Characterization of the Inoculum Effect with *Haemophilus influenzae* and ß-lactams

By

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CHARACTERIZATION OF THE INOCULUM EFFECT WITH *Haemophilus influenzae* AND B-LACTAMS

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TAMARA BALKO

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BLNAR	ß-lactamase Negative Ampicillin Resistant
bp	base pairs
CFU	Colony Forming Units
EDTA	ethylenediaminetetraacetic acid
HTM	Haemophilus Test Media
kb	kilobases
LB	Luria base
MIC	Minimum Inhibitory Concentration
NAD	Nicotinamide Adenine Dinucleotide
NCCLS	National Committee for Clinical Laboratory Standards
OD	Optical Density
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
ROB-1	ß-lactamase gene
sBHI	supplemented Brain Heart Infusion
TEM-1	ß-lactamase gene

ABSTRACT

An inoculum effect is defined as a four-fold or greater increase in minimum inhibitory concentration (MIC) with an increase in bacterial inocula. Using the National Committee for Clinical Laboratory Standards (NCCLS) MIC determination protocol (a turbidimetric method) *Haemophilus influenzae* demonstrated an inoculum effect with ampicillin, cefaclor, loracarbef, cefuroxime, and amoxicillin/clavulanate when initial inocula were increased from $5x10^5$ CFU/ml (low inocula) to $1x10^7$ CFU/ml (high inocula). Using this method an inoculum effect was observed with both ß-lactamase (TEM-1, ROB-1) positive and ß-lactamase negative strains of *H. influenzae*. A viable cell count MIC determination method however, demonstrated that only ß-lactamase positive strains of *H. influenzae* produced an inoculum effect suggesting that turbidimetrically determined MICs using high initial inocula are not reliable when examining the inoculum effect in *H. influenzae*. The magnitude of the inoculum effect with ß-lactamase positive strains was ß-lactam dependent (ampicillin > cefaclor = loracarbef > amoxicillin/clavulanate > cefuroxime). ß-lactam kill-curves confirmed the aforementioned results. Addition of the ß-lactamase inhibitor, clavulanate, completely reversed the inoculum effect in ß-lactamase (TEM-1 and ROB-1) positive strains of *H. influenzae* with all ß-lactams tested. The absence of an inoculum effect in ß-lactamase negative strains of *H. influenzae* and the elimination of the inoculum effect with the addition of clavulanate suggested that the inoculum effect of ß-lactams with *H. influenzae* was due to the activity of ß-lactamase. To test this supposition directly the ß-lactamase gene TEM-1 was inserted into pLS88 and introduced into a ß-lactamase negative strain, *H. influenzae* Rd. This transformation produced an inoculum effect suggesting that the inoculum effect resulted directly from ß-lactamase production.

Characterization of the Inoculum Effect with *Haemophilus influenzae* and ß-lactams

A. INTRODUCTION

1. *Haemophilus influenzae*

a. Laboratory Identification and Morphology

Haemophilus influenzae are small (1 x 0.3 µm), non-motile, non-spore forming, capsule producing Gram-negative rods (54). When Gram stained, their shape can appear pleomorphic, ranging from small coccobacilli to long filaments. When grown on solid media colonies of *H. influenzae* are granular, transparent or slightly opaque and have a circular domed shape. Encapsulated strains have a mucoid appearance (54). *H. influenzae* is a fastidious bacterium requiring two factors, X and V, for growth (54). Iron containing protoporphyrins in the X factor are heat stable and are required for the activity of catalases, peroxidases, and cytochromes of the electron transport chain. V factor is a required coenzyme, nicotinamide adenine dinucleotide (NAD) (54). These two factors are added to chocolate agar (gonococcus agar base and hemoglobin) to facilitate growth of the organism. The requirement of both these factors is used to differentiate *H. influenzae* from other species of *Haemophilus*. Although not essential, some strains of *H. influenzae* prefer to grow in an environment of 5 to 10% CO_2 (54).

b. Pathogenesis

Humans are the only host of *H. influenzae*, a resident of the normal flora of the upper respiratory tract and to a lesser degree, the mucosa of the conjunctiva and the

genital tract (36, 54). The primary virulence factor of *H. influenzae* is capsule production. The capsule provides protection from phagocytosis and complement activation (36). There are six capsular types, a-f, with *H. influenzae* type b being the most invasive (36). However, the other capsular types and non-encapsulated strains may also cause invasive disease (36). Currently a vaccine against type b strains of *H. influenzae* has greatly reduced the occurrence of type b infections and invasive disease. However, the vaccine provides no protection from strains with other capsular types or non-encapsulated strains (29, 36).

The other virulence factors of *H. influenzae* are involved in adherence, colonization, and invasion. The fimbriae are important for attachment to the upper respiratory tract (36). Differences in lipopolysaccharide expression between isolates in the nasopharynx and bloodstream suggest that it may contribute to the virulence of the organism (84). The outer membrane protein P2 of *H. influenzae* type b also appears to play a role in virulence (72). The presence of the iron binding protein TonB has been shown to correlate with the ability of *H. influenzae* to spread systemically in animal models (34) and an IgA protease and a cilotoxin which inhibits ciliary function may also be components involved in the virulence of *H. influenzae* (36).

c. Epidemiology

The majority of people colonized with *H. influenzae* carry non-encapsulated strains and remain healthy, but in some cases disease occurs. The most serious diseases are primarily found in children less than five years of age and are usually caused by type b strains (36, 54). These diseases include meningitis, septic arthritis, epiglotittis, periobital cellulitis and bacteremia (36, 54). More commonly, non-encapsulated strains are

responsible for a significant number of less serious diseases such as otitis media, sinusitis, conjunctivitis, and acute exacerbation of chronic bronchitis and pneumonia (36, 54). Approximately two billion dollars each year is spent in the United States on the management of acute otitis media, one of the most common infections in childhood (5). Non-encapsulated strains of *H. influenzae* have uncommonly been documented to be responsible for serious diseases such as neonatal sepsis, including early onset pneumonia and meningitis (54). In developing countries, non-encapsulated strains of *H. influenzae* are a major cause of acute respiratory tract infections that are associated with childhood mortality (36). These strains can also cause invasive, bacteremic infections in immunocompromised adults (54). Non-encapsulated *H. influenzae* biotype III (formerly *Haemophilus aegyptius*) are thought to be the cause of the fatal childhood disease Brazilian purpuric fever (36).

d. Antibiotic Therapy

In the 1950s infections due to *H. influenzae* were treated with chloramphenicol which was largely replaced in the 1960's by the less toxic and highly effective antibiotic, ampicillin (36). However, ampicillin resistance due to the production of ß-lactamase emerged in 1972 (37). This was followed by the appearance of chloramphenicol resistance mediated by the plasmid mediated enzyme chloramphenicol acetyl transferase in 1979 (37). The incidence of ampicillin resistance due to ß-lactamase production in *H. influenzae* was reported to range from 16.4% (38) to 28.4% (73) in North America between 1989 and 1993. The most recent surveillance study reports *H. influenzae* ß-lactamase production in 36.4% of clinical isolates from North America for 1994 and 1995 (23). In 1997 the recommended treatment for life threatening infections due to *H.*

influenzae is cefotaxime or ceftriaxone while the recommended treatment for non-life threatening illness includes amoxicillin/clavulanate, 2nd or 3rd generation oral cephalosporins, trimethoprim-sulfamethoxazole, azithromycin, clarithromycin or ampicillin/sulbactam (68).

2. ß-lactam Antibiotics

a. History

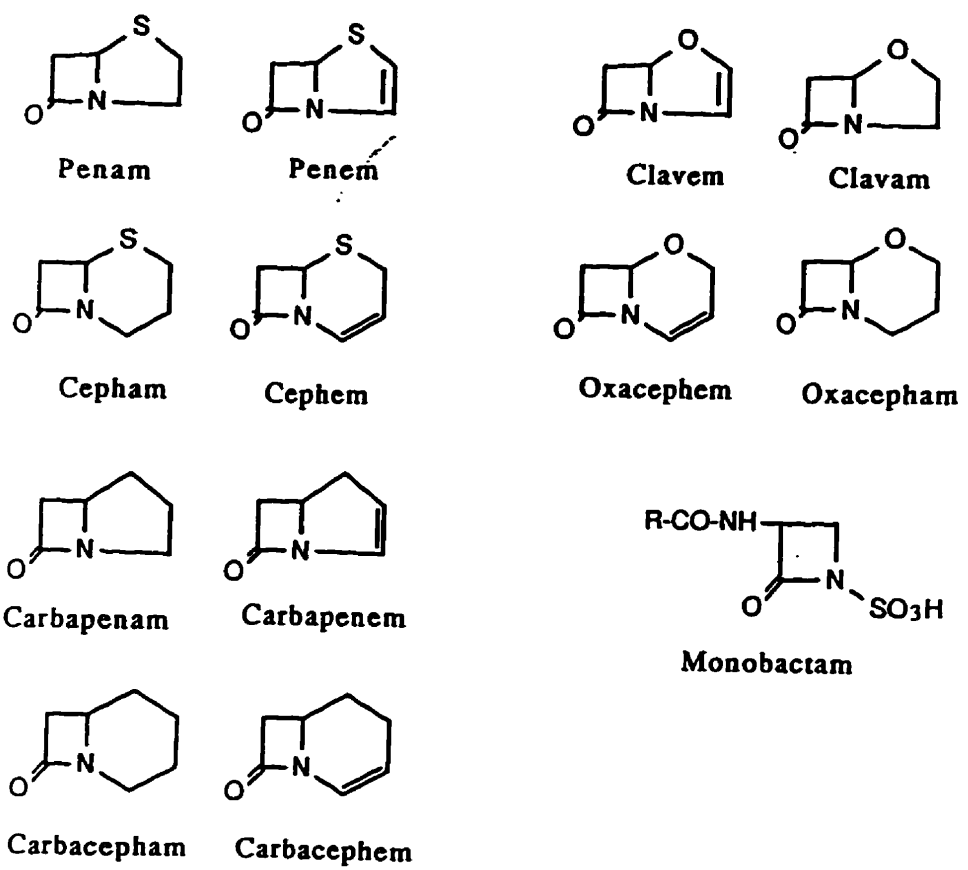
The first ß-lactam antibiotic, penicillin, was also the first antibiotic to be used therapeutically (61). Penicillin was actually first discovered by a French medical student, Ernest Duchesne in 1896, but his work went unrecognized (61). The discovery of penicillin is credited to the Scottish physician Alexander Fleming in 1929 (61). However, Fleming did not initially realize the magnitude of the discovery and in 1939, penicillin was rediscovered by Florey and Chain (61).

b. Structure

Since the discovery of penicillin, there have been many more ß-lactam antibiotics isolated from nature and synthesized in the laboratory (14). In 1957, the identification of the penicillin nucleus, 6-aminopenicillanic acid led to the development of semi-synthetic penicillins (64). The four membered ß-lactam ring is shared by all agents in the ß-lactam class of antibiotics (See Figure 1). This ring may be fused to form a bicyclic ring or exist as a single ring as with monobactams (14). The structure of the nucleus can influence the spectrum of activity of the ß-lactam. Penicillins and cephalosporins have activity against both Gram-positive and Gram-negative bacteria, while monobactams are only effective against aerobic Gram-negative bacteria. Clavulanic acid functions as a ß-lactamase

inhibitor and is used in combination with other ß-lactams (58). Substitutions on the nucleus of the ß-lactam can change the properties of the antibiotic. The addition of an amino group to the side chain of penicillin G prevents acid hydrolysis in the stomach and allows the antibiotic to be taken orally (45). Increased stability to ß-lactamases is produced by substitutions at the 7-α position of cephalosporins. However, substitutions may reduce activity against some bacteria (14). The design of a ß-lactam must balance activity, pharmacokinetics, ß-lactamase stability and toxicity (14). Table 1 presents characteristics of ß-lactams antibiotics examined in this thesis.

