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IN VITRO COMPARISON OF CLARITHROMYCIN, CLARITHROMYCIN PLUS 14-HYDROXY CLARITHROMYCIN, AND AZITHROMYCIN AGAINST *Haemophilus influenzae*

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana

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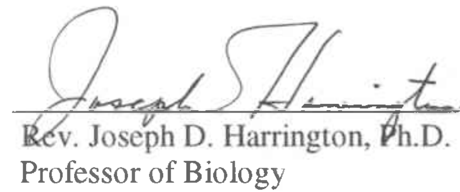
April 1998

SIGNATURE PAGE

This thesis for honors recognition has been approved for the Department of Biology.



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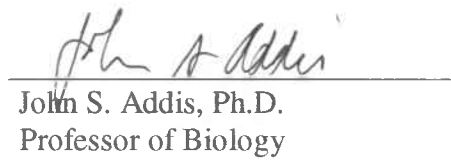

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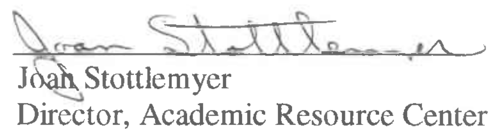

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ABSTRACT

The antibiotic activities of clarithromycin, clarithromycin plus 14-hydroxy clarithromycin and azithromycin against *Haemophilus influenzae* were compared. This comparison was performed because both clarithromycin and azithromycin have proven to be effective against infections due to *H. influenzae*.

H. influenzae is partially responsible for the prevalence of community-acquired pneumonia (CAP) in the United States. Currently, many infectious and non-infectious conditions can mimic CAP, thereby hindering diagnoses of the causative agents and the administration of the most effective treatments. The initial treatment of CAP is crucial in reducing the mortality and morbidity due to CAP, yet the difficulties in diagnosis and administration of appropriate treatments prevent the initial treatment from being precise. Since *H. influenzae* is partially responsible for CAP, the use of an antibiotic that actively inhibits its growth would decrease the prevalence of CAP due to this bacteria.

Minimum inhibitory tests, minimum bactericidal tests, and time kill assays were performed on five different strains of *H. influenzae* in order to determine if one antibiotic was more active over another. The results were not conclusive, but did show that all three antibiotics were bactericidal towards *H. influenzae*. Further studies will need to be performed to determine if one antibiotic over another or a combination of antibiotics should be initially administered to CAP patients in which *H. influenzae* is the responsible pathogen.

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INTRODUCTION AND LITERATURE REVIEW

In the United States, pneumonia is the leading cause of death due to infectious disease (4). Pneumonia can be classified depending on whether the causative agent is acquired in the community or nosocomially (4). Community-acquired pneumonia (CAP) is a common infection in the United States and has associated with it difficulties in diagnosis and treatment (4). In the United States, there are about 4 million cases of CAP annually, approximately 12 cases per 1000 adults per year (3). If hospitalization is necessary, the mortality of CAP increases from 5% to approximately 25% (4). The majority of CAP cases do not require hospitalization, yet approximately 600,000 individuals are hospitalized yearly at a cost of 23 billion dollars (3).

Numerous factors (such as age, immunosuppression, and coexisting illnesses) contribute to the severity of CAP (4). The patient's ability to fight off the infection and/or the virulence of the pathogen greatly influences the incidence and severity of CAP (4). These factors are important in determining the severity of the infection and whether hospitalization will be needed. Hospitalization is considered necessary, only when no signs of improvement have appeared after 48 to 72 hours of antimicrobial therapy (25). The need for hospitalization could be decreased or eliminated if an appropriate treatment was implemented early in the infection (4).

A major problem with CAP is diagnosis (4). The symptoms associated with CAP are not exclusive to pneumonia (4). A fever plus various respiratory symptoms such as a cough, sputum production, pleurisy, or dyspnea may suggest pneumonia is present, but are also associated with acute bronchitis, sinusitis, and some noninfectious diseases (3). Currently, no diagnostic test has the capability to detect every potential

pathogen responsible for causing CAP; therefore the diagnosis of the disease depends on the fulfillment of certain criteria (3, 4). Numerous criteria have been proposed, but the most widely accepted criteria include the presence of infiltrate on a chest radiograph plus two of the following symptoms: fever, cough with sputum production, or leukocytosis (4). These criteria are not absolute. For example, various noninfectious problems including myocardial infarction, pulmonary emboli, pulmonary edema, and pulmonary fibrosis may present symptoms that are clinically indistinguishable from CAP (4). Also, the criteria may not be met by the elderly or debilitated patients with pneumonia who are unable to elicit a response of leukocytosis or a cough with sputum production to certain pathogens, like *Mycoplasma pneumoniae* or *Chlamydia pneumoniae* (4).

If pneumonia is determined to be a plausible diagnosis, physicians start the patient on an empiric antimicrobial therapy (4). Empiric therapies consist of numerous antibiotics in order to combat a wide range of pathogens because in up to 50% of all cases no causative agent is identified even after extensive diagnostic testing (4). The initial administration of an antimicrobial regimen is crucial since it is associated with lower morbidity and mortality (4). In order to guide a physician toward the pathogen responsible, several variables (such as age, the presence of a coexisting illness, severity of the illness at the time of symptom presentation, and whether the patient requires hospitalization) are examined because they tend to influence the types of pathogens encountered (4). These variables make it possible to separate CAP cases into four groups: 1) patients younger than 60 years old, 2) patients over the age of 60 or with coexisting illnesses, 3) patients requiring hospitalization, and 4) patients with severe cases of CAP requiring admission into an intensive care unit (4). Group 1 requires

hospitalization only 10% of the time whereas group 2 requires hospitalization up to 20% of the time (25). Because of these low percentages of hospitalization, these two groups are usually treated as outpatients for CAP (25).

According to Campbell (4), even though the four patient groups can be identified as having CAP, not all antimicrobial treatments are effective; appropriate treatments are only marked by rapid responses. The failure to respond to the treatment administered could be due to a number of reasons. First, the pathogen could be resistant to the antimicrobial therapy. Second, the agent causing the pneumonia may not be bacterial; thus an antibacterial treatment would not eliminate the pathogen. Third, CAP could be caused by an unusual bacterium that is not normally associated with this type of infection, thus preventing the administration of the most precise therapy. Fourth, noninfectious complications such as pulmonary emboli, myocardial infarction, or bronchogenic carcinoma could mimic CAP symptoms or result from the problems associated with CAP. Fifth, complications due to CAP (such as lung abscess, atelectasis, or empyema) may occur, thereby slowing the response to the treatment. Last, the patient may be immunosuppressed. Although these problems can exist and can prevent the antimicrobial treatment from being effective, the initial treatment implemented is crucial in stopping or slowing the activity of the pathogen(s) causing the CAP.

