CHARACTERISATION OF RESISTANCE TO β-LACTAM ANTIBIOTICS IN A POPULATION OF *Haemophilus influenzae*

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February 2008

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Summary

Increased global β-lactam resistance in *H. influenzae* is due to the production of plasmid mediated TEM and ROB β-lactamases and/or reduced affinity penicillin binding proteins (PBPs). β-lactamase-negative, ampicillin resistant (BLNAR) isolates have amino acid substitutions in the transpeptidase region of *ftsI*, the gene encoding PBP3. BLNAR isolates are usually resistant to cefuroxime and other β-lactams.

The resistance phenotypes of 250 clinically significant strains were determined using agar dilution minimum inhibitory concentrations (MICs). β-lactamase production was detected phenotypically in 130 strains; three produced ROB-1 and 90 produced an altered TEM enzyme as determined by PCR amplification. Eighteen BLNAR strains, six β-lactamase positive co-amoxiclav resistant (BLPACR) strains and four isolates with reduced susceptibility to cefotaxime were amongst the phenotypes identified. 71.2% of the clinical isolates were resistant to at least one of ampicillin/amoxicillin, co-amoxiclav and cefuroxime.

An attempt was made to determine the mechanism of imipenem resistance detected in fifteen isolates (MIC range 8-16 mg/L). β-lactamase mediated resistance was discounted and efflux pump investigations were inconclusive. Sequence analysis of the transpeptidase region of *ftsI* of selected clinical isolates revealed four amino acid substitutions (D350N, M377I, A502V and N526K) when compared to the fully susceptible Rd strain. Imipenem resistant mutants were selected by serial passage of ATCC 49247 in imipenem-containing broth. Imipenem resistance was transformed into the susceptible Rd recipient strain, using chromosomal DNA or PCR amplimers of *ftsI* from an imipenem resistant mutant of ATCC 49247. Sequencing of the transpeptidase region of *ftsI* revealed no differences between the parent, mutant and transformant suggesting that imipenem resistance may be due to amino-acid substitutions outside the PBP3 transpeptidase region.

As β-lactam resistance increases, carbapenems are becoming important treatment alternatives for serious *H. influenzae* infections. The detection of imipenem resistance in clinical isolates is a matter of concern.

Key words: *H. influenzae*, imipenem, carbapenem, PBP, *ftsI*

Dedication

In memory of

Derek Edwin Walker
(17.3.1930 – 23.11.1996)

Acknowledgements

I am very grateful to Mrs Jenny Andrews for giving me the opportunity to undertake this study and for her encouragement throughout. I would like to thank Professor Peter Lambert for his enthusiastic support and advice and Dr Adam Fraise for providing funding.

I would especially like to thank Dr Nigel Brenwald for his guidance and tremendous support throughout this study.

I am also grateful to Dr Frank Kaczmarek for providing the efflux pump control strains (Pf 1311 and Pf 1312) and the clinical isolate with the same *ftsI* amino acid substitutions as the City Hospital imipenem resistant clinical strains (Pf 1370); Dr Nigel Saunders for providing the *H. influenzae* Rd strain; Dr Mary Slack for the capsular typing of the fifteen imipenem resistant organisms.

Finally, special thanks to my family and friends, especially Adrian Halliwell, Mary Gilmartin, Jo Morgan and the staff at City Hospital for their sustained support and encouragement throughout the duration of this study.

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Abbreviations

%	percentage
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to
µg	microgram
µL	microlitre
µm	micrometer
A	adenine
AMPS	ammonium persulphate solution
ATCC	American type culture collection
AECB	acute exacerbation of chronic bronchitis
BHIb	brain heart infusion broth
BLNAI	β-lactamase negative, ampicillin intermediate
BLNAR	β-lactamase negative, ampicillin resistant
BLPAR	β-lactamase positive, ampicillin resistant
BLPACR	β-lactamase positive, co-amoxiclav resistant
bp	base-pair
BSAC	British Society for Antimicrobial Chemotherapy
C	cytosine
°C	degree Celsius
$CaCl_2$	calcium chloride
CAP	community acquired pneumonia
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cfu	colony forming units
CLSI	Clinical and Laboratory Standards Institute
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
g	gravity
g	gram
G	guanine
GP	general practitioner
h	hour
HCl	hydrochloric acid
HTMs	Haemophilus test medium supplement
IgA	immunoglobulin A
in	inch
Kb	kilobase
kDa	kilo Dalton
K_2HPO_4	dipotassium hydrogen phosphate
KH_2PO_4	potassium dihydrogen phosphate
lb	pound

L	litre
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MIC	minimum inhibitory concentration
MIC_{50}	minimum inhibitory concentration of 50% of the strains
MIC_{90}	minimum inhibitory concentration of 90% of the strains
min	minute
mins	minutes
mL	millilitre
mM	millimolar
mm	millimetre
MOPS	3-(N-morpholino)propanesulfonic acid
n	number
ng	nanogram
N	normal
NaCl	sodium chloride
NaOH	sodium hydroxide
NAD	nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
nm	nanometre
OD	optical density
OMP	outer membrane protein
PAβN	phe-arg β-naphthylamide dihydrochloride
PBP	penicillin binding protein
PCR	polymerase chain reaction
pH	$\log_{10}$ hydrogen ion concentration
pmol	picomole
RAPD	random amplification of polymorphic DNA
rpm	revolutions per minute
sBHI	supplemented brain heart infusion broth
SDS	sodium dodecyl sulphate
secs	seconds
SRGA	Swedish Reference Group for Antibiotics
T	thymine
TBE	TRIS-borate-EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane
UK	United Kingdom
UV	ultraviolet light
v/v	volume:volume ratio
V	volts
V factor	nicotinamide adenine dinucleotide (NAD)
w/v	weight:volume ratio
X factor	haemin

Chapter 1

Introduction

1.1 Historical background

The first recorded observation of haemophili was made by Robert Koch who described the presence of minute bacilli in pus taken from the eyes of patients with conjunctivitis. Pfeiffer reported the presence of large numbers of small bacilli in the sputum of patients affected by the influenzae pandemic of 1889-92. He was able to culture these organisms and in 1893 described the bacillus as the causative organism of the disease (Campos, 1995). Although it is now known that the influenza virus caused the pandemic, it is still possible that *Haemophilus influenzae* was responsible for secondary infections that contributed towards the high mortality of the 1889-92 and 1918-19 pandemics.

1.2 Characteristics of *Haemophilus influenzae*

1.2.1 Taxonomy

H. influenzae is a member of the family Pasteurellaceae. It is a small (0.3 μm), pleomorphic, Gram-negative cocco-bacillus. It is non-motile, non-spore-forming, fastidious and a facultative anaerobe. It ferments glucose and is catalase and oxidase positive. Its only natural host is man.

1.2.2 Growth requirements

Cultivation of *H. influenzae* is dependent on the presence of an iron source such as haemin or haematin (factor X) and nicotinamide adenine dinucleotide (NAD; Factor V), both of which are present in blood. Optimal growth is achieved in a humid atmosphere in 4 – 6 % CO_2 at 35-37 °C at pH 7.6. After 24 hours incubation on chocolate agar, colonies are small and translucent with a characteristic 'mouse nest' smell.

1.2.3 Genetics

H. influenzae Rd (ATCC 51709), a non-pathogenic derivative of a serotype d strain, was the first free-living organism for which nucleotide sequencing of the entire genome was undertaken (Fleischmann *et al*, 1995). The single circular chromosome consists of 1,830,137 base pairs and has an overall G+C nucleotide content of approximately 38 percent (A, 31 percent; C, 19 percent; G, 19 percent; T, 31 percent) (Genome Sequence DataBase accession number L42023).

1.2.3.1. Transformation of *H. influenzae*

H. influenzae is a naturally transformable bacterium i.e. it possesses specific mechanisms for the uptake of DNA from its environment. Natural transformation is dependent on the presence of a species-specific nucleotide sequence in donor DNA fragments ensuring that only DNA from another strain of *H. influenzae* can be transferred (Sisco and Smith, 1979; Danner *et al*, 1980; Gilsdorf, 1998). Examination of the complete genome sequence of *H. influenzae* Rd has identified 1465 copies of this uptake sequence (5'-AAGTGCGGT-3') in the chromosome (Smith *et al*, 1995).

The recipient organism will only take up DNA when in a state of competence, which may be induced under certain growth conditions. In *H. influenzae,* competence can be induced by transferring logarithmic phase cells from a rich medium into a synthetic medium which allows continued protein synthesis without cell multiplication. During transformation, double-stranded donor DNA binds to the bacterial cell surface in subcellular compartments called transformasomes (Snyder and Champness, 2003). The donor DNA is partially degraded in the cytoplasm and single-stranded donor DNA is incorporated into the recipient chromosome by homologous recombination. The appearance of recombinant strains is evidence of successful incorporation of donor DNA into the genome of the recipient. The generation of recombinant strains of *H. influenzae* by natural transformation is a useful tool in the investigation of issues such as antibiotic resistance.

1.2.3.2. Resistance plasmids of *H. influenzae*

Two different types of resistance plasmid have been described in *H. influenzae* (de Graaff *et al*, 1976). The small (less than 10 Kb), non-conjugative plasmids generally only carry a bla_{TEM} gene conferring β-lactamase mediated ampicillin resistance. The large (greater than 30 Kb), conjugative plasmids are more common and often encode multiple antibiotic resistance (Jahn *et al*, 1979; Leaves *et al*, 2000). The G+C ratio of both plasmid groups is the same as the *H. influenzae* chromosome suggesting that the plasmids are indigenous and probably evolved following the transposition of resistance genes onto existing cryptic plasmids (Laufs *et al*, 1981). A large proportion of antibiotic resistant strains of *H. influenzae* have conjugative resistance plasmids integrated into the chromosome (Stuy, 1980). Excision from the donor chromosome

enables conjugal transfer of the resistance plasmid, which is integrated into the recipient chromosome following extrachromosomal replication.

In addition to ampicillin resistance, the large conjugative plasmids of *H. influenzae* may also confer resistance to one or more of tetracycline, chloramphenicol and kanamycin associated with the tet(B) efflux pump, the production of chloramphenicol acetyltransferase and aminoglycoside-phosphotransferase (3')I respectively (Campos *et al*, 1989; Chopra and Roberts, 2001; Roberts *et al*, 1980).

1.2.4 Pathogenesis

H. influenzae is a common commensal of the human nasopharynx and may also colonise the conjunctiva and genital tract. By five years of age, the majority of children have been exposed to the bacterium, which is spread by direct contact or exhaled respiratory tract droplets. The organism produces IgA protease which degrades the host's secretory IgA, facilitating colonisation of the respiratory tract mucosa. From this site, the organism can spread contiguously to cause infections of the respiratory tract, or disseminate via the blood stream to cause more invasive infections such as meningitis, septicaemia and septic arthritis.

The World Health Organisation (WHO) estimates the organism to be responsible for around three million serious illnesses and 386,000 deaths per year due mainly to meningitis and pneumonia (WHO Fact sheet 294, 2005). In England and Wales, prior to the introduction of an effective vaccine, disease caused by *H. influenzae* affected 1 in 600 children with 30 deaths and 80 cases of permanent brain damage per year (National Health Service, 2007).

The presence of a polysaccharide capsule is a major virulence factor in strains causing serious infections. Distinct capsular polysaccharide antigens separate the encapsulated strains into six serotypes (a-f). Organisms of serotype b are most likely to be associated with serious invasive infections, particularly meningitis, septicaemia and pneumonia and are most commonly observed in children between three months and four years of age.

Although serious invasive infections due to the serotype b strain are declining, *H. influenzae* remains a major causative agent of bronchopulmonary, ear, nose and throat infections in adults and children. These diseases are often caused by non-capsulated (non-typeable) strains of the bacterium amongst which resistance to the β-lactam antibiotics has evolved significantly over recent years.

1.2.5 Vaccination

The *H. influenzae* type b conjugate vaccine (Hib vaccine) contains purified capsular polysaccharide (polyribosylribitol phosphate) covalently bound to a protein carrier. Its introduction in the early 1990s has lead to a significant decrease in serious infections caused by the type b serotype. The vaccines are administered by intramuscular injection and are highly effective. Three doses are usually given to infants at 2, 3 and 4 months, often in conjunction with vaccinations against diphtheria, tetanus, pertussis, measles and polio (WHO Fact sheet 294, 2005).

By the end of 2004, 89 countries were operating systematic childhood vaccination programmes and disease caused by *H. influenzae* has largely been eliminated from the industrialised nations. This is in contrast to Japan where the Hib vaccine has not been introduced and the incidence of *H. influenzae* meningitis is 10 per 100,000 in children under five years of age (Hasegawa *et al*, 2006).

1.3 The Gram-negative Bacterial Cell Wall

1.3.1 Structure

The Gram-negative bacterial cell wall, or envelope, comprises an outer membrane covalently bound to a semi-rigid inner layer of peptidoglycan, which surrounds the cytoplasmic membrane (Figure 1.1). The integrity of the cell wall is essential to the viability of the bacterium; if weakened or damaged the cell is at risk of lysis due to an inflow of water to the hypertonic cytoplasm.

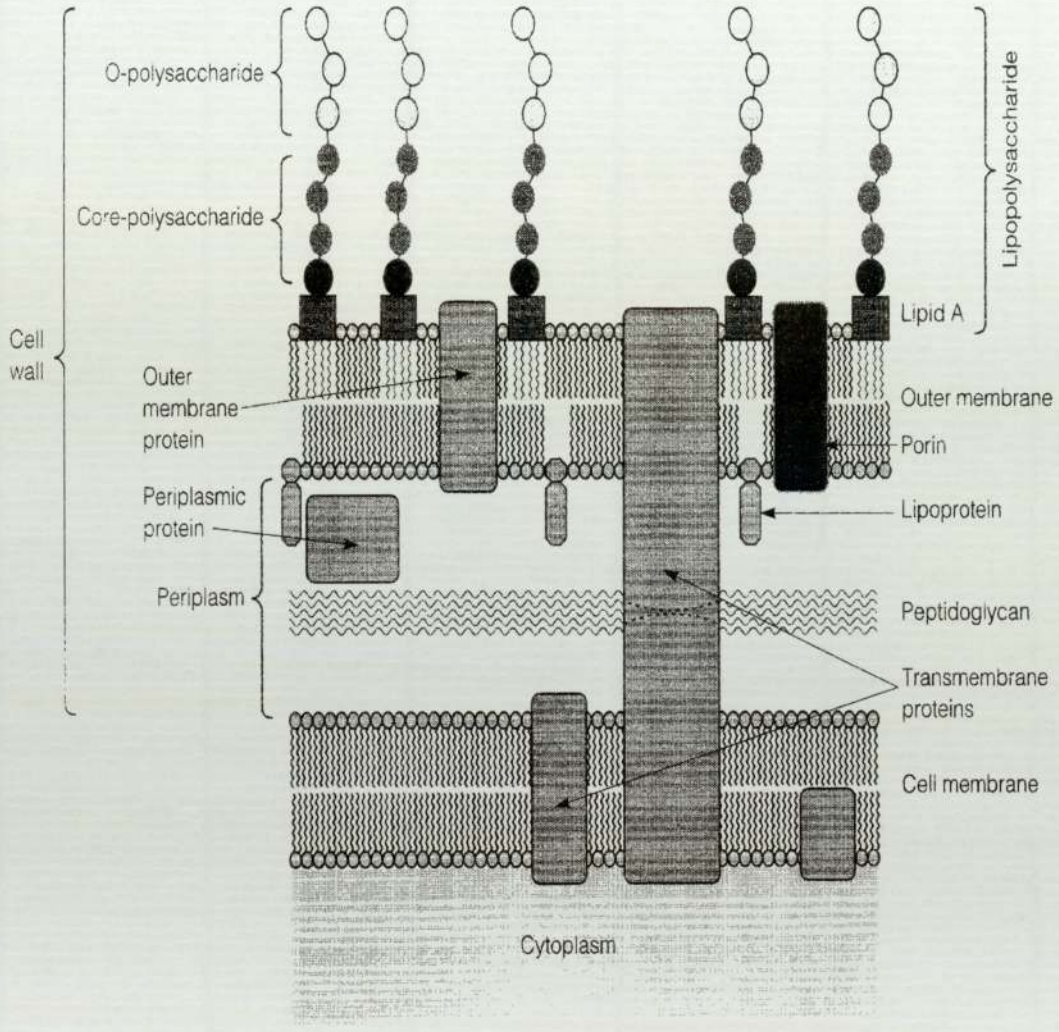


Figure 1.1 The Gram Negative Cell Wall (Baker, S. *et al*, 2007)

The cytoplasmic membrane is a semi-permeable phospholipid bilayer. Proteins spanning the whole, or part of the membrane are associated with the transport of molecules between the cytoplasm and periplasmic space. The enzymes involved in peptidoglycan synthesis are located on the outer surface of the cytoplasmic membrane (Scheffers and Pinho, 2005).

Between the cytoplasmic and cell membranes is the periplasmic space. Various enzymes and proteins associated with cell metabolism are located in the periplasm including the β-lactamase enzymes of Gram-negative bacteria (Livermore, 1995). The semi-rigid peptidoglycan layer is found in the periplasmic space and is connected to the inner surface of the outer membrane by lipoproteins.

The Gram-negative outer membrane is a semi-permeable lipid bilayer that acts as a barrier against the free diffusion of large molecules across the cell envelope. A number of proteins span the outer membrane, some of which form porins, water-filled channels that allow the permeation of low molecular weight solutes. The predominant outer membrane protein in *H. influenzae* is OmpP2 which functions as a porin and is highly permeable to the β-lactam antibiotics (Coulton *et al*, 1983). Strongly antigenic lipopolysaccharides are located on the outer surface of the membrane. Anchored to the membrane by the lipid portion (lipid A), the polysaccharide is made up of two sections: the inner core, or R, polysaccharide (common to all members of a particular genus) and the outer O or somatic antigen. The latter consists of 3 to 5 repeating sugars, dependent on species and is the major antigenic determinant of pathogenic cells.

Capsulated strains of *H. influenzae* secrete a viscous polysaccharide that forms a coating around the outer membrane. The capsule increases the pathogenicity of the bacterium by facilitating adherence to the respiratory mucosa, providing protection against host defences such as antibodies and phagocytes, and acting as a diffusion barrier against some antibiotics.

1.3.2 Peptidoglycan

Peptidoglycan, also known as murein, is a polymer consisting of long glycan chains cross-linked via flexible peptide bridges. It forms a strong, elastic structure that

supports the bacterial cell wall and protects the protoplast from lysis due to the high internal osmotic pressure.

The glycan chain is made up of the sugar derivatives, N-acetylglucosamine (NAcGlc) and N-acetylmuramic acid (NAcMur), linked β, 1-4 in alternating sequence. Polypeptide side chains attached to the carboxyl group of NAcMur link adjacent NAcGlc – NAcMur polymers. The peptide chains generally consist of five amino acid residues in the sequence - L-Alanine – D-Glutamic acid – L-**R** – D-Alanine – D-Alanine where **R** may be one of several amino acids dependent on bacterial species. In *H. influenzae*, **R** is diaminopimelic acid (Burroughs *et al*, 1993).

Peptidoglycan monomers, comprising one each of the NAcGlc and NAcMur amino sugars with the pentapeptide side-chain attached to the NAcMur, are synthesised in the cytoplasm. They are transported across the cell membrane into the periplasmic space by the lipid carrier molecule, bactoprenol. Assembly of the new peptidoglycan molecule occurs on the outer side of the cytoplasmic membrane and is achieved through the action of a group of enzymes collectively known as penicillin-binding proteins (PBPs) due to their binding affinity with the β-lactams. At the point of growth of the existing peptidoglycan molecule, transglycosylase enzymes facilitate the insertion of the new units into the existing glycan chain. Cross-linking of adjacent glycan chains occurs between the diaminopimelic acid at position 3 of the peptide of one glycan chain and the D-alanine at position 4 of the peptide of the adjacent glycan chain (a transpeptidase reaction). The terminal D-alanine of the second polypeptide is then cleaved by a separate carboxypeptidase reaction, thus helping to control the degree of cross-linking between adjacent glycan chains (Scheffers and Pinho, 2005) (Figure 1.2).

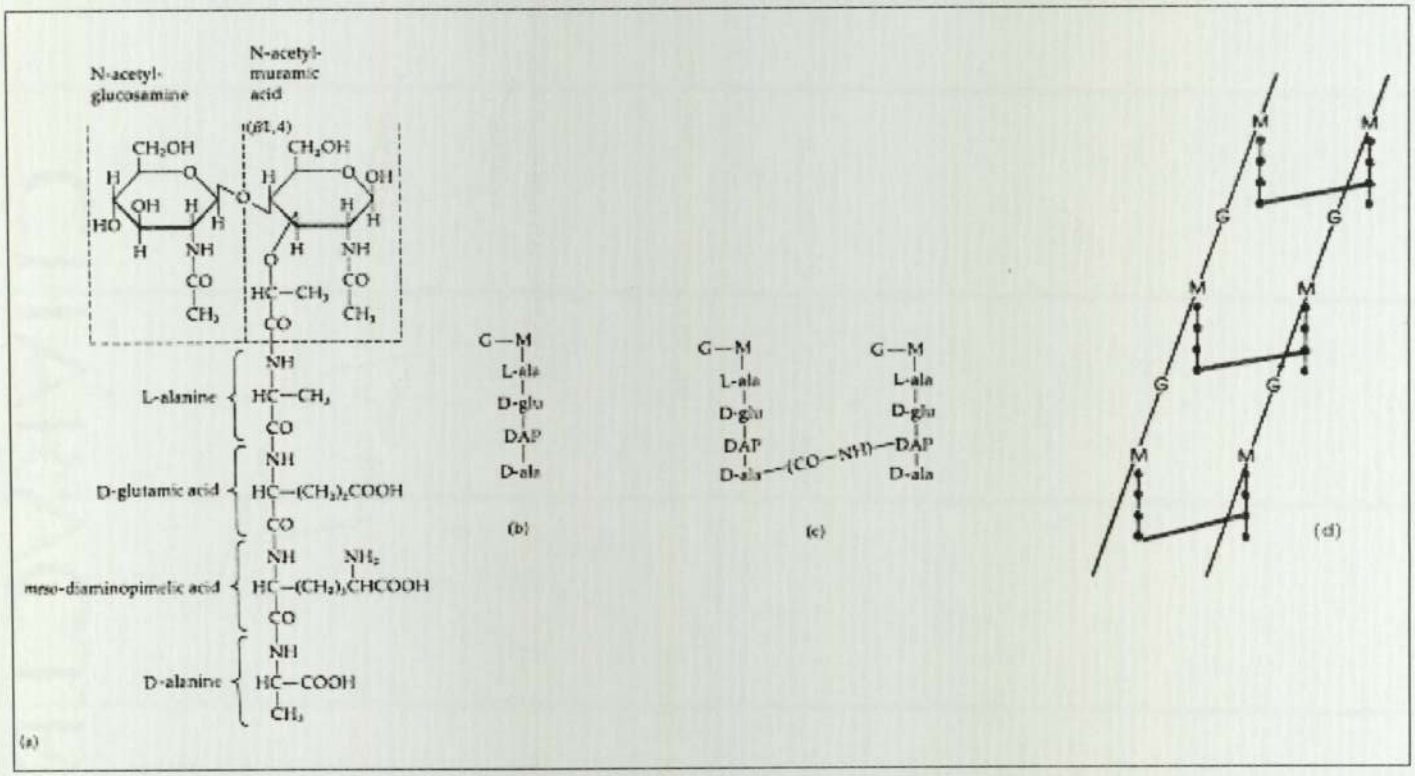


Figure 1.2 The structure of peptidoglycan

(a) The glycan backbone is a repeat polymer of two amino sugars, N-acetylglucosamine (G) and N-acetylmuramic acid (M). Attached to the N-acetylmuramic acid is a tetrapeptide consisting of L-ala-D-glu-DAP-D-ala; (b) Abbreviated structure of the muramic acid subunit; (c) Adjacent side chains are linked by peptide bonds between DAP on one chain and D-ala on the other; (d) The polymeric form of the peptidoglycan molecule.

(www.textbookofbacteriology.net)

1.4 The β-lactam antibiotics

The β-lactams form the largest group of antimicrobial compounds and are the most often prescribed due to their broad spectrum of activity, efficiency and low toxicity to eukaryotes (Georgopapadakou, 1993; Livermore and Williams, 1996). All members of the family are based on a four membered β-lactam ring and are divided into groups based on the nature of the structure to which this is attached (Figure 1.3).

1.4.1 Penicillins

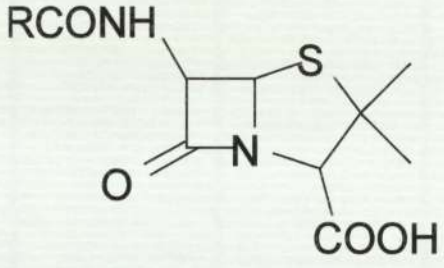
In 1928, Alexander Fleming observed that the mould *Penicillium notatum* produced a substance that was inhibitory to Staphylococci. The substance was named penicillin and is now produced commercially using *Penicillium chrysogenum*. The structure of penicillin is based on 6-aminopenicillanic acid (a β-lactam ring fused to a thiazolidine ring) with semi-synthetic derivatives manufactured by substituting the 6-amino group of the β-lactam ring (Figure 1.3). The broad-spectrum penicillins ampicillin and amoxicillin, and the β-lactamase inhibitor combination co-amoxiclav, are the most often used for treatment of *H. influenzae* infections.

1.4.2 Cephalosporins

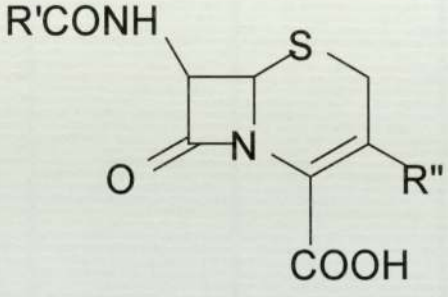
Originally derived from the mould *Cephalosporium acremonium* these compounds are structurally based on a β-lactam ring fused to a dihydrothiazine ring (collectively known as 7-aminocephalosporanic acid). Members of the group differ in the side chains at positions 3 and 7 (Figure 1.3). The cephalosporins are classified in generations, later generations being more stable to inactivation by β-lactamases. Their broad spectrum of activity is utilised in the treatment of serious systemic infections. Cephalosporins have poor penetration of the blood-brain barrier unless the meninges are inflamed; third and fourth generation cephalosporins may be used for the treatment of meningitis caused by *H. influenzae*.

1.4.3 Carbapenems

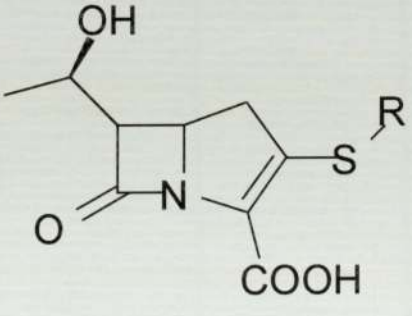
These are semi-synthetic β-lactams derived from thienamycin produced by *Streptomyces* spp. They have the widest spectrum of activity of any currently available group of antibiotics. They are stable to most plasmid and chromosomally mediated β-lactamases due to the structure of their hydroxyethyl side chain.



Penicillin general structure



Cephalosporin general structure



Carbapenem general structure

Figure 1.3 General structures of the penicillins, cephalosporins and carbapenems.

Imipenem is partially inactivated by kidney dihydropeptidase so is administered in combination with cilastatin, a specific enzyme inhibitor. Meropenem is stable to the renal enzyme and is less neurotoxic than imipenem. Ertapenem has a more limited range of activity but is licensed for the treatment of community acquired pneumonia.

1.4.4 Mechanism of action

The β-lactam antibiotics are usually bactericidal and affect only actively growing cells. They are structural analogues of the terminal amino acid residue of the polypeptide side chains of peptidoglycan (Tipper and Strominger, 1965). They inhibit the final stage of peptidoglycan synthesis by covalently binding to the active sites of the D-alanine-D-alanine transpeptidase and carboxypeptidase enzymes associated with the final cross-linking of NAcGlc/NAcMur polymers. This action results in the formation of a defective cell wall and eventual cell lysis (Livermore and Williams, 1996).

1.5 β-lactam therapy of *H. influenzae* infections

Treatment guidelines are dependent on the nature of the *H. influenzae* disease and the resistance profile of the causative organism: susceptibility testing of isolates is necessary due to the high level of antibiotic resistance in the *H. influenzae* population. If the resistance phenotype is known, ampicillin or amoxicillin is standard therapy for a non β-lactamase producing, fully susceptible strain and co-amoxiclav for β-lactamase positive strains (British National Formulary, 2007). The emergence of β-lactamase negative, ampicillin resistant (BLNAR) strains of *H. influenzae,* which have concomitant resistance to the cephalosporins, has lead to the increased use of the carbapenems particularly in Japan (Hasegawa *et al*, 2006).