Figure 1: Core Structures of Different Classes of ß-lactams[a]



a. adapted from Bryan and Godfrey 1991 (14).

Table 1: Characteristics of Selected Oral ß-lactam Antibiotics[a]

Antibiotic	Class	Activity
Ampicillin	Penam (penicillin)	Active against non-ß-lactamase producing *H. influenzae N. gonorrhoeae, N. meningitidis, E. coli, Streptococcus* spp.
Amoxicillin	Penam (penicillin)	Similar to ampicillin but with 2-2.5 times more bioavailability.
Cefuroxime	Oxacepham (cephalosporin)	ß-lactamase stable. Active against *E. coli, Klebsiella* spp., *Enterobacter* spp., *H. influenzae, N. gonorrhoeae, Proteus mirabilis, Staphylococcus* spp. And *Streptococcus* spp.
Loracarbef	Carbacepham (cephalosporin)	ß-lactamase stable. Active against *H. influenzae, Moraxella catarrhalis, E. coli, N. gonorrhoeae, Klebsiella* spp., *Proteus* spp., *Streptococcus pyogenes* and *S. pneumoniae.* Similar to cefuroxime and cefaclor.
Cefaclor	Cephem	ß-lactamase stable. Similar activity as loracarbef
Clavulanic acid	Clavam	ß-lactamase inhibitor used in combination with amoxicillin with activity against ß-lactamase producing organisms (*S. aureus, B. fragilis, E. coli, H. influenzae* etc.)

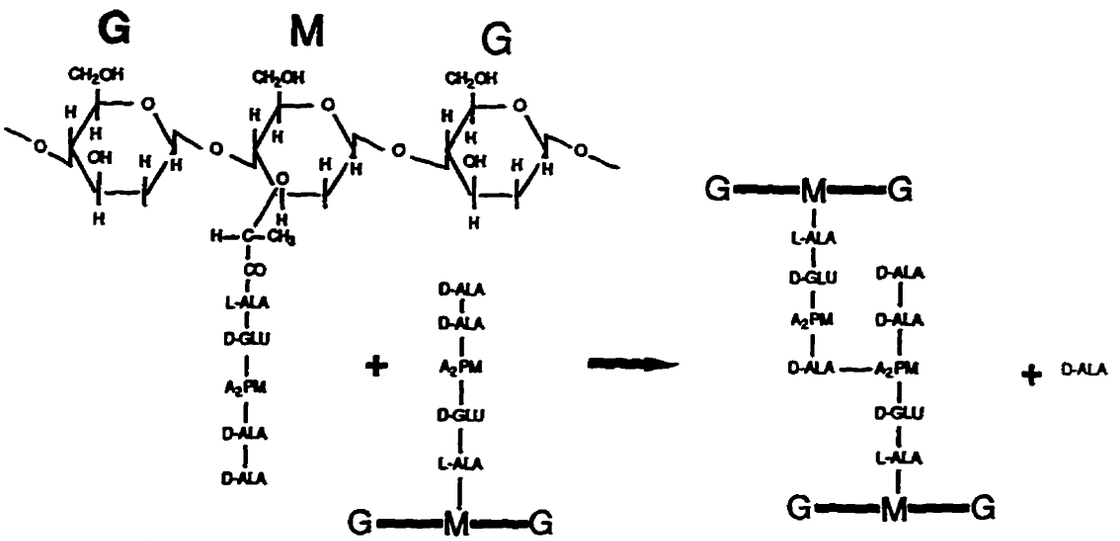
a. From Byran and Godfrey 1991 (14), Hayes and Ward 1986 (33), Krogh 1993 (42).

c. Mode of Action

ß-lactam antibiotics are cell wall active agents that inhibit enzymes involved in the cross-linking of peptidoglycan (14). Peptidoglycan provides structural integrity and rigidity to the cell wall (14, 58). It consists of repeating units of ß-(1-4)-glycoside-linked alternating units of *N*-actetylglucosamine and *N*-acetylmuramic acid which are cross-linked through the pentapeptide attached to *N*-acetylmuramic acid (14) (See Figure 2). Gram-positive bacteria have a thick layer of peptidoglycan with pentapeptides cross-linked via a

glycine interpeptide bridge, while Gram-negative bacteria have a single layer of peptidoglycan with the pentapeptides directly cross-linked (14). These cross-links provide strength to peptidoglycan structure and are important for integrity of the cell wall.

Figure 2: Transpeptidation Reaction: The Cross-linking of Murein in *E.coli*.[a]



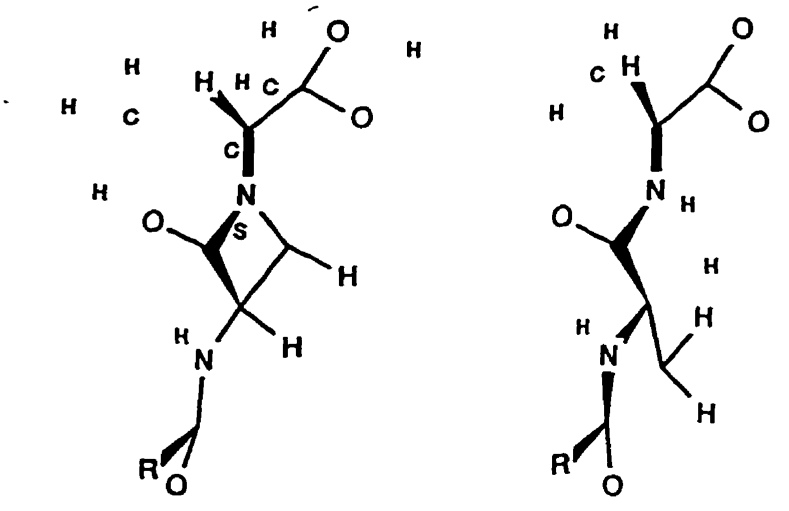
a. adapted from Bryan and Godfrey 1991 (14).

The cross-linking results from the transpeptidase reaction (Figure 2) which results in the linking of the diamino residue at position 3 of the pentapeptide to D-alanine at position 4 of an adjacent pentapeptide (14, 33, 58). The enzymes catalyzing this reaction function as transpeptidases and D,D-carboxypeptidases, reacting with acyl-D-alanyl-D-alanine to form a complex resulting in the elimination of the terminal D-alanine. This complex can react with the free amino group of an adjacent pentapeptide resulting in the

formation of a cross-link (14, 33). These transpeptidases and carboxypeptidases, termed penicillin binding proteins (PBPs), are the target of ß-lactam antibiotics (14). There are a number of PBPs (seven in *E. coli*) with varied enzymatic functions and ß-lactam binding affinities (14).

ß-lactam antibiotics are stereohomologs of acyl-D-alanyl-D-alanine (14) (Figure 3). ß-lactams compete with the natural substrate of the PBPs, interfering with the normal cross-linking (14, 33). This results in disruption of cell wall integrity and eventual cell death. The exact mechanism by which ß-lactams cause cell death is unknown (14). The defective cell wall is less able to withstand osmotic pressures resulting in the rupture of cellular membranes (33). However, this alone cannot account for the lytic effect of ß-lactams on some bacteria. Autolytic enzymes and deregulation of cellular hydrolase(s) may also be responsible for cell lysis upon exposure to ß-lactams (14).

Figure 3: Stereomodels of Penicillin and D-alanyl-D-alanine[a]



Penicillin D-alanyl-D-alanine

a. adapted from Bryan and Godfrey 1991 (14).

d. Mechanisms of Resistance

Resistance to ß-lactam antibiotics can occur through three main mechanisms: target site alteration, decreased permeability of the ß-lactam, and the presence of inactivating enzymes (69).

The mechanisms involving PBP alteration include reduced affinity for ß-lactams, while maintaining enzymatic function, and acquisition of a resistant PBP which may or may not replace the normal PBP (14). Acquisition of the low affinity PBP 2a, in addition to the normal PBPs, is the mechanism of ß-lactam resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) (32). Resistance of ß-lactams due to changes in PBPs have been reported in many bacteria including *H. influenzae* (14, 52, 74).

Resistance due to decreased permeability of ß-lactams occurs primarily in Gram-negative bacteria. Diminished uptake of ß-lactams may be caused by changes in porins or by alterations in the lipopolysaccharide of the outer membrane (14). However, decreased permeability in the absence of ß-lactamase generally has little effect on ß-lactam susceptibility (14). Compared with other Gram-negative bacteria, the outer membrane of *H. influenzae* is very permeable to ß-lactams (21, 51, 83). Decreased permeability is not considered to be a significant mechanism of resistance to ß-lactam antibiotics in *H. influenzae.*

The production of inactivating enzymes is the main mechanism of resistance to ß-lactam antibiotics in Gram-negative bacteria (14). The enzymes, termed ß-lactamases, are produced in more than 99% of ampicillin-resistant clinical isolates of *H. influenzae* (23, 73).

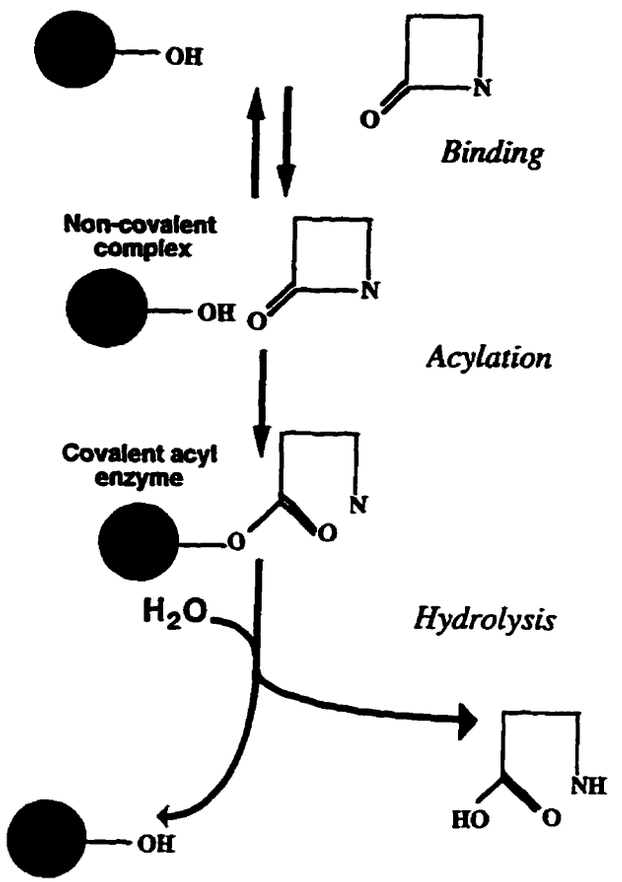
3. ß-lactamases

a. Mechanism of Action

ß-lactamases are located in the periplasmic space of Gram-negative bacteria (14). The enzymes are capable of intercepting and inactivating ß-lactam antibiotics before they reach their target PBPs. Most ß-lactamases of Gram-negative bacteria belong to a superfamily of serine proteases and utilize the serine ester mechanism of hydrolysis (Figure 4) (48, 69). The ß-lactamase non-covalently binds to the ß-lactam substrate and the complex may disassociate or proceed to form a covalent acyl enzyme via an active site hydroxyl. Deacylation results in the release of the biologically inactive open-ringed product (19).

