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β-Lactam Resistance in *Haemophilus influenzae*

by

**LEILA VALI**

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Haemophilus influenzae is the most important pathogen of Haemophilus genus in humans. H. influenzae has been associated with upper and lower respiratory tract as well as invasive infections. Systemic infections such as meningitis and bacteraemia in children are mainly caused by encapsulated strains most frequently by those with type b capsular polysaccharide (Hib), whereas localised infections such as sinusitis and otitis media usually are mediated with nontypeable strains. In adults invasive infections may be caused by both nontypeable and typeable organisms.

Resistance to β-lactams has been documented in this species, however, most β-lactam antibiotics are still widely used for treatment of H. influenzae infections. β-lactamase-mediated ampicillin resistance in H. influenzae is associated with the production of TEM-1 type and ROB-1 β-lactamase enzymes. TEM-1 production accounts for more than 90% of ampicillin resistance globally. Penicillin-binding proteins (PBPs) are targets for β-lactams. Alterations in PBP profile is another mechanism that develops resistance to β-lactam compounds in H. influenzae. Most of the non-β-lactamase mediated ampicillin resistant H. influenzae strains are non-encapsulated and are of respiratory origin. The incidence of non-β-lactamase-mediated resistance has increased in most countries. Since these strains have altered PBPs, cross resistance with other β-lactams with similar targets may jeopardise the treatment.

In order to minimise treatment failure, the anti-microbial efficiency of drugs should be monitored regularly. In this study the prevalence of β-lactam resistance in clinical strains of H. influenzae was determined in Scotland. Six hundred and sixteen isolates were collected from four hospitals during 1993-1995. 20.5% of the strains contained a β-lactamase enzyme. There are marked regional differences in the proportion of β-lactamase producing isolates which ranges from 9.6% in Glasgow Royal Infirmary to 33.3% in Edinburgh Royal Infirmary. The concentrations required for ampicillin,
amoxyccillin/clavulanic acid, cefuroxime, ceftazidime, cefotaxime, cefaclor and imipenem to inhibit 50% and 90% of the isolates were determined and compared to the surveys in other countries. H. influenzae biotypes I, II and III are the most prevalent biogroups in Scotland especially among the over 50 year old adults. β-lactam resistance was observed in all biogroups of H. influenzae.

Among the clinical isolates, one strain from Glasgow Southern General Hospital, although remained relatively sensitive to ampicillin, was found to be β-lactamase positive. A cell free extract of this strain was examined by isoelectric focusing and β-lactamase activity was visualised at a pI of 7.9. This novel enzyme, VAT-1, did not cofocus with any of the controls or any of their extended spectrum derivatives, nor was it indicative of plasmid encoded β-lactamases associated with other respiratory pathogens. Biochemical analysis indicated that VAT-1 had a substrate profile of a cephalosporinase that hydrolysed first generation cephalosporins. Inhibitor analysis demonstrated that VAT-1 was a class C β-lactamase and it is only the third β-lactamase that has been described in H. influenzae.
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DECLARATION

The experiments and composition of this thesis are the work of the author unless otherwise stated.
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ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>%C</td>
<td>total crosslinking monomer concentration per 100 ml</td>
</tr>
<tr>
<td>%T</td>
<td>total monomer concentration per 100 ml</td>
</tr>
<tr>
<td>7-ACA</td>
<td>7-aminocephalosporanic acid</td>
</tr>
<tr>
<td>A</td>
<td>Aadenine</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>BAP</td>
<td>bacterial alkaline phosphatase</td>
</tr>
<tr>
<td>bla</td>
<td>β-lactamase gene</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BPIG</td>
<td>bacterial polysaccharide immunoglobulin dinucleotide</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSAC</td>
<td>British society for antimicrobial chemotherapy</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>Cm</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebro spinal fluid</td>
</tr>
<tr>
<td>dATP</td>
<td>2’-deoxyriboadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>2’-deoxyribocytidine triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>2’-deoxyriboguanosine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dTTP</td>
<td>2’-deoxyribothymidine triphosphate</td>
</tr>
<tr>
<td>dUTP</td>
<td>2’-deoxyribouridine triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>Hib</td>
<td><em>H. influenzae</em> serotype b</td>
</tr>
<tr>
<td>his</td>
<td>histidine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HMW</td>
<td>high molecular weight</td>
</tr>
<tr>
<td>HTM</td>
<td>Haemophilus test media</td>
</tr>
<tr>
<td>ID</td>
<td>inhibitory dose</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>lac</td>
<td>lactose non-fermentor</td>
</tr>
<tr>
<td>lacZ</td>
<td>β-galactoside gene</td>
</tr>
<tr>
<td>LKP</td>
<td>long, thick, hemmagglutination positive</td>
</tr>
<tr>
<td>LOS</td>
<td>lipooligosaccharide</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MDa</td>
<td>megadaltons</td>
</tr>
<tr>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCCLS</td>
<td>national committee for clinical laboratory standards</td>
</tr>
<tr>
<td>NCTC</td>
<td>national collections of type cultures</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OMP</td>
<td>outer membrane protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBP</td>
<td>penicillin-binding protein</td>
</tr>
<tr>
<td>pCMB</td>
<td>para chloromercuribenzoic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>PRP</td>
<td>polyribosyl ribitol phosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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</table>
RNase  ribonuclease
rpm  revolution per minute
rRNA  ribosomal ribonucleic acid
SDS  sodium dodecyl sulphate
Ser  serine
T  thymidine
TEMED  N, N', N'-tetramethyl-ethylenediamine
tet  tetracycline
Thr  threonine
Tn  transposon
trp  tryptophane
UV  ultra-violet light
VIS  visible light
Vmax  maximum rate of hydrolysis
Xgal  5-bromo-4-chloro-3-indoyl-β-galactopyranoside

**Single-Letter Amino Acid Codes**
A  alanine
C  cysteine
D  aspartic acid
E  glutamic acid
F  phenylalanine
G  glycine
H  histidine
I  isoleucine
K  lysine
L  leucine
M  methionine
N  asparagine
P  proline
Q  glutamine
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<td>R</td>
<td>arginine</td>
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<tr>
<td>S</td>
<td>serine</td>
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<tr>
<td>T</td>
<td>threonine</td>
</tr>
<tr>
<td>V</td>
<td>valine</td>
</tr>
<tr>
<td>W</td>
<td>tryptophane</td>
</tr>
<tr>
<td>Y</td>
<td>tyrosine</td>
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PUBLICATIONS


CHAPTER ONE

INTRODUCTION

1.1 *Haemophilus* Species

*Haemophilus*, *Actinobacillus* and *Pasturella* are the members of the family of *Pasteurellaceae*. *Haemophilus*, meaning blood loving, is the most common human pathogen of the family. The *Haemophilus* genus consists of a group of small, gram negative, pleomorphic, non-spore forming, non-motile, facultatively anaerobic bacilli or coccobacilli that require enriched media, usually whole blood or certain of its derivatives (X-factor) and/or NAD (Nicotinamide adenine dinucleotide), or other definable coenzyme like substances (V-factor) for isolation. *Haemophilus* species are differentiated by their growth requirements, biochemical characteristics, pathogenicity and DNA homology (Kilian, 1976). *Haemophilus influenzae* is by far the most important pathogen of this genus in humans. A number of *Haemophilus* species are part of the commensal oral flora and are opportunistic pathogens. Some of these bacteria that are associated with human infections are listed below.

1.2 *H. parainfluenzae*

*H. parainfluenzae* requires V-factor for growth. It may cause endocarditis, meningitis, pneumonia, epiglottitis, septic arthritis and soft tissue abscess. In addition to the respiratory tract, it may be an infrequent pathogen in the genitourinary tract (Albritton, 1982).
1.3 *H. ducreyi*

*H. ducreyi* is responsible for chancroid, a sexually transmitted infection. DNA homology studies indicate that it might have been misclassified as a *Haemophilus* species (Casin *et al.*, 1985). The presence of X-factor is essential for its growth.

1.4 *H. influenzae* biogroup *aegyptius*

*H. influenzae* and *H. aegyptius* appear to be genetically related. *H. aegyptius* is a subgroup of nontypeable *H. influenzae* type III. This organism has been isolated from conjunctiva of patients with ocular disease. *H. influenzae* biogroup *aegyptius* is believed to be the cause of Brazilian purpuric fever, a paediatric infection (Farley *et al.*, 1992b; Brenner *et al.*, 1988).

1.5 *H. aphrophilus*

*H. aphrophilus* is X-factor dependent. It has been isolated from dental plaque and as part of the normal oral flora. There are reports of endocarditis, brain abscess, pneumonia, sinusitis and wound infection caused by this organism (Albritton, 1982).

1.6 *H. paraphrophilus*

*H. paraphrophilus* requires V-factor to grow. Some cases of endocarditis, epiglottitis and pneumonia have been associated with this bacteria (Albritton, 1982).

1.7 *H. influenzae*

*H. influenzae* was first isolated during an influenza pandemic by Pfeiffer in 1892. It was originally regarded to be the causative agent of influenza. This organism is indigenous to humans and is found as part of the normal bacterial flora mainly on the mucous membranes of the upper respiratory tract.
1.7.1 Morphology
In clinical specimens, *H. influenzae* is a small (~1x0.3 μm) coccobacillus, sometimes forming small chains. The morphology depends on age and the medium.

1.7.2 Growth Characteristics
*H. influenzae* requires both haemin (X-factor) and nicotinamide adenine dinucleotide NAD, or phosphate NADP (V-factor) for growth. Haemin is essential for the synthesis of iron-containing respiratory enzymes, such as cytochrome C, cytochrome oxidase and catalase. NAD is a requirement for the oxidation-reduction stage of the metabolism.

1.7.3 Classification
To identify isolates of *H. influenzae* especially for epidemiological purposes, several phenotypic properties such as capsular serotype, biotype, lipopolysaccharide and outer membrane protein patterns, as well as genotypic profiles may be characterised.

1.7.3.A Capsular Serotype
Based on the structure of chemically distinct polysaccharide capsules, *H. influenzae* strains have been categorised into six antigenic serotypes, designated a-f (Pittman, 1931). The strains that do not possess a capsule are referred to as nontypeable. Systemic infections such as meningitis and bacteraemia in children are mainly caused by encapsulated strains of *H. influenzae*, most frequently by those with type b capsular polysaccharide (Hib), whereas localised infections such as sinusitis and otitis media usually are mediated by nonencapsulated strains (Granato et al., 1983). However in adults, invasive infections are associated with both nontypeable (Wallace et al., 1981) and type b organisms (Farley et al., 1992a). *H. influenzae* infection is an important problem in adults with immune suppressed conditions.
More than 70% of the *H. influenzae* present in the normal flora of the upper respiratory tract are nonencapsulated (Moxon & Kroll, 1990), 2-4% are type b (Moxon & Vaughn, 1981) and only 1-2% are of other serotypes.

### 1.7.3.B Biotype Classification

In 1976 Kilian introduced a biotyping classification system for *H. influenzae* based on the enzymatic and biochemical properties such as indole production, urea hydrolysis, and ornitin decarboxylase reactions (Kilian, 1976). According to this classification, *H. influenzae* strains are subdivided into eight biotypes I-VIII excluding *H. influenzae* biotype *aegyptius* (Kilian, 1991). Most type b strains are biotype I. Among nontypeable isolates, biotype II has been frequently recovered from invasive infections. The incidence of β-lactamase production in ampicillin-resistant strains is higher among encapsulated biotype I isolates (Granato et al., 1983; Wallace et al., 1981). Biotypes I, II, and III are commonly isolated from patients. Strains of biotypes VI-VIII account for only a small percentage of isolates. The distribution of certain biotypes of clinical isolates of *H. influenzae* may vary significantly when comparisons are made world-wide (Granato et al., 1983).

### 1.7.3.C Outer Membrane Protein Profiles

The outer membranes of both encapsulated and nonencapsulated strains of *H. influenzae* have various membrane proteins. Outer membrane protein (OMP) composition is a useful tool in epidemiological studies of disease caused by *H. influenzae* especially type b (Loeb & Smith, 1980). The specific functions that most of these proteins provide for the bacterial cell are unclear (Coulton et al., 1992). There are approximately fifteen outer membrane proteins classified as major and minor. Based on the relative positions on sodium dodecyl sulphate (SDS)-polyacrylamide gels, major OMPs have been designated as: P1 (or 'a', 46-47 kDa), P2 (or 'b,c', 39-40 kDa), P5 (or 'd', 37-39 kDa), P4 (or 'e', 28-30 kDa), 'f' (20 kDa) and P6 (or 'g', 15 kDa) (Liu and Smith, 1992; van Alphen et al., 1990). The patterns of these protein bands on SDS-PAGE gels are variable especially among nontypeable strains.
non-capsulated laboratory-derived variants of type b isolate appear to retain the same protein profiles as the parent Hib strain (Barenkamp et al., 1981).

OMPs 'a' or P1 and 'd' or P5 are heat modifiable. Differences in the mobility of P1 is visualised on modified Laemmeli gels. Variants of this protein H (heavy), L (light) and U (unclassified) are the basis of the sub-typing scheme (Barenkamp et al., 1981). P1 is highly conserved among *H. influenzae* type b isolates and only minor amino acid sequence differences exist between the H and L forms of this protein. Munson and Hunt (Munson and Hunt, 1989) have demonstrated that P1 is not required for *in vivo* or *in vitro* growth of the organism. P1 may be a protective antigen but it is not involved in the expression of virulence by *H. influenzae* type b (Hanson et al., 1989).

P2 has porin activity. Lack of expression of P2 in a mutant strain affects the expression of other OMPs. In *H. influenzae* type b, P2 has been described as a growth rate-enhancing physiological component of the outer membrane that is necessary but not sufficient for expression of virulence by this pathogen (Cope et al., 1990). P2 is slightly cation selective with a large exclusion limit, and has a higher permeability toward β-lactam antibiotics compared with that of *E. coli* (Vachon et al., 1986). P2 proteins are antigenically strain specific (Bell et al., 1994; Munson et al., 1983). Expression of antigenically variable OMPs may be a mechanism of host evasion by nontypeable *H. influenzae*.

P6 is a conserved (Barenkamp, 1992) and strongly immunogenic (Liu & Smith, 1992) protein among both capsulated and nontypeable strains.

There are several OMPs that play a role in iron uptake. Free iron is limited *in vivo*, therefore the iron-binding function of these proteins can be regarded as a virulent mechanism (Liu & Smith, 1992).
1.7.3.D Genotypic Classification of *H. influenzae*

The methods mentioned above do not provide the sufficient information that is required for analysis of the genetic structure of the *H. influenzae* population. Multilocus enzyme electrophoresis is based on the relative electrophoretic mobility of chromosomally encoded enzymes. The electrostatic charge and the rate of migration of a protein during electrophoresis are determined by its amino acid sequence. As the result of one or more amino acid substitution, variants of an enzyme, allozymes or electromorphs, would have different electrostatic charges. This is directly correlated with alleles at the corresponding structural gene locus. Therefore the genetic relatedness of two or more strains may be determined by comparing the relative mobility of the enzyme variants (Selander *et al.*, 1986). Characterisation of *H. influenzae* strains by this technique confirmed the substantial genetic diversity present in the *H. influenzae* population (Moxon and Kroll, 1990). Greater genetic diversity is found among nontypeable isolates more than among type b strains (St. Geme III, 1993).

In 1988 another subtyping method was introduced by Stull *et al.* (1988). In this technique *E. coli* or *H. influenzae* ribosomal RNA (rRNA) is used as a probe and provides a broad spectrum of hybridisation with restricted bacterial chromosomal DNA. The hybridisation banding patterns of DNA are then compared. This method (ribotyping) is useful for investigating the molecular epidemiology of genetically diverse bacteria.

In 1994 van Belkum *et al.* introduced another method for genotyping nonencapsulated strains in which the polymerase chain reaction is applied for finger printing of genomic DNA.

1.7.4 Clinical Syndromes

*H. influenzae* has been associated with upper and lower respiratory tract as well as invasive infections.
1.7.4.A Upper Respiratory Tract Infections

1.7.4.A.i Epiglottitis

*H. influenzae* type b occasionally causes severe epiglottitis with obstruction of the airway in young children most commonly between the ages of two to seven (Turk, 1984).

1.7.4.A.ii Acute and Chronic Sinusitis

Infection happens when normal drainage is obstructed. Nontypeable *H. influenzae* and *Streptococcus pneumoniae* account for nearly all bacterial isolates recovered from sinusitis infections in children and adults. The infection can spread to the CNS and cause brain abscesses (St. Geme III, 1993).

1.7.4.A.iii Acute and Chronic Otitis Media

Nontypeable *H. influenzae* is one of the most common organisms that causes otitis media in infants and small children. Most children have one or more episode of the acute form of this infection by the time they are three. *H. influenzae* colonises the middle ear by moving through the Eustachian tube (St. Geme III, 1993).

1.7.4.B Lower Respiratory Tract Infections

*H. influenzae* has been isolated from both acute and chronic infections of the lower respiratory tract.

1.7.4.B i Acute Infections

Acute Bronchitis

Both typeable and nontypeable *H. influenzae* may be the cause of secondary bacterial infection in acute bronchitis (Brook, 1994).
Acute Exacerbation of Chronic Bronchitis

Non-capsulated *H. influenzae* is frequently isolated in the sputum of individuals with chronic bronchitis. Bacterial infection does not initiate the disease, but prolongs the symptoms. Smoking and inhaling dust and fumes can also contribute to this syndrome (St. Geme III, 1993; Brook, 1994).

Pneumonia

Nontypeable *H. influenzae* is an important and common cause of bacterial pneumonia especially among children and elderly. Lung abscess is a complication in this illness. *H. influenzae* type b also has been isolated in this infection (St. Geme III, 1993; van Alphen, 1992; Turk, 1984).

1.7.4.B.ii Chronic Infections

Cystic Fibrosis

Non-capsulated *H. influenzae* strains are among the pathogens that contribute to the respiratory complications in CF patients (Brook, 1994).

1.7.4.C Other Infections

1.7.4.C.i Meningitis

*H. influenzae* type b is the most common type of bacteria responsible for paediatric meningitis. However almost half of the cases of *H. influenzae* in adults are caused by nontypeable strains. Hib disseminates to the meninges by invading the blood stream first, in contrast, nontypeable strains spread by direct extension from a contiguous focus of infection, especially in the presence of predisposing factors such as head trauma, sinusitis or otitis media (St. Geme III, 1993; van Alphen, 1992).
1.7.4.C.ii Septic Arthritis
Secondary Hib infection in a single large joint in young children may cause arthritis. However in older children and adults it may only happen in joints with pre-existing damage or when the individual is immuno-compromised (Brook, 1994).

1.7.4.C.iii Neonatal and Female Genital Tract Disease
There is a group of organisms frequently associated with neonatal-maternal and urogenital infections that has been identified as nontypeable biotype IV *H. influenzae*. These highly virulent organisms are genetically homogeneous, have a distinct multi-locus enzyme genotype and identical outer membrane protein profile. The newly identified species are different from *H. influenzae*. Although they are of biotype IV, they are weakly related to *H. influenzae*. Sometimes, this bacterium causes prenatal infections involving the mother, the baby, or both. In non-pregnant women, usually in the presence of intrauterine devices, or previous damage or infection, it may cause tuboovarian abscess (Quentin et al., 1990; Wallace et al., 1983; Quentin et al., 1993).

There are reports of other illnesses that have been associated with *H. influenzae* such as endocarditis, pericarditis, osteomyelitis, and conjunctiva-otitis syndrome (St. Geme III, 1993).

1.7.5 Pathogenesis of *H. influenzae*
In invasive infections, encapsulated *H. influenzae* strains colonise and replicate on the mucosal surface and eventually invade the respiratory tract epithelium. Subsequently, the bacterium enters the blood stream and its rapid intravascular proliferation results in bacteraemia. Finally, it enters the CNS through the Choroid plexus. The pathogenicity of capsulated *H. influenzae* is determined by an important co-operation between somatic antigens and the polysaccharide capsule (Moxon, 1992; Liu & Smith 1992).
Nontypeable organisms are rapidly removed by mucociliary clearance soon after entering the respiratory tract. However in individuals with impaired clearance, the bacteria remain attached to the mucus layer for longer, where they replicate and subsequently, make contact with the impaired epithelium. The bacteria may then spread through the respiratory tract (Read et al., 1991; Wilson et al., 1992).

The pathogenicity of *H. influenzae* strains may be a reflection of a co-operative effect of several virulence factors. The role of each parameter is discussed further.

### 1.7.5. A Polysaccharide Capsule

The capsule of *H. influenzae*, like other bacteria, is composed of repeating units of polysaccharides. The structure of capsular polysaccharides of serotypes a-f are shown in Figure 1-1 (Moxon & Kroll, 1990).

Although in types a, b, c, and f, phosphoric di-ester links the units, the polymers are still referred to as capsular polysaccharides. A variant serotype e' has also been recognised in which all repeat units of type e polysaccharide, the 3-hydroxyl groups are fructosylated. The significance of this substitution is unknown (Moxon & Kroll, 1990). The a and b capsules are structurally the most closely related of *H. influenzae* capsules, the type b capsule is a linear polymer of ribose ribitol phosphate and the type a capsule consists of glucose ribitol phosphate. The capsular material of type b polyribosyl ribitol phosphate (PRP), is unique among other serotypes in that both of its sugars are pentoses, whereas all of the others contain hexoses. It is postulated that type b polysaccharide is assembled in the cytoplasm; however, there is little known about the site of polymerisation and transportation of the capsule. It is possible that a phospholipid moiety might be responsible for anchoring the capsule to the outer membrane (Moxon & Kroll, 1990).

Ninety percent of invasive diseases are associated with *H. influenzae* type b (Liu & Smith, 1992), other serotypes are rarely responsible for invasive infections. Thus the
PRP capsule may be an important factor in the pathogenicity of type b strains (Moxon & Vaughn, 1981).

The genes encoding the type b capsule, capb, are chromosomal and are clustered in a region of ~50 kb. The capsulation locus consists of a duplication of a 17-18 kb fragment (Moxon, 1992) that are separated by a small 1-1.3 kb bridging region. The bridge region contains a gene, bexA which is essential for transporting carbohydrate to the surface of the cell (Moxon & Kroll, 1990). The configuration of capb locus causes instability in capsule expression and, as a consequence, the frequency of capsule loss in type b isolates to uncapsulated variants has been estimated to be 0.1-0.3% (Hoisteth et al., 1985). This process seems to be irreversible. However in vitro, capsule-deficient isolates could be transformed to encapsulated variants by uptaking the DNA from the environment. Uncapsulated variants (St. Geme III & Falkow, 1991) and nontypeable strains (Read et al., 1992) adhere better to the epithelium than the capsulated isolates. Nontypeable H. influenzae strains do not adhere to the intact epithelium but attach to already damaged epithelial surfaces, proliferate and cause further distraction. Capsulated H. influenzae strains infrequently associate with the epithelial surface; instead they form a thick gel-matrix above the epithelium (Read et al., 1992). Therefore, it is possible that the presence of the capsule prevents adherence to epithelial cells via hydrophobic or electrostatic interaction (St. Geme III & Falkow, 1991). Gal 1 NAC β1-4 Gal is a receptor in various glycosphingolipids to which H. influenzae binds. These receptors may be available only when cells are damaged (Read et al., 1991).

Endothelial cells are located in important areas within the body. Virji et al.,(1991) have demonstrated that both encapsulated and uncapsulated strains are translocated across the endothelial cells and reach the blood by endocytosis. The capsule-deficient bacteria are internalised more rapidly.
Figure 1-1. Structure of Capsular Polysaccharides of *H. influenzae* (Serotypes a through f).

**Type a**

**Type b**

**Type c**

**Type d**

**Type e**

**Type f**

\[\beta D\text{ fructose in type e'}\]
The significance of type b capsule is that it delays clearance, enhances the intravascular survival and increases the virulence of *H. influenzae* in blood (Weller *et al.*, 1977). The rate of survival of *H. influenzae* type b in blood was compared to that of other capsular serotypes by Moxon and Vaughn (Moxon & Vaughn, 1981). After intranasal, intravenous, and peritoneal inoculation of rats with the six serotypes of *H. influenzae* only type b strains survived in blood and caused bactaeremia and meningitis. Non-capsulated isolates were non-invasive. Replication of *H. influenzae* type b in blood is an essential event in the pathogenesis (Moxon, 1992). Phagocytosis especially by the reticuloendothelial system is the crucial mechanism of clearance in blood (Moxon & Kroll, 1990). The Type b capsule confers resistance to the opsonophagocytic mechanisms of the host, such as phagocytic cells, complement components and serum antibodies. (Moxon & Kroll, 1990; Weller *et al.*, 1978).

### 1.7.5.B The Role of Pili in Colonisation

Nasopharyngeal colonisation is the initial process in pathogenesis of disease caused by *H. influenzae* (Turk, 1984). In healthy individuals the non-specific host defences of ciliated respiratory mucous discourages bacterial adherence to epithelial cells. Mucous secretion and co-ordinated, rapid beating drives the mucous towards the oropharynx and creates a turbulence within the preciliary fluid (Wilson *et al.*, 1992; Read *et al.*, 1992). However in the event of reduced host-defences, *H. influenzae* associates with and proliferates within mucous. The increasing number of bacteria eventually damages the already distracted epithelial surface and establishes a more stable colonisation (Wilson *et al.*, 1992). The correlation between the presence of pili or fimbriae in some *H. influenzae* strains and increased adherence to human respiratory epithelial cells was first reported in 1982 (Guerina *et al.*, 1982; Pichichero *et al.*, 1982).

Based on the morphological shape and the ability of hemagglutinating human erythrocytes, the pili of *H. influenzae* have been categorised into four families of which LKP (long, thick, hemagglutination positive) have been extensively studied. LKP pili are common in both nontypeable and type b strains (Brinton *et al.*, 1989).
The expression of pili in *H. influenzae* is subject to phase variation (van Ham *et al.*, 1992; Farley *et al.*, 1990).

Inoculating the human nasopharynx organ culture with piliated *H. influenzae* type b demonstrated an increased adherence over that of non-piliated type b strains (Loeb *et al.*, 1988), but piliation did not significantly increase the epithelial damage (Read *et al.*, 1992). Piliated type b strains adhere to nonciliated epithelial cells and form large clusters of bacteria at the mucosal surface, while the non-piliated strains readily disseminate or invade the areas of mucosal damage (Loeb *et al.*, 1988; Farley *et al.*, 1990).

All non-capsulated *H. influenzae* isolated from the upper respiratory tract are piliated; however, the degree of fimbriation does not correlate with adherence (Read *et al.*, 1991). Fimbriated nontypeable variants adhere more frequently to buccal and mucosal cell culture than nonfimbriated nontypeable strains, but their degree of binding is weaker than fimbriated *H. influenzae* type b isolates (Gilsdorf *et al.*, 1992a; Gilsdorf *et al.*, 1992b). However, non-piliated nontypeable strains show a far more better adherence over non-piliated *H. influenzae* type b (Gilsdorf *et al.*, 1992a). Expression of pili may be a disadvantage in the blood since they stimulate binding to the red blood cells and could thereby promote the clearance of the bacteria. Blood isolates of *H. influenzae* are either non- or poorly piliated (Moxon & Wilson, 1991).

In addition to pili, other non-pilus mediated forms of adherence exist. Barenkamp and Leninger reported the possible role of a group of high molecular weight, HMW, (120 and 125 kDa) surface exposed proteins in promoting attachment of nontypeable *H. influenzae* to epithelial cells via a pilus independent mechanism (Barenkamp & Leininger, 1992). HMW proteins of nontypeable *H. influenzae* recognise and bind specifically to cellular proteoglycans on the surface of the epithelium (Noel *et al.*, 1994). This adhesion may in part be responsible for colonisation of the bacteria in respiratory tract. The highly immunogenic HMW1 and HMW2 are structurally related to the filamentous hemagglutinin of *Bordetella pertussis* (Barenkamp & Leininger, 1992).
1.7.5.C The Production of Haemocin

Haemocin, an extra cellular protein, is a bacteriocin produced only by *H. influenzae* type b. This substance affects nontypeable and non-b capsulated *H. influenzae* as well as certain members of the family of Enterobacteriaceae (Venezia & Robertson, 1975). LiPuma *et al.* (1992) demonstrated that although haemocin production and type b encapsulation are closely associated, they are genetically independent. It is a possibility that the bactericidal activity of type b strains may be an important factor in the nasopharyngeal colonisation and pathogenesis of *H. influenzae* type b infections (LiPuma *et al.*, 1990).

1.7.5.D Immunoglobulin A1 Proteases of *H. influenzae*

If colonising bacteria are to persist in the epithelium, they have to evade the host's local immune system. Immunoglobulin (Ig) A is the primary antibody found in mucosal surface. IgA1 proteases are extracellular bacterial enzymes that specifically hydrolyse human IgA1. These enzymes cleave the hinge region of the heavy polypeptide chain and the resulting products are Fc and Fab (Poulsen, *et al.* 1992; Plaut, 1983). The proteolytic cleavage impairs the function of the IgA1 and facilitates bacterial colonisation. However IgA1 proteases have no effect on IgA2, because the hinge peptide is not present in this molecule (Plaut, 1983). The fact that these enzymes are produced by several clinically important pathogens that infect the mucosal surfaces such as *Neisseria gonorrhoea*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Streptococcus sanguis* and *Haemophilus influenzae* (Plaut, 1983; Kilian *et al.*, 1983; Poulsen, *et al.* 1992), and the absence of IgA1 protease production in closely related but non-pathogenic bacteria, contribute to the role of IgA1 protease in pathogenicity.

*H. influenzae* is the only member of the *Haemophilus* genus that produces serine IgA1 protease (Kilian *et al.*, 1979; Kilian & Poulsen, 1992; Plaut, 1983, Mulks *et al.*, 1982; Poulsen *et al.*, 1992). There are at least two distinct types of IgA1 proteases in *H. influenzae* isolates (Plaut, 1983). IgA1 type1 protease cleaves the Pro-Ser peptide
bond between residues 231 and 232 in the human IgA1 heavy chain. IgA1 type 2 enzyme cleaves the Pro-Thr bond at positions 234-236 (Plaut, 1983; Poulsen et al., 1992; Kilian & Poulsen, 1992). There has also been reports of types 3 and 4 proteases that yield two Fd fragments of different molecular sizes (Mulks et al., 1982).

*H. influenzae* iga genes (encoding IgA1 protease) are chromosomal, in single copies and have a mosaic like structure. The mosaic pattern suggests that genetic elements are horizontally exchanged between *H. influenzae* strains. Thus IgA1 protease is under constant evolutionary selection (Kilian & Poulsen, 1992; Poulsen et al., 1992).

Most capsular serotypes of *H. influenzae* produce only one type of the IgA1 protease, except some isolates of serotypes e and f (Kilian & Poulsen, 1992). Serotypes a, b, d and f secrete protease type 1, whereas serotypes c and e produce type 2. Since IgA1 protease was produced by all five biotypes, no correlation was found between biotype or with the origin of the isolates (Kilian et al., 1979; Mulks et al., 1982). Uncapsulated strains produce either type 1 or 2 or both types simultaneously (Kilian & Poulsen, 1992).

