Investigation of Mechanisms Causing Variable Susceptibility to Macrolides in Clinical Isolates of *Haemophilus influenzae*

By

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AECB	acute exacerbations of chronic bronchitis
AOM	acute otitis media
bp	base pair
CAP	community-acquired pneumonia
CARTI	community-acquired respiratory tract infection
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CFU	colony forming units
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
EPI	efflux pump inhibitor
HCl	hydrogen chloride
Hib	*H. influenzae* type b
HTM	Haemophilus Test Medium
MDR	multiple drug resistance
MFP	membrane fusion protein
$MgCl_2$	magnesium chloride
MIC	minimum inhibitory concentration
MIC_{90}	minimum inhibitory concentration at which 90% of isolates are inhibited
mins	minutes
MLS_B	macrolide-lincosamide-streptogramin B
NaCl	sodium chloride

NaOH	sodium hydroxide
NCCLS	National Committee for Clinical Laboratory Standards
NTHi	nontypeable *H. influenzae*
OM	outer membrane
PCR	polymerase chain reaction
RND	resistance-nodulation-cell division
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
UV	ultraviolet

ABSTRACT

H. influenzae is a Gram negative organism commonly associated with community-acquired respiratory tract infections. Macrolide antibiotics are often used in the empiric treatment of these infections. Clinical isolates of *H. influenzae* can be divided into three phenotypic groups based on their susceptibilities to macrolides: hypersusceptible (clarithromycin MIC <2 μg/mL), susceptible (clarithromycin MIC 2-32 μg/mL), and resistant (clarithromycin MIC >32 μg/mL), with the majority of isolates falling in the susceptible category (clarithromycin MIC_{90} 16 μg/mL).

Resistant isolates are found much less frequently and exhibit higher MICs than those of susceptible strains. The purpose of this project was to determine the mechanisms responsible for the decreased susceptibility to macrolides in these isolates, and compare them to those observed in the other phenotypic groups.

Macrolide resistance is mainly due to modification of the antibiotic target site or active efflux. Various efflux assays were performed in the presence and absence of CCCP, a proton gradient uncoupling efflux inhibitor, in an attempt to observe those strains expressing efflux. Isolates with variable susceptibilities to clarithromycin were studied for the presence of mutations in the genes coding for target sites within the 23S rRNA and ribosomal proteins L4 and L22, using polymerase-chain reaction amplification and genetic sequencing. Strains were also tested for the presence of acquired genes conferring macrolide resistance such as *mef* and *erm*.

Ribosomal mutations are believed to alter the conformation of the drug binding site, therefore causing increased resistance. Such mutations were detected in the majority

of resistant strains with clarithromycin MICs ≥ 32 μg/mL. The efflux assays did not successfully demonstrate the presence of efflux in the study strains, although previous studies have established that an efflux mechanism is present in susceptible and resistant *H. influenzae* clinical strains.

High-level macrolide resistance in clinical isolates of *H. influenzae* is associated with two resistance mechanisms, macrolide efflux and ribosomal mutation. In resistant isolates lacking ribosomal mutations, it has to be assumed that other mechanisms, such as an overexpression of efflux pumps, must be playing a role in resistance to macrolides.

Investigation of Mechanisms causing Variable Susceptibility to Macrolides in Clinical Isolates of *Haemophilus influenzae*

A. INTRODUCTION

1. *Haemophilus influenzae*

a. Characteristics of the Pathogen

Haemophilus influenzae is a small nonmotile, Gram negative bacterium belonging to the family *Pasteurellaceae*. Microscopically, it is small (1 x 0.3 μm) with a shape that is variable, and often described as a pleomorphic coccobacillary form. All members of the *Haemophilus* genus are facultatively anaerobic, and *in vitro* growth requires accessory growth factors contained in blood: hemin (X factor) and nicotinamide adenine dinucleotide (V factor). The growth requirement for these two factors is used in the clinical laboratory to distinguish *H. influenzae* from other *Haemophilus* species, as most others only require one. Strains of this species grow preferentially in a humid atmosphere at 37°C with 5 to 10% CO_2 added (25, 34, 36).

Six major serotypes of *H. influenzae* have been identified. These serotypes are based on antigenically distinct polysaccharide capsules, and are designated serotypes a through f (25, 34, 36). In addition, many strains lack a polysaccharide capsule, and are referred to as nontypeable strains. Of all *Haemophilus* strains identified, type b (Hib) and nontypeable (NTHi) strains are most relevant in the clinical setting.

b. Carriage and Transmission

H. influenzae is an exclusively human pathogen that resides on the mucosa of the upper respiratory tract of adults and children (36). Most infants are colonized by nontypeable strains within one month of birth, and these strains are present in up to 80% of healthy children. Colonization by nontypeable strains is a dynamic process with new strains periodically replacing old strains (25, 36). Type b strains however, colonize the nasopharynx of children at much lower rates. During the first 6 months of life, carriage rates of type b strains are typically below 1% but average 3 to 5% throughout the childhood years (25). These rates may also be considerably higher in settings such as daycare centres, as well as in certain ethnic groups (36). Carriage of Hib in children of developed countries is becoming rare as a result of the antibodies produced following routine administration of Hib conjugate vaccines (34, 36).

c. Colonization and Invasion

Similar to other respiratory pathogens, *H. influenzae* is spread from person to person via airborne droplets or by direct contact with secretions, and readily colonizes mucosal surfaces of the respiratory tract (25, 34, 36). Although the microbial and host determinants of colonization are poorly understood, it has been shown experimentally that prior nasopharyngeal infection with influenza or other viruses can potentiate infection (34). Microbial factors include surface adhesins such as pili which facilitate attachment to epithelial cells *in vitro* (34), as well as lipooligosaccharide (37) and a low molecular weight, heat stable glycopeptide, which have both been shown to inhibit the ciliary activity of human epithelial cells. The polysaccharide capsule is the main

virulence factor of encapsulated strains, as it allows them to resist phagocytosis and complement-mediated lysis. These organisms can then penetrate the epithelium of the nasopharynx and invade blood capillaries directly (34).

d. Clinical Disease

The most serious clinical manifestations caused by *H. influenzae* are invasive infections such as meningitis, epiglottitis, and cellulitis (25, 34, 36). Prior to the implementation of vaccination in many countries, *H. influenzae* was one of the three leading causes of bacterial meningitis worldwide. Most of these infections are usually caused by type b strains (>95% of cases), and occur in young children, less than 2 years of age. In the United States, the annual number of invasive *H. influenzae* type b infections is now below 100, all of which occurred in unvaccinated or incompletely vaccinated children. (25) However, among unvaccinated children, *H. influenzae* type b remains a leading cause of meningitis. It is estimated that at least 3 million cases of serious disease and 400 000 to 700 000 deaths occur in young children worldwide (34). Occasionally, nonencapsulated or capsulated serotype f strains cause invasive infection, however these occur mostly in patients with underlying disease, or those who are otherwise immunocompromised (25, 34). Other important, but less frequent manifestations of *H. influenzae* type b disease include septic arthritis, osteomyelitis and pericarditis (25, 34, 36). Primary *H. influenzae* pneumonia is beginning to occur more frequently in children and adults, and is sometimes complicated by bacteremia. Most isolates from patients with pneumonia are non-type b strains (25).

Nontypeable, or nonencapsulated strains are responsible for the majority of respiratory tract infections due to *H. influenzae* in children and adults. These types of infections are generally less serious than invasive ones, however they occur in greater numbers, and account for substantial morbidity and healthcare costs (37). Unlike type b strains which cause systemic disease, nontypeable strains cause disease by local invasion of mucosal surfaces. Although *H. influenzae* resides mainly in the upper respiratory tract of individuals, it can also colonize the lower airways and cause pneumonia in children and adults, especially in the elderly, and in individuals with established lung disease such as chronic bronchitis or cystic fibrosis (25, 34, 36). *H. influenzae* is the leading cause of acute exacerbations of chronic bronchitis (AECB) (16, 42), and the second most common bacterial cause of community-acquired pneumonia (CAP) in adults, after *Streptococcus pneumoniae* (36, 42). Acute otitis media (AOM) results when bacteria migrate from the nasopharynx to the middle ear (36). It occurs most often in children, and is reported that more than two thirds of children have one or more episodes of AOM by the age of 3 years (34, 37). Nontypeable *H. influenzae* is the second most frequent cause of AOM (25, 36, 37), and is an important cause of sinusitis in both adults and children (16, 25, 34).

e. Treatment of *H. influenzae*

Antimicrobial treatment of community acquired respiratory tract infections (CARTI) has evolved in the past few decades as a result of antimicrobial resistance, however, the bacterial pathogens responsible for these infections have remained virtually the same. *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis* are the organisms most frequently associated with these infections.

Treatment of CARTIs is typically initiated on an empiric basis with broad spectrum oral agents, as the causative pathogen is often unknown. Traditionally, the penicillins formed the foundation of oral therapy for the treatment of RTIs however, with the dramatic emergence of β-lactamase producing pathogens, the focus has shifted from penicillins to other agents. The development of macrolide antimicrobials initially appeared to provide a good alternative to β-lactams. Erythromycin, the first of the macrolides, has been used for many years to treat a variety of respiratory and skin infections (57). Unfortunately, most strains of *S. pneumoniae* with reduced susceptibility to penicillin have become cross-resistant to other classes of drugs, including the macrolides and trimethoprim-sulfamethoxazole (21). Treatment of the Gram negative pathogens *H. influenzae* and *M. catarrhalis*, has also become somewhat problematic as a large proportion of strains worldwide produce β-lactamase, and are consequently resistant to penicillin, ampicillin, and amoxicillin (19, 20, 33). Resistance to trimethoprim-sulfamethoxazole has also reached considerable levels (15-20%) in most regions of the world (19, 20, 33). Most strains however, remain susceptible to other antibiotics frequently used in the treatment of RTIs such as, second generation cephalosporins, macrolides, tetracyclines, amoxicillin-clavulanate, and fluoroquinolones (10, 20, 21, 33). Specific criteria, such as the age of the patient, as well as the presence of other comorbid illnesses, help to guide the selection of treatment agents (10, 21).

Macrolide antibiotics exhibit strong antibacterial activity against *S. pneumoniae* and have traditionally been used for the treatment of CAP because of their spectrum of activity against the major pathogens. They also have good activity against the atypical pathogens, and the newer macrolides, clarithromycin and azithromycin, have improved

activity against *H. influenzae*. For these reasons, some current treatment guidelines advocate their use alone for the empiric treatment of CAP in patients that do not require hospitalization (10, 21, 32).

At present, there is some debate over the use of macrolides to treat infections that may be caused by *H. influenzae*. Global surveillance studies using the National Committee for Clinical Laboratory Standards (NCCLS) breakpoints to measure susceptibility, report that most *H. influenzae* strains remain susceptible to macrolides, with azithromycin yielding the lowest minimum inhibitory concentrations (MIC), followed by erythromycin and clarithromycin (7, 19, 20). However, based on the pharmacokinetic and pharmacodynamic properties of these compounds, and reported bacteriologic failures (21), others remain doubtful regarding their clinical efficacy against this organism (7, 20, 21, 43).

To date, the extent of antibacterial resistance with *H. influenzae* is not as large or diverse as that with *S. pneumoniae*. Nevertheless to prevent further resistance development in the future, it is very important that the choice of antimicrobial agent reflect the resistance patterns of the region.

2. Macrolide Antibiotics

a. Characteristics and Chemistry

Macrolide antibiotics have been used in the treatment of infectious diseases since the late 1950s. Many are naturally produced by several different *Streptomyces* species (57). The main structural component of the macrolides is a large lactone ring that can vary in size from 12 to 16 atoms, and to which one or more sugars are linked (38, 57).

The ring is substituted by alkyl and hydroxyl groups. Erythromycin A, the first macrolide to be used clinically, is comprised of a 14 membered lactone ring with 2 appended sugar moieties (57). Its spectrum of activity includes Gram positive cocci, as well as the atypical pathogens: *Mycoplasma pneumoniae, Legionella pneumophila*, and *Chlamydia pneumoniae*. For many years erythromycin was used to treat respiratory infections and provided a good treatment alternative for patients with β-lactam allergies (57).

The natural macrolides tend to be limited clinically due to gastric instability and/or poor pharmacokinetic properties, and erythromycin is no exception. Several limitations of the drug include: insufficient activity against *H. influenzae*, a short serum half-life, poor oral bioavailability, gastrointestinal side effects, and numerous drug-drug interactions. To overcome many of these limitations, semi-synthetic derivatives of erythromycin were designed through esterification, salt formation, and/or other structural modifications. Modifications of the erythromycin ring at sites prone to degradation, particularly the ketone at C-9, the hydrogen at C-8, the hydroxyl at C-6, and the diol at C-11 and C-12, improved the drugs' stability under acidic conditions (57).