PBPs also belong to the serine protease superfamily and catalyze the same reaction with ß-lactam antibiotics (48). The difference between the two enzymes is found in the deacylation step of the reaction, which proceeds rapidly with ß-lactamases and very slowly or not at all with PBPs (69). This results in ß-lactamases having the ability to hydrolyze a large number of ß-lactam molecules, whereas PBPs are inactivated due to the long-lived acyl-enzyme intermediate (69).

Figure 4: Action of a Serine ß-lactamase. [a]



a. adapted from Livermore 1995 (48).

b. Evolution

An enzyme with the ability to inactivate penicillin was first identified in 1940, before the introduction of the first ß-lactam antibiotics into clinical therapy (2). Sequence analysis suggests that the first ß-lactamases may have evolved from PBPs in response to naturally produced ß-lactam antibiotics (30, 40). Widespread usage of the first ß-lactam antibiotics resulted in dissemination and increased prevalence of ß-lactam resistance due to

ß-lactamase production (69). The use of synthetic ß-lactams has pressured these enzymes into continual evolution (30). First generation cephalosporins were developed in the early 1960s for the purpose of combating ß-lactamase resistance. This lead to the emergence of a variety of plasmid-mediated broad-spectrum ß-lactamases with activity against penicillins and cephalosporins (53). These broad-spectrum ß-lactamases were not capable of hydrolyzing the extended-spectrum ß-lactams (3rd and 4th generation cephalosporins) which were introduced in the early 1980's (17). Evolution through point mutations in broad-spectrum ß-lactamases, and overproduction of chromosomal ß-lactamases (stable derepression) has enabled these enzymes to inactivate even the newest synthetic ß-lactams (24, 48, 59, 60, 70).

c. Distribution

ß-lactamases may be chromosomally encoded or plasmid-mediated. Chromosomal ß-lactamases may be expressed constitutively or inducibly (18, 48) and can be found in a variety of species including *Bacteroides fragilis*, *Klebsiella* spp., *Enterbacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Morganella morganii*, *Pseudomonas aeurginosa*, and several others (48). Plasmid-mediated ß-lactamases are the main mechanism of ß-lactam resistance in Gram-negative bacteria and are common in staphylococci, enterobacteriacea, *H. influenzae*, and *Neisseria gonorrhoeae* (14, 48). There are over 75 different plasmid-mediated ß-lactamases which are generally quite distinct from the chromosomal enzymes (48). Many of the plasmid-mediated ß-lactamase genes are located on transposons enabling the transfer of these genes to a variety of different plasmids and species (48). This likely accounts for the widespread distribution of these enzymes.

d. Classification

Several attempts have been made to effectively classify ß-lactamases. In 1973, Richmond and Sykes (62) used a classification system based on comparison of substrate profiles and inhibition by clavulanic acid and was expanded using isoelectric focusing in 1976 (80). The classification system was further modified by Bush in 1989 (18) using kinetic data of substrate profiles, inhibition profiles and physical characteristics. Table 2 represents a review of the classification system.

Table 2: General classification scheme for bacterial ß-lactamases[a].

Group	Preferred Substrate	Inhibited by: Ca[b]	Inhibited by: EDTA	Representative Enzyme(s) or Organisms and location
1	Cephalosporins	no	no	Gram-negative chromosomal enzymes
2a	Penicillins	yes	no	Gram-positive chromosomal or plasmid
2b	Cephalosporins, Penicillins	yes	no	TEM-1, TEM-2, ROB-1, broad-spectrum, primarily plasmid
2b′	Cephalosporins, Penicillins, cefotaxime	yes	no	TEM-3, TEM-5, extended-spectrum, primarily plasmid
2c	Penicillins, carbenicillin	yes	no	PSE-1, PSE-3, PSE-4, chromosomal or plasmid
2d	Penicillins, cloxacillin	yes	no	OXA-1, PSE-1, primarily plasmid
2e	Cephalosporins	yes	no	*Proteus vulgaris*
3	Variable (Carbenicillin, penicillins, cephalosporins)	no	yes	Metalloenzymes, *Bacillus cereus* II, *Stenotrophomonas maltophilia* L1
4	Penicillins	no	?	*Burkholderia cepacia*

a. adapted from Bush 1989 (16)
b. clavulanic acid

e. ß-lactamase Inhibition

During the development of semi-synthetic penicillins in the 1960s, compounds were discovered that could function as inhibitors of ß-lactamases (65, 78). It was discovered that a combination of a ß-lactamase inhibitor with a ß-lactam antibiotic was synergistic against ß-lactamase producing strains of *E. coli*. However, these initial inhibitors were not effective against plasmid-mediated ß-lactamases and were not clinically successful. The idea of combining a ß-lactam with an inhibitor remained and the search for natural ß-lactamase inhibitors began (64). Screening of microorganisms identified the compound clavulanic acid from *Streptomyces clavuligerus* as a potential ß-lactamase inhibitor (13) and in 1981 the successful formulation of clavulanic acid with amoxicillin was marketed (Beecham Laboratories). Other successful inhibitors include sulbactam, a halogenated derivative of penicillanic acid, and tazobactam, an analogue of sulbactam (64).

The structures of ß-lactamase inhibitors contain the ß-lactam ring and are similar to penicillins and cephalosporins. As with labile ß-lactams, the inhibitor binds the catalytic site of the ß-lactamase resulting in acylation of the enzyme. In the case of the inhibitor, the acyl enzyme is hydrolyzed very slowly or not at all, causing inhibition of the ß-lactamase. Further reactions between the inhibitor and some ß-lactamases, such as clavulanic acid and TEM-1, result in irreversible inhibition (64).

f. The ß-lactamases of *H. influenzae*

TEM-1 and ROB-1 are the only two ß-lactamases that have been identified in *H. influenzae* (37). These enzymes are both plasmid-mediated broad-spectrum ß-lacatamases

which fall into group 2b of the Bush classification system (18) (See Table 2). TEM-1 and ROB-1 share little sequence homology (55.63%) but have similar substrate profiles and relative rates of hydrolysis of ampicillin (18, 39). Generally, only one of the two enzymes is produced by a single isolate of *H. influenzae* (22, 73). Ampicillin-resistance in *H. influenzae* due to the TEM-1 ß-lactamase was first documented in 1975 (79) and the TEM-1 enzyme continues to be the major ß-lactamase in *H. influenzae*, accounting for approximately 93% of ß-lactamase production (22, 73). The ROB-1 enzyme was first identified in an ampicillin-resistant isolate of *H. influenzae* in 1981 (66) and is now responsible for about 7% of ß-lactamase production (22, 73). Detection of the ROB-1 enzyme poses a problem in clinical laboratories that utilize conventional ß-lactamase tests that incorporate nitrocefin which is only weakly labile to the ROB-1 enzyme (37, 48).

4. Inoculum Effect

a. Definition

The inoculum effect has been defined as a four-fold or greater increase in the minimum inhibitory concentration (MIC) of an antibiotic due to an increase in bacterial inocula (1). An inoculum effect may result in a strain of bacteria testing susceptible to an antibiotic when the standard bacterial MIC inoculum, $5x10^5$ CFU/ml is used (56), but testing as resistant to the same antibiotic when a higher inoculum, such as $1x10^7$ CFU/ml is used (12). The standard inoculum of $5x10^5$ CFU/ml often underestimates the true concentration of organisms at a site of infection (11, 12, 25, 46, 49). In certain cases, it may be more clinically relevant to interpret susceptibilities at an inoculum that better reflects the situation in vivo.

b. Clinical Significance

Although the clinical significance of the inoculum effect remains enigmatic, in theory, it may be an important factor in managing infections that have bacterial concentrations which exceed the standard inoculum used for MIC testing (35).

c. Antibiotics and Bacteria Demonstrating Inoculum Effects

The inoculum effect most often occurs with ß-lactamase producing organisms and ß-lactam antibiotics (12). In ß-lactamase producing strains of *Staphylococcus aureus*, an inoculum effect is present with ß-lactams susceptible to ß-lactamase hydrolysis, but is absent with ß-lactamase stable penicillins, cephalosporins and other classes of antibiotics (12, 67). An inoculum effect has also been observed with ß-lactamase producing *Enterococcus faecalis* with penicillin, ampicillin and piperacillin (55). ß-lactamase producing strains of *Neisseria gonorrhoeae* demonstrate an inoculum effect with several penicillins and 2nd and 3rd generation cephalosporins (31). Enterobacteriaceae and *Pseudomonas aeruginosa*, which possess a class 1 chromosomal ß-lactamase, exhibit an inoculum effect with 3rd generation cephalosporins(12, 35).

An inoculum effect can occur with other classes of antibiotics. Quinolone antibiotics have been shown to demonstrate an inoculum effect with various strains of *P. aeruginosa*, Enterobacteriaceae, *Bacteroides fragilis, Clostridium* spp. and *Fusobacterium* spp. (27, 71). Sulfonamide antibiotics also demonstrate an inoculum effect with Enterobacteriaceae and *Streptococcus pneumoniae* (1).

d. The Inoculum Effect of *H. influenzae* with ß-lactams

Haemophilus influenzae demonstrates an inoculum effect with ß-lactams (15, 86), however, inconsistencies exist in the literature concerning the relevance of ß-lactamase

production and the spectrum of ß-lactams producing the inoculum effect. There have been reports of *H. influenzae* exhibiting an inoculum effect regardless of ß-lactamase production (4, 9, 20, 26, 28, 29, 43, 44, 87) and other studies indicating that the inoculum effect is present only with ß-lactamase positive isolates of *H. influenzae* (15, 41, 75, 76, 81).

The factors responsible for producing the inoculum effect of *H. influenzae* with ß-lactams are not clearly defined (12). In Gram-positive bacteria, such as *Staphylococcus aureus*, the inoculum effect is most commonly associated with the cumulative effect of ß-lactamase (35). However, extrapolation to *H. influenzae* does not explain reports of an inoculum effect with ß-lactamase negative strains of *H. influenzae* (4, 9, 20, 26, 28, 29, 43, 44, 87).

In 1995, Zhanel et al. (87) reported that *H. influenzae* demonstrated an inoculum effect with ampicillin, cefuroxime, loracarbef, cefaclor, and amoxicillin/clavulanate, regardless of ß-lactamase production. 50 isolates of ß-lactamase positive and 50 isolates of ß-lactamase negative *H. influenzae* were shown to have a significant increase in microbroth dilution MICs when the inoculum was increased from $5x10^5$ CFU/ml to $1x10^7$ CFU/ml with the aforementioned ß-lactams. This data suggested that the production of a detectable ß-lactamase was not the main mechanism of the inoculum effect in *H. influenzae*.

5. Thesis Objective

The objective of this thesis was to characterize the inoculum effect of *H. influenzae* with ß-lactams and attempt to clarify the mechanism(s) involved. To achieve

this objective, the inoculum effect of ß-lactamase negative and ß-lactamase positive clinical isolates of *H. influenzae* was first evaluated by MIC and kill-curve methods using both low ($5x10^5$ CFU/ml) and high ($1x10^7$ CFU/ml) initial inocula. The effect of the ß-lactamase inhibitor, clavulanate, on the inoculum effect was also determined. Finally, the ß-lactamase gene, *TEM-1* was transformed into a ß-lactamase negative strain of *H. influenzae* and its impact on the inoculum effect then analyzed.