While some believe in the possible role of IgA1 proteases as one of the parameters determining pathogenicity, others (Turk, 1984) suggest that the lack of sufficient evidence questions the ability of the IgA1 protease as a virulent factor.

**1.7.5.E Modulation of Virulence by Lipopolysaccharide**

Lipopolysaccharide (LPS) or more accurately lipoooligosaccharide (LOS) of *H. influenzae* is endotoxic and has biological activity comparable to that of the extensively characterised Enterobacterial endotoxins (Flesher & Insel, 1978). The LOS of *H. influenzae* is rough in nature and lacks the repeating terminal side chain, O-antigen. It consists of a lipid A moiety and neutral sugars; glucose, galactose and heptose (Zwahlen et al., 1986).
Based on the compositional studies of *H. influenzae* LOS, there are quantitative differences in the amount of oligosaccharides present in the core region (Zamze & Moxon, 1987). It appears that the balance between the amount of galactose and glucose is important in virulence (Maskell *et al.*, 1992; Zwahlen *et al.*, 1986; Zamze & Moxon, 1987). *H. influenzae* LOS is characterised by both inter- and intra-strain heterogeneity of the surface-exposed, neutral sugars. The intra-strain variation is in part the result of antigen switching referred to as 'phase variation'. Spontaneous antigenic and phenotypic variation of LOS happens at a relatively high frequency in some strains of Hib (Kimura & Hansen, 1986; Weiser *et al.*, 1990). The structure of one of the epitopes, Gal α1-4 Gal β, is identical to a sugar residue found on the glycosphingolipids of some human epithelial cells (Weiser *et al.*, 1990; Virji *et al.*, 1990). Three chromosomal loci, *lic1*, *lic2*, and *lic3* mediate the biosynthesis and control the *H. influenzae* LOS phase variation (Weiser *et al.*, 1990).

Characterisation of Hib LOS by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), developed a classification system based on the mobility of two to four LOS bands that vary among different strains. This method is sensitive for epidemiological investigation of Hib disease (van Alphen *et al.*, 1983; Inzana & Pichichero, 1984). The LOS pattern of nontypeable strains are less variable, therefore this method is not recommended for subtyping those isolates (Inzana & Pichichero, 1984).

Kimura & Hansen (1986) suggested that the full expression of Hib virulence is associated with the higher molecular weight type of LOS, and Zwahlen *et al* reported a link between the genes involved in determining the structure of the capsule and LOS expression (Zwahlen *et al.*, 1983; Zwahlen *et al.*, 1986).

The lipid A component of LOS is responsible for its proinflammatory activity, while the oligosaccharide side chains are responsible for its antigenic diversity. In meningitis, *H. influenzae* LOS induces alteration in the Blood-Brain Barrier permeability by affecting the intracellular regulatory pathways. The target of LOS
appears to be leukocyte response. Leukocytes are sources of interleukin-1 and tumour necrosis factor that separate the endothelial junctions and result in the loss of barrier and therefore tissue injury (Burroughs et al., 1992; Tunkel et al., 1992). Another possible role for LOS in pathogenicity is its potential to enhance serum resistance and therefore survival in both typeable and non-typeable strains (Zwahlen et al., 1986).

1.7.5.F Peptidoglycan Contribution to Meningeal Inflammation

Cell wall components and LPS are released during growth and antibiotic-induced bacterial autolysis (Burroughs et al., 1993b). Although the inflammatory activity of peptidoglycan is independent from LPS, they may be complementary in the induction of meningeal inflammation (Burroughs et al., 1993b; Burroughs et al., 1992). Peptidoglycan subcomponents of _H. influenzae_ are involved in the inflammatory process such as CSF leukocytosis (Burroughs et al., 1993b), increasing blood-brain barrier permeability (Roord et al., 1994), and in particular cytotoxic brain oedema (Burroughs et al., 1993b; Burroughs et al., 1992) by different mechanisms (Burroughs et al., 1993c).

The role of _H. influenzae_ peptidoglycan in the pathology of meningitis has been supported by the chemically and metabolically different cell wall structures of ampicillin-sensitive and non-β-lactamase mediated ampicillin resistant strains (Burroughs et al., 1992; Burroughs et al., 1993a; Burroughs et al., 1993b). In experimental animals, ampicillin-resistant isolates induce more inflammation and have a higher peptidoglycan turn-over than ampicillin-sensitive strains; however the latter release more peptidoglycan after antibiotic therapy (Burroughs et al., 1992; Burroughs et al., 1993b). An increase in the amount of disaccharide tripeptides and a decrease in 1,6 anhydrodimers in peptidoglycan composition indicate the degree of ampicillin resistance and, therefore, the pathogenicity of _H. influenzae_ strains (Burroughs et al., 1992; Burroughs et al., 1993a).
1.7.6 Prevention

The incidence of *H. influenzae* infections is higher in infants, young children who attend day-care facilities, individuals with immunodeficiencies and in certain groups including Alaska Natives, American Indians and children of lower socio-economic status. Invasive infections are usually associated with encapsulated strains, most frequently with *H. influenzae* type b. There is a relationship between age and *H. influenzae* infection. By around four months of age, as the maternally derived antibody declines, the risk of infection increases, until it reaches a peak at five to twelve months of age. After the age of four years, the incidence of *H. influenzae* type b disease decreases dramatically.

In order to prevent the risk of morbidity and mortality that result from *H. influenzae* type b infections, a vaccine was produced. The early vaccines contained purified capsular polysaccharide, polyribosyl-ribitol phosphate (PRP), the main virulent factor of this organism. However, they were poorly immunogenic in children under eighteen months old, the group with the highest risk of infection. Further research lead to the development of 'second generation' vaccines. The immunogenic effect of these vaccines in infants has been improved by conjugating the capsular polysaccharide to a protein. Currently there are four types of conjugated *H. influenzae* type b vaccines available, PRP-D, (conjugated with diphtheria toxoid) appears to be more age-dependent than the rest, PRP-OMP, (conjugated with outer membrane protein complex of *Neisseria meningitidis*) is the least age-dependent vaccine, PRP-HbOC, (conjugated with CRM197, a non toxic mutant diphtheria toxin) and PRP-T (conjugated with tetanus toxoid). *H. influenzae* type b vaccine, reduces the oropharyngeal carriage of these strains, and the risk of person-to-person transmission (Shapiro & Ward, 1991; Campbell & Carter, 1993).

Recently Bacterial Polysaccharide Immunoglobulin (BPIG) has been prepared from the plasma of adult volunteers immunised with the 23-valent pneumococcal vaccine, 4-valent meningococcal vaccine, and PRP-D vaccine. BPIG is administered followed by PRP-OMP at two, four, and twelve months of age. BPIG contains 15-50 times
concentrations of anti-capsular antibodies than that found in standard immune serum globulin (Singleton et al., 1994).

Nontypeable *H. influenzae* are the common cause of localised respiratory tract infection in both children and adults. In addition, nontypeable *H. influenzae* may occasionally invade beyond the respiratory tract and cause infections such as septicaemia and endocarditis. Immunity to nontypeable *H. influenzae* is complicated because of the marked heterogeneity that exists among these isolates. Over the past years, bacterial surface antigens have been the basis of extensive vaccine studies. Outer membrane proteins P6, P4, PCP, and D15 are potential vaccine candidates. The effectiveness of immunisation with hemagglutinating pili is not clear, since pili are phase variable in expression and might not be required for virulence. Other vaccine candidates include high molecular weight proteins that act as epithelial adhesins, and also the proteins that are responsible for iron acquisition.

### 1.7.7 Treatment of Infections Caused by *H. influenzae*

Until the early 1970s the treatment of choice for *H. influenzae* infections was ampicillin. However, the increasing prevalence of ampicillin-resistance in both capsulated and uncapsulated strains has affected the antibiotic therapy for invasive and non-invasive infections (Gunn et al., 1974; Schiffer et al., 1974; Thomas et al., 1974).

The drug of choice for treatment of invasive infections including meningitis caused by ampicillin-sensitive *H. influenzae* is still ampicillin. For ampicillin-resistant *H. influenzae* strains, many cephalosporins have proved to be highly effective. Nevertheless, the limited susceptibility of this species to most first generation cephalosporins and the poor CSF penetration of these drugs restrict their application to only non-invasive infections. The ability of cefaclor to resist the hydrolytic activity of β-lactamases, allows this antibiotic to achieve greater periplasmic concentrations than ampicillin, and sufficient binding to crucial penicillin-binding proteins in *H. influenzae* (Picard & Malouin, 1992). *In vitro*, activity of second-generation
cephalosporins seems to be sufficient and most of the β-lactamase mediated ampicillin-resistant isolates are susceptible to cefuroxime and other second-generation cephalosporins (Goldberg, 1987). However, cefuroxime is not recommended for the treatment of meningitis since it has been associated with slowed bacterial sterilisation from the CSF (Marks, 1991; Vallejo et al., 1991). Third generation cephalosporins, especially cefotaxime and ceftriaxone, are highly effective against meningitis and other invasive infections caused by ampicillin-resistant H. influenzae strains. Ceftriaxone is usually avoided in newborn infants because of its tendency to displace bilirubin. Ceftazidime like cefuroxime has been associated with delayed bacterial sterilisation from the CSF therefore it may not be used for the treatment of bacterial meningitis. Therapeutic failures with cefotaxime are unlikely, since the drug concentrations achieved in the blood, CSF and tissues are such that even non-β-lactamase mediated β-lactam resistant strains can be eradicated.

Administration of imipenem-cilastatin in patients with bacterial meningitis has been related to a high incidence of seizures, therefore it is not recommended for meningitis treatment (Moellering et al., 1989). The most commonly used alternative to β-lactams is chloramphenicol which performs efficiently against ampicillin-resistant H. influenzae strains (Marks, 1991).

Lower Respiratory Tract infections caused by H. influenzae are treated with ampicillin, co-amoxiclav, cefuroxime and oral cephalosporins such as cefaclor and cefpodoxime. Apart from β-lactams, ofloxacin and azithromycin may also be administered (Hopelman et al., 1993; Daniel et al., 1991; Geddes, 1991; Vallejo et al., 1991). For the treatment of acute exacerbation of chronic obstructive pulmonary disease tetracycline and trimethoprim-sulfamethoxazole are the drugs of choice (Murphy & Sethi, 1992).

The antimicrobial agents found to be most effective in the treatment of the upper respiratory tract infections caused by H. influenzae are amoxycillin, co-amoxiclav, cefaclor, cefuroxime, cefixime, cefpodoxime, cefprozil, loracarbef and trimethoprim-
sulfamethoxazole (Gehanno et al., 1994; Johnson et al., 1991; Giebink, 1994; Giebink & Canafax, 1991).

1.8 \( \beta \)-Lactam Antibiotics

\( \beta \)-lactam antibiotics are among the safest, most effective and most widely used drugs in clinical practice. Penicillins, cephalosporins, carbapenems and monobactams are the members of the family of \( \beta \)-lactam antimicrobials. This family can be divided into bicyclic Penicillins (penams, penems carbapenems, oxapenams), Cephalosporins (cephems, cephamycins, oxacephems, carbacephems) and the monocyclic monobactams. The possession of an intact \( \beta \)-lactam ring is essential to their antibacterial activity. Differences in side chains of the basic molecules influence pharmacological properties and spectra by determining permeability into the bacterial cell, affinity for enzymes involved in cell wall synthesis, and susceptibility or resistance to inactivation by \( \beta \)-lactamase enzymes. \( \beta \)-lactam antibiotics are usually bactericidal to the growing susceptible bacteria (Neu, 1986).

The mode of action of \( \beta \)-lactams is mainly interference with biosynthesis of the peptidoglycan structure. The amide group of the \( \beta \)-lactam ring is stereochemically similar to the D-alanyl-D-alanine end of the peptidoglycan pentopeptide. Therefore, the bacteria mistakes the agent for its normal substrate. To reach its site of action, the \( \beta \)-lactam agent must penetrate the outer structures of the bacterial cell wall, survive the action of periplasmic \( \beta \)-lactamases if present and finally bind to and acylate critical penicillin-binding proteins. Penicillin-binding proteins (PBPs) are a number of specific enzymes located on the inner membrane of the cell wall that catalyse the reactions that lead to the cross-linkage between the peptide chains. PBPs are the sites at which \( \beta \)-lactams bind to, and thus terminate the peptide chain linkage and therefore inhibit the formation of normal peptidoglycan structure (Tipper, 1985), (see section 1.10.2).
β-lactams also activate the bacterial autolysis system that can disrupt the structural integrity of the cell wall. However, our understanding of how the interaction between penicillin-binding proteins and β-lactams leads to suicidal autolytic activity in a bacterial cell is still incomplete. Major differences exist among species in the regulation of cell wall synthesis. Therefore, depending on the species of the bacteria and the nature of the β-lactam, several different antimicrobial targets may be present in the cells (Tomasz, 1979).

### 1.8.1 Penicillins

In 1929 Alexander Fleming isolated a culture of a strain of *Penicillium notatum* that produced a substance that inhibited the growth of *Staphylococcus aureus*. This substance was called 'Penicillin'. The initial crude 'Penicillin' extracted comprised of several closely related compounds termed penicillins F, K, X and G. Benzyl penicillin (penicillin G) was chosen for further development as it was the most active compound and also because it could be exclusively produced by *Penicillium chrysogenum*. It was not until 1941 that penicillin G was introduced for the management of bacterial infections. Since then many other penicillins have been discovered or synthesised, and now this group is one of the most important classes of antibiotics. This family has a wide spectrum of antibacterial activity and because of the therapeutic efficacy, pharmacological properties, relatively non-toxicity and lower cost, penicillins are preferred for the treatment of infections caused by sensitive organisms over the new antibiotics. However, scientists still continue to search for a penicillin with excellent penetration properties through the bacterial cell wall, stability to β-lactamases and high affinity for penicillin-binding proteins.

The basic structure of penicillins, 6-amino penicillanic acid, comprises a thiazolidine ring, an attached β-lactam ring, and a side chain group. Manipulations of this structure, especially the side chain, have produced compounds that offer advantages over the parent molecule. Penicillins have been classified in to six groups.
1.8.1.1 Group 1: Natural Penicillins

Benzyl penicillin is the parent drug of the family and is still administered parenterally in serious infections when high serum drug concentrations are required. Benzyl penicillin is effective for treating infections caused by most gram-positive and some gram-negative pathogens as well as some spirochetes. However benzyl penicillin is susceptible to all β-
procaine penicillin are insoluble salts of benzyl penicillin that act as intramuscular depots for the prolong release of benzyl penicillin (Wright & Wilkowske, 1991; Nathwani & Wood, 1993).

1.8.1.2 Group 2: Oral Penicillin Derivatives
The first major success in improving the pharmacological properties of penicillin was achieved with phenoxyethyl penicillin, penicillin V. This compound is stable in gastric acid and it may substitute Penicillin G in mild to moderate streptococcal respiratory tract infections as well as skin and soft tissue infections. It may also be used as a form of prophylaxis against rheumatic fever and recurrent pneumococcal meningitis.

Phenicillin (Phenethicillin) and Propicillin are the phenoxyethyl and phenoxypropyl derivatives of Penicillin G respectively. These oral penicillins have pharmacological properties similar to those of Penicillin V (Wright & Wilkowske, 1991; Nathwani & Wood, 1993).

1.8.1.3 Group 3: Penicillinase-Resistant Anti Staphylococcal Penicillins
The emergence of penicillinase mediated benzyl penicillin-resistant Staphylococci led to the development of semi-synthetic penicillins that were stable to these enzymes. The mechanism of resistance to the action of penicillinase is believed to be steric hindrance that is the result of the configuration of the acyl side chain preventing the opening of the β-lactam ring. However, these compounds are not resistant to other β-lactamases. Methicillin, oxacillin, and nafcillin are available for parenteral use and oxacillin, nafcillin, cloxacillin, dicloxacillin and flucloxacillin are administered orally. For cloxacillin, dicloxacillin and flucloxacillin, the addition of the halogen groups to the phenyl group improves the oral absorption of the compounds. These agents are mainly used for the treatment of infections caused by
penicillinase-producing Staphylococci (Wright & Wilkowske, 1991; Nathwani & Wood, 1993; Wright et al., 1987).

1.8.1.4 Group 4: 'Broad-Spectrum' Penicillins

The extension of spectrum of benzyl penicillin to provide activity against some of the gram-negative organisms was achieved by the addition of an amino group to the side chain. However these extended spectrum penicillins are not resistant to the action of penicillinase enzymes, therefore, they are not effective against penicillinase producing Staphylococci and some gram-negative organisms. These compounds have been subdivided into two groups.

1.8.1.4.i Aminopenicillins

Aminopenicillins are the first penicillins that had activity against *H. influenzae*, *E. coli*, *Salmonellae* and *Shigellae* as well as other penicillin-sensitive gram-negative bacteria. When ampicillin (D-α-aminobenzyl penicillin) was initially introduced, it had extensive indications. However, more recently, with the dissemination of bacterial resistance, treatment with ampicillin is not considered as reliable as before.

Esters, condensates and analogues of ampicillin have been developed to increase the oral absorption of ampicillin. Carboxyl esters of ampicillin or so-called prodrugs are inactive until they are hydrolysed by non-specific tissue esterases in the intestinal mucous to release ampicillin following or during absorption. Pivampicillin, bacampicillin, and talampicillin are examples of esterified prodrugs. Condensates of ampicillin such as hetacillin and metampicillin spontaneously hydrolyse to release ampicillin. The spectrum of activity of all the drugs mentioned so far is similar to ampicillin.

Improved absorption has also been achieved by minor modifications to the ampicillin molecule to produce amoxycillin and cicalcillin. Amoxycillin is the para-hydroxy derivative of ampicillin. It is more completely absorbed in comparison with the fifty
percent absorption rate of ampicillin. The antibacterial activity of amoxycillin is closely related to ampicillin; however, owing to its greater absorption, less drug is left in the intestinal tract, markedly reducing its efficacy in shigellosis. The therapeutic applications of amoxycillin is limited with the spread of resistance to aminopenicillins. Ciclacillin has the similar therapeutic indication as amoxycillin except for its lesser activity than ampicillin or amoxycillin against S. pneumoniae and H. influenzae (Wright & Wilkowske, 1991; Nathwani & Wood, 1993).

1.8.1.4.ii Amdinocillin

Amdinocillin (Mecillinam) contains a substituted amidino group (N-CH=N) instead of an acyl (CO-NH) at the 6-position of the penicillianic acid. It is highly active against gram-negative bacteria whereas its activity against gram-positive organisms is poor. Mecillinam almost exclusively interacts with PBP3 in H. influenzae and PBP2 in other gram-negative bacteria and causes the organism to become spherical and to lyse. Although β-lactamases hydrolyse mecillinam slower than other β-lactam antibiotics, but because other extended-spectrum penicillins are more active than mecillinam, its use in the treatment of infections may be of limited value (Wright & Wilkowske, 1991; Nathwani & Wood, 1993).

1.8.1.5 Group 5: Penicillins Active Against Pseudomonas aeruginosa

All the agents previously mentioned are inactive against P. aeruginosa, an important opportunistic pathogen. However, there are two groups of penicillins, α-carboxypenicillins and the acylureidopenicillins, that are effective against this organism.

1.8.1.5.i Carboxypenicillins

Carbenicillin is a derivative of benzyl penicillin that bears a carboxyl substituent instead of the amino group of the ampicillin. Ticarcillin, the other member of this
group, is identical to carbenicillin except for the replacement of a 3-thienyl ring instead of a phenyl group in the side chain.

The antibacterial spectrum of these drugs is similar to that of ampicillin, with added activity against certain strains of *P. aeruginosa*, the indole-positive *Proteus* and *Enterobacter* species. The presence of a highly ionic group reduces the activity of these agents against gram-positive bacteria.

Carboxypenicillins have largely been replaced by the ureidopenicillins and quinolones for treating *P. aeruginosa* infections because of lesser side effects and higher chances of efficacy (Wright & Wilkowske, 1991; Nathwani & Wood, 1993).

### 1.8.1.5.ii Acylureidopenicillins or Ureidopenicillins

Mezlocillin, azlocillin, piperacillin and apalcillin are the acylureidopenicillins. These agents are semi-synthetic derivatives of ampicillin with side chain adaptations. The side chains of mezlocillin and azlocillin are acyl derivatives of the urea molecule. Piperacillin has a piperazine side chain on the ampicillin parent molecule. Acylureidopenicillins are generally bactericidal, except for certain *Pseudomonae*, *E. coli* and *Klebsiella* strains. They act by inhibiting cell wall synthesis of dividing bacteria. Their structure allows them to penetrate extensively into the cell wall and bind selectively to penicillin-binding proteins. Ureidopenicillins are susceptible to the action of many β-lactamases, are subject to a marked inoculum effect, and compared with ticarcillin they are less bacteriolytic.

The important advantage of the ureidopenicillins is their increased activity against *P. aeruginosa* and *Klebsiella*. These agents are less active against gram-positive and anaerobic bacteria, β-lactamase producing *Staphylococci* and *H. influenzae*. Therefore, they should not be used as drugs of choice for infections caused by these organisms (Wright & Wilkowske, 1991; Nathwani & Wood, 1993).
1.8.1.6 Group 6: β-Lactamase Resistant Penicillins

In search for a penicillin with greater β-lactamase resistance, compounds with modifications at the 6α-position of the structural nucleus were developed. Temocillin is the 6α-methoxy and formidacillin (foramidocillin) is the 6α-foramido derivative of ticarcillin. These agents are highly resistant to β-lactamases.

Temocillin is more active than carbenicillin or ticarcillin against many gram-negative bacteria including the Enterobacteriaceae, β-lactamase producing H. influenzae and M. catarrhalis, but is less active against P. aeruginosa, Acinetobacter and Serratia species as the result of poor cell wall penetration. Gram-positive bacteria and anaerobes are not inhibited by this agent. Temocillin is reserved for cephalosporin-resistant Enterobacteriaceae or for when extended-spectrum β-lactamases become more prevalent.

The 6α-formamido group substitution has allowed the preservation of both β-lactamase-resistance and antibacterial activity in formidacillin. In vitro, this drug is highly active against Enterobacteriaceae, H. influenzae and N. gonorrhoeae. Its high activity against P. aeruginosa is more superior than piperacillin, aztreonam and ceftazidime. However, it is inactive against gram-positive bacteria or anaerobes (Wright & Wilkowske 1991; Nathwani & Wood, 1993).

1.8.2 Cephalosporins

The first cephalosporin, known as cephalosporin C, was discovered by Brotzu from cultures containing the fungus Cephalosporium acremonium (reviewed by Abraham, 1987). The parent nucleus of cephalosporins is 7-aminocephalosporanic acid (7-ACA) that consists of a β-lactam ring bound to a 6-member dihydrothiazine cephem ring. This structure induces inherent resistance to degradation caused by β-lactamases. Cephalosporins have similar mechanism of action as the penicillins. They inhibit penicillin-binding proteins and interfere with the formation of the bacterial cell wall, leading to the weakening of the cell wall and ultimately, cell lysis.
Cephalosporins are bactericidal to microorganisms that contain autolysis enzymes and bacteriostatic to the ones that lack autolysins (Gustaferro & Steckelberg, 1991).

Modification of the basic structure, 7-ACA, has produced a variety of compounds with differences in spectrum of activity, pharmacokinetics, and toxicity (Christ, 1991). Modifications at the 7-α position of the β-lactam ring alter antimicrobial activity and stability to β-lactamases. Cefazolin, cefotaxime, ceftriaxone and cefuroxime contain 7-α substitutions. Substitutions at position three of the dihydrothiazine ring account for changes in pharmacokinetic properties and toxicity of the compound. Cefamandole, cefazolin, cefoperazone, cefuroxime and moxalactam are examples of position-3 modifications (Goldberg, 1987). Cephalosporins that contain a 7-α methoxy group are referred to as cephamycins. The presence of this grouping confers β-lactamase stability as the result of steric hindrance at the price of reduction in affinity for PBPs (Eriksen & Blanco, 1992). These compounds are inducers of chromosomally mediated β-lactamases. Cefoxitin and cefotetan are examples of this group. Furthermore, it is possible to replace the sulphur atom in the ring system with an oxygen and thus increase the reactivity of the molecule and produce oxacephems such as moxalactam. Oxacephems endure a major decrease in β-lactamase stability and a minor decrease in activity against gram-positive organisms (Eriksen & Blanco, 1992). Carbacephems are the other closely related cephalosporin derivatives in which the sulphur atom is replaced by a methylene group. Loracarbef is the first carbacephem and is administered orally and is largely ineffective (Cooper, 1992; Eriksen & Blanco, 1992).

Most orally absorbed cephalosporins that have adequate antibacterial activity contain an α-amino group on the 7-β-acyl substituent and a small uncharged group at position three. All oral cephalosporins lack activity against *P. aeruginosa* and *Bacteroides* (Wise, 1992).
Figure 1-3. Structure of Cephalosporins.

(7-aminocephalosporanic acid)
Cephalosporins have a broad spectrum of activity, they are generally efficient and demonstrate a relatively low profile of toxicity, therefore, they are widely used to treat infections caused by both gram-positive and gram-negative bacteria.

1.8.2.1 First-Generation Cephalosporins

First generation compounds include the parenterally administered cefazolin, cephamirin, cephradine, and cephalothin and the orally administered cephaalexin, cephradine, cefadroxil, and cefaclor. These agents have reasonable activity against susceptible strains of Staphylococci and Streptococci, but not against most strains of Enterococci. The orally administered compounds are used in the treatment of minor infections of the respiratory tract, urinary tract, bones, joints, skin and soft tissues caused by susceptible organisms. Cefazolin has a long half-life therefore it is preferred over other first generation cephalosporins for antimicrobial prophylaxis for most surgical procedures (Gustaferro & Steckelberg, 1991). The first generation agents fail to achieve therapeutic levels in the cerebrospinal fluid, even when the meninges are inflamed, thus they should never be used against CNS infections (Eriksen & Blanco, 1992).

1.8.2.2 Second-Generation Cephalosporins

The parenterally administered antibiotics in this group consist of cefamandole, cefonicid, ceforanide, cefuroxime and three cephemycins including cefmetazole, cefotetan and cefoxitin. The orally administered drugs are cefuroxime axetil and cefprozil. Among the second-generation cephalosporins differences exist in their ability to penetrate the CNS and their spectrum of action. Generally they are less active against Staphylococci and Streptococci than first-generation compounds, but are more active against selected gram-negative bacteria and anaerobes (Gustaferro & Steckelberg, 1991).

Cefuroxime possesses an α-oxy imino group at position 7 that provides a high level of β-lactamase stability against most plasmid and chromosomal β-lactamases.
Cefuroxime is active against *H. influenzae*, *M. catarrhalis* and *N. gonorrhoeae*, *S. pneumoniae*, *N. meningitis* and *S. aureus*. It is particularly useful in the treatment of lower respiratory tract infections. Cefotaxin and cefotetan are efficient in treating community-acquired infections caused by mixed anaerobes and aerobic organisms, however, they have poor activity against Staphylococci and Streptococci. Cefmetazole possesses similar spectrum of activity as cefoxitin (Eriksen & Blanco, 1992).

1.8.2.3 Third-generation Cephalosporins

The parenterally administered drugs include cefoperazone, cefotaxime, ceftazidime, ceftizoxime, ceftriaxone, cefpirome, cefepime and the oxacephem, moxalactam. The orally administered third-generation cephalosporin available is cefixime.

These agents have an enhanced spectrum of action against gram-negative aerobic bacilli and multiple resistant gram-negative bacteria. Ceftazidime and to a lesser extent cefoperazone provide anti-pseudomonal activity. Most third-generation cephalosporins have less Staphylococcal and Streptococcal potency than do first- or second-generation compounds. Compared to cefoxitin, the anaerobic activity of this group varies from none to modest (Gustaferro & Steckelberg, 1991). Common uses of these drugs include nosocomial pneumonia, post-operative wound infections, urinary tract infections, community-acquired pneumonia and meningitis (Goldberg, 1987).

Two cephalosporins, cefpirome and cefepime have been classified as ‘fourth-generation’ cephalosporins. It has been reported that they have higher potencies against members of the *Enterobacteriaceae* and that both compounds are resistant to β-lactamase over-producing gram-negative strains that previously inactivated the third-generation cephalosporins (Hancock & Bellido, 1992).
1.8.3 Carbapenems

Like penicillins, carbapenems have a β-lactam ring fused to a five-membered ring. Their structure differs from the penam nucleus of penicillins in having a carbon atom instead of a sulphur at position 1 and in having an unsaturated bond between carbon atoms 2 and 3 in the five-membered ring (Cooper, 1992). Carbapenems include the thienamycins, olivanic acids, carpentimycins, asparenomycins, pleuracidomycins, and other natural and semi-synthetic compounds. The olivanic acid produced by *Streptomyces olivaceus* is the parent compound of the oxapenam, clavulanic acid (Rolinson, 1991).

In contrast to the penicillins and cephalosporins which exist in the *cis* configuration, thienamycin contains a hydroxyethyl side chain in the *trans* (α) configuration at position six that accounts for its resistance to a variety of β-lactamase enzymes. The basic alkylthio group at position two is the source of its activity against *P. aeruginosa*. Thienamycin is highly unstable; however, its N-formimidoyl derivative, known as imipenem, has proved to be stable and potentially effective against a broad spectrum of micro-organisms. *In vivo*, imipenem is hydrolysed by a renal dehydropeptidase, therefore it may be co-administered with cilastatin, an inhibitor of this enzyme to overcome the instability (Wise, 1986).

Meropenem is another carbapenem that is similar to imipenem in having a 6-α-hydroxyethyl group, however, it contains a methyl group at position one that accounts for its resistance to dehydropeptidases. The presence of a dimethyl carbamoyl pyrrolidinethio side chain attached at C2 is associated with the anti-pseudomonal activity. Compared to imipenem, meropenem is slightly less active against gram-positive bacteria but exhibits more activity against gram-negative organisms, and is equally effective against anaerobes (Moellering *et al.*, 1989).

The mode of action of carbapenems is similar to that of other β-lactams, however, they have the broadest spectrum of activity of all β-lactam antibiotics which appears to be associated with three properties; their small molecular structure that enables
them to readily penetrate through the bacterial cell wall, to their high affinity for certain penicillin-binding proteins (PBPs), and to their resistance to most bacterial β-lactamases (Cooper, 1992).

Carbapenems are active against Streptococci, Staphylococci, ampicillin-resistant β-lactamase-positive and -negative strains of *H. influenzae* and gonococci, other gram-negative bacilli and obligate anaerobes. However, some strains of *P. aeruginosa* develop mutational resistance to carbapenems during therapy (Cooper, 1992). Carbapenems may induce various Richmond & Sykes (1973) group I chromosomal β-lactamases (Jorgensen *et al.*, 1991) (Figure 1-4).