1.5.1 Meningitis and other serious life-threatening infections

Empirical treatment of meningitis with a broad spectrum β-lactamase resistant antibiotic is usual. Once it has been established that *H. influenzae* is the causitive organism, treatment with the third generation cephalosporin cefotaxime is recommended in the UK (British National Formulary, 2007). This may be substituted with meropenem where β-lactam allergy is an issue. Treatment of other serious life-threatening infections e.g. epiglottitis, is similar to meningitis. In areas

where the Hib vaccine is not routinely administered and childhood pneumonia and bacteraemia are still common, high dose amoxicillin or co-amoxiclav is used as the first line therapy for non-hospitalised patients whilst third generation cephalosporins are used for in-patients (Tristram *et al*, 2007).

1.5.2 Acute exacerbation of chronic bronchitis (AECB)

Non-typeable *H. influenzae* is the most common cause of AECB and recommendations for treatment depend on the underlying condition of the patient. Where there are no complications, amoxicillin or a broad-spectrum cephalosporin may be recommended whilst an additional agent may be added where co-morbidity exists.

1.5.3 Adult pneumonia

Ampicillin is used for the treatment of uncomplicated adult community acquired pneumonia (CAP) and cefuroxime, if the pneumonia is severe or of unknown aetiology. When the pneumonia is hospital acquired, a broad-spectrum cephalosporin (e.g. cefotaxime or ceftazidime) is usually recommended.

1.5.4 Sinusitis, otitis media and other upper respiratory tract infections

Sinusitis may affect adults or children and is often caused by non-typeable *H. influenzae*. Treatment is usually reserved for persistant or severe infections and ampicillin is amongst the drugs of choice.

Otitis media mainly affects children under 3 years old and may be caused by *H. influenzae*. Most uncomplicated cases resolve without antibacterial treatment but where necessary, amoxicillin may be perescribed.

H. influenzae may also be the causitve agent of otitis externa and purulent conjunctivitis. Topical therapy with an aminoglycoside or chloramphenicol is usual but, if the infection is serious or the patient is unwell, systemic treatment with an oral β-lactam may be appropriate.

1.6 Mechanisms of β-lactam resistance in *H. influenzae*

In *H. influenzae*, the phenotypic expression of resistance to the β-lactams is generally a combination of the production of inactivating enzymes such as β-lactamases, the affinity of the antibiotic for the penicillin binding protein (PBP) target site, the presence of an efflux pump mechanism and decreased outer membrane permeability (Tristram *et al*, 2007).

1.6.1 β-lactamase mediated resistance

Ampicillin resistance in *H. influenzae* has been well documented since 1974 when it was associated with the production of a TEM-1 β-lactamase previously only reported in Enterobacteriaceae (Matthew, 1979; Farrar and O'Dell, 1974). A second enzyme, ROB-1, was reported in an apparently β-lactamase negative (by chromogenic cephalosporin assay) *H. influenzae* type b strain following ampicillin treatment failure in a meningitis case (Rubin *et al*, 1981). Both enzymes are plasmid-mediated class A serine β-lactamases which rapidly hydrolyse ampicillin and amoxicillin but not cefuroxime or the carbapenems, and are inactivated by enzyme inhibitors such as clavulanic acid. Isolates producing the ROB-1 enzyme are generally more resistant to cefaclor (Karlowsky *et al*, 2000; Farrell, *et al*, 2005) and less resistant to ampicillin or amoxicillin (Daum *et al*, 1988) than TEM-1 producers. The two enzymes were traditionally distinguished by isoelectric focusing; TEM-1 has an isoelectric point of 5.4, ROB-1 of 8.1 (Rubin *et al*, 1981). More recently, polymerase chain reaction (PCR) based methods of detection have been employed (Scriver *et al*, 1994; Farrell *et al*, 2005).

The recent Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin (PROTEKT) study investigating nearly 15,000 *H. influenzae* isolates from 38 countries reported an overall β-lactamase positivity of 15.0% with the incidence in individual countries ranging from none in Venezuela and Peru to as high as 67.9% in Taiwan (Farrell *et al*, 2005). TEM-1 was the predominant β-lactamase by a ratio of approximately 19:1 with ROB-1 producing isolates almost exclusively found in North America.

It is usual for an individual isolate to produce only one type of β-lactamase although rare isolates producing both TEM-1 and ROB-1 have been reported (Scriver *et al*, 1994; Farrell *et al*, 2005). Recent studies undertaken in Spain and Australia have detected deletions (Molina *et al*, 2003: Tristram *et al*, 2005) or insertions (Tristram and Nichols, 2006) in the promoter region of the bla_{TEM} gene in *H. influenzae*. Altered bla_{TEM} genes were also described in clinical *H. influenzae* isolates collected for the PROTEKT study.

The activity of a β-lactam against β-lactamase positive strains is maintained when prepared in combination with a β-lactamase inhibitor, e.g. co-amoxiclav. However, rare β-lactamase positive isolates that are resistant to co-amoxiclav (BLPACR) are emerging, which combine PBP3 mutations with β-lactamase production to confer β-lactam resistance (Doern *et al*, 1997; Matic *et al*, 2003). These organisms often demonstrate reduced susceptibility to the cephalosporins.

1.6.2 Altered penicillin binding proteins

1.6.2.1 Defining β-lactamase negative ampicillin resistant *H. influenzae*

Non-β-lactamase-producing ampicillin resistant isolates of *H. influenzae* (BLNARs) were first described in the 1980s (Markowitz, 1980). Since then, they have been isolated worldwide with increasing frequency, particularly in Japan (Farrell *et al*, 2005; Felmingham, *et al*, 2005).

The precise definition of a BLNAR strain is difficult to ascertain as the term varies slightly between groups. This is largely due to the use of susceptibility testing methodology recommended by different national bodies and their corresponding Minimum Inhibitory Concentration (MIC) breakpoints (Table 1.1). The majority of studies follow the methods of the Clinical and Laboratory Standards Institute (CLSI, formally the National Committee for Clinical Laboratory Standards, NCCLS) where susceptibility of *H. influenzae* to ampicillin is defined by the following MIC breakpoints: $\leq$ 1 mg/L, susceptible; 2 mg/L, intermediate; $\geq$ 4 mg/L, resistant (CLSI, 2006). Accordingly, researchers following CLSI methodology have reported two populations of BLNAR; β-lactamase negative isolates with intermediate susceptibility to ampicillin as BLNAIs (or low-level ampicillin resistant strains) and isolates with an ampicillin MIC of greater than or equal to 4 mg/L as true BLNARs. Kaczmarek *et al*

(2004) identified an additional population of *H. influenzae* with MICs of ampicillin of greater than or equal to 8 mg/L (high-level BLNARs). These distinctions would not be possible using the methodology described by the British Society for Antimicrobial Chemotherapy (BSAC) where no intermediate category exists, or when comparing MIC data with a fully susceptible population distribution model such as that used by the Swedish Reference Group for Antibiotics (SRGA).

The picture is further complicated by the nature of MIC determination where repeat testing may give values of plus or minus one doubling dilution. Thus, isolates with borderline susceptibility may be considered susceptible on one occasion but resistant on subsequent testing. To overcome this problem, some authors express the MIC as a geometric mean calculated from values obtained from repeat testing (Kaczmarek *et al*, 2004).

For a large number of routine hospital laboratories, disc diffusion testing is the primary method for detecting reduced susceptibility in clinical isolates. Although standardized testing methods are now widely employed, susceptibility testing *H. influenzae* is still problematic; results are notoriously inoculum-dependent and endpoints of agar dilution methods can be difficult to read (James *et al*, 1993). The detection of BLNAR strains can be especially problematic as even slight changes in media composition can alter the quality of growth leading to variations in interpretation of disc susceptibility tests (Barry *et al*, 2001). It has been suggested that the incidence of BLNAR strains may well be greater than currently believed due to problems of detection using the ampicillin disc diffusion test (Mendelman *et al*, 1986; Barry *et al*, 1993). BLNAR strains have reduced susceptibility to β-lactams other than ampicillin, especially the cephalosporins. Cefaclor and cefuroxime have been suggested as better indicators of non-β-lactamase type resistance in *H. influenzae* than ampicillin (Livermore *et al*, 2004; James *et al*, 1993). As clavulanate is inactive against BLNAR strains, these organisms are also resistant to co-amoxiclav (Matic *et al*, 2003).

Table 1.1 β-lactam MIC interpretative guidelines for *Haemophilus influenzae*

Antibiotic	BSAC [1] MIC BP (mg/L)		CLSI [2] MIC BP (mg/L)			EUCAST [3] MIC BP (mg/L)		EUCAST [3] Wild Type population (mg/L)	SRGA [4] Wild Type population (mg/L)
	R >	S ≤	R ≥	I	S ≤	R >	S ≤		
Ampicillin [5]	1	1	4	2	1	-	-	≤ 2	0.125 – 0.5
Amoxicillin[5]	1	1	-	-	-	-	-	≤ 2	0.125 – 0.5
Co-amoxiclav[5]	1	1	8/4	-	4/2	-	-	≤ 2	0.125 – 0.5
Cefuroxime	1	1	16	8	4	2	1	≤ 1	0.25 - 1
Cefaclor	1	1	32	16	8	-	-	-	-
Cefotaxime	1	1	-	-	2	0.125	0.125	≤ 0.064	0.008 – 0.032
Imipenem	4	4	-	-	4	2	2	≤ 2	0.064 – 0.25
Meropenem	4	4	-	-	0.5	2	2	-	0.064 – 0.5
Ertapenem	2	2	-	-	0.5	0.5	0.5	≤ 0.125	0.032 – 0.125

[1] British Society for Antimicrobial Chemotherapy, Andrews (2004); [2] Clinical Laboratory Standards Institute (2006); [3] European Committee on Antimicrobial Susceptibility Testing (2006); [4] Swedish Reference Group for Antibiotics (2007); [5]EUCAST MIC breakpoints for penicillins not yet available.

1.6.2.2 Penicillin Binding Proteins of *H. influenzae*

The number and structure of penicillin binding proteins (PBPs) varies between bacterial species. The PBPs of *H. influenzae* were first characterised in 1981 when eight major PBPs were detected and named PBP1 to PBP8 in order of decreasing molecular weight (Makeover *et al*, 1981). Parr and Bryan (1984) referred to the eight PBPs they detected as PBPs 1A, 1B, 2, 3A, 3B, 4, 5 and 6 based on the similarities of their binding capacities to various β-lactams and those of the PBPs of *E. coli*.

Later studies reported a variable number of PBPs dependent on the growth phase of the cells studied; fully susceptible isolates in the logarithmic growth phase had eight PBPs whilst stationary phase cells from the same isolates had six detectable PBPs (Mendelman *et al*, 1984; Mendelman *et al*, 1990). BLNAR strains were found to express marked heterogeneity with 5 to 10 PBPs detected in logarithmic phase cells (Mendelman *et al*, 1987). In view of this variability, comparison of the earlier work on the PBPs of *H. influenzae* can be problematic. In particular, what are now known as PBP3a and 3b were frequently referred to as PBPs 4 and 5 (Table 1.2).

Penicillin binding studies have identified PBP3a and 3b as the major targets for the β-lactams in *H. influenzae*. Encoded by the *ftsI* gene, PBP3 functions as a transpeptidase enzyme in septal peptidoglycan synthesis and is essential for normal cell division (Malouin *et al*, 1990; Livermore and Williams, 1996).

Table 1.2 The number and apparent molecular weight of the PBPs of ampicillin-susceptible *Haemophilus influenzae* reported in different studies

Makover *et al* (1981) Log phase cells		**Parr and Bryan (1984) Log phase cells**		**Mendelman *et al* (1984) Stationary phase cells**		**Mendelman *et al* (1990) Log phase cells**	
Molecular weight (kDa)	PBP	Molecular weight (kDa)	PBP	Molecular weight (kDa)	PBP	Molecular weight (kDa)	PBP
90	1	85	1a	90	1	90	1
84	2	80.5	1b	80	2	80	2
75	3	74	2	71	3	71	3
68	4	68	3a	62	ND	62	4
64	5	65.5	3b	59	4	59	5
48	6	48	4	45	ND	45	6
41	7	44	5	36	5	36	7
27	8	41	6	25.5	6	25.5	8

ND, Not detected in stationary phase cells

1.6.2.3 Altered Penicillin Binding Proteins

Mendelman *et al* (1984) studied four BLNAR clinical isolates from New Zealand with high-level resistance to ampicillin (MIC of 32 mg/L). As a 23-megadalton plasmid was detected in two of the four strains studied, conjugal transfer of resistance was attempted but unsuccessful suggesting a conjugative plasmid was not involved in BLNAR resistance. Only one strain demonstrated reduced permeability whilst changed outer membrane proteins were detected in all four strains in comparison with the ampicillin susceptible control. Chromosomal DNA from the BLNAR strains was used to transform competent cells of the laboratory MAP strain of *H. influenzae*. Radiolabelled penicillin binding studies detected a reduced binding affinity for PBP 3, 4 and 6 in the ampicillin-resistant transformants. In addition, all the transformants demonstrated a decreased affinity of PBP4 (known as PBP3 in later studies) for ampicillin compared to the susceptible recipient. The group concluded that altered penicillin-binding proteins were the major mechanism of ampicillin resistance in these organisms although membrane permeability may also be involved.

Parr and Bryan (1984) confirmed the association between altered PBP3 and the BLNAR phenotype. Chromosomal DNA from a BLNAR strain isolated from an immunocompromised adult with pneumonia was used to transform the ampicillin susceptible Rd strain to a BLNAR phenotype. Both the transformant and clinical BLNAR strain demonstrated a major reduction in penicillin binding affinity of PBP 3a and 3b compared to the fully susceptible Rd strain.

A study of Canadian BLNARs (Clairoux *et al*, 1992) identified three groups of organisms based on their MIC of ampicillin; values of 0.5 to 1, 2 to 4 and greater than or equal to 8 mg/L were assigned to groups I, II and III respectively. Ampicillin resistant transformants of the Rd strain were prepared using chromosomal DNA from representatives of the three BLNAR groups. Transformants incorporating DNA from groups I and II had ampicillin MICs equal to the donor strains and demonstrated a reduced affinity for β-lactams in PBPs 3A and 3B. Group III transformants also demonstrated reduced binding affinities in PBPs 3A and 3B, but the ampicillin MICs of the transformants were lower than the donor strains. The authors concluded that whilst reduced affinity PBPs accounted for low-level ampicillin resistance in BLNAR strains, additional resistance mechanisms were involved in high-level resistance.

1.6.2.4. Mutations of the *ftsI* gene

Once it was established that altered PBPs were the major cause of reduced ampicillin susceptibility in BLNAR strains, a genetic basis for the resistance was sought. Penicillin-binding studies of 25 Japanese clinical BLNAR strains detected reduced affinities for PBP3a in all isolates. Some of the isolates also expressed reduced affinity for penicillin in PBP3b and PBP4. Nucleotide sequencing of the *ftsI* and *dacB* genes (encoding PBP3 and PBP4 respectively) of these isolates and comparison with the published sequence of *H. influenza*e Rd determined several common amino acid substitutions in the transpeptidase region of the *ftsI* gene but not the *dacB* gene (Ubukata *et al*, 2001). The role of the altered *ftsI* gene in the BLNAR phenotype was confirmed by transforming *H. influenzae* Rd with the PCR-amplified *ftsI* gene of several of the BLNAR strains producing transformants with the same β-lactam MICs as the donor strains.

Amino acid substitutions were detected in the *ftsI* gene of clinical BLNAR strains studied in France and America (Dabernat *et al*, 2002; Kaczmarek *et al*, 2004) and also in BLPACR and β-lactamase negative cefuroxime resistant clinical isolates from Japan and the UK respectively (Matic *et al*, 2003; Straker *et al*, 2003). Comparison of the amino acid substitutions detected in these studies illustrates the heterogeneity of the *ftsI* gene amongst β-lactam resistant *H. influenzae* worldwide (Table 1.3). Whilst the Asn-526-Lys (N526K) substitution was detected in all strains other than those from the Ubukata group1 BLNAR organisms, no single substitution was observed in all the isolates studied. However, using three-dimensional crystallographic models of PBP3, Ubukata (2001) reported that every amino acid substitution detected in the *ftsI* gene of the Japanese isolates occurred in the active-site pocket surrounded by the Ser-327-Thr-Val-Lys (S327-T-V-K), Ser-379-Ser-Asn (S379-S-N) and Lys-513-Thr-Gly (K513-T-G) conserved motifs.

Table 1.3 Comparison of the deduced amino acid substitutions detected in the transpeptidase region of the *ftsI* gene of β-lactam resistant *H. influenzae* from different studies.

	Sequence variations from genomic sequence of *H. influenzae* Rd											
	D350N	**S357N**	**M377I**	**S385T**	**L389F**	**A437S**	**I449V**	**G490E**	**A502V**	**A502T**	**R517H**	**N526K**
Ubukata, 2001												
Group 1 BLNAR	(*)	(*)	(*)	(*)							*	
Group 2 BLNAR	*	(*)										*
Group 3 BLNAR	*	*	*	*	*							*
Dabernat, 2002												
Group 1 BLNAR	(*)	(*)								(*)	*	
Group 2a BLNAR												*
Group 2b BLNAR	(*)	(*)				(*)		(*)	*			*
Group 2c BLNAR	(*)									*		*
Group 2d BLNAR							*					*
Matic, 2003												
BLNAR	(*)	(*)	(*)	(*)	(*)					(*)		*
BLPACR	*	*	(*)	*	(*)				(*)			*
Straker, 2003												
BLNCR	(*)	(*)	(*)						(*)	(*)	(*)	(*)
Kaczmarek, 2004												
Group 1 BLNAR		*									*	
Group 2 BLNAR	(*)	(*)	(*)				(*)	(*)	(*)		(*)	*

BLNAR, β-lactamase negative, ampicillin resistant; BLPACR, β-lactamase positive, co-amoxiclav resistant; BLNCR, β-lactamase negative, cefuroxime resistant; *, substitution observed in all strains; (*), Substitution observed in some but not all strains.

1.6.3 Efflux mediated resistance

In common with other Gram-negative bacteria, *H. influenzae* is known to operate active efflux transporters (Poole, 2004). The complete genome sequencing of *H. influenzae* Rd identified a three-gene complex homologous to the *acrRAB* cluster of *E. coli*; HI0893 (an *acrR* homolog), HI0894 (an *acrA* homolog) and HI0895 (an *acrB* homolog) (Fleischmann *et al*, 1995). It has since been demonstrated that this efflux mechanism is normally expressed in the Rd strain and contributes to the organism's baseline resistance to antimicrobials including erythromycin, dyes and detergent (Sanchez *et al*, 1997).

In *E. coli*, the AcrAB efflux pump is composed of the inner membrane transporter AcrB, the membrane fusion protein AcrA, and the outer membrane channel protein TolC. Trepod and Mott (2004) detected a TolC homologue (HI1462) in *H. influenzae* and demonstrated that it works together with AcrA and AcrB to form a single primary efflux pump. The same study demonstrated that inactivation of the *acrA*, *acrB* and *tolC* genes of *H. influenzae* Rd causes a fourfold reduction in the MIC of ampicillin. Mutations in the AcrAB repressor gene, *acrR*, in combination with altered PBP3 have been demonstrated in high level BLNAR strains (Kaczmarek *et al*, 2004).

1.6.4 Altered Outer Membrane Permeability

A change in outer membrane permeability has been associated with altered susceptibility to antibiotics in many bacteria (Nikaido, 2003). In *H. influenzae*, the major outer membrane protein P2 (OmpP2) functions as a porin, forming non-specific diffusion channels for small water-soluble molecules, including the β-lactams (Vachon *et al*, 1985; Coulton *et al*, 1983). A number of studies of non-capsulated *H. influenzae* isolated from patients with persistent respiratory infections have identified strain-to-strain diversity in the amino acid sequence of *ompP2*, the gene encoding OmpP2, compared to *H. influenzae* type b. These variations were particularly noticeable in isolates from cystic fibrosis patients who are often treated with prolonged courses of β-lactams. Although Regelink *et al* (1999) proposed that point mutations in *ompP2* of these organisms might cause reduced permeability of the porin channel to β-lactam antibiotics, Möller *et al* (1998) determined that reduced susceptibility to the β-lactams occurred irrespective of OmpP2 changes. In a study examining the nature of high-level ampicillin resistance in a population of BLNARs, Kaczmarek *et al* (2004) concluded

that variations in OmpP2 could not account for the difference in susceptibility between the ampicillin susceptible control and ampicillin resistant clinical strains.

1.7 Study background

Numerous national and international surveys have illustrated the global increase in the incidence of resistance to the β-lactams amongst clinical isolates of *H. influenzae*. The detection of this resistance in diagnostic laboratories is dependent on the use of a reliable method of testing. A majority of clinical laboratories in the UK use disc diffusion testing as their primary method for the detection of antibiotic resistance (Andrews *et al*, 1996). This method of susceptibility testing is known to be unreliable for the detection of resistance to certain antibiotics in *H. influenzae* and may lead to the inaccurate reporting of the suitability of therapeutic agents.

Following a standardized disc susceptibility testing method (Andrews, 2004), the diagnostic laboratory at City Hospital, Birmingham, noted an increase in the incidence of *H. influenzae* with zones of inhibition close to the zone diameter breakpoints for β-lactams. Some of these isolates appeared to have unusual susceptibly patterns e.g. β-lactamase positive organisms that were resistant to both amoxicillin and cefuroxime and β-lactamase negative isolates that were susceptible to amoxicillin and co-amoxiclav but resistant to cefuroxime. Whilst the reduced β-lactam susceptibility in these isolates may be due to the resistance mechanisms previously described, there is also the possibility that a novel resistance mechanism may be responsible.

1.8 Study aims

This study was undertaken to investigate the prevalence of β-lactam resistance and the range of different resistance phenotypes amongst the local population of *H. influenzae*. Using MIC determination, β-lactamase detection and identification, and nucleotide sequencing of the bla_{TEM} and *ftsI* genes, an attempt was made to characterize the mechanisms of resistance in these isolates. In addition to therapeutic agents currently used in the UK, the antibiotics studied included the carbapenems imipenem, meropenem and ertapenem.

Chapter 2

Materials and Methods

2.1 Organism selection

2.1.1 Isolation and identification

Clinically significant isolates of *H. influenzae* were collected between January 2000 and October 2003 at City Hospital, Birmingham. Organism identification was confirmed by colonial morphology and dependency on X and V factors. Two hundred and fifty organisms were selected on the basis of resistance to amoxicillin and / or cefuroxime using the British Society for Antimicrobial Chemotherapy (BSAC) standardized disc susceptibility testing method (Andrews, 2004). Isolates were stored in 13 % glycerol Tryptone Soya broth (Oxoid Ltd, Basingstoke, UK) at –70°C until required.

2.1.2 Specimen distribution

The age distribution of the patients from whom the study organisms were isolated is illustrated in Figure 2.1. The majority of the isolates were derived from respiratory specimens (61.8%); the remainder from eyes (35.9%) and blood cultures (1.2%) with one each from an ear swab, a vulval swab and pus from a neck abscess. Four out of five isolates originated from community-acquired infections (i.e. an infection in a patient in the community who, if recently hospitalised, has been discharged for at least the preceding 48 hours), 44% of which were from children of five years or younger. The majority (67%) of nosocomial *H. influenzae* infections (i.e. isolated from hospitalised patients in whom infections occurred at least 48 hours post admission) were detected in patients that were at least 60 years old.

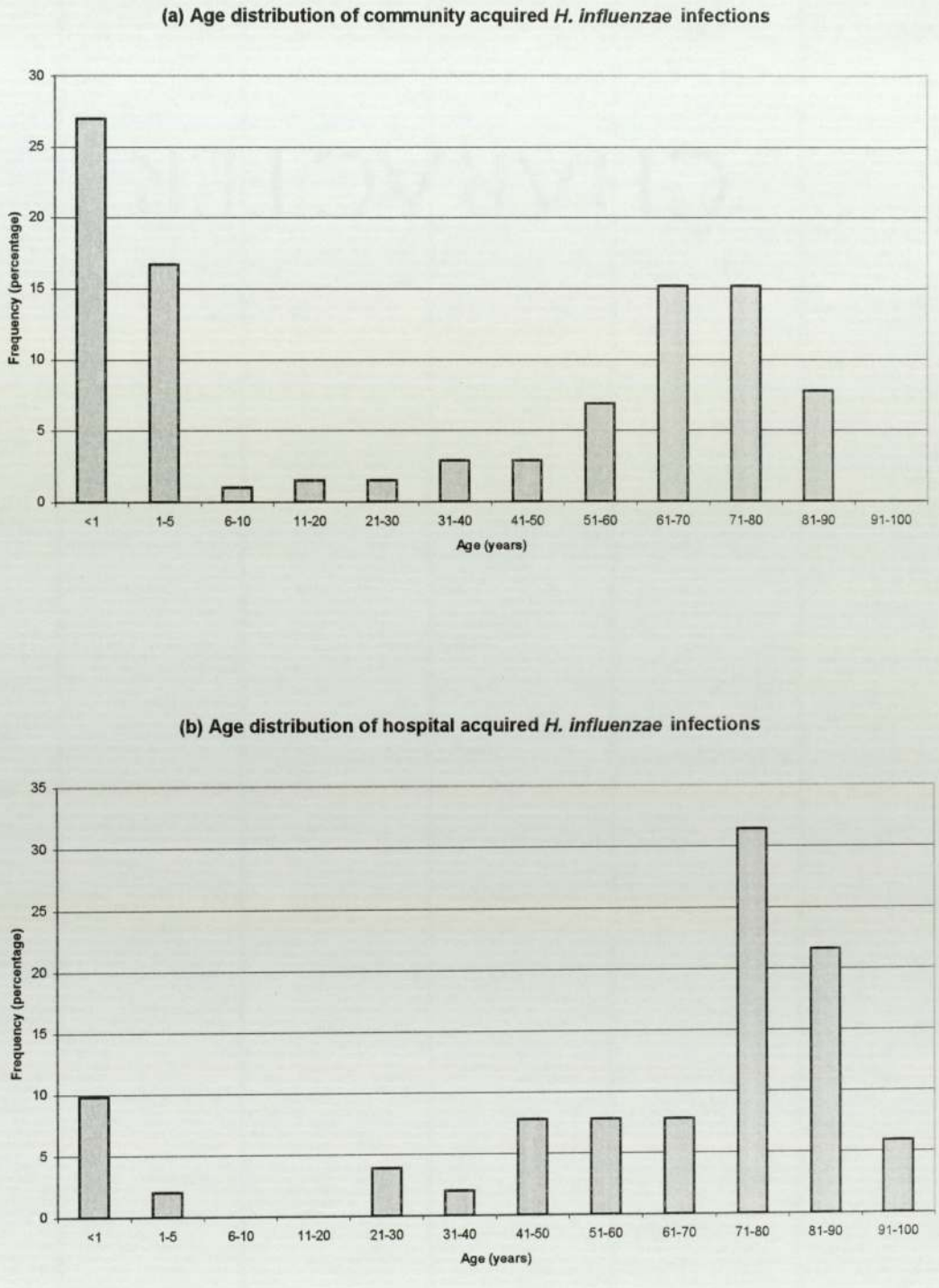


Figure 2.1 Age distribution of patients from whom the study strains of *H. influenzae* were isolated; (a) community acquired infections; (b) hospital acquired infections.

2.2 Antimicrobial susceptibility testing

Throughout this study, interpretation of susceptibility was determined using BSAC MIC (Andrews, 2006) and zone diameter (Andrews, 2004) breakpoints.

2.2.1 Disc susceptibility testing

Zone diameters for amoxicillin, co-amoxiclav and cefuroxime formed the basis for the selection of the population of *H. influenzae* investigated. Additional disc susceptibility testing against imipenem was undertaken on selected strains (Section 4.1.2). All disc susceptibility testing was performed following BSAC methodology (Andrews, 2004). Each new batch of discs and media were controlled using *H. influenzae* ATCC 49247 and NCTC 11931.

2.2.1.1 Preparation of agar plates

Iso-Sensitest agar (Oxoid Ltd., UK) was prepared according to the manufacturer's instructions. When cooled to 42°C, the molten agar was supplemented with 5% v/v defibrinated horse blood (TCS Biosciences Ltd., Botolph Clayton, UK) and NAD (20 mg/L) (Mast Diagnostics, Bootle, UK). The agar was poured into 90 mm sterile plastic Petri dishes to give a depth of 4 mm and allowed to set. The plates were dried in a fan-assisted drying cabinet and stored at 4 – 8°C for a maximum of one week.

2.2.1.2 Preparation of inoculum

Colonies of the clinical isolates and control strains were picked from fresh overnight cultures and suspended in sterile deionised water to give an optical density equivalent to a 0.5 McFarland standard. The suspensions were further diluted 1:100 in sterile deionised water providing an organism suspension of 10^6 cfu/mL.

2.2.1.3 Inoculation and disc application

The inoculum was evenly applied to the surface of the supplemented Iso-Sensitest plate using a sterile cotton-wool swab. The surface of the plate was allowed to dry before application of the antibiotic discs, ensuring that discs were applied within 15 minutes of inoculation.

All antibiotic discs were obtained from Oxoid Ltd. and handled according to the manufacturer's instructions. A maximum of 6 discs were applied to each inoculated 90 mm Petri dish ensuring that contact with the agar was even.