B. MATERIALS AND METHODS

1. Bacterial Strains and Plasmids

Thirteen clinical isolates of *H. influenzae* originally collected in a cross-Canada susceptibility study were used (73). The isolates had previously been classified according to capsule type and ß-lactamase production (73). ß-lactamase positive organisms were further classified as either TEM^+ or ROB^+ by PCR amplification (73, 82). Selection of the isolates was based upon type of ß-lactamase production. The thirteen strains consisted of 5 ß-lactamase negative strains and 8 ß-lactamase positive strains. Of the ß-lactamase producing strains, 4 produced the TEM-1 enzyme and 4 produced the ROB-1 enzyme (See Table 3). An additional strain isolated by the Department of Clinical Microbiology at the Health Sciences Centre in Winnipeg, Canada, was also tested. It was a ß-lactamase negative ampicillin-resistant (BLNAR) (549) strain of *H. influenzae*. Strain 549 had an MIC to ampicillin of 8 µg/ml and tested negative for ß-lactamase production using Cefinase (nitrocefin) (BBL Microbiology Systems, Cockeysville, Md.). Nitrocefin is a cephalosporin which allow rapid colorimetric detection of ß-lactamase activity in Gram-positive and Gram-negative bacteria. *H. influenzae* strains were stocked at -70° in skim milk and subcultured twice on chocolate agar plates before use. Plates were incubated at 35°C with 5% CO_2. *H. influenzae* ATCC 49247 (ß-lactamase negative), *H. influenzae* 103 (a clinical laboratory control strain for ß-lactamase testing, TEM+) and *H. influenzae* 546 (73) were used as controls for MIC determinations and PCR testing. The plasmids pLS88 (85) and pLS88AR were used to transform *H. influenzae* Rd and *E. coli* DH5α. *H. influenzae* Rd is as nonpathogenic, nonencapsulated derivative of a serotype d strain

which does not produce ß-lactamase (3, 8). Plasmid pBR322 (Gibco, Burlington, Ontario) was used as a source of the TEM-1 ß-lactamase (77).

Table 3: Bacterial Strains and Plasmids

H. influenzae strain	Capsular Type	ß-lactamase	Source/Reference
248K	type b	negative	73
309J	type b	negative	73
469	non-typeable[a]	negative	73
478	type b	negative	73
863J	type b	negative	73
301	non-typeable	positive (TEM^+)	73
310	non-typeable	positive (TEM^+)	73
314	non-typeable	positive (TEM^+)	73
10K	type b	positive (TEM^+)	73
322	non-typeable	positive (ROB^+)	73
376	non-typeable	positive (ROB^+)	73
382	type b	positive (ROB^+)	73
408	non-typeable	positive (ROB^+)	73
549	non-typeable	negative- ampicillin resistant	this study
Rd	non-typeable	negative	3, 8
ATCC 49247		negative control	73
103		positive control (TEM^+)	clinical laboratory control strain
546		positive control (ROB^+)	73
Plasmids	**Resistance Markers**	**Characteristics**	**Source/Reference**
pBR322	ampicillin, tetracycline	*E.coli* plasmid, contains *TEM-1* gene	77
pLS88	kanamycin, streptomycin, sulfonamides	shuttle vector between *E.coli* and *H. influenzae*	85
pLS88AR	kanamycin, streptomycin, sulfonamides, ampicillin	pLS88 construct containing *TEM-1* gene	this study
E. coli strain DH5α		used for manipulation of pLS88	

a. non-encapsulated

2. PCR of *TEM-1* and *ROB-1* ß-lactamase Genes

To confirm their previous identification all isolates were tested for ß-lactamase production using Cefinase and screened for the presence of the genes encoding for TEM-1 and ROB-1 enzymes using previously described PCR protocol (73, 82). The primers were synthesized with an Oligo 1000 DNA Synthesizer (Beckman). The TEM-1^1 primer sequence, 5'-TGG GTG CAC GAG TGG GTT AC-3', and the TEM-1^2 primer sequence 5'-TAA TCC GCC TCC ATC CAG TC-3', amplify a 529 bp sequence of the TEM-1 gene (77). The ROB primers, ROB-1^1 5'-CGC CAA ATT CTG TTC ATT-3' and ROB-1^2 5'-GTT GAT ATT GTT CCA CGC-3', amplify a 434 bp sequence (47). The master mix for each PCR reaction contained 5µl 10x PCR buffer (15 mM Mg), 8 µl dNTPs (1.25 mM), 1 µl of either TEM-1^1 or ROB-1^1 , 1 µl of the corresponding second primer, 0.5 µl Taq polymerase (Pharmacia, Baie d'Urfe, Quebec), and 24.5 µl H_2O. The DNA template was obtained by suspending a loopful of organism in 100 µl of water and heating 20 µl of the suspension to 94°C for 5 minutes. Ten microlitres template was used for each reaction. Thermocycling conditions for the TEM primer pairs were as follows: 94°C for 5 minutes; 30 cycles of 94°C for 2 minutes, 57°C for 1 minute, 72°C for 2 minutes; and 72°C for 10 minutes (82). Thermocycling conditions for the ROB primer pairs were as follows: 94°C for 5 minutes; 30 cycles of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds; and 72°C for 10 minutes (73). *H. influenzae* ATCC 49247 was used as a negative control for both TEM-1 and ROB-1. *H. influenzae* ATCC 49766 was used as a positive control for TEM-1 and *H. influenzae* strain 546 (73) was used as a positive control for ROB-1. PCR products were electrophoresed on 1 % agarose gels run in 1x

Tris-borate-EDTA buffer for approximately 1 hour at 100 V. Bands were visualized with ethidium bromide staining.

3. Antibiotics

Ampicillin, amoxicillin, kanamycin (Sigma, St. Louis, Missouri), loracarbef, cefaclor (Eli Lilly, Scarborough, Ontario), clavulanate (Smith Kline Beecham, Oakville, Ontario) and cefuroxime (Glaxo-Wellcome, Montreal, Quebec), powders were used to prepare concentrated antibiotic stock solutions which were stored at -70°C. Concentrated antibiotic stock solutions were thawed as needed and used the same day. Unless otherwise specified, amoxicillin and clavulanate were used in combination with a 2:1 ratio. The concentration of ampicillin used for selection of transformants was 50 µg/ml and 2 µg/ml for *E. coli* and *H. influenzae*, respectively. The concentration of kanamycin used for selection was 30 µg/ml for both bacteria.

4. Media

Haemophilus Test Media (HTM) consisting of 5 g/L yeast extract, 15 µg/ml hematin and 15 µg/ml nicotinamide adenonine dinucleotide (NAD) in Mueller Hinton broth supplemented with 25 µg/ml $CaCl_2$ and 12.5 µg/ml $MgCl_2$ was used for all MIC determinations and kill-curve experiments (57). Luria (LB) broth and Luria (LB) agar was used for the growth of *E. coli* DH5α. S.O.C. media (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was used in *E. coli* transformation. Brain heart infusion agar supplemented with

2 µg/ml NAD and 10 µg/ml hemin (sBHI) was used for selection on *H. influenzae* transformants.

5. Colony Counts

Ten and 100 µl aliquots, of serial ten-fold dilutions made in sterile 4°C 0.85% NaCl, were spread onto chocolate agar, the plates incubated, and colony counts noted after 18 to 24 hours (56).

6. MIC Determinations

Initially, MIC determination was performed according to NCCLS recommended guidelines (56). One milliliter of HTM broth was added to a series of 10 ml test tubes, omitting the first tube. Stock concentrations of antibiotic were diluted in HTM broth to give a final concentration equal to twice the desired concentration at the beginning of the series. One milliliter of the diluted antibiotic was added to the first tube of the series and to the remainder of the tubes by 1 ml two-fold serial dilutions. One or two colonies of freshly sub-cultured bacteria were added to 20 ml HTM broth and incubated with shaking at 37°C for 1.5 hours. The culture was adjusted to the 0.5 McFarland standard ($1x10^8$ CFU/ml), diluted 1:100 or 1:5 in HTM and 1 ml was added to each tube in the series, for an initial inocula of $5x10^5$ CFU/ml (low inoculum) or $1x10^7$ CFU/ml (high inoculum). Colony counts were performed to determine the exact initial inoculum. Tubes were incubated at 35°C for 18-24 hours and the MIC was determined to be the lowest concentration of antibiotic without visible turbidity. Viable cell count MICs were produced by removing aliquots from each turbidimetric macrobroth MIC tube, performing

serial 10-fold dilutions if necessary, plating dilutions on chocolate agar and determining the number of viable cells following 18 to 24 hours of incubation. The viable count MICs was defined as the lowest concentration of antibiotic which resulted in colony counts less than or equal to the initial inoculum of $1x10^7$ CFU/ml (± 0.5 log). An inoculum effect was defined as a four fold or greater increase in MIC when the initial inoculum was increased from $5x10^5$ to $1x10^7$ CFU/ml.

7. Cell Morphology

Cultures of *H. influenzae* with initial bacterial concentrations of $5x10^5$ CFU/ml and $1x10^7$ CFU/ml where incubated in the presence and absence (control culture) of 10 x MIC (low inoculum MIC of the ß-lactam) with each ß-lactam. Following 24 hours of incubation, cells were Gram-stained and viewed using light microscopy (1000x magnification).

8. Kill-Curve Method

Kill-curves were performed to monitor ß-lactam killing of *H. influenzae* during 24 hours of antibiotic exposure. A previously described kill-curve method was used (87). Briefly, 10 ml of HTM broth containing 4x MIC (low inoculum MIC of ß-lactam) and *H. influenzae* at initial concentrations of $5x10^5$ and $1x10^7$ CFU/ml were incubated in a shaking water bath at 37°C for 24 hours. A culture containing only HTM and *H. influenzae* was also prepared as a growth control for each experiment. Aliquots were removed at 0, 2, 4, 6, 8, and 24 hours and colony counts determined. All kill-curve experiments were performed at least in duplicate on separate occasions with ß-lactamase

negative strains (248K, 309J, 469, 478, 863J), TEM+ strains (10K, 301, 310), ROB+ strains (322, 408) and *H. influenzae* Rd with and without pLS88AR. Kill-curves were performed using ß-lactam concentrations of 4 x MIC to allow for the inherent two fold variability of MIC determinations (56).

9. Experiments with Clavulanate

To demonstrate that the presence of ß-lactamase is a major contributing factor to the inoculum effect, viable cell count MICs and kill-curve experiments were repeated with the addition of the ß-lactamase inhibitor clavulanate. Preliminary experiments indicated that a 1:1 ratio of ß-lactam with clavulanate was the minimum ratio required to eliminate the inoculum effect. Clavulanate was used in a 1:1 ratio with cefuroxime, loracarbef, cefaclor, amoxicillin and ampicillin to assess the viable cell count MICs of the TEM+ strain 310, ROB+ strain 408 and *H. influenzae* Rd containing pLS88AR. However, a 1:1 ratio of ß-lactam to clavulanate could not be used with kill-curves at 4 x MIC, as the concentration of clavulanate in some combinations interfered with bacterial growth. The concentration of 4 µg/ml of clavulanate was determined to be the highest concentration that did not inhibit bacterial growth. Therefore, kill-curves for strains 310, 408 and *H. influenzae* Rd with pLS88AR were performed using 4 µg/ml of clavulanate in combination with ß-lactams.

