease, viral respiratory infection and smoking

Adhesion of bacteria to the host cells is essential for infectivity (Beachey, 1981). Attachment of Hib to the oropharyngeal mucosa is thought to be the initial stage in the development of invasive Hib disease (Takala 1993). Any changes to the respiratory mucosa will affect the colonising potential of Hib in the oropharynx and also result in a concomitant increased production of respiratory secretions (Takala, 1994a). As a result, transmission of Hib will be facilitated.

Istre et al (1985), Cochi et al (1986) and Takala et al (1989) found that smoking was more common among parents of patients with invasive Hib disease than among parents of controls. Takala (1994a) states that tobacco smoke damages the respiratory mucosa leading to enhanced attachment of Hib and subsequent increased risk of invasive disease.

It has been suggested that one of the mechanisms by which viruses predispose to bacterial infection is by affecting the interaction between bacteria and cells of the respiratory mucosa. Fainstein et al (1980) demonstrated increased adherence of
19

*H. influenzae* early during the experimental infection of volunteers with influenza virus. They also found that bacterial flora of the pharynx did not change during the study period. The results of a study by Krasinski *et al* (1987) suggest that primary infection with adenoviruses and possibly influenzae B precedes development of bacterial meningitis and could be a predisposing factor. It is possible that increased host susceptibility could be due to alteration of mucosal immunity or bacterial flora (Vadheim and Ward, 1994).

In Finland, Takala *et al* (1989) found that the occurrence of frequent upper respiratory tract infections in general and otitis media specifically were risk factors for invasive Hib disease. In a study among American Indian children, (Takala *et al*, 1993), it was found that 48% of carriers of Hib had symptoms of respiratory infection at the time of sampling compared to 30% in non-carriers, thus demonstrating that upper respiratory tract infection was associated with Hib carriage. Michaels *et al* (1976) also found an elevated Hib carriage rate among children with respiratory infection. In the Gambia, (Bijlmer *et al*, 1990), acute lower respiratory tract infections were found frequently before or concurrently with *H. influenzae* meningitis.

(d) Breast-feeding

Breast-feeding appears to be protective in infants less than 6 months of age (Cochi *et al*, 1986), and was found to be not protective in 6 to 11-month-old infants alone. Cochi *et al* suggest that breast-feeding of infants less than 6 months of age could reduce the risk of Hib disease by 92% in them. Data from studies by Istre *et al* (1985) and Takala *et al* (1989) support this finding. Takala *et al* (1989) found that breast-feeding for more than 6 months was only marginally protective.

In a study of risk factors among Alaskan Eskimos (Peterson *et al*, 1991), 37% of Hib cases were breast-fed compared with 52% of their matched controls. However, the mechanisms for this protective effect were not elucidated. It may involve the presence of anti-PRP antibodies in human milk resulting in an
enhancement of a breast-fed infant's response to Hib. Another possible explanation was that infants who were breast-fed remained in the care of their natural mothers for a longer period of time and were therefore less likely to be exposed to outside contacts and Hib (Takala, 1994a).

(e) Carriage

The association of carriage with disease is a debatable issue. Studies have shown that carriage does not identify children who are in danger of being infected. Individuals who have not been identified as carriers may develop Hib meningitis whilst carriers may remain unaffected (Glode et al, 1976; Dajani et al, 1979). However, Takala et al (1989) suggest that since Hib carriage is highest in children younger than 7 years, contact between these carriers and younger siblings increases the probability of developing invasive Hib disease.

Many factors have been shown to influence the development of invasive Hib disease. A great degree of variation has been shown in the possible influence on disease of different risk factors in different geographic regions and in different population groups. In view of this, it is possible that socioeconomic factors may be the most important determinant of Hib disease since these factors could result in increased transmission of infection (Makela et al, 1992).

2.2 CARRIAGE

A preliminary step in the pathogenesis of invasive disease is the adherence of the organism to the mucosa (Beachey, 1981). In the case of Hib disease this would be the adherence of Hib to the nasopharyngeal epithelium. This is followed by colonisation with Hib and by unknown mechanisms colonisation may be followed by invasive disease. However, studies have shown that carriage of Hib is no indicator of possible disease as carriers may or may not develop disease and those that develop disease are not necessarily carriers (Glode et al, 1976; Michaels and Norden, 1977; Dajani et al, 1979; Granoff and Daum, 1980).
2.2.1 Factors affecting colonisation

Many of the factors affecting colonisation are the same as the risk factors for the development of Hib disease. Therefore, reference will be made to preceding sections.

2.2.1.1 Host factors

(a) Age

Oropharyngeal colonisation with Hib is rare in healthy children during the first 6 months of life (Takala, 1994b) with a frequency of 0.7% during the first 6 months and an average of 3 to 5% throughout the rest of childhood (Turk, 1963; Michaels et al, 1976). Nasopharyngeal carriage was highest in children less than 7 years of age (Michaels et al, 1976).

In situations where young children are in contact with each other for long and regular periods of time eg. day-care centres, orphanages, hospital wards, etc, this carriage rate is much higher than in open communities (Turk, 1963; Mpairwe, 1970; Glode et al, 1976; Takala et al, 1989). In a study in Uganda, Mpairwe (1970) demonstrated a carriage rate between 6 to 53% for Hib in an orphanage as compared to a rate of 17 to 70% seen by Turk (1963).

Carriage rates in open communities are lower as demonstrated by Bijlmer (1989). He found that the carriage rate of Hib in the Gambia was 0-16% for children under 5 years of age. This was higher than in Uganda (Mpairwe, 1970) where a carriage rate of 4.5% was demonstrated for outpatient children living in various parts of Kampala city.

In Navajo and Apache Indian children, oropharyngeal carriage of Hib was detected earlier in infancy (Takala et al, 1993) compared to that reported among Caucasian children (Michaels et al, 1976). Carriage rates greater than 3% were observed in Indian infants 3 to 5 months of age (Takala et al, 1993). This is in
accordance with the high incidence of invasive Hib disease early in infancy among Navajo and Apache Indian children (Losonsky, 1984).

(b) Immunological and host defence factors

Very little is known about immunological and host defence factors that affect oropharyngeal colonisation with Hib. Makela et al (1992) suggest that impaired synthesis of Hib antibodies could be associated with a particular genotype and that the genotype might be more prevalent in some populations thus resulting in an increased incidence of invasive Hib disease. It is possible that high serum antibodies to Hib might lead to increased levels of IgG at the mucosal surface level (Takala 1993). At present it is unknown to what extent this mucosal immunity contributes to protection from invasive Hib infections.

The preventive effect of breast-feeding infants less than 6 months of age on the incidence of invasive Hib disease has been cited in a number of studies (Istre et al, 1985; Cochi et al, 1986; Peterson et al, 1991; Takala et al, 1994a). Prevention of adherence of Hib to respiratory mucosal cells by breast milk could be a mechanism by which colonisation is delayed. However, this mechanism is independent of antibody production and is possibly due to the anti-adhesive activity of human casein (Andersson et al, 1986; Aniansson et al, 1990).

(c) Concurrent viral and other respiratory infection

The effect of concurrent viral infection has been discussed as a risk factor. The factors that affect adherence of Hib to the pharyngeal mucosa will have a concomitant bearing on carriage of Hib and subsequently possibly affect the incidence of invasive disease. (Beachey, 1981; Takala et al, 1993). Studies have shown that carriage of Hib is greater in individuals who have an upper respiratory tract infection compared to those who do not have upper respiratory infection (Michaels et al, 1976; Takala et al, 1993). Experimental infection of volunteers with influenzae viruses resulted in increased adherence of Hib to the mucosa thus increasing carriage (Fainstein et al, 1980).
Increased carriage was also demonstrated in tuberculosis patients in Cape Town, South Africa (Hussey et al, 1994a). Hospitalised TB patients had an Hib carriage rate of 37.1% which was higher than the rate for tuberculosis patients attending an outpatient clinic. This could be due to prolonged hospitalisation and chemoprophylaxis resulting in subsequent immunosuppression.

(d) Day-care attendance and siblings

Attendance at a day-care centre increases the likelihood of contact with young children who are shown to have a high carriage rate of Hib. Any situation which results in prolonged and regular contact between children younger than 7 years increases the risk of disease due to increased carriage in this age group (Turk, 1963; Takala et al, 1989). See 2.1.2.1 (a)

(e) Contact with patient with Hib disease

Studies of Hib disease in enclosed populations have shown an increased carriage rate among contacts, (Glode et al, 1976; Granoff and Daum, 1980) and that these contacts were less than 24 months of age. Glode et al (1976) report a high carriage rate of 20% as well as a high incidence of Hib meningitis. Carriage tends to persist for longer periods of time in closed populations and children frequently remain colonised for many weeks to months. A carriage rate of 41% for Hib among families of patients with Hib meningitis was found by Turk (1963). Carriage rates of 2.4% and 2.9% were found among normal groups.

(e) Antimicrobial treatment

Patients with invasive Hib disease who are treated with antibiotics intravenously have demonstrated a reduction in nasopharyngeal colonisation with Hib within 24 hours of commencement of therapy (Gilsdorf 1986). Specimens obtained and cultured after more than 25 hours did not yield any Hib. The antibiotics used were ampicillin alone, chloramphenicol alone or a combination of ampicillin and chloramphenicol.
Eradication of colonisation using chemotherapeutic agents was suggested in the study of an outbreak of Hib bacteraemia in an intermediate care hospital (Bachrach, 1988). The use of rifampicin in patients and staff and the initiation of strict infection control measures abruptly halted the outbreak. It has been suggested that rifampicin administration eradicates carriage in 90% of treated children (Granoff and Daum, 1980). However, reacquisition may occur after termination of the antibiotic.

The effective reduction of nasopharyngeal carriage of Hib in four day care centres was achieved using rifampicin (Campos et al, 1987). Eradication was achieved in 95.5% of carriers of multiply resistant strains of Hib. Several untreated carriers remained culture positive and could be a reservoir for the organisms. Contradictory findings were reported from Thailand (Simasathien et al, 1980). The carriage rate of Hib in children in an orphanage remained unchanged after treatment with rifampicin and an increased percentage of isolates were resistant to ampicillin and chloramphenicol. They conclude that rifampicin does not eradicate carriage. However, Campos et al (1987) suggest that subtherapeutic doses of rifampicin were used in the Thailand study, and that the results were based on a small number of carriers.

In view of the availability of safe Hib conjugate vaccines that are effective for use in infancy, the value of the use of rifampicin is questionable or less important in the prevention of primary Hib disease (Takala, 1994b). However, it is still of value in the prevention of secondary infections in non-vaccinated individuals.

2.2.1.2  Bacterial factors

To detect differences between strains of Hib, serologic and electrophoretic characterisation of the outer membrane proteins and lipopolysaccharides have been performed. Although subtypes of Hib have been determined based on outer membrane protein analysis (van Alphen, 1983a), the overall pattern is closely
similar in all Hib strains. Different electrophoretic types of Hib have been determined based on the electrophoretic behaviour of a large number of bacterial enzymes but the different Hib strains can be categorised into a small set of closely related electrophoretic types.

Van Alphen (1993) states that the outer membrane subtype designated 13L was more frequently isolated from carriers in an open community suggesting that this subtype (13L) was less virulent than others. The subtype 1H was reported to be more frequently associated with secondary cases of disease in day-care centres (Barenkamp et al, 1981b). Outer membrane proteins are discussed in greater detail under section 2.3.

2.2.2 Oropharyngeal vs nasopharyngeal carriage

There has been considerable debate about whether collection of oropharyngeal or nasopharyngeal swabs was more efficient for the isolation of Hib in carriers. Publications focusing on carriage of Hib are based on studies of nasopharyngeal rather than oropharyngeal cultures (Turk, 1963; Mpairwe, 1970; Glode et al, 1976;). Later investigations on the effect of immunisation on carriage focus on oropharyngeal cultures only (Takala et al, 1991; Takala et al, 1993) and this could be ascribed to the fact that oropharyngeal cultures have been shown to be more efficacious in the isolation of Hib (Michaels et al, 1976).

A comparative study of nasopharyngeal and oropharyngeal culture (Michaels et al, 1976) clearly demonstrates the greater efficiency of oropharyngeal cultures in detecting Hib colonisation. The results of this comparison are illustrated in Table V (Michaels et al, 1976). The study population was a group of 31 childhood siblings of patients with invasive Hib disease. The age range was 6 months to 10 years. Only 4 oropharyngeal Hib carriers were over 8 years of age and were negative for nasopharyngeal carriage.
TABLE V: COMPARISON OF EFFICACY OF OROPHARYNGEAL VERSUS NASOPHARYNGEAL SWABBING FOR THE ISOLATION OF *H. INFLUENZAE* TYPE b (N = 31) (Michaels et al, 1976)

<table>
<thead>
<tr>
<th>Nasopharynx</th>
<th>Throat</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>11</td>
</tr>
<tr>
<td>-</td>
<td>6</td>
</tr>
</tbody>
</table>

Analysis of data from a study to determine the prevalence of ampicillin resistant *H. influenzae* in 249 children (Schwartz et al, 1983) clearly indicated a higher yield from the oropharynx compared to the nasopharynx. Also, 69% of patients who had been treated with ampicillin recently were found to be oropharyngeal carriers in contrast to 44% of patients who had not been exposed to ampicillin. More patients with prior exposure to ampicillin carried ampicillin resistant strains compared to non exposed patients, 28.5% and 7.0% respectively. In contrast, Hussey et al (1994a), found that nasopharyngeal carriage was twofold higher than oropharyngeal carriage.

2.3 IDENTIFICATION AND CHARACTERISATION

2.3.1 General Microbiology

Members of the genus *Haemophilus* are gram negative facultative anaerobes with a morphology that varies from coccobacilli to filamentous rods (Kilian 1991). For *in vitro* growth, the organism requires two growth factors viz. X and V factors. The X factor is a group of heat-stable compounds that are provided by several iron-containing pigments such as haemin and haematin (Koneman et al, 1988). The V factor is nicotinamide adenine dinucleotide (NAD) and is produced by several micro-organisms eg. *Staphylococcus aureus*. Thus, on blood agar, growth of *H. influenzae* is enhanced around colonies of *S. aureus*; a phenomenon termed 'satellitism' (Koneman et al, 1988).
Although both growth factors are contained in blood cells, only the X factor is available in blood agar in adequate amounts. *H. influenzae* will therefore not grow on ordinary blood agar. V factor may be liberated by lysis of the red cells by gentle heating, a process which also serves to inactivate enzymes which destroy the V factor (Kilian, 1991). The resulting chocolate agar contains both X and V factors and will support the growth of *H. influenzae*. Chocolate agar supplemented with bacitracin has been found to give excellent recovery of the organism from nasopharyngeal sites (Kilian, 1991).

Inoculated agar plates are incubated at 35 to 37°C in a moist atmosphere supplemented with 5 to 10% CO₂ (Kilian, 1991). Colonies on chocolate agar appear greyish, semi-opaque and are usually visible after 18 to 24 hours. Strains producing indole usually have a characteristic pungent odour.

Further identification of possible *H. influenzae* colonies is by demonstration of the requirement for both X and V factor. This may be accomplished using discs impregnated with these factors and basal medium lacking X and V. Confirmation of the species is obtained by performance of the porphyrin test (Kilian, 1991), which is a more rapid and accurate test for X factor requirement. Strains requiring this factor, lack the enzymes required in the biosynthesis of haem. Thus, when the substrate δ-aminolaevulinic acid is provided, these strains do not produce the intermediates of the haem biosynthetic pathway viz. porphobilinogen and porphyrin. Subsequently, methods used for the detection of porphyrin and porphobilinogen, the use of a Wood’s lamp or Kovac’s reagent respectively, yields a negative reaction. *H. parainfluenzae* demonstrates a positive reaction and is used as a control.

### 2.3.2 Serotyping

In 1931, Pittman (cited by Turk, 1963) demonstrated the presence of both capsulate and non-capsulate strains of *H. influenzae*. The capsulate strains could be further distinguished into six serotypes viz. a, b, c, d, e and f. Pittman
associated serotype b with acute infections such as meningitis, pneumonia, epiglottitis and suppurative arthritis. Serotyping is now routinely performed in most diagnostic microbiology laboratories for distinguishing between clinical *H. influenzae* isolates.

Studies on the chemical composition of the capsule of type b strains revealed that type b was different from the other five types. This led to the conclusion that the type b capsule was a major virulence factor (Turk, 1984). The type b capsule is a polymer of polyribosyl-ribitol-phosphate (PRP) and is the only type to contain pentoses; the remaining five capsule types have a hexose or a hexose derivative (Mendelmen and Smith, 1987). The risk of invasive disease is associated with the lack of antibodies to PRP which has been found to be a poor immunogen (van Alphen, 1993). Serotypes other than b as well as non-typable strains of *H. influenzae* have been associated with disease (cited by Munson et al, 1989). Studies in animals have demonstrated that only type a and b are invasive (Robbins and Schneerson, 1987). The virulence of type b strains has been attributed to the capsule of these strains (Moxon, 1992) but the mechanism by which this effect is exerted is not known.

Some bacteria have been found to have surface structures that cross-react with antiserum to the capsule of Hib viz. diphtheroids, lactobacilli, *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus epidermidis* and certain pseudomonads. A strain of *Escherichia coli*, K100, has a capsular polysaccharide identical to that of Hib (Mendelman and Smith, 1987). It is thought that exposure to these non-pathogenic organisms with cross-reacting antigens could be responsible for natural immunity to Hib in the absence of direct contact with the organism (Robbins and Schneerson, 1987).

Table VI illustrates the composition of the capsules of the six serotypes.
TABLE VI: CARBOHYDRATE COMPOSITION OF *H.influenzae* CAPSULES (Mendelman and Smith, 1987)

<table>
<thead>
<tr>
<th>Type</th>
<th>Sugar</th>
<th>N-acetyl</th>
<th>Phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>glucose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>b</td>
<td>ribose, ribitol</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>c</td>
<td>galactose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>d</td>
<td>hexose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>e</td>
<td>hexosamine</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>f</td>
<td>galactosamine</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Capsular serotyping may be performed by the slide agglutination method using type specific antiserum (Turk, 1963; Dimopoulou *et al*, 1993; Hussey *et al*, 1994b), latex agglutination (Heikkela, 1987) and coagglutination (van Alphen *et al*, 1987). Countercurrent-immunoelectrophoresis (CIE) has also been used to determine the capsular type of the organism (Dimopoulou *et al*, 1993) but the slide agglutination method has been found to be more practical than CIE for serotyping. In a study of 130 isolates of *H.influenzae* (Capper, 1988), serotyping was performed using the slide agglutination technique. Twenty-four isolates were found to be type b and were studied further using CIE. Only 50% were shown to be positive by CIE and on the basis of this result, Capper (1988) concludes that whilst CIE is a sensitive method, its application lies in identification of serotypes directly from clinical specimens. Once the organism has been isolated, the most practical method for serotyping is slide agglutination (Kilian, 1991). In epidemiologic studies on oropharyngeal carriage of Hib (Takala *et al*, 1991), detection of type b strains was facilitated by the use of Levinthal-bacitracin agar plates containing 2.75 ml Hib antiserum in 45 ml of agar. Colonies of Hib were surrounded by a halo of precipitation. This medium has also been recommended by Dajani *et al* (1979).