The first derivative, roxithromycin, was produced as a result of substituting the ketone at C-9 with an oxime group. Dirithromycin is also produced through changes at the same position, and although these changes improve their acid stability, they do not appear to affect the *in vitro* activities of either drug. The replacement of the hydrogen at position C-8 with a fluoride atom yields the macrolide flurithromycin (57). Another commercially available macrolide is clarithromycin, which is produced through the alkylation of the C-6 hydroxyl group. This modification prevents degradation to hemiketal and spiroketal metabolites, which is important as it is the hemiketal

metabolites that are thought to be responsible for the adverse gastrointestinal effects (57). The *in vitro* activity of clarithromycin is equal or greater than erythromycin against common respiratory pathogens, and exhibits increased activity against *H. influenzae*. This increased activity is due to the synergistic interaction of the 14- hydroxyl metabolite with the parent compound. Azithromycin consists of an enlarged, 15 membered ring that is formed by the insertion of a methyl substituted nitrogen at the C-9a position (30, 57). The addition of this second nitrogen atom forms an azalide structure which prevents degradation of the drug, and improves the activity against *H. influenzae*, as well as other Gram negative and atypical pathogens. Other advantages of this structural modification include a significantly prolonged serum half-life, and superior tissue penetration of the drug (57).

Josamycin, kitasamycin (Leucomycin), spiramycin, tylosin and midecamycin are some examples of naturally occurring 16 membered macrolides. Several of these compounds have been chemically altered to enhance their activity against some resistant organisms, and improve their pharmacokinetic properties. Rokitamycin is produced by the acylation of the 3'-hydroxyl group of leucomycin, and miokamycin is a derivative of midecamycin (57).

Erythromycin, roxithromycin, clarithromycin and azithromycin are currently in use in all developed countries of the world, as well as in many developing countries. The 16 membered ring agents have a more limited distribution. Josamycin is available in France and certain other European countries, whereas spiramycin is registered for use in some European, Asian and Latin American countries (26). Tylosin is used mainly for veterinary purposes (27, 30).

With the emergence of multidrug resistance among respiratory pathogens, new agents are required that retain the improved properties of the semi-synthetic macrolides while being effective against strains resistant to β-lactam and other macrolide drugs. The ketolides, a new class of macrolides, were designed specifically to combat respiratory pathogens that have acquired resistance to macrolides (62). The ketolides are derivatives of Erythromycin A, and retain the 14-membered macrolactone ring, as well the D-desosamine sugar at position 5 (57). The distinguishing feature of this group of compounds is the replacement of the 3-L-cladinose moiety with a keto group. To achieve this, the cladinose is removed from erythromycin to leave a 3-hydroxyl group which is subsequently oxidized to form the 3-keto derivative (8, 57, 62). The removal of the L-cladinose sugar is advantageous as it increases both acid stability and antibacterial potency against bacteria resistant to macrolides by means of efflux (57). To further improve acid stability, a methyl group is attached at the 6-O position, as in clarithromycin. In the absence of other modifications, removal of the L-cladinose sugar from clarithromycin, azithromycin and roxithromycin leads to a loss of antimicrobial activity. However, modifications at other positions of the macrolactone ring can easily compensate for the loss of the sugar (8, 57, 62). This is the case for the ketolides, telithromycin and ABT-773, which both contain an 11,12 cyclic carbamate linkage replacing the two hydroxyl groups of erythromycin A, as well as an arylalkyl or arylallyl extension linked to the molecule. This structure has been shown to enhance their activity to be greater than or equal to the newer macrolides (62).

b. Mode of Action

Macrolide antibiotics exert their activity by preventing the bacterial ribosome from translating its messenger RNAs (mRNA) to synthesize new proteins (8). The ribosome is made up of two subunits, a small 30S subunit and a larger 50S subunit. The latter is composed of 23S ribosomal RNA (rRNA) and some 34 proteins. The secondary structure of 23S rRNA is folded due to base pairing, and forms six domains numbered I to VI, while the tertiary structure of the molecule is maintained by its interactions with proteins (30).

There are several steps involved in the synthesis of new proteins. First, the small 30S subunit interacts with mRNA, and in conjunction with transfer RNAs (tRNA), decodes the information on the mRNA. Each time the 30S subunit recognizes that a tRNA gives a correct fit on an mRNA codon, the amino acid attached to the tRNA is positioned to fit into the peptidyltransferase centre, on the large 50S subunit. At this location, a peptide bond is formed between the amino acid and the newly forming peptide chain. With each additional amino acid, the growing peptide chain passes through the peptide exit tunnel, and emerges on the back of the ribosome (8). Macrolide drugs inhibit synthesis by binding to a single site near the entrance to the peptide exit tunnel. They do not impede the activity at the peptidyltransferase centre, but prevent the extension of the peptide chain by blocking the peptide exit tunnel and promote the premature dissociation of peptidyl tRNA from the ribosome (8, 18, 30).

The macrolide / ketolide binding site is composed primarily of RNA. Two segments of 23S rRNA, the central loop of domain V, and the hairpin 35 loop of domain II, are believed to be the major regions of drug interaction with the ribosome (8, 18, 62).

As only one molecule of drug binds per 50S subunit, the 23S rRNA is presumably folded so that these two regions are proximal in the tertiary structure, and contribute to a single binding pocket (9, 62). This is further supported by the results of RNA footprinting experiments which revealed that macrolide and ketolide antibiotics interact specifically with nucleotides A2058 and A2059 (*E.coli* numbering) of domain V, and protect them from chemical modification. In similar experiments, macrolide and ketolide antibiotics were shown to affect the accessibility of nucleotides in the hairpin loop of domain II to chemical modification. The ketolides strongly protected A752 in domain II, while erythromycin enhanced the accessibility of that residue to modification (8, 9, 53).

Although rRNA appears to be the major component of the macrolide binding site, the ribosomal proteins L22 and L4 also form part of the surface of the tunnel to which the macrolides bind, however it remains unclear whether these proteins are directly involved in the drug binding (18, 30).

More recently, a second and equally important inhibitory activity of macrolides and ketolides has been discovered. Formation of new ribosomes requires the coordinated assembly of rRNAs with the many ribosomal protein components to create the two subunits. Results of experiments by Champney and Burdine suggest that macrolides and ketolides interact with partially assembled 50S subunit precursors to block the assembly process, and lead to the nucleolytic degradation of the unassembled precursor particles (5, 8, 57, 62).

c. Mechanisms of Resistance

i. Target Site Modification

Macrolide resistance is mainly due to alteration of the ribosomal target site or active efflux, and rarely to antibiotic inactivation. In *S. pneumoniae* and other Gram positive cocci, macrolide resistance occurs by two mechanisms, target site modification and/or active efflux. In the most common form of target site modification, a specific adenine residue (A2058) in the 23S rRNA is dimethylated by an rRNA methylase encoded by the *erm* genes. This methylation leads to a conformational change in the ribosome resulting in decreased binding of all macrolide, lincosamide and streptogramin B antibacterials, or so-called MLS_B resistance (15, 21, 29, 57). By another mechanism, macrolides are pumped directly out of the cell by a membrane-associated efflux pump. This pump is encoded by the *mef* genes (*mefE* in *S. pneumoniae* and *mefA* in *S. pyogenes*), and confers resistance to 14- and 15-membered ring macrolides only (15, 21, 29, 57). *mefA* and *mefE* are 90% identical (63) and will be referred to in the text as *mefA/E*.

In recent years, *in vitro* studies have demonstrated that target modification can also be achieved by mutations in the domain II and V of 23S rRNA, and/or in highly conserved amino acids of ribosomal proteins L4 and L22 (7, 43, 44, 48). Changes at specific residues in the peptidyl transferase loop were initially identified to confer resistance to macrolides in various species of laboratory mutants, however, such mutations have now been detected among clinical isolates worldwide (14, 43, 44, 48, 49). Peric *et al.* investigated 31 clinical isolates of *H. influenzae* that showed a high-level resistance (azithromycin MIC > 4 μg/mL, clarithromycin MIC > 64 μg/mL) to both

azithromycin and clarithromycin and found that 28 of 31, or 90%, had modifications in 23S rRNA and/or ribosomal proteins L4 and L22. Mutations occurred via insertion, deletion and/or substitution of amino acids, and some common changes observed were T64K in L4 protein, and G91D in L22 protein (43). Although L4 and L22 primarily bind to domain I of 23S rRNA, mutations in these proteins cause a change in the conformation in domains II, III, and V, affecting the action of the macrolides for domain V of 23S rRNA (41). All detected mutations were in a highly conserved region of the ribosomal protein. Two strains had a replacement of adenine by guanine at position 2058 (*E.coli* numbering), a key residue in the peptidyl transferase centre of 23S rRNA. Other 23S rRNA mutations were in positions 2160 to 2164, which is not in the peptidyl transferase centre but is proximal to the E site (43).

ii. Antibiotic efflux

Active efflux of toxic compounds out of cells is a general mechanism that bacteria have developed to protect themselves against the adverse effects of their environments (45). Both prokaryotic and eukaryotic cells possess families of membrane proteins that act to remove amphiphilic molecules from within the cell. Many antibiotics are amphiphilic in nature, with hydrophobic properties that facilitate cell penetration, and hydrophilic properties that allow for tissue distribution of these compounds throughout the body (45). Macrolides have consistently higher MICs in Gram negative organisms than those in Gram positive ones, and it is widely accepted that this resistance is due to the presence of intrinsic efflux pumps, in combination with slower penetration through the outer membrane (43, 63).

For *H. influenzae*, the major efflux pump responsible for macrolide resistance is the three component, AcrAB-TolC, an efflux system first identified in *E.coli* (46). Virtually all strains of *H. influenzae* have this pump, which spans the inner and outer membranes, and expels substrates directly into the external medium (63).

3. AcrAB-TolC in *H. influenzae*

Efflux of antibiotics out of cells is broadly recognized as a major component of bacterial resistance to many classes of antibiotics. In Gram negative bacteria, most multiple drug resistance (MDR) pumps that contribute to the resistance of clinically beneficial antibiotics are three component structures that extrude antimicrobial agents across both membranes directly in the extracellular milieu (56). They are composed of (i) a pump embedded in the cytoplasmic membrane, (ii) a periplasmic accessory protein belonging to the membrane fusion protein (MFP) family, and (iii) an outer membrane channel belonging to the outer membrane (OM) protein family (12, 54).

Several classes of MDR pumps have been identified based on sequence comparisons, but most of the inner membrane components of clinically relevant efflux systems from Gram negative organisms belong to the resistance-nodulation-cell division (RND) transporter family. A key feature of these pumps is the wide range of structurally unrelated compounds that are recognized and expelled by the single pump protein (1, 12, 13, 56).

The AcrAB-TolC system is the major, constitutively expressed MDR mechanism in *E.coli*, and genome sequencing of *H. influenzae* has identified a three-gene complex that is homologous to the *acrRAB* cluster of *E.coli*. These genes include HI0893, an *acrR*

homolog; HI0894, an *acrA* homolog; and HI0895, a homolog of *acrB* (46). (For clarity, they will be referred to as *acrR*, *acrA*, and *acrB*) Unlike homologous systems in *Pseudomonas aeruginosa*, in which all the components of the tripartite system are located within a single operon, in *H. influenzae* the *acrAB* genes are located in tandem as an operon, whereas *tolC* is located elsewhere on the genome (12).

The acrAB operon codes for two proteins, the inner membrane efflux transporter AcrB, and the periplasmic lipoprotein AcrA, both of which are essential for drug efflux (2, 12). Genetic studies have provided evidence that the multifunctional outer membrane channel TolC is the third component of this transporter, since mutations in *tolC* completely abolished the AcrAB-dependant multidrug resistance phenotype (17, 55). Together, they form a functional complex and confer resistance to a variety of lipophilic and amphiphilic drugs, dyes, and detergent molecules that include: tetracycline, chloramphenicol, fluoroquinolones, β-lactams, erythromycin, fusidic acid, ethidium bromide, crystal violet, and bile salts (11, 13, 46). AcrB, a proton motive force dependant transporter, is large in comparison to members of other transport families such as the major facilitator superfamily, and forms a trimer made up of three individual 1049 amino acid protomers (13, 35). Each protomer contains 12 α-helical transmembrane domains (TMD) and two large periplasmic loops of approximately 300 amino acid residues each between TMD 1 and 2 and TMD 7 and 8. It has recently been determined that the two large loops play a major role in the recognition and binding of substrates (11, 13, 35). The elucidation of the crystal structure of AcrB revealed three openings, called vestibules, at the junctions of the protomer headpieces just outside the plane of the membrane. These vestibules are hypothesized to be conduits for substrate entry from

either the periplasm or the outer leaflet of the cytoplasmic membrane (11, 12, 35). AcrA is a membrane lipoprotein comprised of 397 amino acid residues, and belongs to the membrane fusion protein family. In its mature form, it carries a diacylglycerol group and a palmitic acid chain linked to the N-terminal cysteine residue (2). These lipid moieties are believed to anchor the protein to the outer leaflet of the inner membrane (2, 12, 24, 55). The hydrophobic nature of the C terminus suggests that AcrA may also be attached to the outer membrane and bridge the periplasmic space (12).

Secondary structural predictions have suggested that AcrA and other homologs from the membrane fusion protein family have extended α-helical regions that have a high probability to form coiled coils (56). These regions are often involved in protein oligomerization, such as in another MFP of *E.coli*, HlyD, which was found to be trimeric (56). Chemical cross-linking experiments with AcrA and AcrB have demonstrated that AcrA might form a trimer when associated with the inner membrane, even in the absence of AcrB or TolC. Evidence also suggests that only oligomeric forms of AcrA can interact with AcrB and form a stable complex. In addition, the assembly of the AcrAB complex occurs independently of the outer membrane channel TolC (55). The fact that highly homologous MFPs cannot be interchanged among the tripartite complexes reinforces the notion that a specific interaction must occur between the periplasmic protein, and its corresponding inner membrane transporter (56).