2.2.1.4 Incubation

The plates were incubated within 15 minutes of disc application at 35-37°C in 4-6% CO_2 in air for 18-20 h.

2.2.1.5 Measuring zones and interpretation

The zone diameters were measured to the nearest mm using a ruler. Zone diameters for the control strains were confirmed as being within acceptable limits before interpretation of the test results.

2.2.2 Agar dilution minimum inhibitory concentrations (MICs)

Agar dilution MICs of a panel of 10 β-lactam antibiotics were performed on all clinical isolates. The antibiotics were selected to represent the β-lactam groups used therapeutically for the treatment of *H. influenzae* infections: penicillins (ampicillin and amoxicillin), β-lactamase inhibitor combinations (co-amoxiclav), second-generation cephalosporins (cefuroxime), third-generation cephalosporins (cefotaxime) and carbapenems (imipenem, meropenem and ertapenem). In addition, The MICs of the second-generation cephalosporin cefaclor and the cephamycin cefoxitin were determined; cefaclor to distinguish between ROB and TEM β-lactamase producers (Karlowsky *et al*, 2000) and cefoxitin as it is used as an indicator for β-lactamase mediated resistance in other organism groups (Moritz and Carson, 1986; Skov *et al*, 2003). BSAC methodology (Andrews, 2006) was followed and *H. influenzae* ATCC 49247, NCTC 11931 and ATCC 51907 (Rd) were included for quality control purposes.

2.2.2.1 Preparation of antibiotic stock solutions

Immediately prior to use, a 1000 mg/L stock solution was prepared for each antibiotic using a known potency powder and was further diluted to provide the range of stock solutions required (Table 2.1).

Table 2.1 Preparation of antibiotic stock solutions for agar dilution MIC testing of *H. influenzae*.

Antibiotic	Supplier	Solvent	Diluent	Stock solutions (mg/L)	Dilution Range (mg/L)
Ampicillin	GlaxoSmithKline, Stevenage, UK	2	Water	1000, 100, 10	0.06 - 128
Amoxicillin	GlaxoSmithKline, Stevenage, UK	2	Water	1000, 100, 10	0.06 - 128
Co-amoxiclav[1]	GlaxoSmithKline, Stevenage, UK	3	Water	1000, 100, 10	0.06 - 128
Cefuroxime	GlaxoSmithKline, Stevenage, UK	Water	Water	1000, 100	0.12 - 32
Cefaclor	Sigma-Aldrich UK Ltd., Poole, UK	Water	Water	1000, 100	0.5 - 128
Cefoxitin	Sigma-Aldrich UK Ltd., Poole, UK	Water	Water	1000, 100	0.25 - 32
Cefotaxime	Aventis, Cedex, France	Water	Water	1000, 100, 10, 1	0.004 - 1
Imipenem	Merck Sharp and Dohme, Hoddesdon, UK	4	4	1000, 100, 10	0.015 - 32
Meropenem	Astra Zeneca, Macclesfield, UK	Water	Water	1000, 100, 10, 1	0.008 - 32
Ertapenem	Merck Sharp and Dohme, Hoddesdon, UK	4	4	1000, 100, 10, 1	0.008 - 32
Erythromycin[6]	Sigma-Aldrich UK Ltd., Poole, UK	5	Water	1000, 100	0.25 - 128
Ethidium Bromide[6]	Sigma-Aldrich UK Ltd., Poole, UK	Water	Water	1000	1 - 128

[1] Solvent/diluent details given are for clavulanic acid. A fixed concentration of 1 mg/L of clavulanic acid was used for the co-amoxiclav plates; [2] Saturated $NaHCO_3$ solution; [3] 0.1M pH6.0 phosphate buffer; [4] 0.5M pH6.8 MOPS buffer; [5] Ethanol; [6] Efflux pump studies.

2.2.2.2 Preparation of agar dilution plates

For each antibiotic, sterile plastic universal containers and Petri dishes were labelled with the antibiotic name and concentration (mg/L) for the range of dilutions required. An antibiotic free control plate was included in each batch of MIC plates prepared. Volumes of antibiotic stocks were dispensed into the labelled universal containers as stated in Table 2.2. An appropriate volume of Iso-Sensitest agar (Oxoid Ltd., UK) was prepared following the manufacturer's instructions and allowed to cool to 42°C. The media was supplemented with 5% v/v defibrinated horse blood (TCS Biosciences Ltd., UK) and NAD (Mast Diagnostics, UK) (20 mg/L). Agar was dispensed into the antibiotic-containing universals, mixed gently by inverting the container and poured into the appropriately labelled Petri dish. Once set, the plates were dried for approximately 10 minutes in a fan-assisted hot air drying cabinet. Plates were used on the day of manufacture and stored at 4-8°C, protected from UV light, until inoculated.

Table 2.2 Volume of antibiotic stock solutions dispensed to achieve required concentration in 20 mL agar plates.

Final concentration (mg/L)	Volume of stock (µL)	Stock concentration (mg/L)
128	2560	1000
64	1280	1000
32	640	1000
16	320	1000
8	160	1000
4	80	1000
2	40	1000
1	20	1000
0.5	100	100
0.25	50	100
0.12	25	100
0.06	120	10
0.03	60	10
0.015	30	10
0.008	160	1
0.004	80	1

2.2.2.3 Preparation of inoculum

Colonies of the clinical isolates and control strains were picked from fresh overnight cultures and suspended in sterile deionised water to give an optical density equivalent to a 0.5 McFarland standard. The suspensions were further diluted 1:10 in sterile deionised water providing an organism suspension of 10^7 cfu/mL.

2.2.2.4. Inoculation and incubation

A multipoint inoculator (Mast Diagnostics, UK) was used to deliver 1 µL of each organism suspension onto the surface of the agar plates. The inoculum was allowed to dry before the plates were incubated at 35-37°C in 4 – 6 % CO_2 in air for 18-20 h.

2.2.2.5. Reading and interpretation

After incubation, the antibiotic-free control plates were examined to ensure that all organisms had grown. The MIC was defined as the lowest concentration of antibiotic at which there was no visible growth of the organism; a fine film of growth or the presence of one or two colonies was disregarded. The test MIC results were only accepted if the control MIC values were within one doubling dilution of the expected concentration.

2.2.3 Comparison of MIC and disc susceptibility testing

MIC determination is considered the Gold Standard method for establishing the susceptibility of an organism. The 250 study organisms were selected on the basis of reduced susceptibility to one or more of amoxicillin, co-amoxiclav and cefuroxime as determined by disc susceptibility testing (Andrews, 2004). The MIC values of the study organisms were compared to their respective disc susceptibility testing results and the correlation between MIC and disc testing data determined.

2.2.4 Use of MICs for efflux pump studies

The MIC of ethidium bromide, a known efflux pump substrate (Sanchez *et al*, 1997), was determined for the 250 clinical isolates using an agar dilution method (Section 2.2.2.). The MIC of ethidium bromide for the fully susceptible Rd strain has been variously reported as 1-4 mg/L (Sanchez *et al*, 1997; Trepod and Mott, 2004). MICs of ampicillin, ethidium bromide and erythromycin in the presence of the efflux pump inhibitors reserpine (Sigma-Aldrich Co. Ltd., UK) and phe-arg β-naphthylamide dihydrochloride (PAβN) (Sigma-Aldrich Co. Ltd., UK) were performed on clinical isolates with MICs of ethidium bromide of greater than or equal to 8 mg/L i.e. at least one doubling dilution higher than the expected value for *H. influenzae* Rd.

Reserpine (10 mg) was dissolved in 1 mL chloroform in a glass container and added to 1 L molten Iso-Sensitest agar (Oxoid Ltd., UK) providing a final concentration of reserpine of 10 mg/L. The agar was swirled in a well-ventilated area until the chloroform had fully evaporated. PAβN (10 mg) was dissolved in a minimal amount of sterile deionised water and added to 500 mL of molten Iso-Sensitest agar (Oxoid Ltd., UK) providing a final concentration of PAβN of 20 mg/L. In both cases, 5% v/v defibrinated horse blood (TCS Biosciences Ltd., UK) and 20 mg/L NAD (Mast Diagnostics, UK) were added and the agar used immediately to pour the required MIC plates.

In addition to the Rd strain and ATCC 49247, the MICs were controlled with Pf 1311 and Pf 1312; two strains of *H. influenzae* with high-level BLNAR and known mutations in *acrR*, the AcrAB repressor gene (Kaczmarek *et al*, 2004).

2.3 β-lactamase detection and identification

2.3.1 β-lactamase detection

2.3.1.1 Chromogenic method (Nitrocefin)

Nitrocefin is a chromogenic cephalosporin that changes from yellow to red on hydrolysis by a β-lactamase (O'Callaghan *et al*, 1972). It is reported as being the most sensitive test for the detection of β-lactamases with the exception of staphylococcal penicillinase and the ROB-1 enzyme of *H. influenzae* (Livermore and Brown, 2001).

Nitrocefin powder was obtained in kit form (Oxoid Ltd., UK) and prepared according to the manufacturer's instructions. 5 μL aliquots of reconstituted reagent were dispensed onto filter paper strips in a Petri dish. Using a sterile loop, a single colony of the test organism was applied to the impregnated paper. A colour change of yellow to red within 15 minutes indicated the presence of β-lactamase. Each batch of tests included known positive and negative controls.

2.3.1.2 Acidimetric method (Intralactam)

The hydrolysis of benzyl-penicillin by a β-lactamase generates penicilloic acid. The resulting acidity may be detected using a pH indicator. Intralactam strips (Mast Diagnostics, UK) are commercially prepared filter paper strips impregnated with benzyl-penicillin and the pH indicator bromocresol purple. This acidimetric test is suitable for the detection of β-lactamase in *H. influenzae* (Livermore and Brown, 2001).

The Intralactam strips were placed in a Petri dish and moistened with deionised water before use. A single colony of the test organism was applied to the paper strip using a sterile loop. The development of a yellow colour within 10 minutes indicated the presence of β-lactamase. Positive and negative control organisms were included with each batch of tests.

2.3.2 Detection of *bla*$_{TEM}$ and *bla*$_{ROB}$ by the polymerase chain reaction (PCR)

2.3.2.1 Template DNA preparation

DNA was prepared from all β-lactamase positive isolates. Ten colonies from an overnight culture were suspended in 100 µL sterile deionised water in a 0.5 mL capped micro tube. The organism suspensions were heated to 95 °C for 15 minutes in a heating block then centrifuged at 13,000 *g* for 2 minutes. The supernatants were frozen at –20°C and diluted 1:5 in sterile deionised water for use.

2.3.2.2. Amplification of *bla*$_{TEM}$ and *bla*$_{ROB-1}$ by PCR

All PCR reactions were carried out in thin-walled domed cap 0.2 mL PCR tubes using a programmable ThermoHybaid Omn-E thermocycler (Hybaid, Middlesex, UK). TEM1, TEM2, ROB1 and ROB2 primers (Table 2.3) (Scriver *et al*, 1994) were obtained from Alta Bioscience (University of Birmingham, UK). Individual deoxynucleotide triphosphates (dNTPs), HotStar Taq DNA polymerase and reaction buffer (including $MgCl_2$) were obtained from Qiagen Ltd. (Crawley, UK).

The PCR reactions were carried out in 30 µL volumes comprising 15 µL reaction mixture (including Taq DNA polymerase), 2 µL template DNA and 13 µL distilled water. Known *bla*$_{TEM-1}$ (*H. influenzae* ATCC 33930) and *bla*$_{ROB-1}$ (*H. influenzae* Glaxo 2499E) positive controls were included in each batch of PCR reactions and distilled water replaced template DNA for the negative control. The sequence of the primers, details of the reaction mixture and PCR conditions are provided in Table 2.3.

The TEM1 primer is complementary to a sequence of DNA outside the *bla*$_{TEM}$ gene whilst TEM2 is complementary to an intragenic region. Using the whole chromosome nucleotide sequence of *H. influenzae* Rd (Genome Sequence Database accession number L42023) additional primers, TEM3 and TEM4 (Alta Biosciences, UK), were designed in-house (personal communication, Dr N. P. Brenwald) to amplify an intragenic region of *bla*$_{TEM}$. The TEM3 and TEM4 primers were used to perform additional PCR on all β-lactamase positive isolates with negative PCR results using the TEM1, TEM2, ROB1 and ROB2 primers.

Table 2.3 Primers, reaction mixture and PCR conditions used for the amplification of *bla*$_{TEM}$ and *bla*$_{ROB}$ in β-lactamase positive *H. influenzae*.

Primers

bla$_{TEM}$	(*600 bp amplicon*)	
	TEM1 (forward)	5' –GTG TTA TCA CTC ATG GTT ATG- 3'
	TEM2 (reverse)	5' –GAA TTC TTG AAG ACG AAA GGG- 3'
Intragenic *bla*$_{TEM}$	(*351 bp amplicon*)	
	TEM3 (forward)	5' –CCG CAT ACA CTA TTC TCA GA- 3'
	TEM4 (reverse)	5' –GCC TCC ATC CAG TCT ATT AA- 3'
bla$_{ROB}$	(*434 bp amplicon*)	
	ROB1 (forward)	5' –CGC CCA ATT CTG TTC ATT- 3'
	ROB2 (reverse)	5' –GTT GAT ATT GTT CCA CGC- 3'

Reaction mixture

(*sufficient for 3 PCR reactions*)

10 µL	dNTP mixture	2mM of each dNTP
10 µL	Reaction buffer	As supplied by Qiagen Ltd. (*contains 15 mM $MgCl_2$*)
1 µL	HotStar Taq	As supplied by Qiagen Ltd.
19 µL	Sterile distilled water	
5 µL	Forward primer	20 pmol/mL
5 µL	Reverse primer	20 pmol/mL

PCR conditions

95°C	5 minutes	1 cycle
55°C	45 seconds }	
72°C	45 seconds }	30 cycles
95°C	45 seconds }	
72°C	10 minutes	1 cycle

2.3.2.3. Visualisation of PCR products

Agarose gel electrophoresis and ethidium bromide staining were used to detect and estimate the size of the amplified DNA fragments. PCR product and gel loading dye (Qiagen Ltd., Crawley, UK) were mixed in the ratio 5:1 and loaded onto a 1% electrophoresis grade agarose gel (Sigma-Aldrich Co. Ltd., UK) dissolved in 0.5X TRIS-Borate-EDTA buffer (TBE buffer; 89 mM TRIS; 89 mM boric acid; 2 mM EDTA). Electrophoresis was performed for 30 minutes at 100 V and included a 1Kb GeneRuler™ DNA ladder (Fermentas, York, UK). Gels were stained in 0.5 μg/mL ethidium bromide solution for 30 minutes and the DNA bands visualised using a UV transilluminator with photographs taken where necessary.

2.3.3 Nucleotide sequencing of *bla*$_{TEM}$

2.3.3.1 Purified template DNA preparation

Purified template DNA was prepared from overnight cultures of selected organisms using a Qiagen QIAamp® DNA mini kit (Qiagen Ltd., Crawley, UK) following the manufacturer's instructions for tissue samples, and stored at –20°C until required.

2.3.3.2 Amplification of *bla*$_{TEM}$ by PCR

The PCR reactions were carried out as for section 2.3.2.2 using TEM1 and TEM2 primers and purified template DNA.

2.3.3.3 Purification of PCR products

The amplified PCR products were purified using a QIAquick® PCR purification kit (Qiagen Ltd., UK) following the protocol for micro centrifugation. The size and yield of the purified DNA fragments was analysed by agarose gel electrophoresis, running the samples alongside a GeneRuler™ 1 kb DNA ladder (Fermentas, UK). The purified products were stored at -20°C.

2.3.3.4. Nucleotide sequencing of *bla*$_{TEM}$

The nucleotide sequence of the amplified *bla*$_{TEM}$ was determined using automated fluorescence sequencing (MWG-Biotech UK Ltd., Milton Keynes, UK). The deduced amino-acid sequence was determined using the ExPASy proteomic server (www.expasy.org/ tools) and compared with the published sequence data for TEM

enzymes in *H. influenzae* (Molina *et al*, 2003; Tristram *et al*, 2005; Tristram *et al*, 2007).

2.4 Detection of Penicillin Binding Proteins using Bocillin™ FL

The commercially available synthetic fluorescent penicillin Bocillin™ FL (Invitrogen, Paisley, UK) (Figure 2.2) has been reported as a viable alternative to radioactive labelling agents for the detection and study of penicillin binding proteins (PBPs) (Zhao *et al*, 1999).

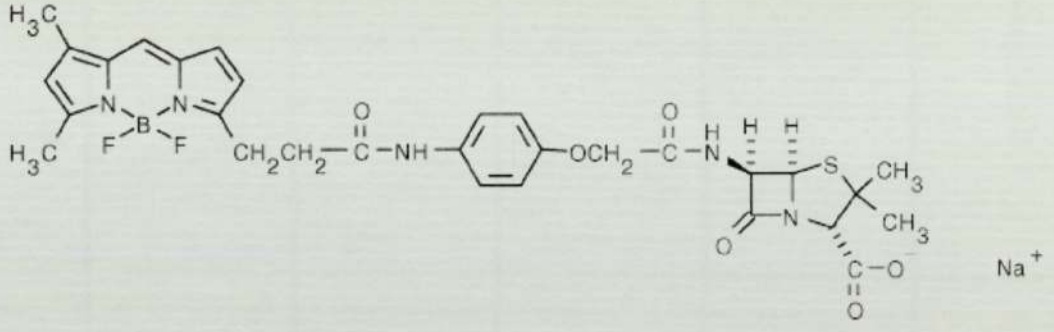


Figure 2. 2 The structure of Bocillin™ FL

2.4.1 Membrane preparation

Cell membrane preparations were manufactured using an adaptation of the method described by Morikawa *et al* (2004). Overnight plate cultures of selected *H. influenzae* clinical isolates and the ATCC 49247 and Rd controls were harvested to make heavy organism suspensions in 10 mL cold 1/15 M phosphate buffer (pH 7.0) in sterile plastic universals. The cells were disrupted by passing three times through an Aminco French press at 7000 – 10000 lb/in^2. Following centrifugation at 6000 *g* for 5 minutes at 4°C, the supernatant was transferred to fresh tubes and spun at 15000 *g* for 1 h at 4°C. The supernatant was discarded and the pellet resuspended in 0.5 mL cold 1/15 M phosphate buffer (pH 7.0). These crude membrane preparations were divided into 100 µL aliquots and frozen at -20°C until required.

2.4.2 Bocillin™ FL assay

A 1.5 mM stock solution of Bocillin™ FL was prepared and stored at -20°C, protected from UV light, until required. The stock was diluted 1:15 in sterile distilled water (0.1 mM) for use. 15 µL of membrane preparation was mixed with 5 µL 0.1 mM Bocillin™ FL in a 200 µL capped micro tube and incubated at 37°C for 30 minutes. Following the addition of 20 µL of sample denaturing buffer (Table 2.4) the mixture was heated to 100°C for 3 minutes in a heating block. This mixture could then be refrigerated overnight, frozen at –20°C or directly loaded onto an electrophoresis gel.

2.4.3 Preparation of the SDS-PAGE system

Reagents were prepared as indicated in Table 2.4 leaving the addition of TEMED and ammonium persulphate solution (AMPS) until immediately before use. A 10 % acrylamide separating gel was cast using a Mini Protean system (BioRad, Hemel Hempstead, UK). When set, stacking gel was added and appropriate combs placed to cast loading wells.

2.4.4 Running the SDS-PAGE system

The gels were positioned in a Mini Protean 3 electrophoresis tank (BioRad, UK) and immersed in electrode buffer before loading with 15 µL of each sample and ProtoMarkers™ protein standard (Geneflow, Fradley, UK). Electrophoresis was performed at 200 V until the marker dye reached the end of the gel (approximately 40 minutes). The gels were washed in distilled water for one hour before the fluorescent bands were observed using a Gene Genius Bioimaging system (Syngene, Cambridge, UK).

Table 2.4 Reagents used to prepare the SDS-PAGE system.

	Separating gel	**Stacking gel**	**Sample denaturing buffer**	**Electrode (running) buffer**
Stock 1[1]	4.8 mL	-	-	-
Stock 2[2]	-	2.5 mL	-	-
10% w/v SDS[3]	0.5 mL	150 µL	5.0 mL	20 mL
1.5M Tris-HCl buffer (pH 8.8)	6.2 mL	-	-	-
0.5M Tris-HCl buffer (pH 6.8)	-	3.8 mL	2.5 mL	-
Double-distilled water	8.2 mL	8.0 mL	5.0 mL	$\leq$ 2000 mL
TEMED[4]	50 µL	40 µL	-	-
10% v/v AMPS[5] (freshly prepared)	70 µL	50 µL	-	-
Glycerol	-	-	2.5 mL	-
2-mercaptoethanol	-	-	250 µL	-
Bromophenol blue (5% w/v)	-	-	200 µL	-
Tris	-	-	-	6.0 g
Glycine (biochemical grade)	-	-	-	28.8 g

[1] Stock 1 Solution of 44% w/v acrylamide and 0.8% w/v bis(N,N[1]-methylene) bisacrylamide

[2] Stock 2 Solution of 30% w/v acrylamide and 0.8% w/v bis(N,N[1]-methylene) bisacrylamide

[3] SDS Sodium dodecyl sulphate

[4] TEMED N,N,N',N' - Tetramethylethylenediamine

[5] AMPS Ammonium persulphate solution

All reagents obtained from Sigma-Aldrich Co. Ltd.

2.5 Mutant selection

Using the methodology described by Clark (Clark *et al*, 2002), serial sub-culture in imipenem containing broth media was used to select imipenem resistant mutants from two parent strains with known mutations in the transpeptidase region of *ftsI* (the BLNAR control strain ATCC 49247 and clinical strain Pf 1370 (Kaczmarek *et al*, 2004)) and the fully susceptible *H. influenzae* Rd.

2.5.1 Preparation of supplemented broth

Brain heart infusion broth (BHIb) (Oxoid Ltd., UK) was made according to manufacturer's instructions and dispensed into glass bijoux in 5.0 mL aliquots. Immediately prior to use, Haemophilus Test Medium supplement (HTMs) (Oxoid Ltd., UK) was reconstituted according to manufacturer's instructions and added to the BHIb to provide a supplemented broth (sBHI) with a final concentration of NAD and haemin of 15 mg/L.

2.5.2 Preparation of imipenem-containing broth

A 1000 mg/L stock solution of imipenem was made in 0.5 M MOPS buffer pH 6.8 from known potency powder (Table 2.1.). The stock was dispensed into 1.0 ml aliquots in 2.0 ml screw topped cryo-tubes and stored at –70°C for a maximum of one month (Andrews, 2006). For use, the 1000 mg/L stock was diluted as required in 0.5 M MOPS buffer pH 6.8 (Table 2.5).

For each parent strain, a series of sBHI was prepared providing imipenem concentrations three doubling dilutions either side of the MIC (Table 2.6). Each series also contained an antibiotic-free control broth. On subsequent days, a fresh series of five broths were prepared with doubling dilutions of imipenem centred on the concentration supporting the same density of growth as the antibiotic free control.

Table 2.5 Volume of antibiotic stock solutions dispensed to achieve required concentration of imipenem in 5.0 mL aliquots of supplemented Brain Heart Infusion broth (Oxoid Ltd., UK)

Final concentration (mg/L)	Volume of stock (µL)	Stock concentration (mg/L)
16	80	1000
8	40	1000
4	20	1000
2	100	100
1	50	100
0.5	25	100
0.25	125	10
0.12	60	10
0.06	30	10

Table 2.6 Initial range of imipenem containing sBHI prepared for each parent strain.

Parent strain	MIC of imipenem (mg/L)	Dilution range (mg/L)
ATCC 49247	0.5	0.06, 0.12, 0.25, 0.5, 1.0, 2.0, 4.0
Pf 1370	0.5	0.06, 0.12, 0.25, 0.5, 1.0, 2.0, 4.0
Rd	0.25	0.03, 0.06, 0.12, 0.25, 0.5, 1.0, 2.0

2.5.3. Inoculum preparation

For each parent strain, five colonies taken from an overnight purity plate were used to inoculate an antibiotic-free 5.0 mL sBHI and purity plate. Following confirmation of culture purity, the freshly prepared series of imipenem-containing sBHI were inoculated with 25 µL of overnight broth culture (i.e. approximately 5 x 10^5 cfu). A purity plate was made from each inoculum and plates and broths incubated for 24 hours at 35 - 37°C in 4-6% CO_2.

2.5.4. Serial passages

Serial passages were performed on a maximum of 45 occasions or until the MIC of imipenem of the selected organism was at least four-fold higher than that of the parent strain.

The inoculum for each passage was taken from the broth with the highest imipenem concentration supporting growth equivalent to the antibiotic-free control. A fresh series of imipenem-containing sBHI was prepared on each occasion, using the imipenem concentration of the inoculum broth as the centre point for the dilution series.

If necessary, a break in passages was arranged by centrifuging the culture to be used as the next inoculum at 3000 *g* for 5 minutes, resuspending the cell pellet in 100 µL of 13 % glycerol Tryptone Soya broth (Oxoid Ltd., UK) and storing at –70°C until required. For use, an overnight plate culture of the recovered organism was used to prepare an organism suspension of 10^8 cfu/mL by comparison with a 0.5 McFarland standard (Andrews, 2004). Each selection broth was inoculated with 40 µL of the organism suspension i.e. an inoculum of approximately 5 x 10^5 cfu.

2.6 Transformation of *H. influenzae* Rd

Following the methodology of Barcak *et al* (1991), chromosomal DNA and amplified *ftsI* PCR product were prepared from donor strains and used to transform competent Rd cells.

2.6.1. Preparation of chromosomal DNA

Purified chromosomal DNA was prepared from selected donors as described in Section 2.3.3.1.

2.6.2. Amplification of *ftsI*

Template DNA was prepared as in Section 2.3.2.1. PCR reactions were carried out in thin-walled domed cap 0.2 mL PCR tubes using a programmable ThermoHybaid Omn-E thermocycler (Hybaid, UK). LongFTS1 and LongFTS2 primers (Table 2.7) were designed in-house (personal communication, Dr N. P. Brenwald) to include the whole *ftsI* motif and approximately 1Kb either side of *ftsI* (total fragment size 4.486 Kb). The primers were obtained freeze-dried from Alta Bioscience. HotStar Taq DNA polymerase, reaction buffer (including $MgCl_2$) and individual deoxynucleotide triphosphates (dNTPs) were supplied by Qiagen Ltd.

The PCR reactions were carried out in 30 µL volumes comprising 15 µL reaction mixture (including Taq DNA polymerase), 5 µL template DNA, and 10 µL water. *H. influenzae* Rd was used as a positive control and sterile distilled water replaced template DNA for the negative control. The sequence of the primers, details of the reaction mixture and PCR conditions are provided in Table 2.7.

Agarose gel electrophoresis and ethidium bromide staining were used to visualise the PCR products (Section 2.3.2.3). The PCR products were purified (Section 2.3.3.3) and stored at -20°C until required.

Table 2.7 Primers, reaction mixture and PCR conditions used for the amplification of the whole *ftsI* gene in *H. influenzae*.

Primers

Whole *ftsI*	(*4486 bp amplicon*)	
	LongFTS1 (forward)	5' – ATTGGCTCCAGCACAGATTT- 3'
	LongFTS2 (reverse)	5' –TGGCTTTGTGATTCTGTTCG- 3'

Reaction mixture

(*sufficient for 3 PCR reactions*)

10 µL	dNTP mixture	2 mM of each dNTP
10 µL	Reaction buffer	As supplied by Qiagen Ltd. (*contains 15 mM $MgCl_2$*)
1 µL	HotStar Taq	5 units/µL
19 µL	Sterile distilled water	
5 µL	LongFTS1 primer	20 pmol/µL
5 µL	LongFTS2 primer	20 pmol/µL

PCR conditions

95°C	5 minutes	1 cycle
54°C	30 seconds }	
72°C	5 minutes }	10 cycles
95°C	15 seconds }	
54°C	30 seconds }	
72°C	5 minutes + 5 seconds }	20 cycles
95°C	15 seconds }	
72°C	10 minutes	1 cycle

2.6.3 Preparation of M-IV transformation medium

M-IV transformation medium was prepared following the protocol of Barcak *et al* (1991). Fresh medium was manufactured for each transformation experiment from stock solutions prepared as indicated in Table 2.8; solution 21 was dispensed into 50 mL aliquots and 0.5 mL each of solutions 22, 23, 24 and 40 added immediately before use.

2.6.4 Induction of competence in *H. influenzae* Rd

Ten colonies from an overnight culture of Rd were used to inoculate 35 mL of sBHI in a sterile 500 mL glass bottle. The broth was shaken at 180 rpm at 35-37°C. At intervals, 1mL aliquots were removed and the optical density at 650 nm (OD_{650}) determined using a PerkinElmer Lambda 2 spectrophotometer (PerkinElmer LAS (UK) Ltd., Beaconsfield, UK). When the OD_{650} reached 0.2 (after approximately 3.5 h), 10 mL of the exponential culture was transferred to a sterile plastic universal container and centrifuged at 5000 *g* for 5 min at room temperature. The supernatant was discarded and replaced with 10 mL fresh M-IV medium. The cell suspension was transferred to a sterile 250 mL glass bottle and shaken at 100 rpm for 100 minutes at 37°C. The competent cells were stored in 1mL aliquots at -70°C in 13% sterile glycerol. For use, the cells were defrosted and centrifuged at 5000 *g* for 2 minutes. The supernatant was discarded and the cells resuspended in fresh M-IV medium.