The value of serotyping for epidemiological purposes is limited in view of the fact that most strains causing invasive disease, especially in children, are
identified as serotype b. Also, the type b polysaccharide is not the only virulence factor associated with disease since type a strains have been isolated from the blood and CSF in Papua New Guinea (Montgomery et al, 1990). In addition, one third of blood isolates from children with pneumonia in Pakistan have been shown to be non-typeable (Weinberg et al 1989). Several other methods for typing of H. influenzae have been documented viz. biotyping (Kilian, 1976 cited by Kilian 1985), outer membrane protein typing (Loeb et al, 1981; Barenkamp et al, 1981a; van Alphen et al, 1983b) and multilocus enzyme electrophoresis (Selander et al, 1986). Some of these methods and their applications will be discussed further.

2.3.3 Biotyping

H. influenzae can be subdivided into 8 biotypes (Table VII) on the basis of indole production, urease activity and ornithine decarboxylase activity according to the scheme proposed by Kilian in 1976 (cited by Kilian, 1985). Specific biotypes have been associated with different sources of isolation and different infections. This method of typing has limited value in epidemiologic studies since only four biotypes are frequently found (van Alphen, 1993). Invasive disease isolates have been found to be mainly type I and II.

TABLE VII: KEY TO THE DIFFERENTIATION OF BIOTYPES OF H. INFLUENZAE

<table>
<thead>
<tr>
<th>BIOTYPE</th>
<th>INDOLE PRODUCTION</th>
<th>UREASE ACTIVITY</th>
<th>ORNITHINE DECARBOXYLASE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>II</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VI</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VII</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VIII</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
In a study of 1015 strains of *H. influenzae* isolated from the CSF of patients aged 23 days to 56 years, Landgraf and Vieira (1993) found that 99.4% were of serotype b. Of these, 70.9% were biotype I, 27.5% biotype II, 0.6% biotype III and IV and 0.4% were biotype V. They also found that the age distribution of biotypes was statistically significant with biotype I predominating in patients under 10 years of age. A similar distribution of biotypes I and II were found by Musser *et al* (1985) who studied 177 isolates of Hib of which 77.0% were biotype I and 21.0% were biotype II.

The study by Musser *et al* (1985) was more detailed since they typed the organism by using outer membrane protein analysis and multilocus enzyme electrophoresis as well. By combining the results of these analyses, they demonstrated that biotype diversity was not correlated with outer membrane protein diversity. In a study among White Mountain Apaches, Losonsky *et al* (1984) also found a predominance of biotype I and biotype II in cases of invasive disease. Similarly, Weinberg *et al* (1981) found that of 61 Hib isolates, 58 were biotype II and 3 biotype I.

Righter and Luchsinger (1988) conclusively state that biotype I was associated with invasive disease since 4 out of 5 blood/CSF isolates were of this biotype. The rate of β-lactamase production was highest among biotype III isolates, but these results were not statistically significant. Capper (1988) studied 130 isolates of *H. influenzae* and found that 24 (18%) of the isolates were serotype b of which only 4 produced β-lactamase. Three of the β-lactamase producing Hib were of biotype II and one was of biotype III. In view of the fact that 30% of Biotype III isolates accounted for invasive infection but were not of serotype b, Capper (1988) suggests that certain biotypes might have an increased prevalence for invasive disease irrespective of the serotype. This is verified by Dimopoulou *et al* (1992) in a study of three epidemiologically unrelated clusters of *H. influenzae* one of which was identified as Hib biotype I. The other two clusters were nonencapsulated strains of biotypes II and III. Strains from all three clusters produced both β-lactamase and chloramphenicol acetyltransferase.
2.3.4 Outer membrane proteins (OMP)

*H. influenzae* has a cell envelope which is typical of that found in gram-negative bacteria and consists of a cytoplasmic membrane, a peptidoglycan layer and an outer membrane. The outer membrane contains protein, lipopolysaccharides, and phospholipid (Mendelman and Smith, 1987). However, studies have also revealed some differences between Hib and other gram-negative bacteria viz. increased permeability to ampicillin exhibited by Hib compared to *Escherichia coli* (Loeb et al, 1981). Loeb et al (1981) also found that the inner membranes were unusually fragile and that there was a tendency for the inner and outer membranes to hybridize.

Using sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE), more than 30 outer membrane proteins (OMP) have been identified of which six account for more than 80% of the total OMP (Loeb and Smith, 1982). A gradient and a normal SDS-PAGE system may be used for analysis of the proteins (Barenkamp et al 1981a; van Alphen et al, 1983b). Granoff and Munson (1986) described the proteins using numerical numbering P1 to P6 whereas van Alphen et al (1983b) used alphabetical numbering a to e.

The major OMP's described and the respective molecular weights of the proteins (in kilodaltons (kD)) are OMP a [or P1] 43-50 Kd; b,c [or P2] 39-42kD; d [or P5] 37-39 Kd; e [or CP4] 30kD and OMP P6, 16,6 Kd. The properties of these major OMP's are shown in Table VIII (van Alphen et al, 1983b). Protein a and d are heat modifiable and may easily be identified in SDS (van Alphen et al, 1983b). Protein b,c, a porin which facilitates the diffusion of nutrients and some antibiotics through the outer membrane, is coded by a gene for which only one copy has been found (van Alphen, 1994). Protein e (P4) is a conserved non-immunogenic protein and protein P6 (no alphabetic number) is a lipoprotein.
TABLE VIII: PROPERTIES OF THE MAJOR OUTER MEMBRANE PROTEINS OF *H. INFLUENZAE* (van Alphen *et al*, 1983b)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Weight x 10^3</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>43 - 50</td>
<td>Heat modifiable, extracted by Triton X-100-MgCl&lt;sub&gt;2&lt;/sub&gt;, sensitive to trypsin</td>
</tr>
<tr>
<td>b</td>
<td>39 - 42</td>
<td>Reduced in cell envelopes, insensitive to trypsin, not heat modifiable</td>
</tr>
<tr>
<td>c</td>
<td>39 - 42</td>
<td>Insensitive to trypsin, not heat modifiable, peptidoglycan associated in 0.5% Triton X-100-0.2% SDS</td>
</tr>
<tr>
<td>d</td>
<td>37 - 39</td>
<td>Heat modifiable, sensitive to trypsin, immunologically related to the protein of <em>E. coli</em> K-12, absent in LiCl-EDTA extracts</td>
</tr>
<tr>
<td>e</td>
<td>30</td>
<td>Extracted in Triton X-100-MgCl&lt;sub&gt;2&lt;/sub&gt;, sensitive to trypsin, not heat modifiable</td>
</tr>
</tbody>
</table>

OMP subtyping is based on the differences in mobility of the major OMP's a, b, c, and d which show diversity in molecular weight. Maximum resolution is obtained when samples are analyzed after boiling in SDS as well as being treated at 37°C. This is due to the heat-modifiable nature of proteins a (P1) and d (P5). Protein b, c (P2) sometimes appears as multiple bands when unheated (van Alphen, 1994). Protein e (P4) is a conserved protein and its migration in SDS-PAGE does not change after heating. Plate 1 is a photograph of OMP subtypes indicating the major proteins.

Barenkamp *et al* (1981c) describe 21 different OMP patterns for Hib isolates from patients with invasive disease in the United States. Van Alphen and Bijlmer (1990) describe 9 OMP patterns for Hib isolates in the Netherlands. Three of the subtypes from the Netherlands are identical to three subtypes described by...
Barenkamp et al. viz. van Alphen types 1, 2 and 3 are the same as Barenkamp types 3L, 2L and 6U respectively.

Plate 1: Photograph of major outer membrane proteins after SDS-PAGE (photograph supplied by Dr. L. van Alphen)

In a study in the Gambia (Bijlmer et al., 1992), it was found that a distinct pattern of OMP subtypes, different from other parts of the world, was prevalent. As a result of the study in the Gambia, 4 additional subtypes have been designated as new numbers in the OMP subtyping system described by van Alphen which now has 13 subtypes. Plate 2 is a photograph of OMP subtypes of Barenkamp et al. (photograph supplied by van Alphen) as analyzed by SDS-PAGE.
In addition to describing 21 subtypes, Barenkamp et al (1981a) also differentiate between heavy (H), light (L) and unclassified (U) forms of protein a (P1). This can only be distinguished using a gradient gel (Barenkamp et al 1981a) or a modified ELISA method using monoclonal antibody 5HA5 to which all L-type strains react (Abdillahi and Poolman, 1987). This ELISA method identifies L-types but does not distinguish H and U types and results are therefore reported as subtype L or non-L subtypes.

2.3.4.1 Worldwide distribution

With reference to the worldwide distribution of OMP subtypes, van Alphen (1994) comments that in most countries one or a few subtypes are predominant. Also, subtypes do not spread easily across natural borders, like seas and this may be attributed to the fact that each population has its own stable reservoir of Hib.
strains genetically linked to that population or that spread may be diminished due to the lack of close contact between carriers. The percentage distribution of common OMP’s in the different continents is shown in Table IX.

TABLE IX: COMMON OMP SUBTYPE DISTRIBUTION (van Alphen, 1994) EXPRESSED AS %

<table>
<thead>
<tr>
<th>OMP</th>
<th>North America</th>
<th>South America</th>
<th>Europe</th>
<th>Asia</th>
<th>Africa</th>
<th>Australia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H</td>
<td>26</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1L</td>
<td>5</td>
<td>0</td>
<td>14</td>
<td>28</td>
<td>8</td>
<td>48</td>
</tr>
<tr>
<td>2H</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2L</td>
<td>23</td>
<td>50</td>
<td>13</td>
<td>6</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td>3L</td>
<td>26</td>
<td>25</td>
<td>71</td>
<td>45</td>
<td>21</td>
<td>48</td>
</tr>
<tr>
<td>6U</td>
<td>4</td>
<td>25</td>
<td>1</td>
<td>18</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>8H</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13L</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

In the US, five subtypes accounted for 87% of all Hib isolates (Barenkamp et al, 1981c), and 39% of disease isolates were of the subtype 1H. This was the most commonly observed subtype in the US (Munson et al, 1989) and was not found in isolates from developing countries. van Alphen (1994) suggests that the H subtypes might be more virulent than the L subtypes. The 3L subtype was found to be the common subtype in Western Europe (van Alphen et al, 1987; Munson et al, 1989) and represented 16% of isolates from Papua New Guinea, 55% of isolates from South and Central America, and 33% of isolates from Thailand and the Philippines. The subtype 6U accounted for 48% of isolates from Papua New Guinea and was also observed in isolates from Thailand, Nigeria and Kenya (Munson et al, 1989).

In the Netherlands, van Alphen et al (1983a) analyzed 80 Hib strains from patients with meningitis and found that 75% of the strains were characterised as
OMP subtype 3L. In a later study (van Alphen et al, 1987) 93% of Hib strains from patients with invasive disease in the Netherlands were found to be subtype 3L. This subtype also predominated in Western Europe with the exception of Iceland where subtype 2L predominated and were characterised by lipopolysaccharide (LPS)-9 whilst 2L subtypes in other parts of the world were characterised by LPS-2.

It was thought that this subtype was peculiar to Iceland and that the circulation of Hib among Icelandic children was facilitated by an increased number of day-care centres (van Alphen et al, 1987). However, with an increase in the population in summer due to tourism, it was interesting to note that Iceland retained its particular strain of Hib (van Alphen, 1994) and it was possible that host factors affecting colonisation were important for distribution of the subtype.

2.3.4.2 OMP subtypes as disease markers

In a study by Hampton et al (1983), 24 carriers of Hib were identified in a study population of 1,448 healthy children under 5 years old. The children had no known previous contact with a patient with Hib disease, had not attended a day-care centre and had not received antibiotic therapy in the week prior to commencement of the study.

The OMP subtypes of these strains were compared to those of 50 isolates from patients with Hib disease. The five most common subtypes found were 1L, 1H, 2L, 2H and 3L and there was no significant difference in the distribution of these subtypes among carriage and disease strains. However, there was a significant difference in the distribution of the unusual 13L subtype which had a higher frequency among carriage strains, suggesting that Hib strains with this subtype might be less virulent than strains with other subtypes.

Outer membrane protein analysis has been used to study the transmission of Hib in day-care centres with cases of invasive disease (Barenkamp et al, 1981c; Edmonson et al, 1982). Secondary cases were caused by strains with the same
OMP subtype as the strain from the index case. The subtype identified was 1H indicating that a single strain of Hib was responsible for the respective initial and subsequent cases of disease in day-care centres. Edmonson et al (1982) also found that recurrent infection was caused by the same subtype of Hib indicating that patients did not develop protective immunity.

Another study on children in day-care (Murphy et al, 1987) found that subtype 6U was associated significantly with cases among black children. This subtype was rare in the US and has been isolated in Pakistan (Weinberg et al 1989) where 95% of Hib isolates from blood of children with lower respiratory tract infection were identified as subtype 6U/12U.

Losonsky et al (1984), characterised Hib from cases of systemic disease among Apache Indian children using OMP and LPS subtyping. They demonstrated that particular OMP subtypes were repeatedly implicated in causing disease in Apache Indians, although they did not identify the specific subtypes. It was also emphasised that OMP or LPS subtyping alone was not sufficiently discriminatory as isolates with the same OMP subtype had differences in LPS subtype and vice versa.

A study of unrelated strains of multiresistant Hib (Dimopoulou et al, 1992), revealed indistinguishable patterns within a geographical region but different patterns between regions. In a hospital in Durban, Peer (1988, unpublished), isolated 16 multiple antibiotic resistant strains of Hib from patients with invasive disease and found that 2 strains exhibited the subtype 2L and the remaining 14 were designated as 2L'.

Analysis of disease strains in the United Kingdom (Urwin et al, 1995) and Australia (Hansman and Lawrence, 1993) indicate a predominance of subtype 3L which is the most abundant strain isolated in Western Europe. Bijlmer et al (1992), found that in the Gambia subtype 5 and 9 were found more often in healthy carriers whilst subtypes 2, 4 and 8 were associated with invasive disease. These were subtypes classified according to van Alphen's scheme (1983b).
Rocephin®

ceftriaxone

THE POWER OF ONE

Reg. No's: Ceftriaxone 350 mg: R/20.1.1/44; Ceftriaxone 500 mg: R/20.1.1/45; Ceftriaxone 1 g: R/20.1.1/46; Ceftriaxone 2 g: R/20.1.1/47.

Full details of composition, indications, contra-indications, side-effects, dosage and precautions are available on request from Roche Products (Pty) Ltd, PO Box 4595, Johannesburg 2000.
Temporal shifts in distribution of subtypes causing disease in a community may occur as evidenced by the fact that IH and 3L subtypes accounted for 76% of disease strains compared to an earlier study in which they accounted for only 47% (Hampton et al., 1983). Musser et al. (1985) suggest that temporal variation in frequency may be due to modification in demographic features of the host population rather than a genetic change in the bacterium.

Whilst these results clearly indicate subtype differences between disease and carrier isolates, subtypes causing disease have been found in the throat of healthy individuals (van Alphen, 1993). McCarty et al. (1986) isolated a strain of Hib resistant to rifampicin from a child whose sibling attended a day-care facility where another child had developed Hib meningitis earlier. Subsequently, specimens were cultured from the oropharynx of 12 individuals who were family members or day-care contacts of the patient. Hib was isolated from both parents and the sibling. OMP analysis was performed on the Hib isolates from both patients and the three carriers. All 5 isolates had identical OMP profiles indicating that they represented a single Hib strain. Thus, carriers can be regarded as reservoirs for Hib and therefore, elimination of carriage is thought to be an important step in the control of Hib disease.

2.4 ANTIMICROBIAL SUSCEPTIBILITY

Treatment and cure of Hib bacteraemia and its complications requires treatment with antimicrobials that will penetrate the blood-brain barrier and reach effective concentrations. Treatment should be of adequate duration to eliminate the organism from the primary site of infection as well as any secondary foci (Mendelman and Smith, 1987). Therefore, the choice of antibiotic must take into account the 'regional' antibiotic susceptibility patterns of disease isolates (Mendelman and Smith, 1987).

2.4.1 Susceptibility testing

In 1987 an improved medium for antimicrobial susceptibility testing of
*H. influenzae* was described by Jorgensen et al. The development of this medium was a response to the need for a complex growth medium which would satisfy the growth requirements of the organism but pose a minimal risk of antagonism of antimicrobial agents (Jorgensen *et al.*, 1987). Haemophilus test medium (HTM), incorporates Mueller-Hinton medium with additions growth-promoting additives viz. 15 μg/ml haematin, 15 μg/ml NAD and 5 mg/ml of yeast extract. Jorgensen *et al* (1987) found that on initial testing, virtually all isolates grew and the accuracy of susceptibility tests was not adversely affected.

When compared with other media, the advantages of HTM included optical clarity, stability, utility for testing trimethoprim and sulphonamide and its commercial availability (Doern and Jones, 1988).

However, in a comparative study by Mendelman *et al* (1990) it was found that not all strains of *H. influenzae* grew adequately. Two preparations of HTM were used, laboratory prepared (PHTM) and commercial (CHTM). The percentage of strains that did not grow on PHTM and CHTM were 37 and 8% respectively.

When an inoculum size of $10^5$ CFU was used, three of seven ampicillin resistant non-β-lactamase producing strains were incorrectly reported as being susceptible but were found to be resistant when supplemented brain heart infusion (BHI) agar was used (Mendelman *et al.*, 1990). Also, CHTM was not cost effective when compared with supplemented Mueller-Hinton agar. The routine use of HTM for susceptibility testing of *Haemophilus* was therefore not recommended.

Jones *et al* (1993) clearly state that whilst HTM was a medium recommended by the NCCLS for the testing of *H. influenzae*, it was functioning comparably but not superiorly to other described media. The use of Oxoid Diagnostic Sensitivity Test (DST) agar supplemented with NAD and lysed horse blood was an acceptable alternative (L van Alphen, personal communication). In a study of 190 strains in The Gambia between 1985 and 1987 (Thesis, Bijlmer *et al.*, 1992), in vitro susceptibility testing was performed using isosensitest agar supplemented with lysed horse blood and NAD.
2.4.2 Antibiotic resistance

Antibiotic resistance in the species *H. influenzae* is of clinical importance especially since serotype b is responsible for severe invasive disease in children. Prompt treatment with effective antibiotics is essential (Rotimi and Turk, 1981). During the 1950's chloramphenicol and tetracycline were used for treatment of infections due to *H. influenzae* (Williams and Moosdeen, 1986). In 1961 ampicillin was introduced and rapidly became the drug of choice due to its efficacy and also because of concern about the effect of chloramphenicol and tetracycline on bone marrow and teeth respectively.

Resistance to ampicillin was first reported in 1972 by Mathies (cited by Needham, 1988) followed by other similar reports (Turk, 1974; Thomas *et al.*, 1974; Khan *et al.*, 1974; Price and Boswell, 1974; MMWR, 1974). In South Africa, the first ampicillin resistant strain of *H. influenzae* was isolated from cerebrospinal fluid (CSF) in 1976 (cited by Block *et al.*, 1980).

Since the emergence of ampicillin resistance, ampicillin together with chloramphenicol have effectively been used in the initial treatment of bacterial meningitis (Kenny *et al.* 1980). However, with the emergence of strains resistant to chloramphenicol (Long and Phillips, 1977; Mendelman and Smith, 1987) as well as strains resistant to both antibiotics (Kenny *et al.*, 1980; Simasathien *et al.*, 1980; Garvey and McMullin, 1983; Coovadia *et al.* 1986; Hoosen *et al.*, 1987), alternative therapeutic agents are essential.

In three surveys in the United Kingdom carried out by Williams and Moosdeen (1986), the gradual increase in the number of strains resistant to different antibiotics is apparent. The results of these surveys are shown in Table X.