Unlike the MFPs, the outer membrane components of Gram negative multidrug efflux systems are interchangeable between different systems. In addition, any one OM protein may function with several different pumps. For example, the OM protein TolC of *E.coli*, is essential for the drug export activity of AcrAB, as well as the protein exporters

HlyB and CvaB (56). Recent crystalline structure studies have revealed that TolC is seen as a long tunnel of approximately 140 angstroms, which spans the outer membrane and periplasm. The homotrimeric TolC is comprised of a 100 angstrom long α-helical barrel projecting into the periplasm, anchored in the OM by a 40 angstrom β-barrel domain (1). The α barrel consists of 12 α helices (four from each monomer), packed in an antiparallel arrangement to form a hollow cylinder. In the lower half of the α barrel, neighboring helices form six pairs of two-stranded coiled coils. At the periplasmic end of the tunnel, three of these coiled coils fold inwards to constrict the entrance to a resting closed state. The proposed mechanism by which TolC transitions to an open state is thought to be achieved by the realignment of the inner coils with the outer coils, therefore enlarging the aperture size (1).

The TolC family of OM proteins is ubiquitous throughout Gram negative organisms, and is central to the Type 1 secretion of toxins and proteins, as well as the efflux of a wide range of antibacterial compounds (1). With Type 1 protein secretion, the recruitment of TolC into the secretion complex appears to occur only after the exported protein has assembled with the transporter and the periplasmic component. Similarly, TolC may be recruited into the efflux complex on a transient basis when the substrate molecule is near to be exported (1).

The actual transport mechanism through which drug molecules are exported is still unclear, as is the precise role of AcrA. Experimental evidence provided by Zgurskaya and Nikaido suggests that AcrA brings the inner and outer membranes into close apposition for subsequent substrate transfer (56). Additionally, structural features of AcrA, such as its highly asymmetric shape, the presence of coiled coil domains, and the

two hydrophobic regions, also support the model that AcrA could act as a true MFP under conditions found in intact cells (56).

The resolution of the crystal structure of AcrB brought forth another theory about the coordinated function of the tripartite complex. Murakami *et al.* revealed that the headpiece of the AcrB, which is formed by the two large periplasmic loops, is almost equal to the TolC bottom, with a diameter of 100 angstroms. As well, the sum of the periplasmic length of AcrB and TolC is approximately 170 angstroms, or enough to cross the periplasmic space, which indicates that they might be directly docked with one another (35).

4. Mechanism of Action of Ketolides

The ketolides represent a new class of antibacterials that have been developed specifically for the optimal empiric treatment of upper and lower respiratory tract infections. The ketolides are derived from 14-membered ring macrolides, and retain the improved characteristics of the second generation macrolides, yet they have certain innovative structural modifications such as a 3-keto group replacing the L-cladinose moiety, and a methoxy substituent at C6, that set them apart. In addition, several of the ketolides under development possess a carbamate side chain at positions C11/C12. Telithromycin, the first ketolide to be approved for use, has an alkyl-aryl extension on the carbamate; and cethromycin (formerly ABT-773) which is progressing through clinical trials, has a similar extension placed at the 6-position of the ring (8, 29, 58, 62).

The ketolides, like the macrolides, exert their antibacterial effects by binding to the bacterial ribosomes and inhibiting protein synthesis (8, 28, 57, 62). Initially, the

possibility of cross-resistance between these agents was raised due to the similarity in their mechanism of action, however, recent data have revealed fundamental mechanistic differences from the macrolides, most notably in terms of the nature and strength of the ribosomal binding (28). The macrolides bind to two sites within the ribosome, domain II and V of 23S rRNA, with a relatively weak interaction at domain II. The ketolides also bind to both domains, but appear to bind far more tightly to the bacterial ribosome than do the macrolides. Two ketolides, HMR 3004 and telithromycin, were found to bind approximately 10-fold more tightly to the bacterial ribosome than erythromycin, and it is thought that the C11/C12 carbamate extension specifically plays a role in this enhanced binding through its interaction with domain II (8, 28, 57).

The spectrum of activity of the ketolides is similar to the macrolides (57, 58, 62). They are active against the majority of Gram positive aerobic bacteria, including strains of *S. pneumoniae* that have become resistant to macrolides via *mef*-encoded efflux or by possessing an inducible or constitutive *erm* gene that confers resistance to MLS_B drugs (8, 29, 57, 58, 62). The ketolides with a C11/C12 carbamate extension remain active against most strains that are resistant to erythromycin because of changes in the drug binding site, probably because of their improved contact with the rRNA. Footprinting experiments have shown that an A2058G mutation in the 23S rRNA reduces the binding of erythromycin and clarithromycin to ribosomes approximately 10 000 fold. The binding of ketolides is also reduced, but remains about 20 fold higher than that of erythromycin. The difference in the binding affinities of these drugs is probably due to the interaction of the ketolides at position A752 at domain II (8).

The majority of Gram negative aerobes have proven to be intrinsically resistant to the macrolides, and subsequently the ketolides, however, these agents are active against a number of clinically relevant Gram negative species. In *H. influenzae*, the ketolides display similar or greater *in vitro* activity as azithromycin (28, 62). In addition, the ketolides display good activity against *M. catarrhalis*, *Neisseria* spp., and *Bordetella pertussis* (57, 62). The ketolides are very effective against the important intracellular and atypical pathogens *Chlamydia pneumoniae*, *Legionella pneumophila*, *Mycoplasma pneumoniae*, and *Ureaplasma urealyticum* (58, 62).

In addition to improved ribosomal binding, the ketolides possess several additional advantageous properties. They lack the ability to induce resistance mechanisms, as previously observed with macrolides in organisms such as *S. pneumoniae*. This is linked to the absence of the L-cladinose moiety which contributes to the strong induction effect of the macrolides in strains that have inducible *erm* genes (8, 28, 29, 57, 62).

In vitro and *in vivo* experiments have shown that telithromycin has a low potential to select for resistance or cross-resistance, and that the exposure of streptococci to ketolides is less likely to result in resistance than upon exposure to macrolides. Only slight increases in MIC were observed with the selection experiments, and mutations occurred at frequencies lower than those obtained for macrolides (28, 29, 62).

Lastly, ketolides have been shown to accumulate at a greater rate than macrolides in bacterial cells, and in human cells giving them potential activity against intracellular pathogens (62).

5. Development of Efflux Pump Inhibitors

Many clinically useful antibiotics are expelled from bacterial cells by a mechanism termed efflux. Recently, efflux has been identified as a relevant contributor to bacterial resistance, and is recognized as one of the most important causes of intrinsic antibiotic resistance in Gram negative bacteria (45, 51, 52). With the recognition of efflux as a major factor in bacterial resistance, several companies have invested in the identification and development of bacterial efflux pump inhibitors. Among those companies, Microcide, Pfizer, and Paratek are in the process of exploring efflux pump inhibitors from synthetic and natural products, and peptidomimetics (45). The development of pump inhibitors able to restore activity to an antibiotic subject to efflux represents an interesting area of discovery, similar to the use of β-lactamase inhibitors to combat β-lactamase production by bacteria.

To date, most research has focused on the Mex efflux pumps of *Pseudomonas aeruginosa* and inhibitors of these. The first inhibitor identified to be active against multiple RND transporters in Gram negative bacteria was MC-207,110, which was found to be active against the MexAB-OprM, MexCD-OprJ, and MexEF-OprN pumps of *P. aeruginosa*. This inhibitor lowered the MIC values to fluoroquinolones for both sensitive and resistant strains, and it also dramatically decreased the frequency of selection of fluoroquinolone-resistant strains (31, 52). This inhibitor was also shown to be active against RND pumps in representative strains of Enterobacteriaceae, *N. gonorrhoeae*, and *H. influenzae* (31).

Another inhibitor was discovered which could inhibit the efflux of non-quinolone antibacterials, as long as they were effluxed by an RND-class pump. MC-04,124, a

dipeptide amide analog of MC-207,110 developed by Microcide, was shown to enhance the *in vitro* efficacy of macrolides against the Gram negative bacteria: *E.coli*, *H. influenzae*, *Klebsiella pneumoniae*, and *P.aeruginosa* (3, 45). In *H. influenzae*, the addition of the inhibitor resulted in the reduction in MIC_{90} values from 1 to 0.25 μg/mL for azithromycin, 16 to 4 μg/mL for clarithromycin, and 16 to 2 μg/mL for erythromycin (6).

These inhibitors are broad spectrum against multiple bacteria as they inhibit more than one pump of the same class, as well as other pumps of the same class. As well, they have shown effectiveness in lowering the MIC values of more than one class of antibacterials known to be effluxed by these systems (45). There are still several challenges to be met in order for inhibitors to be used in future combination therapy. One problem that needs to be addressed is the potential effects on efflux transporters also present in eukaryotic cells. Most inhibitors currently available display strong pharmacological activities in eukaryotic cell systems, and are therefore unusable in clinical practice (51). Another difficulty is that the pharmacokinetic/pharmacodynamic properties of the pump inhibitors will need to closely match those of the companion antibiotic (51).

6. Hypothesis

Gram negative bacteria tend to be more resistant to lipophilic and amphiphilic compounds than Gram positive bacteria. Such compounds include dyes, detergents, and antibiotics, such as the macrolides. This intrinsic resistance has often been attributed

entirely to the low permeability of the outer membrane, although recent studies have shown that multiple drug efflux pumps also play a major role in this resistance.

Macrolide MICs are consistently higher in *H. influenzae* than *S. pneumoniae* with reported MIC distribution values for clarithromycin ranging from 0.25 - ≥128 μg/mL (MIC_{90} 16 μg/mL), as compared to 0.03 to ≥128 μg/mL (MIC_{90} 0.25 μg/mL). Clinical isolates of *H. influenzae* can be divided into three phenotypic groups based on their susceptibilities to macrolides: hypersusceptible (clarithromycin <2 μg/mL, azithromycin <0.25 μg/mL), susceptible (clarithromycin 2-32 μg/mL, azithromycin 0.25-8 μg/mL), and high-level resistant (clarithromycin >32 μg/mL, azithromycin >8 μg/mL). The majority of *H. influenzae* isolates (>90%) have macrolide MICs that are deemed susceptible, and not hypersusceptible or high-level resistant (43, 60).

The objective of my thesis project was to examine the role that efflux played in clinical isolates of *Haemophilus influenzae* with varying susceptibilities to macrolides. A major goal was to determine specific differences in the isolates of different phenotypes that would cause such a range in susceptibilities to the macrolides. Several hypotheses about the varying macrolide susceptibilities were put forward.

First, I hypothesized that all strains of *H. influenzae* possess the genes that encode the intrinsic multidrug efflux pump AcrAB-TolC, but that perhaps these genes were subject to different levels of expression among the different isolates. Higher expression of these genes might lead to higher expression of the protein components of the pump. This would cause more antibiotic to be effluxed from the cells, therefore increasing the MIC. A second hypothesis was that inhibition of the H^+-driven efflux pump would cause more antibiotic to accumulate in the cells, resulting in a decrease in MIC values. Finally,

structural changes in the binding site of macrolides on the ribosome results in fewer drug molecules binding to inhibit bacterial protein synthesis (7, 43, 44, 48). Therefore, the last hypothesis was that isolates with changes in the ribosomal structure require greater concentrations of antibiotic to inhibit growth, and thus have increased MICs.

In order to prove the hypotheses of this project, several objectives were set:

1. To confirm the presence of the genes encoding the AcrAB pump in all strains of *H. influenzae*, PCR experiments were utilized to amplify the genes encoding the structural proteins of the pump.
2. To identify strains which expressed an efflux mechanism, macrobroth and agar dilution experiments were performed in the presence and absence of a proton gradient uncoupling inhibitor, CCCP.
3. To determine if the decrease in susceptibility is due to structural changes in the macrolide target site, the genes encoding ribosomal proteins L4 and L22, and domain V of 23S rRNA were sequenced from isolates with variable susceptibility to clarithromycin.

B. MATERIALS AND METHODS

1. Isolate Selection

Thirty-four isolates of *H. influenzae* were selected from more than 7500 isolates obtained as part of the Canadian Respiratory Organism Susceptibility Study (CROSS), an ongoing, national surveillance study. All organisms were submitted to, or isolated by the Department of Clinical Microbiology, Health Sciences Centre, Winnipeg, Canada, between October 1997 and June 2002 (60). The identity of all *H. influenzae* isolates was confirmed by standard methodologies such as colonial morphology, Gram stain characteristics, and X and V factor requirements (60).

Selection of isolates was based on clarithromycin MICs, which were previously determined by the National Committee for Clinical Laboratory Standards (NCCLS) - recommended broth microdilution method (39). Five macrolide hypersusceptible (clarithromycin MIC <2 μg/mL), 24 macrolide susceptible (clarithromycin MIC 2-32 μg/mL), and 5 macrolide resistant (clarithromycin MIC >32 μg/mL) strains were randomly chosen to represent a *H. influenzae* cohort with various susceptibilities to clarithromycin. The strains used in this study originated from 14 different medical centres representing most geographic regions of Canada. The source of the majority of isolates was from sputum samples, as well as several from throat, bronchoalveolar lavage, and endotracheal specimens.