Table 2.8 Preparation of M-IV competence-inducing medium.

Component	Concentration
Solution 21	
Deionised water	850 mL
L-Aspartic acid	4.0 g
L-Glutamic acid	0.2 g
Furmaric acid	1.0 g
NaCl	4.7 g
Tween 80	0.2 mL
K_2HPO_4	0.87 g
KH_2PO_4	0.67 g
Adjust to pH 7.4 with 4N NaOH. Make up to 1000 mL with deionised water. Aliquot in 50 mL volumes and sterilise by autoclaving.	
Solution 22	
L-Cystine	0.04 g
L-Tyrosine	0.1 g
Dissolve in 10 mL 1N HCl at 37°C. Make up to 10 ml with deionised water then add:	
L-Citrulline	0.06 g
L-Phenylalanine	0.2 g
L-Serine	0.3 g
L-Alanine	0.2 g
Sterilise by filtering with a 0.22 μm membrane	
Solution 23	
$CaCl_2$	0.1 M solution
Sterilise by autoclaving.	
Solution 24	
$MgSO_4$	0.1 M solution
Sterilise by autoclaving	
Solution 40	
5% (w/v) solution of vitamin assay casamino acids (Difco) in deionised water. Sterilise by filtering with a 0.22 μm membrane	

NaCl, sodium chloride; K_2HPO_4, dipotassium hydrogen phosphate; KH_2PO_4, potassium dihydrogen phosphate; NaOH, sodium hydroxide; HCl, hydrochloric acid; $CaCl_2$, calcium chloride; $MgSO_4$, magnesium sulphate.

2.6.5. Preparation of selective plates

Fresh selective plates containing imipenem, meropenem, ertapenem and ampicillin were prepared as for MIC testing (Section 2.2.2.2) using Columbia agar (Oxoid Ltd., UK) supplemented with 5% v/v defibrinated horse blood (TCS Biosciences Ltd., UK) and 20 mg/L NAD (Mast Diagnostics, UK). The range of antibiotic concentrations used is given in Table 2.9.

Table 2.9 Dilution range for the antibiotics used in the preparation of selective plates for the transformation of *H. influenzae* Rd.

		MIC (mg/L)	
Antibiotic	**Dilution range (mg/L)**	**Rd**	**ATCC 49247**
Imipenem	0.12, 0.25, 0.5, 1.0, 2.0, 4.0	0.25	0.5
Meropenem	0.008, 0.015, 0.03, 0.06, 0.12	0.03	0.25
Ertapenem	0.008, 0.015, 0.03, 0.06, 0.12	0.03	0.25
Ampicillin	0.12, 0.25, 0.5, 1.0, 2.0	0.25	4

2.6.6. Transformation

2.6.6.1. Control of transformation

To differentiate between the generation of spontaneous mutants and genuine transformants, positive (competent Rd cells with chromosomal ATCC 49247 DNA) and negative (competent Rd cells with no additional DNA) controls were included in each experiment.

2.6.6.2. Transformation of *H. influenzae* Rd

For each test and the positive control, approximately 1 µg of chromosomal DNA or 250 ng of purified *ftsI* PCR product was added to 1 mL of competent Rd cells and mixed gently. Following incubation at 35 -37°C for 15 min, the transformation mixes

were spun at 5000 *g* for 2 min and the cell pellet resuspended in 600 µL of the supernatant. Viable counts were performed on all cell suspensions (Miles *et al*, 1938). Known volume aliquots of the cells were spread evenly over the surface of selective plates using sterile disposable plastic loops and allowed to air dry before incubating at 35–37 °C in 4-6% CO_2. All plates were examined after 24 and 48 h and the number of colonies recorded. Representative colonies growing on selective plates of an antibiotic concentration at least one doubling dilution higher than the negative control were selected at random and sub-cultured onto fresh selective plates of the same antibiotic concentration. Organisms surviving subculture were considered to be transformants and were preserved in 12% glycerol Tryptone soya broth (Oxoid Ltd., UK) at –70°C.

The frequency of transformation was defined as the number of transformants per mL of transformation mixture divided by the number of competent cells per mL of transformation mixture.

The MICs of imipenem, ertapenem, meropenem, ampicillin, cefuroxime and cefotaxime of randomly selected transformants were determined and compared with those of *H. influenzae* Rd and the appropriate DNA donor.

2.7 Nucleotide sequencing of the transpeptidase region of *ftsI*

For selected clinical isolates and parent/mutant/transformant groups, nucleotide sequencing of the transpeptidase region of *ftsI* was performed and a comparison of the deduced amino acid sequences undertaken.

2.7.1. Amplification of the transpeptidase region of *ftsI* by PCR

Purified template DNA was prepared as in Section 2.3.3.1. PCR reactions were carried out in thin-walled domed cap 0.2 mL PCR tubes using a programmable ThermoHybaid Omn-E thermocycler (Hybaid, UK). HINF1 and HINF2 primers (Dabernat *et al*, 2002) were obtained freeze-dried from Alta Bioscience. HotStar Taq DNA polymerase, reaction buffer (including $MgCl_2$) and individual deoxynucleotide triphosphates (dNTPs) were supplied by Qiagen Ltd.

The PCR reactions were carried out in 30 µL volumes comprising 15 µL reaction mixture (including Taq DNA polymerase), 5 µL template DNA, and 10 µL water. *H. influenzae* Rd was used as a positive control and sterile distilled water replaced template DNA for the negative control. The sequence of the primers, details of the reaction mixture and PCR conditions are provided in Table 2.10.

2.7.2. Visualisation of PCR products

Agarose gel electrophoresis and ethidium bromide staining was used to visualise the PCR products as in Section 2.3.2.3.

2.7.3 Purification of PCR products

The amplified *ftsI* PCR products were purified and the size and yield of the DNA fragments determined as in Section 2.3.3.3. The purified products were stored at -20°C.

2.7.4. Nucleotide sequencing of the transpeptidase region of *ftsI*

Nucleotide sequencing of the purified PCR products was performed by automated fluorescence sequencing (MWG-Biotech UK Ltd, Milton Keynes, UK). The deduced amino-acid sequence was determined using the ExPASy proteomic server (www.expasy.org/ tools) and compared with published sequence data for *ftsI* of *H. influenzae* Rd.

Table 2.10 Primers, reaction mixture and PCR conditions used for the amplification of the transpeptidase region of *ftsI* in *H. influenzae*.

Primers		
***ftsI* (transpeptidase region)**	(*705 bp amplicon*)	
	HINF1 (forward)	5' –GTTAATGCGTAACCGTGCAATTACC- 3'
	HINF2 (reverse)	5' –ACCACTAATGCATAACGAGGATC- 3'
Reaction mixture		
(*sufficient for 3 PCR reactions*)		
10 µL	dNTP mixture	2 mM of each dNTP
10 µL	Reaction buffer	As supplied by Qiagen Ltd. (*contains 15 mM $MgCl_2$*)
1 µL	HotStar Taq	5 units/µL
19 µL	Sterile distilled water	
5 µL	HINF1 primer	20 pmol/µL
5 µL	HINF2 primer	20 pmol/µL
PCR conditions		
94°C	5 minutes	1 cycle
55°C	30 seconds }	
72°C	30 seconds }	30 cycles
94°C	30 seconds }	
72°C	10 minutes	1 cycle

2.8 Random amplification of polymorphic DNA (RAPD) profiling

2.8.1. Template DNA preparation

Template DNA was prepared for selected strains as described in Section 2.3.2.1.

2.8.2 PCR for RAPD analysis

PCR reactions were carried out in 25 µL volumes using puRe*Taq*™ Ready-To-Go™ PCR beads (Amersham Biosciences UK Ltd., Little Chalfont, UK), which include dNTPs, puRe*Taq*™ DNA polymerase and reaction buffer. Freeze-dried RAPDA and RAPDB primers (Mitsuda *et al*, 1999) were obtained from Alta Bioscience. Distilled water replaced template DNA for the negative control. Amplification was performed using a programmable ThermoHybaid Omn-E thermocycler (Hybaid, UK). Details of the Reaction mixture and PCR conditions are given in Table 2.11.

2.8.3. Visualization of PCR products

Agarose gel electrophoresis and ethidium bromide staining was used to visualise the PCR products as in Section 2.3.2.3. The isolates were divided into groups on the basis of similarity of their DNA banding patterns.

2.9 Capsular typing

Selected clinical isolates were sent to Dr Mary Slack at the Haemophilus Reference Unit, Health Protection Agency, London, for capsular genotyping using PCR methodology (Falla *et al*, 1994; Van Ketel *et al*, 1990).

Table 2.11. Primers, reaction mixture and PCR conditions used for RAPD typing of *H. influenzae*.

Primers	
RAPDA	5' – TGCCCGTCGT- 3'
RAPDB	5' –GTAGACCCGT- 3'

Reaction mixture		
(*sufficient for a single PCR reaction*)		
Ready-To-Go™ PCR bead containing:		
	dNTPs	200 µM in 10 mM Tris-HCl
	$MgCl_2$	1.5 mM
	puReTaq™ DNA polymerase	2 units
Primer (12.5 pmol/µL)		2 µL
Template DNA		2 µL
Water		21 µL

PCR conditions		
94°C	5 minutes	1 cycle
36°C	1 minute }	
72°C	2 minutes }	40 cycles
94°C	1 minute }	
72°C	7 minutes	1 cycle

Chapter 3

Results for all clinical isolates

3.1 Determination of susceptibility by MIC

Table 3.1 provides a summary of the MIC results for all 250 organisms studied; a complete table of results is provided in Appendix 1. Resistance to one or more of ampicillin/amoxicillin, co-amoxiclav and cefuroxime was demonstrated in 71.2 % of the clinical isolates.

The activity of the agents tested differed little between the 130 β-lactamase producers and 120 β-lactamase non-producers with the expected exceptions of ampicillin and amoxicillin; the β-lactamase positive isolates all had MICs of greater than 1 mg/L for both ampicillin and amoxicillin, whilst 3.3 % of the non β-lactamase producers were resistant to ampicillin and 15.0 % were resistant to amoxicillin. There was a tendency for the MIC of amoxicillin to be equal to, or one doubling dilution higher than, the MIC of ampicillin.

Six (4.6 %) of the β-lactamase producers had reduced susceptibility to co-amoxiclav (MICs of 2 mg/L), so-called β-lactamase positive, co-amoxiclav resistant strains (BLPACR). Interestingly, four of these organisms also had reduced susceptibility to imipenem (MIC of 8 mg/L) and cefuroxime (MIC range 4 - 8 mg/L). Fourteen (11.7 %) of the β-lactamase negative isolates had co-amoxiclav MICs of 2 mg/L with concomitant resistance to amoxicillin; the majority were also resistant to cefuroxime.

β-lactamase production had no effect on the range of MICs of cefuroxime (0.25 – 8 mg/L) although more β-lactamase negative organisms (34.2 %) were resistant to the agent than β-lactamase producers (11.5 %). Of the forty-one cefuroxime resistant β-lactamase negative isolates, 12 (29.3 %) were also resistant to ampicillin and/or amoxicillin.

No high-level BLNAR strains (ampicillin MIC ≥ 4 mg/L) were identified. Four β-lactamase negative isolates (A982, A1267, A1447 and A1758) had ampicillin and amoxicillin MICs of 2 mg/L, placing them in the low level BLNAR or β-lactamase negative ampicillin intermediate (BLNAI) category (CLSI, 2006). All four isolates were resistant to cefaclor (MICs 4-8 mg/L) and susceptible to cefotaxime and the carbapenems. Three of the four isolates were resistant to co-amoxiclav and two to

cefuroxime. An additional 14 β-lactamase negative organisms had MICs of ampicillin of 1 mg/L and MICs of amoxicillin of 2 mg/L; all were resistant to cefaclor (MIC range 4-16 mg/L), and susceptible to cefotaxime and the carbapenems. Twelve of the isolates were resistant to co-amoxiclav (all had MICs of 2 mg/L) and ten were resistant to cefuroxime (MIC range of 2-4 mg/L).

The β-lactamase positive isolates were all susceptible to cefotaxime (MIC range 0.008 – 0.06 mg/L). Whilst all the β-lactamase negative organisms had MICs of cefotaxime below the MIC breakpoint, four of the isolates (A1135, A1267, A1608 and A1758) had MICs above the expected range for the normal wild sensitive population (SRGA, 2006). A1267 and A1758 were BLNAR strains and A1608 was resistant to amoxicillin and co-amoxiclav. Three of the isolates were resistant to cefuroxime and all four were resistant to cefaclor but susceptible to the carbapenems.

Cefaclor and cefoxitin were the least active agents tested; there was an overall cefaclor resistance rate of 92.4% whereas the MIC_{50} / MIC_{90} of cefoxitin (for which there are no MIC breakpoints for *H. influenzae*) were 2/4 mg/L respectively.

The carbapenems were the most active group of β-lactams tested; all the isolates were susceptible to meropenem and ertapenem. The MIC of imipenem was raised in fifteen (6.0 %) of the 250 organisms tested (Range 8-16 mg/L). Although imipenem resistance has been documented in Japan, little work has been carried out elsewhere to determine the distribution or nature of resistance in *H. influenzae*. Consequently, it was decided to dedicate much of the remaining study time to this group of fifteen organisms (Chapter 4. Imipenem resistant *H. influenzae*).

Table 3.1. Summary of MICs of ten β-lactam antibiotics for 250 clinical isolates of *Haemophilus influenzae*.

	Antibiotic	MIC Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	% Resistance
All isolates (n = 250)	**Ampicillin**	0.12 - 64	2	16	53.6
	Amoxicillin	0.25 - 64	2	16	59.2
	Co-amox	0.12 - 2	0.5	1	8.0
	Cefuroxime	0.25 - 8	1	2	22.0
	Cefaclor	≤ 0.5 - 64	4	16	92.4
	Cefoxitin	≤ 0.25 - 32	2	4	*
	Cefotaxime	≤ 0.004 - 0.25	0.015	0.06	0.0
	Imipenem	0.12 - 16	0.5	4	6.0
	Meropenem	≤ 0.008 - 0.5	0.06	0.12	0.0
	Ertapenem	≤ 0.008 - 0.25	0.06	0.12	0.0
BLN (n = 120)	**Ampicillin**	0.12 - 2	0.5	1	3.3
	Amoxicillin	0.25 - 2	1	2	15.0
	Co-amoxiclav	0.12 - 2	1	2	12.5
	Cefuroxime	0.25 - 8	1	2	34.2
	Cefaclor	≤ 0.5 - 16	4	16	93.3
	Cefoxitin	≤ 0.25 - 8	2	8	*
	Cefotaxime	≤ 0.004 - 0.25	0.03	0.06	0.0
	Imipenem	0.12 - 8	0.5	4	3.3
	Meropenem	≤ 0.008 - 0.5	0.06	0.12	0.0
	Ertapenem	≤ 0.008 - 0.25	0.06	0.12	0.0
BLP (n = 130)	**Ampicillin**	2 - 64	16	32	100.0
	Amoxicillin	2 - 64	16	32	100.0
	Co-amoxiclav	0.25 - 2	0.5	1	4.6
	Cefuroxime	0.25 - 8	0.5	2	11.5
	Cefaclor	≤ 0.5 - 64	2	16	91.5
	Cefoxitin	0.5 - 32	2	4	*
	Cefotaxime	0.008 - 0.06	0.015	0.03	0.0
	Imipenem	0.12 - 16	0.5	2	8.5
	Meropenem	0.015 - 0.5	0.06	0.12	0.0
	Ertapenem	0.015 – 0.12	0.06	0.12	0.0

BLN, β-lactamase non-producers; BLP, β-lactamase producers; *There is no BSAC MIC breakpoint for cefoxitin.

3.2. Comparison of MIC and disc susceptibility testing

Correlation between MIC and disc testing data for amoxicillin, co-amoxiclav and cefuroxime ranged between 63.9 – 72.7%. False resistance was greatest with amoxicillin (35.3 %) and false susceptibility greatest with cefuroxime (3.2 %) (Table 3.2). Where there was a discrepancy between the two methods of testing, the majority of the isolates had MIC values that were within a doubling dilution of the breakpoint.

Disc susceptibility testing correctly detected the eighteen β-lactamase negative isolates with raised MICs of amoxicillin (Section 3.1). All the β-lactamase positive organisms were resistant to amoxicillin by disc testing and MIC determination.

Half of the six β-lactamase producing, co-amoxiclav resistant organisms (BLPACR) identified by MIC determination were resistant by disc susceptibility testing. Disc testing data for co-amoxiclav were not recorded for all isolates but for those organisms with results available, 28 β-lactamase producers were considered resistant by disc susceptibility testing but susceptible by MIC determination.

Table 3.2. Comparison of MIC and disc testing data for 250 clinical isolates of *Haemophilus influenzae*.

	Antibiotic		
	Amoxicillin	**Cefuroxime**	**Co-amoxiclav**
BSAC MIC breakpoint (mg/L)	1	1	1
% Correlation between MIC and disc testing	63.9	72.7	67.7
% False resistance[a]	35.3	24.1	29.6
MIC range of falsely resistant[a] isolates (mg/L)	0.12 – 1	0.25 - 1	0.25 - 1
% Falsely resistant[a] isolates with MIC ± one doubling dilution of breakpoint	93.3	95.1	91.1
% False susceptibility[b]	None	3.2	2.6
MIC range of falsely susceptible[b] isolates (mg/L)	NA[c]	2 - 8	2
% Falsely susceptible[b] isolates with MIC ± one doubling dilution of breakpoint	NA[c]	50.0	100

NA, not applicable; [a] False resistance, resistant by disc testing but susceptible by MIC; [b] False susceptibility, susceptible by disc testing but resistant by MIC; [c] All isolates susceptible to amoxicillin by disc testing were also susceptible by MIC testing.

3.3 Efflux pump studies by MIC

Three (1.2 %) out of the 250 clinical isolates (A1146, A1425 and A1528) had raised MICs of ethidium bromide (MIC of 16, 8 and 32 mg/L respectively). All three organisms were β-lactamase producers and were resistant to ampicillin and cefaclor. Isolate A1146 had reduced susceptibility to cefuroxime (MIC of 2 mg/L) and A1425 was resistant to co-amoxiclav (MIC of 2 mg/L). All three isolates were susceptible to cefotaxime and the three carbapenems.

The clinical isolates and control organisms grew well on the reserpine and PAβN control plates. In all cases, the MICs of ampicillin and ethidium bromide did not alter by more than one doubling dilution in the presence of either pump inhibitor (Table 3.3). In contrast, there was at least a twofold reduction in the MIC of erythromycin in the presence of PAβN for all organisms. However, the MIC of erythromycin was unaffected by the presence of reserpine.

These results suggest possible efflux pump involvement in the reduced susceptibility of these organisms to erythromycin but there is little evidence of this resistance mechanism playing a significant part in their ampicillin resistance.

Table 3.3 Effect of efflux pump inhibitors on three clinical *H. influenzae* isolates with raised MICs of ethidium bromide.

		MIC (mg/L)								
Organism	**β-lactamase**	**AMP**	**AMP + Reserpine**	**AMP + PAβN**	**ERY**	**ERY + Reserpine**	**ERY + PAβN**	**EtBr**	**EtBr + Reserpine**	**EtBr + PAβN**
Rd	Negative	0.25	0.25	0.25	8	8	2	2	2	2
ATCC 49247	Negative	4	4	4	8	8	4	4	4	4
Pf 1311*	Negative	16	8	8	4	4	2	4	4	8
Pf 1312*	Negative	4	4	8	16	16	4	16	8	8
A1146	Positive	32	32	32	8	8	4	16	8	16
A1425	Positive	4	2	4	8	8	2	8	8	16
A1528	Positive	4	4	8	16	16	8	32	32	32

AMP, ampicillin; ERY, erythromycin; EtBr, ethidium bromide; PAβN, phe-arg β-naphthylamide dihydrochloride; * BLNAR strains with known efflux pump mutations (Kaczmarek *et al*, 2004)

3.4 β-lactamase mediated resistance

3.4.1 β-lactamase detection

β-lactamase production was detected in 130 (52.0 %) of the 250 isolates using both Nitrocefin and Intralactam methods; no difference was observed between the two detection methods.

3.4.2 β-lactamase identification

Gel visualisation of the PCR products indicated that nineteen (14.6 %) of the β-lactamase positive isolates had the bla_{TEM-1} gene and three (2.2 %) had the bla_{ROB-1} gene. Ninety isolates had bla_{TEM} gene amplification products, which on gel visualisation, appeared to produce shorter DNA fragments than the TEM-1 control suggesting the deletion of a section of the bla_{TEM} gene (Figure 3.1). Henceforth, the β-lactamase produced by these 90 isolates will be referred to as TEMdel, encoded by the bla_{TEMdel} gene.

Eighteen (13.8 %) of the β-lactamase positive isolates had negative results following PCR with the TEM1, TEM 2, ROB1 and ROB2 primers. However, the bla_{TEM} gene of these organisms was successfully amplified using the TEM3 and TEM4 primers. Henceforth, these isolates will be referred to as TEM producers, or as expressing the bla_{TEM} gene in order to differentiate them from the isolates expressing the bla_{TEM-1} or shortened bla_{TEMdel} genes.

None of the 130 organisms tested were found to produce both TEM and ROB-1 enzymes. PCR for bla_{TEM} and bla_{ROB-1} was not performed on isolates with negative results for β-lactamase production with the Nitrocefin and Intralactam tests.

3.4.3 Nucleotide sequencing of the bla_{TEMdel} gene

The nucleotide sequence of the PCR product amplified using the TEM1 / TEM2 primer pair from both A1437 and A1575, showed a 135 bp deletion in the TEM-1 promoter region. The sequence was compared with published data for truncated TEM genes in *H. influenzae* and found to be identical to the 135 bp deletion described by Tristram *et al* (2005).

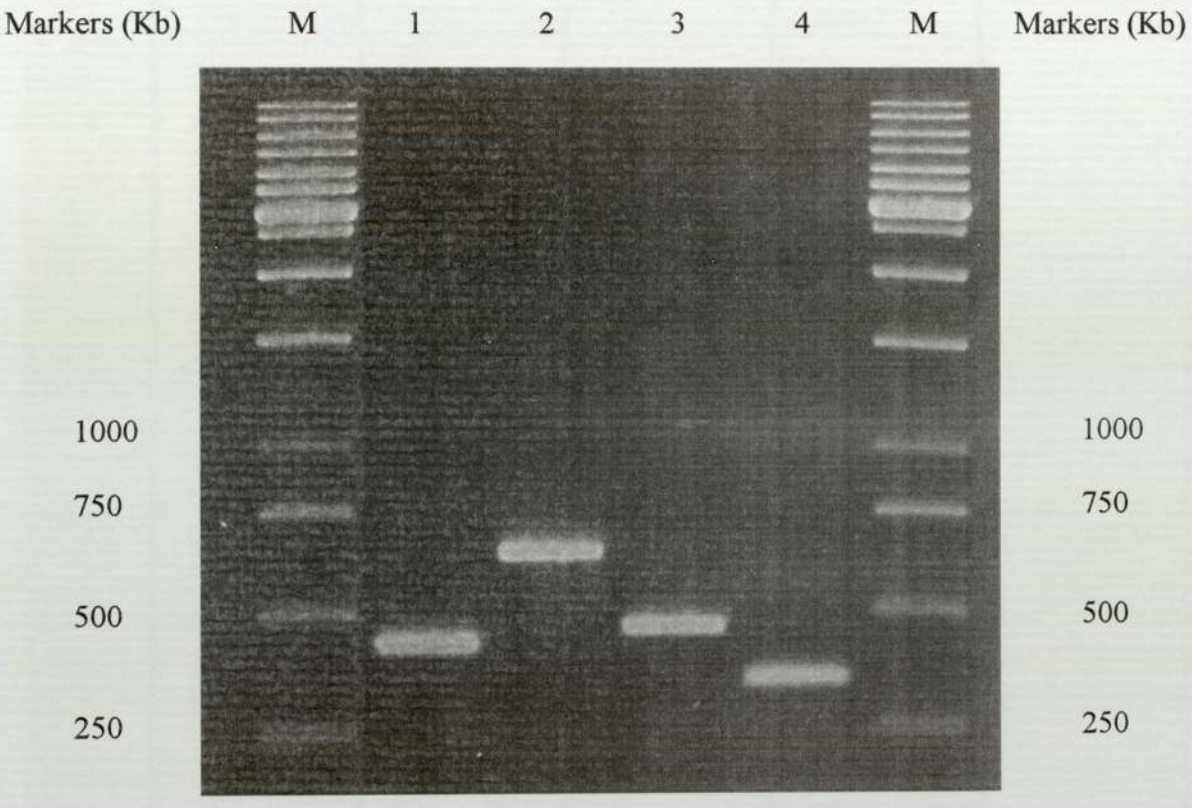


Figure 3.1 Gel visualisation of amplification products following PCR for *bla*$_{TEM}$ and *bla*$_{ROB}$.

Lane 1, *bla*$_{ROB}$ using ROB1 and ROB2 primers; lane 2, *bla*$_{TEM-1}$ using TEM1 and TEM2 primers; lane 3, *bla*$_{TEMdel}$ using TEM1 and TEM2 primers; lane 4, *bla*$_{TEM}$ using TEM3 and TEM4 primers.

3.4.4 MIC distribution in relation to β-lactamase types

Comparison of the antibiotic susceptibility profiles associated with the different β-lactamase types was restricted due to the small number of bla_{ROB} positive isolates (Table 3.4). All the β-lactamase producing isolates were resistant to ampicillin and amoxicillin and susceptible to ertapenem, meropenem and cefotaxime.

Reduced cefuroxime susceptibility was observed in the TEMdel and TEM-1 groups; thirteen of the TEMdel producers and two of the TEM-1 producers had cefuroxime MICs of greater than 1mg/L. All the ROB-1 producers and isolates with bla_{TEM} detected with the intragenic primers were susceptible to cefuroxime.

The β-lactamases of the six BLPACR organisms (Section 3.1) were not restricted to one enzyme type; two produced TEM-1, three had the bla_{TEMdel} gene and one had the bla_{TEM} gene detected with the intragenic primers. The ROB-1 producers were all susceptible to co-amoxiclav.

Only one of the three isolates expressing the bla_{ROB-1} gene was resistant to cefaclor. All the bla_{TEM} producers, 91.1% of the bla_{TEMdel} producers and 84.2% of the bla_{TEM-1} producers were resistant to cefaclor.

There are no MIC breakpoints for cefoxitin and *H. influenzae*; the agent was included in the study as it is a useful tool for detecting β-lactam resistance in other organism groups. From the limited data available, it appears that ROB-1 producers are more susceptible to cefoxitin than the TEM producing group as a whole, whilst the TEMdel producers have the greatest MIC range (1 – 32 mg/L).

Eleven of the fifteen imipenem resistant isolates were β-lactamase producers; ten possessed the bla_{TEMdel} gene; the other strain produced the TEM-1 enzyme. Isolates expressing the bla_{ROB-1} and bla_{TEM} genes were all susceptible to imipenem.

Table 3.4 Distribution of different β-lactamase genes detected by PCR and associated MICs of ten β-lactams.