### 1.8.4 Monobactams

In contrast to the bicyclic β-lactams, monobactams, the product of a variety of soil bacteria, are monocyclic compounds. With the advent of monobactams, it has been realised that a bicyclic structure is no longer necessary for antibacterial activity (Brewer & Hellinger, 1991). Similar to other β-lactams, modifications on the side chain promotes alteration in the spectrum of activity. The sulfonic acid group on the nitrogen at the N-1 position activates the β-lactam carbonyl group and assists in the acetylation process of the penicillin-binding proteins. Monobactams have reduced affinity for PBPs of gram-positive bacteria and anaerobes, therefore their spectrum of activity is limited to aerobic and facultatively anaerobic gram-negative organisms such as *P. aeruginosa*, most *Enterobacteriaceae*, *Neisseria* spp. and *Haemophilus* spp. The side chains at position 4 contribute to their enhanced activity against gram-negative bacteria and provide stability against most β-lactamases (except for the ones that are able to hydrolyse cefotaxime or ceftazidime such as TEM-3, TEM-5, TEM-7 and SHV-2) (Brewer & Hellinger, 1991). Monobactams include aztreonam, carumonam and tigemonam. The spectrum of activity of aztreonam is limited to infections caused by susceptible gram-negative aerobic organisms. Aztreonam appears to be a suitable agent for the treatment of most *H. influenzae* infections for which parenteral therapy is required (Powell & Williams, 1987) (Figure 1-4).
Figure 1-4: Structure of Carbapenems & Monobactams.

Nuclear Structure of Carbapenems

Imipenem

Meropenem

Nuclear Structure of Monobactams

Aztreonam
1.8.5 β-Lactamase Inhibitors

The dissemination of β-lactamase-mediated resistance in bacteria has prompted continuous efforts to stabilise the β-lactam antibiotics against β-lactamases in the pursuit of more efficient treatment. Therefore, in addition to newer β-lactamase-resistant drugs, compounds capable of inhibiting β-lactamases were also developed. Some penicillins and cephalosporins like methicillin and isoxazolyl penicillins that inhibit staphylococcal β-lactamases, function as β-lactamase inhibitors. Since the penetration of these compounds through the porin channels of gram-negative bacteria is poor, they cannot inhibit gram-negative β-lactamases. However, there are a number of other β-lactam compounds that exhibit little or no antimicrobial activity, but are capable of binding irreversibly to β-lactamase enzymes and thus inhibit their activity. Combining a β-lactamase inhibitor with a β-lactam antibiotic extends the spectrum of antimicrobial activity of the already well known and safe β-lactam agent.

β-lactamase-inhibitory activity was first detected in a strain of *Streptomyces olivaceus* that produced a family of β-lactam compounds termed olivanic acids (reviewed by Rolinson, 1991). These agents are classified as carbapenems. Olivanic acids were not able to penetrate sufficiently through the bacterial cell wall and *in vivo* they were metabolised rapidly, therefore they were never used in clinical practice. Shortly thereafter, potent β-lactamase-inhibitory activity was discovered in a culture of *Streptomyces clavuligerus* (Reading & Cole, 1977). A bicyclic β-lactam molecule called clavulanic acid was found to be responsible for inhibiting the effect of β-lactamases (Sutherland, 1990).

The structure of clavulanic acid, like that of other β-lactam compounds, contains a β-lactam ring but with no side chain attached to it. It has an oxygen atom in place of the sulphur atom of penicillins and contains a hydroxyethylidene substitution on the oxazolidine ring. The similarity between the back bone structure of clavulanic acid to other bicyclic β-lactam antibiotics accounts for its ability to inactivate β-lactamase enzymes. Clavulanic acid fits into the catalytic centre of the β-lactamase enzyme and
forms a relatively stable acyl-enzyme complex through its β-lactam carbonyl group. This complex hydrolyses at a relatively slow rate, therefore the β-lactamase is virtually irreversibly inactivated analogous to the reaction in which β-lactam agents bind to PBPs (reviewed by Rolinson, 1991; Sutherland, 1990).

Sulbactam and tazobactam are semi-synthetic penicillanic acid sulfones and like clavulanic acid irreversibly inactivate their target enzymes (Livermore, 1993; Inttiaz et al., 1994). All three compounds readily inhibit Staphylococcal penicillinases and Richmond & Sykes (1973) classes II-V β-lactamas in addition to chromosomal β-lactamas of Bacteroides species. However, the poor affinity prevents clavulanic acid and sulbactam to inactivate class I Richmond & Sykes (1973) β-lactamas. Tazobactam seems to have a moderate ability to inhibit some class I β-lactamas (Akova et al., 1990; Rolinson, 1991).

The in vitro activity of tazobactam is comparable with that of clavulanic acid. Sulbactam appears to be the least potent inhibitor of them all (Livermore, 1993; Payne et al., 1994; Coleman et al., 1989).

The combination of clavutanic acid with amoxycillin or ticarcillin, sulbactam with ampicillin or cefoperazone, and tazobactam with piperacillin or apalcillin, inhibits a wide range of β-lactamas and therefore exhibits a broad spectrum of activity (Sutherland, 1990; Livermore, 1993). These combinations are not recommended when a high quantity of cephalosporinas are likely to be produced especially by Enterobacteriaceae, since these β-lactamase inhibitors especially clavulanic acid induce Richmond & Sykes (1973) class I β-lactamas (Toomer et al., 1991; Kazimierczak et al., 1990; Zhou et al., 1993).

Recently, a C6-triazolyl methylene penem, BRL 42715, with an inhibitory activity that extends to all class I cephalosporinas has been introduced (Coleman et al., 1989). BRL 42715 is an extremely potent suicide inhibitor of a broad spectrum of β-lactamas. After penetrating the outer membrane, it readily inhibits bacterial
cephalosporinases as well as penicillinase enzymes and allows the \(\beta\)-lactam antimicrobial agent to bind to its target (Zhou et al., 1993). When combined with penicillins (amoxyccillin or piperacillin) or cephalosporins (cephalothin or cefoperazone), BRL 42715 demonstrates a significant synergic effect against many \(\beta\)-lactamase producing bacteria (Zhou et al., 1993; Muratani et al., 1993). In contrast to the other \(\beta\)-lactamase inhibitors, there is no evidence of induction of chromosomal cephalosporinases by BRL 42715 (Zhou et al., 1993).

Other \(\beta\)-lactamase inhibitors include 6-\(\beta\)-bromo- and 6-\(\beta\)-iodo penicillanic acid and methylene penicillanate pivalate. The 6-\(\beta\)-halopenicillanic acids inhibit Richmond & Sykes (1973) type III as well as the staphylococcal \(\beta\)-lactamases (Wise et al., 1981). Alkyl derivatives of penicillanic acid such as methylene penicillanate pivalate also exhibit ability to inhibit different \(\beta\)-lactamases (Adam et al., 1992).

Resistance to the combinations of \(\beta\)-lactams with \(\beta\)-lactamase inhibitors has been observed in clinical isolates. The hyper-production of unaltered \(\beta\)-lactamases usually encoded by multi-copy plasmids has been associated with the reduction in bacterial susceptibility to the \(\beta\)-lactam/\(\beta\)-lactamase inhibitor combinations (Martinez et al., 1989). This applies not only to TEM-1 and SHV-1 but also to the chromosomal class IV enzymes found in \(K.\) oxytoca (Sanders et al., 1988). Additionally, altered outer membrane protein profile may also be responsible for a decrease in the permeability and poor penetration of the inhibitor through the bacterial outer membrane (Reguera et al., 1991). Finally, point mutations in the amino acid sequence of the TEM \(\beta\)-lactamase are believed to be responsible for \(\beta\)-lactamase inhibitor resistance. IRTs (Inhibitor-Resistant TEM-\(\beta\)-lactamases) are variants of TEM-1 that are not inactivated as efficiently as their parent enzyme by inhibitors (Brun et al., 1994; Blazquez et al., 1993). Production of IRTs may have been resulted as the consequence of natural selection allowing the bacteria to survive (Sanders & Sanders, 1992).
Figure 1-5. Structure of β-Lactamase Inhibitors.

Clavulanic Acid

Tazobactam

Sulbactam

BRL 42715
1.9 β-Lactamase- Mediated Resistance in Bacteria

In bacteria the production of β-lactamase enzymes is the most important mechanism of resistance to β-lactam antibiotics. The presence of enzymes with β-lactamase activity was first identified by Abraham and Chain in cell extracts of *Escherichia coli* before the extensive use of penicillins in 1940 (Abraham & Chain, 1940). Shortly thereafter a number of other bacteria were reported to possess the ability of destroying penicillin by virtue of producing 'Penicillinase'. In 1944, Kirby demonstrated that β-lactamase activity was responsible for penicillin resistance in *Staphylococcus aureus*. Since then, with the development of the semi-synthetic penicillins and the availability of the broad-spectrum β-lactam antibiotics, selective pressure has influenced the prevalence and wider significance of β-lactamase production. β-lactamases are widely distributed in both gram-positive and gram-negative bacteria as well as among *Mycobacteria* and *Nocardia*. The production of β-lactamase enzymes may be plasmid- or chromosomally-mediated. In gram-positive bacteria the enzyme functions extra-cellularly, whereas in gram-negative organisms β-lactamase activity is detected in the periplasmic space.

1.9.1 Mode of Action of β-Lactamases

The majority of β-lactamases harbour a serine at the active site that forms a non-covalent complex (E.I) with the β-lactam compound. Acylation then follows in which the carboxyl group of the β-lactam ring is linked to the hydroxyl moiety of the serine side chain (E-I). Finally the ester is hydrolysed liberating the active enzyme and the hydrolysed and therefore inert β-lactam (Livermore, 1993).
Figure 1-6. Mode of Action of β-Lactamases.
Apart from the crucial role of the serine active site in the process of \( \beta \)-lactam acylation, the interaction of other amino acid residues with the substrate is also of great importance. In TEM-1 the hydroxyl group of Ser-68 binds to the \( \beta \)-lactam molecule. However, Arg-244, Ser/Thr-235 and Ser-130 also contribute to the existence of multiple hydrogen bonding interactions with the substrate carboxylate (Zafaralla et al., 1992; Imtiaz et al., 1993).

1.9.2 Classification of \( \beta \)-Lactamases

\( \beta \)-lactamases differ in a number of parameters including their genetic origin, biochemical and physiochemical properties (substrate profiles, inhibitory characteristics, and electrophoretic mobility). Therefore classification has proved to be important and necessary for identification and characterisation of these enzymes. Those classification schemes that are well established and are widely used, will be discussed.

1.9.2.1 Richmond & Sykes Classification Scheme

Based on the substrate and inhibitory profile, molecular weight and the location of the gene encoding the \( \beta \)-lactamases, Richmond and Sykes classified the \( \beta \)-lactamases into five categories (Richmond & Sykes, 1973).

Class I includes both inducible and constitutive chromosomal cephalosporinases, typically produced by Enterobacter species and \textit{Pseudomonas aeruginosa}. Class I has been sub-divided into four subtypes, a, b, c, and d. Class II contains chromosomally-mediated penicillinas that are inhibited by cloxacillin but not by carbenicillin. Class III consists of plasmid-borne enzymes with equal activity against penicillins and cephalosporins. These enzymes are sensitive to cloxacillin inhibition and are resistant to \( p \)-Chloromercuribenzoate (\( p \)CMB) and other sulphhydryl inhibitors. \( p \)CMB inhibits enzymes containing essential cysteine residues. Plasmid-mediated TEM- and SHV-type \( \beta \)-lactamases are classified as group III enzymes. Class IV contains plasmid- and chromosomally-mediated \( \beta \)-lactamases with equal activity
against penicillins and cephalosporins. These β-lactamases are resistant to cloxacillin and are sensitive to p-Chloromercuribenzoate. Chromosomal β-lactamases produced by *Klebsiella pneumoniae* are placed in this group. Class V comprises of plasmid-mediated penicillinases that are resistant to inhibition by sulphydryl agents. Some enzymes of this group hydrolyse cloxacillin faster than benzyl penicillins while others are neither activated nor inhibited by this compound. Plasmid-mediated oxacillinases in *E. coli* are classified in this group. Class VI was later added by Neu (1986) and includes chromosomal β-lactamases of *Bacteroides*.

### 1.9.2.2 Ambler Classification Scheme

Ambler proposed a molecular classification based on amino acid sequence homologies of the β-lactamase enzymes (Ambler, 1980).

*Class A* consists of plasmid- or chromosomally-encoded serine β-lactamases with extensive sequence homologies that prefer penicillin substrates over that of cephalosporins. TEM- and SHV-type enzymes belong to class A β-lactamases. *Class B* or metalloenzymes require a metal co-factor, Zinc II, to restore activity. *Class C* β-lactamases are chromosomal serine cephalosporinases of gram-negative bacteria. These enzymes show no homologies with class A or D-alanine carboxypeptidases. Class C was introduced in 1981 by Jaurin & Grundström. *Class D* was proposed and added to Ambler classification scheme by Houvinen *et al.* (1988). It contains oxacillin-hydrolysing serine β-lactamases. *Class E* defines metalloenzymes from *Xanthomonas maltophilia* that have no sequence homology with class B. This extension has been proposed by Sanders (1989).

### 1.9.2.3 Bush Classification Scheme

The criteria used for characterisation of β-lactamases in this classification system include substrate and inhibitor profiles in addition to physical data for both plasmid- and chromosomally-mediated enzymes. Four groups of enzymes have been introduced (Bush, 1989a-c).
Group 1 comprises of cephalosporinases from gram-negative bacteria. These enzymes are not sensitive to inhibition by clavulanic acid (10 μM), sulbactam and tazobactam. However, BRL 42715 readily inhibits their activity (Coleman, 1989; Zhou et al., 1993). Most cephalosporinases in this group appear to be chromosomal and inducible.

Since Group 2 enzymes are β-lactamases with diverse substrate profiles, they have been sub-classified into six sub-types. Group 2a represents many of the penicillinases from gram-positive bacteria. Group 2b contains broad-spectrum β-lactamases that hydrolyse both penicillins and cephalosporins. The most prevalent plasmid-mediated β-lactamases, TEM-1 and SHV-1 belong to this group. The extended-spectrum β-lactamases have been classified as class 2b’. Many of these enzymes are derivatives of TEM-1, TEM-2 and SHV-1 that differ from their parent enzyme by point mutational substitutions. Group 2b’ confers resistance to the third-generation cephalosporins and other extended-spectrum β-lactam compounds. Group 2c includes those β-lactamases that hydrolyse carbenicillin and tend to hydrolyse cephalosporins at slower rates than they hydrolyse penicillins. These enzymes have poor affinity for cloxacillin and aztreonam. PSE-1, PSE-3 and PSE-4 are categorised in this class. Within group 2d are those enzymes that hydrolyse cloxacillin faster than benzyl penicillin. These enzymes like other group 2 β-lactamases are inhibited by clavulanic acid. OXA-type β-lactamases belong to this group. Group 2e comprises those cephalosporinases that are inhibited by low concentrations of clavulanic acid.

Metalloenzymes have been classified in Group 3. They hydrolyse imipenem as well as other β-lactam antibiotics. These β-lactamases require Zinc (II) for their activity therefore they may readily be inhibited by EDTA. However, they are not sensitive to clavulanic acid inhibition. Metalloenzymes are usually chromosomal.

Group 4 enzymes include penicillinases with reduced sensitivity to clavulanic acid.

This classification scheme has recently been revised by Bush et al (1995).
1.9.2.4 Payne & Amyes Classification Scheme For Extended-Spectrum β-Lactamases

TEM-1, TEM-2 and SHV-1 are the most prevalent plasmid-borne β-lactamases in gram-negative bacteria. They belong to class A and III in the classification schemes of Ambler (1980) and Richmond & Sykes (1973) respectively. These enzymes exhibit very similar biochemical properties, they are able to hydrolyse penicillins and early cephalosporins.

The gene encoding TEM-2 differs from that of TEM-1 by only a single functionally silent amino acid substitution at position 37 (glutamic acid to lysine) and evidently the substrate profile and inhibitor susceptibility of TEM-2 is indistinguishable from TEM-1 (Ambler & Scott, 1978; Sutcliffe, 1978). The TEM-13 variant has an additional Thr→Met alteration at position 261. This modification does not have any impact on the substrate spectrum either (Mabilat & Courvalin, 1990). SHV-1 is more common in Klebsiella spp. The gene expressing SHV-1 shares about 68% sequence homology with TEM-1 gene.

Extended-spectrum β-lactamases are plasmid-mediated enzymes that confer resistance to oxyimino-β-lactam antibiotics that were designed to remain effective in the presence of β-lactamase enzymes such as cefotaxime, ceftazidime and aztreonam. Most extended-spectrum β-lactamases are derivatives of TEM-1, TEM-2 and SHV-1 that differ from their parent molecule by virtue of only a few amino acid changes (Philippon et al., 1989). These modifications occur far from the serine active site at position 68 for TEM-type enzymes and 66 for SHV β-lactamases, however, they are capable of altering the substrate profile of the enzyme. The extended-spectrum β-lactamases may produce more hydrogen bonding with the substrate or may facilitate electrostatic interactions with the negative charges on the substrate molecule or even alter the conformation of the area around the enzyme’s active site in order to have more access to the substrate (Jacoby & Medeiros, 1991; Sowek et al., 1991; Labia et al., 1988; Huletsky et al., 1990).
Based on the substrate hydrolysis properties, Payne & Amyes (1991) proposed a method consisting of four groups for classifying the extended-spectrum β-lactamases.

*Group 1* contains the extended-spectrum β-lactamases that hydrolyse both cefotaxime and ceftazidime poorly. In most instances they hydrolyse cefotaxime slightly faster than ceftazidime, although they confer greater resistance to ceftazidime. TEM-7, TEM-E1 and TEM-E2 (now TEM-12) are such examples. *Group 2* consists of enzymes that hydrolyse ceftazidime with greater efficiency than cefotaxime, such as TEM-10 and TEM-26. In *Group 3* there are enzymes that hydrolyse cefotaxime with more efficiency than ceftazidime. Similar to group 1, they confer a greater resistance to ceftazidime. Group 3 enzymes have been categorised into three subgroups. Group 3a are TEM-derived β-lactamases like TEM-3, TEM-4 and TEM-5. Group 3b are SHV-derived enzymes such as SHV-2, SHV-3 and SHV-4. Group 3c consists of enzymes of unknown origin and includes DJP-1 and FEC-1. *Group 4* contains enzymes that confer resistance to all generations of cephalosporins, however, they are not able to hydrolyse cefotaxime or ceftazidime. Unlike groups 1,2 and 3, the β-lactamases in group 4 are resistant to inhibition by clavulanic acid. BIL-1 is categorised in this group.

Although the plasmids encoding the extended-spectrum β-lactamases are transmissible, only local dissemination with limited wide-range spread of these enzymes has been reported (Jacoby & Medeiros, 1991). Therefore they have not yet been determined as major threats, nevertheless where selective pressure is present, every bacteria that expresses TEM-1 or SHV-1 may become a potential extended-spectrum β-lactamase producer (Payne & Amyes, 1991; Jacoby & Medeiros, 1991).

**1.9.3 Cephalosporinas**

Cephalosporinas have been classified by Richmond & Sykes (1973) as group I β-lactamases. Almost all the cephalosporinas in gram-negative bacteria are encoded chromosomal; however, plasmid-mediated β-lactamases with chromosomal
cephalosporinase characteristics have also been identified (Tzouvelekis et al., 1993). The active site of cephalosporinases contain a serine residue. These enzymes hydrolyse both penicillins and preferentially cephalosporins. Nearly every gram-negative bacteria produces a chromosomally-mediated cephalosporinase. In most species the enzyme is constitutively produced at such low concentrations that can not confer resistance. In a number of other gram-negative organisms, such as *P. aeruginosa*, *Enterobacter* species, *Citrobacter freundii* and other non-fermentative bacilli, cephalosporinases are inducible.

There are two methods associated with enzyme induction, first, is when an enzyme inducer, usually a β-lactam, increases the level of the expression of the enzyme for as long as the intact β-lactam compound persists in the environment. The second mechanism is when exposure to a β-lactam enzyme inducer results in spontaneous mutation of the wild type to a stable induced state. Despite of removal of the inducer, the mutants remain to produce high levels of β-lactamase (Sanders & Sanders, 1988).

Various β-lactam antibiotics, particularly imipenem and cefoxitin, are able to induce cephalosporinases. Therefore emergence of resistance and failure in therapy among gram-negative bacteria possessing inducible β-lactamases is common (14%-56%) (Sanders & Sanders, 1988). The concurrent administration of two β-lactam agents or the addition of an aminoglycoside to a cephalosporin does not seem to prevent the emerging resistance in clinical strains (Sanders & Sanders, 1988). It has been reported that the incidence of emergence of multi-resistant gram-negative bacilli correlates with the extent of use of the newer cephalosporins (Ben & Kemp, 1984). Some induced β-lactamases may exhibit differences in Isoelectric points (pI), substrate profiles and kinetic parameters from the un-induced phenotype (Gates et al., 1986).

In gram-positive bacteria, the mechanism of β-lactamase induction comprises a signal pathway. It initiates when the cell registers the presence of a β-lactam molecule at the external side of the cytoplasmic membrane. This information is
received by the intra-cellular regulatory component that has the ability of controlling the expression of the enzyme (Bennett & Chopra, 1993).

In gram-negative bacteria, the genetic organisation responsible for inducible β-lactamases is similar to the ampC gene in E. coli. Two models have been proposed by Bennett & Chopra (1993) for β-lactamase induction in gram-negative organisms. The initial step in both models is the binding of the β-lactam agent to the critical penicillin-binding protein and the disruption of the peptidoglycan synthesis that leads to accumulation of cell wall precursors. In the first model AmpG, which is assumed to be part of a membrane transport system, uptakes the peptidoglycan fragment. This fragment acts as an inducing ligand and combines with the repressor form of the transcriptional regulator, Amp R, and converts it to the active AmpR. Active AmpR stimulates the high-level expression of ampC. However, another protein, AmpD serves to divert the inducing peptidoglycan ligand away from Amp R and only when AmpD is saturated, induction happens. In the second model, AmpG acts as an integrated membrane protein that plays the role of a sensor. After sensing the peptidoglycan precursors, it interacts directly with the depressed form of AmpR and probably by protein modification generates the active AmpR which in turn stimulates the ampC gene. Similar to the first model, AmpD serves as the induction inhibitor.

1.10 Mechanisms of Resistance to β-Lactam Compounds in H. influenzae

1.10.1 β-Lactamase-Mediated Resistance

1.10.1.i TEM-1 Type β-Lactamase

Ampicillin-resistance in clinical isolates of H. influenzae was first reported in 1974 (Gunn et al., 1974; Schiffer et al., 1974; Thomas et al., 1974). Soon thereafter Khan et al., (1974) detected the presence of β-lactamase activity in the resistant strains that resembled enzymes found in other species of gram-negative bacilli (Farrar & O'Dell,
These strains were characterised as TEM-1 type β-lactamase producers. The genetic material for this enzyme is contained within a transposon belonging to the highly related transposon A (TnA) family that in *H. influenzae* is carried by plasmids with the molecular mass of 30 MDa or in some cases 3 MDa (Elwell *et al.*, 1975; Heffron *et al.*, 1975). In addition the determinant transposon may integrate into the chromosome through a process of recombination (Stuy, 1980). Chromosomally integrated plasmids are not as efficient as plasmids at the dissemination of the resistant genes, however, they are uniformly stable in the *H. influenzae* gene pool (Willard *et al.*, 1982). The 3 and 30 MDa plasmids of *H. influenzae* are indigenous to this species and are of independent origin, therefore, it has been speculated that the R-factors in *H. influenzae* arose as a result of the trans-location of TnA from a donor R-factor onto a native and cryptic recipient plasmid and caused ampicillin-resistance (De Graaff *et al.*, 1976; Laufs *et al.*, 1981; Brunton *et al.*, 1986).

Regional variation in antibiotic resistance elements has been observed. Mendelman *et al.*, (1985) have reported ampicillin-resistant *H. influenzae* isolates from Alaska where 40 MDa plasmids harbour the β-lactamase genes. The results of Musser *et al.*, (1990) indicate that extensive genetic diversity exists among the plasmid encoding antibiotic resistance genes and also among the chromosomal genotypes of the resistant *H. influenzae*.

TEM-1 production accounts for more than 90% of ampicillin-resistant *H. influenzae* strains globally (Scriver *et al.*, 1994; Sanders & Sanders, 1992; Powell *et al.*, 1992). Hyper-production of β-lactamase in *H. influenzae* is currently a matter of concern, as this pattern of resistance may diminish the effectiveness of most β-lactams, especially oral cephalosporins and combination of β-lactam/β-lactamase inhibitors. Nevertheless with such high frequency of TEM-1 production, the possibility of point mutation evolution of this enzyme to become an extended spectrum β-lactamase that is active against third-generation cephalosporins should not be ruled out.
1.10.1.ii ROB-1 β-Lactamase

Until 1981, β-lactamase-mediated ampicillin-resistance in \textit{H. influenzae} strains was exclusively associated with the production of TEM-1 type β-lactamase. In 1981, Rubin \textit{et al.}, reported the identification of another broad-spectrum β-lactamase referred to as ROB-1 in a clinical isolate.

In \textit{H. influenzae} the genes encoding ROB-1 β-lactamase are located on a 4.4 kb plasmid. Further studies demonstrated that plasmid DNA extracted from the strains producing this enzyme does not cross hybridise with other \textit{bla} TEM genes (Levesque \textit{et al.}, 1987) and that the genetic elements encoding ROB-1 and TEM-1 enzymes differ considerably in their DNA sequences (Juteau & Levesque, 1990).

On a 10% polyacrylamide isoelectric focusing gel, ROB-1 focuses as a single band at an isoelectric point (pI) of approximately 8.1 that differs extensively from that of TEM-1 β-lactamase (pI 5.4) often detected in \textit{H. influenzae} (Rubin \textit{et al.}, 1981).

ROB-1 β-lactamase consists of 305 amino acids with an estimated molecular weight of 30,424 (Juteau & Levesque, 1990). The substrate hydrolysis profile demonstrated that ROB-1 hydrolyses cephalosporins and preferentially penicillins and is sensitive to inhibition by clavulanic acid (Rubin \textit{et al.}, 1981; Medeiros \textit{et al.}, 1986). Based on these data ROB-1 was identified as a class 2b enzyme in Bush classification scheme (1989b) and a typical class A β-lactamase in the classification system of Ambler (1980).

The sequence analysis of the ROB-1 structural gene exhibits the presence of a conserved serine active site at position 130, critical for all class A β-lactamases (Juteau & Levesque, 1990). This residue is able to interact simultaneously with the carboxyl group of the β-lactam substrate and result in penicillin and cephalosporin hydrolysis (Juteau \textit{et al.}, 1992). Sequence analysis and evolutionary studies revealed that ROB-1 has higher homology values with β-lactamases of gram-positive bacteria.
rather than β-lactamases of gram-negative organisms and therefore it has been postulated that ROB-1 has not originated in *H. influenzae*. However, it could have been acquired by lateral transfer between bacterial species (Jutaeu & Levesque, 1990; Medeiros et al., 1986).

ROB-1 has also been detected in other members of *Pasteurellaceae* family. ROB-1-mediated ampicillin resistant *Actinobacillus pleuropneumoniae* was reported to harbour a 5.4 kb plasmid responsible for the enzyme production (Hirsh et al., 1982; Medeiros et al., 1986). In other *Pasteurella* strains of human (Rosenau et al., 1991) and animal (Livrelli et al., 1988) origin, ROB-1 is carried either on a 4.4 kb plasmid or on the chromosome. However, in *H. ducreyi* a 5.4 kb plasmid encodes the expression of this enzyme (Maclean et al., 1992). Although the plasmids encoding the ROB-1 β-lactamase are not identical, they share multiple homologous fragments suggesting that these plasmids have all derived from a single common plasmid (Azad et al., 1992; Livrelli et al., 1991). It is likely that the ROB-1 β-lactamase detected in *H. influenzae* has originated from *Pasteurella* strains, possibly from an animal reservoir (Livrelli et al., 1988; Livrelli et al., 1991; Medeiros et al., 1986).

The data regarding the prevalence β-lactamase-mediated ampicillin-resistant *H. influenzae* in North America shows that 7% of the isolates contain ROB-1 whereas 93% produce TEM-1 (Scriver et al., 1994; Daum et al., 1988).

### 1.10.2 Penicillin-Binding Proteins & β-Lactam Resistance in *H. influenzae*

Penicillin-Binding Proteins (PBPs) are cell membrane bound proteins that are present in almost all bacteria. These proteins are associated with cell wall synthesis and are important in cell functions such as division and cell differentiation processes. Major enzymatic activities of PBPs include peptidoglycan transpeptidase and DD-
carboxypeptidase. PBPs are targets for β-lactams. Binding of β-lactam antibiotics to PBPs leads to cell lysis, death or growth arrest (Tomasz, 1979; Tipper, 1985).

Alterations in PBP profile is one of the mechanisms that develops resistance to β-lactam compounds. The number, amount and size of PBPs as well as their affinity for β-lactam antibiotics vary from species to species (Makover et al., 1981). Since the outer membrane of *H. influenzae* is highly permeable to β-lactams, it has been suggested that β-lactam resistance in non-β-lactamase producing strains of *H. influenzae* is acquired only by modifications in PBPs (Clairoux et al., 1992; Coulton et al., 1983).

As the result of natural variations that exist among the strains of *H. influenzae*, the interpretation of PBP studies is complicated. In *H. influenzae*, PBP functions variably depending on growth conditions and the physical status of the bacterium. Compared to the relatively homogenous PBP profile of ampicillin-sensitive *H. influenzae* strains, ampicillin-resistant non-β-lactamase producing isolates exhibit marked heterogeneity.

Unique to *H. influenzae*, PBP expression is growth-phase dependent. There are eight detectable major PBPs designated PBP 1 to PBP 8 in ampicillin susceptible *H. influenzae* during the logarithmic phase of growth, however, when cells enter the stationary phase, two of the major proteins, PBPs 4 (62 kDa) and 6 (45 kDa) become undetectable. Therefore it has been suggested that a regulatory mechanism involving a periodic modulation of PBP activity may be functional in this species (Mendelman & Chaffin, 1985; Makover et al., 1981). In non-β-lactamase mediated ampicillin-resistant strains, five to ten PBPs may be detected. PBPs 1 (95 kDa), 7 (40 kDa) and 8 (25 kDa) are always detectable, whereas the penicillin-binding activity of the rest is significantly variable (Mendelman et al., 1987).