A reference strain of *H. influenzae* (ATCC 49247) was included as a control for antibiotic susceptibility tests, as well as for PCR amplification methods.

2. Antibiotics

All antibiotics were obtained as laboratory-grade powders from their respective manufacturers: clarithromycin and ABT-773 (Abbott, Abbott Park, Illinois, USA); azithromycin (Pfizer, Groton, Connecticut, USA), and telithromycin (Aventis, Romainville, France). Stock solutions were prepared and dilutions were made according to NCCLS guidelines (39). The activity of all antibiotic stock solutions was confirmed by the broth macrodilution method with quality control strains *H. influenzae* ATCC 49247 and *Staphylococcus aureus* ATCC 29213.

3. Susceptibility Testing

a. Broth Microdilution

Following two subcultures from frozen stocks, susceptibilities to clarithromycin, azithromycin, telithromycin and ABT-773, were determined for each isolate of *H. influenzae* using the NCCLS microbroth dilution method (39). By this method, a custom-designed panel was inoculated with 5 x 10^6 CFU/mL organism, into a final panel well volume of 100 μL, yielding a final inoculum of 5 x 10^5. The inoculum was prepared from growth on chocolate agar plates incubated for 18-24 hours. All panels were incubated in ambient air at 35 °C for 20 to 24 hours prior to reading of the results. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic required to inhibit visible growth. Periodically, colony counts were determined to confirm inocula. All susceptibility testing was performed in triplicate using cation-supplemented Haemophilus Test Medium (HTM).

b. Broth Macrodilution

Susceptibilities to clarithromycin were determined for select isolates using the broth macrodilution method described by NCCLS (39). Briefly, a series of tubes with doubling dilutions of antibiotic in broth, were inoculated with 1 x 10^6 CFU/mL organism, for a final volume of 2.0 mL. Several control tubes were also included: broth only, broth and organism, and broth and antibiotic. Tubes were incubated at 35 °C for 20 to 24 hours in ambient air before determining the endpoint. The MIC is defined as the lowest concentration of antimicrobial that completely inhibits growth of the organism in the tubes. Colony counts were also performed on the inoculum suspension to ensure that the inoculum was approximately 5 x 10^5 CFU/mL (39).

4. *In vitro* Pharmacodynamic Modeling

The *in vitro* pharmacodynamic model used has been previously described (61). Logarithmic phase cultures were diluted into fresh Haemophilus Test Medium to achieve a final inoculum of approximately 1x 10^6 CFU/mL. This inoculum was then introduced into the central compartment (volume 610 mL) of the *in vitro* pharmacodynamic model, and exposed to azithromycin, clarithromycin (with its 14-hydroxy metabolite), and telithromycin simulating free serum concentrations obtained after standard dosing in healthy volunteers. Pharmacodynamic experiments were performed in ambient air at 37°C. Antibiotic solutions were added at 0 hours, and at -1, 0, 1, 2, 4, 6, 12, and 24 hours, samples were drawn from the central compartment, and bacterial counts performed by plating serial 10-fold dilutions onto chocolate agar. Plates were incubated for 18-24 hours

at 37°C in CO_2. The lowest dilution plated was 0.1 mL of undiluted sample, and the lowest level of detection was 200 CFU/mL. Protein free serum concentrations were simulated using known protein binding fractions, and clearance was simulated using reported serum half-lives of antibiotics in healthy volunteers.

5. Efflux Pump Inhibition

Two different techniques were employed to measure inhibition of the efflux pump in the *H. influenzae* isolates. Both approaches involved the addition of an efflux pump inhibitor to a standard susceptibility testing method to determine the MIC endpoints in the presence or absence of inhibitor. A protonophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP), was used as the inhibiting compound to dissipate the proton gradient needed for proper functioning of the efflux pump. Growth experiments were performed first to determine a concentration that would inhibit the efflux pump, without hindering growth of the organism.

a. Growth Experiment Procedures

i. 1:100 Dilution in 80 mL Broth

A 1:100 dilution of the CCCP solution (0.8 mL in 80 mL HTM broth) was performed to achieve final CCCP concentrations of 0, 0.05, 0.10, 0.25, 0.5, 0.75, and 1 μM. The bottles were inoculated with 1 x 10^6 CFU/ mL organism, and aerated by shaking in a water bath at 37°C. Samples were taken at time 0, 2, 8, and 24 hours. One hundred microlitre samples were plated and colony counts performed after 24 hour incubation. A broth and acetone control (equal to 1 μM) was included as a negative control as acetone

was the solvent used in the CCCP solution. NOTE: When growth was measured spectrophotometrically, all isolates were grown under these conditions, and samples taken at appropriate time intervals. One milliliter samples were placed in cuvettes, and their optical density measured at 600 nanometers using an UltroSpec 2100pro spectrophotometer.

ii. 1:100 Dilution in 2 mL Broth

A 1:100 dilution of the CCCP solution (0.02 mL of inhibitor in 1.980 mL HTM broth) was performed to achieve final CCCP concentrations of 0, 0.05, 0.10, 0.25, 0.5, 0.75, 1 μM. Tubes were inoculated with 1 mL of 1 x 10^6 CFU/mL organism and incubated in ambient air at 37°C for 24 hours. A growth tube, a broth and acetone tube (equal to 1 μM), and broth and inhibitor tube, were included as controls for all experiments.

iii. Doubling Dilution of CCCP in 2 mL Broth

In place of a 1:100 dilution, CCCP solutions were made up at double the desired CCCP concentration, and 1mL of this solution placed in a tube. Tubes were inoculated with 1 mL of 1 x 10^6 CFU/mL organism and incubated in ambient air at 37°C for 24 hours. A growth tube, a broth and acetone tube (equal to 1 μM), and broth and inhibitor tube, were included as controls for all experiments.

b. Macrobroth Dilution Assay with Inhibitor

A macrobroth dilution experiment was set up as previously described in Section 3 part b, of the Materials and Methods. Concurrently, another series of tubes containing the same range of antibiotic dilutions in broth was prepared. A stock solution of CCCP was also prepared, using HTM broth, at twice the desired CCCP concentration. The organism (1×10^6 CFU/mL) was added to this solution, and 1 mL of CCCP and organism added to the 1 mL of antibiotic. Both sets of tubes were incubated for 24 hours in ambient air at 35°C, before MIC endpoints could be determined. For each non–CCCP assay, control tubes were prepared, including a growth control, a broth control, and a drug control. Control tubes for the CCCP assay included a broth control, growth control, solvent and broth only, and inhibitor and broth only. NOTE: At the time that these experiments were first attempted, no positive or negative *H. influenzae* efflux strains were available. These were later acquired, though the experiments were not repeated.

c. Agar Dilution Assay with Efflux Inhibitor

This assay was a modification of the NCCLS-described method for agar dilution, where media plates containing a range of antibiotic dilutions are used to determine MIC values (39). For each experiment, two sets of media were prepared, one containing clarithromycin only, and one set with clarithromycin and CCCP. The range of antibiotic dilutions included 1 dilution higher than the highest MIC value, and 4 dilutions lower than the lowest MIC value of the isolates, to allow observation of efflux inhibition. The CCCP was added at a final concentration of 0.25 μg/mL, into HTM agar, or chocolate agar (depending on the experiment). Using a Steer's replicator, bacterial suspensions

were delivered onto the plates, with a final inoculum of approximately 1 x 10^4 CFU per spot. *H. influenzae* ATCC 49247 and *E.coli* ATCC 25922 were included as quality control reference strains. A chocolate agar plate was inoculated as a growth control plate prior to inoculation of the antibiotic plates, and a second chocolate agar plate was inoculated at the end to ensure no contamination or antibiotic carry-over during inoculations. All plates, with and without CCCP, were run in duplicate. Plates were incubated at 37°C in 5% CO_2 for 18-24 hours. Efflux inhibition was defined as a 2 dilution or greater decrease in MIC.

6. Polymerase Chain Reaction (PCR) - Based Detection of HI0894-HI0895 Genes

a. DNA Isolation

H. influenzae cultures were grown overnight on chocolate agar plates incubated at 35 °C in 5% CO_2. Several colonies were resuspended in 2 mL sterile saline, and the suspension was adjusted to a 3.0 McFarland standard (9 x 10^8 CFU/mL). Cell suspensions were then boiled (95-100°C) for 15 minutes in a dry bath, and left to cool at room temperature. The boiled lysate was then used as the DNA template for the PCR reaction. NOTE: A phenol extraction and ethanol precipitation method was also used to obtain purified DNA from the isolates, though the crude lysis method described here was more rapid and equally successful.

b. PCR Reaction

The amplification reaction was carried out in 50 µL reaction mix containing 5 µL 10X PCR buffer [15 mM $MgCl_2$] (Amersham Pharmacia Biotech Inc.), 0.8 µL 100 mM

dNTPs (Amersham Biosciences), 0.5μL each of (10 μM) forward and (10 μM) reverse primers (Invitrogen), 0.5 μL of 5U/μL Taq DNA polymerase (Amersham Biosciences), 37.7 μL of sterile distilled H_2O, and 5 μL of DNA template. The primers used to amplify the *acrA* and *acrB* homologs of *H. influenzae* were custom ordered based on the previously published primers: 5'- CCT CAG AAT TCT TAT CAC TCA AAA TAG G – 3' and 5'- GTC CAG AAT TCA ACG GTT GCA ATA TCA CG-3' (listed in Table 1) (46). The PCR product was 2.8 kilobases in length which contained the entire HI0894 and a 5' terminal segment of HI0895 (46). PCR amplification of HI0894 and HI0895 genes was performed using the MJ Research Minicycler PCR system, and the PCR conditions were as follows: 94°C for 7 min for 1 cycle; 94°C for 1 min, 57°C for 1 min, 72°C for 2 min, for 30 cycles; and 72°C for 10 min for 1 cycle. An identical reaction mixture using sterile distilled water in place of the DNA template, as well as *E. coli* ATCC 25922 both served as negative controls and were included in each PCR reaction. ATCC strain *H. influenzae* 49247 was included as a positive control.

Table 1. Oligonucleotide Primers used in Polymerase Chain Reactions and Cycle Sequencing Reactions

Gene Product	Primer Name	Primer Sequence	Product Size (bp)	Reference
HI-0894/0895	HFLU 0894 HFLU 0895	5'- CCT CAG AAT TCT TAT CAC TCA AAA TAG G – 3' 5'- GTC CAG AAT TCA ACG GTT GCA ATA TCA CG-3'	2800	46
mef(A/E)	Mef-F Mef-R	5'– AGT ATC ATT AAT CAC TAG TGC – 3' 5'– TTC TTC TGG TAC TAA AAG TGG – 3'	348	47
erm(B)	Erm-F Erm-R	5' – GAA AAG GTA CTC AAC CAA ATA – 3' 5' – AGT AAC GGT ACT TAA ATT GTT TAC – 3'	639	47
L4	HL4-F HL4-R	5' – TTA AGC CGG CAG TTA AAG C – 3' 5' – CAC TTA GCA AAC GTT CTT G – 3'	662	43
L22	HL22-F HL22-R	5' – CGG CAG ATA AGA AAG CTA AG – 3' 5' – TGG ATG TAC TTT TTG ACC C – 3'	296	43
23S rRNA	H23S-F H23S-R	5' – CGG CGG CCG TAA CTA TAA CG – 3' 5' – TTG GAT AAG TCC TCG AGC TAT T – 3'	1001	43
23S rRNA	HF2330 HF2520	5' – GTA TAA GCA AGC TTA ACT G – 3' 5' – GAT GTG ATG AGC CGA CAT CG – 3'	190	7, 43
23S rRNA	H23S-F 23S8-R	5' – CGG CGG CCG TAA CTA TAA CG – 3' 5' – GAT GCG ACG AGC CGA CAT CG – 3'	620	43
Sequencing Primers				
L22	L22-SF L22-SR	5' – CCC ATT GCT ATT CCT CTA C – 3' 5' – GAA ATA AGA GCT AAA GAG ATG G – 3'	ND*	This study
L4	L4-SF L4-SR	5' – AGG AGA TAG AGA TGG AAT TAC – 3' 5' – GTT CTT GAC TCA TGC TAG G – 3'	ND*	This study

* ND = not determined

c. PCR Product Detection

All PCR products were run on 1% agarose gels (Invitrogen) made with 0.5X Tris-Borate-EDTA (TBE) buffer, at 94 volts. Ethidium bromide was added to gels to allow amplified products to be visualized under (UV) ultraviolet light. A 500 base pair (bp) ladder (Invitrogen) was used as a molecular weight marker.