Because an uncertainty exists about the type of empiric treatment appropriate for CAP, guidelines have been established. Two groups responsible for establishing guidelines are the British Thoracic Society and the American Thoracic Society (3). The groups differ in the type of treatment that should be initially administered to CAP patients because each country considers a different spectrum of pathogens as its focus for the

treatments (3). For example, the British Thoracic Society recommends an empiric treatment that uses *Streptococcus pneumoniae* as its focus bacterium therefore penicillin or amoxicillin is administered (3). The American Thoracic Society recommends macrolides, cephalosporins, trimethoprim-sulfamethoxazole, and beta-lactam-beta-lactamase inhibitors (3). These antibiotics are active against or reduce the activity of a variety of pathogens including *Haemophilus influenzae* and *Streptococcus pneumoniae*. In North America, *H. influenzae* is not as prevalent as *S. pneumoniae* (3-11% versus 20-60%), yet the treatment recommended by the American Thoracic Society is effective against these bacteria and others (3, 22). Since this empiric treatment seems to eliminate or reduce the activity of a spectrum of pathogens, time for identification of the pathogen and subsequent administration of a more precise treatment is possible.

Before the availability of antibiotics, *Streptococcus pneumoniae* was responsible for approximately 95% of all CAP cases (22). Currently, *Haemophilus influenzae* is considered the second most causative agent, responsible for up to 11% of cases (22). These bacteria are still considered the primary pneumonia-causing pathogens, especially in patient groups 1, 2, and 3 (4).

A study conducted on the treatment of upper respiratory infections revealed that these infections are the primary cause of antibiotic overuse (11). The study found antibiotics to be clinically beneficial only for patients with nasopharyngeal secretions consisting of *Haemophilus influenzae*, *Moraxella catarrhalis*, or *Streptococcus pneumoniae* (11). The majority of patients treated with antibiotics commonly had side effects and failed to benefit from the treatment. This study emphasized the fact that

antibiotics are used too often in attempts to eradicate any and all types of respiratory pathogens.

CAP could be a prime candidate for antibiotic overuse. Because the identification of the pathogens responsible for CAP is difficult, the introduction of an antibiotic may not necessarily eliminate or affect the pathogen, but cause the pathogen to become more resistant to the antibiotic. The overuse of antibiotics could and does produce bacteria resistant to particular antibiotics. As bacteria acquire resistance, this resistance is passed on to succeeding generations. The process of replication is rapid therefore the bacteria can become resistant in a short amount of time in the infected host. The increase of resistant bacteria could ultimately increase the prevalence of CAP by making it more and more difficult to treat. In order to reduce the amount of antibiotic overuse in CAP and other respiratory infections, less frequent or lower dosages of antibiotics are needed. A solution to the overuse of antibiotics could be found in the use of new antibiotics or new combinations of antibiotics.

One approach to the reduction in bacterial resistance was displayed in a study conducted in Finland. By establishing countrywide guidelines, Finland was able to effectively manage antibiotic overuse (5). For example, reductions in erythromycin use on the erythromycin-resistant group A streptococci were followed by a decrease in the numbers of streptococcal bacteria resistant to this antibiotic (5). By reducing erythromycin use countrywide, Finland was able to decrease the number of resistant isolates by 50% in only four years (5). This study showed that increasing numbers of resistant bacteria are accompanied by a need for changes in current treatments.

Finland was merely able to decrease the use of an antibiotic to satisfy its need; yet, the same results could have occurred had more effective antibiotics been discovered or used. A different antibiotic capable of attacking a particular bacterium would be, in effect, efficient in treating bacteria otherwise resistant; such a drug could be used as an effective treatment as long as it, too, was not overused.

Since resistant bacteria are inevitable with the prolonged use of any one type of antibiotic, new or modified antibiotics are beginning to be utilized. Such is the case with macrolides, which are a class of antibiotics that have been used to treat various infections such as streptococcal and pneumococcal infections (17). Macrolides act against bacteria by interfering with protein synthesis (17). By binding to the 50S subunit of the 70S bacterial ribosome, macrolides inhibit the translocation of amino acids from the aminoacyl transfer RNA to the growing polypeptide chain (17). The first macrolide used was erythromycin (figure 1), which has been used to treat infections since 1952 because of its very low toxicity and the rarity of allergic reactions (14, 17). With few

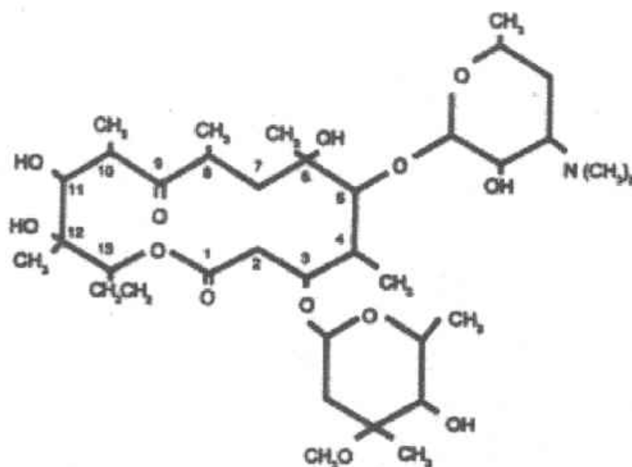

Figure 1. Chemical structure of erythromycin (7)

complications and excellent outcomes, erythromycin has been the most popular antibiotic used to treat respiratory pathogens, until recently.

In 1991, the Food and Drug Administration approved two new macrolides, clarithromycin and azithromycin (known as Biaxin and Zithromax, respectively), to treat both respiratory and skin infections (23). These drugs are considered new, yet they are simply a modification of the parent compound erythromycin (17). While both compounds are classified as macrolides, azithromycin is further classified in a subclass as an azalide compound (7, 16). Both compounds work well against a variety of common respiratory pathogens like *Streptococcus pneumoniae, Moraxella catarrhalis, Legionella pneumonophilia*, and some atypical bacteria (10). These new macrolides are considered more effective or active than erythromycin because they have broader activity spectrums, a lower number of adverse effects, and are required in smaller amounts and fewer dosages (10, 18).

A few characteristics separate clarithromycin and azithromycin from erythromycin. Their slight structural differences increase their stability to acid degradation within the upper gastrointestinal (GI) tract, thus allowing for better absorption into the GI tract (10, 17). This stability enhances their activity range plus increases their availability to the body (17). This increased availability is apparent in the higher intracellular and tissue concentrations when compared to erythromycin (17).

Because clarithromycin and azithromycin penetrate inflammatory and phagocytic cells well, they have important clinical uses against cell-associated and intracellular pathogens, such as *Legionella* (10). Similar to erythromycin, the new macrolides are active against many respiratory infections, but unlike erythromycin, they are active

against *Haemophilus influenzae*, a bacterium that is responsible for one-third of all respiratory infections (18). An effective treatment against *H. influenzae* is needed not merely due to its prevalence, but also because of its increasing resistance to penicillins and oral cephalosporins (10).

Closer examinations of clarithromycin and azithromycin are being conducted especially since they were proven to be more active against *H. influenzae*. Clarithromycin (figure 2) is structurally different from erythromycin at carbon 6 where a methoxy group replaces a hydroxy group on the 14-membered erythronolide ring (18). It is this substitution that prevents the stomach acids from inactivating the antibiotic. When clarithromycin is administered without food there are no negative consequences, but when taken with food the bioavailability of the drug (the extent to which a drug circulates throughout the body and becomes actively available to the target tissues) increases by 25 percent (1, 7).