10. Construction of pLS88AR

To observe the role of ß-lactamase production in the inoculum effect of ß-lactams with *H. influenzae*, a ß-lactamase encoding gene was inserted into a plasmid (pLS88) and

transformed into a ß-lactamase negative strain, *H. influenzae* Rd. The inoculum effect was evaluated using the previously described methods of MIC determination, kill-curves and clavulanate experiments in the wild type and transformed *H. influenzae* Rd.

a. Plasmid Transformation in *H. influenzae*

pLS88 was transformed into *H. influenzae* Rd by modification of the method of calcium-induced artificial competence for plasmid transformation (8). *H. influenzae* Rd in 10 ml of HTM broth was grown up to an OD of 0.3, cooled on ice and centrifuged for 10 minutes at 5000 x g and 4°C. The cells were resuspended in an equal volume of 25 mM $CaCl_2$, centrifuged again at 4°C, resuspended in 1 ml of 75 mM $CaCl_2$ and incubated on ice for 1 hour. The cells were harvested by centrifugation at 4°C and resuspended in 0.2 ml of 75 mM $CaCl_2$. Approximately 1 µg of plasmid DNA was added and the mixture was incubated on ice for 30 minutes. The cells were then heat-shocked at 37°C for 3 minutes and returned to ice for a further 10 minutes. A volume of 1.8 ml of HTM was added and the cells were incubated at 37°C with shaking for 1-2 hours. One hundred microlitre aliquots were plated on sBHI agar containing 30 µg/ml kanamycin for selection of transformants.

b. Amplification of the gene encoding for TEM-1

The entire TEM-1 gene, including promoter sequences, was PCR amplified from pBR322. *Eco*R1 sites and a GC cap were incorporated into the 5' end of both primers (Gibco, Burlington, Ontario) to facilitate further manipulations. Primer 1 (5' GCG CGA ATT CTT GAA GAC GAA AGG G) corresponds to positions 4361-4345 of the sequence of pBR322 in GenBank, and primer 2 (5' GCG CGA ATT CAA GCA GCA GAT TAC G) corresponds to positions 3084-3101. Amplification yields a 1287 base pair fragment

that includes the sequence 211 bp upstream and 258 bp downstream from the 858 bp amino acid coding region of TEM-1 (77). The master mix for each PCR reaction contained 5 µl 10 x PCR buffer (15mM Mg), 8 µl dNTPs (1.25mM), 1 µl of primer 1, 1 µl of primer 2, 0.5 µl Taq polymerase (Pharmacia), and 24.5 µl H_2O. 40 µl of master mix was added to 10 µl of the template [3 µl of pBR322 purified from *E. coli* DH5α using Wizard Mini-Preps (Promega, Madison, Wisconsin), diluted in 7 µl H_2O]. Thermocycling conditions were optimized at 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 3 minutes, and 72°C for 10 minutes. Five µl of PCR product was electrophoresed on a 1% agarose gel and run in 1 x Tris-borate-EDTA buffer at 100 V for 1 hour. The remainder of the PCR product which produced the desired fragment was further purified using the Wizard PCR purification system (Promega).

c. Preparation of Ligation Reaction

i. pLS88

pLS88, which contains a unique *Eco*R1 site, was purified from *H. influenzae* Rd using the Wizard Mini-Prep Plasmid Purification System (Promega). The purified plasmid was digested with the restriction enzyme *Eco*R1 in the following reaction: 30 µl pLS88, 5 µl One-Phor-All Plus buffer (Pharmacia), 2 µl *Eco*R1 (Pharmacia), and 13 µl H_2O. The reaction was incubated overnight at 37°C and the enzyme was then inactivated by 10 minutes of incubation at 65°C. Alkaline phosphatase was used to prevent self-ligation of the sticky ends produced by *Eco*R1 digestion. One half microlitre of alkaline phosphatase (1000 units/ml) (Pharmacia) was added and the reaction was incubated at 37°C for 1 hour. Twenty minutes of incubation at 85°C was used to inactivate the enzyme. The reaction was electrophoresed in a 0.7% agarose gel at 80 V for approximately 1 hour. The single

band corresponding to the digested plasmid at 4.8 kb was excised from the gel and purified from the agarose using Prep-a-gene (Bio-Rad, Mississauga, Ontario).

ii. PCR Product containing TEM-1 Gene

The PCR product was digested with *Eco*R1 to facilitate insertion into the *Eco*R1 site of pLS88. Twenty-five and a half microlitres of PCR product, 3 µl One-Phor-All Plus Buffer and 2 µl *Eco*R1 were incubated overnight at 37°C. The reaction was placed at 65°C for 10 minutes to inactivate the enzyme.

iii. DNA Purification: Phenol Extraction and Ethanol Precipitation

pLS88 and the PCR product were further purified using phenol extraction and ethanol precipitation (7). DNA was diluted to a volume of 100 µl with water in a 1.5 ml micro-centrifuge tube and 100 µl of phenol / chloroform / isoamyl alcohol (25: 24: 1) was added. The mixture was vortexed vigorously for 10 seconds and centrifuged at 13,000 x g for 1 minute. The aqueous (top) layer was removed and set aside. One hundred microlitres of 1x Tris-borate buffer was added to the organic (bottom) layer, the phenol / chloroform /isoamyl alcohol extraction was repeated and the aqueous layers pooled. Twenty microlitres of 3 M sodium acetate (one tenth the volume of the aqueous layers) was added to the aqueous layers and mixed by vortexing. Five hundred microlitres of ice-cold 100% ethanol was added, vortexed and placed at -80°C for 30 minutes. The mixture was then centrifuged for 5 minutes at 13 000 x g and the supernatant was discarded. One millilitre of 70% ethanol at room temperature was added and mixed by inverting the tubes which were then centrifuged for 5 minutes at 13 000 x g. The supernatant was removed and the pellet was allowed to air-dry. The pellet containing the purified DNA was resuspended in 20 µl of distilled water.

d. Ligation Reaction

The ligation reaction consisted of 5 µl purified pLS88, 9 µl purified PCR product, 2 µl 10x Ligation Buffer (Pharmacia), 1 µl T4 DNA Ligase (Pharmacia) and 3 µl distilled water incubated overnight at 15°C. The DNA was purified using the previously described method of phenol extraction and ethanol precipitation and resuspended in 10 µl of water prior to electroporation into *E. coli.*

11. Transformation of pLS88AR into *E.coli* (CCC)

a. Preparation of *E. coli* DH5α for Electroporation

A single colony of DH5α was inoculated into 5 ml of LB broth and incubated overnight at 37°C with shaking. Two and a half millilitres of the overnight culture were diluted in 500 ml of LB broth and grown at 37°C with shaking to an optical density at 600 nm of 0.5-0.6. The cells were placed on ice for 15 minutes, transferred to a pre-chilled 1 liter centrifuge bottle and spun for 20 minutes at 10 000 x g and 2°C. The supernatant was removed and the pellet was resuspended in 5 ml of ice-cold distilled water. A further 500 ml of ice-cold distilled water was added and the cells were again centrifuged as before. Resuspension with 500 ml ice-cold water and centrifugation was repeated and the final pellet was resuspended in the liquid remaining after decanting the supernatant. Forty millilitres of ice cold 10% glycerol was added and the suspension was placed in a pre-chilled 50 ml polypropylene tube and centrifuged at 10 000 x g for 10 minutes. The pellet was resuspended in an equal volume of 10% glycerol and stored at -80°C in aliquots of 40 µl.

b. Electroporation

A 40 µl aliquot of prepared DH5α cells was slowly thawed and 1 µl of purified DNA from the ligation reaction was added on ice. The mixture was transferred to pre-chilled cuvettes with a 0.1 cm electrode gaps and electroporated using the Pulse-gene apparatus (Bio-Rad, Mississauga, Ontario) adjusted to 1.8 kV, 25 µF and 200 Ω. After electroporation, 1 ml S.O.C. media pre-warmed to 37°C, was added and the cells were incubated at 37°C with shaking for 1 hour. One hundred microlitres were plated on LB agar containing 50 µg/ml of ampicillin and incubated overnight at 35°C.

c. Selection of Transformants

Colonies growing on the ampicillin plate were sub-cultured on LB agar containing 30 µg/ml kanamycin. Plasmids from colonies able to grow on both ampicillin and kanamycin were isolated using the Wizard Mini-Preps Plasmid Purification System for transformation into *H. influenzae* Rd.

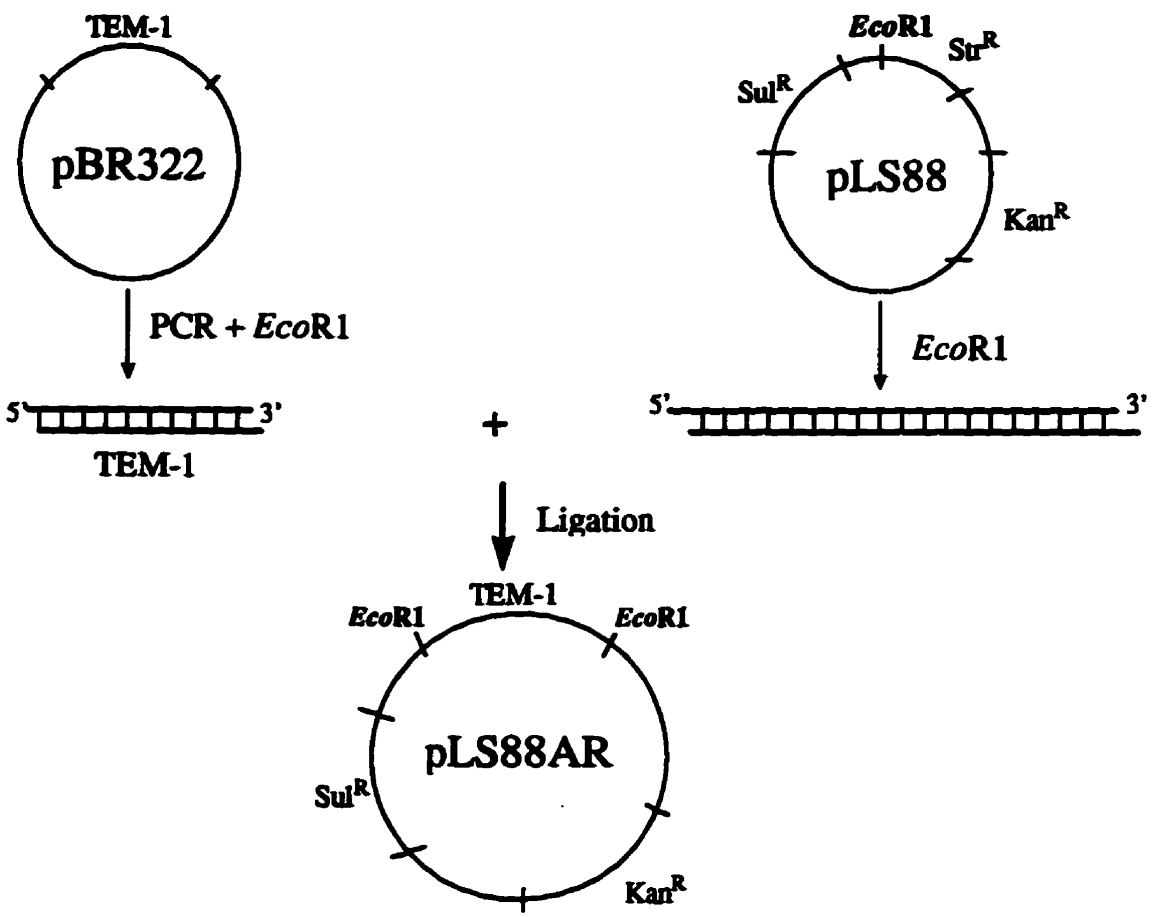
12. Transformation of pLS88AR into *H. influenzae* Rd.