<table>
<thead>
<tr>
<th>AGENT</th>
<th>PERCENTAGE OF STRAINS RESISTANT TO ANTIMICROBIAL AGENT IN INDICATED YEAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>10,0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>0</td>
</tr>
<tr>
<td>Sulphonamide</td>
<td>1,5</td>
</tr>
</tbody>
</table>

2.4.2.1 Ampicillin resistance

The increase in reports on isolation of ampicillin resistant strains of *H. influenzae* resulted in greater interest in and investigations on the resistant strains (Needham, 1988). It became evident that resistance was due to the production of a β-lactamase enzyme (Farrar and O'Dell, 1974) which was plasmid mediated. The enzyme closely resembled β-lactamase found in other gram-negative bacteria especially *Klebsiella pneumoniae* and was identical to the β-lactamase produced by *Escherichia coli* (Needham, 1988).

The gene coding for enzyme production, TEM-1, was located on the chromosome and the plasmid, when this was present or on the chromosome alone in the absence of a plasmid (Willard *et al.*, 1982; Ling *et al.*, 1993). The mechanism of resistance for non-β-lactamase producing ampicillin resistant strains is thought to be due to the alteration of penicillin-binding proteins (PBP’s) resulting in decreased affinity of penicillin-binding protein 5 and, in some strains, penicillin-binding protein 4 (Thorne and Farrar, 1979; Williams and Moosdeen, 1986; Mendelman and Smith, 1987; Needham, 1988). PBP’s are enzymes involved in cell wall synthesis and are present in bacterial cell membranes. These proteins are able to form covalent bonds with penicillin and related antibiotics resulting in the initiation of the bactericidal effect of β-lactam antibiotics (Needham, 1988).
Ampicillin resistance mediated by β-lactamase was found to be the most common form of resistance encountered and enzyme production was higher among type b strains compared to non-type b strains (Nissinen et al, 1995). Resistance due to non-β-lactamase producing strains was rare and it has been postulated that the virulence of these strains was decreased due to the presence of abnormal PBP's (Nissinen et al, 1995).

Syriopoulou et al (1978), studied 1,208 isolates of *H. influenzae* in the US over a three year period and found that the annual incidence of ampicillin resistance increased from 1.4 to 5.3% in the first year to 14 to 16% in the third year. The prevalence of resistance among type b strains and non-b isolates was equal and patients who were recently treated with ampicillin were more likely to harbour resistant strains.

Schwartz et al (1983) investigated the prevalence of oropharyngeal carriage of ampicillin resistant strains of Hib in a geographic region which had a high prevalence of infections due to ampicillin-resistant Hib. Recent exposure to ampicillin was associated with an increase in the recovery of ampicillin-resistant strains.

A 5-year study of the epidemiology of invasive disease due to *H. influenzae* in the US revealed an increase in β-lactamase positive strains from 4.2% to 31% (Istre et al, 1984). No comment was made on the number of different strains isolated.

A study by Kristensen and Mortensen (1991) in Denmark yielded 135 strains of Hib isolated from cases of invasive disease over a period of 15 months. Seven strains (5.2%) produced β-lactamase and were resistant to ampicillin. No resistance to chloramphenicol was detected.

### 2.4.2.2 Chloramphenicol resistance

The first strain of Hib which was confirmed by the Centre for Disease Control, USA, to be resistant to chloramphenicol was isolated from an infant with meningitis in 1976 (Long and Phillips, 1977). The MIC's for both
chloramphenicol and tetracycline were 32 μg/ml. Resistance to both these antibiotics was plasmid mediated (van Klingeren et al., 1977) as demonstrated by loss of resistance by strains when treated with acridine orange. It was also found that resistance markers to chloramphenicol and tetracycline were transferred to other strains of *H. influenzae* as one single unit. Occasionally, the tetracycline marker was lost in the process of transfer (van Klingeren *et al.*, 1977).

A study of nine chloramphenicol-resistant strains of *H. influenzae* revealed the presence of conjugative plasmids in all nine strains (Roberts *et al.*, 1980). The plasmids coded for resistance to chloramphenicol and tetracycline or chloramphenicol, tetracycline and ampicillin. These isolates all produced chloramphenicol acetyltransferase (CAT) and it was concluded that chloramphenicol resistance was via plasmid-mediated production of chloramphenicol acetyltransferase.

CAT catalyses the diacetylation of the antibiotic with acetyl coenzyme A (Needham, 1988). In a rapid method for the detection of CAT (Azemun *et al.*, 1981), free coenzyme A was measured spectrophotometrically as a measure of the amount of chloramphenicol that was inactivated.

A second, less common, mechanism for chloramphenicol resistance has also been reported (Williams and Moosdeen, 1986; Needham, 1988). This type of resistance is chromosomally mediated and is due to a decrease in the uptake of the antibiotic by the cell. Burns *et al.*, 1985 (cited by Needham *et al.*, 1988) postulated that changes in the relative permeability of the outer membrane to the antibiotic mediated this type of resistance. This change in permeability was associated with the loss of an outer membrane protein viz. a porin (Mendelman and Smith 1987).

A National Collaborative study conducted in the US (Doern *et al.*, 1988) revealed that only 0.5% (14) of 2 811 clinical isolates of *H. influenzae* were reported as resistant to chloramphenicol as well as tetracycline. Of these 14 strains, only 2 were type b and both also produced β-lactamase. A similar survey of 21
Hospitals in Wales revealed 1,533 strains of *H. influenzae* of which 1.2% (19) were resistant to chloramphenicol (Howard *et al.*, 1986). In this survey as well the number of resistant type b strains was minimal as only 1 type b strain was isolated and was found to be resistant to ampicillin, tetracycline and trimethoprim in addition to chloramphenicol. In a British study (Powell *et al.*, 1986), 1.7% of 2,400 strains were reported to be chloramphenicol resistant. This was slightly higher than the percentage reported earlier ((Philpott-Howard and Williams, 1982) in which a prevalence rate of 1.03% was found. In South Africa one strain of chloramphenicol resistant Hib was isolated from the cerebrospinal fluid of a 4-month old child (Krajewska *et al.*, 1986). The isolate also produced β-lactamase and the MIC for chloramphenicol using the agar dilution method was 9.6 μg/ml.

### 2.4.2.3 Tetracycline, sulphamethoxazole, rifampicin and trimethoprim resistance

The incidence of tetracycline resistance among strains isolated in the UK (Powell *et al.*, 1986), decreased slightly from 3.1% in 1982 to 2.7% in 1987. However, a significant increase in the resistance to trimethoprim was noted viz. 1.4% in 1981 to 4.2% in 1986. Resistance to sulphamethoxazole was 3.5%. Only 3 type b strains were resistant to these antibiotics.

Two mechanisms for resistance to trimethoprim have been documented (Mendelman and Smith, 1987), viz. decreased penetration of the antibiotic and production of a variant target enzyme, dihydrofolate reductase, which was not inhibited by the drug.

In experiments performed by de Groot *et al* (1988), the gene for trimethoprim resistance was cloned and they concluded that the mechanism of trimethoprim resistance in *H.influenzae* was overproduction of chromosomally located dihydrofolate reductase

The National Collaborative study in the US (Doern *et al.*, 1988) revealed the following prevalence of resistance: tetracycline 2.3% (20 type b strains),
trimethoprim-sulphamethoxazole 0,9% (5 type b isolates) and rifampicin 0,7%. Only 0,1% were susceptible to erythromycin. Methods for detection of resistance to erythromycin have been problematic. Mendelman and Smith (1987) suggest that high level resistance could be a one-step mutation and that this was chromosomally determined.

Rifampicin was used in a day-care centre as a preventive measure after a child developed Hib meningitis (McCarty et al, 1986). Subsequently, a sibling of one of the attendees of the day-care centre also developed Hib meningitis. The OMP subtypes of the Hib strains isolated were identical but the strain isolated from the second child was resistant to rifampicin. The virulence of these isolates were studied further in infant rats. The conclusion from this study was that mutation of H. influenzae to rifampicin resistance was a hazard of rifampicin chemoprophylaxis.

2.4.2.4 Multiple antibiotic resistance

A strain of H. influenzae which was resistant to chloramphenicol, ampicillin and tetracycline was reported in Canada in 1978 (Bryan, 1978). In 1979 in Thailand (Simasathien et al, 1980), three children died of invasive Hib disease caused by strains resistant to ampicillin and chloramphenicol. Subsequent studies on nasopharyngeal carriage of resistant strains yielded a carriage rate of 47% for resistant Hib. Attempts at eradication of carriage using rifampicin were unsuccessful. Strains that were resistant to chloramphenicol and ampicillin demonstrated a one-fold increase in the MIC for rifampicin (MIC 0,4 vs 0,2 μg/ml).

In the United States (Kenny et al, 1980) a resistant strain of Hib was isolated from a 12-month old infant in 1979. The organism demonstrated in vitro resistance to ampicillin, chloramphenicol, tetracycline and carbenicillin. The MIC for each of the antibiotics was 8 μg/ml or greater. The isolate was found to be sensitive to rifampicin for which it demonstrated an MIC of 0,5 μg/ml.
The first British case of Hib resistant to chloramphenicol and ampicillin occurred in 1983 (Garvey and McMullin, 1983) in a 30-month old child. The organism produced both β-lactamase and CAT. The MIC's for ampicillin and chloramphenicol were 128 and 12 μg/ml respectively. The isolate was sensitive to sulphamethoxazole and trimethoprim.

A high prevalence of ampicillin and chloramphenicol resistant strains of Hib were reported from Spain and it was concluded that it had become an endemic problem (Campos et al, 1984a; Campos et al, 1986). Susceptibility studies were performed on 77 multiply resistant strains of *H. influenzae* between 1981 and 1983 (Campos et al, 1984b). Of these, 39 were serotype b and were isolated from the following clinical specimens: cerebrospinal fluid, 16; blood, 4; ear, 7 and nasopharynx, 12. The majority of the isolates, 94.8%, were resistant to ampicillin, chloramphenicol, tetracycline, sulphamethoxazole and trimethoprim. These resistant strains were susceptible to rifampicin.

In another study in Spain (Campos et al, 1986) between 1981 and 1984, 57% of Hib strains isolated from cases of community acquired meningitis were resistant to both chloramphenicol and ampicillin. A high percentage of these resistant strains were also resistant to tetracycline, trimethoprim and sulphamethoxazole. All strains were found to be susceptible to rifampicin. In a further study in Spain (Campos et al, 1987), the emergence of multiply resistant strains of Hib in 4 day-care centres prompted a study on the efficacy of rifampicin in eradicating carriage of these strains. It was concluded that rifampicin was effective in reducing carriage of strains that were susceptible to rifampicin.

In South Africa the first case of Hib resistant to both chloramphenicol and ampicillin was reported in 1985 (Coovadia et al, 1986). The organism was isolated from cerebrospinal fluid of a 9-month old infant. Subsequently, Krajewska et al (1986) reported a similar case in Pretoria. Further cases of multiply resistant Hib were reported from various parts of the world viz. Mexico, (Guiscafre et al, 1986), Britain (Fraise et al, 1986), Saudi Arabia (Karrar et al, 1987), Durban (Hoosen et al, 1987) and the US (Givner et al, 1989).
Jorgensen et al. (1988) conducted a nationwide antimicrobial surveillance study in the US and recovered 2,811 isolates of *H. influenzae*. A group of 106 strains (4%) were identified as being multiply resistant strains. Only 35 strains produced β-lactamase of which 7 were serotype b and 2 of these were resistant to ampicillin, chloramphenicol and tetracycline.

Multiple resistance to antibiotics in non-encapsulated, non-pathogenic strains of *H. influenzae* were shown to be transmissible *en bloc* to *Escherichia K12*, and at a lower frequency to *H. influenzae* type d. A single, 62Mdal plasmid was found in the donor strain (Rotimi and Turk, 1981). This finding is of epidemiological concern since the possibility exists that the plasmid could be transferred to virulent type b strains.

2.4.3 Susceptibility to other antibiotics

2.4.3.1 Cephalosporins

Strains of *H. influenzae* that produce β-lactamase were found to be susceptible to second and third generation cephalosporins (Jorgensen et al., 1988). However, strains that were resistant to ampicillin but did not produce β-lactamase demonstrated reduced susceptibility to second generation cephalosporins viz. cefuroxime and cefonicid. These strains were susceptible to third generation cephalosporins viz. cefotaxime, cefizoxime, ceftiraxone, ceftazidime, moxalactam and cefixime.

Ceftriaxone was found to be highly efficacious in the treatment of meningitis due to Hib (Aronoff et al., 1984) with 90% of strains being inhibited at a concentration of 0.06 μg/ml. Cefaclor was found to be the least active against Hib with no difference in MIC's between β-lactamase positive and negative strains (Doern and Chapin, 1986). Similar results were obtained by Campos and Garcia-Tornel (1987), Nash et al. (1991), Barry et al. (1993) and James et al. (1993).
2.4.3.2 Quinolones

All multiply antibiotic resistant strains were highly susceptible to ciprofloxacin and pefloxacin (Jorgensen et al, 1988) with MIC values equal to or less than 0.25 μg/ml.

2.4.3.3 Macrolides

The activity of macrolides against gram-negative bacteria is influenced by pH of the medium. Lower MIC’s are shown with increase in pH up to 8.5. The presence of rRNA methylases is the primary mechanism of resistance of macrolides.

Erythromycin was also found to be ineffective against H. influenzae and MIC’s greater than 8 μg/ml were demonstrated (Doern and Chapin, 1986). The newer macrolide, azithromycin, demonstrated excellent activity against H. influenzae with 90% of strains being inhibited by 1 μg/ml. Two other new macrolides, clarithromycin and roxithromycin, were four- to eightfold less active (Neu, 1991). Goldstein et al (1990) demonstrated 100% inhibition with azithromycin compared to 16% with erythromycin and 5% with roxithromycin.

2.5 IMMUNISATION

2.5.1 Vaccine development

2.5.1.1 Polysaccharide vaccine

Evidence of the importance of antibodies in the host defence against Hib infections first came from Fothergill and Wright who, in 1933, demonstrated that the blood of young children lacked bactericidal activity against Hib. The blood of older children and adults was bactericidal (cited by Kayhty et al 1994a). Interest in the development of a vaccine against Hib centred around the capsular polysaccharide of type b strains viz. polyribosylribitol-phosphate (PRP). The
polysaccharide was purified and characterised and its immunogenicity demonstrated in laboratory and clinical studies (Rodrigues et al. 1971; Anderson et al. 1972; Robbins et al. 1973; Anderson and Smith 1977).

The PRP vaccine was registered in the United States in 1985 based on the results of a Finnish efficacy trial (Peltola et al. 1977), and was the first vaccine available for the prevention of Hib disease. This trial was conducted to establish the efficacy of a Group A meningococcal vaccine and Hib PRP vaccine was used in the control group. Consequently, it was established that efficacy of the vaccine was 90% in children between 18 months and 2 years of age (Peltola et al., 1977). However, the vaccine was not effective in children less than 18 months old and was not completely immunogenic or protective even in older children (Ward, 1988). Serum antibody levels in the first two years of life were found to be below the accepted protective level of 1 µg/ml and persisted for a shorter duration compared with induced antibody levels in older children. More importantly, the antibodies were mainly of the IgM class and a second dose of the vaccine did not elicit a memory response, suggesting that antibodies to PRP were T-independent (Parke, 1987). The age related antibody response to the vaccine is illustrated in Table XI (Peltola, 1977).

### TABLE XI: SERUM ANTIBODY LEVELS IN IMMUNISED FINNISH CHILDREN (Peltola, 1977)

<table>
<thead>
<tr>
<th></th>
<th>LEVEL (µg/ml) BY AGE IN MONTHS</th>
<th>% WITH LEVELS &lt;0,15 µg/ml IN MONTHS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-17</td>
<td>18-23</td>
</tr>
<tr>
<td>Hib PRP vaccine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preimmunisation</td>
<td>0,10</td>
<td>0,25</td>
</tr>
<tr>
<td>Postimmunisation</td>
<td>0,36</td>
<td>3,17</td>
</tr>
<tr>
<td>1,5 years after</td>
<td>0,36</td>
<td>0,72</td>
</tr>
<tr>
<td>Immunisation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Percentages for age groups 3 to 6, 6 to 12 and 12 to 17 months.

Results of studies in the United States (Murphy, 1987; Shapiro et al., 1988) show concordance with the results obtained in Finland. However, in a case-control day care-based study, Harrison et al. (1988) demonstrated a 45% level of efficacy,
which was less than expected. They also emphasised the usefulness of case-control studies for monitoring vaccine efficacy. Another case-control study (Osterholm et al, 1988) indicated that the vaccine had a minimal or no effect on the prevention of invasive Hib disease. The protective efficacy level obtained in this study was -32%.

Overall, the rate of efficacy of the vaccine was lower in the US than in Finland. The use of case-control based methodology has been suggested as a possible reason for the lower figures obtained (Kayhty et al, 1994a) since this could lead to underestimation of vaccine efficacy.

2.5.1.2 Conjugate vaccines

Licensure of the polysaccharide vaccine laid the groundwork for the development of conjugate vaccines in the early 1980's. Eskola et al (1985) evaluated a vaccine containing PRP conjugated to diphtheria toxoid (PRP-D) and compared their findings with serological data collected in connection with the PRP trial in Finland in 1974. They found that antibody levels greater than 1 μg/ml were induced in 50% of the children immunised at 3, 5, and 7 months and in 57% of those vaccinated at 7 and 9 months. These responses were almost as high as that obtained in children aged 18 to 23 months using PRP only. The major difference between the vaccines was the antibody response after the second dose was administered - a booster response was evident. Although Eskola et al (1985) concluded that inclusion of PRP-D in the immunisation schedule might be justified, they were of the opinion that clinical efficacy had to be proven before this could occur.

Two clinical trials in adults and similar trials in children aged 2 to 7 months, 7 to 14 months, 7 to 9 months and 15 to 24 months were carried out (Lepow, 1987). PRP-D was found to be consistently immunogenic in all age groups and was licensed for use in the US in December 1987. Since then, a number of other conjugate vaccines have been licensed for approval (Table XII, Murphy et al 1993a).
A different protein carrier is used by each of the manufacturers of conjugate vaccines. HbOC (HibTITER, Lederle) is a conjugate of oligosaccharides of PRP i.e., less than 30 repeat units of the ribose sugar conjugated to a mutant diphtheria toxin (CRM\textsubscript{197}) which does not require inactivation with formalin since toxicity is reduced due to a point mutation (Ellis, 1994).

**TABLE XII: *HAEMOPHILUS INFLUENZAE TYPE b VACCINES IN THE UNITED STATES* (Murphy *et al*, 1993a; MMWR, 1993)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Licensure or approval</th>
<th>Recommended age months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysaccharide (PRP)</td>
<td>April 1985</td>
<td>24-59 (18-23*)</td>
</tr>
<tr>
<td><strong>Conjugate vaccines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRP-D</td>
<td>December 1987</td>
<td>18</td>
</tr>
<tr>
<td>HbOC</td>
<td>December 1988</td>
<td>18</td>
</tr>
<tr>
<td>PRP-D, HbOC, PRP-OMP</td>
<td>December 1989</td>
<td>15</td>
</tr>
<tr>
<td>HbOC</td>
<td>October 1990</td>
<td>2 (multidose)</td>
</tr>
<tr>
<td>PRP-OMP</td>
<td>December 1990</td>
<td>2 (multidose)</td>
</tr>
<tr>
<td>PRP-T</td>
<td>March 1993</td>
<td>2 (multidose)</td>
</tr>
<tr>
<td>DTP-HbOC</td>
<td>March 1993</td>
<td>2 (multidose)</td>
</tr>
</tbody>
</table>

*Children at increased risk of invasive Hib disease.