β-lactamase gene	Antibiotic	MIC Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	% Resistance
bla_{ROB-1}	**Ampicillin**	8 – 16	16	16	100.0
	Amoxicillin	8 – 16	16	16	100.0
(n=3)	**Co-amoxiclav**	0.25 – 0.5	0.25	0.5	0.0
	Cefuroxime	0.5	0.5	0.5	0.0
	Cefaclor	1 – 4	1	4	33.3
	Cefoxitin	1	1	1	-
	Cefotaxime	0.008 – 0.015	0.008	0.015	0.0
	Imipenem	0.25	0.25	0.25	0.0
	Meropenem	0.03	0.03	0.03	0.0
	Ertapenem	0.03	0.03	0.03	0.0
bla_{TEM-1}	**Ampicillin**	2 – 64	8	32	100.0
	Amoxicillin	2 – 64	8	32	100.0
(n=19)	**Co-amoxiclav**	0.25 – 2	0.5	1	10.5
	Cefuroxime	0.25 – 4	0.5	1	10.5
	Cefaclor	≤ 0.5 – 32	2	8	84.2
	Cefoxitin	0.5 – 16	2	4	-
	Cefotaxime	0.008 – 0.06	0.015	0.03	0.0
	Imipenem	0.12 – 8	0.5	2	5.3
	Meropenem	0.015 – 0.12	0.03	0.06	0.0
	Ertapenem	0.015 – 0.12	0.03	0.06	0.0
bla_{TEM}	**Ampicillin**	4 – 32	16	16	100.0
	Amoxicillin	4 – 64	16	16	100.0
(n=18)	**Co-amoxiclav**	0.25 – 2	0.5	0.5	5.6
	Cefuroxime	0.25 – 1	0.5	0.5	0.0
	Cefaclor	2 – 16	2	2	100.0
	Cefoxitin	1 – 8	2	2	-
	Cefotaxime	0.015 – 0.03	0.015	0.015	0.0
	Imipenem	0.25 – 4	0.5	0.5	0.0
	Meropenem	0.03 – 0.12	0.06	0.06	0.0
	Ertapenem	0.03 – 0.06	0.03	0.06	0.0
bla_{TEMdel}	**Ampicillin**	2 – 64	16	32	100.0
	Amoxicillin	2 – 64	16	32	100.0
(n=90)	**Co-amoxiclav**	0.25 – 2	0.5	1	3.3
	Cefuroxime	0.25 – 8	0.5	2	14.4
	Cefaclor	1 – 64	2	16	91.1
	Cefoxitin	1 – 32	2	4	-
	Cefotaxime	0.008 – 0.06	0.015	0.03	0.0
	Imipenem	0.12 – 16	0.5	8	11.1
	Meropenem	0.03 – 0.5	0.06	0.12	0.0
	Ertapenem	0.015 – 0.12	0.06	0.12	0.0

bla_{TEM}, *bla_{TEM}* gene amplified using the intragenic primers TEM3 and TEM4; *bla_{TEMdel}*, *bla_{TEM}* gene with 135 bp deletion

Chapter 4

Imipenem resistant *H. influenzae*

The MIC of imipenem was raised for fifteen (6.0 %) of the 250 study organisms (MIC range 8-16 mg/L) (Table 4.1). This chapter is dedicated to the study of these fifteen clinical isolates and the efforts made to determine the nature of their reduced susceptibility to imipenem.

4.1 Susceptibility testing of clinical isolates

4.1.1 MIC determination

Of the15 imipenem resistant isolates 11 (73.3 %) were TEM producers with MICs of ampicillin and amoxicillin of 16-64 mg/L. One of the β-lactamase positive isolates produced the TEM-1 β-lactamase whilst the shortened bla_{TEMdel} gene was detected in the other ten strains. Four of the β-lactamase producers had reduced susceptibility to co-amoxiclav (MICs of 2 mg/L) and all eleven strains were resistant to cefuroxime (MIC range 2-8 mg/L).

The four β-lactamase negative imipenem resistant isolates had MICs of ampicillin, amoxicillin and co-amoxiclav that were below the MIC breakpoint but above the range for the wild sensitive population of *H. influenzae*. Similarly, the MICs of cefuroxime for the four strains were on or above the MIC breakpoint of 1 mg/L.

Cefaclor resistance was demonstrated in all fifteen isolates (MIC range 8-64 mg/L) and the MIC of cefoxitin was at least double that of the fully susceptible Rd strain. The group were all susceptible to cefotaxime, meropenem and ertapenem.

4.1.2 Imipenem disc susceptibility testing

In all cases, imipenem disc susceptibility testing produced zones of inhibition that were greater than the zone diameter breakpoint of 20 mm (Andrews, 2004) i.e. 100% false susceptibility for the imipenem resistant organisms (Table 4.2.).

Table 4.1. Susceptibility of fifteen imipenem-resistant clinical isolates of *H. influenzae* to ten β-lactam compounds.

		MIC (mg/L)									
Isolate	β-lactamase	IPM	MEM	EPM	AMP	AMX	AMC	CXM	CEC	CTX	FOX
Rd	-	0.25	0.06	0.03	0.25	0.5	0.25	0.5	2	0.008	2
A979	-	8	0.12	0.12	1	1	1	2	16	0.03	8
A1091	-	8	0.12	0.12	0.5	1	1	1	8	0.03	4
A1234	-	8	0.12	0.12	1	1	1	2	16	0.06	4
A1243	-	8	0.06	0.06	0.5	1	0.5	1	8	0.03	8
A1464	+ (TEM-1)	8	0.12	0.12	64	64	2	4	32	0.06	16
A1380	+ (TEMdel)	8	0.12	0.12	32	32	1	2	8	0.06	4
A1437	+ (TEMdel)	8	0.12	0.12	32	32	2	4	16	0.06	4
A1545	+ (TEMdel)	8	0.12	0.12	32	32	1	2	16	0.06	8
A1651	+ (TEMdel)	8	0.12	0.12	32	64	2	8	32	0.06	8
A1736	+ (TEMdel)	8	0.12	0.12	16	32	2	4	16	0.06	4
A965	+ (TEMdel)	16	0.12	0.12	32	64	1	8	32	0.06	16
A1366	+ (TEMdel)	16	0.12	0.12	32	64	1	4	16	0.03	8
A1397	+ (TEMdel)	16	0.12	0.12	32	64	1	2	16	0.03	16
A1525	+ (TEMdel)	16	0.12	0.12	32	16	1	4	32	0.03	16
A1575	+ (TEMdel)	16	0.12	0.12	32	64	1	8	64	0.03	32

IPM, imipenem; MEM, meropenem; EPM, ertapenem; AMP, ampicillin; AMX, amoxicillin; AMC, co-amoxiclav; CXM, cefuroxime; CEC, cefaclor; CTX, cefotaxime; FOX, cefoxitin.

Table 4.2. Zone of inhibition to imipenem 10 µg disc in relation to MIC of imipenem (mg/L) for fifteen imipenem resistant clinical isolates of *H. influenzae*.

Imipenem MIC (mg/L)	Zone diameter (mm)														
	20[b]	21	22	23	24	25	26	27	28	29	30	31	32	33	34
0.25															
0.5															
1															
2															
4[a]															
8						1	3	4	2						
16			1	2	1	1									

Analysis based on BSAC guidelines (version 3) (Andrews, 2004); [a] MIC breakpoint for imipenem is 4 mg/L; [b] zone diameter breakpoint for imipenem is 20 mm

4.2 Study of PBPs using Bocillin FL

Attempts were made to examine the PBPs in membrane preparations of selected imipenem resistant organisms using Bocillin FL. Labelled PBP bands were detected on the agarose gels but their clarity and resolution were not of the standard reported by other workers (Zhao *et al*, 1999; Morikawa *et al*, 2004) and interpretation of the profiles was not possible. This was most likely to be a result of the standard of centrifugation equipment available for membrane preparation. Further work would be required to improve the clarity of the results before this technique could be used to investigate the PBP profiles.

4.3 Efflux pump studies

Table 4.3 shows the effect of efflux pump inhibitors on the MIC of 13 imipenem resistant clinical strains; two of the clinical isolates were omitted from this investigation. The ampicillin susceptibility of *H. influenzae* Rd and the ATCC 49247 control strain were unaffected by either reserpine or PAβN. The MIC of ampicillin of the efflux pump control strain Pf 1312 was reduced by one doubling dilution in the presence of both pump inhibitors; both concentrations of PAβN gave the same results. Whilst the ampicillin MIC of efflux pump control Pf 1311 was unaffected by reserpine, the organism grew poorly on the PAβN control plate and the MIC of ampicillin was reduced in the presence of this compound, most notably at the higher concentration. The MICs of ampicillin for the imipenem resistant clinical isolates were unaffected by the presence of either pump inhibitor.

The imipenem susceptibility of the Rd, ATCC 49247 and Pf 1312 control strains was unaffected by either pump inhibitor. Reserpine had no effect on the MIC of imipenem for the efflux pump control Pf 1311. However, as mentioned previously, the strain grew poorly in the presence of PAβN and had a reduced MIC of imipenem compared with the imipenem only control. The MIC of imipenem of all the clinical strains was reduced by two to fourfold in the presence of reserpine. All the clinical isolates grew well on the PAβN control plate and had reduced MICs of imipenem (four- to sixteen fold) in the presence of this compound.

Table 4.3. Effect of efflux pump inhibitors on ampicillin and imipenem susceptibility in imipenem resistant *H. influenzae* clinical isolates.

Organism	β-lactamase	MIC (mg/L)							
		AMP	AMP + Reserpine	AMP + PAβN (20 mg/L)	AMP + PAβN (40 mg/L)	IMI	IMI + Reserpine	IMI + PAβN (20 mg/L)	IMI + PAβN (40 mg/L)
Rd	Negative	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
ATCC 49247	Negative	4	4	4	4	0.5	0.5	0.5	0.5
Pf 1311*	Negative	8	8	2	≤ 0.25	1	1	0.25	≤ 0.12
Pf 1312*	Negative	8	4	4	4	0.5	0.5	0.5	0.5
A979	Negative	1	0.5	1	1	16	8	4	1
A1234	Negative	1	1	1	1	8	2	1	0.5
A1243	Negative	0.5	0.5	1	1	4	2	1	0.5
A965	Positive	32	32	32	64	16	4	4	4
A1366	Positive	32	32	32	32	16	8	1	1
A1380	Positive	32	32	32	64	8	4	1	1
A1437	Positive	32	32	32	32	8	4	1	1
A1464	Positive	64	64	64	64	16	8	4	1
A1525	Positive	32	32	32	64	16	8	8	4
A1545	Positive	32	32	32	32	8	4	1	1
A1575	Positive	64	64	64	64	16	8	2	2
A1651	Positive	32	32	32	32	8	4	1	1
A1736	Positive	32	32	32	32	8	4	1	1

AMP, ampicillin; IMI, imipenem; PAβN, phe-arg β-naphthylamide dihydrochloride; *BLNAR strains with known efflux pump mutations (Kaczmarek *et al*, 2004).

Although these results suggest that there may be efflux pump involvement in the imipenem resistance of the clinical isolates, the results for the two efflux pump control strains are inconclusive.

4.4. Selection of imipenem resistant mutants

After 42 serial subcultures in imipenem-containing broth media, the MIC of imipenem of *H. influenzae* ATCC 49247 was raised more than fourfold (increase in geometric mean MIC from 0.46 mg/L to 2.44 mg/L) whilst no change in susceptibility was achieved in *H. influenzae* Rd. The MIC of imipenem of the clinical strain Pf 1370 was increased after only five passages (increase in geometric mean MIC from 0.63 mg/L to 1.78 mg/L).

The ATCC 49247 imipenem resistant mutant, A42, also had reduced susceptibility to meropenem and ertapenem compared to the parent strain whilst the susceptibility of the Pf 1370 imipenem resistant mutant, Pf 70-5, was unchanged (Table 4.4.). In neither case were the susceptibilities to ampicillin, cefuroxime or cefotaxime altered.

Table 4.4. Geometric mean MIC of six β-lactams for *H. influenzae* Rd and parent and mutant pairs of ATCC 49247 and Pf 1370.

		Geometric mean MIC (mg/L)					
Organism		**IMI**	**MEM**	**EPM**	**AMP**	**CXM**	**CTX**
Rd		0.22	0.03	0.02	0.16	0.45	0.01
Parent	**ATCC 49247**	0.46	0.21	0.21	4.42	7.13	0.12
Mutant	**A42**	2.44	1.00	1.59	3.36	5.66	0.12
Parent	**Pf1370**	0.63	0.12	0.10	0.87	2.00	0.06
Mutant	**Pf70-5**	1.78	0.12	0.10	0.84	1.41	0.04

IMI, imipenem; MEM, meropenem; EPM, ertapenem; AMP, ampicillin; CXM, cefuroxime; CTX, cefotaxime.

4.5 Transformation of *H. influenzae* Rd

Transformation experiments were undertaken using competent Rd cells and donor DNA from *H. influenzae* ATCC 49247 (transformation control), the A42 and Pf 70-5 imipenem resistant mutants and the A 1234 imipenem resistant clinical isolate.

The use of imipenem-containing selection media was unsuccessful; inoculation with the transformation mixture resulted in a confluent growth of tiny colonies from which no viable growth was recovered on sub-culture onto fresh imipenem-containing media. Ampicillin-containing selection media was the most successful with transformation frequencies of between 10^{-4} and 10^{-7} cfu (Table 4.5).

Agar dilution MICs of imipenem, meropenem, ertapenem, ampicillin, cefuroxime and cefotaxime were performed on randomly selected transformants. Irrespective of selection media, the MICs of ampicillin, cefuroxime and cefotaxime of all the transformants were at least fourfold that of the DNA recipient, *H. influenzae* Rd, determined on the same occasion.

Using ampicillin-containing selection media, three imipenem resistant transformants were produced using DNA from the ATCC 49247 mutant, A42; one was manufactured using chromosomal DNA (tRdA42c) and two using purified *ftsI* PCR product (tRdA42f-1 and tRdA42f-3). Reduced susceptibility to ertapenem was transferred in all three cases and tRdA42c was also resistant to meropenem. Selection of carbapenem resistant transformants on ertapenem-containing media was unsuccessful but transformants resistant to ertapenem were selected on meropenem-containing media using both chromosomal DNA and purified *ftsI* PCR product (Table 4.5).

Imipenem resistant transformants were also produced using chromosomal DNA and purified *ftsI* PCR product from the Pf 70-5 imipenem resistant mutant and chromosomal DNA from the A1234 imipenem resistant clinical strain (purified *ftsI* PCR product was not manufactured from this organism); all three transformants were selected on ampicillin-containing media and were susceptible to meropenem and ertapenem.

Table 4.5. Transfer of β-lactam resistance[1] from selected DNA donors into competent *H. influenzae* Rd cells.

Donor		DNA	Selection β-lactam	Tranformation Frequency[2] (cfu)	Number studied	Resistance transferred					
						IPM	MEM	EPM	AMP	CXM	CTX
Parent	**ATCC 49247**	Chromosomal	AMP 0.5 mg/L	10^{-7}	2	0	0	0	2	2	2
			EPM 0.06 mg/L	10^{-7}	1	0	0	0	1	1	1
			MEM 0.06 mg/L	10^{-5}	2	0	0	0	2	2	2
		ftsI	AMP 0.5 mg/L	$10^{-5} - 10^{-7}$	4	0	0	0	4	4	4
			EPM 0.06 mg/L	10^{-5}	2	0	0	0	2	2	2
			MEM 0.06 mg/L	10^{-5}	3	0	0	0	3	3	3
Mutant	**A42**	Chromosomal	AMP 0.5mg/L	$10^{-6} - 10^{-7}$	4	1	1	2	4	4	4
			EPM 0.06 mg/L	10^{-6}	3	0	0	0	3	3	3
			MEM 0.06 mg/L	10^{-5}	2	0	0	1	2	2	2
		ftsI	AMP 0.5 g/L	10^{-6}	3	2	0	3	3	3	3
			EPM 0.06 mg/L	10^{-5}	3	0	0	0	3	3	3
			MEM 0.06 mg/L	10^{-5}	3	0	0	1	3	3	3
Mutant	**Pf 70-5**	Chromosomal	AMP 0.25 mg/L	10^{-7}	1	1	0	0	1	1	1
		ftsI	AMP 0.25 mg/L	10^{-4}	1	1	0	0	1	1	1
Clinical isolate	**A 1234**	Chromosomal	AMP 0.25 mg/L	10^{-7}	1	1	0	0	1	1	1

[1] resistance defined as an increase in MIC of at least fourfold compared to *H. influenzae* Rd tested on the same occasion; [2] transformation frequency defined as the number of transformants per mL of transformation mixture divided by the number of competent cells per mL of transformation mixture; IPM, imipenem; MEM, meropenem; EPM, ertapenem; AMP, ampicillin; CXM, cefuroxime; CTX, cefotaxime; A42, imipenem resistant mutant of ATCC 49247; Pf 70-5, imipenem resistant mutant of Pf 1370; 0, less than a fourfold increase in MIC of transformant compared to *H. influenzae* Rd tested on the same occasion.

4.6. Sequencing of the *ftsI* gene

4.6.1 Clinical isolates

Nucleotide sequencing was performed on the transpeptidase region of *ftsI* for three selected imipenem resistant clinical isolates (A1234, A1437 and A1575). All three organisms had the same deduced amino acid substitutions in the *ftsI* gene compared to the sequence of *H. influenzae* Rd i.e. Asp-350-Asn (D350N), Met-377-Ile (M377I), Ala-502-Val (A502V) and Asn-526-Lys (N526K) (Table 4.6).

4.6.2. Parent, mutant and transformant groups

Nucleotide sequencing was performed on the transpeptidase region of the *ftsI* gene of the imipenem susceptible ATCC 49247 parent strain, the imipenem resistant A42 mutant and the imipenem resistant transformants tRdA42c and tRdA42f-1. All four organisms had the N526K amino acid substitution compared to the sequence of *H. influenzae* Rd (Table 4.6.).

Nucleotide sequencing of the transpeptidase region of *ftsI* of Pf 1370 (imipenem susceptible parent) and Pf 70-5 (imipenem resistant mutant) disclosed the same deduced amino acid substitutions as detected in the three imipenem resistant clinical isolates i.e. D350N, M377I, A502V and N526K.

Thus, the imipenem susceptible Pf1370 strain has the same four amino acid substitutions as the imipenem resistant clinical isolates and Pf70-5 mutant, yet only the N526K substitution is present in the imipenem resistant A42 mutant and tRdA42c and tRdA42f-1 transformants. The transformants had been manufactured using either chromosomal DNA or the whole *ftsI* gene (4486 bp) of the imipenem resistant A42 mutant yet only the transpeptidase region of *ftsI* (705 bp) of the transformants had been sequenced. This suggests that any amino acid substitutions associated with imipenem resistance are likely to be located outside the transpeptidase region of *ftsI*.

Table 4.6. Deduced amino acid sequencing of the transpeptidase region of *ftsI* of control strains and imipenem resistant mutants, transformants and clinical isolates.

Organism		MIC (mg/L)						Amino acids[1]
		IPM	MEM	EPM	AMP	CXM	CTX	
Rd		0.25	0.06	0.03	0.25	0.5	0.008	SDSAMSLAIGARN
Parent	**ATCC 49247**	0.5	0.25	0.25	4	8	0.12	SDSAMSLAIGARK
Mutant	**A42**	2	1	2	4	4	0.12	SDSAMSLAIGARK
Transformant	**tRdA42c**	1	0.12	0.06	0.5	8	0.06	SDSAMSLAIGARK
	tRdA42f-1	1	0.06	0.06	1	2	0.03	SDSAMSLAIGARK
	tRdA42f-3	0.5	0.06	0.06	1	2	0.06	SDSAMSLAIGARK
Parent	**Pf 1370**	0.5	0.12	0.12	1	2	0.06	SNSAISLAIGVRK
Mutant	**Pf70-5**	2	0.12	0.12	1	1	0.03	SNSAISLAIGVRK
Clinical isolates	**A1234**	8	0.12	0.12	1	2	0.06	-NSAISLAIGVRK
	A1437	8	0.12	0.12	32	4	0.06	-NSAISLAIGVRK
	A1575	16	0.12	0.12	32	8	0.03	-NSAISLAIGVRK

IPM, imipenem; MEM, meropenem; EPM, ertapenem; AMP, ampicillin; CXM, cefuroxime; CTX, cefotaxime.[1] Deduced amino acids at positions 327, 350, 357, 368,377, 385, 389, 437, 449, 490, 502, 517 and 526 of the transpeptidase region of *ftsI*.; substitution compared to sequence of *H. influenzae* Rd in boldface

4.7. Random Amplification of polymorphic DNA (RAPD) profiling

RAPD typing was utilised to determine whether the imipenem resistant clinical isolates were clonal in nature. In addition, the imipenem susceptible parent / imipenem resistant mutant pairs and the transformant / donor / recipient groups were examined to ensure that the organisms being studied were those intended, as far as could be determined using this technique. In all cases, gel visualisation of the PCR products manufactured using the RAPDB primer disclosed very few bands and did not improve on the information derived from using the RAPDA primer alone.

4.7.1 RAPD typing of imipenem resistant clinical isolates

Gel visualisation of the RAPD PCR products divided the fifteen imipenem resistant clinical isolates into four groups of similar banding patterns, all of which were significantly different to the ATCC 49247 and Rd control strains (Figure 4.1). Examination of the specimen details disclosed no common epidemiological factor (Table 4.7). Although four of the RAPD group 4 isolates were derived from sputum samples collected in 2002 from non-hospitalised patients of at least 60 years of age, the patients lived in different areas of Birmingham and attended different GP surgeries.

4.7.2 RAPD typing of parent, mutant and transformant groups

Gel visualisation of the RAPD PCR products of the imipenem resistant mutants A42 and Pf70-5 and their susceptible parent strains, ATCC 49247 and Pf1370, demonstrated banding patterns sufficiently similar to confirm that the parents and mutants were of the same origin. Similarly, RAPD typing demonstrated that the imipenem resistant transformants had the same banding pattern as the competent Rd recipient (Figure 4.2).

4.8 Capsular typing

The fifteen imipenem resistant clinical isolates of *H. influenzae* were all non capsulated as determined using PCR methodology.

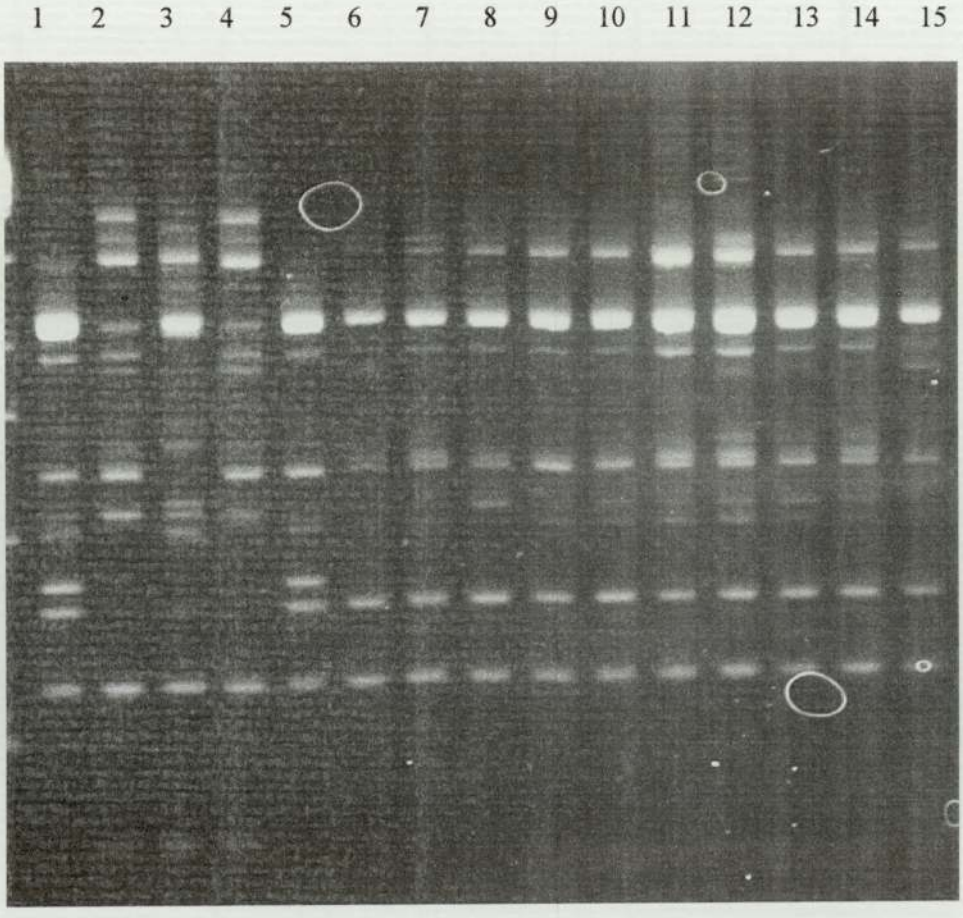


Figure 4.1. RAPD typing of the fifteen imipenem resistant clinical isolates.

Lane 1, A979; lane 2, A1091; lane 3, A1234; lane 4, A1243; lane 5, A1464; lane 6, A1380; lane 7, A1437; lane 8, A1545; lane 9, A1651; lane 10, A1736; lane 11, A965; lane 12, A1366; lane 13, A1397; lane 14, A1525; lane 15, A1575.

Table 4.7. Specimen details and RAPD typing group for the fifteen imipenem resistant clinical isolates

Organism	RAPD group	Specimen type	Patient age	Patient location	Year of isolation
A 1464	1	Eye	9 years	Community	2002
A 979	1	Sputum	64 years	Community	2000
A 1243	2	Eye	42 weeks	Community	2001
A 1091	2	Sputum	41 years	Hospital	2000
A 1234	3	Sputum	74 years	Hospital	2001
A 1380	4	Eye	Not known	Community	2002
A 1437	4	Eye	2 weeks	Community	2002
A 1545	4	Eye	84 years	Hospital	2002
A 1651	4	Eye	3 years	Community	2003
A 1736	4	Eye	90 years	Hospital	2003
A 965	4	NPA	32 weeks	Community	2000
A 1366	4	Sputum	74 years	Community	2002
A 1397	4	Sputum	60 years	Community	2002
A 1525	4	Sputum	62 years	Community	2002
A 1575	4	Sputum	78 years	Community	2002

NPA, naso-pharyngeal aspirate; Eye, eye swab; Community, patient in the community who, if recently hospitalised, has been discharged for at least the preceding 48 hours; Hospital, hospitalised patients in whom infections occurred at least 48 hours post admission.

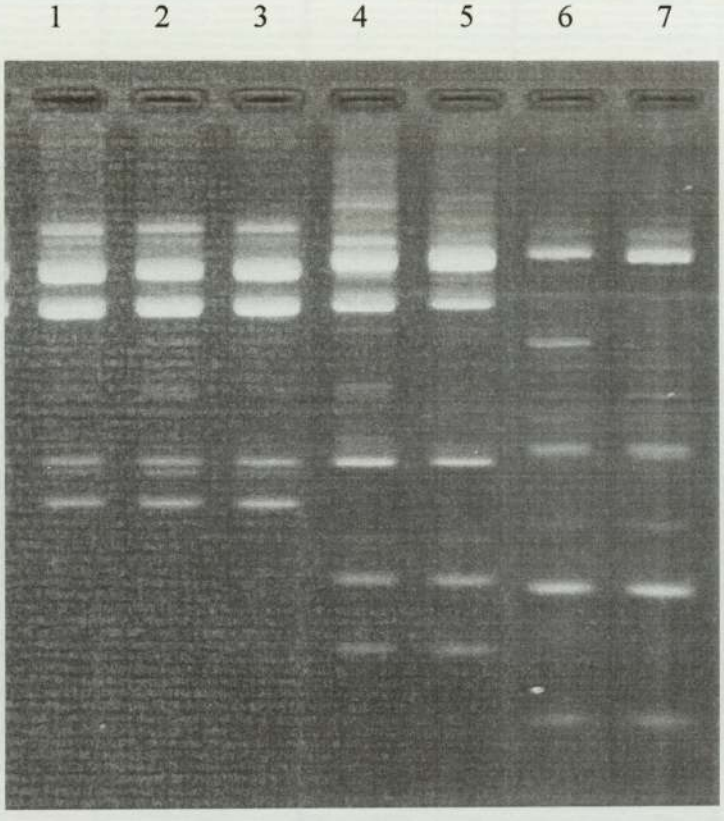


Figure 4.2 RAPD typing of control strains and parent, mutant and transformant groups.

Lane 1, Rd (ATCC 51907); lane 2, transformant tRdA42c; lane 3, transformant tRdA42f;
lane 4, ATCC 49247; lane 5, ATCC 49247 mutant A42; lane 6, Pf 1370; lane 7, Pf 1370 mutant Pf 70-5.

Chapter 5

Discussion

5.1. β-lactamase positive, ampicillin resistant phenotype (BLPAR)

The predominant resistance phenotype detected amongst the population studied was β-lactamase mediated resistance to ampicillin and amoxicillin (BLPAR). A majority of the β-lactamase positive isolates expressed a bla_{TEM} gene. No attempt was made to distinguish between TEM-1 and TEM-2 β-lactamases; TEM-2 differs from TEM-1 in the substitution of C for A at position 4046 of the TEM-1 gene (Scriver *et al*, 1994).

PCR for bla_{TEM} revealed three different sized amplimers amongst the β-lactamase positive clinical isolates. Ninety of the β-lactamase positive isolates had DNA fragments shorter than the TEM-1 control suggesting a deletion in the bla_{TEM} gene. Nucleotide sequencing of bla_{TEM} for two of these organisms using primers TEM1 and TEM2 showed that both had the same 135 bp deletion, within the promoter region of TEM-1, as described by Tristram *et al* (2005). Nucleotide sequencing was not performed on the eighteen organisms for which bla_{TEM} PCR was positive using intragenic primers (TEM3 and TEM4); it is likely that the gene encoding β-lactamase production for these strains is located on a different resistance plasmid than the genes amplified using the TEM1 and TEM2 primers.