Studies on the binding of several β-lactam antibiotics to PBPs have indicated that PBP 2 (80 kDa) and temperature-sensitive PBP 3 (75 kDa), correspond to PBPs 1a
and 2 of *E. coli* in structure and in functional activities (Malouin & Bryan, 1988; Makover *et al.*, 1981). PBP 3 contributes to the catalysis of cell wall incorporation, therefore, the loss of this protein correlates with reduced sensitivity to ampicillin (Mendelman & Chaffin, 1987; Mendelman *et al.*, 1990). Furthermore, at higher growth temperatures (42°C), the number of PBP 1a (90 kDa) would increase and therefore PBP 1a becomes a target of choice for β-lactam antibiotics. The higher degree of antibacterial activity of penicillin G at 42°C against *H. influenzae* may be the result of this phenomenon (Malouin & Bryan, 1988).

PBP 4 or 3a (62 kDa) and PBP 5 or 3b (59 kDa) are vitally important in cell wall incorporation and septum formation (Mendelman *et al.*, 1990; Malouin *et al.*, 1990). These proteins function as transpeptidases and are therefore primary targets for β-lactam antibiotics (Mendelman & Chaffin, 1987). Reduced sensitivity and resistance to β-lactam compounds in all *H. influenzae* strains studied so far have been associated with PBP 4 and PBP 5 (Mendelman *et al.*, 1990).

Overall, it appears that several PBPs are responsible for β-lactam resistance in non-β-lactamase producing strains of *H. influenzae*. In general, alterations in PBPs 3, 4 and 5 have been associated with the resistant phenotypes (Clairoux *et al.*, 1992).

The genes encoding PBP modification are located on the chromosome. The fact that the complete transfer of the ampicillin-resistant phenotype in *H. influenzae* can be achieved in a single step, suggests that the elements encoding resistance are either located on one gene or most probably are closely linked (Parr & Bryan, 1984; Mendelman *et al.*, 1984). Inter-species recombinational events that are responsible for penicillin resistance in other pathogens including *S. pneumoniae*, has not yet been studied in *H. influenzae*.

Most of the non-β-lactamase mediated ampicillin-resistant *H. influenzae* strains are non-encapsulated and are of respiratory origin (Mendelman *et al.*, 1987). However, this type of resistance may also be detected in other serologically typeable isolates as
well (Clairoux et al., 1992). Non-β-lactamase-mediated ampicillin-resistant nontypeable strains are diverse and therefore it seems that they have not evolved from a single clone, although among all of them methionine requirement is a common marker (Mendelman et al., 1987).

Decreased virulence has been reported with acquisition of this type of resistance which is more related to impaired in vivo multiplication than efficient bactericidal activity of neutrophils or serum (Rubin et al., 1991).

Although the incidence of inherent resistance in H. influenzae remains in the range of 5% in most countries, however, since these strains have altered PBPs, cross resistance with other β-lactams with similar targets may easily occur (Yeo & Livermore, 1994; Jorgensen et al., 1988).

### 1.10.3 The Role of Outer Membrane Permeability of H. influenzae in β-Lactam Resistance

In gram-negative bacteria outer membrane is considered as a barrier against the free diffusion of compounds across the cell envelope. Porins, highly specialised proteins that form water-filled channels, are responsible for the selective permeability of molecules. However, the outer membrane of H. influenzae does not seem to act as a significant barrier against the free diffusion of solutes, and therefore, the passage of β-lactam antibiotics into the cell is not delayed to any degree (Medeiros & O'Brian, 1975). Compared with E. coli and P. aeruginosa, H. influenzae has intermediate pore size with a higher content of basic, positively charged amino acids, that are directed towards the lumen of the hydrophilic pore. Hydrophilic β-lactam compounds can therefore readily enter the channels and penetrate into the bacterium (Coulton et al., 1983; Vachon et al., 1985).

Outer membrane protein profiles of both capsulated and non-encapsulated H. influenzae strains, have proved to be diverse and variant (Loeb & Smith, 1980).
suggested that intrinsic resistance to ß-lactams in *H. influenzae* may only be caused by altered PBPs (Clairoux *et al.*, 1992; Coulton *et al.*, 1983), however, Reid *et al.* (1987) implied that outer membrane profile differences exist between ampicillin-susceptible and ampicillin-resistant, ß-lactamase-negative *H. influenzae* isolates of the same biotype. However, no specific protein change was accounted for this finding.
The Aims of This Thesis

1. To evaluate the biochemical properties of *H. influenzae* strains that cause non-invasive infections in Scotland.

2. To identify the distribution patterns of biotypes in relation to the site of isolation, age of the host, and capsulation in non-invasive infections.

3. To determine the incidence of resistant *H. influenzae* strains isolated from the local population of Scotland to a range of β-lactams.

4. To determine the prevalence of β-lactamase production in this species.

5. To determine the presence of any extended-spectrum β-lactamases in strains resistant to β-lactams.
2.1 Bacterial Strains

Six hundred and sixteen clinical *H. influenzae* isolates were collected from four hospitals in Scotland during the period of 1992-1995. To reflect accurately the incidence of various biotypes and antimicrobial resistance, this study contained non-duplicate specimens that were not selected on any specified criteria. The purity and integrity of the strains were initially verified by colony morphology on Chocolate Blood Agar plates which were incubated at 37°C in an atmosphere of air containing 5% carbon dioxide.

The standard bacterial strains and the strains expressing standard β-lactamases used in experiments are listed in Tables 2-1, 2-2 & 2-3.

2.2 Storage of Cultures

*H. influenzae* strains were stored at -70°C in 10% skimmed milk solution (Oxoid, Unipath). Strains were always subcultured from the stock on solid media and were not passaged.

2.3 Information Storage

Information regarding the clinical *H. influenzae* isolates were recorded and stored on the database programme dBase III (Ashton-Tate, Milton Keynes).
2.4 Statistical Analysis

Statistical analysis on the raw data was kindly performed by Mr. W.H. Adams, Department of Medical Statistics, Medical School, University of Edinburgh. The Pearson Chi Square values for the contingency tables were calculated and the probability values $p \leq 0.05$ were indicated as significant.

Table 2-1, Standard Bacterial Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> K12 J62</td>
<td><em>pro</em>’, <em>his</em>’ <em>trp</em>’, <em>lac</em>’</td>
<td>S.G.B Amyes</td>
<td>Bachmann, 1972</td>
</tr>
<tr>
<td><em>E. coli</em> K12 TG2</td>
<td>tet’F[proAB’lacI’] <em>lacZΔM15</em></td>
<td>S.G.B Amyes</td>
<td>Sambrook et al., 1989</td>
</tr>
<tr>
<td><em>H. influenzae</em> NCTC 11315</td>
<td>Amp’, bla’</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>H. influenzae</em> NCTC 11931</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2-2, Standard Bacterial Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (kb)</th>
<th>Markers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18</td>
<td>2.7</td>
<td><em>Ap</em>’, <em>lacZ</em>’</td>
<td>Norrander et al., 1983</td>
</tr>
<tr>
<td>pSU18</td>
<td>2.3</td>
<td><em>Cm</em>’, <em>lacZ</em>’</td>
<td>Bartolomé et al., 1991</td>
</tr>
<tr>
<td>pROB</td>
<td>4.4</td>
<td><em>Ap</em>’</td>
<td>Rubin et al., 1981</td>
</tr>
</tbody>
</table>
Table 2-3, Bacterial Strains Producing Standard \( \beta \)-Lactamases

<table>
<thead>
<tr>
<th>( \beta )-Lactamases</th>
<th>pI</th>
<th>Bacterial Strain</th>
<th>Plasmid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1</td>
<td>5.4</td>
<td><em>E. coli</em> J53-2</td>
<td>R6K</td>
<td>Norrander <em>et al.</em>, 1983</td>
</tr>
<tr>
<td>TEM-2</td>
<td>5.6</td>
<td><em>E. coli</em> J62-2</td>
<td>RP4</td>
<td>Hedges <em>et al.</em>, 1974</td>
</tr>
<tr>
<td>TEM-10</td>
<td>5.6</td>
<td><em>E. coli</em> J53-2</td>
<td>pJPQ100</td>
<td>Payne, 1990</td>
</tr>
<tr>
<td>TEM-E4</td>
<td>5.6</td>
<td><em>E. coli</em> J53-2</td>
<td>pUK724</td>
<td>Payne, 1990</td>
</tr>
<tr>
<td>SHV-1</td>
<td>7.6</td>
<td><em>E. coli</em> J53-2</td>
<td>R1010</td>
<td>Petrocheilou <em>et al.</em>, 1977</td>
</tr>
<tr>
<td>SHV-3</td>
<td>7.0</td>
<td><em>E. coli</em> J53-2</td>
<td>pUD18</td>
<td>Spenser <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>OXA-1</td>
<td>7.4</td>
<td><em>E. coli</em> J53-2</td>
<td>R455</td>
<td>Dale &amp; Smith, 1974</td>
</tr>
<tr>
<td>OXA-4</td>
<td>7.5</td>
<td><em>E. coli</em> 7529</td>
<td>pMG203</td>
<td>Medeiros <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>TEM-8</td>
<td>6.0</td>
<td><em>E. coli</em> J-53-2</td>
<td>pCFF34</td>
<td>Chanal <em>et al.</em>, 1988</td>
</tr>
<tr>
<td>OHIO-1</td>
<td>7.0</td>
<td><em>E. coli</em> C600:75</td>
<td>pDO75</td>
<td>Shlaes <em>et al.</em>, 1986</td>
</tr>
<tr>
<td>TLE-1</td>
<td>5.5</td>
<td><em>E. coli</em> 7604</td>
<td>pMG204b</td>
<td>Medeiros <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>BRO-1</td>
<td>5.6</td>
<td><em>M. catarhalis</em></td>
<td>not detected</td>
<td>Wallace <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>BRO-2</td>
<td>6.5</td>
<td><em>M. catarhalis</em></td>
<td>not detected</td>
<td>Wallace <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>ROB-1</td>
<td>8.1</td>
<td><em>E. coli</em> DH1 3022</td>
<td>R\text{ROB}</td>
<td>Azad <em>et al.</em>, 1992</td>
</tr>
</tbody>
</table>

2.5 Materials

2.5.1 Reagents

All chemicals and reagents were supplied by Sigma Chemical Co. Ltd. (Poole, Dorset) unless otherwise stated.

2.5.2 Media

Growth media were sterilised by autoclaving at 15 lbs/in\(^2\) for 15 minutes at 121°C unless otherwise stated.
2.5.3 Complex Media
The following complex media were used; Nutrient Agar, Nutrient Broth, Mueller Hinton Agar, Mueller Hinton Broth, SOB and SOC. All materials were supplied by Oxoid, Unipath (Basingstoke, Hants) unless otherwise stated.

2.5.4 Davis-Mingioli Minimal Salts Media (DM)
Double strength Minimal Salts media was prepared (Table 2-4) as described by Davis & Mingioli (1950). A single strength minimal salts medium was prepared by dilution of double-strength DM with an equal volume of sterile distilled water. Single-strength DM was used for the dilution of bacterial cell suspensions.

Table 2-4, Double Strength Davis & Mingioli Minimal Salts

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g) in Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$HPO$_4$</td>
<td>14.0</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>6.0</td>
</tr>
<tr>
<td>Na$_3$ Citrate</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.2</td>
</tr>
<tr>
<td>(NH$_4$)$_2$ SO$_4$</td>
<td>2.0</td>
</tr>
</tbody>
</table>

2.5.5 Chocolate Blood Agar
Mueller Hinton Agar was supplemented with 5g/l yeast extract (Oxoid, Unipath) and prepared according to the manufacturer’s instructions. The media was cooled to 65°C prior to the addition of 5% v/v defibrinated horse blood.
2.5.6 Supplemented Mueller Hinton Broth

5g/l yeast extract was added to the appropriate amount of Mueller Hinton broth, the media was sterilised, cooled and supplemented with *Haemophilus* Test Medium (HTM) (Oxoid, Unipath).

2.5.7 SOB Medium

The constituents of one litre of SOB broth were 20g Bacto tryptone (Difco), 5g Bacto yeast extract (Difco), 10mM NaCl, 2.5M KCl, 10mM MgCl$_2$, and 10mM MgSO$_4$. The pH was adjusted to 7.0 with NaOH.

2.5.8 SOC Medium

SOC contains the same ingredients as SOB plus 20mM glucose.

2.5.9 Luria-Bertani (LB) Media

One litre LB broth was prepared with 10g Bacto peptone (Difco), 5g Bacto yeast extract (Difco), and 5g NaCl. LB Agar was prepared as above except 15g Bacteriological Agar No. 1 (Oxoid) was added to the medium.

2.5.10 Antimicrobial Agents

Antimicrobial agents were supplied as sterile powders from Sigma Chemicals Co. Ltd. (Poole, Dorset) unless indicated (Tables 2-5 & 2-6). Drugs were stored as dry powders in darkness at 4°C. The antimicrobial solutions were freshly prepared with sterile ultra pure deionised water when required.
### Table 2-5, Antimicrobial Agents

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td>Bencard</td>
</tr>
<tr>
<td>Benzyl Penicillin</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>Lilly Industries</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>Dista</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>Roussel Laboratories</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>Merck Sharp Dohme</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>Glaxo Laboratories</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Cephalaxin</td>
<td>Glaxo Laboratories</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>Lilly Industries</td>
</tr>
<tr>
<td>Cephradine</td>
<td>Bristol-Meyers Squibb</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Bayer plc</td>
</tr>
<tr>
<td>Imipenem</td>
<td>Merck Sharp Dohme</td>
</tr>
<tr>
<td>Nitrocephin</td>
<td>SmithKline Beecham &amp;</td>
</tr>
<tr>
<td></td>
<td>Glaxo Research Group</td>
</tr>
</tbody>
</table>

### Table 2-6, β-Lactamase Inhibitors

<table>
<thead>
<tr>
<th>β-Lactamase Inhibitors</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRL 42715</td>
<td>SmithKline Beecham</td>
</tr>
<tr>
<td>Clavulanic Acid</td>
<td>SmithKline Beecham</td>
</tr>
<tr>
<td>Sulbactam</td>
<td>Pfizer</td>
</tr>
<tr>
<td>Tazobactam</td>
<td>Lederle Laboratories (Cyanamid)</td>
</tr>
</tbody>
</table>
2.6 Methods

2.6.1 Identification of *H. influenzae* Strains

*H. influenzae* were identified as gram negative cocco-bacilli that required both haemin (X-factor, Sigma) and β-NAD (V-factor, Sigma) for growth and isolation. Therefore test strains were seeded on Nutrient Agar plates and disks (Medical Wire & Equipment Co., Bath) containing X-factor, V-factor and both were firmly placed on the plates. The plates were incubated at 37°C in air overnight. The isolates were confirmed as *H. influenzae* by visualising the growth zone around the XV disk providing no growth around X or V disks.

2.6.2 Identification of Biochemical Properties of *H. influenzae*

Biotyping was based on three biochemical reactions consisting of; Indole production, Urea hydrolysis and Ornithine decarboxylation (Kilian, 1976).

2.6.2.i Indole Production

The method described by Clarke & Cowan (1952) was performed for indole production tests. Kovács' reagents was added to the tubes after 3 hours of incubation at 37°C.

2.6.2.ii Urease Activity

Detection of urease activity was carried out as described by Lautrop & Lacey (1960). The results were read after incubation for 4 hours and 24 hours at 37°C.

2.6.2.iii Ornithine Decarboxylase Activity

The medium used was similar to that recommended by Møller (1955). After inoculation, each test was covered with sterile mineral oil and incubated at 37°C for 4 hours. The results were read after 4 hours and after 24 hours at room temperature.
2.6.2.iv Biotype Classification

The classification of *H. influenzae* isolates closely followed the scheme introduced by Kilian (1991). Table 2-7 displays the biochemical differentiation of biotypes of *H. influenzae*.

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Indole Production</th>
<th>Urease Activity</th>
<th>Ornithine Decarboxylation</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>

*The table has been adopted from Kilian (1991) in Manual of Clinical Microbiology, Washington D.C., American Society for Microbiology.*

2.6.3 Identification of Encapsulated Strains

Strains were streaked out on Nutrient Agar plates containing 5% Fildes’ extract (Oxoid, Unipath) and were incubated at 37°C in an atmosphere of 95% air and 5% carbon dioxide for 18 hours. In strong obliquely transmitted light, encapsulated strains are slightly opaque and iridescent, whereas the unencapsulated strains are translucent and non-iridescent (Pittman, 1931). However, it was found difficult to distinct the typeable and nontypeable isolates from fresh cultures by this conventional method. Therefore, in order to demonstrate capsules, Hiss’s staining method
(Cheesbrough, 1992) was found most useful. Colonies were suspended in a drop of normal saline. The dried smear was fixed with absolute ethanol and stained with crystal violet (1% w/v). The smear was heated gently until the steam began to rise. The slide was left for one minute and the excess of crystal violet was washed with CuSO₄ (20% w/v) and air dried. One drop of immersion oil was added on the slide and the test strain was visualised with a 100x objective lens. In stained preparations, the encapsulated bacterial cells were dark purple surrounded by pale blue capsules. They appeared as short rods, almost uniform in size and larger than non-encapsulated isolates.

### 2.6.4 Antibacterial Susceptibility Testing [Minimum Inhibitory Concentrations (MICs) mg/l]

Antimicrobial susceptibility tests were performed on Mueller Hinton Agar plates containing 5% yeast extract supplemented with 5% horse blood. MICs were determined by agar dilution of the antibacterial agents. Stock solutions of antibiotics were prepared immediately prior to use and two-fold dilutions of each antibiotic were prepared in Chocolate Blood Agar plates. The media containing antimicrobial agents were used within 24 hours.

Bacterial strains were grown overnight in HTM supplemented Mueller Hinton Broth at 37°C with continuous shaking. A 10⁻² serial dilution of the overnight culture was made in single strength DM to an approximate concentration of 10⁵ cfu/ml. The surface of Chocolate Blood Agar plates containing the antibacterial agents were then inoculated with a 2μl volume of the freshly diluted bacterial suspension by a Denley, multipoint inoculator (Denley, Billinghurst, Surrey) to give a final concentration of 2 x 10⁵ cfu per spot. *H. influenzae* NCTC 11931 and 11315 were used as control organisms. A plate with no added antibiotic was inoculated as a positive control. All plates were incubated at 37°C in a 5% CO₂ atmosphere for 18-24 hours. The first dilution of the antimicrobial agent to inhibit all visible growth was determined as the MIC (mg/l).
2.6.5 β-Lactamase Preparation

2.6.5.i Small Scale Preparation of Crude Cell Free Extracts

The test strain was inoculated onto the surface of a Chocolate Blood Agar slope and incubated at 37°C in a 5% CO₂ incubator overnight. Bacteria were washed off the surface of the agar with 1ml of 50mM Sodium Phosphate buffer (pH 7.0) and transferred to an ice cold container. The cells were cooled on ice and disrupted by sonication (MSE Soniprep 150, MSE Instruments, Crawley, Sussex) at 4 x 15 seconds pulses of 8µm amplitude separated by a 15 seconds cooling period. The cell lysate was cleared by centrifugation at 4°C in an MSE Microcentaur centrifuge at 11,600 g (13,000 rpm) for 20 minutes. The cell free supernatant was stored at -20°C until required.

Alternatively, small scale β-lactamases were prepared from a 10ml HTM supplemented Mueller Hinton Broth. The overnight culture was centrifuged for 15 minutes at 3,000 rpm (Heraeus Christ Bactifuge). The pellet was washed with sodium phosphate buffer (pH 7.0) and resuspended in the same buffer for sonication. The sonicated extract was centrifuged in an MSE Microcentaur centrifuge for 15 minutes at 4°C at 13,000 rpm and the supernatant was stored at -20°C.

2.6.5.ii Large Scale Preparation of Crude Cell Free Extracts

10ml of Mueller Hinton Broth supplemented with HTM was inoculated with the test strain and incubated at 37°C overnight with vigorous shaking (200 rpm). The culture was transferred to a 2 litre flask containing 1 litre Mueller Hinton Broth with HTM and was grown with shaking at 37°C overnight. The cells were harvested by centrifugation at 6,000 g for 15 minutes at 4°C (Sorvall RC-5B). The pellet was resuspended and washed in 20ml of 50mM sodium phosphate buffer (pH 7.0) and recentrifuged at 6,000 g for 15 minutes at 4°C. The pellet was resuspended in 1ml of the same buffer and disrupted by sonication at 8µM amplitude (MSE Soniprep 150) with constant cooling for 4 x 15 seconds with 15 seconds cooling intervals. The cell
lysate was cleared by centrifugation at 4°C at 32,000 g for 45 minutes. The supernatant was stored at -20°C until required.

2.6.6 Assessment of β-Lactamase Activity, Nitrocephin Spot Test

The reaction time for a 30μl volume of the β-lactamase preparation to change the colour of 100μl of nitrocephin solution (50mg/l) from yellow to red was taken as an indication of the β-lactamase activity of the enzyme preparation.

2.6.7 Partial Purification of β-Lactamases by Sephadex G-75 Gel Filtration

2.6.7.i Preparation of Sephadex G-75 Gel Filtration Column

Column preparation was performed according to the method described by Andrews (1964). Fine grade Sephadex G-75 (Pharmacia) was swollen with the appropriate volume of 50mM sodium phosphate buffer (pH 7.0) at 100°C for 3 hours. After the sephadex was cooled down to 4°C, it was carefully poured into a 2cm² x 90cm acrylic column (Amicon Ltd, Stonehouse, Glos.). When the column was fully packed, it was connected to an LKB 10200 peristaltic pump (Pharmacia) and a flow rate of 2ml/10min was established. The gel was washed and allowed to equilibrate for 48 hours with 50mM sodium phosphate buffer (pH 7.0) prior to use. After each use, the column was washed with two volumes of the same buffer (180 ml) and maintained with continuous recycling of buffer. The fractions containing the β-lactamase activity were determined by testing samples with a nitrocephin solution (50mg/l).

2.6.7.ii Calibration of Sephadex G-75 Gel Filtration Column

The sephadex column was calibrated as described by Andrews (1964). A 1ml aliquot of three proteins (10mg/ml each) of known molecular weight comprising Cytochrome C, Chymotrypsinogen, and Ovalbumin (Table 2-8), were applied to the
column and eluted at a rate of 2ml/10min in 50mM sodium phosphate buffer (pH 7.0).

The eluted fractions were collected in 2ml volumes with an LKB 2070 Ultrarac II fraction collector (Pharmacia). The elution volume of the standard proteins was determined by measuring absorbance of the fractions at the wave length of 280nm with a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer (Beaconsfield, Bucks). The absorbance of each fraction was plotted against the fraction number and the position of the protein markers was determined. Subsequently a standard curve of Molecular Weight versus fraction number was plotted.

<table>
<thead>
<tr>
<th>Protein Standards (Sigma)</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>45,000</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>25,500</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>12,384</td>
</tr>
</tbody>
</table>

2.6.8 Molecular Weight Determination of β-Lactamases

2.6.8.i Determination of the Molecular Weight of the β-Lactamases by Gel Filtration

1ml of the crude large scale β-lactamase preparation was applied to the calibrated column and eluted with 50mM sodium phosphate (pH 7.0). The flow rate and the settings of the fraction collector were similar to the calibrated procedure. Fractions were collected overnight until a volume of buffer equivalent to the total column volume had passed through (180 mls). The fractions containing the β-lactamases activity were determined by testing samples from each fraction with nitrocephin solution (50mg/l). These fractions were then assayed by the spectrophotometric
method of O'Callaghan et al. (1969). β-lactamase activity was compared on the standard curve to establish the molecular weight of the particular sample. Fractions containing significant activity were pooled and used for further assays or stored at -20°C until required.

2.6.8.ii Determination of the Molecular Weight of β-Lactamas by SDS-PAGE

The molecular weight of SDS-denatured proteins were measured on the Phastsystem (Pharmacia) using Phast Gel gradient media and the low molecular weight Pharmacia Calibration kit. Partially purified β-lactamase extracts were treated with 2.5% SDS with 5% β-mercaptoethanol and 2.5% SDS at 100°C for 5 minutes before electrophoresis on a Phast gel gradient 10 to 15 minigel. The protocol closely followed the manufacturer's instructions. β-lactamase activity in the gels was detected after renaturing the proteins by soaking the gels in phosphate buffer (pH 7.0) containing 1% Triton X-100 for 2-4 hours prior to staining with nitrocephin. The relative migration of the β-lactamase band was compared with the mobilities of the low molecular weight protein standards that were run simultaneously under the same conditions. The standard markers were stained with Coomassie brilliant blue R250 (Sigma). The molecular weight of the β-lactamase was estimated by comparing the gel containing the molecular weight standards with the gel containing the renatured enzyme.

2.6.9 Analytical Isoelectric Focusing

β-lactamases were identified by analytical isoelectric focusing (IEF) as described by Matthew et al., (1975). β-lactamase extracts were focused on a thin layer polyacrylamide gel containing broad range ampholines (pH 3.5-10). The composition of the gel mixture is shown in Table 2-9.
Table 2-9, The Composition of the Isoelectric Focusing Gel

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
<th>Volume (ml)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% v/v Tetramethyl-ethylenediamine (TEMED) in distilled water</td>
<td>Sigma</td>
<td>0.2</td>
<td>0.25 mg/l</td>
</tr>
<tr>
<td>40% w/v ampholines pH 3.5-10</td>
<td>Sigma</td>
<td>2</td>
<td>2% w/v</td>
</tr>
<tr>
<td>Acrylamide (100g) &amp; methyl bisacrylamide (2.7g) in 300 ml distilled water</td>
<td>BDH</td>
<td>9</td>
<td>acrylamide 76g/l, bisacrylamide2g/l</td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Riboflavin (20mg/l)</td>
<td>Sigma</td>
<td>4</td>
<td>2mg/l</td>
</tr>
</tbody>
</table>

The polyacrylamide gel solution was poured between two glass plates that were 1mm apart. Prior to use, one of the two glass plates was coated with a binding solution comprising 0.5% w/v gelatine and 0.5% w/v chromium potassium sulphate dodecahydrate (both Aldrich Chemical Co. Ltd, Gillingham, Dorset) dissolved in sterile distilled water. It was left to dry in air to promote adhesion of the polyacrylamide gel. The other glass plate was siliconised with Sigmacote siliconising solution to reduce adhesion. After the polyacrylamide gel solution was poured, it was left to polymerise and set in the presence of ultra-violet light. The reaction was catalysed by riboflavin.
Samples of the β-lactamase enzymes were applied close to the anode on the surface of the gel. The amount of β-lactamase preparation (in μl) applied to the gel was equivalent to the time (in seconds) taken for the nitrocephin spot test to show a positive reaction. Up to a maximum of 50μl of the β-lactamase was loaded to any one lane.

Isoelectric focusing was carried out at a constant power of 1W, 500V and 20mA overnight. The focused β-lactamase bands were visualised by overlaying the surface of the gel with sheets of filter paper (Whatman No. 54, BDH), previously soaked in nitrocephin solution (500mg/l). The bands of β-lactamase activity appeared red on a yellow background. Isoelectric points of novel enzymes were estimated from the focused bands of enzymes of known pI or 5μl of wide-range isoelectric point markers, pI 4.7-10.6 (BDH, Poole) were loaded to quantify the gradient. Photographs of focused β-lactamases were taken with a Polaroid camera (setting B4, F8) with a Wratten 58 green filter.

2.6.10 Protein Concentration of the β-Lactamase Preparation

Protein concentration of the β-lactamase preparation was determined by the method described by Waddel & Hill (1956). The concentrations of proteins (mg/ml) were diluted 1:1000 and measured at two wavelengths; 215nm and 225nm on a Perkin Elmer Lambda 2 spectrophotometer. The difference of absorbance between the two measurements was multiplied by the correction factor for the spectrophotometer.

2.6.11 Specific Activities of β-Lactamases

Specific activity of a β-lactamase was measured by determination of rate of nitrocephin hydrolysis against protein concentration and was expressed as nanomoles of nitrocephin hydrolysed per minute per mg of protein.
2.6.12 Hydrolytic Activity of β-Lactamases

Hydrolytic activity against several β-lactam compounds were evaluated spectrophotometrically. Fresh substrate solutions of penicillins at $10^{-2}\text{M}$, cephalosporins and carbapenems at $10^{-3}\text{M}$ and nitrocephin at $10^{-4}\text{M}$ were prepared in 50mM sodium phosphate buffer (pH 7.0). Assays were performed at the wavelength ($\lambda$) of maximal absorbance for the β-lactam ring of each compound (Table 2-10).

Table 2-10, Optimal Wavelength for the Measurement of β-lactam Hydrolysis

<table>
<thead>
<tr>
<th>β-lactam Agent</th>
<th>Molecular Weight</th>
<th>Wavelength ($\lambda$ max) nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzyl Penicillin</td>
<td>356.4</td>
<td>238</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>371.4</td>
<td>238</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>385.8</td>
<td>270</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>477.4</td>
<td>265</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>446.4</td>
<td>260</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>347.4</td>
<td>260</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>415.5</td>
<td>255</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>418.4</td>
<td>260</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>636.6</td>
<td>260</td>
</tr>
<tr>
<td>Imipenem</td>
<td>316.4</td>
<td>299</td>
</tr>
<tr>
<td>Nitrocephin</td>
<td>516</td>
<td>384</td>
</tr>
</tbody>
</table>

The rate of hydrolysis for a fixed concentration of substrate ($10^{-5}\text{M}$ for nitrocephin, $10^{-4}\text{M}$ for cephalosporins and $10^{-3}\text{M}$ for penicillins) was measured to give an initial indication of enzyme activity. The enzyme preparation was adjusted so that the β-lactamase would completely hydrolyse the antibiotic. A 100μl aliquot of β-lactamase
preparation was added to a 3ml cuvette containing 0.3ml substrate and 2.6ml 50mM phosphate buffer (pH 7.0). A Control cuvette did not contain the substrate. The rate of decrease in absorbance over time (in minutes) was monitored from the linear part of the curve to obtain a value for $\Delta A/t$. The rate of hydrolysis was defined as micromoles of substrate hydrolysed per minute, per millilitre of enzyme preparation (O'Callaghan et al., 1972).

$$R = \frac{\Delta A \times n \times d}{A_0 \times t}$$

where:  
$R$ = initial rate of hydrolysis (µmoles / minute / ml of the enzyme solution)  
$\Delta A$ = change in absorbance over time (minute)  
$n$ = number of micromoles of substrate added to cuvette (3 for penicillins, 0.3 for cephalosporins, and 0.03 for nitrocephin)  
d = enzyme dilution adjusted to 1ml  
$A_0$ = Absorbance of intact substrate  
t = time in minutes

### 2.6.13 Evaluation of Kinetic Parameters

In order to determine $K_m$ and $V_{max}$, the initial velocity of hydrolysis was measured for substrates at limiting concentrations in a Perkin-Elmer Lambda 2 double beam UV/Vis spectrophotometer at a final reaction volume of 1ml as described by Reid & Amyes (1986).