PRP-D indicates Hib PRP coupled to diphtheria toxoid

PRP-OMP is Hib PRP coupled to *Neisseria meningitidis* outer membrane proteins

PRP-T is Hib PRP coupled to tetanus toxoid

DTP-HbOC is diptheria-tetanus toxoids - pertussis coupled to Hib oligosaccharide

HbOC is Hib oligosaccharide of PRP coupled to a mutant diphtheria toxin

### 2.5.2 Protective efficacy of conjugate vaccines

In non-immunised individuals minimum antibody levels of 0.15 µg/ml have been
established as being protective. After immunisation with a polysaccharide vaccine, 1 μg/ml is associated with protection (Anderson, 1984). In a letter to the Lancet, Anderson (1984) argues that a peak level of 1 μg/ml is necessary to assure a minimal level of 0.1 μg/ml during the course of one year. He pointedly states that there can be no assumption of vaccine efficacy based only on a 'protective level' achieved a few weeks after immunisation. Of greater importance was the continued long term protection conferred by circulating minimum levels of antibody viz. 0.1 μg/ml after immunisation.

In a study of a high risk Navajo population, the immunogenicity of HbOC was compared to that of combined HbOC and bacterial polysaccharide immune globulin (BPIG) (Letson et al, 1988). It was found that combined passive and active immunisation at 2 months maintained protective antibody levels of 0.15 μg/ml up to 6 months. Also, there was no interference with the active antibody response when infants were subsequently immunised with HbOC only at the age of 4 and 6 months. However, 2 month old infants who received only HbOC did not respond favourably to the first dose and only 27% demonstrated an increase in antibody after the first dose. The conclusions from this study were that a single dose of HbOC vaccine induced a protective antibody response in 18 month old children and that 2 and 7 month old infants required 3 and 2 doses respectively to achieve a similar response.

In 1991 the conjugate vaccine Hib-OMP was evaluated among Navajo and Apache infants and children (Santosham et al, 1991) and was found to be safe and highly immunogenic. One dose was administered to children aged 12 to 60 months. Infants were immunised at 2 months and between 12 and 15 months after the initial dose. All the children who received one dose only were found to have protective antibody levels of at least 1 μg/ml one month after immunisation. Infants responded poorly to the first dose and only 11% of the Apaches and 8% of the Navajos had protective antibody levels. However, these percentages progressively increased with subsequent vaccinations and after the booster dose, all children had protective antibody levels.
Similar antibody levels were obtained by Mulholland et al (1993) in a study using PRP-OMP to immunise Gambian children aged 1 to 4 months. However, Mulholland et al (1993) argue in favour of a booster dose for children aged 12 to 15 months especially in developing countries. This is supported by evidence in their study that after 18 months, the children did not have protective antibody levels. They also suggest that protection might not necessarily be indicated by antibody level once immunisation had been achieved.

In Alaskan infants, the PRP-D vaccine was found to have limited efficacy (Ward et al, 1990). After three doses of the vaccine the efficacy was only 35% and was not related to any factors such as age and maternal antibodies. Control groups were used in the study and it was found that antibody levels in the immunised group increased only after administration of the second dose. Even after the third dose only 48% of the infants demonstrated protective levels of 0.1 µg/ml. In contrast to these results, Eskola et al (1990) reported that PRP-D was found to be effective in children in Finland.

In 1991 a study of the impact of combined active and passive immunisation on Alaskan infants was initiated (Singleton et al, 1994). A dramatic decrease in the incidence of disease was observed in this high risk population. Unlike the study on Navajo infants by Letson et al (1988), all Alaskan babies were given bacterial polysaccharide immune globulin (BPIG) at birth and every 3 months thereafter until the age of 10 months (Singleton et al, 1994). The conjugate vaccine used was PRP-OMP. The conclusion from the study by Singleton et al (1994) concurred with that of Letson et al (1988) viz. that simultaneous administration of BPIG resulted in minimum antibody levels of 0.15 µg/ml.

The safety and protective efficacy of HbOC conjugate vaccine (HibTITRE) was investigated in 1 to 6 month old infants (Madore et al, 1990a) and in 15 to 23 month old infants (Madore et al, 1990b). In the former group, antibody levels 2 months after the first dose of the vaccine were minimal but there was a significant increase after the second dose with 84% having antibody levels greater than 1 µg/ml. More than 98% of these infants had antibody levels greater than
1 μg/ml after the third dose of the vaccine.

In the second study (Madore et al. 1990b), only one dose of the vaccine was administered since all the children were at least 15 months old. One month after immunisation, 99% of the infants had protective antibody levels greater than or equal to 1 μg/ml. Six months later all subjects achieved protective levels of 1 μg/ml.

In a comparative study, Kim et al. (1990) compared the immune responses of children aged 18 to 58 months to the polysaccharide vaccine as well as to a conjugate vaccine. Response to the PRP vaccine was limited and it was suggested that age and ethnicity were contributing factors. Postvaccination antibody concentrations of > 1 μg/ml were observed in 36% of children 18 to 23 months of age and in 59% of children older than 24 months of age. The conjugate vaccine (PRP-OMP) was immunogenic in 94% of children regardless of age.

Vadheim et al. (1993) conducted a controlled clinical trial to determine the efficacy of the conjugate vaccine PRP-T. The study was prematurely terminated after one year but the results indicate that PRP-T was equivalent to other conjugate vaccines in terms of safety and immunogenicity.

Although all conjugate vaccines primed infants to evoke a memory antibody response to PRP, Granoff et al. (1993) suggest that differences in priming may affect the ability of the different vaccines to confer protection against disease. Other factors which could affect efficacy of any of the vaccines were socioeconomic factors, age and ethnicity (Eskola et al., 1990; Kim et al., 1990). PRP-D was less immunogenic than other conjugate vaccines and was recommended for use in children at least 15 months of age (Valdapena et al., 1995).

2.5.3 Effect of immunisation on incidence of disease

The compulsory immunisation of all infants with a conjugate vaccine has been
offered as an explanation for the sharp decline in the incidence of Hib meningitis in Finland from 30 in 1986 to none in 1991 (Peltola, 1992). In the UK, the efficacy of the vaccine has been reported to be greater than 98% with a large decline in the incidence of invasive Hib disease (Slack et al, 1993). In Germany, the incidence of disease after the introduction of large-scale vaccination was 1.9 per 100,000 compared to the Netherlands which was 0.6 per 100,000 after vaccination (von Kries et al, 1994).

2.5.4 Antibody response in compromised hosts

The response to PRP-D vaccine was evaluated in children with acute lymphoblastic leukaemia (Lange et al, 1989). The majority of the children responded to the vaccine with a sustained antibody level of 0.15 μg/ml. However, only 50% achieved a level of 1 μg/ml and were able to retain this level 6 months later. The booster effect could not be demonstrated in these children. A study of the response of chronically ill premature infants to PRP-OMP (Washburne et al, 1993) indicates that these infants do not respond to PRP-OMP as well as term infants. It was suggested that this could be due to delayed maturation of the immune system.

Steinhoff et al (1991) studied the immune response to PRP-CRM in men with human immune deficiency virus (HIV) infection. It was found that immunisation early in infection was likely to confer protection against Hib disease and correlated with a high level of CD4 lymphocytes in the blood. Immunisation using a conjugate vaccine was found to be protective after a second dose was administered to patients having recurrent infections, IgA deficiency or common variable immunodeficiency (Barra et al, 1992). Children with sickle cell anaemia demonstrated protective levels of antibody after immunisation with HbOC (Gigliotti et al, 1989). There does not appear to be any published information on the immune response of children with tuberculosis, children with malnutrition or institutionalised children.
2.5.5 Vaccine efficacy in developing countries

The epidemiology of Hib disease in developing countries is different to that in developed countries and therefore different intervention strategies are necessary (Mulholland and Greenwood, 1994). The peak age related incidence is much lower than in developing countries and most infants are immune by the end of the first year either due to a period of carriage of Hib or colonisation with another cross-reacting organism (Mulholland and Greenwood, 1994). There is a difference in the clinical spectrum of disease between developed and developing countries. Meningitis and epiglottitis are the most common manifestations of Hib disease in developed countries. Meningitis and pneumonia are more prevalent in developing countries whilst epiglottitis is extremely rare (Greenwood, 1992).

As discussed in section 2.1.2, the incidence of Hib disease may vary considerably with varying risk factors that depend on cultural, genetic and socioeconomic factors. Similarly, genetic and environmental factors could affect the performance of Hib vaccines in developing countries. Mulholland and Greenwood (1994) suggest the following possible reasons for differences in vaccine efficacy between developed and developing countries (Table XIII).

**TABLE XIII: REASONS FOR VARIATIONS IN VACCINE EFFICACY BETWEEN DEVELOPED AND DEVELOPING COUNTRIES**

<table>
<thead>
<tr>
<th>Variations in genetic control over responsiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA class 2-related</td>
</tr>
<tr>
<td>Other</td>
</tr>
<tr>
<td>Variations in the prevalence of environmental factors influencing responsiveness</td>
</tr>
<tr>
<td>Protein-calorie malnutrition</td>
</tr>
<tr>
<td>Micronutrient deficiencies</td>
</tr>
<tr>
<td>Suppressive effect of infections</td>
</tr>
<tr>
<td>Variations in the level of exposure</td>
</tr>
<tr>
<td>Route of exposure to infection</td>
</tr>
<tr>
<td>Infective dose</td>
</tr>
</tbody>
</table>
Before any conjugate vaccine can be included in an expanded programme of immunisation in any developing country, it has to be subjected to clinical and protective efficacy trials similar to those conducted in developed countries (Mulholland and Greenwood, 1994).

2.5.6 Effect of conjugate vaccines on carriage

Colonisation with Hib induces both serum and mucosal antibody responses to the Hib capsular polysaccharide. Pichichero and Insel (1983) describe the concomitant development of mucosal and serum antibodies to PRP in 8 out of 10 children aged 18 months to 7 years. The mucosal antibodies were of the IgA type and were thought to afford local protection although the precise mechanism of action was not known. Surface proteins are thought to mediate adherence and colonisation of Hib (Beachey, 1981) but it is not known how mucosal antibodies to PRP could interfere with this step (Takala, 1994a).

The effect of vaccination on oropharyngeal carriage has been evaluated for PRP-D, HbOC and PRP-OMP conjugate vaccines. This information is summarised in Table XIV (Takala, 1994b).

Takala et al (1991) evaluated the effect of PRP-D on oropharyngeal carriage of Hib in 725 healthy children in Finland. An oropharyngeal carriage rate of 3.5% was noted in children who had not been vaccinated prior to swabbing while no carriage was detected in children who had been vaccinated. In a similar study in the US using PRP-D (Murphy et al, 1993b) as well as PRP only, it was found that PRP-D decreased oropharyngeal colonisation while the rate of colonisation after vaccination with PRP was the same as in unvaccinated children. Murphy et al (1993b) argue that their study was a more stringent test of the efficacy of the vaccine in preventing colonisation since it was based on results from children who attended day-care centres. Both vaccinated as well as unvaccinated children were therefore exposed to this risk of colonisation.
The effect of HbOC on oropharyngeal carriage was evaluated by Barbour et al (1993) in the UK. A 4-year follow-up was performed on 120 children who had been enroled in an immunogenicity trial in which 60 of them had received HbOC vaccine. No significant difference was found for the carriage rate of Hib between vaccinated and non-vaccinated children. However, the children who were colonised had higher serum anti-capsular IgG levels than children who were not colonised. Among the children who were colonised, vaccinated children had higher antibody levels than unvaccinated children viz. 53,7 and 2.8 μg/ml respectively. This difference in antibody levels suggests that vaccination had primed the vaccinees so that subsequent mucosal challenge with Hib resulted in a booster response.

Takala et al (1993) evaluated the effect of PRP-OMP on oropharyngeal carriage of Hib among Navajo and Apache Indian children. This was a population that had a high risk for invasive Hib disease. A total of 1423 oropharyngeal swabs were obtained from 1321 children of which 293 were obtained prior to vaccination, 1119 were taken after the primary series of PRP-OMPC vaccinations and 11 were taken after the booster dose was administered. Of the total swabs taken, 40 were positive for Hib and 13% of the carriers had received PRP-OMPC appropriate for age at swabbing compared to 36% of non-carriers. A greater percentage of carriers had respiratory infections at the time of swabbing than non-carriers, 48% and 30% respectively. The presence of respiratory infection increased the risk of carriage 2-fold. Takala et al (1993) concluded that PRP-OMPC vaccine reduced oropharyngeal carriage of Hib, an effect similar to that of PRP-D vaccine.

However, the effect of PRP-OMPC was not as dramatic as the effect of PRP-D in the study in Finland (Takala et al, 1991) in which carriage was eliminated. Also, there was a high rate of carriage at a younger age among Navajo and Apache children in accordance with the high incidence of invasive Hib disease in both these populations.
# TABLE XIV: EFFECT OF CONJUGATE VACCINES ON CARRIAGE OF H. INFLUENZAE TYPE b (Takala, 1994b)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Control group</th>
<th>Mean Age at swab (range)</th>
<th>Effect of vaccine compared to control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRP-D (190)</td>
<td>No vaccine</td>
<td>Finnish 36 mo (32-43)</td>
<td>Reduction of carriage of Hib</td>
</tr>
<tr>
<td>PRP-D (137)</td>
<td>No vaccine</td>
<td>Finnish 36 mo (32-43)</td>
<td>Reduction of carriage of Hib</td>
</tr>
<tr>
<td>PRP-D (89)</td>
<td>No vaccine</td>
<td>Texas 33 mo +13 mo</td>
<td>Reduction of carriage of Hib</td>
</tr>
<tr>
<td>HbOC (60)</td>
<td>No vaccine</td>
<td>British 52 mo (NA)</td>
<td>Reduction of carriage; decreased intensity of carriage; booster response upon carriage</td>
</tr>
<tr>
<td>PRP-OMP</td>
<td>Not vaccinated</td>
<td>Arizona 9.7 mo (3-56)</td>
<td>Reduction of carriage of Hib; Concurrent respiratory infection increased risk for carriage</td>
</tr>
</tbody>
</table>

The information in the above Table indicates that conjugate vaccines did not eradicate carriage of Hib. However, a reduction of carriage occurred with PRP-D and PRP-OMP. The vaccine HbOC did not reduce carriage but reduced the intensity of carriage. Also, carriage of Hib was found to enhance the immune response to HbOC.
CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 ISOLATION AND IDENTIFICATION OF STRAINS

3.1.1 Sampling and immunisation

The collection of specimens and immunisation of children began in July 1993. All children already in the paediatric wards at King George V Hospital were investigated. All swab specimens were collected by the same individual for this initial group. Specimens for subsequent admissions were collected by the nursing staff on admission. The last new patient was sampled and immunised on the 30 November 1993.

Oropharyngeal and nasopharyngeal swabs were collected and immediately inoculated onto two chocolate agar plates one of which contained 10μg/ml bacitracin. The plates were then taken to the laboratory where the inoculum was streaked out and the plates incubated at 37°C in a CO₂ incubator. Venous blood specimens were taken from all the children and the serum separated and stored at -72°C until required. The children were immunised and the number of doses administered was according to the immunisation schedule provided by Lederle Laboratories (appendix A).

One month after immunisation, oropharyngeal and nasopharyngeal swabs as well as blood samples were again collected from the same children. The swabs and blood specimens were processed as above.

Prior to the commencement of specimen collection and immunisation, laboratory staff at King George V Hospital processed blood and CSF specimens from patients with invasive disease. Isolates identified as *Haemophilus influenzae* were further analyzed as in 3.2 and 3.3 below.
3.1.2 Identification of isolates

Strains were identified as *Haemophilus influenzae* by the following criteria: (a) colonial morphology on chocolate agar; (b) Gram stain morphology - gram negative pleomorphic bacilli; (c) the requirement for V factors as demonstrated by the test for satellitism on blood agar using a staphylococcal streak and (d) the requirement for X factor as demonstrated by a negative porphyrin test. (Hausler, Moyer and Holcomb; 1985)

3.2 CHARACTERISATION OF ISOLATES

3.2.1 Serotyping of isolates

All isolates identified as *Haemophilus influenzae* (3.1.2) were serotyped using a commercial slide agglutination kit (Murex Diagnostics, UK) according to the manufacturers instructions. All isolates demonstrating a positive agglutination reaction with type b antiserum were cultured onto chocolate agar plates and incubated overnight at 37°C in a CO₂ incubator. Using a bent sterile pasteur pipette, a heavy inoculum of the growth from the plate was placed into sterile cryotubes having 0.4 ml of brain heart infusion (BHI) broth containing 10% glycerol and sterile glass beads. The tube was vortexed, immersed in liquid nitrogen to snap freeze the culture, and stored at -72°C until required.

3.2.2 Biotyping

The biotype of all type b isolates was determined using the API NH system (Bio Merieux, France) according to the manufacturers instructions (Appendix B). Using this system, which is based on enzymatic reactions and sugar fermentation, the identification of isolates was confirmed as well as the production of penicillinase. The reference strain used as a control was *Haemophilus influenzae* ATCC 10211 (manufacturer's recommendation).
3.2.3 Outer Membrane Protein (OMP) analysis

Outer membrane protein profiles of isolates of *Haemophilus influenzae* type b were determined according to the method of Barenkamp *et al* (1981a). Reference strain A920001, obtained from Dr. van Alphen (Netherlands) was included as a control.

Overnight chocolate agar cultures of isolates were inoculated into 50ml of brain heart infusion broth supplemented with 10μg/ml of NAD and 10μg/ml of haemin (sBHI) (Appendix C). Broths were incubated at 37°C overnight on a rotary shaker to ensure aeration of cultures. Cells were harvested by centrifugation at 10 000 rpm for 20 minutes at 4°C using 50ml Beckman polyallomer centrifuge tubes and a JA-29 rotor in a Beckman J-218 centrifuge. The pellet was resuspended in 10ml of 10mM HEPES pH7.4 then sonicated four times in 15 second bursts in an ice bath using a Brunswick sonicator. The sonicate was centrifuged at 4000 rpm for 20 minutes at 4°C and supernatant was carefully removed into 10ml Beckman polycarbonate tubes and centrifuged at 50 000 rpm for one hour at 4°C using a Beckman 50-Ti rotor and L8-55 ultracentrifuge. A clear gel-like pellet was obtained and resuspended in 1ml of 10mM HEPES pH7.4 to which 1ml of 2% sodium lauryl sarcosinate in 10mM HEPES (pH7.4) was added and the contents incubated at room temperature for 30 minutes. The tubes were again subjected to ultracentrifugation at 50 000 rpm for one hour at 4°C and the sarcosinate insoluble fraction was harvested and resuspended in 150μl sterile distilled water and stored at -20°C.

A mixture of 50μl of protein sample and 50μl sample loading buffer (Appendix D) was boiled at 100°C for 2 minutes and cooled immediately by refrigeration.

For electrophoresis, a 12.5% resolving gel and 5% stacking gel were prepared (Appendix D). A 20 well comb was inserted into the glass slab prior to the stacking gel being poured. On polymerisation of the gel, the comb was removed and the wells washed with sterile distilled water.
Running buffer of pH 8.3 (Appendix D) was poured into the gel tank and 50μl of the boiled sample/loading buffer mixture was carefully loaded into each well. The same volume (50μl) of a mixture of protein molecular weight markers ranging from 170 kDa to 14.3 kDa was also loaded. The gel was electrophoresed at 200V until the samples had passed through the stacking gel after which the voltage was reduced to 50V and the gel electrophoresed overnight.