Three of the 130 β-lactamase positive isolates produced the ROB-1 enzyme as determined by PCR. Previous reports of ROB-1 production in *H. influenzae* have been restricted to North America (Farrell *et al*, 2005). Little is known about the prevalence of these strains in the UK although this study suggests that ROB-1 producers are uncommon. In contrast to reports of the failure to detect ROB-1 production using chromogenic cephalosporins (Rubin *et al*, 1981), all three ROB-1 producers had positive results using nitrocefin to detect β-lactamase production. Also, it does not appear that the previously reported association between ROB-1 production and cefaclor resistance (Karlowsky *et al*, 2000; Farrell, *et al*, 2005) is present in the population studied although data analysis is limited due to the small number of ROB-1 producing isolates. Interestingly, of the four different β-lactamase enzymes detected in the *H. influenzae* population studied, the ROB-1 isolates demonstrated the greatest overall susceptibility to cefaclor (66.6 %); the highest rate of cefaclor resistance was observed in the isolates with bla_{TEM} detected with the intragenic primers (100%) and strains producing the truncated TEMdel β-lactamase (91.1%). Reduced susceptibility to

cefaclor has previously been associated with deletions in the TEM promoter region (Molina *et al*, 2003). Although cefaclor is widely prescribed for infections caused by *H. influenzae*, there is doubt regarding its efficacy in the treatment of respiratory infections (Andrews, 2007), and the BSAC MIC breakpoint has been set to reflect this.

5.2. β-lactamase negative, ampicillin resistant phenotype (BLNAR)

Eighteen β-lactamase negative isolates had raised MICs of ampicillin and /or amoxicillin and were considered BLNAR strains. Although ampicillin resistant, none of the 18 isolates were considered high-level BLNAR as they all had MICs below 4 mg/L. Amoxicillin resistance was detected by disc susceptibility testing in all cases. All 18 BLNAR isolates had zone diameters consistent with cefuroxime resistance although the MICs of cefuroxime for six organisms were 1 mg/L. These findings are in disagreement with James *et al* (1993) who suggested that non-β-lactamase mediated β-lactam resistance might be better detected using cefuroxime (MIC greater than 4 mg/L) than ampicillin.

5.3. Co-amoxiclav resistance and BLPACRs

Twenty (8%) of the study organisms had raised MICs of co-amoxiclav (2 mg/L); six were β-lactamase positive and fourteen were β-lactamase negative. It is not clear whether the β-lactamase producers should be classified as BLPACRs as this term generally refers to isolates with co-amoxiclav MICs of at least 8 mg/L (Tristram *et al*, 2007). Although not yet as common as the BLNAR phenotype, the incidence of BLPACR *H. influenzae* is increasing, especially in Japan (Doern *et al*, 1997; Matic *et al*, 2003; Hasegawa *et al*, 2004). All 20 co-amoxiclav resistant isolates demonstrated concomitant resistant to ampicillin and / or amoxicillin, and the majority were resistant to cefuroxime. Two of the β-lactamase negative isolates had borderline susceptibility to cefotaxime (0.12 mg/L) and four of the β-lactamase positive isolates were resistant to imipenem.

5.4. Cefotaxime resistance

Using the BSAC MIC breakpoints of 2004 (Andrews, 2004), four of the β-lactamase negative clinical isolates had MICs of cefotaxime that were interpreted as susceptible yet ranging between 0.12 and 0.25 mg/L were above those of the wild sensitive

population. In 2007, the BSAC reduced their MIC breakpoints for cefotaxime to 0.12 mg/L as part of the efforts to harmonise MIC breakpoints across Europe (Andrews, 2007). This reduced breakpoint places one of the four clinical isolates in the resistant category and the other three on the borderline.

The MICs of cefuroxime, ampicillin, amoxicillin and co-amoxiclav for all four isolates centred on the breakpoint and were higher than the wild sensitive population. All were resistant to cefaclor. All were isolated from community respiratory samples for which the treatment was unknown. Should these patients have been hospitalised with invasive infections arising from these isolates, β-lactam treatment options would be restricted to the carbapenems to which they were all susceptible.

Reports of *H. influenzae* strains resistant to third or fourth generation cephalosporins are rare in Europe (Fluit *et al*, 2005; Jansen *et al*, 2006; Morrissey *et al*, 2005) but increased MICs of cefotaxime and ceftriaxone have been associated with BLNAR strains in Japan (Hasegawa *et al*, 2006). The MICs of third generation cephalosporins for BLNAR strains may be as much as 32 times greater than those for β-lactamase non-producing ampicillin susceptible strains (Ubukata *et al*, 2001). Matic *et al* (2003) found a correlation between raised MICs of cefotaxime and Leu-389-Phe (L389F) point mutations in *ftsI* in a population of Japanese BLPACR and BLNAR strains. In a recent study of Spanish strains, reduced susceptibility to cefuroxime, cefotaxime and cefixime was detected in 4% of BLNAR strains and all had the Ubukata type-III-like mutations in *ftsI* (Ubukata *et al*, 2001; Garcia-Cobos *et al*, 2007). The authors determined that mutations in the SNN motif of *ftsI*, particularly Met-377-Ile (M377I) and Ser-385-Thr (S385T) and Leu-389-Phe (L389F) were required for the development of cefotaxime and cefixime resistance.

Whilst TEM-derived ESBLs conferring cefotaxime resistance are common amongst the Enterobacteriaceae (Paterson and Bonomo, 2005), β-lactamase mediated resistance to cefotaxime has not yet been reported in *H. influenzae*. In a study to examine the effect of transforming Rd and BLNAR strains with plasmid mediated ESBLs, cefotaxime resistance was transferred to BLNAR strains known to have altered PBPs but not to the fully susceptible Rd strain or TEM producing BLPAR strains (Bozdogan *et al*, 2006).

The authors determined that should this mechanism of resistance occur naturally in *H. influenzae*, it might be detected using a co-amoxiclav disc diffusion test.

5.5. Efflux mediated resistance

The role of efflux pump mechanisms in the ampicillin resistance of the 250 study organisms was largely dispelled although only phenotypic testing was performed. High-level BLNAR in *H. influenzae* is known to combine PBP3 changes with mutations in the *acrR* regulatory gene of the AcrAB efflux pump (Kaczmarek *et al*, 2004). However, efflux mediated resistance has not been associated with increased resistance to other β-lactams in *H. influenzae* (Tristram *et al*, 2007).

The two control strains used for the efflux pump studies (Pf 1311 and Pf 1312) were high-level BLNAR strains with altered *acrR* genes. The effect of the pump inhibitors reserpine and PAβN on their phenotypic resistance has not previously been reported. As reserpine had little effect on the MICs of ampicillin of these two strains it is possible that it may not act as an inhibitor of the acrAB efflux pump. Whilst seeking pump inhibitors suitable for the study of efflux pump-mediated resistance in *H. influenzae* and *E. coli*, it was observed that PAβN may act as a growth inhibitor (Kaczmarek *et al*, 2005); this may explain the inhibitory effect this compound had on the Pf 1311 control strain.

Reserpine and PAβN had no effect on the MICs of ampicillin but reduced the MICs of imipenem in the fifteen imipenem resistant clinical isolates. Although it is possible that an efflux pump mechanism may play a part in the imipenem resistance of these strains, further work using alternative pump inhibitors and / or nucleotide sequencing of the genes encoding the acrAB efflux pump and its associated regulatory mechanisms is necessary.

5.6 Carbapenem resistance

The most unusual β-lactam resistance detected amongst the study population was the reduced susceptibility to imipenem identified in fifteen clinical isolates. At the time of data analysis, the BSAC MIC breakpoint for imipenem was 4 mg/L but has since been amended to 2 mg/L (Andrews, 2007). Re-examination of the MIC results has revealed

that a total of 29 of the 250 study isolates have reduced susceptibility to imipenem. The work carried out to determine the nature of the imipenem resistance expressed in this population of organisms was performed on the original fifteen isolates.

In other Gram-negative bacteria, resistance to the carbapenems is known to be caused by plasmid or chromosomally mediated β-lactamases such as the IMP and VIM metallo β-lactamases and KPC carbapenemase enzymes, porin loss or alterations in PBPs (Paterson and Bonomo, 2005). In this study, β-lactamase hydrolysis of imipenem was discounted as the possible resistance mechanism as four of the fifteen imipenem resistant isolates were β-lactamase non-producers. The evidence for efflux-mediated resistance was inconclusive in this investigation. A recent Italian study investigated the role of efflux pump mechanisms in the imipenem resistance of two invasive nontypeable *H influenzae* strains using the broad spectrum efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). In conjunction with nucleotide sequencing of the *acrR* regulatory gene of the AcrAB efflux pump, the authors concluded that the imipenem resistance observed in their clinical isolates was not efflux mediated (Cerquetti *et al*, 2007). The same group also studied the OMP2 porin of their imipenem resistant strains and detected no apparent differences between the clinical isolates and imipenem susceptible control organisms.

That all fifteen imipenem resistant clinical strains had MICs of cefuroxime above the range for the normal wild population suggested the presence of altered PBP3 (Straker *et al*, 2003). Nucleotide sequencing of the transpeptidase region of *ftsI* of imipenem resistant clinical strains, mutants and transformants established that all these organisms had the Asn-526-Lys (N526K) substitution compared to Rd. This amino acid substitution has been reported in all BLNARs and BLPACRs with the exception of those assigned to the Ubukata group I (Ubukata *et al*, 2001; Dabernat *et al*, 2002; Matic *et al*, 2003; Kaczmarek *et al*, 2004). Using site-directed mutagenesis of PBP 3 in *H. influenzae* Rd, a Japanese group reported that raised MICs of imipenem were dependent on the presence of the N526K mutation (Osaki *et al*, 2005). However, as the N526K substitution was also present in the imipenem susceptible ATCC 49247 and Pf 1370 strains (parents of the A42 and Pf70-5 imipenem resistant mutants respectively), it is unlikely to be the sole cause of imipenem resistance.

Both the ATCC 49247 and Pf 1370 imipenem susceptible strains have amino acid substitutions additional to N526K in *ftsI;* ATCC 49247 has Ser-273-Ala (S273A) and Pf 1370 has Asp-350-Asn (D350N), Met-377-Iso (M377I) and Ala-502-Val (A502V) substitutions in relation to the sequence of Rd (Kaczmarek *et al*, 2004). Imipenem resistant mutants were successfully manufactured from both these strains but not from Rd. That the MIC of imipenem was increased by fourfold in the Pf 1370 mutant (Pf 70-5) after only five serial subcultures compared with the 42 passages required to achieve a similar increase in the ATCC 49247 mutant (A42), suggests that the additional amino acid substitutions present in the *ftsI* gene of Pf 1370 may predispose the organism to the development of carbapenem resistance. Nucleotide sequencing of the transpeptidase region of *ftsI* of three imipenem resistant clinical isolates demonstrated the same four amino acid substitutions as the imipenem susceptible Pf 1370 strain. So, although amino acid substitutions in the transpeptidase region of *ftsI* are not the sole cause of imipenem resistance in *H. influenzae*, they are likely to be a contributing factor.

The transfer of imipenem resistance from the A42 mutant to the susceptible Rd recipient was achieved using both chromosomal DNA and the 4.486 Kb DNA fragment incorporating the whole *ftsI* gene. This suggests that the amino acid substitutions essential for imipenem resistance lie within the 4.486 Kb sequence although outside the transpeptidase region of *ftsI* for the reasons discussed previously. The MICs of imipenem for the resistant transformants were two to fourfold lower than the imipenem resistant donor (A42 mutant) suggesting that either additional genetic material is required for the full expression of imipenem resistance or that introducing the donor DNA into the genetic background of Rd reduces the effect observed in the donor. This observation was also true of the transformants manufactured using donor DNA from the imipenem resistant Pf 70-5 mutant and A1234 clinical isolate. A Japanese study observing the same effect deduced that reduced affinity to PBPs in addition to PBP3 might be responsible for the imipenem resistance observed in their strains (Osaki *et al*, 2005).

Resistance to meropenem and ertapenem was not observed in any of the imipenem resistant clinical isolates. Additionally, resistance to these other carbapenems was not developed in the imipenem resistant Pf 70-5 mutant although the A42 mutant was

resistant to both agents. The transfer of meropenem and ertapenem resistance between the carbapenem resistant A42 donor and Rd recipient was varied; meropenem resistance was transferred on chromosomal DNA whereas ertapenem resistance was transferred on both chromosomal DNA and the 4.486 Kb DNA fragment including the whole *ftsI* gene. Rare, naturally occurring, meropenem resistant clinical strains of *H. influenzae* have been isolated in Japan (Osaki *et al*, 2005) but ertapenem resistance has yet to be reported. Full DNA sequence analysis of all PBPs in the Japanese meropenem resistant clinical isolates did not reveal any mutations other than those already described in the PBP3 of ampicillin and imipenem resistant strains (Osaki *et al*, 2005). It has been suggested that meropenem has a greater affinity for PBPs 4 and 5 than PBP3 in *H. influenzae* (Miyazaki *et al*, 2001).

5.7 Conclusion

Although no high level BLNARs or BLPACRs were identified amongst the group of clinical isolates studied, it was evident that a large proportion of the organisms had reduced susceptibility to at least one of the aminopenicillins, co-amoxiclav and second-generation cephalosporins examined. The resistance profiles of these organisms were generally consistent with previous descriptions of β-lactamase production and / or altered PBPs.

As the incidence of cephalosporin resistance in *H. influenzae* increases, alternative treatment options become necessary and the carbapenems are already being used for nosocomial pneumonia and severe community acquired pneumonia in Japan (Gomi *et al*, 2007). Imipenem has been recommended as an appropriate treatment alternative for invasive infections caused by ampicillin resistant isolates when a third generation cephalosporin cannot be used (Cerquetti *et al*, 2004). This is particularly relevant in the light of the emerging resistance to the third generation cephalosporins. Reports of carbapenem resistance amongst clinical strains of *H. influenzae* are rare and until recently have been restricted to the United States, Korea and Japan (Brown and Traczewski, 2005; Kim *et al*, 2007; Sanbongi *et al*, 2006). This study has illustrated that naturally occurring imipenem resistance already exists in clinical strains of *H. influenzae* in the UK. The selection of the carbapenem resistant mutant, A42, demonstrates that the development of resistance to meropenem and ertapenem is

possible *in vitro* and naturally occurring meropenem resistant strains have already been reported (Osaki *et al*, 2005). It seems inevitable that the increased use of carbapenems will lead to the development and global spread of resistance in clinical strains of *H. influenzae*.

The problems associated with the detection of reduced β-lactam susceptibility in *H. influenzae* have already been discussed. Although generally considered a good indicator of antimicrobial resistance in clinical isolates, disc susceptibility testing may fail to detect organisms with borderline susceptibilities, including BLNAR strains (Mendelman *et al*, 1987). The unreliability of disc susceptibility testing for the detection of imipenem resistant *H. influenzae* has been reported previously (Zerva *et al*, 1996); the fifteen imipenem resistant clinical isolates identified in this study by agar dilution MICs, would have been missed following standardized disc susceptibility testing methodology. It may become necessary for routine clinical laboratories to adopt an alternative method for the detection of β-lactam resistance in clinical isolates of *H. influenzae*.

Increasingly, molecular methods are being used for the detection of antibiotic resistance in clinical isolates e.g. detection of the *mecA* gene in *Staphylococcus aureus* (Brown, 2001). The molecular detection of antibiotic resistance mechanisms is dependent on knowing the genetic cause. As the cause of BLNAR type resistance appears to involve multiple substitutions in *ftsI* and the precise cause of carbapenem resistance is as yet undetermined, molecular techniques for the detection of β-lactam resistance in *H. influenzae* are not yet appropriate. Billal *et al* (2007) proposed that the gradient method of MIC determination might be suitable for the detection of β-lactam resistance in BLNAR strains or those with *ftsI* mutations. The higher inoculum specified for this technique may not provide a true representation of the organism's MIC value but provides a better chance of detecting resistance. Indeed, the recent detection of two Italian strains of *H. influenzae* with reduced susceptibility to imipenem was due to the use of Etest MIC methodology (Cerquetti *et al*, 2007).

5.8 Future work

This study has established that imipenem resistance occurs naturally in clinical isolates and can be selected *in vitro* in organisms known to have amino acid changes in the transpeptidase region of *ftsI*. It has also been established that the genetic cause of imipenem resistance is located in part within the 4.486 Kb DNA fragment transferred from the imipenem resistant A42 donor to susceptible Rd but is outside the transpeptidase region of *ftsI*.

In order to determine the precise character of imipenem resistance in *H. influenzae*, it is proposed that the following nucleotide sequencing is undertaken:

1. Sequencing of the whole 4.486 Kb DNA fragment of the tRdA42f-1 imipenem resistant transformant and comparison with Rd to determine which mutations are unique to the transformant.
2. Sequencing of the whole 4.486 Kb DNA fragment of the imipenem susceptible ATCC 49247 and Pf 1370 DNA parents and their corresponding imipenem resistant mutants (A42 and Pf 70-5) to determine which mutations are unique to the mutants.
3. Sequencing of the whole 4.486 Kb DNA fragment of the imipenem resistant clinical isolates.

It is hoped that comparison of the sequence data for the imipenem resistant clinical isolates, transformant and mutants may disclose amino acid substitutions which are unique to the imipenem resistant strains.

Appendices

Appendix 1. Specimen details, disc and MIC susceptibility testing results and B-lactamase status for 250 clinically signigficant *H. influenzae* isolates

		Patient details			Disc testing results			MIC (mg/L)											B-lactamase detection		
Lab ID	**Specimen**	**Age**	**Sex**	**Source**	**AMX**	**AMC**	**CXM**	**AMP**	**AMX**	**AMC**	**CXM**	**CEC**	**FOX**	**CTX**	**IPM**	**MEM**	**EPM**	**EtBr**	**Nitro**	**Intra**	***bla* gene**
A 866	Sputum	53	M	Hospital	S	S	R	0.25	1	0.5	1	8	8	0.015	2	0.12	0.06	2	neg	neg	
A 898	Eye	83	F	Community	R	S	R	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.03	≤1	pos	pos	*TEM-1*
A 905	Eye	31	M	Community	S	S	R	1	1	1	2	8	2	0.06	0.5	0.12	0.12	≤1	neg	neg	
A 912	Sputum	40	F	Community	S	S	R	0.5	1	1	2	8	2	0.03	0.25	0.06	0.06	≤1	neg	neg	
A 914	Blood	0 days	M	Community	S	S	R	1	1	1	1	4	4	0.03	0.5	0.12	0.12	≤1	neg	neg	
A 929	Sputum	87	M	Hospital	S	S	R	1	1	1	2	8	8	0.06	2	0.12	0.12	2	neg	neg	
A 944	Eye	1	M	Community	S	S	R	0.5	1	1	2	8	2	0.015	0.5	0.06	0.06	≤1	neg	neg	
A 950	Eye	38	F	Community	R		R	1	1	1	2	8	4	0.03	0.5	0.06	0.06	2	neg	neg	
A 951	Eye	1	M	Community	R		R	0.25	0.25	0.12	0.25	1	0.5	≤0.004	≤0.12	NA	NA	≤1	neg	neg	
A 952	Eye	59	M	Community	S	S	R	0.25	0.5	0.5	0.5	8	2	0.015	0.25	0.03	0.03	2	neg	neg	
A 959	Sputum	63	F	Community	R		S	8	8	0.5	1	4	2	0.03	0.5	0.06	0.06	4	pos	pos	*TEMdel*
A 960	Sputum	4	F	Community	R		S	2	2	0.25	0.5	1	2	0.015	0.25	0.06	0.03	≤1	pos	pos	*TEMdel*
A 961	Sputum	78	M	Community	R		S	0.5	0.5	0.5	1	4	2	0.03	0.5	0.06	0.06	≤1	neg	neg	
A 965	NPA	32 weeks	F	Community	R		R	32	64	1	8	32	16	0.06	16	0.12	0.12	2	pos	pos	*TEMdel*
A 968	Eye	5 weeks	F	Community	R		S	8	8	0.5	0.5	4	4	0.008	2	0.03	0.03	2	pos	pos	*TEMdel*
A 969	Eye	5	M	Community	R		S	0.5	1	1	1	8	8	0.03	4	0.12	0.12	2	neg	neg	
A 972	Sputum	76	M	Community	R		S	8	8	0.5	1	2	2	0.03	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 973	NPA	1	M	Community	R		S	2	4	0.25	0.25	1	2	0.008	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 977	Sputum	78	F	Hospital	R		S	0.25	0.5	0.5	1	2	4	0.03	0.5	0.06	0.03	≤1	neg	neg	
A 979	Sputum	64	M	Community	R		R	1	1	1	2	16	8	0.03	8	0.12	0.12	2	neg	neg	
A 980	NPA	32 weeks	M	Community	R		R	1	1	1	1	4	1	0.06	0.25	0.12	0.12	≤1	neg	neg	
A 982	NPA	7 weeks	F	Community	R		R	2	2	2	2	8	2	0.06	2	0.12	0.06	2	neg	neg	
A 983	Sputum	77	M	Hospital	R		R	0.5	1	1	1	8	2	0.03	0.25	0.06	0.06	≤1	neg	neg	
A 984	Sputum	62	M	Community	R		S	1	1	1	4	16	4	0.03	1	0.06	0.06	2	neg	neg	
A 985	Sputum	62	M	Community	R		S	8	8	0.25	0.25	1	1	0.008	0.25	0.03	0.03	≤1	pos	pos	*TEMdel*
A 986	Sputum	73	F	Community	R		S	2	2	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEM-1*
A 987	Sputum	69	M	Community	R	R	R	1	2	1	1	8	4	0.06	1	0.12	0.12	4	neg	neg	
A 990	Eye	45 weeks	M	Community	R		S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 991	Sputum	62	F	Community	R		R	1	1	1	0.5	2	≤0.25	0.03	≤0.12	NA	NA	≤1	neg	neg	
A 992	Sputum	78	M	Community	R		R	0.25	0.5	0.5	1	2	2	0.015	4	0.12	0.12	≤1	neg	neg	
A 993	Sputum	72	F	Hospital	R		R	8	4	1	2	8	2	0.06	0.5	0.12	0.12	2	pos	pos	*TEM-1*
A 994	Eye	1	F	Community	R		S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEM*
A 995	Sputum	74	M	Community	R		R	8	16	1	2	16	4	0.03	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 997	Eye	15 weeks	M	Community	R		S	16	16	0.5	0.5	2	4	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEM*
A 998	Eye	8 days	M	Community	R		S	8	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 999	Eye	1	F	Community	R		R	1	1	1	1	4	2	0.03	0.5	0.06	0.06	≤1	neg	neg	
A 1000	Eye	12 weeks	M	Community	R		S	0.25	0.5	0.5	1	2	4	0.015	0.5	0.06	0.06	2	neg	neg	
A 1002	Eye	57	F	Community	R		R	1	1	1	2	8	4	0.06	2	0.06	0.06	4	neg	neg	
A 1004	NPA	19 weeks	F	Community	R		R	0.5	1	1	1	8	2	0.03	NA	NA	NA	≤1	neg	neg	
A 1014	Eye	32 weeks	M	Community	R		S	8	16	0.5	0.5	2	2	0.015	0.25	0.06	0.06	≤1	pos	pos	*TEM*
A 1015	Eye	1 day	F	Community	R		R	1	2	2	2	4	4	0.03	1	0.12	0.12	2	neg	neg	
A 1016	NPA	39 weeks	M	Community	R		R	1	1	1	2	16	8	0.06	4	0.12	0.12	4	neg	neg	
A 1021	Eye	38 weeks	M	Community	R		S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*

Appendix 1 Continued.

		Patient details			Disc testing results			MIC (mg/L)											B-lactamase detection		
Lab ID	Specimen	Age	Sex	Source	AMX	AMC	CXM	AMP	AMX	AMC	CXM	CEC	FOX	CTX	IPM	MEM	EPM	EtBr	Nitro	Intra	*bla* gene
A 1022	Eye	9 weeks	M	Community	R		S	8	4	0.5	1	4	4	0.03	0.5	NA	NA	4	pos	pos	*TEMdel*
A 1023	Eye	2	M	Community	R		S	0.5	0.5	0.5	1	2	2	0.03	0.5	0.06	0.06	≤1	neg	neg	
A 1029	Spu	74	F	Community	R		R	1	1	1	1	2	2	0.03	0.5	0.12	0.12	≤1	neg	neg	
A 1031	Eye	20 weeks	F	Community	R	S	S	0.25	0.5	0.25	0.5	1	0.5	0.015	≤0.12	0.06	0.06	≤1	neg	neg	
A 1034	Sputum	74	M	Hospital	R		S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1051	Eye	32 weeks	M	Community	S		R	0.5	0.5	0.5	1	4	2	0.03	≤0.12	0.03	0.03	≤1	neg	neg	
A 1056	NPA	1	M	Community	R		R	1	1	1	1	4	2	0.06	0.5	0.12	0.12	≤1	neg	neg	
A 1059	Eye	7 weeks	M	Community	R		R	0.5	1	1	1	8	2	0.03	0.5	0.06	0.06	2	neg	neg	
A 1060	NPA	16 weeks	M	Community	R		S	16	16	0.25	0.5	4	1	0.015	0.25	0.03	0.03	≤1	pos	pos	*ROB-1*
A 1063	Sputum	53	F	Hospital	S		R	0.25	0.25	0.12	1	4	1	0.03	≤0.12	0.008	0.008	2	neg	neg	
A 1070	Sputum	72	F	Community	R		S	8	8	0.5	0.5	2	2	0.015	0.5	0.03	0.03	≤1	pos	pos	*TEMdel*
A 1071	Bronchial lavage	41	F	Community	R		S	2	2	0.5	0.5	2	2	0.015	0.5	0.06	0.03	2	pos	pos	*TEMdel*
A 1074	Eye	1	M	Community	R		S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.03	≤1	pos	pos	*TEM*
A 1080	Eye	76	F	Community	R		S	0.25	0.5	0.5	1	4	2	0.015	0.5	0.06	0.03	≤1	neg	neg	
A 1083	Sputum	55	M	Community	R		R	1	2	1	2	8	2	0.03	0.5	0.12	0.12	≤1	neg	neg	
A 1090	NPA	38 weeks	F	Community	R	R	S	8	8	0.5	0.5	2	2	0.015	0.25	0.06	0.03	≤1	pos	pos	*TEM*
A 1091	Sputum	41	F	Hospital	R		R	0.5	1	1	1	8	4	0.03	8	0.12	0.12	≤1	neg	neg	
A 1096	Sputum	76	M	Community	R		S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEM*
A 1097	Sputum	59	M	Community	R		S	4	4	0.5	0.5	2	2	0.015	0.25	0.03	0.03	4	pos	pos	*TEM-1*
A 1099	Eye	14 days	M	Community	R		S	0.25	0.25	0.25	0.5	2	2	0.015	≤0.12	0.03	0.015	2	neg	neg	
A 1103	Eye	13 weeks	F	Community	R		S	0.25	0.5	0.5	1	4	4	0.015	1	0.06	0.06	2	neg	neg	
A 1104	Eye	52	F	Community	R		S	0.25	0.5	0.5	0.5	2	2	0.015	0.25	0.06	0.03	≤1	neg	neg	
A 1105	Eye	14	M	Community	R		S	0.25	0.5	0.5	0.5	1	2	0.015	0.25	0.06	0.03	≤1	neg	neg	
A 1107	Sputum	65	F	Community	R		S	4	4	0.5	0.5	2	4	0.015	0.25	0.03	0.03	≤1	pos	pos	*TEMdel*
A 1108	Eye	80	F	Community	R		R	0.5	1	0.5	1	4	2	0.03	0.25	0.12	0.06	≤1	neg	neg	
A 1111	Sputum	65	F	Community	R		S	16	16	0.25	0.5	1	2	0.015	1	0.06	0.03	≤1	pos	pos	*TEMdel*
A 1113	Sputum	69	F	Community	R		R	0.5	0.5	0.5	1	2	2	0.03	0.5	0.06	0.06	2	neg	neg	
A 1115	NPA	27 weeks	M	Community	R		R	0.5	1	0.5	1	4	1	0.03	0.25	0.12	0.12	≤1	neg	neg	
A 1116	Bronchial lavage	71	M	Community	R		R	0.5	0.5	0.5	0.5	2	2	0.03	1	0.06	0.06	4	neg	neg	
A 1117	Eye	4 weeks	F	Community	R		S	0.5	0.5	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	neg	neg	
A 1124	ET secretion	1	F	Hospital	R		R	0.5	0.5	0.5	0.5	2	4	0.015	0.5	0.06	0.06	4	neg	neg	
A 1130	NPA	42 weeks	M	Hospital	R	R	R	1	1	1	1	4	1	0.03	0.25	0.25	0.25	≤1	neg	neg	
A 1132	Sputum	67	M	Hospital	R	R	S	8	8	0.5	1	2	2	0.03	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1133	NPA	21 weeks	F	Hospital	R	R	R	0.5	0.5	0.5	0.25	1	1	0.008	0.25	0.06	0.06	2	neg	neg	
A 1135	Sinus washout	39	M	Community	R	S	R	0.5	1	0.5	4	8	2	0.12	0.5	0.06	0.06	2	neg	neg	
A 1140	Sputum	43	M	Community	R	R	S	8	16	0.5	0.5	2	2	0.015	0.5	0.06	0.03	4	pos	pos	*TEM*
A 1144	Sputum	95	F	Hospital	R	R	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1146	Sputum	53	M	Hospital	R	R	R	16	16	1	2	4	2	0.03	0.5	0.12	0.12	16	pos	pos	*TEMdel*
A 1152	Sputum	71	M	Community	R	R	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1153	NPA	1	F	Community	R	R	R	1	1	1	8	16	4	0.06	4	0.06	0.06	2	neg	neg	
A 1155	Sputum	83	F	Community	R	S	S	2	2	0.25	0.5	2	2	0.015	0.25	0.03	0.03	≤1	pos	pos	*TEMdel*
A 1157	Sputum	70	F	Community	R	S	R	16	16	0.5	0.5	1	1	0.008	0.25	0.03	0.03	≤1	pos	pos	*ROB-1*
A 1165	Eye	18 weeks	U	Community	R	R	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*

Appendix 1 Continued.