7. PCR Based Detection of *mef* (A/E) / *erm* (B) Erythromycin Resistance Determinants

a. DNA Isolation

H. influenzae strains were subcultured on chocolate agar plates and incubated for 18-24 hours at 35 °C in 5% CO_2. Lysates were prepared by resuspending several colonies in 2 mL sterile saline, which was adjusted to a 3.0 McFarland (9×10^8 CFU/mL). This solution was then boiled in a dry bath for 15 min, and allowed to cool to room temperature. The boiled lysate was used as a DNA template.

b. PCR Reaction

The amplification reactions of *mef*(A/E) and *erm*(B) were each carried out in 50 µL reaction mixtures containing 5 µL 10X PCR buffer [15 mM $MgCl_2$] (Amersham Pharmacia Biotech Inc.), 0.8 µL 100 mM dNTPs (Amersham Biosciences), 0.5 µL each of (10 µM) forward and (10 µM) reverse primers (Invitrogen), 0.5 µL of 5U/µL Taq DNA polymerase (Amersham Biosciences), 37.7 µL of sterile distilled H_2O, and 5 µL of DNA template. The primers used to amplify the *mef*(A/E) and *erm*(B) genes were custom ordered based on previously published primers (47), and previously used to amplify these

genes in *H. influenzae* (43). For *mef*(A/E), the forward primer was 5'– AGT ATC ATT AAT CAC TAG TGC – 3' and the reverse primer, 5'– TTC TTC TGG TAC TAA AAG TGG – 3'. For *erm*(B), the forward and reverse primers were 5' – GAA AAG GTA CTC AAC CAA ATA – 3' and 5' – AGT AAC GGT ACT TAA ATT GTT TAC – 3', respectively (listed in Table 1). The PCR products were 348 bp in length for *mef*(A/E), and 639 bp for *erm*(B) (47). PCR amplification of the *mef*(A/E) and *erm*(B) genes was performed using a Perkin-Elmer 9700 Gene Amp® PCR system, and the PCR conditions were as follows: 94°C for 2 min for 1 cycle; 94°C for 1 min, 53°C for 1 min, 72°C for 1 min, for 30 cycles; and 72°C for 10 min for 1 cycle. An identical reaction mixture using sterile distilled water in place of the DNA template, as well as *H. influenzae* 49247 served as negative controls for all the *mef*(A/E) and *erm*(B) reactions. An *erm*(B) positive *S. pneumoniae* strain 30420 was included as a positive control for the *erm*(B) reaction, and a negative control for the *mef*(A/E) reaction. The *mef*(A/E) positive *S. pneumoniae* strain 12300 was the positive control in the *mef*(A/E) reaction, and a negative control for the *erm*(B) reaction. NOTE: *S. pneumoniae* positive and negative control strains were available, and were therefore chosen as the control strains instead of *H. influenzae.*

c. PCR Product Detection

All PCR products were run on 1% agarose gels (Invitrogen) made with 0.5X Tris-Borate-EDTA (TBE) buffer, at 94 volts. Ethidium bromide was added to gels to allow amplified products to be visualized under ultraviolet light. A 123 base pair (bp) ladder (Invitrogen) was used as a molecular weight marker.

8. Sequencing of the Genes Encoding Ribosomal Proteins L22, L4 and 23S rRNA

a. DNA Isolation

Several different methods were employed to isolate the DNA for further sequencing:

i. Crude Lysis

H. influenzae cultures were grown overnight on chocolate agar plates incubated at 35 °C in 5% CO_2. Several colonies were resuspended in 2 mL sterile saline, and the suspension was adjusted to a 3.0 McFarland standard (9 x 10^8 CFU/mL). Cell suspensions were then boiled on high (95-100°C) for 15 minutes in a dry bath, and left to cool at room temperature. The boiled lysate was then used as the DNA template for the PCR reaction (5 µL of the lysate into 50 µL total PCR reaction mixture).

ii. Phenol Extraction and Ethanol Precipitation of DNA

H. influenzae cultures were grown overnight on chocolate agar plates, and five colonies were resuspended in 1 mL of sterile saline. Following centrifugation at 13 000 rpm for 10 minutes, the supernatants were removed, and the pellet resuspended in 300 µL of prewarmed lysis solution containing 0.1 M NaOH, 2 M NaCl, and 0.5 % sodium dodecyl sulfate (SDS). This solution was boiled for 15 min in a dry bath. Once cooled (approx 5 min at room temperature), 200 µL of 0.1 M Tris-HCl (pH 8.0) was added, and the solution inverted for proper mixing. For extraction of the genomic DNA, 500 µL of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and again inverted for mixing, before centrifugation at 13 000 rpm for 10 min. The top aqueous layer was then transferred to a new tube, and 1 mL of cold (-20°C) 100% alcohol added. The solution

was mixed and the DNA was allowed to precipitate at -80°C for a minimum of 30 mins. The precipitated DNA was collected by centrifugation at 13 000 rpm for 15 mins at 4 °C. The supernatant was removed and the pellet airdried for a minimum of 30 mins. The pellet was resuspended by vortex in 30 µL of sterile distilled H_20. Lysates were then stored at -20°C, and used as a DNA template. Five microlitres of the lysate was added to the PCR reaction mixture.

iii. Enzymatic Lysis

A lysis solution was made containing 0.1 mL 1 M Tris HCl (pH 7.5), 2.25 µL Nonidet P40 (Fluka Biochemika), 2.25 µL Tween 20 (Sigma), 8.8 µL Proteinase K (25mg/mL) (Sigma), and 0.889 mL sterile distilled H_20, and stored at -20°C. *H. influenzae* cultures were grown overnight on chocolate agar plates, and 1 colony was resuspended in 30 µL of lysis solution in a 200 µL PCR tube. The tube was placed in a thermocycler and cycled at the following conditions: 10 min at 60°C, 5 min at 95°C, and cooled to 4°C. The solution obtained was used as the DNA template. Only 2 µL of this solution was added to the PCR reaction mixture.

b. PCR Reaction

The PCR reaction for sequencing of L4 was carried out in 50 µL reaction mixture containing 5 µL of 10x PCR buffer (15 mM $MgCl_2$) (Amersham Pharmacia Biotech Inc.), 1 µL 25 mM $MgCl_2$ (Amersham Pharmacia Biotech Inc.), 0.8 µL 100 mM dNTPs (Amersham Biosciences), 0.5 µL each of forward primer (10 µM) (5' – TTA AGC CGG CAG TTA AAG C – 3') and reverse primer (10 µM) (5' – CAC TTA GCA AAC GTT

CTT G – 3') (Invitrogen), 0.5 μL of Taq polymerase (Amersham Biosciences), 2 or 5 μL of DNA template (depending on DNA isolation method), and sterile distilled H_2O to make up the difference to 50 μL.

The reaction components were identical for the amplification of L22 and 23S rRNA, with the exception of the primers. The forward primer for L22 was 5' – CGG CAG ATA AGA AAG CTA AG – 3' and the reverse primer 5' – TGG ATG TAC TTT TTG ACC C – 3'. For the 23S rRNA amplification, the following primers were used: forward 5' – CGC CGG CCG TAA CTA TAA CG – 3' and reverse 5' – TTG GAT AAG TCC TCG AGC TAT T– 3'. All primers were custom-ordered based on previously published primers by Peric *et al.* (43).

The PCR conditions for the L4, L22 and 23S rRNA reactions were as follows: 94°C for 5 min for 1 cycle; 94°C for 30 sec, 53°C for 30 sec, 72°C for 45 sec, for 35 cycles; and 72°C for 7 min for 1 cycle. Additionally, for reaction optimization, the annealing temperature for the 23S rRNA reaction was adjusted to 55°C instead of 53°C, and the total $MgCl_2$ concentration in the reaction was modified to either 20 or 25 mM.

An identical reaction mixture using sterile distilled water in place of the DNA template served as a negative control, and ATCC *H. influenzae* 49247 served as a positive control strain for all reactions.

c. PCR Product Purification

Following the PCR reaction, all products were run on a 1% agarose gel with ethidium bromide, and visualized under UV light to confirm proper amplification. The PCR products were subsequently purified using Microcon centrifugal filter units

(Millipore). First, products were centrifuged through the membrane at 2300 rpm for 15 min to remove all impurities. To retrieve the DNA, the filters were inverted into a new tube and 30 µL of Tris-EDTA (TE) buffer was added before centrifugation at 2600 rpm for 3 min. Purified PCR products were then quantitated using an UltroSpec 2100 pro spectrophotometer. Five microlitres of the product was diluted into 95 µL H_2O (1:20 dilution), placed into Eppendorf Uvettes®, and read on the DNA setting. Purified PCR products were stored at -20°C.

d. Cycle Sequencing Reaction

All sequencing reactions were performed using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit. Each sample reaction contained 4 µL of premix solution, 1.6 µL of 0.1 µM primer, 40 ng/µL of purified PCR product, and a variable amount of sterile distilled H_2O for a total volume of 10 µL. For each series of samples to be loaded on the sequencer, one control reaction was included that consisted of 4 µL of premix solution, 2 µL of the designated primer from the kit, 1.5 µL of sterile distilled H_2O, and 2.5 µL of template included in the kit. The primers used for the sequencing reactions were the same as for the initial amplification reactions; however, a 1:100 dilution was carried out to achieve a concentration that would be consistent with the other components.

The cycle sequencing was performed using a Perkin-Elmer 9700 Gene Amp® PCR system at the following conditions: 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min, for a total of 25 cycles.

e. Sequencing Product Purification

The majority of sequencing products were purified using the ethanol / sodium acetate method. This was carried out by combining 1 μL of 3M sodium acetate (pH 4.6), 25 μL of 95 % ethanol, and 10 μL of the sequencing product. These were mixed by vortexing briefly, and left in the dark at room temperature for no less than 15 mins, but no more than 24 hours. The mixture was then centrifuged for 20 minutes at 13 000 rpm using an Eppendorf 5417C centrifuge. The supernatant was then carefully removed, and the pellet rinsed with 125 μL of 70 % ethanol. After a brief vortexing, the pellet is centrifuged for 5 mins at 13 000 rpm. The supernatant was again gently removed, and the pellet dried by placing the tube with the lid open at 90°C for 1 min. The dried pellet was reconstituted in 16 μL of template suppression reagent (TSR), and stored at -20°C for no longer than 1 week.

Several reactions were purified by the ethanol/ EDTA precipitation method. Ten microlitres of sequencing product was added to 30 μL of 100 % ethanol and 2.5 μL of 125 mM EDTA in a 1 mL eppendorf tube. After a brief vortex, samples were incubated at room temperature for 15 mins. Tubes were centrifuged for 30 minutes at 6000 rpm. The supernatant was removed and the pellet rinsed with 30 μL of 70 % ethanol. After a short vortex, samples were again centrifuged for 15 mins at 4500 rpm, though at 4°C instead of room temperature. The final steps of drying, reconstitution, and storage of the pellet proceeded as described above in the ethanol and sodium acetate purification method.

f. Sequencing

All samples were denatured by heating at 90-95°C for 2 mins, and placed immediately on ice. Sequencing of the ribosomal proteins L4 and L22 was performed using an ABI PRISM 310 Genetic Analyzer.

g. Sequence Analysis

Forward and reverse sequence fragments for each isolate and respective gene were arranged into contigs using Lasergene's (DNA Star Inc., Madison, WI) SeqMan II program. Each consensus sequence was compared to a published reference sequence, for both L4 and L22, and aligned using Lasergene's MegAlign module.

C. RESULTS

Part I. Antibiotic Susceptibility Testing of *H. influenzae*

All strains selected for this project, were isolated from an ongoing respiratory organism surveillance study encompassing all regions of Canada. As part of the study, all isolates underwent microbroth dilution susceptibility testing of various antibacterial agents, including the macrolides and ketolides. The results of this testing formed the basis for the selection of isolates with variable susceptibility to macrolides. Isolates were chosen based on their clarithromycin MICs, and formed three distinct susceptibility phenotypes. These phenotypes are observed across different surveillance studies (43), and were previously described by Peric *et al.* (4, 43). Isolates with clarithromycin MICs of <2 μg/mL were considered hypersusceptible (HS), 2 – 32 μg/mL susceptible (S), and >32 μg/mL resistant (R). Twenty-four susceptible, 5 resistant, and 5 hypersusceptible strains were randomly chosen, with the hypersusceptible strains occurring less frequently.

Once strains were selected, microbroth dilution testing was performed in triplicate on all isolates to confirm their susceptibilities to clarithromycin, azithromycin, telithromycin and ABT-773. (Table 2) Several hypersusceptible strains grew poorly and/or inconsistently in the microbroth panels, therefore their susceptibilities to clarithromycin only was measured using the macrobroth dilution technique. (Table 2) The MICs for clarithromycin ranged from 0.25 μg/mL in the hypersusceptible strains to >64 μg/mL in the resistant strains. The MICs for azithromycin were 4 to 8 fold lower than the clarithromycin MICs, typical for strains of *H. influenzae*. The ketolides, telithromycin and ABT-773, had similar MIC values to azithromycin. An acceptable MIC

range for *H. influenzae* 49247 by macrodilution is 4 – 16 μg/mL, therefore this isolate was run simultaneously in the macrobroth experiments to ensure appropriate drug dilutions.