The gel was removed and stained for 30 minutes in 0.125% Coomassie Brilliant Blue R250 dissolved in water:methanol:acetic acid (5:5:1). It was destained first in the same solvent for one hour and then destained exhaustively in 7% acetic acid, 5% methanol until the gel was clear.

Some strains were analyzed further in the Microbiology laboratory at the Academic Medical Centre of the University of Amsterdam. The method used (unpublished) was developed by Dr. L. van Alphen. Overnight cultures on chocolate agar were used to prepare bacterial suspensions having an optical density of 1 at 549nm using phosphate buffered saline pH7.4. Reference strains 760705, 770235 and 770277 of *Haemophilus influenzae* were included. Equal volumes of the sample, 400 μl, and sample loading buffer (without mercaptoethanol) were added to duplicate eppendorf tubes. One set of tubes was heated at 100°C for 10 minutes. The resolving and stacking gel were prepared as in Appendix F. A volume of 20 μl of sample mixture was loaded onto the gels and electrophoresed at 50mA for 4 hours. A protein molecular weight marker, 8 μl, was also loaded. After 4 hours, the gel was removed and stained for 16 hours on a shaker in 0.1% Coomassie blue R250 in 10% acetic acid:25% methanol:65% water. The stain was poured off and replaced with destaining solution (solvent used to make up the stain) and the gel destained for 6 hours during which the destaining solution was replaced with fresh solution hourly.

A modified ELISA method (Abdillahi and Poolman, 1987) using a monoclonal antibody 5HA5 was used to determine whether protein bands were of the L or non-L type.
3.3 ANTIMICROBIAL SUSCEPTIBILITY TESTING

3.3.1 Enzyme tests

All isolates identified as *Haemophilus influenzae* type b were tested for chloramphenicol acetyltransferase production using the method of Slack *et al* (1977). Tests for β-lactamase production were performed using Intralactam strips (Mast Diagnostics, UK) according to the manufacturers instructions (Appendix B).

3.3.2 Disc susceptibility tests

All isolates were tested for susceptibility to ampicillin, erythromycin, tetracycline, chloramphenicol, and rifampicin on chocolate agar using the Kirby-Bauer method. Plates were incubated at 37°C overnight. Results were recorded as sensitive (S), moderately sensitive (ms) or resistant (R) based on zone sizes. With the exception of rifampicin, all the discs contained 10 µg of antibiotic. The concentration of the rifampicin was 5 µg/disc.

3.3.3 Test for Minimum Inhibitory Concentration (MIC)

The MIC's were determined by the agar dilution method.

3.3.3.1 Bacterial strains

All previously confirmed isolates of *Haemophilus influenzae* type b were tested for their *in vitro* susceptibility to antimicrobial agents. Eighteen hour chocolate agar cultures of stored isolates were used. Three control strains were included with every test batch. These were reference cultures of *Haemophilus influenzae* ATCC 49247, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213.
3.3.3.2 Antimicrobial agents

The antimicrobial agents tested were powders of stated potencies for laboratory use supplied by pharmaceutical companies. All antibiotics were dissolved in appropriate solvents to prepare stock solutions. These were then diluted in water to provide a final range of concentration from 128μg/ml to 0.008μg/ml when tested. The antibiotics tested and their sources are shown in Table XV.

3.3.3.3 Culture media

DST agar (Oxoid) supplemented with 10 μg/ml NAD and 0.25% lysed horse blood was prepared (appendix C) and kept molten at 50°C. Freshly prepared, appropriately diluted antibiotic solutions in 5ml amounts were added to 20ml of molten agar. This was mixed thoroughly and dispensed into 9cm sterile petri dishes. The agar medium was allowed to solidify at ambient temperature and dried at 37°C for 30 minutes. All plates were kept at 4°C and used within 24 hours of preparation. Antibiotic free plates were used for growth control.

3.3.3.4 Inoculum preparation

Colonies of *Haemophilus influenzae* type b from a 18 hour chocolate agar culture were suspended in 5ml Mueller Hinton broth (MHB) and the turbidity adjusted to match that of a 0.5 MacFarland standard. A 1 in 10 dilution was then prepared in MHB for inoculation onto agar containing antibiotics. Inoculation of media was made within 30 minutes of adjusting the inoculum.
TABLE XV: ANTIBIOTICS TESTED

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ampicillin</td>
<td>SmithKline Beecham</td>
</tr>
<tr>
<td>2. Chloramphenicol</td>
<td>Parke-Davis</td>
</tr>
<tr>
<td>3. Rifampicin</td>
<td>Mer National</td>
</tr>
<tr>
<td>4. Cefotaxime</td>
<td>Roussel</td>
</tr>
<tr>
<td>5. Ceftriaxone</td>
<td>Roche</td>
</tr>
<tr>
<td>6. Amoxicillin</td>
<td>SmithKline Beecham</td>
</tr>
<tr>
<td>7. Clavulanic acid</td>
<td>SmithKline Beecham</td>
</tr>
<tr>
<td>8. Sulphamethoxazole</td>
<td>Roche</td>
</tr>
<tr>
<td>9. Cefuroxime</td>
<td>Glaxo</td>
</tr>
<tr>
<td>10. Cefazolin</td>
<td>Eli-Lilly</td>
</tr>
<tr>
<td>11. Gentamicin</td>
<td>Sigma</td>
</tr>
<tr>
<td>12. Amikacin</td>
<td>Bristol-Myers Squibb</td>
</tr>
<tr>
<td>13. Erythromycin</td>
<td>Abbott</td>
</tr>
<tr>
<td>14. Azithromycin</td>
<td>Pfizer</td>
</tr>
<tr>
<td>15. Roxithromycin</td>
<td>Roussel</td>
</tr>
<tr>
<td>16. Pefloxacine</td>
<td>MayBaker</td>
</tr>
<tr>
<td>17. Ofloxacine</td>
<td>Hoechst</td>
</tr>
<tr>
<td>18. Ciprofloxacin</td>
<td>Bayer</td>
</tr>
<tr>
<td>19. Tetracycline</td>
<td>Lawrence Laboratories</td>
</tr>
<tr>
<td>20. Imipenem</td>
<td>Logos</td>
</tr>
<tr>
<td>21. Aztreonam</td>
<td>Bristol-Myers Squibb</td>
</tr>
<tr>
<td>22. Doxycycline</td>
<td>Pfizer</td>
</tr>
<tr>
<td>23. Minocycline</td>
<td>Lederle Laboratories</td>
</tr>
<tr>
<td>24. Trimethoprim</td>
<td>Fisons</td>
</tr>
<tr>
<td>25. Lomefloxacin</td>
<td>Searle</td>
</tr>
</tbody>
</table>

3.3.3.5 MIC determination

The diluted suspensions were applied to agar plates using a Cathra replicator (Rousseau and Harbec, 1987). The final inoculum applied to the testing media
contained approximately $10^5$ colony forming units (CFU). Antibiotic free plates were inoculated at the beginning and end of each set of antibiotic plates to ensure viability of organisms and to check for potential contamination. All plates were incubated at 37°C in a CO$_2$ incubator for 18 hours. After incubation, plates were examined for growth. The growth on the antibiotic plates was compared to the growth on the antibiotic free plates. Very fine hazy growth or single colonies were considered to indicate no growth.

The MIC of each antibiotic was defined as the lowest concentration of an antibiotic which inhibited growth of the organism.

### 3.4 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

#### 3.4.1 Preparation of antigen and reference sera

HbO-HA antigen (1 mg/ml), a reference standard serum having a value of 70 µg/ml total immunoglobulin and a reference control serum with an immunoglobulin value of 4.75 µg/ml were supplied by Prof. Porter Anderson from Rochester University, USA. Dilutions of the reference sera, patients' sera and the conjugate (Protein G peroxidase) were prepared using 3% skimmed milk (Lactogen) in 0.5 M Tris - 0.1 M NaCl, pH 8.0 (Tris/saline).

#### 3.4.2 Determination of antigen concentration for coating of plates

Corning, flat bottom, disposable, sterile polystyrene ELISA plates with 96 wells were used. 1 ml of sterile, pyrogen-free water was added to a vial of HbO-HA antigen containing 1 mg antigen. Doubling dilutions of the antigen were prepared in 0.05M bicarbonate buffer (pH 9.6). The dilutions ranged from 1 in 125 to 1 in 32 000. 100 µl of the dilutions were added to a row of wells beginning with the 1 in 125 dilution and ending with the 1 in 32 000 dilution. This was done in quadruplicate. The ELISA plate was covered and placed in a moist chamber at room temperature overnight then washed with 0.05% Tween 20 in Tris-saline (TST).
cupboard at room temperature for 30 minutes. Thereafter the reaction was stopped with 100 μl 2M H₂SO₄. The plate was read as before, and the reference control value at different dilutions was determined against the reference standard using the ELISA+ (Meddata Inc) computer software available at the Natal Blood Transfusion Services.

### 3.4.4 Determination of concentration of secondary standard

Pooled serum from three paediatric patients was used to prepare a secondary standard. Dilutions of the reference standard were prepared and added to triplicate wells as in 3.4.3 above. The pooled serum was diluted beginning with a 1 in 2 dilution and ending with a 1 in 256 dilution. 50 μl of each dilution was added to triplicate wells beginning with the 1 in 256 dilution and 50 μl of milk was added to three wells as blanks. The procedure followed was the same as in 3.4.2, and the concentration of antibody in the pooled serum, at different dilutions, was determined using the reference standard. This pooled serum was thereafter used as a secondary standard with the established value.

### 3.4.5 Determination of total antibody concentration of all patients’ sera

All tests were performed in triplicate and dilutions of the secondary standard were prepared as in 3.4.4. The reference control was diluted 1 in 2, and the procedure followed was as in 3.4.2. Total immunoglobulin concentration was determined against the secondary standard using the software available.
CHAPTER 4

4.0 RESULTS

The study was conducted amongst patients admitted to the paediatric wards of a tuberculosis hospital (King George V) between July and November 1993. Specimens collected were oropharyngeal and nasopharyngeal swabs as well as venous blood. These specimens were collected prior to immunisation and one month after immunisation.

4.1 DEMOGRAPHIC DATA

The total number of patients studied was 135, 89 of whom were already in the wards at the commencement of the study and 46 were admitted subsequently over the study period.

The sex distribution of the patients indicated a ratio of 3:1 of which 97 (72.4%) were males and 38 (27.6%) females. The patients ranged in age from 4 months to 14 years 8 months. The mean and median ages were 52.8 and 37.5 months respectively. Table XVI illustrates the age distribution of the patients. The vaccine was administered according to the recommended schedule (Lederle, appendix A) which was based on the age of the children. Three patients required 3 doses of the vaccine, four required 2 doses and the remainder required one dose only.

4.2 CLINICAL DATA

During the 5 month study period, none of the patients who had been immunised developed invasive disease due to Haemophilus influenzae type b (Hib). Of a total of 135 patients, 8 were known to be HIV positive.

The patients who were studied were all receiving treatment for tuberculosis which included rifampicin. Nine children had chickenpox at the time of immunisation.
Of the 89 patients who were in the wards at the commencement of the study, 10 were being treated for infections other than tuberculosis. Five of these patients received treatment for infection due to Hib and 4 of the strains isolated were β-lactamase positive. Nine other patients had a previous history of isolation of Hib.

Of the total of 46 subsequent admissions to the study wards, 15 were receiving antibiotics at the time of admission. The antibiotics were either amoxycillin, penicillin VK, amoxycillin/clavulanic acid, amikacin, flucloxacillin, fucidic acid, cloxacinil, pipracillin, cotrimoxazole or ampicillin.

4.3 ORO- AND NASOPHARYNGEAL CARRIAGE

The carriage of *Haemophilus influenzae* in the oro- and nasopharynx of patients before and after immunisation is illustrated in Table XVII. Pre- and post-immunisation isolates of *Haemophilus influenzae* were 81 and 109 respectively. The isolation rate for nasopharyngeal carriage only was low, 0 and 2% in pre- and post-immunisation specimens respectively. The yield from the oropharynx only was 19% prior to immunisation and 14% after immunisation.

A total of 25 patients had received antibiotics prior to immunisation and 6 of these patients were carriers of *Haemophilus influenzae* type b before receiving the vaccine. Of these, 3 carried multiple antibiotic resistant strains of Hib and had been previously treated with either amoxycillin or amoxycillin/clavulanic acid. One month after immunisation, 11 of these patients were colonised by Hib and only 1 still carried a resistant strain.
# TABLE XVI: AGE DISTRIBUTION OF PATIENTS (N = 135)

<table>
<thead>
<tr>
<th>AGE</th>
<th>NO. PATIENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 6 months</td>
<td>3</td>
</tr>
<tr>
<td>7 - 11 months</td>
<td>4</td>
</tr>
<tr>
<td>12 months - 2 years</td>
<td>30</td>
</tr>
<tr>
<td>2 - 3 years</td>
<td>27</td>
</tr>
<tr>
<td>3 - 4 years</td>
<td>20</td>
</tr>
<tr>
<td>4 - 5 years</td>
<td>10</td>
</tr>
<tr>
<td>5 - 10 years</td>
<td>28</td>
</tr>
<tr>
<td>10 - 15 years</td>
<td>13</td>
</tr>
</tbody>
</table>
TABLE XVII: ISOLATION OF *HAEMOPHILUS INFLuenzAE* FROM ORO- AND NASOPHARYNX

<table>
<thead>
<tr>
<th></th>
<th>PREIMMUNISATION (N = 135)</th>
<th>POSTIMMUNISATION (N = 135)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO.</td>
<td>%</td>
</tr>
<tr>
<td>Nasopharynx only</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Oropharynx only</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>Naso- and oropharynx</td>
<td>55</td>
<td>41</td>
</tr>
<tr>
<td>Negative for naso-</td>
<td>54</td>
<td>40</td>
</tr>
</tbody>
</table>

TOTAL ISOLATES:

PREIMMUNISATION = 81

POSTIMMUNISATION = 109
4.4 CHARACTERISATION OF ISOLATES

4.4.1 Serotype distribution

Prior to immunisation 23 (28%) isolates were non-typable whilst after immunisation this was reduced to 22 (20%). Isolation of other serotypes was minimal as indicated in Table XVIII.

Of a total of 81 and 109 pre- and post - immunisation isolates of *Haemophilus influenzae* respectively, type b numbered 51 (63%) and 84 (77%) respectively with 34 patients being colonised by Hib both before and after immunisation. Fifty-one patients did not harbour Hib either before or after immunisation. The overall carriage rate of Hib is illustrated in Table XIX.

There was no observed increase in carriage of *Haemophilus influenzae* type b (Hib) by any particular age group. Colonisation of children aged between 0 -6 months was not detected either before or after immunisation. Table XX illustrates the distribution of Hib carriage by age. Of the 51 patients colonised by Hib prior to immunisation, 50% were from 7 to 36 months old. Colonisation in this age group decreased to 44% after immunisation. In children older than 5 years, post immunisation carriage increased to 34% compared to a carriage rate of 25% before immunisation.

4.4.2 Biotypes

Table XXI illustrates the random distribution of biotypes of *Haemophilus influenzae* type b. Post immunisation isolates were not analyzed since it was apparent from the results of the pre- immunisation biotypes that this did not provide additional information. Common biotypes were observed between β-lactamase producing and non-producing strains.
**TABLE XVIII: SEROTYPE DISTRIBUTION OF ISOLATES**

<table>
<thead>
<tr>
<th>SEROTYPE</th>
<th>PRE - IMMUNISATION (N = 80)</th>
<th>POST - IMMUNISATION (N = 108)</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>51 (64%)</td>
<td>84 (78%)</td>
</tr>
<tr>
<td>a</td>
<td>0</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>d</td>
<td>2 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>e</td>
<td>1 (1%)</td>
<td>0</td>
</tr>
<tr>
<td>f</td>
<td>3 (4%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>non-typable</td>
<td>23 (29%)</td>
<td>22 (20%)</td>
</tr>
</tbody>
</table>
TABLE XIX: CARRIAGE OF *H.*INFLUENZAE* TYPE b BY STUDY PATIENTS BEFORE AND AFTER IMMUNISATION (N = 135)

<table>
<thead>
<tr>
<th>Colonisation Status</th>
<th>Number of Patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonised before and after immunisation</td>
<td>34 25</td>
</tr>
<tr>
<td>Colonised before immunisation only</td>
<td>17 13</td>
</tr>
<tr>
<td>Colonised after immunisation only</td>
<td>50 37</td>
</tr>
</tbody>
</table>
TABLE XX: DISTRIBUTION OF *HAEMOPHILUS INFLUENZAE* TYPE b BY AGE

<table>
<thead>
<tr>
<th>AGE</th>
<th>PREIMMUNISATION (N = 51)</th>
<th>POSTIMMUNISATION (N = 84)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 6 months</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7 -11 months</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>12 months - 2 years</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>2 - 3 years</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>3 - 4 years</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>4 - 5 years</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>5 - 10 years</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>10 - 15 years</td>
<td>5</td>
<td>11</td>
</tr>
</tbody>
</table>
TABLE XXI: BIOTYPES OF *HAEMOPHILUS INFLUENZAE* TYPE b

<table>
<thead>
<tr>
<th>BIOTYPE</th>
<th>NO.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 51)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>II</td>
<td>18</td>
<td>33</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>VI</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VII</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>VIII</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
4.5 CHARACTERISATION OF ISOLATES FROM PATIENTS WITH INVASIVE DISEASE

Prior to and during the study period, *Haemophilus influenzae* type b was isolated from 8 patients who had developed invasive Hib disease. Seven strains were isolated from blood culture of which 6 were multi-resistant producing both β-lactamase and chloramphenicol acetyltransferase. The remaining isolate from blood culture did not produce either enzyme. One multi-resistant strain was isolated from cerebrospinal fluid and produced both β-lactamase and chloramphenicol acetyltransferase. All 8 strains were of biotype II.

4.6 ANTIMICROBIAL SUSCEPTIBILITY TESTS

4.6.1 Tests for enzyme production

All isolates of *Haemophilus influenzae* were tested for the production of β-lactamase and chloramphenicol acetyltransferase. Table XXII illustrates the results of the enzyme tests. In general, there was a reduction in the number of post-immunisation isolates that produced either or both of the enzymes. The number of *H. influenzae* strains that did not produce any enzymes was 75% and 95% respectively for pre- and post-immunisation isolates.

The results of tests for enzyme production by type b strains are illustrated in Table XXIII. The number of type b strains producing both enzymes decreased from 25% to 4% from pre- to post-immunisation isolates respectively and production of β-lactamase only decreased from 2% to 1%. None of the isolates produced only chloramphenicol acetyltransferase either before or after immunisation. Prior to immunisation 73% of the isolates did not produce any enzymes. This figure increased to 95% after immunisation.
TABLE XXII: β-LACTAMASE (βL+) AND CHLORAMPHENICOL ACETYLTRANSFERASE (CAT+) PRODUCTION BY *HAEMOPHILUS INFLUENZAE*.