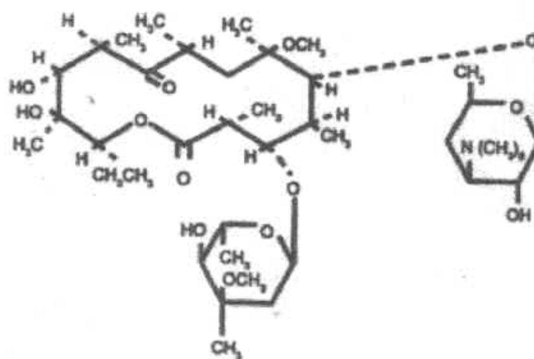

Figure 2. Chemical structure of clarithromycin (7)

The structural difference of clarithromycin also affects the development of active metabolic by-products (10). Clarithromycin is actively metabolized to 14-hydroxy clarithromycin. In vivo studies show that clarithromycin when combined with 14-hydroxy clarithromycin (that is parent compound plus metabolite) seems to have an enhanced antibiotic activity when compared to clarithromycin (the parent compound) against common respiratory pathogens like *Haemophilus influenzae, Streptococcus pneumoniae,* and *Moraxella catarrhalis* (10). A study conducted to determine the differences in bioavailability of clarithromycin plus 14-hydroxy clarithromycin against clarithromycin revealed the oral bioavailability of the former to be 85% versus 55% for the antibiotic clarithromycin alone (7). This higher percentage of bioavailability indicates that 30% more of the parent compound-metabolite combination is retained in the serum (7).

In vitro studies further showed clarithromycin plus 14-hydroxy clarithromycin to be four times more potent than erythromycin (10). Not only is it more potent than erythromycin but fewer dosages are required for an effective treatment (18). Clarithromycin has a higher serum half-life than erythromycin, thereby eliminating the need for more dosages per day (18). For example, on average erythromycin is taken four times daily, but with clarithromycin the number of dosages taken per day is reduced to two (18). Clarithromycin and clarithromycin plus 14-hydroxy clarithromycin continue to gain in popularity because of their high levels of microbiologic activities against respiratory infections, especially against the pathogens commonly responsible for CAP (19).

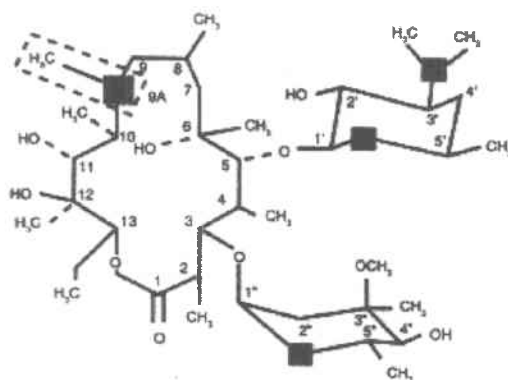

Figure 3. Chemical structure of azithromycin (7)

As clarithromycin becomes increasingly used against respiratory infections so does azithromycin. Azithromycin (figure 3) is a 15-membered aglycone ring bearing one substituted aza-methyl group at position 9 on the ring (15). Although its bioavailability is only 37%, it differs from other systemic antibiotics in that it has an extensive tissue distribution, in spite of its low serum concentration (7). Phagocytes have the ability to accumulate azithromycin (12). When bacteria is introduced to the phagocytic cell, the cells release their azithromycin reserves. The bioavailability of azithromycin is decreased if it is administered with food because the drug has a higher affinity to bind food than to absorb through the intestinal wall (12). Similarly, azithromycin should not be taken with antacids containing aluminum or magnesium (12).

The renal elimination of azithromycin is low with approximately 5% of the drug excreted unchanged, whereas 20 to 30% of clarithromycin is excreted unchanged (7). The low percentage of excreted azithromycin further validates the fact that the drug extensively penetrates tissues. With a low percentage of drug excreted unchanged, the

suggested dosage is once daily, yet at the beginning of a treatment first dosage is usually doubled (12). Azithromycin is unusual in that it is retained in tissues for up to four days after the initial dose (17). Azithromycin shares a few characteristics with clarithromycin, which explains why both these drugs are increasingly administered to patients with respiratory infections.

Because both clarithromycin and azithromycin have a lower incidence of adverse effects, longer half-lives, and are well tolerated, they are more likely to be chosen over erythromycin in treatments against CAP, especially when the causative agent is found to be *H. influenzae* (14, 18). As these new macrolides are prescribed, the uncertainty of the type of treatments that should be initially administered to CAP patients should be eliminated. The use of the most effective and efficient treatments possible should significantly reduce the prevalence of CAP and antibiotic overuse due to respiratory infections.

My study was conducted to compare the in vitro activity of clarithromycin, clarithromycin plus 14-hydroxy clarithromycin, and azithromycin against *H. influenzae*. Previous studies have been conducted using these three compounds, but most of the studies have utilized in vivo models or had methodological problems. A better understanding on the potency of these antibiotics should be revealed through minimum inhibitory concentration tests, minimum bactericidal concentration tests, and time kill assays. More precise treatments can be administered to patients with CAP if more is known about the potency of these drugs.

MATERIALS AND METHODS

BACTERIAL STRAINS. The five strains of *Haemophilus influenzae* used were obtained from the clinical laboratory at the University of Nebraska Medical Center. The ATCC 10211 strain of *H. influenzae* was from the American Type Culture Collection (ATCC) in Rockville, MD. ATCC 10211 is a strain of *H. influenzae* type b originally from the Walter Reed Army Medical Center in Washington, DC (24). The strains indicated as Bullock, Holstrom, Kelly, and Moris were obtained from patients at the medical center. All five strains were grown on chocolate agar and incubated at 37 ° C in a carbon dioxide incubator. In order to maintain good growing colonies, the chocolate agar plates were restreaked every two to three days. Each strain was stored at -70°C in cryogenic tubes with 1 mL of whole sheep blood (hemolysed or non-hemolysed). A generous swab of bacteria was gently swirled in the blood in the cryogenic tube. If needed later, these frozen samples were available as back-up to maintain actively growing strains.

ANTIBIOTICS. Clarithromycin, azithromycin, and the 14-hydroxy metabolite were obtained in crystalline form from Abbott Laboratories, North Chicago, IL. Abbott Laboratories manufactured a 14-hydroxy clarithromycin compound so that it mimics the 14-hydroxy metabolite that is metabolized from clarithromycin in the human body. Stock solutions were prepared and later diluted to the desired concentrations to be tested. Clarithromycin was prepared at a 2 mg/mL concentration by adding 10 mg of clarithromycin powder to 2 mL of methanol to help dissolve the powder and 3 mL of haemophilus test broth (HTB) (Becton Dickinson and Co., Cockeysville, MD). The amount of methanol needed to dissolve clarithromycin may vary. Therefore, in order to

get the desired stock concentration, methanol was added first to dissolve the chemical then it was brought to volume using the HTB. Azithromycin was prepared at a 2 mg/mL concentration by adding 10 mg of azithromycin to 1 mL of methanol and 4 mL HTB. The 14-hydroxy metabolite was prepared at a 1 mg/mL concentration by adding 5 mg of the powder to 1 mL of methanol and 4 mL of HTB. A stock solution of clarithromycin plus 14-hydroxy clarithromycin was not prepared. To get the desired concentrations of clarithromycin plus its14-hydroxy metabolite, the appropriate amounts of clarithromycin (2 mg/mL) and 14-hydroxy metabolite (1 mg/mL) were combined. The stock solutions of the antibiotics are stable for only two weeks.