Purified plasmids from *E. coli* potentially containing the desired pLS88AR plasmid were transformed into *H. influenzae* Rd by the previously described method of calcium-induced-artificial competence (8). Colonies able to grow on sBHI containing 30 µg/ml kanamycin were sub-cultured to sBHI with 2 µg/ml ampicillin.

13. Confirmation of pLS88AR Transformation

Plasmids were purified as before from colonies able to grow in the presence of both ampicillin and kanamycin. Purified plasmids were digested with *Eco*R1, as previously described and the resultant digests were electrophoresed on a 1% agarose gel for 1 hour at 100 V. Digests containing pLS88AR will contain two bands, one at 4.8 kb, corresponding to pLS88, and the other at 1287 bp, corresponding to the TEM-1 gene. A single colony containing pLS88 was selected for further experiments.

Figure 5: Construction of pLS88AR



Kan = kanamycin

Str = streptomycin

Sul = sulfonamide

14. Statistical Analysis

Standard analysis of covariance calculations were performed to identify significant changes in ß-lactam killing between low ($5x10^5$ CFU/ml) and high ($1x10^7$ CFU/ml) inocula. ß-lactam killing was defined as the log CFU/ml of the initial inoculum minus log CFU/ml at each measured time point. Negative values indicated regrowth. Statistical significance was established when $P < 0.05$.

C. RESULTS

To characterize the inoculum effect of *H. influenzae* with ß-lactams, 14 clinical strains of *H. influenzae* were studied. The strains were first classified in regards to ß-lactamase production by Cefinase and PCR. MICs were determined using low ($5x10^5$ CFU/ml) and high ($1x10^7$ CFU/ml) initial inocula and evaluated turbidimetrically and by viable cell counts. Kill-curve experiments using the low and high initial inocula were also performed. The results of MIC and kill-curves experiments indicated that the inoculum effect was present only in ß-lactamase producing clinical strains of *H. influenzae*. To investigate the role of ß-lactamase production in the inoculum effect, high inoculum MICs and kill-curves were repeated with the addition of a ß-lactamase inhibitor. Finally, to confirm that ß-lactamase was responsible for the inoculum effect of ß-lactams in *H. influenzae*, the *TEM-1* ß-lactamase gene was transformed into the ß-lactamase negative strain, *H. influenzae* Rd.

1. PCR of *TEM-1* and *ROB-1* ß-lactamase Genes

ß-lactamase production in the clinical strains of *H. influenzae* was characterized by Cefinase and PCR. Figure 6 shows the PCR products for the control strains of *H. influenzae*. Both sets of primers produced negative results for the ß-lactamase negative strain ATCC 49247 (lanes 2 and 3). The TEM-1 primer set produced negative results for the ROB^+ control strain (546) and the TEM^+ control strain (ATCC 49766) was negative with the ROB-1 primers (lanes 6 and 7). PCR products were the expected length, 526 bp for TEM-1 primers and 434 bp for ROB primers with their respective positive control strains.

The results of Cefinase testing for ß-lactamase production and PCR of *TEM-1* and *ROB-1* ß-lactamase genes are presented in Table 4. All strains of *H. influenzae* which tested positive for ß-lactamase production were also PCR positive for either *TEM* or *ROB*, but not both. None of isolates classified as ß-lactamase negative by Cefinase produced positive PCR results for *TEM* or *ROB*.

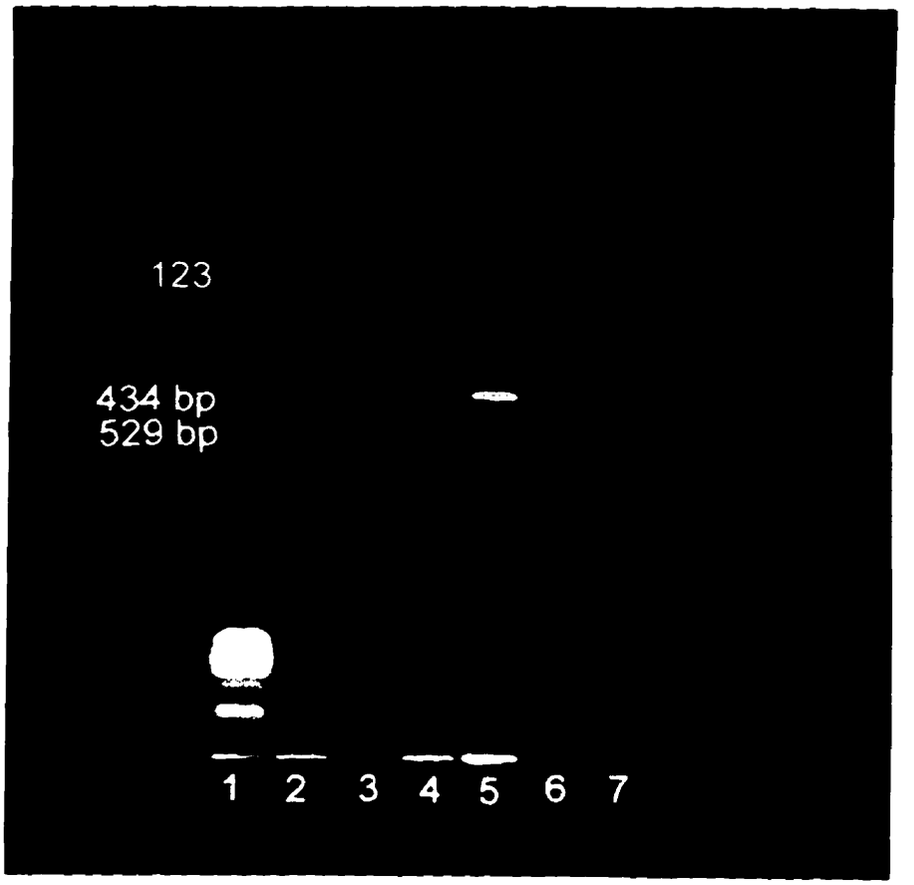


Figure 6: PCR Control Products from *TEM* and *ROB* ß-lactamase Genes. Lane 1: 123 bp ladder. Lane 2: *H. influenzae* ATCC 49247 with TEM-1 primers (negative control). Lane 3: *H. influenzae* ATCC 49247 with ROB-1 primers (negative control). Lane 4: *H. influenzae* 103 with TEM-1 primers (positive control). Lane 5: *H. influenzae* 546 (ROB^+) with ROB-1 primers (positive control). Lane 6: *H. influenzae* ATCC 49766 with ROB-1 primers. Lane 7: *H. influenzae* 546 (ROB^+) with TEM-1 primers.

Table 4: ß-lactamase Production and PCR of *TEM* and *ROB* Genes

H. influenzae strain	ß-lactamase Production (Cefinase)[a]	*TEM*[b]	*ROB*[c]
248K	-	-	-
309J	-	-	-
469	-	-	-
478	-	-	-
863J	-	-	-
301	+	+	-
310	+	+	-
314	+	+	-
10K	+	+	-
322	+	-	+
376	+	-	+
382	+	-	+
408	+	-	+
549	-	-	-
ATCC 49247	-	-	-
103	+	+	-
546	+	-	+
H. influenzae Rd	-	-	-

a. (+) colour change, (-) no color change
b. (+) 529 bp band visible with *TEM* primers, (-) no band visible with *TEM* primers
c. (+) 434 bp band present with *ROB* primers, (-) no band visible with *ROB* primers

2. Turbidimetric MICs

NCCLS macrobroth susceptibility testing uses an initial inoculum of $5x10^5$ CFU/ml with the MIC defined as the lowest concentration of antibiotic resulting in no visible growth (56). The MICs for ß-lactamase negative and ß-lactamase positive strains of *H. influenzae* are presented in Tables 5 and 6, respectively. When the initial inoculum was increased to $1x10^7$ CFU/ml and the MICs evaluated turbidimetrically, the MICs were greater than 256 µg/ml for all antibiotics with all ß-lactamase negative and ß-lactamase positive strains of *H. influenzae* tested. (Tables 5 and 6).

3. Viable Cell Count MICs

When high initial inocula ($1x10^7$ CFU/ml) MICs were evaluated using the viable cell count method, no significant increases in ß-lactam MICs were seen for any of the ß-lactamase negative strains tested (Table 5). In contrast, ß-lactamase positive strains generally demonstrated greater MICs with the high initial inocula when assayed by viable cell counts (Table 6). The magnitude of the increase in MIC varied with the ß-lactam tested (ampicillin >cefaclor = loracarbef > amoxicillin/clavulanate > cefuroxime) (Table 6). Cefuroxime did not demonstrate an inoculum effect with TEM^+ strains of *H. influenzae* but did for 3 (322, 376, 408) of the 4 ROB^+ strains (Table 6). Loracarbef MICs for 3 (301, 310, 10K) of 4 TEM^+ and for all ROB^+ strains tested demonstrated an inoculum effect when compared with turbidimetrically determined MICs with a starting inocula of $5x10^5$ CFU/ml (Table 6). Similarly, cefaclor MICs for all TEM^+ and ROB^+ strains tested demonstrated an inoculum effect (Table 6). An inoculum effect was also noted for amoxicillin/clavulanate with 3 (322, 376, 408) of 4 ROB^+ and all TEM^+ strains tested

(Table 6). The BLNAR strain did not demonstrate an inoculum effect with cefuroxime, loracarbef, cefaclor, amoxicillin/clavulanate or ampicillin when viable cell count MICs were compared with turbidimetrically determined MICs. It was also observed that capsular production did not influence the inoculum effect evaluated both turbidimetrically and by viable cell counts.

Table 5. MIC (µg/ml) ranges for the 4 ß-lactamase negative strains of *H. influenzae* using low and high initial inocula.

Antibiotic	MIC Range (µg/ml) Low Inoculum[a]	MIC Range (µg/ml) High Inoculum[b] Visually Determined	MIC Range (µg/ml) High Inoculum Viable Cell Counts
Cefuroxime	0.5-1	>256	0.5-1
Loracarbef	0.5-2	>256	0.5-2
Cefaclor	1-4	>256	1-4
Amox/Clav[c]	0.25-0.5	>256	0.25-0.5
Ampicillin	0.125-0.25	>256	0.125-0.25

a. $5x10^5$ CFU/ml
b. $1x10^7$ CFU/ml
c. Amoxicillin/clavulanate (2:1)

Table 6. MIC (µg/ml) ranges for the 8 ß-lactamase positive strains of *H. influenzae*[a] using low and high initial inocula.