Measurements were carried out at 37°C in 50mM sodium phosphate buffer (pH 7.0). The reciprocal of the substrate concentration against the reciprocal of the rate of hydrolysis was plotted according to the Lineweaver-Burk (1934) method. The efficiency of hydrolysis was calculated by dividing the $V_{max}$ by the $K_m$ values.
2.6.14 Inhibition Studies

Determination of the concentrations required to inhibit 50% of the β-lactamase activity (ID$_{50}$) were performed spectrophotometrically as described by O’Callaghan et al., (1969). Nitrocephin was the test substrate for inhibition studies. It was prepared at 10$^{-4}$M in pipes (Sigma) buffer (pH 7.0) for Zinc and in sodium phosphate buffer (pH 7.0) for other assays.

Crude enzyme preparations were standardised to obtain similar rates of nitrocephin hydrolysis. The initial rate of hydrolysis was determined for nitrocephin in the absence of inhibitor as described previously (Section 2.6.12). Various concentrations of β-lactamase inhibitor were incubated with equal volumes of enzymes at 37°C for 10 minutes before addition of nitrocephin to a final concentration of 10$^{-5}$M. This procedure was repeated until inhibition approached 100%. The ID$_{50}$ values were determined from plotting a graph of the inhibition percentage against log$_{10}$ of the inhibitor concentration.

2.6.15 β-Lactamase Induction Studies

Bacterial test strains that were grown overnight in HTM supplemented Mueller Hinton Broth at 37°C with continuous shaking were diluted 10-fold into fresh broth. After 2-4 hours incubation in a rotary shaker at 37°C, imipenem was added at the concentration equal to one-fourth the MIC, and incubation continued for a further 4 hours. Cells were harvested by centrifugation, washed in 50mM sodium phosphate buffer (pH 7.0) and sonicated. Cell debris was removed by centrifugation and the crude enzyme extract in the supernatant was stored at -20°C until required. β-lactamase activity was assayed by chromogenic nitrocephin.
2.6.16 Curing and Elimination of Plasmids by Ethidium Bromide

A number of agents intercalate between base-pairs of DNA and inhibit DNA polymerase reactions and thus increase the rate of spontaneous segregation of stable plasmids (Bouanchaud et al., 1969). Intercalating dyes including ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide) (Sigma) are employed to isolate plasmid-free cells. “Curing” was performed with a series of tubes containing HTM supplemented Mueller Hinton Broth and ethidium bromide at concentrations ranging from 0.06mg/l to 32mg/l. Tubes were inoculated with $10^4$-$10^5$ organisms per ml and incubated at 37°C overnight with continuous shaking. The culture tube that contained the highest concentration of ethidium bromide and still allowed visible growth was plated on Chocolate Blood Agar plates. Isolated colonies were subsequently subcultured on Chocolate Blood Agar slopes and β-lactamase was prepared and detected as described in Sections 2.6.5.i & 2.6.6.

Loss of β-lactamase activity was taken as an indication for cured or deleted plasmid that mediated the production of the β-lactamase enzyme.

2.6.17 Isolation and Purification of DNA

2.6.17.i Isolation of Total Genomic DNA

Total genomic DNA was prepared by the method of Zyskind & Bernstein (1989). 1.2 ml of an overnight culture of *H. influenzae* was spun down in an MSE Micro centaur centrifuge at 13,000 rpm and the pellet was resuspended in HTE buffer (50mM Tris-HCl pH 8.0; 20mM EDTA). Cells were lysed by 2% sarcosyl in HTE buffer (pH 8.0) and RNA was removed by adding 1mg/ml RNase. The mixture was incubated for 15 minutes at 37°C and lysis was completed with the addition of pronase (0.5 mg/ml) and incubation for a further 1 hour at 50°C. DNA was extracted by phenol/chloroform and ether and precipitated with sodium acetate and isopropanol. The mixture was frozen at -70°C overnight before centrifugation at 13,000 rpm for 20
minutes. The pellet was washed with 70% ethanol three times, dried and resuspended in TE buffer (10mM Tris-HCl pH 8.0; 0.1mM EDTA). The DNA was checked for purity on the spectrophotometer and stored at -20°C until required.

2.6.17.ii Isolation of Plasmid DNA

Plasmid DNA from *H. influenzae* strains were prepared by employing the method described by Meyers *et al.*, (1976) or Hanson & Olsen (1978). Extraction of plasmids from other organisms was carried out by the alkaline lysis method of Takahashi & Nagano (1984).

2.6.18 Gel Electrophoresis of DNA

Intact plasmid DNA, chromosomal DNA, restriction endonuclease digested DNA and PCR-amplified DNA were electrophoresed on 0.4-1.2% w/v agarose gels in TAE buffer (40mM Tris-acetate pH 7.6; 1mM EDTA). Electrophoresis was performed on horizontal slab gels in a Bethesda Research Laboratories Horizon 20.25 gel tank (Life Technologies Inc., Petersburg, Florida, USA), Pharmacia GNA-100 minitank or Biocad Mini Sub Cell at a constant voltage (between 70V overnight to 100V for 1 hour).

Each sample was mixed with loading buffer (0.01% w/v bromphenol blue, 50% v/v glycerol) to a ratio of 5:1 prior to loading onto the gel. λ Phage-DNA digested with *Hind III* (Gibco-BRL) was loaded alongside the samples during electrophoresis as an indicator of molecular weight. After electrophoresis was completed, the DNA was stained with ethidium bromide solution (50 μg/l) for an hour and viewed on a UV transilluminator (UV Products, Cambridge). Photographs were taken with a Polaroid camera system (setting F8) for 3 minutes with an orange filter.
2.6.19 Determination of Concentration and Purity of Double-Stranded DNA

The DNA was diluted 1:50 in TNE buffer (10mM Tris-HCl pH 8.0; 10mM NaCl; 0.1mM EDTA) and the optical density was measured at 260nm and 280nm on a Lambda 2 spectrophotometer. The concentration and the purity of the DNA were assessed according to the method described by Zyskind & Bernstein (1989) where the absorbance at 260nm in a 1cm quartz cuvette of a 50µg/ml solution of double stranded DNA is equal to one. The ratio between the O.D. 260nm and O.D. 280nm provides an estimate of purity. The ratio of 1:1.65 to 1:1.85 is an indication of pure DNA preparation.

2.6.20 Dot-Blot Hybridisation

2.6.20 i Preparation of Whole Cell DNA for Hybridisation

Whole cell DNA was prepared from *H. influenzae* isolates (Section 2.6.17 i) and the nucleic acid concentration of the samples was adjusted to 25µg/ml. 20µl of each test sample was denatured by adding 20µl of 1M NaOH to a final concentration of 0.5M.

2.6.20.ii Preparation of Plasmid Probes

Plasmid DNA from *H. influenzae* strain A87 containing pROB was isolated as described by Meyers *et al.*, (1976). pUC18 containing bla gene was prepared from *E. coli* J62-2 by employing the method of Takahashi & Nagano, (1984). After electrophoresis, based on the instructions recommended by the manufacturer of Geneclean II kit (Bio 101 Inc., LaJolla, California), plasmid probes were removed and purified from 0.6% agarose gels. The probes were diluted in water to a final concentration of 20µg/ml and were denatured by heating for 5 minutes in boiling water. Probes were labelled with fluorescein using the reagents and the protocol supplied with the ECL™ random prime labelling and detection system kit (Amersham, Life Sciences). In the ECL™ random prime system, fluorescein-11-dUTP (Fl-dUTP) was used as the label that partially replaced TTP in the reaction.
The fluorescein residues within the DNA were subsequently detected by an enzyme-linked anti-fluorescein antibody (anti-fluorescein horseradish peroxide conjugate) that was provided with the kit.

2.6.20.iii Hybridisation

Prior to hybridisation, denatured whole cell DNA samples were loaded onto nylon filters, Hybond™-N+ (Amersham International). Unlabelled plasmids were used as positive controls. The filters were air-dried and baked in a vacuum gel drier at 80°C for 2 hours. Hybridisation was performed according to the instructions recommended by the manufacturer of the ECL™ random prime, labelling system (Amersham, Life Sciences). Labelled plasmid probes were hybridised under stringent conditions to whole cell DNA bound to the membrane. Stringency was increased by raising the overnight hybridisation temperature to 65°C and washing the hybridised filters in 0.1 x SSC; 0.1% SDS and 0.05 x SSC; 0.1% SDS at 65°C for 20 minutes (20 x SSC contained 0.3M sodium citrate; 3M NaCl, pH 7.0). Bound labelled sequences were determined by autoradiography at room temperature for 1 hour (Hyperfilm MP X-ray film, Amersham International plc). The exposed film was then removed and developed with Kodak Developer & Fixer (Sigma).

2.6.21 Cloning DNA Fragments

2.6.21.i Preparation of Vector DNA

pSU18, a chloramphenicol-resistant pUC-derived 2.3 kb vector (Bartolomé et al., 1991) was prepared by the alkaline lysis method of Takahashi & Nagano (1984). The extracted DNA was subjected to electrophoresis at 60 V overnight in 0.7% agarose in TAE buffer (pH 8.0). The DNA was visualised by staining with ethidium bromide (50mg/l). A 2.3kb DNA fragment was purified from the agarose gel by GeneClean II kit (Bio 101 Inc., La Jolla, California, USA). Concentration and purity of DNA was assessed by reading the O.D. at 260nm and 280nm (Section 2.6.19).
The vector DNA was digested with *Bam*H I (Gibco-BRL, Life Sciences) restriction endonuclease at 37°C for 2 hours in "React" buffer system supplied with the enzyme. The digested DNA was treated with Bacterial Alkaline Phosphatase (BAP) (Gibco-BRL) to remove 5' phosphate groups and to prevent recircularisation of the vector during ligation. BAP was removed from the reaction mixture prior to ligation by phenol/chloroform extraction and vector DNA was precipitated with ethanol. To examine the restricted DNA, electrophoresis was carried out with 0.8% agarose and TAE buffer (pH 8.0).

### 2.6.21.ii Preparation of Insert DNA

Genomic DNA from *H. influenzae* LG629 was isolated (Zyskind & Bernstein, 1989) and its purity and concentration were assessed spectrophotometrically (Section 2.6.19). Whole cell DNA was partially digested with *Sau*3AI endonuclease enzyme (Promega, Madison, USA) employing the method recommended by Silhavy *et al.*, (1984) for 45 minutes at 37°C. The reaction was terminated (with 2μl of 0.5M EDTA) and analysed by electrophoresis at 2V/cm for 18 hours through a 0.4% agarose gel in TAE buffer (pH 8.0). Partially digested DNA was extracted with 1 volume of phenol/chloroform and was precipitated with ethanol.

### 2.6.21.iii Ligation of Vector and Insert DNA

The ligation reaction was carried out in 20μl reaction volume containing ligation mix buffer (Gibco-BRL), vector DNA fragment and partially digested genomic DNA. 0.1 unit of T4 DNA ligase (Gibco-BRL) was added to the reaction and the mix was incubated overnight in a 12°C water bath. A portion of the ligation products were transformed into the host cell.
Preparation of Competent Cells and Transformation

For blue/white colour screening of the pSU18 plasmid, *E. coli* TG2 that carries lacZ ΔM15 on an F' episome was used as the host cell. Cloning was carried out as described by Sambrook *et al.*, (1989).

*E. coli* TG2 was grown in 10ml of SOB broth for 18 hours at 37°C. 1ml of this culture was inoculated into 100ml of SOB broth containing 10mM MgCl₂. The cells were grown at 37°C with vigorous shaking for 2 hours to approximately mid-log phase (4-7 x 10⁷ CFU/ml). The culture was harvested by centrifugation at 2,500x g for 12 minutes at 4°C (RC-5B Sorval) and resuspended in 40ml of ice-cold sterile 10mM CaCl₂ solution and stored on ice for 15 minutes. The cells were harvested at 2,000x g for a further 10 minutes and resuspended in 8ml of ice-cold 100mM CaCl₂ before dispensing into 0.2ml aliquots. 9μl of the ligation mix was added to the cells. The tube was then stored on ice for 30 minutes. The mixture was heat shocked at 42°C for 1 minute and cooled on ice for 1-2 minutes. 0.8ml pre-warmed (37°C) SOC Broth was added before incubation at 37°C for 2 hours.

For blue/white selection of recombinant clones, 5-bromo-4-chloro-3-indoyl-β-galactopyranoside, X-gal, (40μl of a 20mg/ml solution in N,N-dimethyl formamide) and isopropyl-β-D-thiogalactopyranoside, IPTG, (10μl of a 200mg/ml aqueous solution) were spread onto LB agar plates containing 25mg/ml chloramphenicol. The plates were allowed to absorb the components for 30 minutes at 37°C prior to plating out the transformation mix. After overnight incubation at 37°C, colonies containing β-galactosidase activity were blue and pin-point in size, whereas possible recombinants were white and larger in size. White colonies were subcultured onto LB Agar plates containing 25mg/ml chloramphenicol. The plasmid was extracted (Section 2.6.17.ii) and analysed by restriction digest. The efficiency of transformation was evaluated by transforming a known concentration of religated pSU18 DNA and counting the number of transformants.
2.6.22 DNA Sequencing

2.6.22.i Amplification of DNA With Polymerase Chain Reaction (PCR)

PCR amplification of the TEM structural gene was performed in a 0.5ml microfuge tube in a total volume of 0.1ml with a Techne PHC-2 Dri-block Cycler (Cambridge, Cambs) as previously described by Du Bois (1993). The reaction components are listed in Table 2-11.

The reaction mixtures were covered with two drops of mineral oil to reduce evaporation. The tubes were smeared with a thin film of silicone vacuum grease to improve thermal contact with the heating block of the thermal cycler. The amplification program was performed as described in Table 2-12.

After the PCR reaction was completed, the samples were placed on ice. PCR products were verified as approximately 900 bp fragments by electrophoresis on a 1.2% agarose gel. The samples were stored at -20°C until required. The biotinylated primer, Biotin-Bla 4' was used for the amplification of DNA fragment in order to perform direct sequencing with the Dynabeads M-280 streptavidin (Dynal, Norway).

2.6.22.ii Preparation of PCR Products for Sequencing

Dynabeads M-280 Streptavidin (Dynal, Norway) was used as a magnetic solid phase to capture and purify PCR products and to prepare immobilised single-stranded DNA template for sequencing. In order to convert the double stranded DNA into a single-stranded template, the PCR product was end labelled with biotin. Therefore biotinylated-Bla 4' primer was used instead of the Bla 4' oligonucleotide. Biotin- Bla 4' had the same DNA sequence as the Bla 4' plus the addition of a biotin ligand at its 5'-terminus. PCR was performed as previously described (Section 2.6.22.i).
Table 2-11, PCR Reaction Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Supplier</th>
<th>Quantity per Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x Taq Reaction Buffer</td>
<td>Promega, Southampton, Hants</td>
<td>10µl</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>Promega</td>
<td>10µl</td>
</tr>
<tr>
<td>4mM dNTP stock solution*</td>
<td>Boehringer Mannheim</td>
<td>5µl</td>
</tr>
<tr>
<td>Bla 3' PCR Primer¹</td>
<td>Oswel Laboratory, Dept. of Chemistry, Edinburgh University</td>
<td>10 pmoles</td>
</tr>
<tr>
<td>Bla 4' PCR Primer²</td>
<td>Oswel Laboratory</td>
<td>10 pmoles</td>
</tr>
<tr>
<td>Genomic DNA Preparation</td>
<td></td>
<td>2µl</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>Promega</td>
<td>1 unit</td>
</tr>
<tr>
<td>Deionised Distilled Water</td>
<td></td>
<td>to 100µl</td>
</tr>
</tbody>
</table>

*1mM dATP, 1mM dTTP, 1mM dGTP, 1mM dCTP in 10mM Tris-HCl

1 Bla 3': 5' - CTC TCT AGA AAA AGG AAG AGT ATG AGT ATT-3'
2 Bla 4': 5' - CTC GCA TGC GTA AAC TTG GTC TGA CAG TTA-3'
Table 2-12, The PCR Heating Cycle Protocol

<table>
<thead>
<tr>
<th>Segment</th>
<th>Repeats</th>
<th>Temperature (°C)</th>
<th>Time (sec)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>x 1</td>
<td>96</td>
<td>30</td>
<td>denaturation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>60</td>
<td>Primer annealing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>90</td>
<td>Primer extension</td>
</tr>
<tr>
<td>2</td>
<td>x 24</td>
<td>96</td>
<td>15</td>
<td>denaturation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>30</td>
<td>Primer annealing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>90</td>
<td>Primer extension</td>
</tr>
<tr>
<td>3</td>
<td>x 1</td>
<td>72</td>
<td>5 x 60</td>
<td>Final extension</td>
</tr>
</tbody>
</table>

20μl of Dynabeads M-280 streptavidin (10mg/ml) was removed and used for each template preparation. The beads were first washed with 20μl of 0.1% BSA (Albumin Bovine, Sigma) in 1 x PBS buffer (pH 7.2). One litre of 1 x PBS consisted of 8.5g NaCl; 1.07g Na₂HPO₄; and 0.39g NaH₂PO₄. The pH was adjusted to 7.2 with H₃PO₄ or NaOH. The tube containing the beads was placed in the magnetic separator (Dynal MPC) where the beads adhered to the side of the tube and allowed the buffer to be removed. The beads were then washed with 20μl of Binding & Washing (B&W) buffer which contained 10mM Tris-HCl, pH 7.5; 1mM EDTA; and 2.0 M NaCl and were resuspended in 40μl of the same buffer at a final concentration of 5mg/ml.

To immobilise the PCR product, 40μl of the pre-washed Dynabeads was added to 40μl of each PCR reaction. The tube was incubated at room temperature for 20
minutes with gentle agitation. The supernatant was removed and the beads were washed with 40μl B&W buffer. The duplex DNA was denatured by eluting the beads in 8μl 0.15M NaOH solution and incubation at room temperature for 10 minutes. The DNA strands were separated by removing the NaOH supernatant containing the non-biotinylated strand to another tube. The alkali supernatant was neutralised with 4μl 0.3M HCl and 1μl 1M Tris-HCl (pH 8.0). The volume was adjusted to 20μl with deionised distilled sterile water. The pellet containing the biotin-labelled strand was collected on the side of the tube and was washed once with 50μl of 0.15M NaOH, then with 50μl B&W buffer (pH 7.5), and finally with 50μl TE (10mM Tris-HCl, pH 8.0; 1mM EDTA). The beads were resuspended in 20μl TE (pH 8.0) and were submitted to the dideoxy sequencing procedure.

2.6.22.iii Single-Stranded DNA Sequencing

The sequencing of single-stranded template DNA was performed with a USB Sequenase version 2.0 DNA sequencing kit (Cambridge, Bioscience) and [35S]α-dATP label (Amersham International plc) based on the chain-termination method of Sanger et al., (1977).

The reactions were set up according to the protocol recommended by the USB sequenase version 2.0 kit manufacturer. The products were separated by electrophoresis in a denaturing polyacrylamide gel (8%T, 5%C acrylamide/bisacrylamide, 7M Urea) (Table 2-13) with the Sequi-Gen Nucleic Acid Sequencing Cell (BioRad, Watford, Herts) in an electrolyte gradient. The bottom of the electrophoresis gel was sealed with the plug solution consisting of 20ml 8%T, 5%C acrylamide/ bisacrylamide; 7M Urea stock solution; 100μl of 25% w/v ammonium persulphate and 100μl TEMED (Table 2-13). The polyacrylamide gel solution was prepared with 50ml of the stock solution (8%T, 5%C acrylamide/bisacrylamide; 7M Urea), 80μl 25% w/v ammonium persulphate and 80μl TEMED. The gel was poured and left overnight to polymerise. The inner plate chamber and the lower chamber of the Sequi-Gen Cell were filled with 0.6 x TBE
buffer (10 x TBE buffer contained 0.89M Tris borate, pH 8.3; 20mM EDTA). The gel was pre-run for 1 hour at a constant power (3000V, 50mA, 60W) to warm the apparatus to 50-55°C. The samples were loaded and electrophoresis proceeded for 2-3 hours. The gel was removed and soaked in a solution of 10% v/v acetic acid and 20% v/v methanol for 45 minutes before drying at 80°C for 2 hours under vacuum in a flat-bed gel drier. The gel was exposed to Hyperfilm MP X-ray film (Amersham International plc) and autoradiographed for 24-48 hours at room temperature. The film was kindly developed by the X-ray department at the Edinburgh Royal Infirmary.

Table 2-13, The Components of the Denaturing Acrylamide/Bisacrylamide Stock Solution

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Stock Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>7M</td>
</tr>
<tr>
<td>10 x TBE*</td>
<td>single strength</td>
</tr>
<tr>
<td>40%T, 5%C acrylamide/N,N'-methylene-bisacrylamide</td>
<td>8%T, 5%C</td>
</tr>
</tbody>
</table>

*0.89M Tris-borate, pH 8.3; 20mM EDTA
CHAPTER THREE

RESULTS

3.1 Isolation of Clinical *H. influenzae* Strains

Six hundred and sixteen non-duplicate *H. influenzae* isolates were obtained over the period of three years (1992-1995) from four hospitals in Scotland. Table 3-1 represents the number and the origin of the strains.

<table>
<thead>
<tr>
<th>Hospital of Isolation</th>
<th>No. (%) of Strains Collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aberdeen Royal Infirmary</td>
<td>63 (10.2)</td>
</tr>
<tr>
<td>Edinburgh Royal Infirmary</td>
<td>81 (13.1)</td>
</tr>
<tr>
<td>Glasgow Royal Infirmary</td>
<td>62 (10.1)</td>
</tr>
<tr>
<td>Glasgow Southern General</td>
<td>410 (66.6)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>616</strong></td>
</tr>
</tbody>
</table>

Of the 616 *H. influenzae* strains, 10.2% were collected from Aberdeen Royal Infirmary, 13.1% from Edinburgh Royal Infirmary, and 10.1% from Glasgow Royal Infirmary. The highest number of isolates were obtained from Glasgow Southern General Hospital (66.6%).
The data accommodating the physiological site of recovery of the isolates, the age and the sex of the hosts were not provided for all 616 strains; however; the results concerning the factors mentioned will be presented with regard to the known and unknown details.

3.2 Clinical Sites of Isolation

Clinical sites of isolation for 479 (77.8%) *H. influenzae* strains were known, however; the site of recovery for 137 (22.2%) remained unknown (Table 3-2).

<table>
<thead>
<tr>
<th>Site of Isolation</th>
<th>Number of Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye</td>
<td>3</td>
</tr>
<tr>
<td>Sputum</td>
<td>459</td>
</tr>
<tr>
<td>Nasopharynx</td>
<td>7</td>
</tr>
<tr>
<td>Tracheal Aspirate</td>
<td>7</td>
</tr>
<tr>
<td>Catheter Tip Suction</td>
<td>3</td>
</tr>
<tr>
<td>Unknown</td>
<td>137</td>
</tr>
</tbody>
</table>

The vast majority of the isolates, 476 (99.4%) were recovered from respiratory tract, from which 96% (450) accounted for sputum cultures and only 0.6% (3) of the specimens were recovered from the eye. Information regarding the clinical diagnosis was not provided.
3.3 The Age & the Sex of the Patients

The data representing the age of the hosts was available for 64% (394) of the isolates. Table 3-3 illustrates the distribution of the isolates in four age groups with respect to the sex of the patients.

In all categories (male, female and unknown) the incidence of *H. influenzae* strains recovered was significantly higher among patients older than 50 years of age (72.8%). The two age groups of 16-24 and 25-49 year olds contained 2% (8) and 22.7% (89) of the patients respectively. The paediatric group (0-15 year olds) consisted of 10 (2.6%) hosts.

Table 3-3, Distribution of the Isolates in each Age Group with Respect to the Sex of the Hosts

<table>
<thead>
<tr>
<th>Sex of the Host</th>
<th>No. of Isolates</th>
<th>No. of Isolates of each Age Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-15</td>
</tr>
<tr>
<td>Female</td>
<td>140</td>
<td>0</td>
</tr>
<tr>
<td>Male</td>
<td>228</td>
<td>10</td>
</tr>
<tr>
<td>Unknown</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>394</td>
<td>10</td>
</tr>
</tbody>
</table>

3.4 The Relationship Between Sites of Isolation and the Age of the Hosts

The data illustrating both the age of the hosts and the biological sites of isolation of the *H. influenzae* strains was available for 389 (63.1%) strains. In Table 3-4, the distribution of isolates in each age group has been associated with the physiological sites of recovery. The highest proportion of the strains (70.4%) were sputum specimens isolated from patients older than 50 years of age.
Table 3-4, Age Distribution of the 389 *H. influenzae* Isolates Recovered from Different Clinical Sites

<table>
<thead>
<tr>
<th>Site of Isolation</th>
<th>No. of Strains</th>
<th>0-15</th>
<th>16-24</th>
<th>25-50</th>
<th>&gt;50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>375</td>
<td>8</td>
<td>8</td>
<td>85</td>
<td>274</td>
</tr>
<tr>
<td>Nasopharynx</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Tracheal Aspirate</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Eye</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>389</strong></td>
<td><strong>10</strong></td>
<td><strong>8</strong></td>
<td><strong>88</strong></td>
<td><strong>283</strong></td>
</tr>
</tbody>
</table>

3.5 Biochemical Characteristics of the Isolates

The biochemical intra-species differentiation was based on three biochemical properties; indole production, urea hydrolysis, and ornithine decarboxylation. The isolates were non-selectively included in the study to reflect accurately the normal incidence of the various biotypes. All six hundred and sixteen clinical *H. influenzae* isolates were categorised into seven biotypes. Table 3-5 shows the distribution of the strains in biotypes for each hospital.
Table 3-5, Distribution of 616 *H. influenzae* Isolates among Classes of Biotypes.

<table>
<thead>
<tr>
<th>Hospital</th>
<th>No. of Strains</th>
<th>I  (No.)</th>
<th>II (No.)</th>
<th>III (No.)</th>
<th>IV (No.)</th>
<th>V  (No.)</th>
<th>VI (No.)</th>
<th>VII (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aberdeen Royal Infirmary</td>
<td>63</td>
<td>17.5 (11)</td>
<td>30.2 (19)</td>
<td>28.6 (18)</td>
<td>15.9 (10)</td>
<td>1.6 (1)</td>
<td>1.6 (1)</td>
<td>4.8 (3)</td>
</tr>
<tr>
<td>Edinburgh Royal Infirmary</td>
<td>81</td>
<td>26 (21)</td>
<td>45.7 (37)</td>
<td>19.8 (16)</td>
<td>8.6 (7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glasgow Royal Infirmary</td>
<td>62</td>
<td>37 (23)</td>
<td>17.7 (11)</td>
<td>16.1 (10)</td>
<td>24.2 (15)</td>
<td>3.2 (2)</td>
<td>1.6 (1)</td>
<td>0</td>
</tr>
<tr>
<td>Glasgow Southern General</td>
<td>410</td>
<td>23.9 (98)</td>
<td>28.5 (117)</td>
<td>27.1 (111)</td>
<td>15.4 (63)</td>
<td>3.2 (13)</td>
<td>1.7 (7)</td>
<td>0.2 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>616</td>
<td>24.8 (153)</td>
<td>29.9 (184)</td>
<td>25.2 (155)</td>
<td>15.4 (95)</td>
<td>2.6 (16)</td>
<td>1.5 (9)</td>
<td>0.6 (4)</td>
</tr>
</tbody>
</table>
In Aberdeen Royal Infirmary the largest group of isolates were of biotype II with 30.2%. This was followed by biotype III (28.6%) and biotype I (15.9%). Biotype IV consisted of 15.9% and biotype VII harboured 4.8% of the strains isolated from this hospital. Biotypes V and VI equally contained 1.6% of the strains.

In Edinburgh Royal Infirmary, the highest proportion of strains were also of biotype II with 45.7%. Biotypes I and III were represented by 26% and 19.8% of the strains and biotype IV consisted of 8.6% of the isolates. In this hospital no strains were recovered that exhibited the biochemical properties of biotypes V-VII.

In contrast to the other hospitals, a higher proportion of the strains isolated from Glasgow Royal Infirmary were of biotype I (37%), and 17.7% of the strains were classified as biotype II and 16.1% as biotype III. Biotype IV contained a higher number of isolates (24.2%) than other centres, and biotypes V and VI consisted of 3.2% and 1.6% of the strains.

In Glasgow Southern General hospital from the 410 strains collected, 23.9% were categorised as biotype I, 28.5% as biotype II, 27.1% as biotype III and 15.4% as biotype IV. The distribution of strains in biotypes V, VI, and VII was 2.6%, 1.5%, and 0.6% respectively.

The distribution of biotypes in the collaborative hospitals varied significantly (p=0.0042). Overall, from the 616 H. influenzae isolates provided, 24.8% were classified as biotype I and 25.2% as biotype III. Biotype II contained the largest number of the isolates with 35.9%. Biotype IV consisted of 15.4%, and 2.6% of the strains were of biotype V. Finally only 1.5% and 0.6% of the specimens resembled the biochemical characteristics of biotypes VI and VII. Biotype VIII was not isolated from any of the hospitals.
3.6 The Prevalence of Capsulated and Non-Capsulated \( H. \text{influenzae} \) Strains in Hospitals of Isolation

\( H. \text{influenzae} \) isolates were examined for the presence of capsule by Hiss’ staining method (Section 2.6.3). According to this method, 14% (86) of the 616 strains were categorised as capsulated. The distribution of typeable strains in each hospital is shown in Table 3-6.

Table 3-6, Prevalence of Capsulated and Non-Capsulated \( H. \text{influenzae} \) Strains

<table>
<thead>
<tr>
<th>Hospital</th>
<th>No. of Strains</th>
<th>% (No.) Capsulated</th>
<th>% (No.) Non-Capsulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aberdeen Royal Infirmary</td>
<td>63</td>
<td>6.3 (4)</td>
<td>93.7 (59)</td>
</tr>
<tr>
<td>Edinburgh Royal Infirmary</td>
<td>81</td>
<td>21 (17)</td>
<td>79 (64)</td>
</tr>
<tr>
<td>Glasgow Royal Infirmary</td>
<td>62</td>
<td>11.3 (7)</td>
<td>88.7 (55)</td>
</tr>
<tr>
<td>Glasgow Southern General</td>
<td>410</td>
<td>14.1 (58)</td>
<td>85.9 (352)</td>
</tr>
</tbody>
</table>

The highest incidence of encapsulated organisms was amongst the strains provided by Edinburgh Royal Infirmary with 21%. The lowest proportion (6.3%) was obtained from Aberdeen Royal Infirmary.

3.7 The Relationship Between Biotypes & Serotypeable Strains

Table 3-7 displays the prevalence of biotypes of \( H. \text{influenzae} \) among capsulated and non-capsulated isolates. In general, there were insignificant differences in the pattern of distribution of capsulated and non-capsulated strains among biotypes \((p=0.266)\). Biotype II comprised the majority of the isolates in both capsulated (36%) and non-capsulated (28.9%) groups.
Table 3-7, Distribution of Capsulated & Non-Encapsulated Strains in Biotypes

<table>
<thead>
<tr>
<th>Type of Strain</th>
<th>No. of Strains</th>
<th>% (No.) of Isolates of Each Biotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Capsulated</td>
<td>86</td>
<td>27.9 (24)</td>
</tr>
<tr>
<td>Non-Capsulated</td>
<td>530</td>
<td>24.3 (129)</td>
</tr>
</tbody>
</table>
3.8 Biotypes & Clinical Origin of the Strains

Table 3-8 displays the relationship between the biotypes of *H. influenzae* and their clinical sites of recovery. As previously mentioned, the physiological origin of 137 specimens remained unknown throughout the study.