<table>
<thead>
<tr>
<th></th>
<th>βL+ AND CAT+</th>
<th>βL+ ONLY</th>
<th>CAT+ ONLY</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PRE-IMMUNISATION</strong> (N = 81)</td>
<td>18 (22%)</td>
<td>1 (1%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td><strong>POST-IMMUNISATION</strong> (N = 109)</td>
<td>4 (4%)</td>
<td>1 (1%)</td>
<td>0</td>
</tr>
</tbody>
</table>
TABLE XXIII: β-LACTAMASE (βL+) AND CHLORAMPHENICOL ACETYLTRANSFERASE (CAT+) PRODUCTION BY *HAEMOPHILUS INFLUENZAE* TYPE b

<table>
<thead>
<tr>
<th></th>
<th>βL+ AND CAT+</th>
<th>βL+ ONLY</th>
<th>CAT+ ONLY</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE-IMMUNISATION (N = 51)</td>
<td>13 (25%)</td>
<td>1 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>POST-IMMUNISATION (N = 84)</td>
<td>3 (4%)</td>
<td>1 (1%)</td>
<td>0</td>
</tr>
</tbody>
</table>
4.6.2 Disc susceptibility tests (Kirby-Bauer method)

Resistance of Hib to ampicillin, erythromycin, tetracycline and chloramphenicol was lower in post immunisation isolates ie. 5%, 2%, 2% and 4% respectively, compared to pre-immunisation isolates which were 27%, 16%, 25% and 25% respectively. A high prevalence of resistance to rifampicin was noted in both pre- (92%) and post-immunisation (100%) Hib isolates. Two pre-immunisation isolates were sensitive to rifampicin and two demonstrated moderate sensitivity. Table XXIV illustrates these results.

4.6.3 Minimum inhibitory concentration (MIC)

One hundred and thirteen isolates (113) of Haemophilus influenzae type b were tested against 24 antibiotics. A number of strains (30) did not grow following storage and after repeated unsuccessful attempts to revive these strains, they were excluded. Four of these isolates had demonstrated sensitivity to rifampicin (4.6.2 above).

The MIC range, MIC$_{50}$ and MIC$_{90}$ of the 113 strains of Haemophilus influenzae type b tested are shown in Table XXV. The cumulative percentages of strains inhibited at each concentration for the various antimicrobial agents tested are shown in Figures 1 to 6. Twenty-two of these strains produced both β-lactamase and chloramphenicol acetyltransferase. The MIC range, MIC$_{50}$ and MIC$_{90}$ of these 22 strains are shown in Table XXVI.

The quinolones were the most effective of all the antibiotics tested and all the strains were within the sensitive range. The MIC$_{90}$'s of ciprofloxacin, lomefloxacin, pefloxacin and ofloxacin were 0,03, 0,25, 0,25 and 0,12 µg/ml respectively. When tested against β-lactamase producing strains, the MIC$_{90}$ for ciprofloxacin was fourfold greater whereas that for lomefloxacin, pefloxacin and ofloxacin were only twofold greater than was observed for non-β-lactamase producing strains.
TABLE XXIV: ANTIMICROBIAL SUSCEPTIBILITY PROFILE OF PRE- AND POST IMMUNISATION STRAINS OF *HAEMOPHILUS INFLUENZAE* TYPE b USING A DISC DIFFUSION METHOD (KIRBY-BAUER)

<table>
<thead>
<tr>
<th>ANTIBIOTIC</th>
<th>PRE-IMMUNISATION (N = 51)</th>
<th>POST-IMMUNISATION (N = 84)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPICILLIN</td>
<td>14 (27%)</td>
<td>4 (5%)</td>
</tr>
<tr>
<td>ERYTHROMYCIN</td>
<td>8 (16%)</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>TETRACYCLINE</td>
<td>13 (25%)</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>CHLORAMPHENICOL</td>
<td>13 (25%)</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>RIFAMPICIN</td>
<td>47 (92%)</td>
<td>84 (100%)</td>
</tr>
<tr>
<td>ANTIBIOTIC</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/ml)</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Amox/Clav</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Imipenem</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>2.0</td>
<td>128</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>1.0</td>
<td>16</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.008</td>
<td>0.01</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.5</td>
<td>8.0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>64</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>64</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Amikacin</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Lomefloxacin</td>
<td>0.06</td>
<td>0.25</td>
</tr>
<tr>
<td>Pefloxacin</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td>Minocycline</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

* Amox/clav = amoxycillin : clavulanic acid (2:1)
**TABLE XXVI: IN-VITRO SUSCEPTIBILITIES OF β-LACTAMASE AND CAT+ PRODUCING STRAINS OF *H. INFLUENZAE* TYPE b (N = 22)**

<table>
<thead>
<tr>
<th>ANTIBIOTIC</th>
<th>MIC$_{50}$ (µg/ml)</th>
<th>MIC$_{90}$ (µg/ml)</th>
<th>RANGE (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>4</td>
<td>32</td>
<td>1 - 64</td>
</tr>
<tr>
<td>Amox/Clav</td>
<td>2</td>
<td>2</td>
<td>0,25 - 4</td>
</tr>
<tr>
<td>Imipenem</td>
<td>1</td>
<td>8</td>
<td>0,5 - 16</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>0,06</td>
<td>0,12</td>
<td>0,03 - 0,12</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>1</td>
<td>16</td>
<td>0,008 - 128</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>2</td>
<td>2</td>
<td>0,01 - 8</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0,01</td>
<td>0,03</td>
<td>0,008 - 0,06</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0,008</td>
<td>0,01</td>
<td>0,008 - 0,03</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>16</td>
<td>16</td>
<td>0,5 - 64</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>64</td>
<td>128</td>
<td>8 - &gt;128</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
<td>2 - &gt;128</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>64</td>
<td>64</td>
<td>0,5 - 64</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>4</td>
<td>8</td>
<td>2 - 8</td>
</tr>
<tr>
<td>Amikacin</td>
<td>16</td>
<td>32</td>
<td>4 - 32</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0,01</td>
<td>0,12</td>
<td>0,008 - 0,25</td>
</tr>
<tr>
<td>Lomefloxacin</td>
<td>0,06</td>
<td>0,5</td>
<td>0,03 - 1</td>
</tr>
<tr>
<td>Pefloxacin</td>
<td>0,06</td>
<td>0,25</td>
<td>0,01 - 0,05</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>0,06</td>
<td>0,25</td>
<td>0,01 - 0,05</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>4</td>
<td>8</td>
<td>2 - 16</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>1</td>
<td>2</td>
<td>0,5 - 2</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>16</td>
<td>32</td>
<td>4 - 32</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>16</td>
<td>16</td>
<td>0,5 - 32</td>
</tr>
<tr>
<td>Minocycline</td>
<td>1</td>
<td>4</td>
<td>0,12 - 4</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>2</td>
<td>4</td>
<td>0,12 - 4</td>
</tr>
</tbody>
</table>

* Amox/clav = amoxycillin : clavulanic acid (2:1)
FIGURE 1:
Cumulative % minimum inhibitory concentrations for *H. influenzae* type b to ampicillin, amoxycillin:clavulanic acid, imipenem, rifampicin and aztreonam.
FIGURE 2:
Cumulative % minimum inhibitory concentrations for *H. influenzae* type b to cefazolin, cefuroxime, cefotaxime and ceftriaxone.
FIGURE 3:
Cumulative % minimum inhibitory concentrations for *H. influenzae* type b to chloramphenicol, sulphanethoxazole, trimethoprim, gentamicin and amikacin.
FIGURE 4:
Cumulative % minimum inhibitory concentrations for *H. influenzae* type b to ciprofloxacin, lomefloxacin, pefloxacin and ofloxacin.
FIGURE 5:
Cumulative % minimum inhibitory concentrations for *H.influenzae* type b to erythromycin, azithromycin and roxithromycin.
FIGURE 6:
Cumulative % minimum inhibitory concentrations for *H. influenzae* type b to tetracycline, doxycycline and minocycline.
Of the cephalosporins tested, cefazolin was the least effective having an MIC$_{90}$ of 128 $\mu$g/ml. All strains tested were sensitive to cefotaxime and ceftriaxone whereas the MIC$_{90}$ for cefuroxime was 16 $\mu$g/ml, which is the breakpoint for resistance based on NCCLS standards.

A high incidence of resistance to tetracycline was prevalent. The MIC$_{90}$ for tetracycline was 16 $\mu$g/ml and 49% of strains tested demonstrated MIC's greater than the NCCLS recommended breakpoint for resistance which is 8 $\mu$g/ml. The MIC$_{90}$'s for minocycline and doxycycline were 4 and 2 $\mu$g/ml respectively. There was no difference in the activity of tetracycline and minocycline against $\beta$-lactamase producing strains since the MIC$_{90}$'s were the same. However, the MIC$_{90}$ for doxycycline was two-fold higher for $\beta$-lactamase producing strains compared to non-$\beta$-lactamase producers.

Of the three macrolides tested, roxithromycin was the least active and had an MIC$_{90}$ of 32 $\mu$g/ml. Azithromycin showed a lower MIC than roxithromycin and erythromycin, demonstrating an MIC$_{90}$ of 2 $\mu$g/ml with 37% of the strains tested having MIC's between 1 and 4 $\mu$g/ml. The MIC$_{90}$ for erythromycin was 8 $\mu$g/ml. There was no difference in the activity of the macrolides against strains which produced $\beta$-lactamase and those that did not.

A high prevalence of resistance to sulphamethoxazole, trimethoprim and rifampicin was evident since the MIC$_{90}$'s were $>128$, 64, and $>128$ $\mu$g/ml respectively, irrespective of whether the strains produced $\beta$-lactamase or not. The MIC$_{90}$ of chloramphenicol was one-fold higher for $\beta$-lactamase producing strains.

The aminoglycosides tested did not show any difference in activity against strains producing $\beta$-lactamase. However, the MIC$_{90}$ of amikacin was two-fold higher than that of gentamicin.

Aztreonam showed the lowest MIC of all the $\beta$-lactam antimicrobials tested having an MIC$_{90}$ of 0,12 $\mu$g/ml for all strains tested. Imipenem and the combination of amoxycillin/clavulanic acid showed no difference in the
concentration required to inhibit 90% of strains whether they produced β-
lactamase or not. However, as expected, a marked difference in activity was
noted for ampicillin when the MIC<sub>90</sub> of β-lactamase producing strains was
compared with that of strains not producing the enzyme. Strains producing the
enzyme were inhibited by a three-fold greater concentration of the antibiotic.

4.7 OUTER MEMBRANE PROTEIN (OMP) ANALYSIS

Outer membrane proteins were extracted from 143 strains. Thirty-four patients
colonised with Hib before and after immunisation accounted for 68 strains. Of
the remaining strains, 8 were from patients with invasive disease, 17 from
patients colonised prior to immunisation only and 50 from patients who were
colonised after immunisation.

Of the 34 patients colonised before and after immunisation, only 5 were
colonised with the same outer membrane protein type. The remaining 29 patients
were colonised by different OMP types after immunisation. Plate 3 is a
photograph of a gel showing the diversity of outer membrane protein profiles
among the isolates. The profile in lane 3 is that of a β-lactamase producing
strain, and analyses carried out in the Department of Medical Microbiology (Academic Medical Centre, University of Amsterdam) under the expert guidance
of Dr. Loek van Alphen, revealed that this strain was type 1H.

Analysis of the OMP profiles of the remaining isolates from the immunised
patients indicated that of the 50 strains isolated from patients who were colonised
after immunisation, 8 different types of OMP profiles were prevalent. The most
prevalent type accounted for 32% of the isolates and a photograph of the relevant
gel is shown in plate 4. The reference strain is in lane 5 and protein molecular
weight markers in lane 6. The OMP profiles of isolates in lanes 1 to 3, 9 to 16,
18 and 20 are identical. The remaining OMP types accounted for the following
percentages in decreasing order of prevalence viz. 22%, 18%, 12%, 10%, and
2% each for the remaining 3 types.
Plate 3: Photograph of gel after SDS-PAGE showing OMP profiles of strains isolated before and after immunisation. Lane 5 is the reference strain A920001 (1L) and lane 6 is the protein molecular weight marker. Lanes 1 to 4 and 7 to 20 are paired samples from patients colonised before and after immunisation eg. lanes 1 and 2 are from the same patient but represent strains isolated before and after immunisation respectively.
Plate 4: Photograph of gel after SDS-PAGE showing OMP profiles of strains colonising patients after immunisation only. In lane 5 is the reference strain A920001 and lane 6 is the protein molecular weight marker.
Further analysis in Amsterdam of representative strains from this group was inconclusive. A more detailed study of the OMP types was required as a study on its own.

Analysis of the OMP profiles of the 17 isolates colonising patients before immunisation revealed 3 OMP types. One type accounted for 50% of these strains and included 5 β-lactamase producing strains. The remaining isolates were equally distributed between the remaining two OMP types. Plate 5 is a photograph of the relevant gel showing some of these strains. Lanes 11 to 19 are OMP profiles of 9 of these strains. Strains in lanes 11, 13, 14, 15 and 17 produced the enzyme β-lactamase. The profiles in lanes 11, 15 and 17 are identical.

The isolates from patients with invasive disease had identical OMP profiles with the exception of the strain that was negative for β-lactamase production (plate 5). Lanes 1 to 4 and 7 to 9 illustrate the OMP profiles of the resistant strains. Lane 10 is the sensitive strain. In this photograph, a resistant strain isolated from a patient who was colonised (lane 14), has the same protein pattern as the resistant strains causing invasive disease.

Further analysis in Amsterdam identified the resistant strains causing invasive disease as OMP type 1H. Plate 6 is a photograph of the gel showing the identical patterns demonstrated by a representative resistant strain causing invasive disease and a resistant strain from oropharyngeal colonisation. Both are type 1H. Scrutiny of OMP patterns of resistant strains from carriage revealed that 2 were type 1H.

Analysis of OMP patterns of representative colonisation strains, performed in Amsterdam, were inconclusive. A high degree of diversity was observed and it was thought that at least 13 types were prevalent. A more detailed study only of OMP patterns was necessary in order to type the organisms.
Plate 5: Photograph of gel after SDS-PAGE showing OMP profiles of invasive strains. Reference strain A920001 is in lane 5 and protein molecular weight markers in lane 5. Lanes 1 to 4 and 7 to 10 are protein patterns of isolates from cases of invasive disease. Protein patterns in lanes 11 to 19 are from strains isolated before colonisation only. Lane 20 is the protein pattern of an isolate from a patient colonised after immunisation only. Protein patterns from strains producing $\beta$-lactamase are represented in lanes 1 to 4, 7 to 9, 11, 13 to 15, and 17.
Plate 6: Photograph of a gel after SDS-PAGE (Amsterdam). The protein molecular weight marker is in lane 1. The protein patterns of the resistant strains from a colonised patient and a patient with invasive disease as well as the pattern of the reference strain type 1H are shown in lanes 22, 23 and 24 respectively. The protein pattern of reference type 7H is shown in lane 12. OMP patterns in lanes 2 - 5, 8, 9, 13 - 21 and 25 - 26 are from pre-immunisation isolates. Protein patterns of post-immunisation isolates are shown in lanes 6, 7, 10, 11 and 27.
4.8 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

4.8.1 Concentration of antigen for coating of plates

An antigen coating curve of optical density vs antigen concentration was plotted to determine the optimum concentration of antigen required to coat the ELISA plates (figure 7). A concentration of 1 in 100 of the antigen was used since the curve was almost at a plateau at a concentration of 1 in 125. The optimum concentration is that at which the curve just reaches a plateau.

4.8.2 Dilution of control serum and patients' serum

At a 1 in 2 dilution, the control serum antibody concentration was 4,86 µg/ml. The concentration indicated on the vial was 4,75 µg/ml. All serum samples were therefore diluted 1 in 2.

4.8.3 Concentration of internal standard

The concentration of the internal standard was determined against that of the reference standard and was calculated to be 16,48 µg/ml. Where the concentration of antibody in a patient's serum was found to exceed this value, the serum was diluted as necessary.

4.8.4 Concentration of antibody in patients' serum samples

A total of 133 paired serum samples were analyzed. Serum samples of 2 patients had leaked and were therefore excluded. Additional serum samples were taken from 7 patients after they had received a second dose of the vaccine. Only 6 samples were available for analysis since one had leaked in storage. Interpretation of results was based on the criteria that (a) in non-immunised individuals, antibody levels of 0,15 µg/ml confer short term immunity and (b) in immunised individuals a minimum antibody level of 1 µg/ml is associated with long term protection.
FIGURE 7:
Antigen coating curve used to determine concentration of antigen required to coat ELISA plates.
Prior to immunisation, 117 (88%) patients had antibody levels less than 0.15 μg/ml while only 16 (12%) had levels that were equal to or greater than 0.15 μg/ml. The mean and median values were 0.18 μg/ml and less than 0.10 μg/ml respectively. Only one child in the age group 4 to 24 months had a minimum antibody level of 0.15 μg/ml. Of the 16 children with minimum antibody levels of 0.15 μg/ml, 8 (50%) were over 8 years of age.

Postimmunisation, antibody levels increased but still 88 patients (66%) showed levels less than 1.0 μg/ml. Of these, 16 had antibody levels greater than or equal to 0.15 μg/ml. The number of patients that had antibody levels greater than 1.0 μg/ml was 45 (34%). The mean and median antibody levels after immunisation were 3.16 μg/ml and 0.3 μg/ml respectively.

Of the 45 children who demonstrated antibody levels greater than or equal to 1.0 μg/ml after immunisation, 50% were 7 years and older and 3 (7%) were in the age group 4 to 24 months. The age distribution of children having the recognised minimum protective antibody levels before and after immunisation is illustrated in Table XXVII. Of the total number of 133 children, only 11 children had minimum antibody levels of 0.15 and 1.0 μg/ml before and after immunisation respectively. None of these children were aged 4 to 24 months and 50% were 9 years and older. Table XXVIII illustrates the overall antibody levels of patients before and after immunisation.

The number of patients who showed the ideal antibody levels i.e. minimum antibody levels of 0.15 and 1.0 μg/ml before and after immunisation respectively was only 11. Of these, 7% were 30 months old and the remainder were 5 years and older. Patients who had received two doses of the vaccine i.e. children between 4 to 11 months, demonstrated an increase in antibody concentration after the second dose of the vaccine. Their response ranged from 0.4 to 35 μg/ml with a mean value of 10.68 μg/ml and median value of 4.75 μg/ml. None of these patients had a minimum antibody level of 0.15 μg/ml prior to immunisation or that of 1.0 μg/ml after the first dose of the vaccine.
TABLE XXVII: AGE DISTRIBUTION AND ANTIBODY RESPONSE BEFORE AND AFTER IMMUNISATION (N = 133)

<table>
<thead>
<tr>
<th>AGE</th>
<th>NO.</th>
<th>P only No (%)</th>
<th>R only No (%)</th>
<th>PR No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6 months</td>
<td>3</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>7-11 months</td>
<td>4</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>12-24 months</td>
<td>30</td>
<td>1 (3)</td>
<td>3 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2-3 years</td>
<td>27</td>
<td>0 (0)</td>
<td>4 (15)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>3-4 years</td>
<td>19</td>
<td>1 (5)</td>
<td>6 (32)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>4-5 years</td>
<td>10</td>
<td>2 (20)</td>
<td>3 (30)</td>
<td>1 (10)</td>
</tr>
<tr>
<td>5-10 years</td>
<td>28</td>
<td>1 (4)</td>
<td>12 (43)</td>
<td>5 (18)</td>
</tr>
<tr>
<td>10-15 years</td>
<td>12</td>
<td>0 (0)</td>
<td>5 (42)</td>
<td>4 (33)</td>
</tr>
</tbody>
</table>

KEY:
The percentage response is calculated as a percentage of each age group and not of the total number of children

**P only** = number of children demonstrating minimum antibody levels of 0,15 μg/ml prior to immunisation

**R only** = number of children demonstrating minimum antibody levels of 1,0 μg/ml after immunisation

**PR** = number of children demonstrating minimum antibody levels of 0,15μg/ml and 1,0 μg/ml before and after immunisation respectively.
4.9 CORRELATION BETWEEN CARRIAGE AND ANTIBODY LEVELS

Prior to immunisation, 51 children were colonised by Hib. Of these, 4 had minimum antibody levels equal to or greater than 0.15 µg/ml. The median antibody concentration for these colonised patients was less than 0.06 µg/ml. Of the 82 patients who were not colonised by Hib prior to immunisation, 11 demonstrated minimum antibody levels equal to or greater than 0.15 µg/ml. The median antibody concentration for these patients was also less than 0.06 µg/ml.