McFARLAND STANDARD. The McFarland Standard was used to determine the approximate number of bacteria by nephelometry. Different standards can be prepared depending on the number of bacteria desired. According to the National Committee for Clinical Laboratory Standards (NCCLS) (13), the McFarland 0.5 Standard is prepared by adding 0.5 mL $BaCl_2$ (1.175%) to 99.5 mL H_2SO_4 (1%). The 0.5 standard represents 1.5×10^8 bacteria per mL. The turbidity of a cell suspension is compared to the McFarland Standard by using a Wickerham Card, which contains numerous lines of varying thickness. The Wickerham Card provides a constant background to facilitate the visible comparison of the two solutions. The McFarland Standard uses comparable tubes and equal volumes to compare turbidities. A visible comparison may be used but greater accuracy requires a spectrophotometer. The McFarland 0.5 Standard at a wavelength of 625 nm has an acceptable optical density (absorbance) of 0.08 to 0.10 (8). The optical density of the bacterial suspension can be compared to the optical density of the

McFarland Standard giving a bacterium count closer to what the McFarland standard represents .

INOCULUM PREPARATIONS. The following guidelines for the susceptibility tests were established by NCCLS and the Handler Journal of Antimicrobial Susceptibility Testing (8, 13). At least 24 hours prior to the day the tests will be conducted, each strain of *H. influenzae* was streaked on chocolate agar plates. The day of the minimum inhibitory concentration test (MIC) or time kill assay, an initial suspension of was prepared by transferring approximately 5 colonies from the agar plates 5 mL of HTB. The number of colonies transferred may varied because the initial goal was to make the inoculated broth as turbid as the McFarland Standard. Once the turbidity matched the standard, 0.2 mL or 0.3 mL of the inoculated broth was transferred into another 5 mL of HTB. This second suspension was incubated on a mechanical shaker in a CO_2 incubator at 37°C ± 2°C until the turbidity again matched the McFarland Standard. This takes at least 5 hours for *H. influenzae*. This type of preparation was designed to correspond to a mid-logarithmic growth phase for *H. influenzae*, which was the phase that should be used to conduct both the MIC and time kill assay (8).

MINIMUM INHIBITORY CONCENTRATION (MIC). The MIC is used to determine the in vitro activity an antibiotic has against a bacterial strain. The MIC is the lowest concentration of an antibacterial agent that can prevent bacterial growth. Use of the MIC microdilution method provided a way for numerous concentrations of antibiotic to be tested at one time.

In order to do the MIC test, the guidelines found in the Handler Journal (8) were followed. Sterile U-bottom microdilution trays with 96 wells were used for the MIC test.

The MIC tray has 12 columns therefore 9 concentrations can be tested leaving 2 columns for antibiotic free controls and 1 column for a broth control. The MIC tray has 8 rows, providing a way to test 4 different strains of *H. influenzae* on one tray. The MIC tray was initially prepared by adding 100 µL of HTB to each well. The antibiotic stock solutions were diluted to the highest concentration to be tested, and then serial dilutions were done from that concentration. In order to do the serial dilutions, columns one and two were filled with the highest concentration of antibiotic to be tested. Serial dilutions were done by using the microwell dilution technique and a 100 µL micropipettor. The range of clarithromycin concentrations tested was 64 µg/mL to 0.25 µg/mL. The range of azithromycin concentrations tested was 8 µg/mL to 0.03125 µg/mL.

The MIC tray was inoculated with the *H. influenzae* and HTB suspension that was allowed to grow for at least five hours or until it matched the McFarland Standard. Prior to inoculating the MIC tray, 0.8 mL of the bacterial suspension was added to 25 mL deionized water. This dilution must be utilized within 15 minutes of its preparation. All the wells, except for the broth controls, were inoculated with 10 µL of the 25.8 mL solution, which was mixed with the antibiotic solution by aspirating the micropipettor at least 7 times.

After 24 hours of incubation, the MIC trays were read and the MIC was determined. Using MIC trays with U-bottoms allowed for the presence of growth to be easily detected as a white or yellowish pellet in the bottom of the well. If none of the wells containing the antibiotic showed growth, the range of concentrations used was too high, an adjustment was made and the MIC test was repeated. From each antibiotic free

well, 100 μL were plated to verify bacterial growth. The wells showing no growth were used to determine the minimum bactericidal concentration (MBC).

MINIMUM BACTERICIDAL CONCENTRATION (MBC). The MBC test was performed to determine the lowest concentration of an antibiotic that kills bacterial cells. The MBC test used the wells from the MIC that showed no growth. From each well, 100 μL were plated on chocolate agar and incubated in a CO_2 incubator at 37°C (8). After 24 hours, the colonies were counted.

TIME KILL ASSAY. Time kill assays were used to determine the killing effects of a certain antibiotic concentration over time. This is another way to look at an antibiotic's antimicrobial potential. The concentrations tested against *H. influenzae* were 30 μg/mL of clarithromycin, 1.86 μg/mL of clarithromycin plus 14-hydroxy clarithromycin, and 2.5 μg/mL of azithromycin.

Once the inoculum was prepared and grown in HTB until the turbidity matched the McFarland standard, 0.4 mL of bacterial suspension was added to 0.6mL of phosphate buffer saline (PBS) at a pH between 7.2 and 7.4 (8). Only 100 μL of this dilute suspension were used to inoculate 10 mL of the antibiotic, which was diluted from the stock solution. The antibiotic-bacteria suspension was incubated at 37°C in a CO_2 incubator. Samples of 100 μL were taken at times 0, 1, 3, 6, and 24 hours after the antibiotic was introduced to the bacterial solution. This sample was diluted four-fold. The 100 μL sample was added to 0.9 mL of PBS then 100 μL of this 1 mL sample and PBS solution was removed and added to another 0.9 mL of PBS. This process continued until four dilutions were obtained. Between each dilution, the solution was vortexed to obtain a homogeneous suspension. It was crucial to keep the speed of the vortex on low

to reduce the possibility of bacteria cell lysis. Only 100 µL of each dilution were plated on chocolate agar. These plates were read after 24 hours of incubation.

RESULTS

MINIMUM INHIBITORY CONCENTRATION (MIC). In order to determine the MIC, the trays were read after 24 hours of incubation. The MIC value is the lowest antibiotic concentration in the microdilution tray showing no growth. The tray should reveal a transition point from a column of no bacterial growth to the adjacent column showing growth it is this area of the tray that reveals the MIC concentration. For *H. influenzae*, the MIC values ranged from 1.0 to 2.0 μg/mL for clarithromycin (Table 1) and 0.0625 to 0.5 μg/mL for azithromycin (Table 2). In this study, the MIC tests were only performed on clarithromycin and azithromycin. Time was not spent on the MICs because the data varies for each strain; therefore it is nearly impossible to report the MIC for clarithromycin against *H. influenzae*. For example, in this study the MIC for 4 strains was 1 μg/mL (Table 1) whereas other studies have reported MICs (for 90% of the strains) as 4 μg/mL or ranging from 2 to 8 μg/mL (10). The same is true for azithromycin MICs therefore in this study clarithromycin plus 14-hydroxy clarithromycin was not tested since MICs are not very conclusive and there is no standard to compare it to.