		Patient details			Disc testing results			MIC (mg/L)										B-lactamase detection		
Lab ID	Specimen	Age	Sex	Source	AMX	AMC	CXM	AMP	AMX	AMC	CXM	CEC	FOX	CTX	IPM	MEM	EPM	EtBr	Nitro	Intra	*bla* gene
A 1172	Bronchial lavage	37	M	Hospital	R	R	R	1	1	1	2	4	4	0.03	0.5	0.12	0.12	≤1	neg	neg	
A 1173	Peritonsillar abscess	47	M	Community	R	S	S	2	4	0.5	0.5	4	4	0.015	0.5	0.03	0.06	2	pos	pos	*TEM-1*
A 1175	Sputum	90	F	Community	R	S	S	0.12	0.25	0.25	0.5	1	2	0.008	0.25	0.03	0.06	≤1	neg	neg	
A 1178	Eye	4	M	Community	R	R	R	0.5	1	1	1	4	2	0.03	0.5	0.03	0.06	2	neg	neg	
A 1180	Sputum	87	M	Community	R	S	S	16	16	0.25	0.5	2	4	0.015	0.25	0.03	0.03	4	pos	pos	*TEM*
A 1181	Sputum	65	M	Community	S	S	R	1	1	1	2	8	4	0.06	4	0.06	0.06	4	neg	neg	
A 1183	Eye	7 weeks	F	Community	R	S	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1191	Eye	0 days	M	Hospital	S	S	R	0.5	1	0.5	1	8	2	0.03	0.25	0.06	0.06	≤1	neg	neg	
A 1192	Sputum	63	M	Hospital	S	S	R	0.5	0.5	0.5	1	4	2	0.03	0.5	0.06	0.06	≤1	neg	neg	
A 1195	NPA	19 weeks	F	Hospital	R	R	S	8	16	0.5	1	8	4	0.03	2	0.06	0.06	4	pos	pos	*TEMdel*
A 1196	Blood	21	F	Community	S	S	R	0.5	1	0.5	1	8	2	0.03	0.25	0.06	0.06	≤1	neg	neg	
A 1197	Eye	46 weeks	F	Community	R	R	S	8	8	0.5	1	4	2	0.03	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1198	Eye	20 weeks	M	Community	R	R	S	8	8	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1199	Eye	91	M	Hospital	R	R	S	8	8	0.5	1	4	2	0.03	0.25	0.06	0.06	2	pos	pos	*TEMdel*
A 1203	Sputum	67	F	Community	R	S	S	8	4	0.25	0.25	2	1	0.015	0.25	0.06	0.06	≤1	pos	pos	*TEM*
A 1207	NPA	1	F	Community	R	S	R	0.25	0.5	0.5	0.5	1	2	0.015	0.25	0.06	0.03	2	neg	neg	
A 1208	Sputum	65	M	Community	R	S	R	0.25	0.5	0.5	1	4	2	0.015	4	0.12	0.12	≤1	neg	neg	
A 1209	Eye	13 weeks	M	Community	R	S	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.03	≤1	pos	pos	*TEM*
A 1214	Sputum	70	F	Community	R	S	S	8	8	0.25	0.5	1	1	0.008	0.25	0.03	0.03	≤2	pos	pos	*ROB-1*
A 1220	Eye	25 weeks	F	Community	S	S	R	1	1	1	2	8	4	0.06	2	0.06	0.06	2	neg	neg	
A 1234	Sputum	74	M	Hospital	S	S	R	1	1	1	2	16	4	0.06	8	0.06	0.06	4	neg	neg	
A 1238	NPA	35 weeks	F	Community	R	S	S	32	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1243	Eye	42 weeks	M	Community	R	S	S	0.5	1	0.5	1	8	8	0.03	8	0.06	0.06	2	neg	neg	
A 1246	Eye	23 weeks	F	Community	R	S	R	1	1	1	1	4	4	0.03	4	0.12	0.12	≤1	neg	neg	
A 1248	Eye	56	M	Community	R	S	S	8	4	0.5	0.5	2	1	0.015	0.25	0.06	0.03	4	pos	pos	*TEMdel*
A 1252	Eye	73	F	Hospital	R	S	S	32	16	0.25	0.5	2	2	0.015	0.25	0.06	0.03	≤1	pos	pos	*TEMdel*
A 1255	Corneal scrape	71	M	Community	R	S	R	0.25	0.5	0.5	1	2	2	0.015	0.5	0.06	0.06	2	neg	neg	
A 1259	Neck pus	7	M	Community	R	R	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1260	Eye	65	M	Community	R	S	S	0.25	0.5	0.5	0.5	2	2	0.015	0.25	0.06	0.03	2	neg	neg	
A 1261	Eye	NK	M	Community	R	R	S	8	4	0.5	0.5	2	1	0.015	0.25	0.03	0.03	2	pos	pos	*TEMdel*
A 1262	Eye	NK	M	Community	R	S	S	8	8	0.5	0.5	4	4	0.015	2	0.03	0.03	≤1	pos	pos	*TEMdel*
A 1263	Eye	24 weeks	F	Community	R	R	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.03	≤1	pos	pos	*TEM*
A 1264	Eye	6 weeks	F	Community	R	S	S	16	16	0.5	0.5	1	2	0.008	0.5	0.06	0.03	≤1	pos	pos	*TEMdel*
A 1266	Eye	75	M	Community	R	S	S	8	8	0.5	0.5	4	2	0.015	0.5	0.06	0.06	4	pos	pos	*TEMdel*
A 1267	Sputum	77	M	Community	R	R	R	2	2	1	2	8	2	0.25	0.5	0.12	0.12	≤1	neg	neg	
A 1268	Sputum	81	M	Community	R	S	R	0.5	0.5	0.5	2	8	4	0.015	2	0.06	0.03	4	neg	neg	
A 1270	Sputum	71	M	Hospital	R	S	R	0.25	0.5	0.5	0.5	2	2	0.015	0.5	0.06	0.03	2	neg	neg	
A 1271	Sputum	90	F	Community	R	S	R	0.25	0.5	0.5	0.5	2	2	0.015	0.5	0.06	0.03	2	neg	neg	
A 1272	Sputum	29	F	Community	R	R	R	0.25	0.5	0.25	0.25	1	0.5	≤0.004	0.25	0.06	0.06	4	neg	neg	
A 1273	NPA	4 weeks	F	Community	R	S	R	0.5	0.5	0.5	1	2	2	0.015	0.25	0.06	0.06	≤1	neg	neg	
A 1280	Sputum	55	F	Community	R	R	R	0.25	0.5	0.5	1	2	2	0.03	1	0.06	0.03	≤1	neg	neg	
A 1287	Sputum	68	M	Community	R	R	S	8	8	0.5	1	2	2	0.03	0.5	0.06	0.06	4	pos	pos	*TEMdel*
A 1288	NPA	41 weeks	M	Community	R	S	S	16	32	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*

Appendix 1 Continued.

		Patient details			Disc testing results			MIC (mg/L)											B-lactamase detection		
Lab ID	Specimen	Age	Sex	Source	AMX	AMC	CXM	AMP	AMX	AMC	CXM	CEC	FOX	CTX	IPM	MEM	EPM	EtBr	Nitro	Intra	*bla* gene
A 1290	Sputum	14	M	Community	R	R	R	1	1	1	1	4	2	0.03	0.5	0.12	0.12	≤1	neg	neg	
A 1304	Sputum	72	M	Community	R	S	S	4	8	0.25	0.5	≤0.5	0.5	0.008	≤0.12	0.015	0.015	2	pos	pos	*TEM-1*
A 1307	Sputum	81	F	Community	R	S	S	4	8	0.25	0.25	1	1	0.008	0.25	0.03	0.03	4	pos	pos	*TEMdel*
A 1308	Sputum	73	M	Hospital	R	R	R	0.25	0.5	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	neg	neg	
A 1309	NPA	27 weeks	F	Hospital	R	R	R	0.25	0.5	0.5	0.5	2	2	0.015	0.25	0.06	0.03	≤1	neg	neg	
A 1310	NPA	43 weeks	M	Community	R	S	R	0.25	0.5	0.5	1	4	2	0.015	0.25	0.06	0.03	4	neg	neg	
A 1311	NPA	13 weeks	M	Community	R	S	R	0.25	0.5	0.5	0.5	2	2	0.03	0.5	0.06	0.06	≤1	neg	neg	
A 1314	NPA	1	M	Community	R	R	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1317	Sputum	71	F	Hospital	R	R	R	0.25	0.5	0.5	0.5	2	2	0.015	0.5	0.06	0.03	≤1	neg	neg	
A 1318	Sputum	66	M	Community	R	S	R	0.25	0.5	0.5	0.5	2	2	0.015	0.25	0.03	0.03	≤1	neg	neg	
A 1320	NPA	8 weeks	M	Community	R	R	R	0.25	0.5	0.5	1	2	2	0.015	0.25	0.06	0.03	2	neg	neg	
A 1323	Brochial lavage	65	F	Community	R	R	S	4	4	0.25	0.5	2	2	0.008	0.12	0.03	0.03	2	pos	pos	*TEMdel*
A 1324	Sputum	54	F	Community	R	S	S	16	16	0.5	1	2	2	0.03	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1330	NPA	27 weeks	F	Community	R	S	S	0.5	0.5	0.5	1	2	4	0.03	0.25	0.06	0.03	≤1	neg	neg	
A 1332	Sputum	28	M	Hospital	R	S	R	1	2	2	2	8	8	0.03	4	0.12	0.12	2	neg	neg	
A 1336	NPA	20 weeks	F	Community	R	S	S	0.25	0.25	0.25	1	4	2	0.015	2	0.015	0.015	2	neg	neg	
A 1337	Sputum	75	F	Hospital	R	R	S	0.12	0.25	0.25	0.25	≤0.5	1	0.008	0.25	0.03	0.03	≤1	neg	neg	
A 1339	NPA	1	F	Community	R	R	S	8	8	0.5	1	8	2	0.03	2	0.06	0.06	2	pos	pos	*TEMdel*
A 1341	NPA	31 weeks	M	Community	R	R	S	8	8	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1346	Eye	16 weeks	M	Community	R	R	R	0.5	1	1	2	8	2	0.03	0.5	0.12	0.12	4	neg	neg	
A 1347	Eye	3	M	Community	R	R	R	0.5	1	0.25	1	2	0.5	0.03	≤0.12	0.12	0.12	2	neg	neg	
A 1356	NPA	26 days	M	Community	R	R	S	32	16	1	1	8	2	0.06	0.5	0.12	0.12	≤1	pos	pos	*TEMdel*
A 1359	NPA	14 weeks	F	Community	R	R	R	1	1	1	2	8	2	0.03	0.5	0.25	0.12	≤1	neg	neg	
A 1362	Sputum	73	M	Community	R	S	S	16	16	0.5	0.5	2	2	0.015	0.5	0.03	0.03	2	pos	pos	*TEM*
A 1366	Sputum	74	M	Community	R	R	R	32	64	1	4	16	8	0.03	16	0.12	0.12	≤1	pos	pos	*TEMdel*
A 1370	NPA	22 weeks	F	Community	R	R	S	0.5	0.5	0.5	0.5	2	0.5	0.015	≤0.12	0.06	0.06	≤1	neg	neg	
A 1371	Sputum	77	F	Community	R	S	S	32	32	0.5	1	4	2	0.03	0.5	0.06	0.06	≤1	pos	pos	*TEM-1*
A 1372	Sputum	71	M	Community	R	S	S	8	8	0.5	1	4	2	0.03	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1374	Sputum	26	M	Community	R	R	R	0.25	0.5	0.5	0.5	2	2	0.015	1	0.06	0.03	4	neg	neg	
A 1376	Brochial lavage	73	M	Community	R	R	R	0.5	1	1	1	4	2	0.015	0.5	0.12	0.12	≤1	neg	neg	
A 1380	Eye	NK	M	Community	R	S	R	32	32	1	2	8	4	0.06	8	0.12	0.12	≤1	pos	pos	*TEMdel*
A 1386	Eye	2 days	M	Community	R	S	R	0.5	1	1	1	4	4	0.03	2	0.12	0.12	≤1	neg	neg	
A 1387	Brochial lavage	68	F	Community	R	R	R	1	1	1	1	4	2	0.03	0.5	0.12	0.12	≤1	neg	neg	
A 1388	Sputum	61	F	Community	R	S	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1390	Sputum	81	F	Hospital	R	S	S	32	32	0.5	1	16	2	0.03	2	0.06	0.06	≤1	pos	pos	*TEM-1*
A 1391	Eye	2	F	Community	R	S	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.03	2	pos	pos	*TEM*
A 1393	Eye	75	F	Community	R	S	S	8	8	0.25	0.5	2	1	0.015	0.25	0.06	0.03	≤1	pos	pos	*TEM-1*
A 1395	Sputum	45	F	Community	R	R	R	1	1	1	2	16	4	0.06	4	0.12	0.12	2	neg	neg	
A 1396	Sputum	80	M	Hospital	R	R	R	0.5	1	1	1	4	2	0.03	0.5	0.12	0.06	≤1	neg	neg	
A 1397	Sputum	60	F	Community	R	R	R	32	64	1	2	16	16	0.03	16	0.12	0.12	≤1	pos	pos	*TEMdel*
A 1399	Sputum	69	M	Community	R	S	S	8	8	0.5	0.5	2	2	0.03	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1400	Eye	75	F	Community	S	S	S	1	1	1	2	8	2	0.03	0.5	0.12	0.12	≤1	neg	neg	
A 1401	Eye	2	F	Community	R	S	S	16	32	0.5	0.5	2	2	0.015	0.5	0.06	0.03	2	pos	pos	*TEM*

Appendix 1 Continued.

		Patient details			Disc testing results			MIC (mg/L)											B-lactamase detection		
Lab ID	Specimen	Age	Sex	Source	AMX	AMC	CXM	AMP	AMX	AMC	CXM	CEC	FOX	CTX	IPM	MEM	EPM	EtBr	Nitro	Intra	*bla* gene
A 1404	Sputum	75	M	Community	R	S	S	0.25	0.5	0.5	0.5	2	2	0.015	0.25	0.03	0.03	4	neg	neg	
A 1407	Eye	87	F	Hospital	R	S	S	32	32	0.5	1	8	2	0.03	0.5	0.06	0.06	≤1	pos	pos	*TEM-1*
A 1413	Sputum	55	M	Community	R	S	R	0.5	1	0.5	1	4	2	0.03	0.5	0.06	0.06	2	neg	neg	
A 1421	Eye	2	F	Community	R	R	R	1	2	2	2	8	8	0.03	2	0.12	0.12	2	neg	neg	
A 1422	Eye	1	M	Community	R	R	R	1	2	2	2	8	4	0.06	2	0.12	0.12	2	neg	neg	
A 1425	Sputum	52	F	Community	R	S	S	16	32	2	1	2	2	0.015	2	0.03	0.03	8	pos	pos	*TEM-1*
A 1428	Sputum	53	F	Community	R	R	S	32	64	2	1	16	8	0.03	4	0.12	0.06	≤1	pos	pos	*TEM*
A 1430	Eye	86	M	Community	R	S	S	8	8	0.25	0.5	1	2	0.008	0.25	0.03	0.03	2	pos	pos	*TEM-1*
A 1434	Eye	7 days	F	Community	S	S	R	1	1	1	4	16	4	0.06	2	0.06	0.12	2	neg	neg	
A 1436	Eye	NK	NK				R	0.5	1	1	2	8	2	0.03	0.5	0.06	0.06	≤1	neg	neg	
A 1437	Eye	2 weeks	M	Community	R	S	S	32	32	2	4	16	4	0.06	8	0.12	0.12	≤1	pos	pos	*TEMdel*
A 1438	Eye	77	F	Community	R	S	S	16	16	0.5	0.5	2	4	0.015	0.5	0.06	0.03	2	pos	pos	*TEMdel*
A 1439	Eye	32	F	Community	R	S	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1441	Eye	17 weeks	M	Community	R	R	R	1	2	2	4	16	4	0.03	2	0.25	0.12	2	neg	neg	
A 1447	Eye	1	F	Community	R	S	R	2	2	2	1	4	8	0.06	4	0.5	0.25	2	neg	neg	
A 1449	Eye	1	F	Community	R	S	S	16	32	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1456	Sputum	75	M	Hospital	R	R	R	1	1	1	4	16	8	0.06	4	0.12	0.12	4	neg	neg	
A 1458	Eye	1	M	Community	R	S	S	8	8	0.5	1	4	2	0.03	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1460	Eye	81	F	Hospital	R	R	S	8	8	0.5	0.5	1	1	0.015	1	0.06	0.03	≤1	pos	pos	*TEMdel*
A 1464	Eye	9	F	Community	R	S	R	64	64	2	4	32	16	0.06	8	0.12	0.12	2	pos	pos	*TEM-1*
A 1468	Sputum	54	F	Community	R	R	S	16	16	0.5	0.5	2	2	0.015	0.12	0.03	0.03	2	pos	pos	*TEM-1*
A 1469	NPA	1	F	Community	R	R	R	1	1	1	0.5	4	2	0.03	0.5	0.12	0.12	≤1	neg	neg	
A 1473	Sputum	67	F	Community	R	R	R	1	2	2	2	16	8	0.03	4	0.12	0.12	2	neg	neg	
A 1475	Eye	84	F	Community	R	R	R	1	1	1	2	4	2	0.06	0.25	0.12	0.12	2	neg	neg	
A 1476	Brochial lavage	78	F	Hospital	R	R	S	8	4	1	1	4	2	0.03	0.5	0.12	0.06	4	pos	pos	*TEMdel*
A 1482	Eye	61	M	Community	R	S	S	8	8	0.5	0.5	2	4	0.015	1	0.03	0.03	2	pos	pos	*TEMdel*
A 1488	Sputum	88	M	Hospital	R	S	S	2	2	0.5	0.5	2	1	0.015	0.5	0.06	0.03	4	pos	pos	*TEM-1*
A 1489	Sputum	77	F	Community	R	S	R	0.5	1	1	1	8	8	0.03	2	0.12	0.06	4	neg	neg	
A 1491	Sputum	81	F	Community	R	R		16	32	0.5	0.5	2	2	0.03	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1525	Sputum	62	M	Community	R	S	S	32	16	1	4	32	16	0.03	16	0.12	0.12	2	pos	pos	*TEMdel*
A 1528	Sputum	50	M	Community	R	S	S	8	8	0.5	1	4	4	0.015	1	0.03	0.03	32	pos	pos	*TEMdel*
A 1531	Sputum	50	M	Hospital	R	S	S	16	16	1	0.5	4	2	0.015	0.5	0.12	0.06	4	pos	pos	*TEMdel*
A 1538	Sputum	81	M	Hospital	R	R	R	1	2	2	1	4	2	0.03	2	0.25	0.12	≤1	neg	neg	
A 1545	Eye	84	F	Hospital	R	S	S	32	32	1	2	16	8	0.06	8	0.12	0.12	≤1	pos	pos	*TEMdel*
A 1547	Sputum	74	M	Hospital	R	S	S	16	32	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1555	Sputum	72	M	Community	R	S	S	8	8	0.5	0.5	4	4	0.03	0.25	0.06	0.03	≤1	pos	pos	*TEMdel*
A 1558	Brochial lavage	62	M	Community	R	S	S	8	4	0.5	0.5	2	2	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1575	Sputum	78	M	Community	R	S	S	32	64	1	8	64	32	0.03	16	0.12	0.12	2	pos	pos	*TEMdel*
A 1576	Sputum	78	F	Community	R	S	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1588	Sputum	86	M	Community	R	S	S	2	2	0.5	0.5	4	1	0.015	0.25	0.5	0.06	≤1	pos	pos	*TEMdel*
A 1604	Blood	86	F	Community	R	S	S	16	16	1	2	8	4	0.03	0.5	0.12	0.12	4	pos	pos	*TEMdel*
A 1605	Sputum	95	M	Hospital	R	S	S	8	8	1	1	4	4	0.03	0.5	0.12	0.12	≤1	pos	pos	*TEMdel*
A 1607	Sputum	68	M	Hospital	R	S	S	2	4	0.5	0.5	2	2	0.015	0.5	0.06	0.06	4	pos	pos	*TEMdel*

Appendix 1 Continued.

		Patient details			Disc testing results			MIC (mg/L)											B-lactamase detection		
Lab ID	Specimen	Age	Sex	Source	AMX	AMC	CXM	AMP	AMX	AMC	CXM	CEC	FOX	CTX	IPM	MEM	EPM	EtBr	Nitro	Intra	*bla* gene
A 1608	Brochial lavage	67	F	Community	R	R	R	1	2	2	2	8	1	0.12	0.25	0.12	0.12	4	neg	neg	
A 1616	Eye	1	M	Community	R	R	R	1	2	2	2	16	4	0.06	2	0.12	0.12	≤1	neg	neg	
A 1620	Ear	3	F	Community	R	S	S	8	8	0.25	0.5	2	2	0.015	0.5	0.06	0.03	2	pos	pos	*TEM*
A 1624	Sputum	62	M	Community	R	S	S	16	16	0.5	0.5	2	4	0.015	2	0.06	0.03	2	pos	pos	*TEMdel*
A 1645	Sputum	64	F	Community	R	S	S	32	32	0.5	1	NA	NA	0.03	2	0.06	0.06	2	pos	pos	*TEMdel*
A 1650	Sputum	47	M	Hospital	R	S	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.03	≤1	pos	pos	*TEMdel*
A 1651	Eye	3	F	Community	R	R	R	32	64	2	8	32	8	0.06	8	0.12	0.12	≤1	pos	pos	*TEMdel*
A 1652	Eye	21 weeks	M	Community	R	S	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1656	Eye	12	F	Community	R	R	R	1	2	2	1	8	8	0.03	2	0.12	0.12	≤1	neg	neg	
A 1657	Eye	1	M	Community	R	R	R	1	1	1	2	16	4	0.06	2	0.12	0.12	≤1	neg	neg	
A 1658	Brochial lavage	63	M	Community	R	R	R	0.5	1	1	2	16	4	0.03	0.5	0.06	0.06	2	neg	neg	
A 1659	Sputum	81	M	Community	R	S	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.03	≤1	pos	pos	*TEMdel*
A 1670	Eye	2	F	Community	R	R	R	1	1	1	2	8	4	0.06	0.5	0.06	0.06	4	neg	neg	
A 1671	Eye	36 weeks	F	Community	R	R	R	1	2	2	2	8	2	0.06	2	0.12	0.12	≤1	neg	neg	
A 1672	Sputum	58	M	Hospital	R	S	S	8	8	0.5	1	4	1	0.015	0.25	0.03	0.03	≤1	pos	pos	*TEM*
A 1675	Sputum	50	F	Hospital	R	S	S	4	8	0.5	0.5	2	2	0.015	0.5	0.03	0.03	4	pos	pos	*TEM-1*
A 1678	Sputum	76	M	Hospital	R	S	S	16	16	0.5	0.5	2	4	0.015	0.5	0.06	0.03	≤1	pos	pos	*TEMdel*
A 1679	Sputum	84	M	Hospital	R	S	S	16	32	0.5	0.5	2	2	0.015	0.5	0.06	0.06	4	pos	pos	*TEMdel*
A 1680	Sputum	86	F	Community	R	S	S	16	16	0.5	0.5	2	4	0.015	1	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1683	Sputum	86	M	Community	R	S	S	1	1	1	2	8	4	0.06	2	0.12	0.12	2	neg	neg	
A 1688	Sputum	41	M	Community	R	S	S	64	64	0.5	0.5	NA	NA	0.015	0.5	0.06	0.06	≤1	pos	pos	*TEMdel*
A 1700	Eye	1	M	Community	R	S	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.03	≤1	pos	pos	*TEM*
A 1718	Sputum	75	F	Community	R	S	S	8	8	0.5	0.5	1	2	0.015	0.5	0.03	0.03	≤1	pos	pos	*TEMdel*
A 1736	Eye	90	F	Hospital	R	R	R	16	32	2	4	16	4	0.06	8	0.12	0.12	≤1	pos	pos	*TEMdel*
A 1745	Eye	36 weeks	M	Community	R	S	S	8	8	0.5	1	4	2	0.03	0.5	0.06	0.06	4	pos	pos	*TEMdel*
A 1749	Eye	2	F	Community	R	S	S	16	32	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1757	Sputum	70	M	Community	R	S	S	8	8	0.5	0.5	2	2	0.03	0.5	0.06	0.06	2	pos	pos	*TEMdel*
A 1758	Brochial lavage	71	F	Community	R	R	R	2	2	2	1	4	1	0.12	0.5	0.12	0.25	≤1	neg	neg	
A 1761	Sputum	65	M	Hospital	R	S	S	4	8	0.5	0.5	2	2	0.015	0.5	0.06	0.06	2	pos	pos	*TEM*
A 1769	Sputum	66	M	Community	R	S	R	1	2	2	1	8	8	0.03	4	0.12	0.12	2	neg	neg	
A 1772	Eye	1	F	Community	R	R	S	16	16	0.5	0.5	2	2	0.015	0.5	0.06	0.06	4	pos	pos	*TEMdel*
A 1781	Vulval swab	3	F	Community	R	S	S	8	2	0.25	0.5	2	2	0.015	0.25	0.03	0.015	2	pos	pos	*TEMdel*
A 1783	Sputum	81	M	Community	R	S	S	8	8	0.5	0.5	4	2	0.015	0.5	0.03	0.03	2	pos	pos	*TEM-1*
A 1805	Eye	1	M	Community	R	S	S	16	32	0.5	0.5	1	1	0.008	0.25	0.03	0.03	≤1	pos	pos	*TEM-1*
A 1807	NPA	6 weeks	M	Community	R	S	R	1	1	1	2	16	4	0.06	0.5	0.12	0.06	≤1	neg	neg	

AMP, ampicillin; AMX, amoxicillin; AMC, co-amoxiclav; CXM, cefuroxime; CEC, cefaclor; FOX, cefoxitin; CTX, cefotaxime; IPM, imipenem; MEM, meropenem; EPM, ertapenem; EtBr, ethidium bromide; Nitro, Nitrocefin; Intra, Intralactam; NPA, naso-pharyngeal aspirate; ET secretion, endo-tracheal secretion; F, female; M, male; R, resistant; S, susceptible; NA, not available; shaded cells, MICs above BSAC MIC breakpoint (Andrews, 2004).

Appendix 2. Nucleotide sequence of *Haemophilus influenzae* Rd from position 1196452 bp to 1200937 bp (Genome Sequence DataBase accession number L42023).