Table 2. Antibiotic Susceptibility of 34 *H. influenzae* Study Isolates to Macrolide and Ketolide Antibiotics

Isolate #		Phenotype[a]	Microbroth MIC[b] (μg/mL)			
Stock	Experimental		CLARI	AZI	TELI	ABT-773
23812	1	HS	0.25 [c]	0.25*	0.12*	0.12*
36305	2	HS	0.25 [c]	0.5*	0.06*	0.12*
30872	3	HS	0.25 [c]	0.5*	0.03*	0.5*
32326	4	HS	0.25 [c]	0.25*	0.12*	0.12*
30149	5	HS	0.5 [c]	0.25*	0.06*	0.12*
23905	6	S	2	0.5	0.5	1
23067	7	S	4	1	1	1
23168	8	S	4	1	1	1
23629	9	S	8	1	2	2
24315	10	S	8	2	2	4
24764	11	S	8	1	2	2
27894	12	S	8	1	2	2
23342	13	S	8	2	4	2
23344	14	S	8	2	2	2
23356	15	S	8	2	2	4
23430	16	S	8	1	2	4
23469	17	S	8	2	2	4
23498	18	S	8	2	2	4
23505	19	S	8	1	2	4
23622	20	S	8	1	2	4
23750	21	S	16	2	4	4
24457	22	S	16	2	2	4
24786	23	S	16	2	4	8
26339	24	S	16	2	4	8
29306	25	S	16	1	2	4
22961	26	S	16	4	4	4
23343	27	S	16	4	4	4
23633	28	S	32	8	16	8
23748	29	S	32	4	4	2
28715	30	R	64	4	8	4
23866	31	R	>64	>64	>32	4
25025	32	R	>64	32	>32	8
23144	33	R	>64	8	8	4
23460	34	R	>64	16	32	8

Footnote:
All MIC values, with the exception of those denoted with [c] or * are an average of 3 repeated experiments; all others were tested once
* Results from CROSS Study

[a] HS, Hypersusceptible; S, Susceptible; R, Resistant
[b] CLARI, clarithromycin; AZI, azithromycin; TELI, telithromycin. Breakpoints defined by NCCLS for microbroth dilution (in μg/mL) are as follows: Clarithromycin: susceptible ≤8 μg/mL ; intermediate 16 μg/mL; resistant ≥32 μg/mL Azithromycin: susceptible ≤4 μg/mL Telithromycin: susceptible ≤4 μg/mL; intermediate 8 μg/mL; resistant ≥16 μg/mL ABT-773 does not have established breakpoints.
[c] MICs determined by macrobroth dilution method

Part II. *In Vitro* Pharmacodynamic Modeling of Macrolides and Ketolides Against Hypersusceptible *H. influenzae* Strains

To assess the *in vitro* pharmacodynamic activity of the macrolide clarithromycin, and the ketolide, telithromycin, against macrolide hypersusceptible *H. influenzae* strains, an *in vitro* modeling system was used. Three hypersusceptible strains (Experimental isolates numbered 1, 2, and 3) were studied. Clarithromycin (alone and in combination with its 14-OH metabolite) and telithromycin were added simulating free epithelial lining fluid pharmacokinetics after standard doses. All experiments ran for either 24 or 48 hours, with telithromycin being dosed once daily, and clarithromycin dosed twice daily.

Telithromycin at a concentration of 17 μg/mL was bactericidal (≥ 3 $\log_{10}$ killing over a 24-hour period of time) against all three isolates by 4 hours, with no regrowth at 24 hours (Table 3). Telithromycin at a concentration of 2 μg/ml was bactericidal against Isolate 1 at 24 hours with bacterial numbers being below the level of detection, however there was subsequent regrowth at 48 hours. Telithromycin (2 μg/ml) was only used against this one isolate, and represented the total serum concentration after standard dosing.

Clarithromycin (14 μg/ml) and 14-OH clarithromycin (3.5 μg/ml) used in combination were bactericidal at 6, 12, and 24 hours against all three isolates. Bacterial numbers were below the level of detection for all three isolates at 12 and 24 hours. There were no signs of regrowth with any three of the isolates (Table 3).

When 14-OH clarithromycin (3.5 μg/ml) was used alone against Isolate 1, bactericidal activity was observed at 6, 12, and 24 hours with bacterial numbers being

below the level of detection at 12 hours, with very little regrowth at 24 hours. Similar results occurred when clarithromycin (14 μg/ml) was used against Isolate 1, although at this concentration the drug was bactericidal at 4 hours, up to 24 hours with no regrowth (Figure 1). Clarithromycin (14 μg/ml) and 14-OH clarithromycin (3.5 μg/ml) were only used separately against this one isolate.

Figure 1. ***In vitro*** **Pharmacodynamic Modeling of Clarithromycin (14μg/mL) or 14-OH Clarithromycin (3.5 μg/mL) against** ***H. influenzae*** **23812**

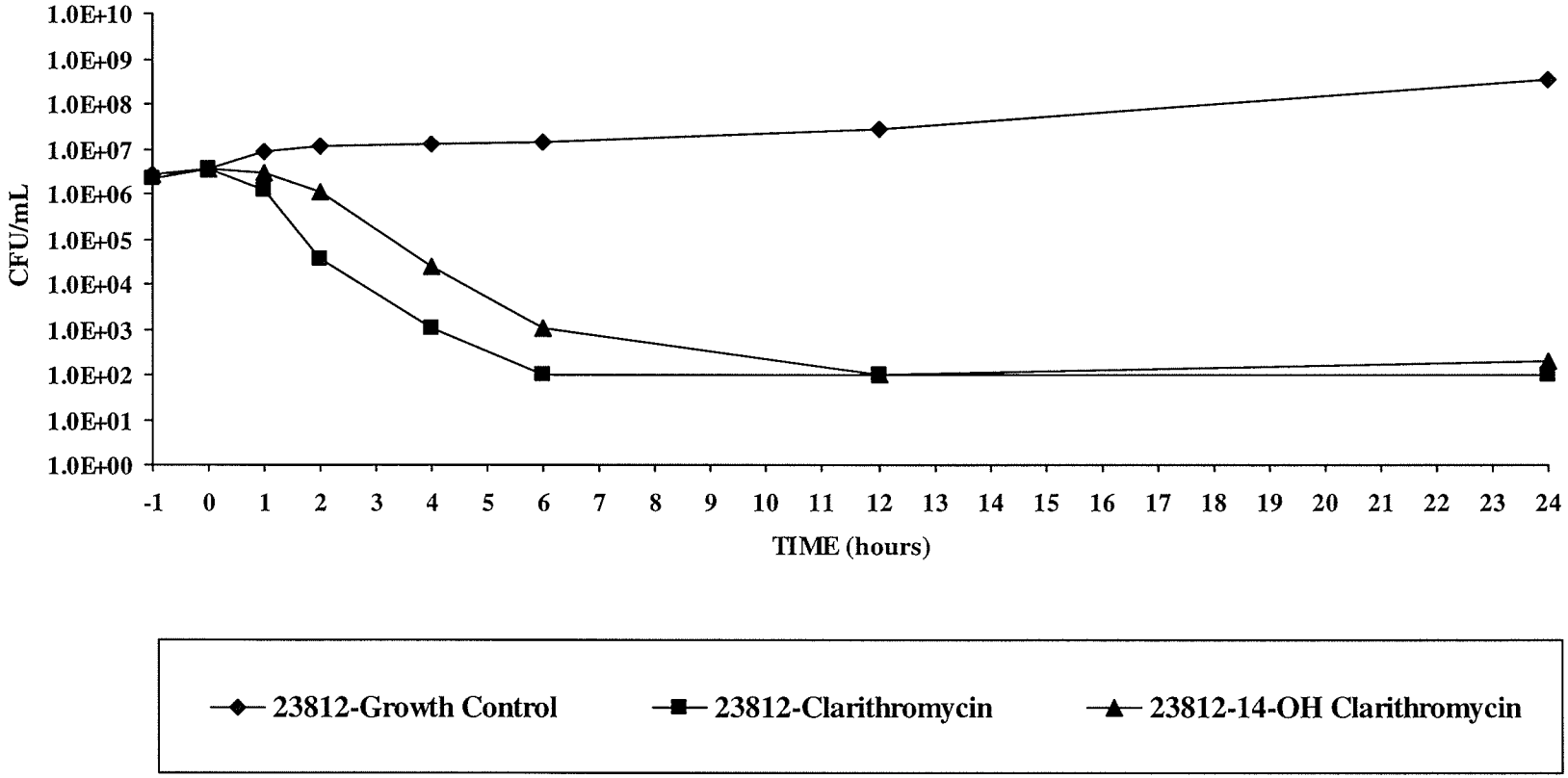


Table 3. Antibiotic Killing of Hypersusceptible *H. influenzae* Simulating Free Serum Concentrations[a]

Antibiotic / Antibiotic Combination	Cpmax (µg/mL)	Experimental Isolate(s) Tested	Log_{10} reduction of the original inoculum at time (hr)				
			0 hr	4	6	12	24
Clarithromycin	14	1	-	>4.0	>4.0	>4.0	>4.0
14-OH Clarithromycin	3.5	1	-	2.0	3.0	4.0	4.0
Clarithromycin / 14-OH Clarithromycin	14 / 3.5	1	-	2.0	3.0	>4.0	>4.0
		2	-	4.0	>4.0	>4.0	>4.0
		3	-	4.0	4.0	>4.0	>4.0
Telithromycin	17	1	-	3.0	>4.0	>4.0	>4.0
		2	-	4.0	4.0	>4.0	>4.0
		3	-	>4.0	>4.0	>4.0	>4.0
Telithromycin	2	1	-	2.0	2.5	>4.0	>4.0

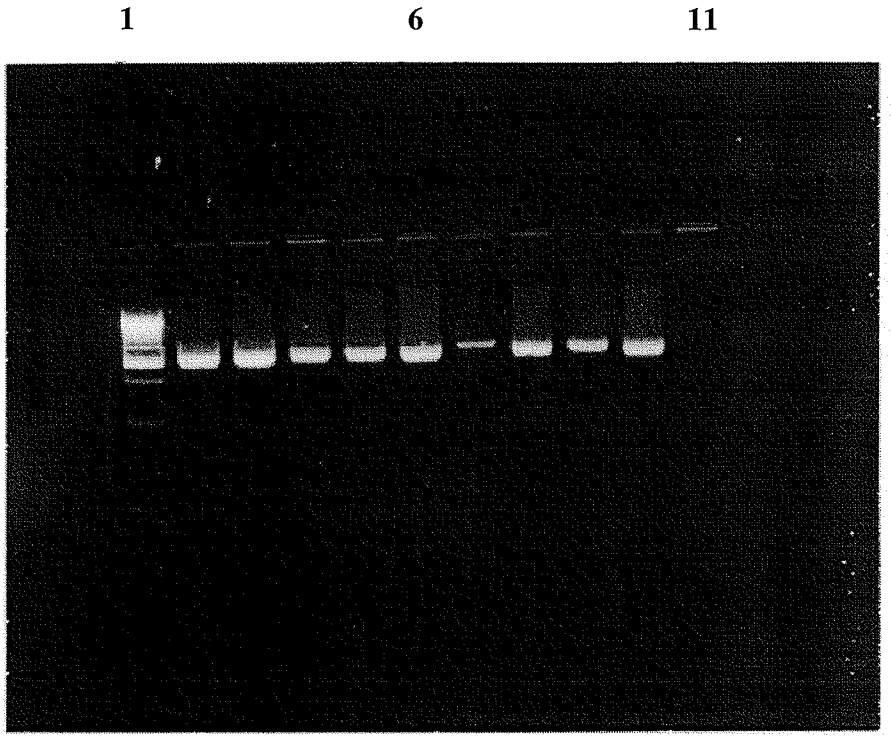
Footnote:
killing = decrease log_{10} CFU/mL
[a] = free (unbound fraction only)

Part III. Detection of HI0894-0895 Genes (*acrAB* homolog)

Basic PCR methods were used to confirm the presence of the HI0894 and HI0895 genes in all study isolates. All isolates were positive for the genes (Figure 2).

Figure 2. PCR Amplification of *acrAB* Homolog in Representative Susceptible and Hypersusceptible *H. influenzae* Strains

Lane 1, 1 kb ladder; Lane 2 through 8, contain isolates 7, 8, 11, 12, 2, 4, 3, 5 respectively (for actual isolate numbers refer to Table 2); Lane 9, ATCC *H. influenzae* 49247; Lane 10, ATCC *E.coli* 25922 (Positive Control); Lane 11, ATCC *S. aureus* 29213 (Negative Control)



Part IV. Detection of *mef* (A/E) / *erm* (B) Erythromycin Resistance Determinants

To confirm the absence of the resistance genes, *mef (A/E)* and *erm (B)*, PCR was performed on the resistant isolates with clarithromycin MICs ≥ 32 μg/mL. All isolates were negative for both resistance genes, and the control strains were positive for their respective genes.

Part V. Inhibition of Macrolide Efflux Pump

After determining that all isolates possessed the genes that encode the AcrAB efflux pump, it was hypothesized that inhibiting this proton motive force-dependant pump would result in more drug accumulating in the cells, thus decreasing MICs. In order to test this, a biological assay was proposed that would include the antibiotic(s) to be tested in the presence or absence of an efflux pump inhibitor (EPI). First, it was necessary to determine what concentration of EPI would inhibit the pump while still allowing growth of the organism.

a. Growth Experiments

A series of growth experiments were run testing isolates from each susceptibility phenotype in the presence of the protonophore CCCP. The EPI was dissolved in 100% acetone, with a stock concentration of 1000 μM.

In the first experiment, bottles containing 80 mL broth with a range of CCCP concentrations (see Materials and Methods Section 5 Part a Number i) were inoculated

with isolate 31, and ATCC strain *H. influenzae* 49247. For both isolates, bottles containing 0, 0.05, 0.10, 0.25, 0.5 μM became noticeably cloudy at 8 hours, whereas those with 0.75 and 1 μM CCCP were visibly clear with less growth. Colony counts reflected these observations as well.