MINIMUM BACTERICIDAL CONCENTRATION (MBC). To determine the MBC values, the wells showing no growth from the MIC trays were plated on chocolate agar. After 24 hours, these plates were read by counting the colony forming units (CFU). The plates showing no growth correspond to the antibiotic concentration that kills the bacterial cells. The minimum bactericidal concentration is the last column of the MIC tray plated showing no growth adjacent to the next column showing a large

number of CFUs on the agar plates. For clarithromycin, the MBC values ranged from 2.0 to 64.0 µg/mL (Table 1). For azithromycin, the MBC values ranged from 0.25 to 1.0 µg/mL (Table 2).

Table 1. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of *H. influenzae* and clarithromycin

H. influenzae Strains	MIC (µg/mL)	MBC (µg/mL)
ATCC 10211	1.0	16.0
Bullock	1.0	2.0
Holstrom	1.0	64.0
Kelly	1.0	2.0
Moris	2.0	8.0

Table 2. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of *H. influenzae* and azithromycin

H. influenzae Strains	MIC (µg/mL)	MBC (µg/mL)
ATCC 10211	0.25	0.5
Bullock	0.125	0.25
Holstrom	0.0625	1.0
Kelly	0.125	0.25
Moris	0.5	0.5

TIME KILL ASSAY. After the plates were incubated for 24 hours, the CFUs were counted. For all five strains, the number of CFUs was determined for each dilution at each time period. In order to do an accurate comparison of the antibiotics, the percent change of CFUs from the original inoculum size was calculated (Tables 3, 4, 5, 6, and 7; Figures 4, 5, 6, 7, and 8). The mean CFUs at each dilution was determined and used in the percent change calculations. At time zero, 0% means there is no change from the initial inoculum size whereas -100% means that the plates showed a 100% decrease in the number of CFUs indicating that all the bacteria were killed. Therefore, a negative percentage indicates that the number of CFUs decreased from the original inoculum size whereas a positive percentage indicates a increase in the number of CFUs (Tables 3, 4, 5, 6, and 7; Figures 4, 5, 6, 7, and 8). The percent change was determined because the initial number of CFU for each strain was not the same, therefore graphs of time versus CFU/mL provided a poor representation of the data.

The percent change for each antibiotic does show that the compounds are antibacterial. The control group in which no antibiotic was added proves that the antibiotics were actively killing the bacteria since the bacteria show good growth. The reaction of the bacteria to the 14-hydroxy clarithromycin metabolite seems to differ for each strain. For the ATCC 10211, Holstrom, and Moris strains, 14-hydroxy clarithromycin has no overall bactericidal effect on the bacteria. 14-hydroxy clarithromycin merely slowed their growth which is obvious when it is compared to the control (Figures 4, 6, and 8). The Bullock and Kelly strains show 14-hydroxy clarithromycin as a bactericidal agent (Figures 5 and 7). After 24 hours of incubation,

14-hydroxy clarithromycin did not kill all the bacteria, but it did decrease the number of CFUs in these two strains. Clarithromycin, clarithromycin plus 14-hydroxy clarithromycin, and azithromycin do show antibacterial activity against *H. influenzae*.

Table 3. Percent change of colony forming units of *H. influenzae* with clarithromycin

TIME (hours)	PERCENT CHANGE*				
	ATCC** 10211	Bullock***	Holstrom***	Kelly***	Moris***
0	0.0%	0.0%	0.0%	0.0%	0.0%
1	51.9	-17.7	128.0	51.1	0.0
3	-86.5	-71.3	24.0	-90.3	-40.0
6	-100.0	-99.6	-38.0	-100.0	-58.8
24	--	-100.0	-100.0	--	-90.8

*a negative sign indicates a decrease in colony forming units
**ATCC 10211= American Type Culture Collection 10211
***Bullock, Holstrom, Kelly, and Moris are strains of *H. influenzae* obtained from patients at the University of Nebraska Medical Center clinical laboratory

Table 4. Percent change of colony forming units of *H. influenzae* with clarithromycin plus14-hydroxy clarithromycin

TIME (hours)	PERCENT CHANGE*				
	ATCC** 10211	Bullock***	Holstrom***	Kelly***	Moris***
0	0.0%	0.0%	0.0%	0.0%	0.0%
1	-18.0	-25.3	14.9	-47.6	66.7
3	-93.2	-90.5	80.9	-98.1	28.6
6	-100.0	-99.7	-74.5	-100.0	-44.0
24	--	-100.0	-100.0	--	-99.7

*a negative sign indicates a decrease in colony forming units
**ATCC 10211= American Type Culture Collection 10211
***Bullock, Holstrom, Kelly, and Moris are strains of *H. influenzae* obtained from patients at the University of Nebraska Medical Center clinical laboratory

Table 5. Percent change of colony forming units of *H. influenzae* with azithromycin

TIME (hours)	PERCENT CHANGE*				
	ATCC** 10211	Bullock**	Holstrom***	Kelly***	Moris***
0	0.0%	0.0%	0.0%	0.0%	0.0%
1	-22.3	32.2	2.1	-72.9	-12.5
3	-96.2	-81.5	-58.5	-99.7	-22.5
6	-100.0	-100.0	-99.6	-100.0	-96.9
24	--	--	-100.0	--	-98.0

*a negative sign indicates a decrease in colony forming units
**ATCC 10211= American Type Culture Collection 10211
***Bullock, Holstrom, Kelly, and Moris are strains of *H. influenzae* obtained from patients at the University of Nebraska Medical Center clinical laboratory

Table 6. Percent change of colony forming units of *H. influenzae* with 14-hydroxy clarithromycin

TIME (hours)	PERCENT CHANGE*				
	ATCC** 10211	Bullock***	Holstrom***	Kelly***	Moris***
0	0.0%	0.0%	0.0%	0.0%	0.0%
1	31.4	254.8	-38.9	23.3	-5.4
3	-9.5	6.5	59.7	76.7	137.8
6	395.2	-25.8	203.9	40.0	232.4
24	947.6	--	--	--	--

*a negative sign indicates a decrease in colony forming units
**ATCC 10211= American Type Culture Collection 10211
***Bullock, Holstrom, Kelly, and Moris are strains of *H. influenzae* obtained from patients at the University of Nebraska Medical Center clinical laboratory

Table 7. Percent change of colony forming units of *H. influenzae* without antibiotics