In total, biotype II strains were isolated most frequently (31.7%), from respiratory secretions. This was followed by strains of biotypes III (25%) and I (24.5%). Among nasopharyngeal specimens, biotype I accounted for 42.9% of the isolate recovered. Isolates from eye culture were evenly distributed among biotypes II, III, and IV.

Except for one biotype I encapsulated strain that was recovered from tracheal aspirate, all typeable strains were sputum specimens.

3.9 Relationship Between Age, Source of Isolation & Biotypes

Distribution of biotypes of *H. influenzae* in each age group with regard to the site of isolation is shown in Table 3-9.

The majority (43.8%) of the sputum specimens recovered from patients less than 25 years of age were of biotype III, in the third age group (25-49 year olds), the highest rate of sputum isolates were classified as biotype II (30.6%). However; there were no significant differences ($p=0.45$) in the distribution of specimens among the designated biotypes isolated from the patients. Eye cultures comprising of biotypes II, III and IV were recovered from patients aged 6 months, 1 year and 41 years old. Nasopharyngeal and bronchial aspirate specimens were mainly recovered from older age groups and were largely of biotypes I and III.
Table 3-8, Relationship between Biotypes & Clinical Origin of the Isolates

<table>
<thead>
<tr>
<th>Clinical Source</th>
<th>No. of Specimens</th>
<th>% (No.) of Isolates of Each Biotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Sputum</td>
<td>459</td>
<td>24 (110)</td>
</tr>
<tr>
<td>Tracheal Aspirate</td>
<td>7</td>
<td>42.9 (3)</td>
</tr>
<tr>
<td>Nasopharynx</td>
<td>7</td>
<td>42.9 (3)</td>
</tr>
<tr>
<td>Eye</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Catheter tip Suction</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3-9, Relationship Between Age of the Host, Clinical Source of Isolation & Biotypes.

<table>
<thead>
<tr>
<th>Age</th>
<th>No. of Isolates</th>
<th>Origin</th>
<th>% (No.) of Isolates of Each Biotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>0-15</td>
<td>8</td>
<td>Sputum</td>
<td>12.5(1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Eye</td>
<td>0</td>
</tr>
<tr>
<td>16-24</td>
<td>8</td>
<td>Sputum</td>
<td>0</td>
</tr>
<tr>
<td>25-50</td>
<td>85</td>
<td>Sputum</td>
<td>20 (17)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Nasopharynx</td>
<td>100 (1)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Tracheal Aspirate</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Eye</td>
<td>0</td>
</tr>
<tr>
<td>&gt;50</td>
<td>274</td>
<td>Sputum</td>
<td>26.6 (73)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Nasopharynx</td>
<td>40 (2)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Tracheal Aspirate</td>
<td>50 (2)</td>
</tr>
</tbody>
</table>
3.10 Distribution of Capsulated Strains Among Designated Age Groups

The relationship between serotypeable strains and the age of the hosts is displayed in Table 3-10. 10% of the infections caused by *H. influenzae* in the paediatric group (0-15) was associated with typeable strains. However, none of the isolates recovered from 16-24 year olds were classified as capsulated. The third age group (25-50), contained the highest incidence of typeable strains with 18% and finally 13.6% of the strains isolated from patients older than 50 years of age were serotypeable. The non-capsulated strains showed a similar pattern of distribution among the designated age groups.

Table 3-10, Relationship Between Age Groups & Serotypeable Strains

<table>
<thead>
<tr>
<th>Age Group</th>
<th>0-15</th>
<th>16-24</th>
<th>25-50</th>
<th>&gt;50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsulated</td>
<td>10 (1)</td>
<td>0</td>
<td>18 (16)</td>
<td>13.6 (39)</td>
</tr>
<tr>
<td>Non-Capsulated</td>
<td>90 (9)</td>
<td>100 (8)</td>
<td>82 (73)</td>
<td>86.4 (248)</td>
</tr>
</tbody>
</table>

3.11 Detection of β-Lactamase Producing Strains

The cell-free extracts of the 616 isolates were prepared (Section 2.6.5) and the β-lactamase activity of each preparation was assayed by nitrocephin spot test. The change of colour of nitrocephin solution (10⁻⁴M) from yellow to red after 30 minutes was taken as an indication of β-lactamase activity. Based on this method 20.5% (126) of the *H. influenzae* isolates harboured a detectable β-lactamase enzyme.
3.11.1 The Prevalence of β-Lactamase Production in Clinical H. influenzae Isolates

The prevalence of β-lactamase production in H. influenzae strains isolated in Scotland is shown in Table 3-11. The proportion of β-lactamase positive strains ranged from 9.6% in Glasgow Royal Infirmary to 33.3% in Edinburgh Royal Infirmary.

Table 3-11, Prevalence of β-Lactamase-Positive H. influenzae Strains in Scotland

<table>
<thead>
<tr>
<th>Hospital</th>
<th>No. of Isolates</th>
<th>% (No.) of β-Lactamase Producers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aberdeen Royal Infirmary</td>
<td>63</td>
<td>17.4 (11)</td>
</tr>
<tr>
<td>Edinburgh Royal Infirmary</td>
<td>81</td>
<td>33.3 (27)</td>
</tr>
<tr>
<td>Glasgow Royal Infirmary</td>
<td>62</td>
<td>9.6 (6)</td>
</tr>
<tr>
<td>Glasgow Southern General</td>
<td>410</td>
<td>20 (82)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>616</strong></td>
<td><strong>20.5 (126)</strong></td>
</tr>
</tbody>
</table>

3.11.2 β-Lactamase Production & Biotypes

Table 3-12 presents the number of β-lactamase positive strains that were detected in each biotype. These strains were isolated with greater frequency (37.5%) among biotype V isolates. There were no β-lactamase-positive biotype VII isolate. β-lactamase production significantly varied (p=0.040) among the isolates of each biotype.
β-lactamase producing *H. influenzae* strains isolated from the four hospitals have been categorised according to their biochemical properties. Table 3-13 shows that from the 126 β-lactamase producers, the majority were of biotype I (32.5%). The incidence of β-lactamase production was higher for biotype I strains isolated from Edinburgh Royal Infirmary and Glasgow Royal Infirmary; however, such incidence was not found to be universal, as in Aberdeen Royal Infirmary both biotypes II and III were equally prevalent (27.3%) and in Glasgow Southern General most (33%) β-lactamase positive strains were detected to be of biotype III. In total these differences did not appear to be significant (*p*=0.368).

**Table 3-12, Prevalence of β-Lactamase Production in Each Biotype**

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Total No. of Isolates</th>
<th>% (No.) of β-lactamase producers</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>153</td>
<td>26.8 (41)</td>
</tr>
<tr>
<td>II</td>
<td>184</td>
<td>14.1 (26)</td>
</tr>
<tr>
<td>III</td>
<td>155</td>
<td>23.2 (36)</td>
</tr>
<tr>
<td>IV</td>
<td>95</td>
<td>16.8 (16)</td>
</tr>
<tr>
<td>V</td>
<td>16</td>
<td>37.5 (6)</td>
</tr>
<tr>
<td>VI</td>
<td>9</td>
<td>11.1 (1)</td>
</tr>
<tr>
<td>VII</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3-13. Distribution of β-Lactamase Producing *H. influenzae* Isolates among Biotypes

<table>
<thead>
<tr>
<th>Hospital</th>
<th>β-lactamase producers</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aberdeen Royal Infirmary</td>
<td>11</td>
<td>18.2 (2)</td>
<td>27.3 (3)</td>
<td>27.3 (3)</td>
<td>18.2 (2)</td>
<td>9.1 (1)</td>
<td>0</td>
</tr>
<tr>
<td>Edinburgh Royal Infirmary</td>
<td>27</td>
<td>40.7 (11)</td>
<td>29.7 (8)</td>
<td>18.5 (5)</td>
<td>11.1 (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glasgow Royal Infirmary</td>
<td>6</td>
<td>50 (3)</td>
<td>0</td>
<td>16.7 (1)</td>
<td>16.7 (1)</td>
<td>16.7 (1)</td>
<td>0</td>
</tr>
<tr>
<td>Glasgow Southern General</td>
<td>82</td>
<td>30.5 (25)</td>
<td>18.3 (15)</td>
<td>33 (27)</td>
<td>12.2 (10)</td>
<td>4.9 (4)</td>
<td>1.2 (1)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>126</strong></td>
<td><strong>32.5 (41)</strong></td>
<td><strong>20.6 (26)</strong></td>
<td><strong>28.8 (36)</strong></td>
<td><strong>12.7 (16)</strong></td>
<td><strong>4.8 (6)</strong></td>
<td><strong>0.8 (1)</strong></td>
</tr>
</tbody>
</table>
3.11.3 β-Lactamase Production, Biotypes, & Serotypeable Strains

Of the 126 β-lactamase positive strains, 12.7% (16) were capsulated. The relationship between β-lactamase production and biotypes for the 86 typeable strains is shown in Table 3-14.

The majority (56.3%) of the capsulated β-lactamase producers were of biotype I, whereas in the β-lactamase-negative group the majority of the typeable strains (40%) were categorised as biotype II. None of the encapsulated β-lactamase positive isolates were obtained from Aberdeen Royal Infirmary or Glasgow Royal Infirmary. However, the distribution of β-lactamase-positive group collected from Edinburgh Royal Infirmary and Glasgow Southern General followed a similar pattern where biotype I contained 50% and 60% of the isolates respectively. With the exception of Glasgow Royal Infirmary, where most capsulated β-lactamase-negative strains were of biotype III (42.8%), in other hospitals the majority consisted of biotype II isolates.
Table 3-14, Relationship between β-Lactamase Production, Biotype & Capsulation

<table>
<thead>
<tr>
<th>Hospital</th>
<th>β-Lactamase</th>
<th>No. of Isolates</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aberdeen Royal Infirmary</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>4</td>
<td>25 (1)</td>
<td>50 (2)</td>
<td>0</td>
<td>25 (1)</td>
<td>0</td>
</tr>
<tr>
<td>Edinburgh Royal Infirmary</td>
<td>+</td>
<td>6</td>
<td>50 (3)</td>
<td>33.3 (2)</td>
<td>16.7 (1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>11</td>
<td>27.3 (3)</td>
<td>45.4 (5)</td>
<td>27.3 (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glasgow Royal Infirmary</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>7</td>
<td>0</td>
<td>28.6 (2)</td>
<td>42.8 (3)</td>
<td>28.6 (2)</td>
<td>0</td>
</tr>
<tr>
<td>Glasgow Southern General</td>
<td>+</td>
<td>10</td>
<td>60 (6)</td>
<td>10 (1)</td>
<td>30 (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>48</td>
<td>23 (11)</td>
<td>39.6 (19)</td>
<td>23 (11)</td>
<td>10.4 (5)</td>
<td>4.2 (2)</td>
</tr>
<tr>
<td>Total</td>
<td>+</td>
<td>16</td>
<td>56.3 (9)</td>
<td>18.8 (3)</td>
<td>25 (4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>70</td>
<td>21.4 (15)</td>
<td>40 (28)</td>
<td>24.2 (17)</td>
<td>11.4 (9)</td>
<td>2.9 (2)</td>
</tr>
</tbody>
</table>
3.12 Prevalence of Antimicrobial Resistance in *H. influenzae*

The antimicrobial susceptibilities of β-lactamase-positive and β-lactamase-negative *H. influenzae* isolates were determined for a number of β-lactam compounds consisting of ampicillin, amoxycillin:clavulanic acid (2:1), cefaclor, cefuroxime, ceftazidime, cefotaxime, and imipenem. Interpretative criteria for illustrating the results of the agar dilution tests have been adopted from the standard guidelines defined by the British Society of Antimicrobial Chemotherapy (Phillips *et al.*, 1991). Resistant and susceptible strains were distinguished by the use of *in vitro* break point antibiotic concentrations. Resistant *H. influenzae* strains were those organisms for which the MIC (mg/l) of an antibiotic was above the recommended break-point. Table 3-15, displays the break-points designated by BSAC guidelines for the β-lactam agents used in this study.

<table>
<thead>
<tr>
<th>β-Lactam Agents</th>
<th>Break-Point (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>1</td>
</tr>
<tr>
<td>Amoxycillin Clavulanate</td>
<td>1</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>8</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>4</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>2</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1</td>
</tr>
<tr>
<td>Imipenem</td>
<td>4</td>
</tr>
</tbody>
</table>

*Figures adopted from Phillips *et al.*, 1991*
The concentrations required for the indicated β-lactam agents to inhibit 50% (MIC$_{50}$ mg/l) and 90% (MIC$_{90}$ mg/l) of the isolates have been presented in Tables 3-16 to 3-20.

13.12.1 Minimum Inhibitory Concentrations (MICs) (mg/l) of the Strains Isolated from Aberdeen Royal Infirmary

Table 3-16 provides the results of the antimicrobial susceptibility tests performed on the strains isolated from Aberdeen Royal Infirmary. All 54.5% of the β-lactamase-mediated ampicillin resistant strains were inhibited by 0.5mg/l clavulanic acid. With the exception of ampicillin, β-lactamase producers were susceptible to the antimicrobial activity of all other agents tested. Intrinsic resistance to ampicillin was not detected in β-lactamase-negative strains isolated from this hospital. The MIC$_{50}$ (mg/l) and MIC$_{90}$ (mg/l) of imipenem for the β-lactamase-negative strains were found to be higher than those obtained for the β-lactamase producers (Table 3-16); however; only one strain (3.7%) required concentrations >4mg/l for inhibition and was resistant to this agent.
Table 3-16, β-Lactam Resistance in *H. influenzae* Isolated from Aberdeen Royal Infirmary

<table>
<thead>
<tr>
<th>β-lactam Agent</th>
<th>β-lactamase</th>
<th>MIC$_{50}$ (mg/l)</th>
<th>MIC$_{90}$ (mg/l)</th>
<th>%Resistant</th>
<th>LMIC$^1$</th>
<th>HMIC$^2$</th>
<th>Range (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>+</td>
<td>2</td>
<td>4</td>
<td>54.5 (6)</td>
<td>0.5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
<td>0.12</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Amoxyclavulanate</td>
<td>+</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
<td>0.25</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
<td>0.25</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cefaclor</td>
<td>+</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0.5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>+</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
<td>0.25</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.5</td>
<td>1</td>
<td>0</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>+</td>
<td>0.12</td>
<td>0.12</td>
<td>0</td>
<td>0.06</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.12</td>
<td>0.25</td>
<td>0</td>
<td>0.06</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>+</td>
<td>0.008</td>
<td>0.016</td>
<td>0</td>
<td>0.008</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.016</td>
<td>0.016</td>
<td>0</td>
<td>0.008</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td>+</td>
<td>0.5</td>
<td>1</td>
<td>0</td>
<td>0.25</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>1.9 (1)</td>
<td>0.12</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ Lowest MIC (mg/l)

$^2$ Highest MIC (mg/l)
3.12.2 Minimum Inhibitory Concentrations (MICs) (mg/l) of
the Strains Isolated from Edinburgh Royal
Infirmary

Table 3-17 shows the MICs (mg/l) of the strains isolated from Edinburgh Royal
Infirmary. Generally the MIC_{50}S (mg/l) and MIC_{90}S (mg/l) were higher than those of
Aberdeen Royal Infirmary. 44.4% of the β-lactamase positive strains were
ampicillin-resistant; however; 92% were inhibited by clavulanic acid (0.5mg/l). The
incidence of resistance to cefaclor and imipenem in β-lactamase positive group was
7.4% and 3.7% respectively. One β-lactamase producer strain had reduced
susceptibility to imipenem (MIC>4mg/l) and cefaclor (MIC>8mg/l), as well as
ampicillin (MIC>1mg/l) and amoxycillin clavulanate (MIC>1:0.5mg/l).

In the β-lactamase-negative group, 13% of the isolates showed intrinsic resistance to
ampicillin, and this ratio was reduced to 9.3% when clavulanic acid was combined
with amoxycillin. Resistance was determined as 11.1%, 5.6% and 3.7% for cefaclor
(MIC>8mg/l), cefuroxime (MIC>4mg/l) and ceftazidime (MIC>2mg/l) respectively.
The two strains with reduced sensitivity to ceftazidime were also resistant to other β-
lactams except for cefotaxime and imipenem.

The incidence of imipenem resistance in non-β-lactamase producing isolates was
3.7%, similar to that of β-lactamase-positive group. One imipenem resistant strain
was also resistant to ampicillin, amoxycillin clavulanate, cefaclor and cefuroxime. In
β-lactamase-negative group, the concentration required for cefaclor and cefuroxime
to inhibit 50% and 90% of the strains exceeded those of the β-lactamase-positive
group.
Table 3-17, β-Lactam Resistance in *H. influenzae* Isolated from Edinburgh Royal Infirmary

<table>
<thead>
<tr>
<th>β-lactam Agent</th>
<th>β-lactamase</th>
<th>MIC$_{50}$ (mg/l)</th>
<th>MIC$_{90}$ (mg/l)</th>
<th>%Resistant</th>
<th>LMIC$^1$</th>
<th>HMIC$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>+</td>
<td>0.5</td>
<td>16</td>
<td>44.4 (12)</td>
<td>0.12</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.5</td>
<td>2</td>
<td>13 (7)</td>
<td>0.12</td>
<td>16</td>
</tr>
<tr>
<td>Amoxyclavulanate</td>
<td>+</td>
<td>0.5</td>
<td>1</td>
<td>3.7 (1)</td>
<td>0.12</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.5</td>
<td>1</td>
<td>9.3 (5)</td>
<td>0.12</td>
<td>4</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>+</td>
<td>4</td>
<td>4</td>
<td>7.4 (2)</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>4</td>
<td>16</td>
<td>11.1 (6)</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>+</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0.25</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>4</td>
<td>5.6 (3)</td>
<td>0.5</td>
<td>32</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>+</td>
<td>0.25</td>
<td>0.25</td>
<td>0</td>
<td>0.06</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.25</td>
<td>0.25</td>
<td>3.7 (2)</td>
<td>0.03</td>
<td>4</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>+</td>
<td>0.03</td>
<td>0.06</td>
<td>0</td>
<td>0.008</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.03</td>
<td>0.06</td>
<td>0</td>
<td>0.008</td>
<td>0.12</td>
</tr>
<tr>
<td>Imipenem</td>
<td>+</td>
<td>1</td>
<td>4</td>
<td>3.7 (1)</td>
<td>0.25</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>4</td>
<td>3.7 (2)</td>
<td>0.12</td>
<td>8</td>
</tr>
</tbody>
</table>

$^1$ Lowest MIC (mg/l)

$^2$ Highest MIC (mg/l)

3.12.3 Minimum Inhibitory Concentrations (MICs) (mg/l) of the Strains Isolated from Glasgow Royal Infirmary

Table 3-18 presents the MIC$_{50}$s (mg/l) and MIC$_{90}$s (mg/l) of the strains collected from Glasgow Royal Infirmary. The 6 β-lactamase-positive strains isolated from this hospital were resistant to ampicillin. However, the incidence of ampicillin-resistance decreased to 16.7% when 0.5mg/l clavulanic acid was combined with amoxycillin.
(1mg/l). Other β-lactam agents effectively inhibited the isolates that produced a detectable β-lactamase enzyme.

Amongst the β-lactamase-negative strains, 5.4% showed an intrinsic resistance to ampicillin, and while 1.8% were inhibited by clavulanic acid (0.5mg/l), 3.6% remained sensitive. Apart from the two cefaclor resistant strains, all the isolates were susceptible to other agents tested.

Table 3-18, β-Lactam Resistance in H. influenzae Isolated from Glasgow Royal Infirmary

<table>
<thead>
<tr>
<th>β-lactam Agent</th>
<th>β-lactamase</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (mg/l)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (mg/l)</th>
<th>%Resistant</th>
<th>LMIC&lt;sup&gt;1&lt;/sup&gt;</th>
<th>HMIC&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Range (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>+</td>
<td>8</td>
<td>8</td>
<td>100 (6)</td>
<td>8</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.5</td>
<td>1</td>
<td>5.4 (3)</td>
<td>0.12</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Amoxyclavulanate</td>
<td>+</td>
<td>1</td>
<td>1</td>
<td>16.7 (1)</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.5</td>
<td>1</td>
<td>3.6 (2)</td>
<td>0.12</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Cefaclor</td>
<td>+</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>4</td>
<td>8</td>
<td>3.6 (2)</td>
<td>0.5</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>+</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0.25</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>+</td>
<td>0.25</td>
<td>0.25</td>
<td>0</td>
<td>0.12</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.25</td>
<td>0.25</td>
<td>0</td>
<td>0.03</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>+</td>
<td>0.016</td>
<td>0.06</td>
<td>0</td>
<td>0.016</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.016</td>
<td>0.06</td>
<td>0</td>
<td>0.008</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td>+</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0.12</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Lowest MIC (mg/l)

<sup>2</sup> Highest MIC (mg/l)
3.12.4 Minimum Inhibitory Concentrations (MICs) (mg/l) of the Strains Isolated from Glasgow Southern General Hospital

The MICs (mg/l) of the strains isolated from Glasgow Southern General Hospital are shown in Table 3-19. Both types of resistance to ampicillin were more common in this hospital than elsewhere; 33% of the β-lactamase-mediated ampicillin resistant isolates and only 2% of the strains with non-β-lactamase resistance to this agent were inhibited by 0.5mg/l clavulanic acid.

In contrast to the strains isolated from other hospitals, the incidence of cefaclor (20.7%) and cefuroxime (6.1%) resistance was higher for β-lactamase-positive than for the β-lactamase negative strains (14.6% and 2.7% respectively). Ceftazidime and cefotaxime readily inhibited all the strains and were the most effective β-lactams tested. To the antimicrobial action of imipenem, none of the β-lactamase-positive isolates and only 0.6% of the β-lactamase-negative strains showed reduced sensitivity.
Table 3-19, β-Lactam Resistance in *H. influenzae* Isolated from Glasgow Southern General Hospital

<table>
<thead>
<tr>
<th>β-lactam Agent</th>
<th>β-lactamase</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (mg/l)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (mg/l)</th>
<th>% Resistant</th>
<th>LMIC&lt;sup&gt;1&lt;/sup&gt;</th>
<th>HMIC&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>+</td>
<td>2</td>
<td>32</td>
<td>68.3 (56)</td>
<td>0.5</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>16.5 (54)</td>
<td>0.12</td>
<td>128</td>
</tr>
<tr>
<td>Amoxyclavulanate</td>
<td>+</td>
<td>1</td>
<td>2</td>
<td>33 (27)</td>
<td>0.25</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>14.6 (48)</td>
<td>0.25</td>
<td>4</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>+</td>
<td>4</td>
<td>16</td>
<td>20.7 (17)</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>4</td>
<td>16</td>
<td>14.6 (48)</td>
<td>0.25</td>
<td>32</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>+</td>
<td>1</td>
<td>2</td>
<td>6.1 (5)</td>
<td>0.25</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>2.7 (9)</td>
<td>0.25</td>
<td>16</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>+</td>
<td>0.12</td>
<td>0.25</td>
<td>0</td>
<td>0.06</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.12</td>
<td>0.25</td>
<td>0</td>
<td>0.06</td>
<td>1</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>+</td>
<td>0.03</td>
<td>0.06</td>
<td>0</td>
<td>0.008</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.03</td>
<td>0.06</td>
<td>0</td>
<td>0.008</td>
<td>0.12</td>
</tr>
<tr>
<td>Imipenem</td>
<td>+</td>
<td>1</td>
<td>2</td>
<td>0.6 (2)</td>
<td>0.25</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>0.6 (2)</td>
<td>0.25</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>1</sup> Lowest MIC (mg/l)  
<sup>2</sup> Highest MIC (mg/l)

### 13.12.5 Minimum Inhibitory Concentrations (MICs) (mg/l) of the Strains Isolated from Scotland

Table 3-20 summarises the MIC<sub>50s</sub> (mg/l) and MIC<sub>90s</sub> (mg/l) of the seven β-lactams tested against the 616 *H. influenzae* isolates collected in Scotland. Compared to the β-lactamase-negative group, the β-lactamase-positive group exhibited higher MIC (mg/l) values of ampicillin. This confirmed that β-lactamase production may result in ampicillin inactivation more efficiently. However, other β-lactam compounds did not
appear to be much affected; since the MICs for the β-lactamase producers and non-producers remained similar. Of the 126 β-lactamase-positive isolates, 63.5% (80) were resistant to ampicillin (MIC>1mg/l). Although clavulanic acid dramatically reduced the MIC₉₀ of amoxycillin (from 32mg/l to 2mg/l), 23% of the strains with such resistance still remained insensitive to this compound. Ceftazidime and cefotaxime with concentrations well below the susceptibility break points that are currently used to define clinical resistance (2mg/l for ceftazidime & 1mg/l for cefotaxime (Phillips et al., 1991) readily inhibited the β-lactamase-positive strains. The rate of resistance was 15% to cefaclor, 4% to cefuroxime and 0.8% to imipenem.

The prevalence of non-β-lactamase mediated strains with diminished susceptibility to ampicillin was 13.1%. This figure was reduced to 10.8% when clavulanic acid (1mg/l) was combined with amoxycillin (2mg/l) suggesting that clavulanic acid has the ability to bind to PBPs and inhibit cell wall synthesis reactions. The rates of resistance to cefaclor (11.4%) and cefuroxime (2.4%) were lower in this group than those detected for the β-lactamase-positive strains; however, the incidence of resistance to ceftazidime (0.4%) and imipenem (1%) were slightly higher. Of the β-lactams tested, cefotaxime was the most active agent against both β-lactamase producing and non-producing strains.

In summary, 23.4% (144) of the 616 strains were resistant to ampicillin, of which 55.6% produced a β-lactamase and 44.4% were characterised as intrinsically resistant. Therefore, in Scotland, 13% of the β-lactamase-positive strains and 10.4% of the non-β-lactamase producing isolates were ampicillin resistant; 26.3% of the ampicillin-resistant β-lactamase producers and 40.6% of the ampicillin-resistant non-β-lactamase producers were resistant to at least one other β-lactam compound in addition to ampicillin.
### Table 3-20, β-Lactam Resistance in *H. influenzae* Isolated in Scotland

<table>
<thead>
<tr>
<th>β-lactam Agent</th>
<th>β-lactamase</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>%Resistant</th>
<th>Range (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>+</td>
<td>2</td>
<td>32</td>
<td>63.5 (80)</td>
<td>0.12 128</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.5</td>
<td>2</td>
<td>13.1 (64)</td>
<td>0.12 128</td>
</tr>
<tr>
<td>Amoxyclavulanate</td>
<td>+</td>
<td>1</td>
<td>2</td>
<td>23 (29)</td>
<td>0.12 4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.5</td>
<td>2</td>
<td>10.8 (53)</td>
<td>0.12 4</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>+</td>
<td>4</td>
<td>16</td>
<td>15 (19)</td>
<td>0.5 32</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>4</td>
<td>16</td>
<td>11.4 (56)</td>
<td>0.25 32</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>+</td>
<td>1</td>
<td>2</td>
<td>4 (5)</td>
<td>0.25 16</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>2.4 (12)</td>
<td>0.25 32</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>+</td>
<td>0.12</td>
<td>0.25</td>
<td>0</td>
<td>0.06 0.5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.12</td>
<td>0.25</td>
<td>0.4 (2)</td>
<td>0.03 4</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>+</td>
<td>0.03</td>
<td>0.06</td>
<td>0</td>
<td>0.008 0.12</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.03</td>
<td>0.06</td>
<td>0</td>
<td>0.008 0.12</td>
</tr>
<tr>
<td>Imipenem</td>
<td>+</td>
<td>1</td>
<td>2</td>
<td>0.8 (1)</td>
<td>0.25 8</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>1 (5)</td>
<td>0.12 8</td>
</tr>
</tbody>
</table>

<sup>1</sup> Lowest MIC (mg/l)

<sup>2</sup> Highest MIC (mg/l)

### 3.12.6 β-Lactam Resistance & Biotype Classification

Distribution of β-lactam resistant strains among biotypes is displayed in Table 3-21. Biotypes I, II, and III contained 80% of the ampicillin resistant isolates; however, the distribution of sensitive strains appeared to follow a similar pattern.

The majority of the strains resistant to cefaclor (36%) (MIC>8mg/l) and ceftazidime (100%) (MIC>2mg/l) were of biotype III, whereas 41.2% of cefuroxime resistant strains (MIC>4mg/l) belonged to biotype I. Finally biotype II contained 50% of imipenem-resistant strains (MIC>4mg/l).
<table>
<thead>
<tr>
<th>(\beta)-lactam Agent</th>
<th>No. of Strains</th>
<th>Antibiotic Sensitivity</th>
<th>I (%) (No.)</th>
<th>II (%) (No.)</th>
<th>III (%) (No.)</th>
<th>IV (%) (No.)</th>
<th>V (%) (No.)</th>
<th>VI (%) (No.)</th>
<th>VII (%) (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>144</td>
<td>Resistant</td>
<td>25 (36)</td>
<td>29.2 (92)</td>
<td>25.7 (37)</td>
<td>14.6 (21)</td>
<td>4.2 (6)</td>
<td>0.7 (1)</td>
<td>0.7 (1)</td>
</tr>
<tr>
<td></td>
<td>472</td>
<td>Sensitive</td>
<td>24.8 (117)</td>
<td>30.1 (142)</td>
<td>25 (118)</td>
<td>15.7 (74)</td>
<td>2.1 (10)</td>
<td>1.7 (8)</td>
<td>0.6 (3)</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>75</td>
<td>Resistant</td>
<td>22.7 (17)</td>
<td>14.7 (11)</td>
<td>36 (27)</td>
<td>20 (15)</td>
<td>5.3 (4)</td>
<td>1.3 (1)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>541</td>
<td>Sensitive</td>
<td>25.1 (136)</td>
<td>32 (173)</td>
<td>23.7 (128)</td>
<td>14.8 (80)</td>
<td>2.2 (12)</td>
<td>1.5 (8)</td>
<td>0.7 (4)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>17</td>
<td>Resistant</td>
<td>41.2 (7)</td>
<td>5.9 (1)</td>
<td>29.4 (5)</td>
<td>17.6 (3)</td>
<td>0</td>
<td>5.9 (1)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>599</td>
<td>Sensitive</td>
<td>24.4 (146)</td>
<td>30.6 (183)</td>
<td>25 (150)</td>
<td>15.4 (92)</td>
<td>2.7 (16)</td>
<td>1.3 (8)</td>
<td>0.7 (4)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>2</td>
<td>Resistant</td>
<td>0</td>
<td>0</td>
<td>100 (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>614</td>
<td>Sensitive</td>
<td>24.9 (153)</td>
<td>30 (184)</td>
<td>24.9 (153)</td>
<td>15.5 (95)</td>
<td>2.6 (16)</td>
<td>1.5 (9)</td>
<td>0.7 (4)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>6</td>
<td>Resistant</td>
<td>0</td>
<td>50 (3)</td>
<td>16.7 (1)</td>
<td>33.3 (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>610</td>
<td>Sensitive</td>
<td>25.1 (153)</td>
<td>29.7 (181)</td>
<td>25.2 (154)</td>
<td>15.7 (93)</td>
<td>2.6 (16)</td>
<td>1.5 (9)</td>
<td>0.7 (4)</td>
</tr>
</tbody>
</table>
3.12.7 The Frequency of β-Lactam Resistance Among the Isolates of a Specific Biotype

The prevalence of antibiotic resistance for each biotype is shown in Table 3-22. The highest proportion of β-lactam resistance was observed among the isolates that were of biotype III. Similar to the highest rate of β-lactamase production, the prevalence of resistance to ampicillin (37.5%) and cefaclor (25%) was also detected most frequently among biotype V isolates. All strains of biotype VII were sensitive to cephalosporins and imipenem.