After immunisation, 84 children were found to be colonised by Hib. Of these, 27 demonstrated minimum antibody levels of 1.0 µg/ml. The median value for the antibody concentration was 0.25 µg/ml. Of the 49 patients who were not colonised by Hib after immunisation, 18 demonstrated minimum antibody levels of 1.0 µg/ml. The median antibody concentration for these non-colonised patients was 0.28 µg/ml.

Carriage was not statistically related to antibody concentration. Even after controlling for age, there was still no statistical difference between the mean antibody concentrations of patients who were colonised by Hib and those who were not. These results are illustrated in Table XXIX. The mean antibody level of patients colonised prior to immunisation was 0.18 µg/ml. The standard deviation of 0.44 indicates that antibody levels ranged from very high to very low.

Analysis of variance was done to determine if there was a statistical difference in the mean postimmunisation antibody levels among those patients who were colonised before and after immunisation, those that were colonised after immunisation only, those colonised before immunisation only and those not colonised before or after immunisation. The F statistic value was 0.65 (P < 0.58) indicating that there was no statistical difference among the means. A chi-square analysis of pre- and post immunisation antibody levels and carriage showed no correlation between carriage and antibody levels. The chi-square value was 0.291.
Post-immunisation antibody levels were distributed over a wide range thus resulting in a high standard deviation. 41% of these patients had antibody levels less than 0.15 µg/ml and 11% had levels greater than 10 µg/ml. From this group (11%), 4 patients demonstrated levels greater than 35 µg/ml. Of the 45 patients that demonstrated antibody levels greater than and equal to 1.0 µg/ml after immunisation, 26 were carriers of Hib.
### TABLE XXVIII: ANTIBODY LEVELS IN PATIENTS BEFORE AND ONE MONTH AFTER IMMUNISATION

<table>
<thead>
<tr>
<th></th>
<th>PRE-IMMUNISATION</th>
<th>POST-IMMUNISATION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RANGE</strong></td>
<td>= 133</td>
<td>= 133</td>
</tr>
<tr>
<td>&lt; 0,1 - 2,2 μg/ml</td>
<td></td>
<td>&lt; 0,1 -&gt; 35 μg/ml</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td>0,18 μg/ml</td>
<td>3,16 μg/ml</td>
</tr>
<tr>
<td><strong>MEDIAN</strong></td>
<td>0,1 μg/ml</td>
<td>0,3 μg/ml</td>
</tr>
<tr>
<td>117 &lt; 0,15 μg/ml (88%)</td>
<td></td>
<td>88 &lt; 1,0 μg/ml (66%)</td>
</tr>
<tr>
<td>16 &gt; 0,15 μg/ml (12%)</td>
<td></td>
<td>45 &gt; 1,0 μg/ml (34%)</td>
</tr>
</tbody>
</table>

### TABLE XXIX: CORRELATION BETWEEN CARRIAGE OF Hib AND ANTIBODY RESPONSE TO THE VACCINE (N = 133)

<table>
<thead>
<tr>
<th></th>
<th>Carriage of Hib</th>
<th>Mean antibody concentration</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-immunisation</strong></td>
<td>51 positive</td>
<td>0,18 μg/ml</td>
<td>0,44</td>
</tr>
<tr>
<td></td>
<td>82 negative</td>
<td>0,11 μg/ml</td>
<td>0,18</td>
</tr>
<tr>
<td><strong>Post-immunisation</strong></td>
<td>84 positive</td>
<td>2,51 μg/ml</td>
<td>6,84</td>
</tr>
<tr>
<td></td>
<td>49 negative</td>
<td>4,23 μg/ml</td>
<td>8,65</td>
</tr>
</tbody>
</table>

SD = standard deviation
CHAPTER 5

5.0 DISCUSSION

It has been well documented that *Haemophilus influenzae* is a major cause of systemic disease in infants and young children. The majority of these infections are caused by encapsulated strains of serotype b. *Haemophilus influenzae* type b (Hib) is found in the natural naso- and oropharyngeal flora of many individuals thus posing a threat to the health of vulnerable individuals.

Studies in developed countries have shown that prior to the advent of Hib vaccines, meningitis accounted for more than 50% of all cases of invasive Hib disease. Subsequent to the use of Hib vaccines there has been a dramatic decrease in the incidence of Hib meningitis and all invasive disease due to this organism.

The limited data available from developing countries indicate that the incidence of invasive Hib disease is high compared to developed countries. Furthermore, the use of Hib vaccines is minimal in developed countries and is not included in the immunisation schedule of any country which may be classified as belonging to the Third World.

Crowding and increased contact between young children have been related to increased risk of invasive disease. Studies by Glode *et al* (1976), Hoosen *et al* (Conference presentation, 1976) and Peer *et al* (Conference proceedings, 1987) suggest nosocomial acquisition of Hib by paediatric patients. However, carriage of Hib does not necessarily indicate the probability of disease as studies have shown that carriers may or may not develop disease and vice versa (Glode *et al*, 1976; Michaels and Norden, 1977; Dajani *et al*, 1979; Granoff and Daum, 1980).

The development of a vaccine for Hib gained momentum from 1971 onwards and the first PRP vaccine was registered in the US in 1985. The vaccine was not effective in children less than 18 months of age (Ward, 1988) and did not elicit
a memory response on subsequent exposure to the organism (Parke, 1987). A conjugate vaccine, PRP-D, was subsequently licensed for use in the US in 1987 based on the results of two clinical trials (Lepow, 1987). This vaccine was immunogenic in all age groups and a booster response was evident. Since then, a number of conjugate vaccines have been licensed for approval.

The effect of immunisation on carriage of Hib is controversial. A reduction of carriage has been reported with some of the conjugate vaccines viz. PRP-D (Takala et al., 1991) and PRP-OMP (Takala et al., 1993). The use of PRP-D in Finland eliminated carriage in the predominantly White Finnish population. The effect of PRP-OMP on the Navajo and Apache Indians was not as dramatic in reducing carriage. A possible explanation for this may be ascribed to the high incidence of invasive Hib disease present in this population. The effect of the vaccine on carriage of Hib in children in institutional settings has not been documented. Furthermore, the immune response to Hib vaccine of hospitalised children infected with TB has not been studied anywhere in the world.

The health care system in South Africa has been a differential one with the Black population in the country being disadvantaged with reference to the quality and availability of health care facilities. In the Durban metropolitan area, which has a population of 2.4 million, there has been one dedicated tuberculosis hospital for the Black population. With TB being an endemic condition in South Africa, the wards of this hospital are always full of infected persons. In early 1993, an outbreak of multi-antibiotic resistant Hib occurred predominantly in the paediatric wards of this hospital. A number of infection control measures were undertaken in order to control this outbreak. Amongst these was the decision to use the newly registered HibTITRE vaccine manufactured by Lederle.

In this study, a total of 135 children were immunised according to the manufacturer’s schedule which is based on age. Seven children received more than one dose of the vaccine as they were less than 12 months of age and the remaining 128 received only one dose of the vaccine. With reference to mucosal carriage of Hib, 3 children who were less than 6 months old were not colonised
by Hib either before or after immunisation (Table XX). This is consistent with the findings of Takala (1994a, 1994b) and Turk (1963) who also found that young children, less than 6 months old, were not colonised. It is suggested that maternal antibodies conferred protection on these infants at the mucosal level. Seventy-five percent (75%) of children who were colonised by Hib prior to immunisation were between 6 months and 5 years of age. After immunisation, the carriage rate decreased slightly in this age group from 75% to 67% (Table XX). However, the overall carriage rate for the total study population was high being 38% and 62% before and after immunisation respectively. The percentage of patients who became colonised only after immunisation was 35% and this was statistically significant (McNemar’s test, \( P < 0.01 \)).

At the beginning of the study, there were 89 patients in the paediatric wards. Subsequently, another 46 patients were admitted. Of these new admissions, 20% (9/46) were colonised at the time of entry into the wards. One month after immunisation, 35% (16/46) of the new admissions were colonised indicating that colonisation occurred as a result of contact with colonised children.

It must be noted that the children were all being treated for tuberculosis and were in contact with each other for a long period of time. These children were from a poor socioeconomic environment. In 9 patients, chickenpox was diagnosed at the time of immunisation and 8 were known to be HIV antibody positive. This setting is a closed population, and as such, a high carriage rate is to be expected as also shown by a number of studies (Turk, 1963; Mpairwe, 1970; Glode et al, 1976; Takala et al, 1989). Hussey et al (1994a), in their study of Hib carriage amongst TB children in Cape Town, found a much higher rate in those hospitalised (37%) than those treated at outpatient clinics (3%).

In closed populations, carriage tends to persist for longer periods of time and the children may remain colonised for weeks or months. Also, in the event of carriage being eliminated in some children, there is a greater likelihood of recolonisation occurring due to close and prolonged contact (Granoff and Daum, 1980; Glode et al, 1976).
Gilsdorf (1986), demonstrated a reduction in colonisation within 24 hours of commencement of antibiotic therapy and found that after 24 hours, cultures did not yield any Hib. Twenty-five patients had received antibiotics for infections other than tuberculosis. Three of them were colonised by multi-antibiotic (ampicillin, chloramphenicol, erythromycin, tetracycline and rifampicin) resistant strains of Hib and 3 by antibiotic sensitive strains prior to immunisation. Two of the 3 patients who carried resistant strains had been treated with amoxycillin and one with amoxycillin/clavulanic acid. One month after immunisation, 11 of these patients was colonised with Hib one of whom was still colonised by a resistant strain. Thus, antibiotic therapy could account for a lower colonisation rate before immunisation compared to the colonisation rate after immunisation. With regard to the continued presence of multiple antibiotic resistant strains, the selective pressure of antimicrobial agents could have played an important role. Furthermore, these resistant strains were already present at the commencement of the study and could have been a circulating strain within the hospital environment.

Recommendations by the Immunisation Practices Advisory Committee (ACIP) in the US (MMWR, 1986) for prophylaxis for infected patients, siblings at risk of secondary Hib disease, children in day care centres and for the elimination of carriage in convalescents, has been the use of rifampicin. Campos et al (1987), in a study of children in 4 day care centres in Spain, found that rifampicin was effective in the reduction of carriage of multiple antibiotic resistant Hib isolates. They concluded that rifampicin successfully reduced prevalence of multiple resistant Hib. In an earlier study in an orphanage in Thailand (Simasathien et al, 1980) the use of rifampicin to eliminate antibiotic resistant strains was not successful. Campos et al (1987) suggest that the low dosage of rifampicin used may have contributed to this outcome.

In this study rifampicin was being administered to the children from the date of admission for the treatment of tuberculosis. A high prevalence of Hib resistance to rifampicin was noted in Hib isolates before and after immunisation. Prior to immunisation, 92% of the Hib isolates were resistant to rifampicin and this
increased to 100% after immunisation. The lower percentage of rifampicin resistance prior to immunisation was due to 4 new admissions who had not had prior treatment with rifampicin nor had they had contact with TB patients. Hussey et al (1994a) also found the prevalence of resistance to rifampicin in patients being treated for TB to be 100%. They further state that antimicrobial resistance was a major problem only in children hospitalised with tuberculosis.

The effect of rifampicin on Hib was studied by McCarty et al (1986) who used rifampicin as a preventive measure in a day-care centre after a child contracted Hib meningitis. A sibling of one of the children also contracted Hib meningitis but the isolate was resistant to rifampicin. After further studies of the isolates in infant rats, McCarty et al (1986) concluded that rifampicin resistance was due to a mutation and that this was a hazard of rifampicin chemoprophylaxis. In closed settings such as the one where the study was carried out, the use of rifampicin as stated in the ACIP recommendations are of no value.

The estimated values for minimum serum concentrations of antibody associated with protection against Hib is in the range 0,04 to 0,15 µg/ml (Kayhty et al, 1983). Most authorities consider antibody levels of 0,15 µg/ml as being protective in non-immunised individuals. In immunised individuals, 1,0 µg/ml is associated with protection (Anderson, 1984). Anderson further argues that a peak level of 1,0 µg/ml was necessary to maintain circulating antibody levels of 0,1 µg/ml during the course of one year and that vaccine efficacy could not be based on a protective level achieved a few weeks after immunisation.

The mean antibody levels of children in this study before and after immunisation were 0,18 and 3,16 µg/ml respectively. The respective standard deviations were 0,3 and 7,6. This increase in antibody level from pre- to post immunisation was statistically significant (paired t-test, P < 0,0001). After immunisation, only 45 (34%) patients had antibody levels equal to or greater than 1,0 µg/ml and the remaining 88 (66%) had levels less than this value. Of these 88 patients, 16 had antibody levels equal to or greater than 0,15 µg/ml indicating that an immune response to the vaccine had taken place but that it was inadequate. It is possible
that a booster dose is necessary to elicit antibody levels of at least 1,0 μg/ml.

Children who received only one dose of the vaccine (older than 11 months) numbered 126 (95%). Of these, 45 (34%) showed antibody levels greater than or equal to 1,0 μg/ml one month after immunisation. This is in sharp contrast to the results of Madore et al., (1990b) who found that 99% of children who received only one dose of the vaccine had antibody levels equal to or greater than 1,0 μg/ml after one month. This increased to 100% after 6 months.

The mean antibody level of 51 (38%) colonised children prior to immunisation was 0,18 μg/ml. Although this is greater than the minimum protective level of 0,15 μg/ml expected in non-immunised patients, the standard deviation (0,44) indicates a wide range (Table XXIX). Furthermore, a median value of 0,10 μg/ml for the total population studied indicates that 50% of the patients had antibody levels less than the minimum protective level. Thus, a few patients with levels exceeding this minimum value resulted in an increased mean antibody level of 0,18 μg/ml. Therefore, in this group of 51 colonised children, prior exposure to the organism did not appear to elicit the expected immune response.

After immunisation, the mean antibody level of the 84 patients who were colonised was 2,51 μg/ml and the standard deviation 6,84. Again, very high antibody levels in a few patients resulted in the mean reflecting a value exceeding 1,0 μg/ml. The mean antibody level for patients who were not colonised after immunisation was 4,23 μg/ml with a standard deviation of 8,65.

Analysis of variance was done to determine if there was a statistical difference in the mean antibody levels of patients who were a) colonised before and after immunisation, b) colonised before immunisation only, c) colonised after immunisation only and d) not colonised at all. The F statistic was 0,65 (P < 0,58), which indicates no statistical difference among the means of the antibody levels in these 4 groups. This was corroborated using a chi-square test which showed that there was no statistical significance between mean antibody levels of children who were colonised by Hib and those who were not (P = 0,291).
The vaccine did not result in reduction of carriage of Hib since 51 children were colonised prior to immunisation and this figure increased to 84 after immunisation.

These results are in contrast to results obtained by Barbour et al (1993) who found that children who were colonised had higher antibody levels than children who were not colonised. They suggest that mucosal challenge by Hib subsequent to vaccination results in a booster response. Their study also did not find a reduction in carriage but rather a decrease in intensity of colonisation. The dramatic effect of immunisation on carriage of Hib in Finland (Takala, 1991) could be ascribed to the fact that children are immunised early in life i.e. the immunisation schedule has been expanded to include Hib vaccine. This could have resulted in a decrease in the prevalence of Hib bacteria and would also have induced herd immunity. Therefore, the number of carriers of Hib in the population in Finland would be negligible. In addition, the population was mainly White and this could have further reduced the risk of colonisation. Similarly, Murphy et al (1993b) found that colonisation was prevented in 81% of children immunised with a conjugate vaccine PRP-D. Takala (1993) found that a conjugate vaccine, PRP-OMP, reduced oropharyngeal carriage in the American Indian population which is a high risk population for invasive Hib disease.

After one dose of the vaccine, the protective antibody level of 1.0 μg/ml was not achieved in the vulnerable age group 4 to 24 months. Children under one year, who received a second dose of the vaccine, demonstrated antibody levels greater than or equal to 1.0 μg/ml only after the second dose. These results concur with those of Madore et al, (1990a) who studied the efficacy of HbOC conjugate vaccine (HibTITRE) in infants aged 1 to 6 months.

The poor immune response to the vaccine in this study population could be ascribed to a number of factors mentioned earlier. Peter (1987) suggested that rifampicin prophylaxis could prevent the development of humoral immunity to Hib. Since none of the children developed invasive disease during the study period, the vaccine could possibly have afforded a degree of protection which
was not substantiated by the recognised minimum protective antibody level. This is corroborated by Kayhty (1994b) who states that suggested protective levels of antibody were based on the response to PRP vaccines. It is further stated that after immunisation with a conjugate vaccine, protective antibody levels were lower compared to levels after use of PRP vaccine. In addition, the differences between different conjugate vaccines might have an influence on functional activity.

The role of IgA in affording protection at the mucosal level is poorly understood. It is thought to protect the host from invading organisms by blocking adherence to the mucosal surface. When infection or colonisation occurs, an antigen overload results. In such a setting, the value of antibodies, whose primary strategy is direct neutralisation, is minimised (Underdown and Schiff, 1986).

There is no information available regarding the outcome of immunisation in a comparable population i.e. hospitalised children. In view of the poor immune response by the children in this study, it is highly probable that the supplied immunisation schedule needs to be investigated. Also, efficacy trials should be conducted in developing countries prior to the introduction of new vaccines since genetic and environmental factors could affect the performance of Hib vaccines (Mulholland and Greenwood, 1994). Since case fatality rates for Hib disease in developing countries were high (Bijlmer, 1994), efficacy of Hib vaccine should be evaluated with the view to including it in the immunisation schedule for children.

With regard to carriage of resistant strains of Hib, 25% of patients carried strains producing β-lactamase and chloramphenicol acetyltransferase before immunisation and this decreased to 4% after immunisation. Carriage of strains producing only β-lactamase was 2% and 1% before and after immunisation respectively (Table XXII). Prior to immunisation, 3 patients who were colonised by β-lactamase producing strains had been treated with either amoxycillin or amoxycillin/clavulanic acid. Also, ward files recorded a history of previous isolation of β-lactamase and non-β-lactamase producing strains of Hib in 4 and
9 patients respectively. Thus, resistant strains of Hib were already present at the commencement of the study.

Antibiotic treatment of some of the patients before immunisation eliminated sensitive strains of Hib but could have facilitated the survival of resistant strains. One of the patients received antibiotic treatment other than for TB during the period following immunisation. This could be the reason for the reduction in the number of strains producing chloramphenicol acetyltransferase and β-lactamase since the selective pressure exerted by the antibiotics was no longer present.

Syriopoulou et al. (1978) and Schwartz et al. (1983) found that patients who were colonised by ampicillin resistant strains of *Haemophilus influenzae* were more likely to have had recent exposure to β-lactam antibiotics.