Antibiotic	MIC Range (µg/ml) Low Inoculum[b]		MIC Range (µg/ml) High Inoculum[c] Visually Determined		MIC Range (µg/ml) High Inoculum Viable Cell Counts	
	TEM$^+$	ROB$^+$	TEM$^+$	ROB$^+$	TEM$^+$	ROB$^+$
Cefuroxime	0.25-1	0.5-1	>256	>256	0.25-1	1-4
Loracarbef	0.5-2	2-4	>256	>256	4-32	≥256
Cefaclor	1-4	4-8	>256	>256	16-128	128-256
Amox/Clav[d]	0.5-1	0.5-1	>256	>256	4-16	1-8
Ampicillin	8-16	8-32	>256	>256	>256	>256

a. 4 strains TEM+ and 4 strains ROB+
b. $5x10^5$ CFU/ml
c. $1x10^7$ CFU/ml
d. Amoxicillin/clavulanate (2:1)

4. Cell Morphology

Samples taken from turbid high inoculum MIC tubes are represented in Figures 7, 8, and 9. Figure 7 shows typical *H. influenzae* cell morphology without prior antibiotic exposure. Figures 8 and 9 depict samples taken following 24 hours of incubation with ampicillin at a concentration of 10x MIC with strains 478 (ß-lactamase negative) and 408 (ROB^+), respectively. Aliquots taken from low inocula cultures of ß-lactamase negative and ß-lactamase positive strains, grown in the presence of 10x MIC for 24 hours, were free of intact cells, large aggregates and cell fragments. Similar cultures at the high initial inocula were turbid at 24 hours of incubation. Large aggregates and cell fragments predominated in cultures of ß-lactamase negative strains (Figure 8), whereas, in cultures of ß-lactamase positive strains although large aggregates were present, there were a variable number of intact cells also present (Figure 9). The number of resulting intact cells depended upon the high inocula MIC and the ß-lactam tested.

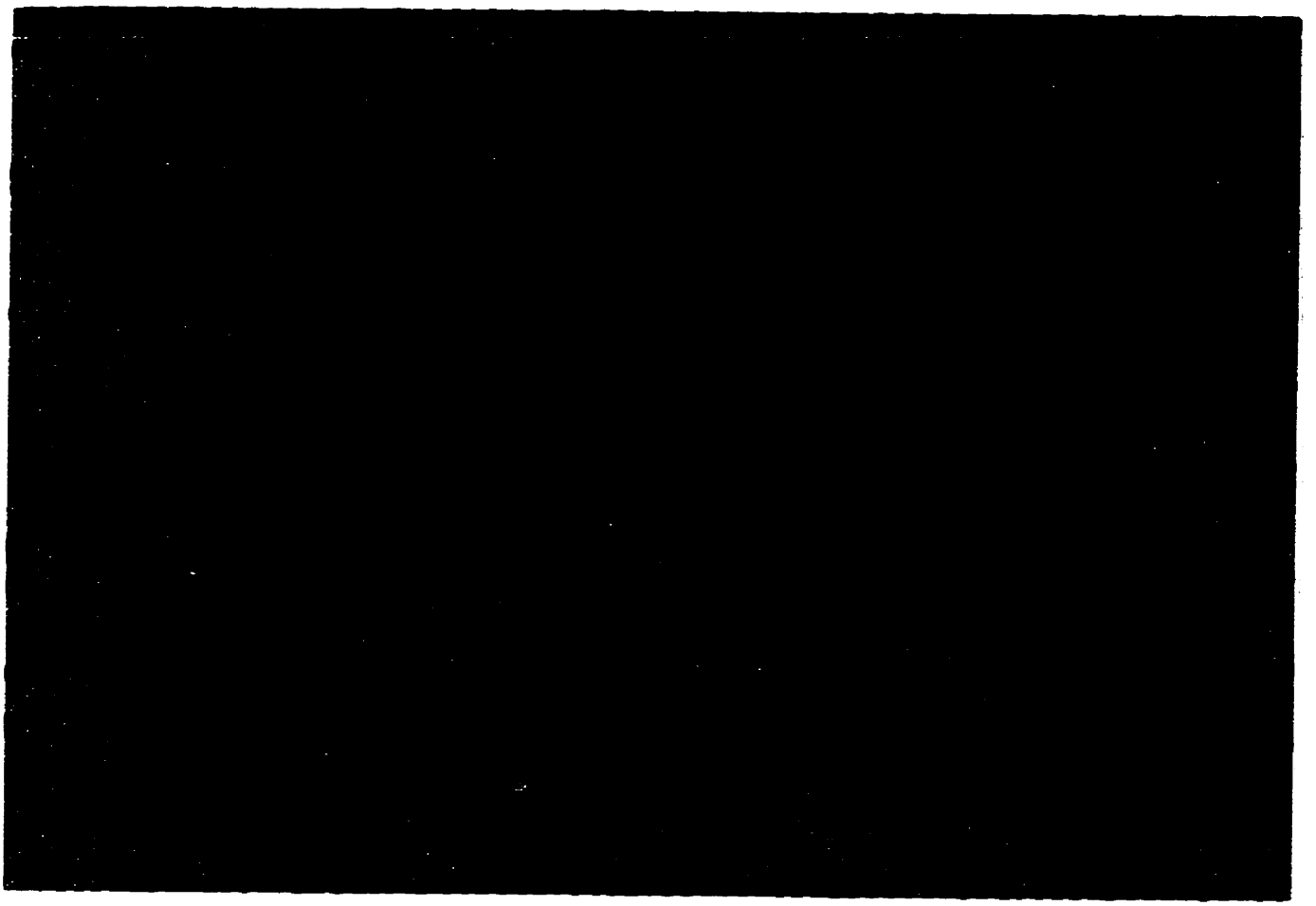


Figure 7: ***H. influenzae*** **typical cell morphology. Gram-stain of ß-lactamase negative strain 478 incubated for 24 hours without antibiotic viewed at 1000x magnification using light microscopy.**

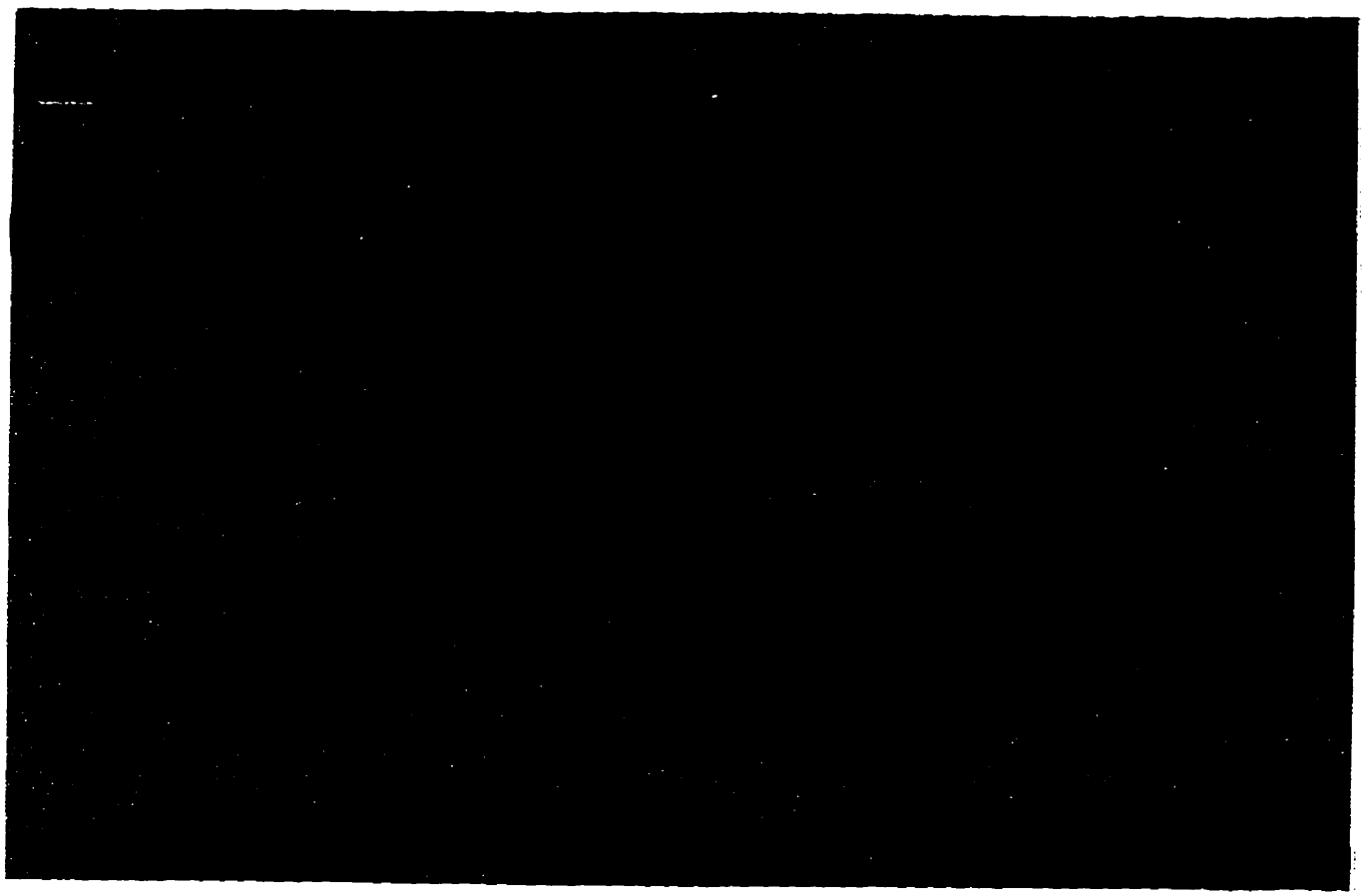


Figure 8: Gram-stain of ß-lactamase negative strain 478 incubated for 24 hours with 10x MIC of ampicillin viewed at 1000x magnification using light microscopy.