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        LongFTS1 primer ->
   1  ATTGGCTCCA GCACAGATTT TGCTGCTGAT GCGTTAAATG ATTTCTCATT
      TAACCGAGGT CGTGTCTAAA ACGACGACTA CGCAATTTAC TAAAGAGTAA

  51  ATAGAAAATA ATGTAGTACT TTTATCTTAA ATTTATGAAT AGCGAAAATT
      TATCTTTTAT TACATCATGA AAATAGAATT TAAATACTTA TCGCTTTTAA

 101  CCTTTTCTTC ATCTGAACAT ATTACAGTTT TACTTCACGA AGCCGTGAAT
      GGAAAAGAAG TAGACTTGTA TAATGTCAAA ATGAAGTGCT TCGGCACTTA

 151  GGCTTGGCAT TGAAGGAGAA TGGCATTTAT ATTGATGGTA CTTTTGGGCG
      CCGAACCGTA ACTTCCTCTT ACCGTAAATA TAACTACCAT GAAAACCCGC

 201  TGGGGGGCAT TCTCGGTTTA TCCTTTCTCA ACTTTCTTCT AATGGTCGTT
      ACCCCCCGTA AGAGCCAAAT AGGAAAGAGT TGAAAGAAGA TTACCAGCAA

 251  TGATAGCTGT AGATCGCGAT CCTCGTGCTA TTGCAGAAGC ACACAAAATC
      ACTATCGACA TCTAGCGCTA GGAGCACGAT AACGTCTTCG TGTGTTTTAG

 301  CAAGACTTGC GTTTTCAGAT TGAACATAAC AGCTTTTCGC ATATTCCTGA
      GTTCTGAACG CAAAAGTCTA ACTTGTATTG TCGAAAAGCG TATAAGGACT

 351  AATTTGTGAC AAATTAAATT TAGTGGGCAA AATTGACGGT ATTTTGCTTG
      TTAAACACTG TTTAATTTAA ATCACCCGTT TTAACTGCCA TAAAACGAAC

 401  ATCTTGGTGT GTCTTCCCCT CAGCTTGATG AAGCAGAACG TGGTTTTAGT
      TAGAACCACA CAGAAGGGGA GTCGAACTAC TTCGTCTTGC ACCAAAATCA

 451  TTTATGAAAG ATGGCCCGCT TGATATGCGT ATGGATACAA CTCAAGGTTT
      AAATACTTTC TACCGGGCGA ACTATACGCA TACCTATGTT GAGTTCCAAA

 501  ATCTGCTGAA GAATGGTTAA AACAAGTGTC CATTGAGGAT TTAACTTGGG
      TAGACGACTT CTTACCAATT TTGTTCACAG GTAACTCCTA AATTGAACCC

 551  TGTTGAAAAC TTTTGGCGAA GAGCGTTTCG CTAAACGTAT TGCCACTGCT
      ACAACTTTTG AAAACCGCTT CTCGCAAAGC GATTTGCATA ACGGTGACGA

 601  ATTGTTGATT TCAATAAAAG TGCGGTAAAA AATGGCACAG AATTTTTATC
      TAACAACTAA AGTTATTTTC ACGCCATTTT TTACCGTGTC TTAAAAATAG

 651  GCGTACCAGT CAATTGGCGG AACTTATTTC ACAGGCAGTT CCTTTTAAAG
      CGCATGGTCA GTTAACCGCC TTGAATAAAG TGTCCGTCAA GGAAAATTTC

 701  ATAAACATAA ACATCCTGCG ACGCGTAGTT TCCAAGCTAT TCGTATTTTT
      TATTTGTATT TGTAGGACGC TGCGCATCAA AGGTTCGATA AGCATAAAAA

 751  ATTAATTCGG AATTAGATGA ATTAGAAAGT CTGCTTAATT CTGCGTTAGA
      TAATTAAGCC TTAATCTACT TAATCTTTCA GACGAATTAA GACGCAATCT

 801  TATGTTAGCA CCAGAAGGTC GTTTATCAAT TATTAGTTTC CATTCTTTAG
      ATACAATCGT GGTCTTCCAG CAAATAGTTA ATAATCAAAG GTAAGAAATC
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 851  AAGATAGAAT GGTGAAACAT TTTATGAAAA AACAAAGTAA GGGCGAGGAT
      TTCTATCTTA CCACTTTGTA AAATACTTTT TTGTTTCATT CCCGCTCCTA

 901  ATTCCCAAAG GTTTACCATT GCGAGAAGAT CAAATTCAGC GTAATCAAAA
      TAAGGGTTTC CAAATGGTAA CGCTCTTCTA GTTTAAGTCG CATTAGTTTT

 951  ATTAAGAATT ATTGGTAAAG CCATTCAGCC AAGTGATGCA GAAATTCAAG
      TAATTCTTAA TAACCATTTC GGTAAGTCGG TTCACTACGT CTTTAAGTTC

1001  CCAATCCTCG TTCAAGAAGT GCCATATTAC GTGTGGCAGA GAGAATTTAG
      GGTTAGGAGC AAGTTCTTCA CGGTATAATG CACACCGTCT CTCTTAAATC

1051  CGATGTCTGA AAATAATAAG CCTCGTTATC CGTTACAGCA GATTTTAGTC
      GCTACAGACT TTTATTATTC GGAGCAATAG GCAATGTCGT CTAAAATCAG

1101  GAAGATTTAT TTTCTTCAAA TAAGTTAGTG GTGTTGCTGT TAATAGGGAT
      CTTCTAAATA AAAGAAGTTT ATTCAATCAC CACAACGACA ATTATCCCTA

1151  TTTAGTTTCT GCAATGGGGA CGATTTGGAT AACCCATAAA ACTCGCCAAT
      AAATCAAAGA CGTTACCCCT GCTAAACCTA TTGGGTATTT TGAGCGGTTA

1201  TAATTTCTGA AAATGGAATG TTAATTTTAC AGCGTCAAGC ACTTGAGAAT
      ATTAAAGACT TTTACCTTAC AATTAAAATG TCGCAGTTCG TGAACTCTTA

1251  GAATACCGTA ATTTACAAGT GCAGGAAGCT ACGGAAGGGG ATAGCACGCG
      CTTATGGCAT TAAATGTTCA CGTCCTTCGA TGCCTTCCCC TATCGTGCGC

1301  AGTAGAATCT ATTGCGATTA GTACATTAAA AATGAAAGTT GTTTCTTCAG
      TCATCTTAGA TAACGCTAAT CATGTAATTT TTACTTTCAA CAAAGAAGTC

1351  AGCAAGAAGT TGAAATTAGA GAATAATAGG TAAAAAAAAT GGTGAAATTT
      TCGTTCTTCA ACTTTAATCT CTTATTATCC ATTTTTTTTA CCACTTTAAA

1401  AATTCCTCGC GTAAATCAGG TAAGTCAAAA AAAACAATTA GAAAATTGAC
      TTAAGGAGCG CATTTAGTCC ATTCAGTTTT TTTTGTTAAT CTTTTAACTG

1451  CGCACCTGAA ACTGTAAAGC AAAACAAGCC TCAAAAGGTG TTTGAAAAAT
      GCGTGGACTT TGACATTTCG TTTTGTTCGG AGTTTTCCAC AAACTTTTTA

1501  GCTTTATGCG TGGACGTTAT ATGCTTTCTA CGGTTCTTAT TTTACTTGGC
      CGAAATACGC ACCTGCAATA TACGAAAGAT GCCAAGAATA AAATGAACCG

1551  CTGTGTGCTT TAGTCGCACG AGCAGCTTAT GTTCAATCTA TTAATGCCGA
      GACACACGAA ATCAGCGTGC TCGTCGAATA CAAGTTAGAT AATTACGGCT

1601  TACGTTATCG AATGAAGCGG ATAAGCGTTC TTTGCGTAAA GATGAAGTAT
      ATGCAATAGC TTACTTCGCC TATTCGCAAG AAACGCATTT CTACTTCATA

1651  TATCGGTGCG TGGTTCTATT TTAGATCGTA ATGGTCAGCT TTTATCTGTA
      ATAGCCACGC ACCAAGATAA AATCTAGCAT TACCAGTCGA AAATAGACAT

1701  AGCGTGCCGA TGAGCGCGAT TGTGGCAGAT CCAAAAACGA TGTTGAAGGA
      TCGCACGGCT ACTCGCGCTA ACACCGTCTA GGTTTTTGCT ACAACTTCCT

1751  AAATTCGCTT GCGGATAAAG AACGAATTGC AGCTTTAGCC GAAGAATTAG
      TTTAAGCGAA CGCCTATTTC TTGCTTAACG TCGAAATCGG CTTCTTAATC
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1801  GTATGACTGA AAATGATTTA GTGAAAAAAA TTGAGAAAAA TTCTAAATCT
      CATACTGACT TTTACTAAAT CACTTTTTTT AACTCTTTTT AAGATTTAGA

1851  GGTTATTTGT ATTTAGCACG TCAAGTTGAA TTAAGTAAAG CTAACTATAT
      CCAATAAACA TAAATCGTGC AGTTCAACTT AATTCATTTC GATTGATATA

1901  TCGTAGATTA AAAATTAAGG GTATTATTTT AGAAACAGAG CATCGCCGTT
      AGCATCTAAT TTTTAATTCC CATAATAAAA TCTTTGTCTC GTAGCGGCAA

1951  TTTATCCTCG TGTAGAAGAA GCTGCACACG TGGTGGGTTA TACGGATATT
      AAATAGGAGC ACATCTTCTT CGACGTGTGC ACCACCCAAT ATGCCTATAA

2001  GATGGAAATG GTATTGAAGG CATTGAGAAA AGTTTTAATT CCCTGCTTGT
      CTACCTTTAC CATAACTTCC GTAACTCTTT TCAAAATTAA GGGACGAACA

2051  TGGTAAAGAC GGTTCACGTA CTGTTCGTAA AGATAAACGT GGGAATATTG
      ACCATTTCTG CCAAGTGCAT GACAAGCATT TCTATTTGCA CCCTTATAAC

2101  TTGCACATAT CTCCGATGAG AAAAAATATG ATGCACAAGA TGTTACCTTA
      AACGTGTATA GAGGCTACTC TTTTTTATAC TACGTGTTCT ACAATGGAAT

2151  AGTATCGATG AAAAATTGCA ATCTATGGTG TATCGTGAGA TTAAAAAGGC
      TCATAGCTAC TTTTTAACGT TAGATACCAC ATAGCACTCT AATTTTTCCG

2201  GGTGTCTGAG AATAATGCTG AGTCTGGTAC TGCGGTGTTA GTTGATGTTC
      CCACAGACTC TTATTACGAC TCAGACCATG ACGCCACAAT CAACTACAAG

2251  GAACAGGGGA AGTGTTAGCT ATGGCGACTG CGCCCTCTTA TAATCCAAAC
      CTTGTCCCCT TCACAATCGA TACCGCTGAC GCGGGAGAAT ATTAGGTTTG

                                        HINF1 primer ->
2301  AACCGTGTCG GCGTGAAATC AGAGTTAATG CGTAACCGTG CAATTACCGA
      TTGGCACAGC CGCACTTTAG TCTCAATTAC GCATTGGCAC GTTAATGGCT

2351  TACTTTTGAG CCAGGTTCTA CGGTAAAACC TTTCGTTGTT TTAACCGCAC
      ATGAAAACTC GGTCCAAGAT GCCATTTTGG AAAGCAACAA AATTGGCGTG

2401  TTCAACGAGG TGTAGTTAAA CGAGATGAAA TTATTGATAC TACGTCCTTT
      AAGTTGCTCC ACATCAATTT GCTCTACTTT AATAACTATG ATGCAGGAAA

2451  AAATTAAGCG GTAAAGAAAT TGTGGACGTT GCACCACGTG CTCAGCAAAC
      TTTAATTCGC CATTTCTTTA ACACCTGCAA CGTGGTGCAC GAGTCGTTTG

2501  TTTAGACGAG ATTTTAATGA ACTCTAGTAA CCGTGGTGTA AGTCGTCTTG
      AAATCTGCTC TAAAATTACT TGAGATCATT GGCACCACAT TCAGCAGAAC

2551  CATTACGTAT GCCACCTAGT GCATTAATGG AAACTTATCA AAATGCAGGT
      GTAATGCATA CGGTGGATCA CGTAATTACC TTTGAATAGT TTTACGTCCA

2601  TTAAGTAAAC CGACAGATTT AGGCTTGATC GGAGAGCAAG TTGGGATTTT
      AATTCATTTG GCTGTCTAAA TCCGAACTAG CCTCTCGTTC AACCCTAAAA

2651  GAATGCAAAT CGTAAACGCT GGGCAGATAT TGAGCGTGCA ACAGTCGCTT
      CTTACGTTTA GCATTTGCGA CCCGTCTATA ACTCGCACGT TGTCAGCGAA

2701  ATGGTTATGG TATTACTGCG ACACCTTTAC AAATTGCTCG TGCCTATGCA
      TACCAATACC ATAATGACGC TGTGGAAATG TTTAACGAGC ACGGATACGT
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2751  ACCCTTGGTA GTTTCGGTGT TTATCGTCCG CTTTCTATCA CTAAAGTTGA
      TGGGAACCAT CAAAGCCACA AATAGCAGGC GAAAGATAGT GATTTCAACT

2801  TCCGCCAGTT ATTGGGAAAC GGGTTTTCTC TGAAAAAATA ACTAAAGATA
      AGGCGGTCAA TAACCCTTTG CCCAAAAGAG ACTTTTTTAT TGATTTCTAT

2851  TTGTGGGAAT TTTAGAGAAA GTAGCAATTA AAAATAAACG CGCAATGGTG
      AACACCCTTA AAATCTCTTT CATCGTTAAT TTTTATTTGC GCGTTACCAC

2901  GAAGGCTACC GTGTCGGCGT AAAAACAGGT ACGGCACGTA AGATTGAAAA
      CTTCCGATGG CACAGCCGCA TTTTTGTCCA TGCCGTGCAT TCTAACTTTT

2951  TGGACATTAT GTAAATAAGT ATGTGGCATT TACTGCGGGT ATTGCACCAA
      ACCTGTAATA CATTTATTCA TACACCGTAA ATGACGCCCA TAACGTGGTT

3001  TTAGTGATCC TCGTTATGCA TTAGTGGTTT TGATCAATGA TCCAAAAGCA
      AATCACTAGG AGCAATACGT AATCACCAAA ACTAGTTACT AGGTTTTCGT
            <- HINF2 primer

3051  GGAGAATATT ATGGTGGTGC GGTTTCTGCC CCTGTATTCT CTAACATTAT
      CCTCTTATAA TACCACCACG CCAAAGACGG GGACATAAGA GATTGTAATA

3101  GGGCTATGCG TTACGTGCAA ATGCTATTCC GCAAGATGCT GAAGCAGCTG
      CCCGATACGC AATGCACGTT TACGATAAGG CGTTCTACGA CTTCGTCGAC

3151  AAAACACAAC AACGAAAAGT GCAAAACGTA TTGTTTATAT TGGCGAACAC
      TTTTGTGTTG TTGCTTTTCA CGTTTTGCAT AACAAATATA ACCGCTTGTG
                                >
3201  AAGAATCAAA AAGTGAATTA AGGAAAAATT ATGAAAAAAC TCACCGCACT
      TTCTTAGTTT TTCACTTAAT TCCTTTTTAA TACTTTTTTG AGTGGCGTGA

3251  TTTTAATTTG CCTGAATTAA AGAATGATAT AGAACTCCAT AATATGGTGT
      AAAATTAAAC GGACTTAATT TCTTACTATA TCTTGAGGTA TTATACCACA

3301  TAGATAGCCG TAAGGTTAAA GCTGGCGATC TTTTTGTGGC GATAAAAGGT
      ATCTATCGGC ATTCCAATTT CGACCGCTAG AAAAACACCG CTATTTTCCA

3351  CATCAGGTGG ATGGAAATCA ATTTATTGAT TCTGCTCTTC ATTCTGGTGC
      GTAGTCCACC TACCTTTAGT TAAATAACTA AGACGAGAAG TAAGACCACG

3401  GAGTGCGGTG GTTTCTGAGA CAGAATTATC CAGCGAGCAT TTAACTGTAG
      CTCACGCCAC CAAAGACTCT GTCTTAATAG GTCGCTCGTA AATTGACATC

3451  CGTTTATCGG GAATGTTCCC GTAGTGAAAT ATTATCAACT TGCACATCAT
      GCAAATAGCC CTTACAAGGG CATCACTTTA TAATAGTTGA ACGTGTAGTA

3501  CTTTCATCTT TGGCGGATGT TTTCTATGAT TCGCCCTCTA ACAATTTAAC
      GAAAGTAGAA ACCGCCTACA AAAGATACTA AGCGGGAGAT TGTTAAATTG

3551  CCTTGTTGGT GTCACGGGGA CAAATGGCAA AACCACTATT TCTCAATTAT
      GGAACAACCA CAGTGCCCCT GTTTACCGTT TTGGTGATAA AGAGTTAATA

3601  TAGCGCAATG GGCGGAATTA TTGGGGCATC GTGCGGCTGT GATGGGAACC
      ATCGCGTTAC CCGCCTTAAT AACCCCGTAG CACGCCGACA CTACCCTTGG

3651  ATTGGTAATG GACTTTTTGG GCAAATTGTA GAAGCTAAAA ATACGACAGG
      TAACCATTAC CTGAAAAACC CGTTTAACAT CTTCGATTTT TATGCTGTCC
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3701  TTCAGCAGTA GAAATTCAGT CATCTCTTTC AGCTTTCAAA CACGCAGGTG
      AAGTCGTCAT CTTTAAGTCA GTAGAGAAAG TCGAAAGTTT GTGCGTCCAC

3751  CAGATTTTAC CTCTATTGAA GTTTCATCAC ACGGTTTGGC GCAGCATCGT
      GTCTAAAATG GAGATAACTT CAAAGTAGTG TGCCAAACCG CGTCGTAGCA

3801  GTAGAAGCCT TGCATTTTAA AGCAGCAATT TTCACGAATT TAACCCGTGA
      CATCTTCGGA ACGTAAAATT TCGTCGTTAA AAGTGCTTAA ATTGGGCACT

3851  TCATCTAGAT TATCATCAAT CTATGGAAAA TTATGCTGCA GCGAAGAAAC
      AGTAGATCTA ATAGTAGTTA GATACCTTTT AATACGACGT CGCTTCTTTG

3901  GCTTGTTCAC TGAATTAGAT ACCCAAATTA AAGTGATTAA TGCTGATGAT
      CGAACAAGTG ACTTAATCTA TGGGTTTAAT TTCACTAATT ACGACTACTA

3951  GAAATTGGAT ACCAATGGCT AACTGAACTA CCTGATGCTA TTGCCGTAAG
      CTTTAACCTA TGGTTACCGA TTGACTTGAT GGACTACGAT AACGGCATTC

4001  TATGAATGCG GATTTTAAAG TAGGTTCACA CCAATGGATG AAAGCAATAA
      ATACTTACGC CTAAAATTTC ATCCAAGTGT GGTTACCTAC TTTCGTTATT

4051  ATATCCATTA TCATTTTAAA GGTGCAGATA TTACTTTTGA ATCTAGCTGG
      TATAGGTAAT AGTAAAATTT CCACGTCTAT AATGAAAACT TAGATCGACC

4101  GGTAATGGTG TTTTGCATAG CCCATTAATT GGTGCTTTTA ATGTAAGTAA
      CCATTACCAC AAAACGTATC GGGTAATTAA CCACGAAAAT TACATTCATT

4151  TTTATTATTA GTAATGACCA CGTTGTTATC GTTTGGTTAC CCATTGGAAA
      AAATAATAAT CATTACTGGT GCAACAATAG CAAACCAATG GGTAACCTTT

4201  ATTTACTCGC TACGGCGAAA TCTTTAAAAG GAGTATGTGG AAGAATGGAA
      TAAATGAGCG ATGCCGCTTT AGAAATTTTC CTCATACACC TTCTTACCTT

4251  ATGATTCAAT ATCCAAATAA ACCAACCGTT ATTGTAGATT ATGCGCATAC
      TACTAAGTTA TAGGTTTATT TGGTTGGCAA TAACATCTAA TACGCGTATG

4301  ACCAGATGCG TTGGAAAAAG CGTTGATTGC TGCGCGTGAA CATTGCCAAG
      TGGTCTACGC AACCTTTTTC GCAACTAACG ACGCGCACTT GTAACGGTTC

4351  GCGAATTATG GTGCATTTTT GGTTGTGGCG GAGACCGTGA TAGAGGCAAA
      CGCTTAATAC CACGTAAAAA CCAACACCGC CTCTGGCACT ATCTCCGTTT

4401  CGTCCGTTAA TGGCACAGGT TGCAGAGCAG TTTGCTGAAA AGATTATTGT
      GCAGGCAATT ACCGTGTCCA ACGTCTCGTC AAACGACTTT TCTAATAACA

4451  GACAAAAGAT AATCCACGAA CAGAATCACA AAGCCA
      CTGTTTTCTA TTAGGTGCTT GTCTTAGTGT TTCGGT
                        <- LongFTS2 primer
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Shaded area, location of the *ftsI* gene; LongFTS1/LongFTS2 primers for amplification of the 4.486 Kb DNA fragment used for transformation; HINF1/HINF2 primers for amplification of the transpeptidase region of *ftsI* used for nucleotide sequencing.

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Publication arising from this thesis

Paper Number E-315

Activity of Carbapenems against Clinical Isolates of *Haemophilus influenzae*.

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Abstract

BACKGROUND
The extensive distribution of β-lactam resistant *Haemophilus influenzae* is well documented, but reports of carbapenem resistance are largely confined to Japan. A study was undertaken to determine the prevalence of carbapenem resistance in 260 ampicillin and or cefuroxime resistant, non-consecutive, clinically significant isolates of *H. influenzae* isolated at City Hospital, Birmingham, UK, between January 2000 and October 2003.

METHOD
Susceptibility to ertapenem, imipenem and meropenem and a range of other compounds were determined by BSAC standardized methodology. β-Lactamase production was detected with nitrocefin and Intralactam and PCR was used to detect the presence of bla_{TEM} and bla_{ROB} in the β-lactamase positive isolates.

RESULTS
Of the 260 organisms tested, 139 (53.5 %) were β-lactamase positive; 136 were bla_{TEM} positive and three were positive for bla_{ROB} by PCR. The MIC_{50}/ MIC_{90} and MIC range of ertapenem, imipenem and meropenem for the isolates were 0.06/0.12 (0.008 - 0.25), 0.5/4 (≤0.12 - 16) and 0.06/0.12 (0.008 - 0.5) μg/mL respectively. Fifteen (5.8 %) of the isolates were resistant to imipenem (IMR) using the BSAC breakpoint of 4.0 μg/mL (MIC of imipenem 8 - 16 μg/mL). All 15 of the IMR isolates were susceptible to ertapenem and meropenem (MIC range 0.06 - 0.12 μg/mL). Eleven of the isolates were β-lactamase producers (TEM-1) and the majority were resistant to cefuroxime (MIC range 1 - 8 μg/mL). None of the IMR isolates were resistant to imipenem when tested by the BSAC standardized disc susceptibility testing method.

CONCLUSION
Carbapenem resistance was rare among the isolates of *H. influenzae* tested; all the isolates were susceptible to ertapenem and meropenem. The finding of imipenem resistance in 5.8 % of the isolates highlights the fact that susceptibility to imipenem cannot be inferred by susceptibility to ertapenem or meropenem. Disc susceptibility testing of carbapenems against *H. influenzae* may be unreliable, as imipenem resistance was not detected using the BSAC methodology.

Introduction

The incidence of resistance to β-lactam antibiotics, such as ampicillin and cefuroxime in *Haemophilus influenzae* is recognised worldwide. Several mechanisms of β-lactam resistance have been described, including two β-lactamase enzymes (TEM-1 and ROB-1) and reduced affinity penicillin binding proteins.

Carbapenems may be an alternative treatment for invasive infections caused by *H. influenzae* where standard β-lactam therapy may no longer be relied upon. Few data, however, are available regarding the activity of carbapenems against *H. influenzae*. Reports of carbapenem resistance in *H. influenzae* have mainly been confined to Japan.[1]

We present data from a study examining the carbapenem susceptibility of clinical isolates of *H. influenzae* with reduced β-lactam susceptibility isolated at City Hospital, Birmingham, UK, between January 2000 and October 2003.

Materials and Methods

Isolates
A total of 260 non-consecutive clinically significant isolates of *H. influenzae* were examined which had been isolated from 256 patients. The majority of isolates were from respiratory samples (61.9%) and conjunctival swabs (35.0%). A further 1.2% isolates were from blood cultures. The isolates were initially selected on the basis of resistance to one or more of amoxicillin, co-amoxiclav and cefuroxime using the standardized BSAC disc susceptibility testing.[2]

Antimicrobial susceptibility
The susceptibility of isolates to imipenem, meropenem, ertapenem, ampicillin, amoxicillin, co-amoxiclav, cefuroxime, cefotaxime, and cefaclor were determined by agar dilution MIC using BSAC methodology. Briefly, dilutions of the antimicrobial compounds were made in Iso-Sensitest agar (Oxoid Ltd., Basingstoke, UK) supplemented with 5% defibrinated horse blood and 20 mg/L NAD. The inoculum was 10^4 cfu/spot and the plates were incubated in 4-6% CO_2 at 35-37°C for 18-20 h.

Disc susceptibility testing of imipenem was carried out using the BSAC standardized disc susceptibility method and imipenem 10μg discs.

β-lactamase detection
The isolates were tested for β-lactamase production using the chromogenic cephalosporin, nitrocefin (Oxoid Ltd., Basingstoke, UK) and the acidimetric method, Intralactam (Mast Diagnostics, Bootle, UK). PCR was used to detect the presence of bla_{TEM-1} and bla_{ROB-1} in the β-lactamase positive isolates using primers and conditions described by Scriver *et al*.[3]

Sequencing of ftsI
PCR was used to amplify part of *ftsI* from representative isolates[4]. Nucleotide sequencing of the PCR products was undertaken using the same primers.

Results

Of the 260 isolates examined 139 (53.5 %) were β-lactamase-positive; 136 were bla_{TEM} positive and three were positive for bla_{ROB-1} by PCR.

The MIC_{50} / MIC_{90} of amoxicillin, co-amoxiclav and cefuroxime for the 139 β-lactamase-positive isolates were 16 / 32, 0.5 / 1 and 0.5 / 2 mg/L, respectively. The remaining 121 isolates were β-lactamase-negative and their MIC_{50} / MIC_{90} of amoxicillin, co-amoxiclav and cefuroxime were 1 / 2, 1 / 2 and 1 / 2 mg/L, respectively.

All the isolates examined were susceptible to cefotaxime (MIC ≤ 0.25 mg/L).

The majority of isolates were susceptible to imipenem (BSAC breakpoint of 4 mg/L). 15 (5.8%) isolates were resistant (MIC of imipenem 8 - 16 mg/L). Table 1 shows the susceptibility of the imipenem resistant isolates.

All the imipenem resistant isolates were susceptible to meropenem and ertapenem.

The 15 imipenem resistant isolates all appeared susceptible to imipenem by disc susceptibility testing having zone diameters of 22 - 29 mm (breakpoint diameter of 20 mm).

Table 1. Susceptibility of imipenem-resistant *H.influenzae* to nine β-lactam compounds.

		MIC (mg/L)								
Isolate	β-lactamase	IPM	MEM	EPM	AMP	AMX	AMC	CXM	CEC	CTX	Amino-acids[a]
Control											
Rd	-	0.25	0.06	0.03	0.25	0.5	0.25	0.5	2	0.008	DSAHSLAIGASN
Clinical isolates											
A979	-	8	0.12	0.12	1	1	1	2	16	0.03	ND
A1091	-	8	0.12	0.12	0.5	1	1	1	8	0.03	ND
A1234	-	8	0.12	0.12	1	1	1	2	16	0.06	NSAISLAIGVSK
A1243	-	8	0.06	0.06	0.5	1	0.5	1	8	0.03	ND
A1464	+(TEM-1)	8	0.12	0.12	64	64	2	4	32	0.06	ND
A1380	+(TEM-1)	8	0.12	0.12	32	32	1	2	8	0.06	ND
A1437	+(TEM-1)	8	0.12	0.12	32	32	2	4	16	0.06	NSAISLAIGVSK
A1545	+(TEM-1)	8	0.12	0.12	32	32	1	2	16	0.06	ND
A1651	+(TEM-1)	8	0.12	0.12	32	64	2	8	32	0.06	ND
A1756	+(TEM-1)	8	0.12	0.12	16	32	2	4	16	0.06	ND
A965	+(TEM-1)	16	0.12	0.12	32	64	1	8	32	0.06	ND
A1366	+(TEM-1)	16	0.12	0.12	32	64	1	4	16	0.03	ND
A1397	+(TEM-1)	16	0.12	0.12	32	64	1	2	16	0.03	ND
A1525	+(TEM-1)	16	0.12	0.12	32	16	1	4	32	0.03	ND
A1575	+(TEM-1)	16	0.12	0.12	32	64	1	8	64	0.03	NSAISLAIGVSK

IPM, imipenem; MEM, meropenem; EPM, ertapenem; AMP, ampicillin; AMX, amoxicillin; AMC, co-amoxiclav, CXM, cefuroxime; CEC, cefaclor; CTX, cefotaxime. [a] Deduced amino-acids at positions 350, 357, 368, 377, 385, 389, 437, 449, 490, 502, 517 and 526 of PBP 3; substitutions, compared with Rd, in boldface. ND, not determined.

Discussion

Imipenem resistance in our isolates appeared not to be associated with β-lactamase production as both producers and non-producers were found to be resistant.

Most of the imipenem resistant isolates were resistant to cefuroxime; two isolates had MICs of cefuroxime equal to the BSAC breakpoint (1 mg/L).

Cefuroxime resistance is associated with mutations of *ftsI*, which encodes penicillin-binding protein 3 (PBP3)[5]. Not all our cefuroxime resistant isolates were resistant to imipenem. This suggests that imipenem resistance occurs either as a result of additional changes in *ftsI*, or by some other mechanism.

The 3 imipenem resistant isolates sequenced had the same deduced amino-acid substitutions in the transpeptidase region of PBP 3. We do not know the significance of these substitutions to imipenem resistance.

All the imipenem resistant isolates remained susceptible to the other two carbapenems tested, meropenem and ertapenem. Meropenem and ertapenem may retain their activity against the imipenem resistant isolates because of differences in their PBP binding activities.

The finding of imipenem resistance in 5.8% of our isolates highlights the fact that susceptibility to imipenem cannot be assumed. Susceptibility testing of *H. influenzae* should be carried out if imipenem is being considered as a treatment.

Caution should be exercised when testing the susceptibility of *H. influenzae* against carbapenems. We found that a single compound cannot be used as an indicator to predict the carbapenem susceptibility of *H. influenzae*; if meropenem or ertapenem were used as the indicator carbapenem resistance to imipenem would not be detected.

Disc susceptibility testing of *H. influenzae* against imipenem does not appear reliable. Previously, doubt has been cast on the reliability of imipenem disc susceptibility testing of *H .influenzae*.[6] Therefore a MIC based method might be more appropriate for determining the imipenem susceptibility of *H. influenzae*.

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