In this study, a few patients were exposed to antibiotics which could have resulted in development of resistance. These resistant strains were therefore prevalent in the hospital environment and acquisition thereof by patients who had not been treated with antibiotics was therefore nosocomial.

There has been considerable debate as to whether swabs from the oropharynx or from the nasopharynx were more efficient in detecting colonisation with *H. influenzae*. Some studies have been carried out using only nasopharyngeal swab specimens whilst others use only oropharyngeal. Few comparative studies have been documented.

The earliest reported study comparing the efficacy of the two types of swab specimens was carried out by Masters et al. (1958) on adults and children. The isolation rate was the same regardless of the site from which the specimen was taken. A comparative study by Michaels et al. (1976) clearly demonstrated the superiority of swabs from the oropharynx for the isolation of Hib from colonised patients. Turk (1963) and Mpairwe (1970) investigated the carriage rate of Hib by culturing of nasopharyngeal swabs only. Similarly, in a study on the carriage of Hib in an enclosed hospital population after an outbreak of meningitis (Glode
et al, 1976), only nasopharyngeal swab specimens were collected and cultured.

In a number of later studies carried out to ascertain the effect of immunisation on carriage of Hib, oropharyngeal swabs only have been cultured (Takala et al, 1991; Takala et al, 1993; Murphy et al, 1993b; Barbour et al, 1993).

This study compared the efficacy of swab specimens from the oropharynx and nasopharynx (Table XVII) for the isolation of H. influenzae. It was found that if only oropharyngeal swabs had been cultured, 2 isolates only would have been excluded. Thus, it would be more cost effective and less time consuming to collect only oropharyngeal swabs from patients.

To study the epidemiology of Hib disease, it is necessary to compare isolates from different parts of the world. These isolates may be compared using various methods for typing of Hib (van Alphen, 1994). Some of the typing methods that have been used are biotyping, outer membrane protein (OMP) subtyping and electrophoretic typing. Biotyping and OMP subtyping are the two methods that were used in this study.

With reference to biotyping, only pre-immunisation isolates of Hib were tested since the results indicated that this could not be used to discriminate between Hib strains. Of the 51 strains of Hib isolated, biotype II accounted for 33%, biotype III 25%, and biotype I 16% (Table XXI). The invasive strains isolated from patients with Hib disease were all of biotype II. Studies on invasive strains of Hib indicate that biotypes I and II predominate (Musser et al, 1985; Weinberg et al, 1989; Landgraf and Vieira, 1993).

Of the 14 isolates that produced β-lactamase prior to immunisation, only 6 were biotype II ie. the same as the strains causing invasive disease. The remaining strains were biotype I, V and VII. None of these strains were biotype III. Biotyping is not a discriminatory method of typing as can be seen by the fact that β-lactamase producing and non-producing strains are of the same biotype. Similarly, isolates causing invasive disease and isolates from patients who were
colonised share the same biotype viz. II. It does not necessarily follow that they are identical. Studies by Musser et al (1985) have shown that biotype diversity was not correlated to outer membrane protein diversity.

Outer membrane protein analysis was performed using two methods. OMP was extracted from all Hib isolates using the method of Barenkamp et al (1981a). This method was time consuming and laborious. Representative strains from groups of isolates that shared the same OMP profile were studied further in Amsterdam (Academic Medical Centre, University of Amsterdam) under the guidance of Dr. L van Alphen. OMP profiles of some strains isolated from patients who were colonised by Hib before and after immunisation were examined as were isolates from patients with invasive disease. The method used (unpublished) was simpler and less time consuming. No expensive equipment eg. high speed refrigerated centrifuge, was required. Thus, using this method, the analysis of OMP’s could be performed in most laboratories.

Of the 8 isolates from patients with invasive Hib disease, 7 produced $\beta$-lactamase and were OMP type 1H. One isolate was negative for $\beta$-lactamase production and was of a different OMP type. Two $\beta$-lactamase producing isolates from patients who were colonised also had the OMP type 1H. The 8 clinical isolates as well as the 2 isolates from colonised patients were of biotype II. These results are corroborated by van Alphen (1993) who found that subtypes causing disease were also present in the throat of healthy individuals. Thus, the strains causing invasive disease could have originated from the patients who were colonised since the OMP is the same.

OMP type 1H was the most commonly observed subtype in the US (Munson et al, 1989; van Alphen, 1994) whilst type 2L was most common in Africa. Peer (South Africa 1988, unpublished) found that invasive, multiresistant strains of Hib were of the subtype 2L and 2L'. In this study, the OMP subtype causing invasive disease was type 1H which differs from Peer’s (1988, unpublished) findings in South Africa. It is clear that more than one OMP type is prevalent in South Africa.
The results of OMP analysis on the isolates from patients who were colonised indicate that there were possibly 13 different subtypes. After immunisation, there was an increase in the number of subtypes that were prevalent. In view of the fact that the increase in carriage after immunisation was statistically significant, the increase in the number of OMP subtypes was not unexpected. Reports on OMP subtypes of Hib strains prevalent in South Africa have not been published. It is a relevant area of epidemiological investigation that has been neglected to date.

Antibiotics used for the treatment of established Hib disease historically has been ampicillin and chloramphenicol. With the emergence of multiple antibiotic resistant strains, this has to be reviewed. The study of susceptibility profiles of isolates in any geographical area allows this.

For this study, Direct Sensitivity Test agar (DST agar, OXOID) supplemented with 10 μg/ml NAD and 0.25% lysed horse blood was used. In personal communication with a world authority on Hib, Dr. L. van Alphen (Amsterdam), it was determined that this was an acceptable alternative. The NCCLS recommends the use of HTM which proved to be too expensive and was not available at the time of the study.

The antibiotics that demonstrated the lowest MIC range were the quinolones. The MIC\textsubscript{90} of ciprofloxacin was the lowest at 0.03 μg/ml. However, when tested against β-lactamase producing strains, the MIC\textsubscript{90} of ciprofloxacin was fourfold greater whereas that for lomefloxacin, pefloxacin and ofloxacin were only twofold greater than was observed for non-β-lactamase producing strains. These results are consistent with the findings of Jorgensen et al (1988) who showed that multiple antibiotic resistant strains of *H. influenzae* were highly susceptible to ciprofloxacin and pefloxacin and that MIC values for ciprofloxacin were higher than those for the other fluoroquinolones.

MIC values for all strains tested to the third generation cephalosporins viz. cefotaxime and ceftriaxone were in the susceptible range. The first generation
cephalosporin cefazolin was the least active with an MIC\textsubscript{90} of 128 \( \mu \)g/ml. Cefuroxime, a second generation cephalosporin, showed reduced activity; the MIC\textsubscript{90} was 16 \( \mu \)g/ml which is the breakpoint for resistance. Only 15% of the strains tested demonstrated this level of resistance to cefuroxime. However, \( \beta \)-lactamase producing strains showed a higher MIC\textsubscript{90} of 16 \( \mu \)g/ml for cefuroxime.

Aronoff \textit{et al} (1984) found that the efficacy of ceftriaxone was comparable to that of ampicillin and chloramphenicol and further recommend the use of ceftriaxone for the treatment of meningitis due to multiple antibiotic resistant Hib. In this study, the MIC\textsubscript{90} was 0.01 \( \mu \)g/ml irrespective of \( \beta \)-lactamase production. In a study of ampicillin and chloramphenicol resistant \textit{H. influenzae} strains against 15 antibiotics, (Campos and Garcia-Tornel, 1987), ceftriaxone, cefotaxime and aztreonam were found to have the lowest MIC\textsubscript{90} values ie. 0.03, 0.06 and 0.25 \( \mu \)g/ml respectively. These findings are very similar to the values obtained in this study.

There was no difference in the activity of tetracycline and minocycline against \( \beta \)-lactamase producing and non-producing strains. However, the MIC\textsubscript{90} of doxycycline was twofold higher for \( \beta \)-lactamase producing strains.

The macrolides also showed no difference in activity against strains which produced \( \beta \)-lactamase and those that did not. The MIC\textsubscript{90} for erythromycin was 8 \( \mu \)g/ml and only 6% of strains tested had MIC values greater than this. This is consistent with the findings of Jorgensen \textit{et al} (1990) who showed the same MIC value for erythromycin. Goldstein \textit{et al} (1990) compared the efficacy of azithromycin, erythromycin and roxithromycin against \textit{H. influenzae} and found that azithromycin was 4 to 8 times more potent than erythromycin and roxithromycin. In this study, azithromycin was found to have the lowest MIC\textsubscript{90} with the values for erythromycin and roxithromycin being 3 to 5 dilutions higher. Roxithromycin had the highest MIC\textsubscript{90} of 32 \( \mu \)g/ml.

The most effective \( \beta \)-lactam antimicrobial tested was aztreonam for which an MIC\textsubscript{90} of 0.12 \( \mu \)g/ml was demonstrated. Imipenem and the combination
amoxyccillin/clavulanic acid showed no difference in the concentration required to inhibit 90% of strains whether they produced β-lactamase or not.

As expected, the activity of ampicillin was markedly different for β-lactamase producing strains compared to non enzyme producers. Strains producing the enzyme were inhibited by a three-fold greater concentration of the antibiotic.
6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

6.1.1 For the duration of the study period, the prevalence of invasive disease in the study wards was reduced subsequent to immunisation.

6.1.2 The accepted minimum protective antibody level after immunisation was not achieved in all children.

6.1.3 Immunisation using the HibTITRE vaccine did not eliminate or reduce carriage.

6.1.4 Multiple antibiotic resistant strains causing invasive disease had the same OMP type and biotype as some resistant strains which colonised the children.

6.1.5 Sampling from the oropharynx was superior to nasopharyngeal sampling for the detection of carriers.

6.1.6 Biotyping of Hib isolates did not discriminate between Hib strains and therefore has limited value for epidemiological purposes.

6.2 RECOMMENDATIONS

6.2.1 The conjugate Hib vaccine should be considered for children in closed settings such as this one as well as in open populations.

6.2.2 The existing dosage schedule for Hib vaccines needs to be revised for local settings such as the study population.
7.0 REFERENCES


Doern GV and Jones RN. Antimicrobial susceptibility testing of *Haemophilus influenzae, Branhamella catarrhalis* and *Neisseria gonorrhoeae*. Antimicrobial Agents and Chemotherapy 1988,32:1747-1753.


Fraise AP, Meeks ACG and Richards JE. Meningitis due to *Haemophilus influenzae* resistant to ampicillin and chloramphenicol. *Archives of Disease in Childhood* 1986;61:1134-1135.

Fraser DW, Geil CC and Feldman RA. Bacterial meningitis in Bernalillo County, New Mexico: A comparison with three other American populations. *American Journal of Epidemiology* 1974;100:1:29-34.


Murphy TV, White KE, Gabriel L, Medley F, Granoff DM and Osterholm MT. Declining incidence of *Haemophilus influenzae* type b disease since introduction of vaccination. *JAMA* 1993(a);269:246-148.


Pichichero ME and Insel RA. Mucosal antibody response to parenteral vaccination with *Haemophilus influenzae* type b capsule. *Journal of Allergy and Clinical Immunology* 1983;72:481-485.


Shapiro ED, Murphy TV, Wald ER and Brady CA. The protective efficacy of *Haemophilus* b polysaccharide vaccine. *JAMA* 1988;260:1419-1422.


Urwin G, Musser JM and Yuan MF. Clonal analysis of *Haemophilus influenzae* type b isolates in the UK. *Journal of Medical Microbiology* 1995;43:45-49.


van Alphen L, Bijlmer HA. Molecular epidemiology of Haemophilus influenzae type b. Paediatrics 1990;85:S636

van Alphen L. The molecular epidemiology of Haemophilus influenzae. Reviews in Medical Microbiology 1993;4:159-166


PERSONAL COMMUNICATION

L van Alphen, Department of Microbiology, University of Amsterdam.

CONGRESS PAPERS AND PROCEEDINGS


THESES


APPENDIX A

HibTITER

Single dose

Haemophilus b Conjugate Vaccine 10μg/dose

IMMUNIZATION SCHEDULE

Please see full prescribing information

<table>
<thead>
<tr>
<th>AGE AT INITIAL IMMUNIZATION</th>
<th>NUMBER OF DOSES</th>
<th>BOOSTER DOSE 15 MONTHS AND OVER (AT LEAST 2 MONTHS AFTER PREVIOUS DOSE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 - 6 MONTHS</td>
<td>3</td>
<td>YES</td>
</tr>
<tr>
<td>(6 - 8 WEEKS APART)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 - 11 MONTHS</td>
<td>2</td>
<td>YES</td>
</tr>
<tr>
<td>(6 - 8 WEEKS APART)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 - 14 MONTHS</td>
<td>1</td>
<td>YES</td>
</tr>
<tr>
<td>15 MONTHS AND OVER</td>
<td>1</td>
<td>NO</td>
</tr>
</tbody>
</table>

Can be administered concurrently with DTP at different injection sites

Store at 2 - 8°C

DO NOT FREEZE

For more information call Lederle Laboratories at (011) 974-5232
1. INTRALACTAM (ET01) (Mast diagnostics)

For the detection of β-lactamase

Mast Intralactam strips (ET01) are filter paper strips 5 - 7 by 0.6 cm which are printed to identify the test, positive control and negative control areas. The strips are impregnated with benzyl penicillin and bromcresol purple at appropriate concentrations.

Method

1. The paper strip was placed on a clean microscope slide and a small drop of distilled water was added to each area of the strip. The paper was moist but not saturated.
2. A sterile swab was used to sweep the surface of the test isolate and was spread onto the test area of the strip.
3. A sample from a known intrinsic β-lactamase producing strain and a non-β-lactamase producing strain was applied to the strip as controls.

β-lactamase detection in *Haemophilus* sp

A change in colour from purple to yellow in up to 10 minutes indicates β-lactamase production.

2. API NH

System for the identification of *Neisseria* and *Haemophilus*

Overnight chocolate agar plate cultures of the isolates were used.

1. The API strip was removed from its wrapping and placed in the prepared incubation box.
2. A suspension of the isolates was prepared using 2 ml of sterile 0.85% NaCl and was used immediately. The turbidity prepared was equivalent to 4 on the McFarland scale.
3. Only the tube part of the first seven microtubes was filled. The tube and
cupule of the last three microtubes were filled.

4. The first seven microtubes were covered with mineral oil.

5. The incubation box was closed and incubated for 2 hours at 37°C in aerobic conditions.

6. The reactions were read by referring to the reading table in the instruction manual.
Appendix C

Reagents and Gel preparation for SDS-PAGE

1. **Acrylamide/bis (30% T, 2.67% C)**
   - 87.6g acrylamide (29.2 g/100 ml)
   - 2.4 g N’N’-bis-methylene-acrylamide (0.8 g/100 ml)
   Make to 300 ml with distilled water. Filter and store at 4°C in the dark (30 days maximum)

2. **1.5 M Tris-HCl, pH 8.8 (running gel buffer)**
   - 27.23 g Tris base (18.15 g/100 ml)
   - ~ 80 ml distilled water
   Adjust to pH 8.8 with 1N HCl. Make to 150 ml with distilled water and store at 4°C.

3. **0.5 M Tris-HCl, pH 6.8 (stacking gel buffer)**
   - 6 g Tris base
   - ~ 60 ml distilled water
   Adjust to pH 6.8 with 1 N HCl. Make to 100 ml with distilled water and store at 4°C.

4. **10% SDS**
   Dissolve 10 g SDS in water with gentle stirring and bring to 100 ml with distilled H2O.

5. **Sample Loading Buffer (store at room temperature)**
   - Distilled water 4,0 ml
   - 0,5 M Tris-HCl, pH 6.8 1,0 ml
   - Glycerol 0,80 ml
   - 10% (w/v) SDS 1,6 ml
   - 2-b-mercaptoethanol 0,4 ml
   - 0,05% (w/v) bromophenol blue 0,2 ml
6. **5X Electrode Buffer, pH 8.3**

- Tris base 9 g
- Glycine 43.2 g
- SDS 3 g

Make to 600 ml with distilled water. Store at 4°C. Warm to 37°C before use if precipitation occurs. Dilute 60 ml 5X stock with 240 ml distilled water for one electrophoretic run.

7. **Running gel**

- 8.37 ml acrylamide mix
- 6.23 ml H₂O
- 5.0 ml running gel buffer
- 200 μl 10% SDS
- 200 μl 10% ammonium persulphate* 
- 8 μl TEMED

8. **Stacking gel**

- 1.66 ml acrylamide mix
- 5.14 ml H₂O
- 2.52 ml stacking gel buffer
- 100 μl 10% SDS
- 100 μl 10% ammonium persulphate* 
- 15 μl TEMED

* ammonium persulphate must be freshly prepared
APPENDIX D

Media

1. **Supplemented Brain Heart Infusion (sBHI) broth (DIFCO)**
   - Calf brains, infusion from
   - Beef heart, infusion from
   - Bacto proteose peptone
   - Bacto dextrose
   - Sodium chloride
   - Disodium phosphate

   The medium was prepared as per manufacturers instructions, sterilised and cooled to 50°C. 10 \mu g/ml haemin and 10\mu g/ml NAD were added. The broth was stored in the refrigerator and used as required.

2. **Supplemented DST agar (OXOID)**
   - Proteose peptone
   - Veal infusion solids
   - Glucose
   - Sodium chloride
   - Disodium phosphate
   - Sodium acetate
   - Adenine sulphate
   - Guanine hydrochloride
   - Uracil
   - Xanthine
   - Aneurine
   - Agar

   The agar was prepared as per manufacturers instructions, sterilised and cooled to 50°C. 10 \mu g/ml NAD and 0.25% lysed horse blood were added.
APPENDIX E

ELISA reagents

1. Chromogenic substrate containing OPD
   a) Freeze-dried OPD
      4-Phenyldiamine.2HCl (Sigma P-1520)
      Pyrogen-free distilled water
   
   b) Buffered urea hydrogen peroxide
      Succinic acid
      Disodium tetraborate
      Hydrogen peroxide urea
      Pyrogen-free distilled water

Buffered urea hydrogen peroxide was added to freeze-dried OPD and used immediately
APPENDIX F

Preparation of gels and buffers according to the method used at the Academic Medical Centre, University of Amsterdam.

1. Running gel buffer
   1.5 M tris HCl pH 8.8
   0.4% SDS

2. Stacking gel buffer
   0.25 M Tris HCl pH 6.8
   0.4% SDS

3. Acrylamide
   30 g acrylamide
   0.8 g bisacrylamide

Add 75 ml H₂O and shake overnight at 4°C. Make up to 100 ml and filter. Store at 4°C in the dark.

4. Resolving gel
   9.17 ml bis/acrylamide
   8.81 ml H₂O
   6.25 ml running gel buffer (appendix C)
   0.63 ml ammonium persulphate* (15 mg/ml)
   50 μl TEMED

5. Stacking gel
   1.25 ml bis/acrylamide
   4.69 ml H₂O
   6.25 ml stacking gel buffer (appendix C)
   0.31 ml ammonium persulphate* (15 mg/ml)
   25 μl TEMED

* ammonium persulphate must be freshly prepared
6. **Electrophoresis buffer 4x concentrated**
   
   0.1 M Tris HCl pH 8.7
   
   0.77 M glycine
   
   0.4% SDS

7. **Staining solution**

   10% acetic acid
   
   25% methanol
   
   65% H₂O
   
   0.1% Coomassie blue R250 (w/v)

8. **Destaining solution**

   As for staining solution but without Coomassie blue