Table 3-22, The Frequency of Resistant Strains in each Biotype

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Ampicillin</th>
<th>Cefaclor</th>
<th>Cefuroxime</th>
<th>Ceftazidime</th>
<th>Imipenem</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>23.5 (36)</td>
<td>11.1 (17)</td>
<td>4.6 (7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>22.8 (42)</td>
<td>6 (11)</td>
<td>0.5 (1)</td>
<td>0</td>
<td>1.6 (3)</td>
</tr>
<tr>
<td>III</td>
<td>23.9 (37)</td>
<td>17.4 (27)</td>
<td>3.2 (5)</td>
<td>1.3 (2)</td>
<td>0.7 (1)</td>
</tr>
<tr>
<td>IV</td>
<td>22.1 (21)</td>
<td>15.8 (15)</td>
<td>3.2 (3)</td>
<td>0</td>
<td>2.1 (2)</td>
</tr>
<tr>
<td>V</td>
<td>37.5 (6)</td>
<td>25 (4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VI</td>
<td>11.1 (1)</td>
<td>11.1 (1)</td>
<td>11.1 (1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VII</td>
<td>25 (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
3.12.8 Relationship Between β-Lactam Resistance, Capsulation & Biotypes

Table 3-23, displays the relationship between the capsulated β-lactam resistant strains and their biochemical properties.

Twenty-four (16.7%) of the 144 ampicillin resistant strains were capsulated. 54.2% of which were resistant to ampicillin produced a β-lactamase and were most commonly of biotypes I (38.5%) or III (38.5%), whereas non-β-lactamase mediated ampicillin-resistant capsulated strains were largely of biotype III (36.4%).

Similar results were obtained with capsulated strains that were resistant to cefaclor. 18.7% of the total of 75 cefaclor resistant strains were capsulated. Although less strains produced a β-lactamase, the prevalence of resistance was still higher for biotypes I and III each containing 40% of the isolates. In β-lactamase-negative group, biotype III comprised the majority of the resistant strains with 55.6%.

One of the capsulated β-lactamase-negative strains (14.3%) was resistant to cefuroxime. The resistant strain was characterised as biotype I.

All capsulated strains were sensitive to ceftazidime as well as cefotaxime. Resistance to imipenem was only observed among capsulated strains and most commonly among isolates of biotype II.

There were no β-lactam resistant capsulated strains that were categorised as biotypes V-VII.
Table 3-23: The Prevalence of Encapsulated Resistant Strains in each Biotype

<table>
<thead>
<tr>
<th>β-Lactam Resistance</th>
<th>No. of Strains</th>
<th>β-Lactamase</th>
<th>% (No.) of Strains in Each Biotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>38.5 (5)</td>
<td>9.1 (1)</td>
<td>22.2 (2)</td>
</tr>
<tr>
<td></td>
<td>23 (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>27.3 (3)</td>
<td>100 (1)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>40 (2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Imipenem</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20 (1)</td>
<td>40 (2)</td>
</tr>
</tbody>
</table>
3.13 Characterisation of β-Lactamases in *H. influenzae*

To identify the β-lactamases in *H. influenzae*, sonicated extracts of the 126 β-lactamase producing isolates were examined by analytical isoelectric focusing (IEF) technique. The cell-free extracts were applied to polyacrylamide gels containing a broad-range ampholine (pH 3.5-10) and were run at 500V, 20mA, 1W overnight. β-lactamases were visualised after overlaying sheets of filter paper soaked in 1mM nitrocephin directly on the gels. Isoelectric points (pI) of the enzymes was determined and compared with preparations of β-lactamases of known pI.

It was demonstrated that 122 (96.8%) strains produced a TEM-1 type β-lactamase that was focused as a single band at pI 5.4, and 2 (1.6%) isolates contained a β-lactamase with a pI 8.1, corresponding to that of ROB-1. The presence of TEM-2 and other extended-spectrum β-lactamases derived from TEM-1 or TEM-2 was ruled out by this technique. However, the focusing patterns of the remaining two (1.6%) β-lactamases differed from those of TEM-1 and ROB-1; the only two β-lactamase enzymes commonly found in this species.

The β-lactamase activity of strain LG629, a non-capsulated biotype III isolate, was revealed as a single band with a pI of 7.9 (Figure 3-1). This novel β-lactamase enzyme was designated VAT-1.
Figure 3-1, IEF Pattern of the β-lactamase Isolated from Strain LG629 (VAT-1) on a 10% polyacrylamide gel containing broad-range ampholines (pH 3.5-10).

Lane 1, ROB-1 (pI 8.1); Lane 2, VAT-1 (pI 7.9), (a novel β-lactamase in H. influenzae); Lane 3, TEM-1 (pI 5.4).
Strain LG646, also a non-encapsulated biotype III isolate, produced VAT-1 and a β-lactamase with pI 5.5 (Figure 3-2).

Figure 3-2, IEF Patterns of the β-lactamases Isolated from Strain LG646 on a 10% polyacrylamide gel containing broad-range ampholines (pH 3.5-10).

Lane 1, TEM-1 (pI 5.4); Lane 2, TEM-2 (pI 5.6); Lanes 3 & 5, β-lactamases isolated from strain LG646; Lane 4, VAT-1 (isolated from strain LG629).
Initial preparations of the β-lactamases isolated from strain LG646 produced 3 bands that focused at pIs of 7.9, 6.3, and 5.5. However, the β-lactamase activity of the band with the pI of 5.5 became faint in extracts obtained after the strain was stored at -70°C (Figure 3-3).

Figure 3-3, IEF of crude enzyme preparations from strain LG646 demonstrating the loss of β-lactamase activity of the band with the pI 5.5, after the strain was stored at -70°C.

Lanes 1, 5, & 6, TEM-1; Lane 2, initial β-lactamase obtained before storage in -70°C; Lane 3, after 3 weeks of storage; Lane 4, after 2 months storage; Lane 7, after 6 months storage; Lane 8, after 1 year storage.
IEF patterns of the two bands at pI 6.3 and 7.9 did not correspond to any of the $\beta$-lactamases tested (Figures 3-4, 3-5 & 3-6).

Figure 3-4, IEF gel demonstrating that $\beta$-lactamases isolated from strains LG629 & LG646 were different from BRO-I & BRO-II extracted from *Moraxella catarhalis* and ROB-1.

Lane 1, VAT-1; Lane 2, BRO-I; Lane 3, BRO-II; Lane 4, $\beta$-lactamases from strain LG646; Lane 5, ROB-1; Lanes 6 & 7, TEM-1.
Figure 3-5, IEF gel demonstrating that the isoelectric points of the novel β-lactamases do not correspond to any of the β-lactamases tested.

Lanes 1, 7, & 9, TEM-1 (pI 5.4); Lane 2, OHIO-1 (pI 7.0); Lane 3, OXA-4 (pI 7.5); Lane 4, OXA-1 (pI 7.4); Lane 5, TLE-1 (pI 5.5); Lane 6, SHV-1 (pI 7.6); Lane 8, β-lactamases from strain LG646.
Figure 3-6, IEF gel demonstrating that the isoelectric points of the novel β-lactamases do not correspond to any of the extended-spectrum β-lactamases tested.

Lane 1, TEM-1 (pI 5.4); Lane 2, TEM-E3 (pI 5.5); Lane 3, TEM-E4 (pI 5.6); Lane 4, TEM-2 (pI 5.6); Lanes 5 & 6, β-lactamases from strain LG646; Lane 7, TEM-4 (pI 5.9); Lane 8, TEM-6 (pI 5.9); Lanes 9 & 10, β-lactamases from strain LG646; Lane 11, TEM-8 (pI 6); Lane 12, SHV-3 (pI 7.0).
3.14 Molecular Mass Determination of VAT-1

3.14.1 Determination of Molecular Mass by Gel Filtration

The crude extract of LG629 was applied to a calibrated Sephadex G-75 column. The apparent molecular size of the β-lactamase of pI 7.9 (VAT-1) was estimated to be 35.5 kDa when compared to the standard proteins.

3.14.2 Determination of Molecular Mass by SDS-PAGE

SDS-PAGE analysis of the partially purified extracts of VAT-1 was performed as described in Section 2.6.8.ii. According to this method the molecular mass of VAT-1 was estimated to be 38 kDa.

3.15 Biochemical Properties of VAT-1

3.15.1 Substrate Profile & Kinetic Parameters of VAT-1

Hydrolytic activity of partially purified VAT-1 was assayed on a Perkin-Elmer Lambda 2 spectrophotometer. VAT-1 showed a greater ability to hydrolyse cephalosporins than penicillins. This enzyme hydrolysed nitrocephin, cephalaxin, cephradine, cephalothin, and cefamandole. However; no detectable hydrolytic activity was observed for cefuroxime and third generation cephalosporins. Penicillins were poor substrates for VAT-1 and only benzyl penicillin was hydrolysed but only at a slow rate. The kinetic parameters (Km & Vmax) and hydrolytic efficiency (Vmax/Km) of VAT-1 were determined by the Lineweaver-Burk method (Table 3-24).
Hydrolytic studies revealed that VAT-1 β-lactamase exhibited a substrate profile similar to those of other cephalosporinase enzymes. Other β-lactam compounds were hydrolysed at a low rate, therefore, Vmax and Km values were not measurable. From the data on the Vmax values, it was revealed that cefaclor was hydrolysed in a higher rate than nitrocephin or cephallexin, although VAT-1 had a greater affinity (lower Km) for nitrocephin. The hydrolytic efficiency (Vmax/Km) suggested that under physiological conditions cefaclor may be hydrolysed more efficiently than cephallexin. Since nitrocephin was hydrolysed with more efficiency than other substrates, it was chosen as the test substrate for inhibition studies.

### 3.15.2 Inhibition Analysis of VAT-1

Table 3-25 shows the effect of several inhibitors on the activity of VAT-1. The concentration of inhibitor that results in a 50% reduction of β-lactamase activity (ID50) was determined for each inhibitor. The solution of the enzyme was preincubated with different concentrations of inhibitor at 37°C for 10 minutes prior to
the addition of nitrocephin at a final concentration of $10^{-5}$M. The prototype TEM-1 from *E. coli* J62-2 and ROB-1 from *E. coli* DH1 (pRM 3022) were used as the reference β-lactamases.

In contrast to TEM-1 and ROB-1 that showed sensitivity to all serine β-lactamase inhibitors, VAT-1 was resistant to the inhibitory effect of clavulanic acid, tazobactam and to a lesser extend to sulbactam. However; BRL 42715 readily inhibited the β-lactamase activity of VAT-1 (ID$_{50}$ $4 \times 10^{-10}$M). Neither the addition of EDTA nor the presence of Zn$^{2+}$ in the reaction buffer resulted in the loss of the β-lactamase property of VAT-1. The absence of a cysteine in the active site of VAT-1 was confirmed by the insensitivity to *para*-Chloromercuribenzoate (pCMB).

On the basis of inhibition studies, VAT-1 had similar β-lactamase characteristics to those enzymes categorised as class C of Ambler’s classification scheme (Ambler, 1980).
Table 3-25, Inhibitory dose (M) of β-lactamase inhibitors that caused 50% reduction in β-lactamase activity (ID$_{50}$) of VAT-1, TEM-1 and ROB-1.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>β-lactamases</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VAT-1</td>
<td>TEM-1$^a$</td>
<td>ROB-1$^b$</td>
</tr>
<tr>
<td>Clavulanic Acid</td>
<td>1.2 x 10$^{-3}$</td>
<td>5 x 10$^{-8}$</td>
<td>2 x 10$^{-8}$</td>
</tr>
<tr>
<td>Tazobactam</td>
<td>1.6 x 10$^{-3}$</td>
<td>1 x 10$^{-7}$</td>
<td>7 x 10$^{-8}$</td>
</tr>
<tr>
<td>Sulbactam</td>
<td>4.5 x 10$^{-5}$</td>
<td>5.2 x 10$^{-6}$</td>
<td>1.2 x 10$^{-6}$</td>
</tr>
<tr>
<td>BRL 42715</td>
<td>4 x 10$^{-10}$</td>
<td>6 x 10$^{-10}$</td>
<td>-</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 x 10$^{-4}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>7 x 10$^{-5}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pCMB</td>
<td>&gt;10$^{-2}$</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ from *E. coli* J62-2

$^b$ from *E. coli* DH1 (pRM3022)
3.16 Induction Studies

An overnight culture of LG629, producing VAT-1 β-lactamase was induced with 0.06 mg/l (1/4 MIC) imipenem. Although according to nitrocephin spot testing, there were no detectable differences in the β-lactamase activity before and after induction; different bands were observed when sonicated extracts were subjected to analytical IEF. In preparations obtained from the induced strains, the major band (pl 7.9) was not detectable; however, in addition to the satellite band (pl 7.6) much of the β-lactamase activity was focused at pl 7 (Figure 3-7).

The induced strain behaved in all regards like that of the un-induced isolate, therefore, different bands probably reflected altered forms of the same enzyme.

Strain LG646 was also subjected to induction with imipenem. Similar to LG629, the induced enzyme exhibited activity at pl 7.6 and pl 7 (Figure 3-7, lane 4). The enzyme with the pl 5.5 that was originally present in this strain, was not detectable after induction.

Induction assays suggested that other commonly used β-lactamase inducers were not as potent as imipenem for inducing VAT-1.
Figure 3-7, IEF gel of VAT-1 β-lactamase prepared from the induced and un-induced cultures of LG629.

Lane 1, TEM-1; Lane 2, VAT-1 prepared from an un-induced culture of LG629; Lane 3, induced form of VAT-1 extracted from LG629; Lane 4, enzyme preparation from induced LG646; Lane 5, TEM-1.
3.17 Identification of Genetic Origin of VAT-1

3.17.1 Elimination of β-Lactamase Production by Ethidium Bromide

To determine the genetic origin of the VAT-1 β-lactamase, curing was performed with ethidium bromide and *H. influenzae* strain LG629. The highest concentration of ethidium bromide (2mg/l) that allowed visible growth was selected and sonicated extracts containing β-lactamase activity were prepared. In spite of the presence of ethidium bromide, VAT-1 was detected with nitrocephin spot testing. Therefore, it appeared that VAT-1 was a chromosomally-encoded cephalosporinase. However; the failure to cure a trait does not necessarily indicate that the phenotype is not plasmid-mediated, since treatment with intercalating dyes may not eliminate the extra chromosomal elements that have integrated into the host chromosome.

3.17.2 Plasmid Screening

To identify the presence of a possible plasmid associated with VAT-1 β-lactamase, several methods were employed for isolation of plasmid DNA (Meyers et al., 1976; Hansen & Olsen, 1978; Takahashi & Nagano, 1984). There were no identifiable plasmids encoding the VAT-1 β-lactamase in strains producing the enzyme. Therefore, VAT-1 was characterised as a chromosomally-mediated cephalosporinase. Strain LG646 harbouring both VAT-1 and the β-lactamase with the pI 5.5 was also plasmid-free. In contrast, clinical *H. influenzae* strains producing ROB-1 contained a stable 4.4 kb plasmid (Figure 3-8).
Figure 3-8, 0.8% agarose gel electrophoresis of the 4.4kb plasmid encoding ROB-1 in *H. influenzae*.

Lane 1, λ digested with *Hind* III endonuclease enzyme, markers correspond to 23.7, 9.5, 6.7, 4.3, 2.3 and 2 kb; Lanes 2 & 3, 4.4kb plasmid encoding ROB-1 β-lactamase isolated from a clinical *H. influenzae* strain (A87). Plasmid DNA has been prepared according to the method proposed by Meyers *et al.* 1976.
3.18 Identification of TEM- or ROB-Derived β-Lactamases

3.18.1 Hybridisation of Whole Cell DNA with Fluorescein-Labelled Plasmid Probes

Plasmid DNA encoding the two different β-lactamases; TEM-1 and ROB-1, were used as molecular probes to classify the enzymes produced by *H. influenzae* strains LG629 and LG646.

The results of autoradiographs of whole cell DNA isolated from strains LG629 and LG646 with fluorescein labelled pROB (isolated from the clinical *H. influenzae* strain A87) and pUC18 DNA are shown in Figures 3-9 & 3-10. Under stringent conditions, isolate LG629 (VAT-1 producer) did not hybridise with any of the probes (pROB or pUC18). However, strain LG646 hybridised intensively with the pUC18 probe and exhibited minimal hybridisation with pROB. Therefore, it may be concluded that VAT-1 was neither TEM- nor ROB-related β-lactamase enzyme. On the other hand strain LG646 contained a TEM-derived β-lactamase that was focused at pI 5.5.
Figure 3-9. Hybridisation of whole cell DNA with fluorescein-labelled pUC18

Samples designated as A, pUC18; B, *H. influenzae* A87 (ROB-1 producer); C, *H. influenzae* LG646; D, TEM-1 producing clinical *H. influenzae* strain LG920; E, *H. influenzae* LG629 (VAT-1 producer) were loaded onto nylon membrane.
Figure 3-10, Hybridisation of whole cell DNA with fluorescein-labelled pROB

Samples designated as A, pROB; B, TEM-1 producing *H. influenzae* LG920; C, *H. influenzae* LG646; D, *E.coli* J62-2; E, *H. influenzae* LG629 (VAT-1 producer) were loaded onto nylon membrane.
3.18.2 Identification of TEM-Derived β-Lactamases by PCR

*H. influenzae* strains, LG629 and LG646, were examined with differential Polymerase Chain Reaction (PCR) technology for the presence of a TEM-derived β-lactamase. TEM-specific primers, Bla 3' and Bla 4' were chosen to amplify a sequence of 860 bp in length. A β-lactamase-negative isolate and a TEM-1 producing strain were included as controls. Target genomic DNA was obtained and PCR was performed under standard conditions (Section 2.6.22.i). PCR-amplified DNA were verified by electrophoresis on 1.2% agarose gels and the fragments were visualised with ethidium bromide (50μg/l) staining.

Strain LG646 generated a PCR fragment of approximately 900 bp in size, therefore it was concluded that the β-lactamase that focused at πL 5.5 was a TEM-derived enzyme. However, the genomic DNA extracted from the VAT-1 producer, strain LG629, did not produce this fragment, strongly suggesting that VAT-1 was not TEM-related (Figure 3-11).
Figure 3-11, 1.2% Agarose gel electrophoresis of PCR amplified fragments

Lane 1, *Hind* III digest of λ (23.7, 9.5, 6.7, 4.3, 2.3 & 2 kb); Lane 2, *E. coli* J62-2 containing pUC18; Lanes 3 & 4, *H. influenzae* LG920 containing TEM-1; Lane 5, *H. influenzae* LG629 containing VAT-1; Lanes 6 & 7, *H. influenzae* LG646 containing a TEM-related β-lactamase.
3.19 Antimicrobial Susceptibility of VAT-1 Producing Strains

Antimicrobial susceptibility tests were performed on the strains producing VAT-1 β-lactamase (LG629 & LG646). The MICs (mg/l) were determined for a variety of β-lactam compounds by the agar dilution method. Table 3-26 shows the results of the susceptibility tests.

Table 3-26, MICs (mg/l) of Various β-Lactam Compounds for Isolates Producing VAT-1.

<table>
<thead>
<tr>
<th>β-lactam</th>
<th>LG629</th>
<th>LG646</th>
<th>Break Point (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0.5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Amoxycillin Clavulanate</td>
<td>0.5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>8</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>2</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>2</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Cephradine</td>
<td>16</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>2</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>2</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>0.5</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0.25</td>
<td>0.25</td>
<td>2</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.03</td>
<td>0.03</td>
<td>1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.06</td>
<td>0.06</td>
<td>1</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.25</td>
<td>0.25</td>
<td>4</td>
</tr>
</tbody>
</table>

The MICs for the VAT-1 producing strains were below the recommended break point values for almost all β-lactams tested except for cephalexin, cephradine, ampicillin...
and amoxycillin clavulanate. Low production of VAT-1 and the TEM-derived β-lactamase may be associated with the relatively low MICs obtained.

3.20 Cloning of the DNA Fragment Encoding VAT-1 β-Lactamase

The recipient plasmid vector, pSU18 was prepared by alkaline lysis method (Takahashi & Nagano, 1984) and was restricted with BamHI endonuclease enzyme. The product was dephosphorylated with alkaline phosphatase to prevent end-ligation. Genomic DNA from the VAT-1 producer isolate, strain LG629, was isolated and partially digested with Sau3AI. The produced fragments were ligated to the BamHI site of pSU18 contained within the lacZ α reporter gene of the vector. pSU18 was a derivative of pUC plasmids in which the bla gene was replaced with a chloramphenicol resistance determinant cassette (Bartolomé et al., 1991). Ligation mixtures were transformed into competent cells of E. coli TG2. Transformants resistant to 25mg/l chloramphenicol that produced a white colony on the surface of the LB agar plates containing X-gal and IPTG were isolated and screened for β-lactamase production. The β-lactamases were analysed spectrophotometrically to identify cefaclor and cephalaxin hydrolysis. As none of the transformants were able to hydrolyse either of the mentioned cephalosporins, the attempt to clone the DNA fragment responsible for the expression of VAT-1 was not successful. The sensitivity of the VAT-1 producing strain prevented applying a selective antibiotic screening strategy.

3.21 DNA Sequencing of the TEM-1 Type β-Lactamase Gene

It has been speculated that the transposon A carrying the genetic elements encoding TEM-1 has entered H. influenzae cells by translocation from gram-negative bacilli donor (De Graaff et al., 1976, Brunton et al., 1986). It is possible that in time the TEM-1 molecule has undergone alterations. It was one of the intentions of this study
to identify the β-lactamases produced by this organism. For this reason the gene encoding the TEM-related β-lactamase was partially sequenced with Dynabeads M-280 Streptavidin (Dynal, Norway) immobilisation technique. Prior to sequencing, total genomic DNA of *H. influenzae* strain LG646 was amplified by the PCR reaction with a biotin-labelled TEM-primer (Biotin Bla-4') and a non-biotinylated oligonucleotide (Bla 3'). The double-stranded DNA fragment generated was mixed with Dynabeads to yield a single-stranded DNA template. Polyacrylamide gel (8%T, 5%C acrylamide/bisacrylamide, 7M Urea) electrophoresis was employed to separate the sequencing reaction products.

A continuous nucleotide sequence of 320 bp was read. The result of the comparison of the partial DNA sequence of the gene producing the TEM-derived β-lactamase (pI 5.5) with TEM *bla* gene (isolated from *E.coli* J62-2), showed that both sequences contained homologous amino acids with two exceptions. At position 81, a highly unusual change from Arginine to Valine was caused by modification of CGT to GTA and another alteration from Histidine to Serine at position 94 was caused by a significant change from CAC to TCC (Figure 3-12).
Figure 3-12. Nucleotide sequences of amplified DNA fragments from *E. coli* J62-2 and *H. influenzae* strain LG646 and their deduced amino acid sequences. Changes have been shown in bold and are underlined.

*E. coli* J62-2 1 ATG AGT ATT CAA CAT TTC CGT GTC GCC CTT ATT CCC TTT TTT GCG GCA TTT TGC CTT CTT GTT

*H. influenzae* 1 ATG AGT ATT CAA CAT TTC CGT GTC GCC CTT ATT CCC TTT TTT GCG GCA TTT TGC CTT CTT GTT

*E. coli* J62-2 64 TTT GCT CAC CCA GAA ACG CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG GGT GCA CGA

*H. influenzae* 64 TTT GCT CAC CCA GAA ACG CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG GGT GCA CGA

*E. coli* J62-2 124 GTG GGT TAC ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT GAG AGT TTT CGC CCC GAA

*H. influenzae* 124 GTG GGT TAC ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT GAG AGT TTT CGC CCC GAA

*E. coli* J62-2 184 GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT CTG CTA TGT GGCG CGC GTA TTA TCC CGT

*H. influenzae* 184 GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT CTG CTA TGT GGCG CGC GTA TTA TCC **GTA**

*E. coli* J62-2 244 GTT GAC GCC GGG CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT TCT CAG AAT GAC TTG GTT

*H. influenzae* 244 GTT GAC GCC GGG CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT TCT CAG AAT GAC TTG GTT

*E. coli* J62-2 304 GAG TAC TCA CCA GTC ACA

*H. influenzae* 304 GAG TAC TCA CCA GTC ACA

*E. coli* J62-2 1 MSIQHFRVALIPFPAFCLPVFAHPETLKVKDADQQLGARVGYIELDNSGKILESRPRPEERFPMMSTFK

*H. influenzae* 1 MSIQHFRVALIPFPAFCLPVFAHPETLKVKDADQQLGARVGYIELDNSGKILESRPRPEERFPMMSTFK

*E. coli* J62-2 72 VLLCGAVLSRVDAGQELQGRIRHYSQNDLVEYSPVT

*H. influenzae* 72 VLLCGAVLS**Y**DAGQELQGRIR**S**YSQNDLVEYSPVT
H. influenzae is an important respiratory pathogen in both children (Robbins et al., 1973) and adults (Musher et al., 1983). β-lactams are among the drugs of choice for treating the infections caused by this organism. However, the emergence of β-lactam resistant strains has influenced the therapeutic efficacy of these compounds. To minimise treatment failures, the antimicrobial activity of β-lactams should be monitored continuously.

Over a three-year study period (1992-1995), 616 strains were isolated concurrently from four hospitals in Scotland. Specimens were isolated from a broad range of ages to evaluate certain aspects of the relationship between biotypes and non-invasive infections, occurrence of β-lactamase production and β-lactam resistance in the local population in Scotland. Therefore, no predetermined criteria were used in selecting the isolates. The proportion of the strains contributed from the four collaborative hospitals were as follows; 66.6% from Glasgow Southern General hospital, 13.1% from Edinburgh Royal Infirmary, 10.2% from Aberdeen Royal Infirmary and 10.1% from Glasgow Royal Infirmary. Isolates were categorised by study hospital, patient age or specimen source. The clinical site of isolation was indicated for 77.8% of the strains, of which 99.4% were recovered from the respiratory tract and only 0.6% (3) were originated from the eye. The age of the infected patients was provided for 64% of the isolates. Most (72.8%) were recovered from adults older than 50 years of age, 22.7% of the hosts were between 25-50 years old, 2% of the specimens were isolated from 16-24 year olds and 2.6% were recovered from paediatrics (0-15 years of age).
In the four designated age groups, the most frequent source from which *H. influenzae* was recovered was sputum.

### 4.1 Biochemical Distribution of the Isolates

In 1976, Kilian developed a system to biochemically differentiate eight biotypes of *H. influenzae* (Kilian, 1991). This scheme has proved to be a potential tool for epidemiological studies of *H. influenzae* strains in correlation with different parameters (Long *et al.*, 1983; Granato *et al.*, 1983; Wallace *et al.*, 1981; Harper & Tilse, 1991). In 14-35% of healthy children, *H. influenzae* is a part of the normal commensal flora of the upper respiratory mucous membranes (Long *et al.*, 1983). To differentiate pathogens from saprophytes Long *et al.*, (1983) have suggested that regardless of encapsulation, there is an association between biotype I and virulence. However, the basis for this finding is unclear. In this study 12.5% of the isolates recovered from patients less than 15 years of age were of biotype I. As the number of the patients in the paediatric age group (0-15) was not sufficient for statistical analysis, no conclusion could be drawn as to whether the cause of the localised infections in children was solely dependent on the presence of biotype I. Nevertheless, 35.7% of the strains isolated from children belonged to biotype III, 25% were of biotype II and other biotypes contained 25% of the strains. Therefore, to demonstrate whether the non-biotype I strains were possibly non-pathogenic and of saprophyte origin, the study was to be extended and compared with the biochemical properties of the specimens recovered from patients after the infection was cleared.

Biotype III was also more prevalent in the age group of 16-24 year olds. However, sputum specimens recovered from other age groups (25-50 & >50 year olds) were mostly of biotype II (*p*=0.451). The majority of nasopharyngeal and tracheal aspirate cultures in all age groups consisted of biotype I.

Distribution of the isolates of each biotype was determined for the hospitals of isolation. In Aberdeen Royal Infirmary the majority of the strains were of biotype II
(30.2%) and biotypes III, I and IV accounted for 28.6%, 17.5% and 15.9% of the isolates respectively. Strains of biotypes V, VI and VII were isolated in comparatively small numbers making up 7.9% of the isolates. In Edinburgh Royal Infirmary, biotype II strains were the cause of most non-invasive infections with 45.7%, followed by biotypes I (26%), III (19.8%), and IV (8.6%). However, no strains of other biotypes were detected. In Glasgow Royal Infirmary, biotype I was isolated most frequently from infected patients (37%) which was followed by biotype IV (24.2%). The number of the strains that were categorised as biotypes II and III were lower than those found in other hospitals accounting for 17.7% and 16.1% respectively. Biotypes V and VI contained only 4.8% of the isolates. Strains of biotype VII were not detected in this hospital. In Glasgow Southern General, similar to Aberdeen Royal Infirmary, the majority of the strains were of biotype II (28.5%) followed by biotypes III (27.1%), I (23.9%) and IV (15.4%). The highest prevalence of biotype I (37.5%) was detected in Glasgow Royal Infirmary and the highest percentage of strains with biotype II characteristics was found in Edinburgh Royal Infirmary.

These results suggested that the distribution of biotypes recovered from clinical specimens may not only vary world-wide (Granato et al., 1983) but also may vary in different hospitals within a restricted geographical area ($p=0.0042$).