A similar experiment was repeated with isolate 2 and isolate 12, however a spectrophotometer was used instead to measure growth quantitatively (Materials and Methods 5 a i). The readings were very irregular for the different time points, and no conclusive results were obtained.

All subsequent growth experiments were performed by measuring growth in tubes (see Materials and Methods Section 5 Part a Number ii and iii). The first experiment was repeated, although the final broth volume was 2 mL instead of 80 mL (Materials and Methods Section 5 Part a Number ii). One isolate from each susceptibility group (Isolate 2, 12, and 31), and ATCC *H. influenzae* 49247 were tested for visible growth. All isolates had growth in tubes 0.05, 0.10, 0.25 μM, susceptible isolate 12 and resistant isolate 31 had growth up to and including 0.5 μM, and *H. influenzae* 49247 had growth up to and including 0.75 μM.

Another growth experiment was performed in which samples were taken at various time points, and colony counts later done, although different control scenarios were included. (Materials and Methods Section 5 Part a Number ii, sampling as per Section 5 Part a Number i) Isolates 2, 12, and 31 were grown in HTM broth, in different concentrations of acetone (the same concentrations as used in inhibitor dilutions), and in different final concentrations of inhibitor (0.25, 0.5, 0.75 and 1 μM). No major

differences in CFU/mL were observed between the strains, the different concentrations of inhibitor, and acetone.

A new CCCP solution was made by dissolving in DMSO instead of acetone, and a series of growth tubes were setup to compare growth with either solvent. NOTE: CCCP concentrations converted to μg/mL for better comparison with concentrations mentioned in the literature (4, 43).

Isolate 12 was grown in 10, 20, and 25 μg/mL final concentration of CCCP, using both CCCP/acetone and CCCP/DMSO (see Materials and Methods Section 5 Part a Number iii). After a 24 hour incubation, no growth was observed in any tube containing inhibitor.

When isolate 9 was grown in the presence of 5, 10, 15, and 20 μg/mL final concentration of CCCP, no growth was observed in any tube containing inhibitor.

Isolate 10 was grown in 1, 2, 5, and 10 μg/mL final concentration of CCCP, and no growth was observed after 24 hours in any of the tubes. There was growth in the DMSO control (DMSO and broth), and in the growth control tube.

A final growth experiment consisted of comparing the growth of isolate 10 at either: final CCCP concentrations of 0.5, 1, 5, 10, 25, 50, 100 μg/mL (Materials and Methods Section 5 Part a Number iii) and a 1:100 dilution of the 0.5, 1, 5, 10, 25, 50, 100 μg/mL inhibitor solutions (Materials and Methods Section 5 Part a Number ii). All tubes with final concentrations of inhibitor had no growth. With the 1:100 dilution tubes, all tubes with the equivalent of 0.005 to 0.25 μg/mL had the same growth as the growth control, the tube equivalent to 0.5 μg/mL had growth, although visibly less than the control, and the tube with the equivalent of 1 μg/mL had no growth.

b. Macrobroth Dilution Assays with Inhibitor

The first inhibition assay involved testing isolates against two different concentrations of CCCP. Isolates 12 and 31 were each placed in tubes containing a range of antibiotic with either 0.25 and 0.5 μM final concentration of CCCP.

Using a modified macrobroth dilution method (see Materials and Methods, Section 5 Part b), isolate 12 (clarithromycin MIC = 8) was tested using both 0.25 and 0.5 μM final concentration CCCP. After a 24 hour incubation period, the MIC for the tubes with 0.25 μM inhibitor was 8 μg/mL, and also 8 μg/mL for those containing 0.5 μM. The MIC for the tubes containing clarithromycin only was 8 μg/mL, as expected for that isolate. There was no decrease in MIC for the different conditions, therefore no inhibition was observed.

For isolate 31 (clarithromycin MIC: >64), at both concentrations of inhibitor (0.25 and 0.5 μM), there was no growth in the tubes with inhibitor at 24 hours incubation, although the tubes with clarithromycin only had MICs at 128 μg/mL. After 48 hours incubation, there was growth with apparent endpoints at 16 μg/mL, for both 0.25 and 0.5 μM CCCP.

An identical experiment was performed with different isolates from the susceptible and resistant groups. Susceptible isolates 9 and 10, and resistant isolates 32 and 33 were subjected to the same inhibitor conditions of 0.25 and 0.5 μM CCCP. There was no decrease in MIC observed for either condition, and the MICs for clarithromycin only were in their appropriate ranges.

An inhibition assay was performed using isolate 9, with a final concentration of 25 μg/mL (equal to 122 μM). No growth was observed after 24 hour incubation in the tubes containing inhibitor.

c. Agar Dilution Assay with Efflux Inhibitor

In another attempt to measure the potential decrease in MIC caused by the addition of an EPI, we chose the agar dilution method of susceptibility testing. A set of plates containing clarithromycin, and a set of plates containing both clarithromycin and CCCP were prepared and tested with various isolates.

In the first experiment, HTM agar plates were used as the growth medium for the plates containing 0.015 – 1024 μg/mL clarithromycin. All experimental isolates were grown on each plate, as well as ATCC strains *H. influenzae* 49247 and *E.coli* 25922. The final CCCP concentration was 0.25 μg/mL, in the plates containing inhibitor. Growth after 24 hour incubation was poor, and very inconsistent for many isolates, on that media. For example, isolate 29, (clarithromycin MIC of 32 μg/mL), did not grow on 0.03, 0.06, 0.5, 1, 2, 8 μg/mL plates, but grew on all others up to 64 μg/mL. All organisms grew well on a chocolate agar plate that was used as a growth control.

This experiment was repeated using chocolate agar in place of the HTM agar plates. Also included as growth controls were commercially prepared HTM plates (Oxoid), HTM plates from the previous experiment, and chocolate agar plates made in-house by the media department. Overall, the growth was much better on chocolate agar than HTM agar, and also much better on the commercially prepared HTM agar than on

the custom-made plates. MIC values were comparable to the micro and macrobroth dilution methods. There was no inhibition (> 2 dilutions) detected in any isolates.

The next time the experiment was carried out, the range of clarithromycin narrowed to 8 – 256 μg/mL, and three sets of CCCP-containing plates were made: 0.25, 0.5, and 1 μg/mL. All isolates with clarithromycin MICs ≥32 μg/mL, as well as 2 isolates with MICs of 16 (Isolate 21 and 24) were chosen for testing. All isolates grew at CCCP concentrations of 0.25 and 0.5 μg/mL, with MICs being within 1 or 2 wells of microbroth MICs. At 1 μg/mL CCCP, 1 resistant isolate (Isolate 30), and both isolates 21 and 24 did not grow. No isolates tested had MICs > 2 dilutions lower in the CCCP plates, therefore no inhibition was observed.

The experiment was repeated with concentrations of CCCP as high as 10 and 20 μg/mL, although several isolates did not grow in those concentrations of inhibitor. No difference in MIC observed between the CCCP and non-CCCP plates.

Part VI. Sequencing of 23S rRNA and Ribosomal Proteins L4 and L22

To reveal any mutations in domain V of 23S rRNA, or in the surrounding ribosomal proteins L4 and L22, sequencing of these ribosomal regions was performed on all resistant and hypersusceptible strains, and several susceptible strains. Sequencing was performed on hypersusceptible and susceptible strains to confirm that mutations were not also present in these strains.

Isolates of the same susceptibility phenotype were grouped together for all sequencing steps. Amplification of the L4 and L22 genes was successful for all isolates

within the resistant group (Figure 3). A single band was observed for each strain, with an estimated size of 662 bp for L4, and 296 bp for L22. Amplification of 23S rRNA was more difficult. Multiple bands were observed for each resistant isolate, under the same amplification conditions as L4 and L22. (Figure 4) The same PCR reaction had to be repeated many times, each time varying the concentration of $MgCl_2$, the annealing temperature, or the type of lysate used. Optimal reaction conditions were not established for amplification of domain V of 23S rRNA, and no sequencing results were obtained.

Initially, the DNA concentration used for the cycle sequencing reaction of L4 was 110 ng/μL. Examination of the electropherograms revealed many overlapping peaks of variable intensities, or "dirty" samples. At various points, the sequencer was unable to distinguish the baseline from the base peaks of the sample, and therefore many bases were not called, or miscalled.

For the subsequent cycle sequencing reaction of L22, a DNA concentration of 120 ng/μL was used. This again produced "dirty", unusable results. To find an optimal DNA concentration for the sequencing of *H. influenzae*, a trial sequencing experiment was setup to compare different DNA concentrations. Two resistant isolates were tested (Isolates 29 and 33) with reactions containing 10, 40, and 100 ng/μL DNA, for both L4 and L22 genes. Overall, the best results were obtained with the reactions containing 40 ng/μL DNA, therefore this was the concentration used for all later reactions.

Isolates with clarithromycin MICs of 0.25 to 4 μg/mL formed the next group. Both genes were successfully sequenced for all 8 isolates of this group using 40 ng/μL DNA.

All isolates with a clarithromycin MIC of 8 μg/mL underwent PCR and product purification. Six of those 12 were selected for sequencing of L4. The DNA concentration for the reaction was lowered to 25 ng/μL DNA, as recommended in sequencing guidelines provided by the manufacturer, however all were unsuccessfully sequenced. The identical reactions were repeated, but did not work. The reactions were repeated with a DNA concentration of 35 ng/μL, only to achieve similar, unusable sequencing results. New PCR products were made following this for a future sequencing attempt. Although sequencing was attempted with new products, no usable sequences were obtained. The cycle sequencing reactions were repeated using 2μL premix with 2μL 5X buffer, however no difference was seen in the electropherograms. A final attempt was made to sequence these isolates using custom-designed, nested primers (Table 1), but this was also futile.

When those 6 isolates had their L22 genes sequenced however, all reactions were successful on the first attempt (DNA concentration - 40 ng/μL DNA).

Ribosomal mutations were detected in strains from all phenotypic groups. (Table 4) Four out of the 5 resistant strains had mutations in either L4 or L22, and 2 susceptible isolates had mutations in L4. The clarithromycin MICs for both susceptible strains with changes was 32 μg/mL, and was 0.25 μg/mL for the hypersusceptible strain. Alteration of the ribosomal proteins occurred by amino acid insertion, deletion, or one amino acid substitution. Two isolates had D to G changes at position 139 of L4, while another two had G to D changes at position 65. One strain had a T64K change, and an RA deletion was observed at position 68 in another isolate. The lone mutation detected in L22 was a 5 amino acid insertion that occurred after position 88 (Table 4).

Figure 3. PCR Amplification of Gene Coding for Ribosomal Protein L22 (*rpL22*) in Representative Susceptible and Resistant *H. influenzae* Strains

Lane 1, 123 bp ladder; Lane 2 through 8, contain isolates 28, 29, 30, 31, 32, 33, 34 respectively (for actual isolate numbers refer to Table 2); Lane 9, ATCC *H. influenzae* 49247 (Positive Control); Lane 10, contamination control (not visible)

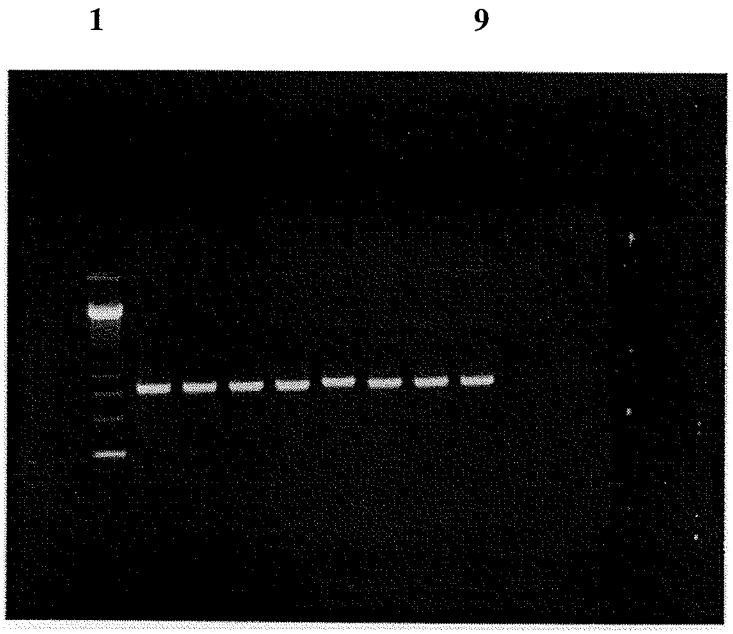


Figure 4. PCR Amplification of Domain V region of 23S rRNA in Representative Susceptible and Resistant *H. influenzae* Strains

Lane 1, 500 bp ladder; Lane 2 through 8, contain isolates 28, 29, 30, 31, 32, 33, 34 respectively (for actual isolate numbers refer to Table 2); Lane 9, ATCC *H. influenzae* 49247 (Positive Control); Lane 10, contamination control (not visible)