TIME (hours)	PERCENT CHANGE[*]				
	ATCC[**] 10211	Bullock[***]	Holstrom[***]	Kelly[***]	Moris[***]
0	0.0%	0.0%	0.0%	0.0%	0.0%
1	107.0	59.4	90.5	42.9	32.0
3	1158.1	247.8	109.5	128.6	360.0
6	+1158.1	+247.8	503.2	+128.6	+360.0
24	++1158.1	++247.8	+503.2	++128.6	++360.0

[*] a negative sign indicates a decrease in colony forming units; + indicates too many colonies to count therefore number of CFUs is greater than last countable plate
[**] ATCC 10211= American Type Culture Collection 10211
[***] Bullock, Holstrom, Kelly, and Moris are strains of *H. influenzae* obtained from patients at the University of Nebraska Medical Center clinical laboratory

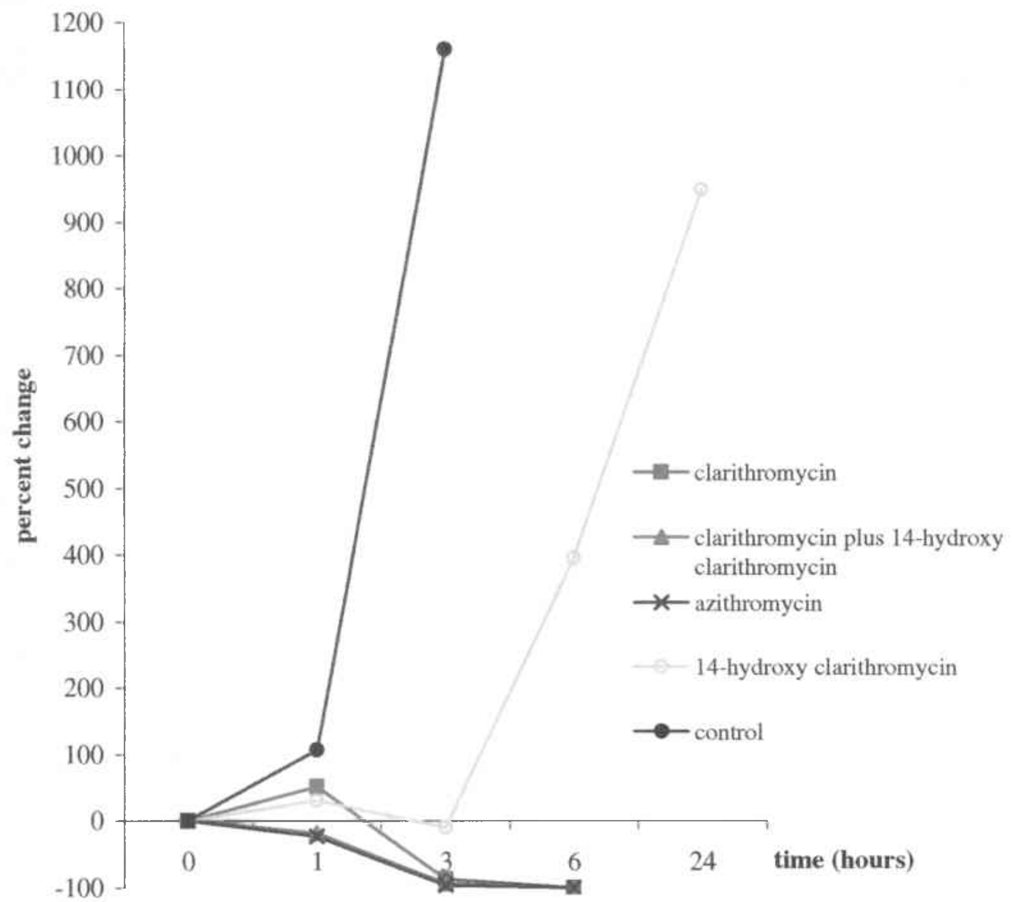

Figure 4. Percent change of the ATCC 10211 *H. influenzae* strain with added compounds.

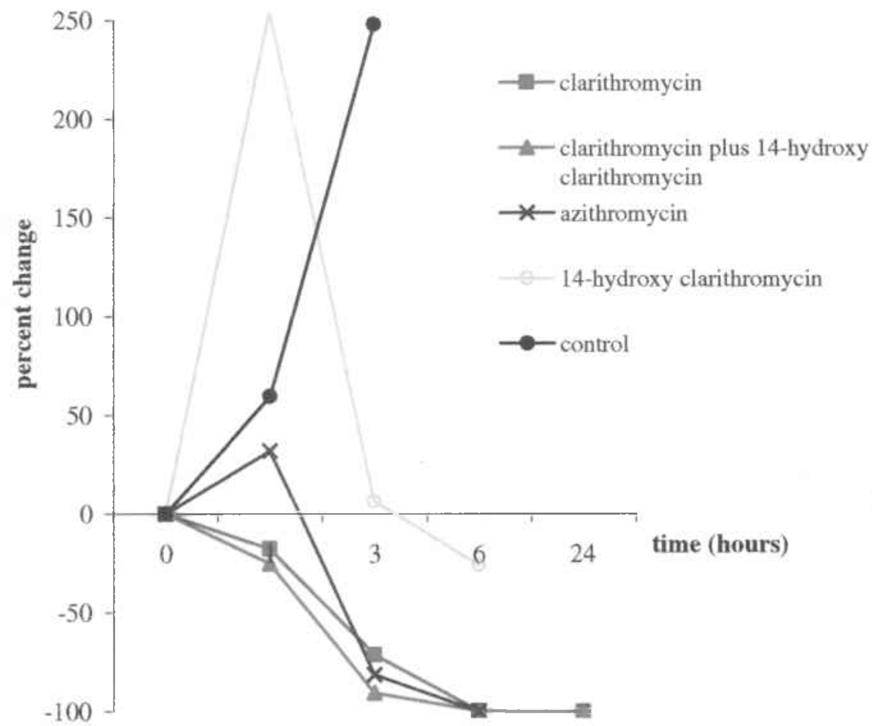

Figure 5. Percent change of the Bulock *H. influenzae* strain with added compounds.