The prevalence of each biotype in Scotland has been compared to the data reported from other countries. In total, the prevalence of *H. influenzae* isolates of each biotype in Scotland suggested that although less common than elsewhere, the majority of non-invasive infections were caused by biotype II isolates (29.9%) which was in agreement with the studies conducted in Canada (57.6%) (Righter & Luchsinger, 1988), United States (46.8%) (Oberhofer & Back, 1979), and Australia (48%) (Harper & Tilse, 1991). Subsequently, the recovery of biotype III isolates was prevalent, accounting for 25.2% of the strains. This finding was also consistent with the results obtained from the United States (27.6%) (Oberhofer & Back, 1979), Canada (26.5%) (Righter & Luchsinger, 1988), and Australia (26%) (Harper & Tilse,
The incidence of infections caused by biotype I (24.8%) was similar to biotype III and higher than elsewhere (21.3% in United States, 9.4% in Canada, and 16% in Australia). Biotype IV contained 15.4% of the 616 clinical strains. This ratio was considerably lower in North America (0.86% in United States and 1.2% in Canada) and Australia (1.9%) (Righter & Luchsinger, 1988; Oberhofer & Back, 1979; Harper & Tilse, 1991). However the proportion of biotype V strains (2.6%) in Scotland was the same as in Canada (2.4%) (Righter & Luchsinger, 1988), and United States (1.7%) (Oberhofer & Back, 1979), and lower than that in Australia (6.1%) (Harper & Tilse, 1991). The high incidence of biotype V in Australia may be explained by the fact that certain biotypes may become endemic to a particular geographical area for a period of time (Granato et al., 1983). Biotype VI was as common in Scotland (1.5%) as was in the United States (1.7%) (Oberhofer & Back, 1979), and Australia (1.3%) (Harper & Tilse, 1991). Finally biotype VII strains were found more frequently (0.6%) in this country than elsewhere. There were no strains of biotype VIII detected throughout this study.

These results confirmed that unlike invasive infections where biotype I and II contain the majority of the pathogenic strains (Oberhofer & Back, 1979; Albritton et al., 1978), there was no correlation between biotypes of *H. influenzae* and non-invasive infections (Harper & Tilse, 1991). Although it was shown that higher rates of respiratory infections occurred with biotype II isolates, strains of other biotypes were also found to be equally capable of causing non-invasive infections especially among the elderly.

The presence of a polysaccharide capsule appears to be one of the most important virulence determinants in *H. influenzae* (Moxon & Vaughn, 1981; Granato et al., 1983). The ratio of capsulated strains in this study varied from 6.3% in Aberdeen Royal Infirmary to 21% in Edinburgh Royal Infirmary \((p=0.080)\). Overall 14% of the strains isolated from patients with localised infections were capsulated whereas nontypeable strains constituted 86% of the isolates. This ratio was in agreement with the previous studies conducted in Canada (13.5%) (Righter & Luchsinger, 1988) and
higher than the results reported from Australia (7%) (Harper & Tilse, 1991). The majority of typeable (31%) and non-typeable (28.9%) strains isolated from patients with non-invasive infections were of biotype II, whereas in invasive infections biotype II was reported to be the most common biotype only among non-typeable isolates and biotype I contained most (>80%) of the typeable strains (Wallace et al., 1981; Granato et al., 1983).

To compare the rate of prevalence of the β-lactamase producing isolates to other countries, it is important to consider the characteristics of the population under investigation. In this study most of the strains were respiratory specimens isolated from adults. The assessment of β-lactamase production by chromogenic nitrocephin revealed that the rate of β-lactamase production varied regionally ranging from 9.6% to 33% \((p=0.038)\). Overall 20.5% of the strains isolated in Scotland produced a detectable β-lactamase enzyme. This rate was similar to the Canadian (25%) (Matsumura et al., 1995a) and American (16.5%) (Jorgensen et al., 1990) studies. In Scotland the distribution of β-lactamase producing strains in each biotype varied significantly \((p=0.04)\). The incidence of β-lactamase-positive isolates was higher among strains that exhibited the biochemical properties of biotype V \((6/16, 35.5\%)\). In total most β-lactamase positive strains were of biotype I \((32.5\%)\) whereas in Canada (Righter & Luchsinger, 1988) biotype III contained the majority of the β-lactamase-producing isolates \((17.1\%)\). In this study only 12.5% of the strains that harboured a β-lactamase enzyme were capsulated, of which the majority \((56.3\%)\) were of biotype I isolates. However, it was biotype II that contained most \((40\%)\) of the capsulated β-lactamase-negative strains.

Similar to the rate of β-lactamase production, distribution of β-lactamase-positive strains among designated biotypes was also subjected to regional trends for both capsulated and non-capsulated strains \((p=0.368)\).
4.2 β-Lactam Resistance Among Clinical Isolates of

*H. influenzae*

β-lactam compounds are among the antibiotics found to be most effective in the treatment of infections caused by *H. influenzae*. To provide a broad view on the prevalence of antibiotic resistance, conducting large surveys, within a defined geographical region and direct comparisons with the data previously observed, is of great value. The knowledge of *in vitro* antibiotic susceptibility patterns assists the clinicians to select the effective antibiotics for the optimal treatment and management of invasive and non-invasive infections caused by this organism. One of the aims of this study was to examine the prevalence of resistance to several β-lactams commonly used against *H. influenzae*. Resistance patterns were determined by susceptibility tests performed on β-lactamase-positive and -negative strains. Strains were considered resistant when the results from sensitivity testing were beyond the range currently defined by BSAC guidelines for sensitive isolates (Phillips *et al*., 1991).

Decreased susceptibility to β-lactams in *H. influenzae* may be attributed to the involvement of altered Penicillin-binding proteins (PBPs) or to hydrolysis by β-lactamase enzymes.

Several PBPs are responsible for decreased affinity for β-lactams in non-β-lactamase-producing strains. In general, alterations in PBPs 3, 4 and 5 have been associated with the resistant phenotypes (Clairoux *et al*., 1992). The contribution of outer membrane permeability has not been proved to be an essential factor in causing β-lactam resistance in *H. influenzae* (Clairoux *et al*., 1992, Reid *et al*., 1987). Although parenteral or oral forms of some β-lactams especially second- and third-generation cephalosporins are relatively resistant to hydrolysis by β-lactamases and are active against invasive and localised infections caused by ampicillin-resistant *H. influenzae*, the presence of modified or low-affinity PBPs may deter the antimicrobial activities of these agents.
The most common mechanism of β-lactam resistance in \textit{H. influenzae} is the production of an enzyme with β-lactamase activity. Ever since the development of broad-spectrum β-lactam antibiotics and β-lactamase inhibitors, the prevalence of β-lactamase-mediated resistance has been under selective pressure.

Geographical variations in the prevalence of β-lactam resistance were observed among strains isolated from different cities. In Aberdeen 54.5% of the β-lactamase-positive strains that were resistant to ampicillin (2mg/l) were inhibited by clavulanic acid (1mg/l). The proportion of β-lactamase-producing ampicillin resistant strains in this region has increased from 2.3% in 1991 (Powell \textit{et al.}, 1992) to 9.5% in 1995. However; the incidence of β-lactamase-negative ampicillin resistance has decreased considerably since then (4.6% versus 0%). All the strains were sensitive to the cephalosporins tested and imipenem was ineffective only against 1.6% (1) of the isolates.

In Edinburgh, a significant increase in the number of ampicillin-resistant β-lactamase-producing isolates was observed, from 2% in 1991 (Powell \textit{et al.}, 1992) to 14.8% and similar to Aberdeen, compared to the survey conducted in 1991 (9.6%) fewer numbers of ampicillin-resistant non-β-lactamase-producing strains (8.6%) exhibited reduced sensitivity to ampicillin. Less than half of the β-lactamase-positive strains (44.4%) were resistant to this agent, of which 3.7% remained insensitive; even when clavulanic acid (1mg/l) was combined with amoxycillin (2mg/l). The frequency of ampicillin resistance among β-lactamase-negative strains was 13% and cross resistance was extended not only to amoxycillin clavulanate (9.3%) but also to cefaclor (11.1%), cefuroxime (5.6%), ceftazidime (3.7%) and imipenem (3.7%).

In Glasgow Royal Infirmary, all six β-lactamase-positive strains were ampicillin-resistant. However, only one strain (16.7%) was not inhibited by 1mg/l clavulanic acid, 5.4% of the non-β-lactamase producing strains were resistant to ampicillin.
(MIC>2mg/l) and 3.6% were resistant to the combination of amoxycillin clavulanate (MIC>2:1mg/l) and to cefaclor (MIC>16mg/l).

In Glasgow Southern General, 68.3% of β-lactamase-positive strains had reduced sensitivity to ampicillin and high proportion (33%) remained insensitive to amoxycillin clavulanate. The number of non-β-lactamase-mediated ampicillin-resistant strains was marginally reduced from 16.5% to 14.6% in the presence of clavulanic acid. -β-lactamase-positive and -negative strains resistant to cefaclor, cefuroxime and imipenem were also isolated from this hospital. In contrast to Edinburgh and Glasgow Royal Infirmary, in Glasgow Southern General strains resistant to cefaclor and cefuroxime were detected with more frequency among β-lactamase-positive isolates.

Although Glasgow Royal Infirmary and Glasgow Southern General are two of the largest hospitals in Glasgow, the incidence of β-lactam resistance was higher in Southern General Hospital. Notably the latter centre is situated in a less affluent area of the city.

Generally, in the Glasgow area, the prevalence of β-lactamase producing ampicillin-resistant strains has decreased from 15.5% reported in 1991 (Powell et al., 1992) to 13.1%. On the other hand the proportion of the strains resistant to ampicillin, without producing any detectable β-lactamase enzyme, has increased from 8.9% in 1991 to 12.1%. It is apparent that during the past few years apart from Glasgow, the incidence of β-lactamase-mediated ampicillin resistance has increased in Aberdeen and Edinburgh and, despite the fall in the proportion of non-β-lactamase-producing ampicillin-resistant strains in those cities (Aberdeen and Edinburgh), this type of resistance has increased in Glasgow.

Variations in testing methodologies carried out in this study compared with the survey performed by Powell et al., (1992) may have affected the results of the sensitivity testings. The disc susceptibility protocol adopted in the 1991 survey has
been shown to be unreliable and, therefore, it is likely that resistance patterns may have been under-estimated (Mendelman et al., 1986).

The present investigation with isolates obtained in Scotland showed that the prevalence of β-lactamase mediated ampicillin resistance (13%) was comparatively higher than the 8.6% and 8.4% recorded in the studies conducted in United Kingdom (Powell et al., 1992) and Wales (Howard & Williams, 1988) respectively.

To extend the antimicrobial spectrum, clavulanic acid was combined with amoxycillin (2:1), however; a high proportion (36.3%) of the β-lactamase-producing ampicillin-resistant strains remained insensitive to amoxycillin. This may be explained by the hyper-production of β-lactamase enzymes in clinical strains which, in turn, is a threat to the effectiveness of the β-lactamase inhibitors currently administered for the treatment of β-lactamase-positive H. influenzae infections.

About 17.2% of the non-β-lactamase-producing ampicillin-resistant strains showed sensitivity to amoxycillin clavulanate (2:1) indicating that clavulanic acid may bind to the PBPs essential to the catalysis of cell wall incorporation (Weber & Sanders, 1990).

The incidence of ampicillin-resistant β-lactamase-negative strains was 10.4%, higher than those reported in the United Kingdom (5.8%) (Powell et al., 1992) Canada (1%, 2.6%) (Matsumura et al., 1995b; Tremblay et al., 1990) and the United States (3.2%) (Doern et al., 1988). The lower incidence of resistance in North America may be explained by the fact that the break points (mg/l) defined by the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (Jorgensen et al., 1992) adopted in those studies are higher than BSAC recommendations (Phillips et al., 1991). Therefore it is more accurate to compare the susceptibility results of a survey to the previous studies conducted within the same geographical region by adopting similar protocols and guidelines.
Overall, 12.2% of the isolates were resistant to cefaclor, 2.8% to cefuroxime, 0.32% to ceftazidime, and 1% to imipenem. There were no strains with reduced sensitivity to cefotaxime. Reduced susceptibility to the agents mentioned occurred with higher frequency among non-β-lactamase-producing strains.

Resistance to cefaclor has dramatically increased over the past years compared to the 1.6% recorded in Wales in 1988 (Howard & Williams, 1988), and the 1.1% in the United States (Jorgensen et al., 1990). The ability of cefaclor to resist hydrolytic activity of β-lactamases and to achieve sufficient periplasmic concentration to bind to crucial PBP targets (Picard & Malouin, 1992) was demonstrated when only 3% of the β-lactamase-producing strains as opposed to the 9% of the non-β-lactamase-producing isolates showed a reduction in sensitivity towards this agent. However, in a recent study conducted in Canada (Matsumura et al., 1995b), 7% of the β-lactamase-positive and 4.3% of the β-lactamase-negative strains showed insensitivity to cefaclor. The differences in the incidence of cefaclor resistance in Canada and Scotland apart from the adopted break points (8mg/l in Scotland versus 32mg/l in North America), may be associated with the nature and the origin of the specimens under investigation.

The increase in the insensitivity of some clinical *H. influenzae* isolates to cefuroxime in Scotland compared to the previous studies; 0.7% (Tremblay et al., 1990) and 0.2% (Jorgensen et al., 1990) in Canada and United States, was in agreement with the figures reported by James et al. (1993).

The rate of resistance to imipenem (1%) has not increased significantly since 1989 (0.9%) (Powell et al., 1992). It was found that 67% of imipenem-resistant isolates (MIC>4mg/l) were also resistant to ampicillin (MIC>2mg/l). In contrast to the earlier speculations (Powell & Williams, 1987; Powell & Livermore, 1990), and in support of Yeo & Livermore (1994), it was observed that there may be a correlation between the factor(s) responsible for causing resistance to imipenem and other β-lactam compounds. Therefore, strains may lose their sensitivity to imipenem and at the same
time become resistant to other β-lactams. However, despite the earlier reports (James et al., 1993; Yeo & Livermore, 1994), no significant relationship was observed between biotype III and insensitivity to carbapenems. Noticeably there was no evidence of the presence of carbapenemases in the strains that conferred resistance to this agent.

In general, the in vitro antimicrobial activity of the cephalosporins tested was not influenced by the β-lactamase production, since the MICs (mg/l) of those agents for β-lactamase-positive and -negative strains were identical. The elevation in MIC₉₀ (mg/l) for the β-lactamase-positive and -negative strains of both ceftazidime and cefotaxime (0.25mg/l for ceftazidime and 0.06mg/l for cefotaxime) indicate that there is a great potential for H. influenzae to develop resistance to third-generation cephalosporins.

There was no apparent association of increased β-lactam resistance in the strains isolated from Scotland with any particular biotype supporting the findings of other studies (James et al., 1993; Yeo & Livermore, 1994).

As predicted (Long et al., 1983; Granato et al., 1983) since this study consisted of strains isolated from non-invasive infections, the majority were characterised as non-capsulated. As the result, not only fewer numbers of typeable strains were found to produce a β-lactamase enzyme but also fewer conferred resistance to β-lactams.

In summary, the β-lactams that showed the most effective in vitro antimicrobial activity against H. influenzae were cefotaxime, ceftazidime, imipenem, cefuroxime, cefaclor, amoxycillin clavulanate, and to a lesser degree ampicillin.

It is likely that the high rate of antibiotic resistance detected in this study was as the result of the age of the hosts. Most specimens were isolated from older patients who were likely to have had repeated exposure to β-lactam antibiotics which had provided
the selective pressure and promoted a flora with reduced susceptibility to these agents (James et al., 1993).

4.3 Identification of β-Lactamases of H. influenzae

The most prominent mechanism of resistance to β-lactam compounds in clinical H. influenzae strains is the production of β-lactamases. The presence of a TEM-1 type β-lactamase activity, detected initially in other gram-negative bacilli (Farrar & O’Dell, 1974), was first reported in ampicillin-resistant H. influenzae isolates in 1974 (Gunn et al., 1974; Khan et al., 1974; Thomas et al., 1974). The genes encoding the TEM-1 type β-lactamase is contained within a transposon closely related to transposon A (TnA) which is carried by plasmids (Elwell et al., 1975; Heffron et al., 1975) or may integrate into the chromosome (Stuy, 1980; Willard et al., 1982). During or after translocation of the genetic elements (De Graaff et al., 1976; Brunton et al., 1986), the resistant genes encoding TEM-1 β-lactamase may undergo alterations. Partial sequencing (320bp) of the TEM-1 molecule extracted from a clinical H. influenzae strain revealed that two silent mutations had occurred in the \( \text{blat}_{\text{TEM}} \) gene. At position 81 Arginine was substituted with Valine and at position 94, Histidine was substituted with Serine. Therefore, it is possible that other mutations in the genes encoding TEM-1-type β-lactamase occur in this species that may alter or diminish the current effectiveness of the β-lactams especially the efficiency of third-generation cephalosporins.

TEM-1 production accounted for 96.8% of the β-lactamase-positive strains isolated in this study similar to the figures obtained world-wide (Scriver et al., 1994; Powell et al., 1992; Sanders & Sanders, 1992). None of the strains contained enzymes identified as TEM-2 or other extended-spectrum β-lactamases derived from TEM-1 or TEM-2.

It was not until 1981 that Rubin et al. (1981) identified a second β-lactamase enzyme, ROB-1, in H. influenzae that was associated with ampicillin resistance.
ROB-1 is not isolated as frequently as TEM-1 type β-lactamase. The rate of the prevalence of ROB-1 in Scotland was 1.6%, whereas in North America higher incidences (7%) of ROB-1 production among clinical *H. influenzae* isolates have been reported (Scriver *et al.*, 1994; Daum *et al.*, 1988). ROB-1 is a plasmid-mediated enzyme (Rubin *et al.*, 1981) and, in contrast to TEM-1, has higher homology values with β-lactamases characterised in gram-positive bacteria. Therefore, *H. influenzae* may have acquired ROB-1 by lateral transfer that occur between bacterial species (Jutaeu & Levesque, 1990; Medeiros *et al.*, 1986).

In this study a novel β-lactamase (VAT-1) was also identified in *H. influenzae*. By virtue of the isoelectric point (7.9), it was initially postulated that VAT-1 was not compatible with TEM-1, ROB-1, or any other β-lactamase tested. This finding was later confirmed when the whole-cell DNA of the VAT-1 producing isolate failed to hybridise with probes derived from pROB gene of *H. influenzae* and bla gene of pUC18. In addition, differentiated PCR with the genomic DNA of the strain producing VAT-1 and TEM oligonucleotide primers did not generate a ~900bp PCR fragment exclusive to TEM-derived enzymes. These negative results reflected that VAT-1 was not the product of evolutionary processes originating from TEM-1 or ROB-1 β-lactamases. Nevertheless, the origin of VAT-1 remained undefined.

Similar to BRO-1 and BRO-2 of *M. catarrhalis* (Wallace *et al.*, 1990), VAT-1 is a relatively weak enzyme, probably as the result of strong cell-association and low amounts of production which in turn explains the low MIC (mg/l) values detected for the VAT-1 producing strain. There were no identifiable plasmids in this strain, therefore preliminary data suggested that the enzyme may be encoded by a chromosomal gene. The apparent molecular size of VAT-1 was estimated by Sephadex G-75 gel filtration and SDS-PAGE electrophoresis to be around 35.5-38 kDa.

VAT-1 preferentially hydrolysed cephalosporins rather than penicillins. In addition to the substrate specificity, the response of VAT-1 to various inhibitors suggested that
VAT-1 resembled the profile of a typical cephalosporinase. This \(\beta\)-lactamase was resistant to clavulanic acid, tazobactam, and to a lesser extent to sulbactam. However; VAT-1 was readily inhibited by BRL 42715, a potential inhibitor of serine \(\beta\)-lactamases (Coleman et al., 1989), additionally, its insensitivity to EDTA, Zn and \(p\)CMB demonstrated that VAT-1 contained a serine residue at its active site. Therefore, VAT-1 most probably belongs to Ambler class C \(\beta\)-lactamases (Ambler, 1980).

VAT-1 was inducible with imipenem. Although when induced it exhibited a slightly different isoelectric point (7.6), the substrates profile and the kinetic parameters were identical to the uninduced phenotype. The change in the isoelectric point may have been caused by physical alteration in the structural form of the enzyme.

In conclusion, VAT-1 is the only cephalosporinase detected in \(H.\ influenzae\) to date.
REFERENCES


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Haemophilus influenzae, β-lactamase, Imipenem.
HAEMOPHILUS INFLUENZAE: IDENTIFICATION OF A NOVEL \( \beta \)-LACTAMASE

L. Vai, C.J. Thomson, S.G.B. Ames, Department of Medical Microbiology, The Medical School, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, UK.

\( \beta \)-Lactamases are one of the most common pathogens responsible for respiratory tract infections. Penicillins and cephalosporins are widely used to treat such infections. However, resistance to these agents can predominantly result from the production of a \( \beta \)-lactamase. To date only two \( \beta \)-lactamases have been detected in \( H.\) influenzae: TEM-1 (Williams et al. 1974) and ROB-1 (Rubin et al. 1981).

A clinical \( H.\) influenzae isolate from Glasgow Southern General Hospital (LG629) was found to be \( \beta \)-lactamase-positive according to a chromogenic cephalosporin assay, although remained sensitive to amoxicillin, with a minimum inhibitory concentration (MIC) of \( 1 \text{mg} L^{-1} \) at \( 10^5 \) colony forming units (cfu), and the cephalosporins tested at the same cfu (Table 1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Minimum Inhibitory Concentration (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amoxycillin</td>
</tr>
<tr>
<td>( 10^6 )</td>
<td>2</td>
</tr>
<tr>
<td>( 10^7 )</td>
<td>4</td>
</tr>
<tr>
<td>( 10^8 )</td>
<td>1</td>
</tr>
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Cell-free extracts were examined by isoelectric focusing and \( \beta \)-lactamase activity visualized with nitrocefin. A novel \( \beta \)-lactamase (\( \text{VAT}-1 \)) was found to be \( \beta \)-lactamase-positive according to a chromogenic cephalosporin assay, although remained sensitive to amoxicillin, with a minimum inhibitory concentration (MIC) of \( 1 \text{mg} L^{-1} \) at \( 10^5 \) colony forming units (cfu), and the cephalosporins tested at the same cfu (Table 1).

Table 1. MIC of \( H.\) influenzae no LG629

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Clavulamic acid (( \mu \text{M} ))</th>
<th>Cefaclor</th>
<th>Cephalexin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clavulamic acid</td>
<td>1.5</td>
<td>30</td>
<td>2000</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>1.0</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>8.5</td>
<td>45</td>
<td>32</td>
</tr>
</tbody>
</table>

These results show the presence of the novel \( \text{VAT}-1 \) \( \beta \)-lactamase in \( H.\) influenzae isolated in Glasgow. This is only the third \( \beta \)-lactamase that has been described in this species.


ANTIBACTERIAL ACTIVITY OF DIOSQUINONE FROM THE ROOT OF DIOPYROS MESPILIFORMIS

Bolani A. Lajubutu, H.A. Odetola, B.A. Ota, R.J. Pinney and M.F. Roberts, Departments of Pharmaceutical Microbiology and Clinical Pharmacy and Botany, University of Ibadan, Nigeria; and Microbiology Section, Department of Pharmaceutics, and Department of Pharmacognosy, The School of Pharmacy, University of London, London WC1N 1AX, UK.

Several ethnopharmacological claims have been made in respect of \( D.\) mespiliformis, including control of leprosy, dysentery, and oral infections (Irving 1961). We report data on the antibacterial activity of diosquinone (Fig. 1), isolated as pure compound from plant root by chloroform extraction and column chromatography.

Minimum inhibitory concentrations (MICs) were determined against a range of Gram-positive and -negative organisms for single cell inocula on nutrient agar. Sensitivities to lethal concentrations of drugs were determined in nutrient broth or in DM buffered salts solution (Davis and Mingioli 1950).

Table 1. MICs on nutrient agar

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S.) aureus NCTC 6571</td>
<td>3</td>
</tr>
<tr>
<td>( S.) pyogenes NCTC 8198</td>
<td>15</td>
</tr>
<tr>
<td>( E.) coli KL16</td>
<td>15</td>
</tr>
<tr>
<td>( P.) aeruginosa NCTC 6750</td>
<td>30</td>
</tr>
<tr>
<td>( S.) paraguanae LT2</td>
<td>45</td>
</tr>
</tbody>
</table>

MICs ranged between 3 and 15 mg L\(^{-1}\) for Gram-positive organisms and between 15 and 45 mg L\(^{-1}\) for Gram-negatives (Table 1). Bactericidal activity against \( S.\) aureus in nutrient broth increased with concentration up to 60 mg L\(^{-1}\) (Fig. 2A). Above this concentration, activity declined, giving a biphasic response. This was not observed with \( E.\) coli, where activity in nutrient broth increased with concentration up to the highest concentration tested (Fig. 2B). The compound was much more active against \( E.\) coli in DM salts solution than in nutrient broth: 30 mg L\(^{-1}\) was highly bactericidal in DM, whereas it was only bacteriostatic in nutrient broth.

Fig. 2. Sensitivities of \( S.\) aureus (A) and \( E.\) coli (B) to diosquinone in nutrient broth. Data are survival levels (percent) after 270 min exposure. (Viable count at time 0 = 100 percent).

Diosquinone has been shown, previously, to be present in \( D.\) micolor (Lillie et al 1973). This is, however, the first report of its antibacterial activity. The low MIC values recorded against a variety of organisms, particularly \( P.\) aeruginosa, make it worthy of further study.

A-68 Development of in vitro Resistance to Meropenem by Pseudomonas aeruginosa from Cystic Fibrosis Patients. J. AKANIN, H. STUTMAN, M. BAYLON and M. MARKS, Memorial Miller Children's Hospital, Long Beach, CA.

The management of pulmonary infections in CF patients due to P. aeruginosa (PA) is characterized by repeated and prolonged courses of antimicrobial therapy. Consequently, resistance can be a major threat to the clinical effectiveness of new agents. Meropenem is a new agent with enhanced antipseudomonal activity. To monitor its propensity for resistance development, 10 isolates of PA from CF patients were serially exposed to increasing concentrations of meropenem and imipenem for a total of 10 passages. Initial and final MIC values were compared and acquired resistance defined as a >16-fold increase in MIC. To determine stability of resistance, organisms from the final transfer were subcultured 5 times onto drug-free media and restested. Determinations of frequency of cross-resistance were made. The criterion for cross-resistance was a >16-fold increase in MIC over preselection values. Against meropenem, stable resistance emerged in 3 PA isolates. In contrast, 1 PA isolate developed stable resistance to imipenem. Cross-resistance to meropenem occurred in 3 PA isolates and cross-resistance to imipenem was noted in 4 PA isolates. These results will help to assign a role for meropenem in the treatment of CF infections due to PA and underscore the need for the inclusion of resistance studies in the preliminary phases of antimicrobial evaluations.

A-69 Individualization of a Susceptibility Pattern to β-Lactams Among Haemophilus strains in Scotland.

E. hermannii was recently individualized on genotyping criteria. Comparatively to E. coli, major species of this genus, biochemical patterns are similar, and may cause a false identification in practice. The susceptibility pattern of E. hermannii (27 strains) was clearly individualized towards 10 β-lactams by agar diffusion method and the principal component analysis (PCA) comparatively to those of E. coli producing or not a penicillinase, Citrobacter diversus, Klebsiella pneumoniae, and K. oxytoca (136 strains).

In vitro activities of 13 β-lactams combined or not with a β-lactamase inhibitor (clavulanate, CA at 2 μg/ml) against E. hermannii (14 strains) were determined by agar diffusion method (geometric mean MIC, μg/ml): amoxicillin, 6.24; aztreonam, 0.25; ticarcillin, 45.2; ticarcillin CA, 0.82; piperacillin, 0.52; piperacillin CA, 0.11; cephalothin, 0.52; cefuroxime, 0.28; ceftazidime, 0.08; cefotaxim, 0.39; cefotaxim, 0.03; cefoperazone, 0.03; cefotaxim, 0.03; cefoperazone, 0.03; imipenem, 0.03. A β-lactamase clavulane-sensitive activity was localized by isoelectric focusing on gel for 13 strains and isoenzyme revelation by benzylpenicillin with various subisoelectric points from 7.0 to 8.5. No cross hybridization with DNA intragenic probes (β-lactamase, SHV, blOL, CAZ and blOXL) was observed by dot blot procedure. Because this low resistance level to penicillins of E. hermannii, such data seem promising for a fast bacteriological diagnosis.

A-70 Incidence of the TEM-1 β-Lactamase in Fecal Flora from Healthy Populations in Africa.

J. APPLETON, G. LINDSAY and S. G. AMES, University of Edinburgh, Edinburgh and Southern General Hospital, Glasgow.

Although increasing evidence suggests that the aerobic gut flora of healthy individuals may act as a reservoir of antibiotic resistance genes, few studies have examined the mechanisms of resistance in such isolates. The carriage of antibiotic resistance amongst the commensal flora of the healthy populations in Africa has now been determined. The genetic and biochemical basis for resistance to β-lactam agents has now been examined. 608 ampicillin resistant strains isolated from the previous study were examined for their production of extended spectrum β-lactamases (ESBLs) and their biochemical characteristics. These studies were performed on 98% of the TEM-1 β-lactamase positive isolates, using the Lactate–Diaminopimelate – Disodium EDTA (LDD) method. The TEM-1 β-lactamase was found in 8% of the examined isolates. The TEM-1 β-lactamase was found to be the most common TEM enzyme detected in both rural and urban isolates.

A-71 β-Lactamase in Haemophilus influenzae isolated in Glasgow University.

E. HERMANNII and S. G. AMES, University of Edinburgh, Edinburgh and Southern General Hospital, Glasgow.

Haemophilus influenzae is a common respiratory pathogen but the prevalence of clinical Haemophilus influenzae strains, resistant to β-lactam antibiotics, has influenced the therapeutic practice. Until recently was usually associated with the production of a β-lactamase which was invariably the ubiquitous TEM-1 enzyme. The discovery of ROB-1 has heralded a new era in the identification of β-lactamase producing Haemophilus influenzae strains. One hundred and ninety-six clinical isolates of Haemophilus influenzae were collected from the Southern General Hospital in Glasgow in 1993. A chromogenic cephalosporin assay revealed that 31 (15.5%) of these strains possessed a β-lactamase. Analytical isoelectric focusing revealed the 36 (17.5%) strains expressing the TEM-1 β-lactamase while the β-lactamases of the remaining 5 (2.5%) of the isolates focused as double bands at isoelectric points of approximately 5.5 and 5.6. These bands did not co-focal with any of the β-lactam controls, neither TEM-1, TEM-2 or their extended-spectrum variants nor were they indicative of ROB-1, BRO-1 or BRO-2. The β-lactamase bands of 5.5 and 7.0 were inhibited by overlapping the gel with 1 μM clavulanic acid and the protein band 5.5 by 15 μM clavulanic acid. Amplification of DNA from the isolates expressing the multiple β-lactamase in the Polymerase Chain Reaction revealed that TEM-derives β-lactamases were present. These results suggest that the Haemophilus influenzae resistant, isolated in Glasgow, encoded a TEM-derived β-lactamase that has never previously been found in this species.