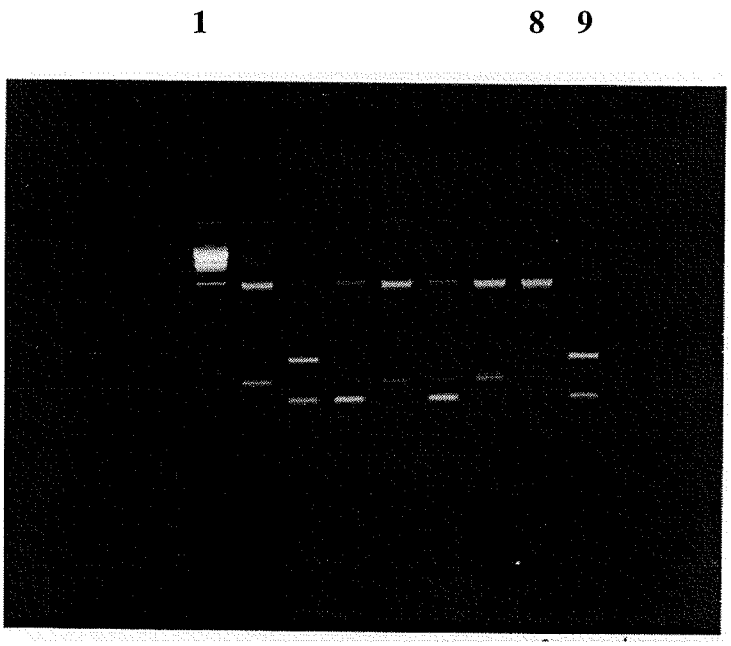


Table 4. Amino Acid Mutations Detected in L4 and L22 Genes of *H. influenzae* Study Isolates

Isolate #		Phenotype[a]	Mutation[b]	
Stock	**Experimental**		**L4**	**L22**
23812	1	HS	**D139G**	None observed
36305	2	HS	None observed	None observed
30872	3	HS	None observed	None observed
32326	4	HS	None observed	None observed
30149	5	HS	None observed	None observed
23905	6	S	None observed	None observed
23067	7	S	None observed	None observed
23168	8	S	None observed	None observed
23629	9	S	NS*	None observed
24315	10	S	NS*	None observed
24764	11	S	NS*	None observed
27894	12	S	NS*	NS*
23342	13	S	NS*	None observed
23344	14	S	NS*	None observed
23356	15	S	NS*	None observed
23430	16	S	NS*	NS*
23469	17	S	NS*	NS*
23498	18	S	NS*	NS*
23505	19	S	NS*	NS*
23622	20	S	NS*	NS*
23750	21	S	NS*	NS*
24457	22	S	NS*	NS*
24786	23	S	NS*	NS*
26339	24	S	NS*	NS*
29306	25	S	NS*	NS*
22961	26	S	NS*	NS*
23343	27	S	NS*	NS*
23633	28	S	**T64K**	None observed
23748	29	S	**G65D**	None observed
28715	30	R	None observed	None observed
23866	31	R	**D139G**	None observed
25025	32	R	None observed	**INS 88GPSMK**
23144	33	R	**G65D**	None observed
23460	34	R	**DEL 68RA**	None observed

Footnote:

NS*: Unable to sequence

[a] : HS, Hypersusceptible; S, Susceptible; R, Resistant

[b] : INS; insertion, DEL; deletion. Position numbers for L4 and L22 are based on the *H. influenzae* numbering system. Amino acid abbreviations: D; aspartic acid, G; glycine, T; threonine, K; lysine, R; arginine, A; alanine, P; proline, S; serine, M; methionine.

D. DISCUSSION

Part I. A Macrolide Efflux Mechanism is Associated with Susceptible and Resistant *H. influenzae* Strains

Antimicrobial treatment of community-acquired respiratory tract infections has evolved over the last 3 decades as a result of antibiotic resistance. *Streptococcus pneumoniae*, a key pathogen involved in CARTIs, has acquired resistance to several classes of antimicrobial agents by a number of mechanisms. Globally, penicillin and macrolide resistance rates are on the rise in this organism, with macrolide resistance exceeding penicillin resistance in many countries (20, 59). In *H. influenzae*, production of β-lactamase is the primary mechanism of resistance to ampicillin and other β-lactam antibacterials. Data from the PROTEKT study showed that the prevalence of β-lactam producing *H. influenzae* varies considerably by region, and a recent surveillance study reported that the mean prevalence of these strains in Canada is approximately 22 % (60).

Although resistance to macrolides has not been the main concern regarding *H. influenzae* to date, clarithromycin-resistant isolates have been emerging worldwide, and it is of concern that these strains may also show resistance to other macrolides (19).

It is well known that macrolides have consistently higher MICs in Gram negative organisms than those in Gram positive bacteria. Results from the CROSS Study report *H. influenzae* MIC distribution values for clarithromycin at 0.25 - ≥128 μg/mL (MIC_{90} 16 μg/mL), as compared to 0.03 to ≥128 μg/mL (MIC_{90} 0.25 μg/mL) for *S. pneumoniae* (59, 60). Current NCCLS *H. influenzae* breakpoints for clarithromycin are: susceptible, ≤ 8 μg/mL and ≥ 32 μg/mL resistant (40). The resistance of most Gram negative bacteria to

macrolide antibiotics is thought to be mainly due to the synergy between limited outer membrane permeability and energy-dependent multidrug efflux.

Sanchez *et al.* (46) were first to describe that the *acrAB* homolog in *H. influenzae* coded for a functional multidrug efflux pump able to pump out noxious dyes, detergents, and lipophilic antibiotics. This group demonstrated that when these genes are expressed, they contribute to the baseline level of resistance of the organism. All *H. influenzae* study isolates possessed these genes, which were confirmed by PCR.

The efflux assays were initially developed as a way to determine which strains were indeed expressing efflux, and what difference in MIC would be noted if the pump were inhibited. There were many obstacles with these experiments as no similar assays were found in the literature. Many published studies have added CCCP in timed assays to measure accumulation of antibiotic over a short period of time (22, 23, 43, 46), although few have reported using it in the growth medium. Valdezate *et al.* (50) employed the agar dilution method with CCCP to investigate the presence of an active efflux mechanism in *Stenotrophomonas maltophilia*, however the experiment did not work well for *H. influenzae*. No decrease in MIC was observed in the experiments, and an efflux mechanism was inferred to be present only if the clarithromycin MIC in the presence of CCCP was at least fourfold less, or 2 doubling dilutions lower than the corresponding MIC in the absence of the inhibitor (50).

The different concentrations of inhibitor used in the experiments were selected based on reported concentrations, however, at these concentrations, no decrease in MIC was noted or cell death occurred. In addition, no conclusions could be drawn from these experiments as positive and negative efflux strains were not included.

Although more time-consuming, an accumulation assay using radiolabeled antibiotic could have been used to reveal which strains expressed efflux pumps. Bozdogan *et al.* (4) used this method to determine the presence of an efflux mechanism in strains with different susceptibility patterns. It was discovered that azithromycin and clarithromycin hypersusceptibility is rare and was found to be associated with the absence of an efflux mechanism. An efflux mechanism is present in susceptible and resistant strains, which represents the majority of isolates (>98%) (4, 43).

Sutcliffe *et al.* (47) described several different mechanisms causing macrolide resistance in other organisms, namely the *mef (A/E)* gene encoding an efflux system in streptococci; and *erm,* an rRNA methylase gene. Specific primers were designed to detect these determinants, and these primers were used to confirm that the decreased susceptibility of the *H. influenzae* isolates was not due to either of these acquired resistance genes.

Part II. Mutations are Detected in Resistant Strains of *H. influenzae* in Ribosomal Proteins and 23S rRNA

More recently, resistance due to mutations in the macrolide target site has been reported in several different organisms, such as *S. pneumoniae*, *N. gonorrhoeae*, and *H. influenzae* (41, 43, 49). *H. influenzae* laboratory mutants with high-level macrolide resistance, produced by exposure to azithromycin and clarithromycin have been shown to be associated with mutations in 23S rRNA or ribosomal proteins L4 and L22 (7). These mutations likely cause a conformational change in the ribosome that decreases the affinity of macrolides.

Many mutations reported in the mutant strains have been detected among clinical isolates, and are similar to those found in other bacterial species (41, 44, 48). Common mutations in domain V of 23S rRNA are point mutations at positions 2058 and 2059 (*E. coli numbering*). In a study by Peric *et al.*, two *H. influenzae* clinical isolates identified with A2058G mutations had azithromycin and clarithromycin MICs > 64 μg/mL (43), with similar mutations causing the highest level of MLS_B resistance in *S. pneumoniae* (30).

One successful part of this project was sequencing the genes encoding the L4 and L22 ribosomal proteins. In the resistant and hypersusceptible isolates that were sequenced, several mutations were identified which corresponded to mutations found in other clinical *H. influenzae* isolates, as well as the mutants created by *in vitro* selection (7, 43). Previously reported amino acid changes such as T64K, G65D, and D139G in L4 were also found in the isolates. Other mutations were found in the study isolates that were

not previously described, however they occurred at similar positions to those mutations reported by Peric *et al.*, in highly conserved regions of the ribosomal proteins (43).

A single resistant isolate (Experimental isolate 30) with a clarithromycin MIC of 64 μg/mL and an azithromycin of 4 μg/mL was found to have no mutations in either L4 or L22. It is possible that it contained an undetected mutation in 23S rRNA which caused the increase in MIC, but similar isolates were observed by the Peric group (43). Several strains with high-level resistance to only clarithromycin were found to have no ribosomal mutations, but the majority of strains for which MICs were high for both azithromycin and clarithromycin had modifications in 23S rRNA and/or ribosomal proteins L4 and L22 (43). Other resistance mechanisms must be involved in the decreased susceptibility of these isolates to clarithromycin

In many *H. influenzae* surveillance studies, macrolide hypersusceptible strains comprise a portion of the distribution of isolates. They appear much less frequently than susceptible strains, and the mechanism by which strains become hypersusceptible to macrolides still remains unknown. Mutational studies performed by Clark *et al.* (7) yielded an *H. influenzae* clone which, after selection by clarithromycin, became hypersusceptible to macrolides. The MICs for erythromycin, azithromycin, and clarithromycin were 0.5, 0.06, and 0.12 μg/mL respectively; instead of 8, 2, and 8 μg/mL respectively in the parent strain (7). This is a particularly interesting finding as this may be a glimpse of what could be also be occurring in a clinical setting.

Another surprising finding was the discovery of a ribosomal mutation (D139G) in a hypersusceptible strain, identical to that found in a high-level resistant strain with azithromycin and clarithromycin MICs of > 64 μg/mL. As previously mentioned,

hypersusceptible strains were shown to not have a functional macrolide efflux system; therefore the antibiotics should accumulate readily in these cells. If sufficient antibiotic is present to kill the cell, perhaps the decreased binding affinity due to that particular mutation would be negligible, and the cell maintains a hypersusceptible phenotype.

High-level macrolide resistant strains are also less common, and results from this and other studies have shown that most of these strains have two resistance mechanisms: ribosomal mutations and the macrolide efflux mechanism also found in susceptible strains. Perhaps the necessity for the accumulation of two resistance mechanisms may explain the infrequency of these high-level macrolide-resistant strains.

From this, one might speculate that MICs increase proportionally with the number of ribosomal mutations present; however this is not the case. Certain mutations can affect the conformation of the drug binding site more than others, therefore creating higher or lower MICs. For example, two clinical strains analysed by Peric *et al.* (43) were found to have a single amino acid substitution at position 2058 of domain V of 23S rRNA, with clarithromycin and azithromycin MICs for both strains of > 64 μg/mL. Also, three isolates were found with mutations in 23S rRNA, L4 and L22, although the MICs for azithromycin ranged from only 8 to 32 μg/mL and the clarithromycin MICs ranged from 32 to >64 μg/mL (43).

The sequencing of the genes of interest, 23S rRNA, L4, and L22, was quite challenging as we encountered many obstacles. The problems that arose surrounding the initial amplification reaction of 23S rRNA may have been due to the fact that 6 copies of the gene exist in the *H. influenzae* genome. All copies should have been individually amplified with specific primers for each allele. In addition, the region that was being

amplified with the designated primers was very large, nearly 1 kilobase in length. Overall, the sequencing of the L22 gene was superior to that of the L4 gene, with a greater number of isolates being successfully sequenced and analyzed. The length of the L4 gene was nearly twice that of the L22 gene, therefore perhaps the concentrations of the reaction components were not ideal for the size of the product.

Many questions remain surrounding the cause of the decreased susceptibility of these isolates, and several different experiments would have to be conducted in order to fully characterize these strains. It is possible that these strains have mutations in domain V of 23S rRNA that we were unable to detect that may be contributing to their resistance. Additionally, the overexpression of efflux systems due to mutations in regulatory regions of the pump is another mechanism worth investigating as it has been reported in clinical isolates (41), as well as in mutants generated in the laboratory (22).

E. CONCLUSION

In conclusion, clinical isolates of *H. influenzae* can be divided into three phenotypically distinct categories based on their macrolide susceptibility: hypersusceptible, susceptible, and resistant. The majority of isolates fall into the susceptible category with hypersusceptible and resistant isolates occurring more rarely. All isolates possess the genes encoding acrAB, a multidrug efflux pump, though it is only expressed in susceptible and resistant strains. A second macrolide resistance mechanism, target site modification due to ribosomal mutation was detected in most resistant isolates. Mutations occurred in highly conserved regions of the ribosomal proteins, and appeared to have an impact on the MICs of the macrolides, clarithromycin and azithromycin; and the ketolide, telithromycin.

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