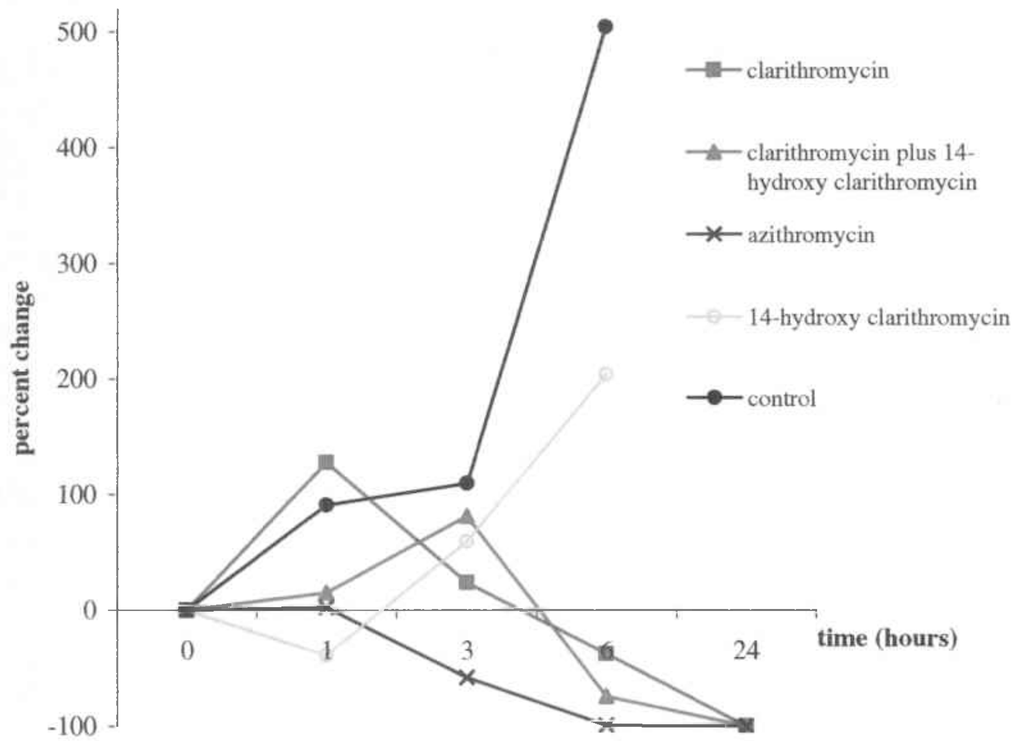

Figure 6. Percent change of the Holstrom *H. influenzae* strain with added compounds.

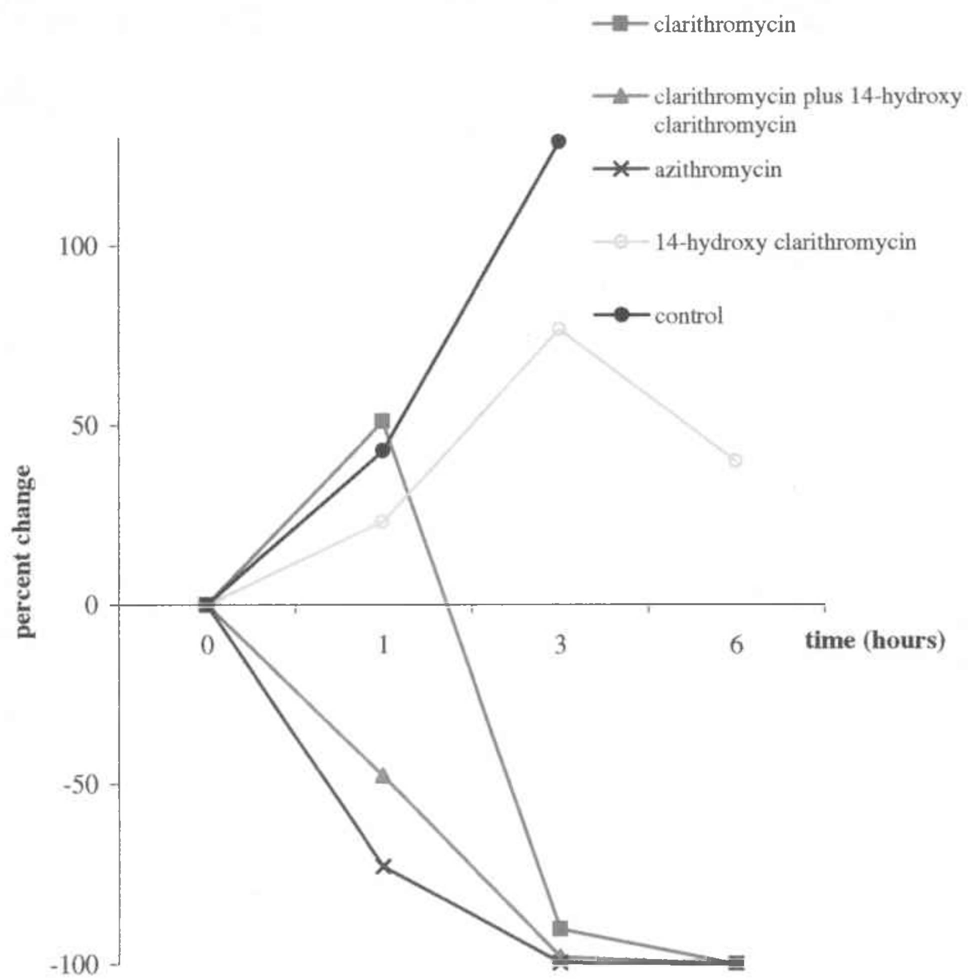

Figure 7. Percent change of the Kelly *H. influenzae* strain with added compounds.

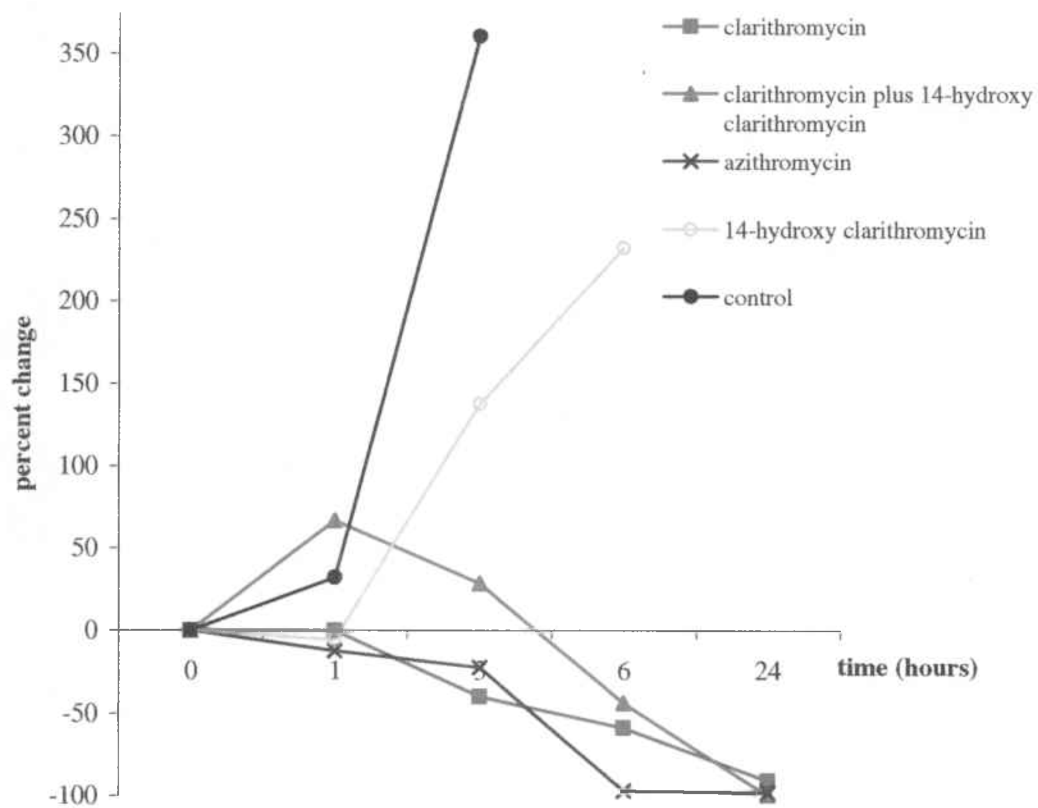

Figure 8. Percent change of Moris *H. influenzae* strain with added compounds.

DISCUSSION AND CONCLUSIONS

Similar susceptibility studies were performed on clarithromycin, clarithromycin plus 14-hydroxy clarithromycin, and erythromycin against *H. influenzae*. Some methodological problems occurred in these studies, thereby providing reasons to repeat them.

One study compared clarithromycin and erythromycin against *H. influenzae*, but it failed to test clarithromycin plus 14-hydroxy clarithromycin and the antibiotic concentrations used were high at 15 μg/mL (2). At this concentration, the study concluded that both clarithromycin and erythromycin were similarly bactericidal toward *H. influenzae* (2). From these in vitro results, this conclusion seems valid, but this conclusion cannot be applied to individual human patients. Antibiotic levels of 15 μg/mL are unattainable in the human serum (2). In order to mimic serum concentrations, the study could have used the steady-state peak serum clarithromycin concentration, which ranges from 1 to 3 μg/mL, depending on the dosing, either 250 mg or 500 mg every 12 hours (20). These steady-state peak serum concentrations reflect the levels attained in the body after at least two days of antibiotic therapy (20). For erythromycin, the peak serum levels range from 1.13 to 1.68 μg/mL after three hours of administration, then quickly decline to 0.30 to 0.42 μg/mL in 6 hours due to its short half life (21).

Through the reduction in MIC values, another study showed clarithromycin plus 14-hydroxy clarithromycin to be twice as active as clarithromycin against *H. influenzae* (9). With this increased activity, it would also appear as that the clarithromycin plus 14-hydroxy clarithromycin would have greater bactericidal activity than azithromycin (9). Even though the MICs of clarithromycin and azithromycin are equal in some studies,

azithromycin would not be as active because of its lower bioavailability (10). This lower bioavailability refers to the fact that it has low serum concentrations. Therefore, it would not seem to be the best option in treating extracellular pathogens like *H. influenzae* or other similar pathogens involved in causing CAP.

MICs and MBCs were performed as a standard procedure for susceptibility studies. This data shows azithromycin to be more potent against *H. influenzae*, but how much more potent it is compared to clarithromycin was not determined. For each strain it was possible to determine the potency of azithromycin to clarithromycin, but the overall potency differences against *H. influenzae* varies for each strain. Both clarithromycin and azithromycin show that higher concentrations of antibiotic are required to kill *H. influenzae* but the concentration again varies with each strain. This variation could be due to resistance *H. influenzae* acquired during the MIC test. The MIC test puts the bacteria in an environment where the antibiotic is continuously present, thereby inhibiting growth. The exposure to the antibiotic could have given some bacteria the chance to become resistant.

When the MBCs were performed, the antibiotic-bacterial suspensions were plated, which reduced the antibiotic saturation on the bacteria. Also, the plate provided fresh growth media lacking antibiotics. This new environment allowed some bacteria to grow, thereby indicating some bacteria were more resistant than others are, which could explain why the MBC values are higher than the MIC values (Tables 1 and 2).

In order to perform the time kill assay, different antibiotic concentrations were tested: clarithromycin at 30 μg/mL, clarithromycin plus 14-hydroxy clarithromycin at 1.86 μg/mL, and azithromycin at 2.5 μg/mL. These concentrations are found in the

bronchial fluid or lung epithelial fluid of patients who have taken full regimens of the drugs and most closely mimic physiological conditions (6). Because *H. influenzae* is not an obligate intracellular pathogen, the antibiotic concentrations found in alveolar macrophages were not utilized. Also, this study did not use the peak serum concentrations since they probably would not accurately test azithromycin due to low serum concentrations. The level of azithromycin in the serum may not be high enough to affect *H. influenzae*; therefore results from a previous study on the drug levels in the lung fluid were used.

To compare the antibiotics, the data were expressed in percent change from the initial inoculum size at time zero. The data on the 14-hydroxy clarithromycin suggests that it has no significant antibacterial activity, but compared to the control it did slightly affect bacterial growth (Tables 6 and 7). The data for clarithromycin, clarithromycin plus 14-hydroxy clarithromycin, and azithromycin do suggest significant antibacterial activity (Tables 3, 4, and 5).

The effectiveness of clarithromycin, clarithromycin plus 14-hydroxy clarithromycin, and azithromycin was difficult to determine from this data (Tables 3, 4, and 5; Figures 4, 5, 6, 7, and 8). From the results, it can be immediately concluded that the potency of one antibiotic over another is not significant since the differences in the percentages are not great. One antibiotic does not seem to stand out above the rest; this is especially apparent in the graphical representation of the data (Figures 4, 5, 6, 7, and 8). Both clarithromycin plus 14-hydroxy clarithromycin and azithromycin seem to have better activity against the five strains of *H. influenzae* when compared to clarithromycin. For example, by examining the percent change at 3 hours, a comparison was made. The

ATCC strain showed the differences between clarithromycin and azithromycin as 9.7%, clarithromycin and clarithromycin plus 14-hydroxy clarithromycin as 6.7%, and clarithromycin plus 14-hydroxy clarithromycin and azithromycin as only 3%. This example shows that the amount of bacteria killed by each antibiotic does not differ significantly. These data do not show a two-fold difference between clarithromycin and clarithromycin plus 14-hydroxy clarithromycin as the other study suggested. If there had been a two-fold difference between these two compounds, the amount of time it took to kill the bacteria should have been less.

For one or more antibiotic, each *H. influenzae* strains showed an increase of CFUs for the initial samples that were taken (Tables 3, 4, and 5). This initial increase may be due to the time it takes for the compound to get into the bacterial cell and act. Since the compounds are known to act at the level of RNA translation by binding to the 50S subunit of the ribosome, it would take time for the compound's effects to show.

By examining all the data, the greatest overall difference seems to exist between clarithromycin and the combination of clarithromycin and 14-hydroxy clarithromycin. The data also suggest that clarithromycin plus 14-hydroxy clarithromycin is comparable to azithromycin when used against *H. influenzae*. The reason for the enhanced activity of clarithromycin when it is combined with 14-hydroxy clarithromycin cannot be explained by this study and has yet to be examined.

Even though the time kill assays did not show one antibiotic to be significantly better than another, especially in the case of azithromycin and clarithromycin plus 14-hydroxy clarithromycin, further studies are needed. Clarithromycin plus 14-hydroxy clarithromycin may be more effective than azithromycin as shown by a post-antibiotic

effect assay. The post-antibiotic effect (PAE) is the period of time after which the drug has been completely removed and bacterial growth is still limited. By doing this test, a different conclusion could be made on which drug is more readily retained in an active state in the tissue or in the bacteria. It is the PAE data that is often used in determining the optimal drug dosages and schedules.

Until the PAE data are collected, the data presented in my study are not conclusive on whether clarithromycin, clarithromycin plus14-hydroxy clarithromycin, or azithromycin is the best antibiotic to use against *H. influenzae*. Furthermore, this study gives no indication as to which antibiotic should be used in the initial treatment for patients suffering from *H. influenzae* induced CAP.

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