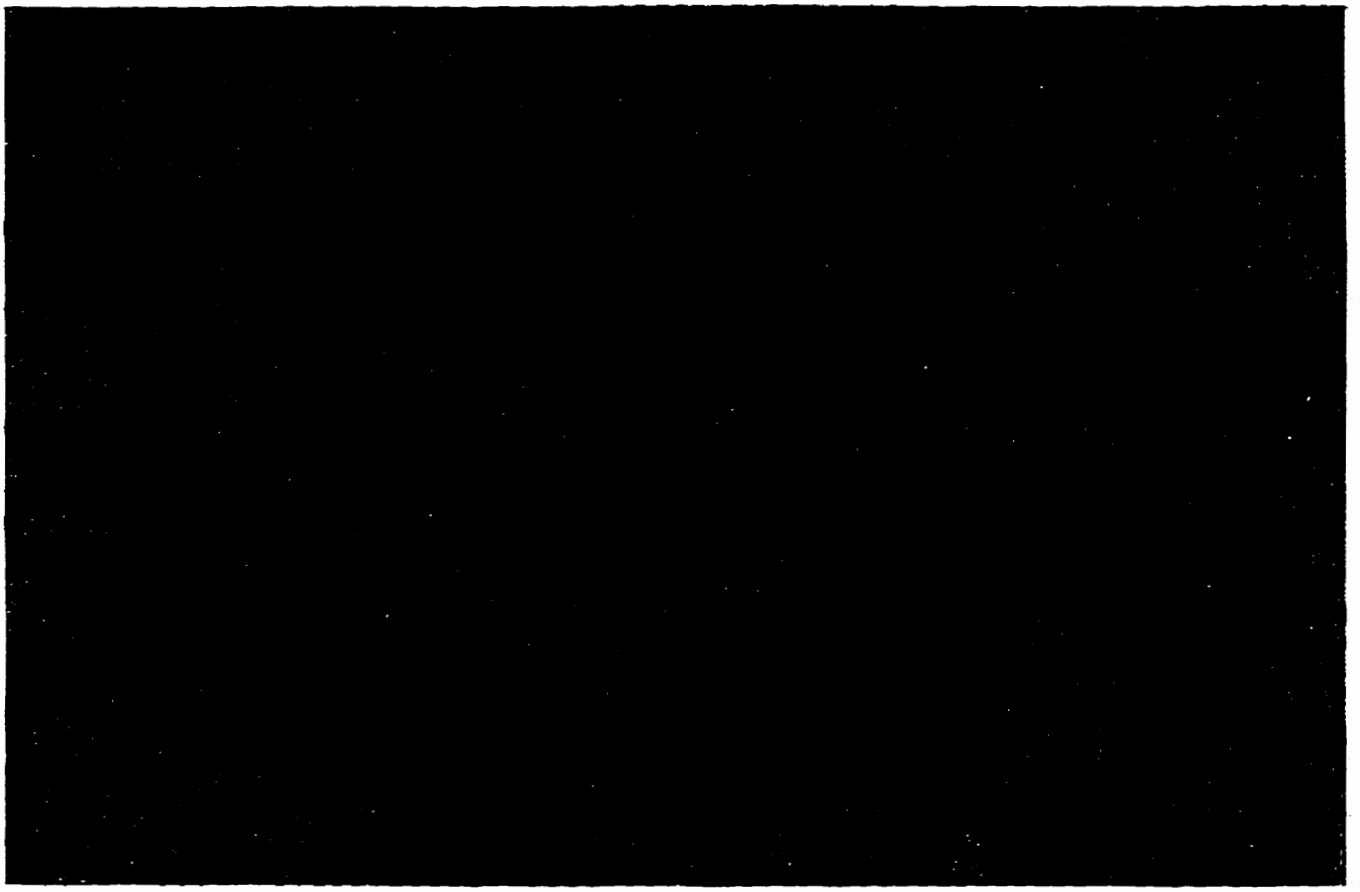


Figure 9: Gram-stain of ß-lactamase positive strain 408 (ROB^+) incubated for 24 hours with10x MIC of ampicillin viewed at 1000x magnification using light microscopy.

5. Kill-Curves

To observe the effect of increased inocula on ß-lactam killing over time, kill-curves were performed at low ($5x10^5$ CFU/ml) and high ($1x10^7$ CFU/ml) inocula. Figure 10 presents representative kill curves for *H. influenzae*. Kill-curve sets A (low inoculum) and B (high inoculum) from Figure 10 depict ß-lactam killing of the ß-lactamase negative strain 309J. The other ß-lactamase negative strains produced similar kill-curve sets. Analysis of covariance of all ß-lactamase negative strains demonstrated no significant difference in rate of bacterial killing over time between low and high initial inocula (P=0.9673) for all ß-lactams.

Figures 11 and 12 present the results for ß-lactamase positive strains 310 and 408. Analysis of covariance of all the ß-lactamase positive kill-curve data showed significant differences between low and high inocula for all ß-lactams (P=0.0010). Incubation with cefuroxime produced a 3.12 ± 0.26 log decrease in CFU/ml (log kill) at 8 hours with the low initial inoculum for all TEM^+ and ROB^+ strains (Figures 11A and 12A). At the high initial inocula, all TEM^+ and ROB^+ strains demonstrated a 1.46 ± 0.33 log kill (Figures 11B and 12B) at 8 hours. Incubation with cefaclor for 8 hours produced 2.66 ± 0.42 log kill with the lower inoculum for strains containing either ß-lactamase (Figures 11A and 12A). When a high inoculum was used regrowth occurred in 4 of 5 ß-lactamase positive strains at 8 hours (Figures 11B and 12B). Results from kill-curves with loracarbef were very similar to those of cefaclor (Figures 10 and 11). After 8 hours of incubation with amoxicillin/clavulanate, all strains exhibited 3.68 ± 0.32 log kill when the lower inoculum

was used, whereas at the higher inoculum there was a 2.36 ± 0.66 log kill at 8 hours (Figures 11 and 12).

Figures 11A, 11B, 12A and 12B demonstrate differences in killing of loracarbef and cefaclor between the TEM^+ (310) and ROB^+ (408) strains. At the high initial inocula, growth of the TEM^+ was inhibited during the first 6 hours of incubation with loracarbef or cefaclor (Figure 11B), while the CFU/ml of the ROB^+ strain increased with incubation with both ß-lactams over the same time period (Figure 12B). As well, the ROB^+ strains exhibited regrowth after 24 hours of incubation with loracarbef or cefaclor at the low initial inoculum (Figure 12A) whereas, the TEM^+ strains did not (Figure 11A). Similar results were demonstrated by all TEM^+ and ROB^+ strains.

Figure 10: Representative ß-lactam kill-curves for ß-lactamase negative *H. influenzae* at 4x MIC with low ($5x10^5$ CFU/ml) and high ($1x10^7$ CFU/ml) initial inocula. A, ß-lactamase negative strain 309J with a low initial inoculum ; B, ß-lactamase negative strain 309J with a high initial inoculum; Growth control (•), cefuroxime(■), loracarbef (▲), cefaclor (Δ), amoxicillin/clavulanate (o) and ampicillin (□).

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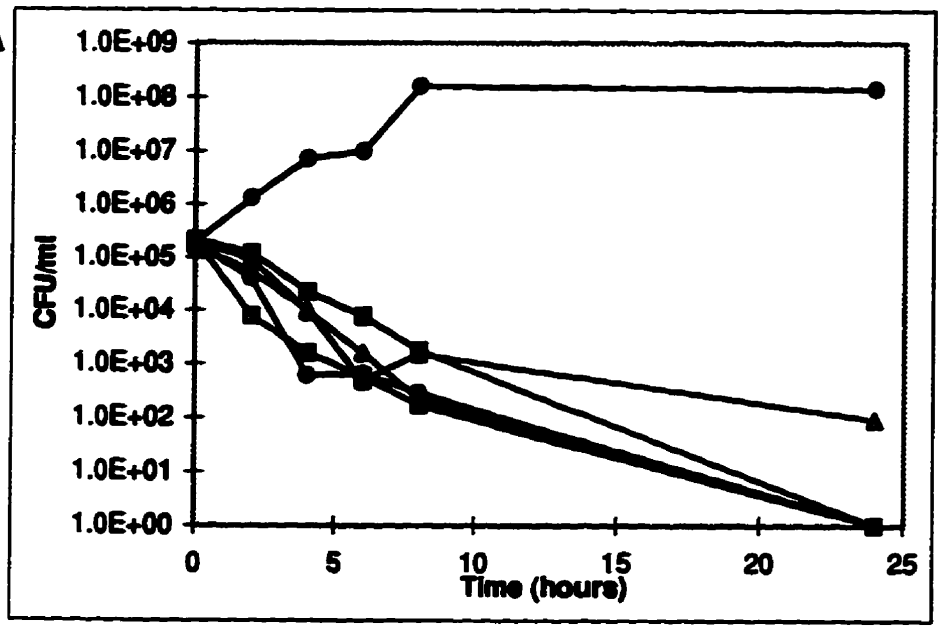
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CFU/ml
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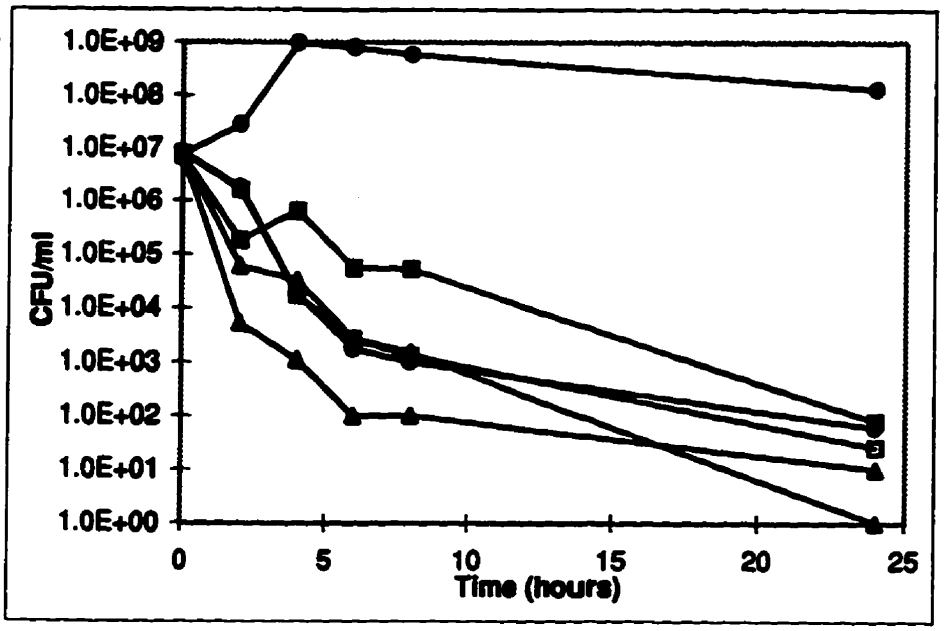
Time (hours)

B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CFU/ml
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Time (hours)

Figure 11: Representative ß-lactam kill-curves for ß-lactamase positive (TEM$^+$) *H. influenzae* at 4x MIC with low ($5x10^5$ CFU/ml) and high ($1x10^7$ CFU/ml) initial inocula: A, TEM+ strain 310 with a low initial inoculum; B, TEM+ strain 310 with a high initial inoculum; Growth control (•), cefuroxime (■), loracarbef (▲), cefaclor (Δ), amoxicillin/clavulanate (○) and ampicillin (□).

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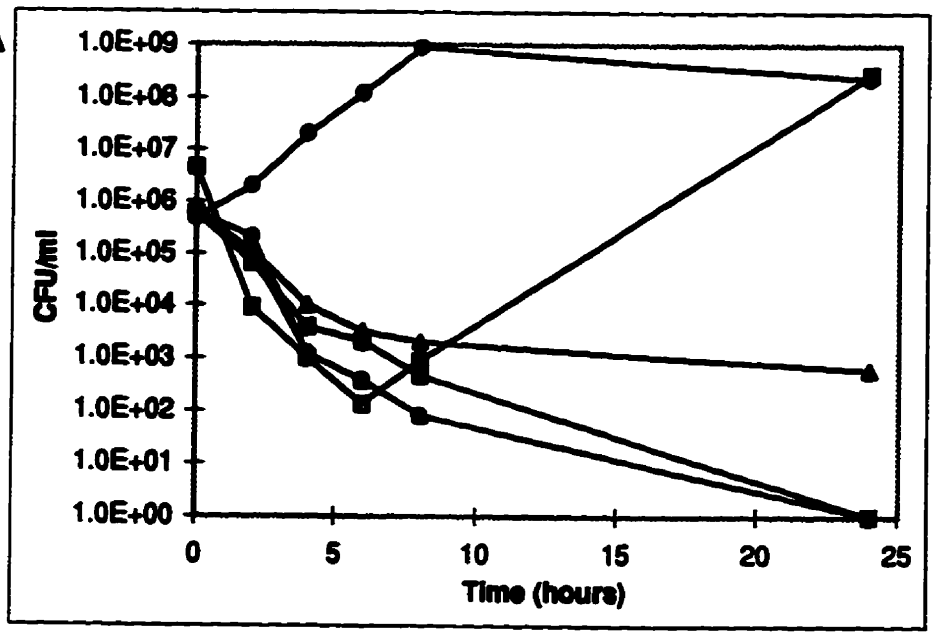
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Time (hours)

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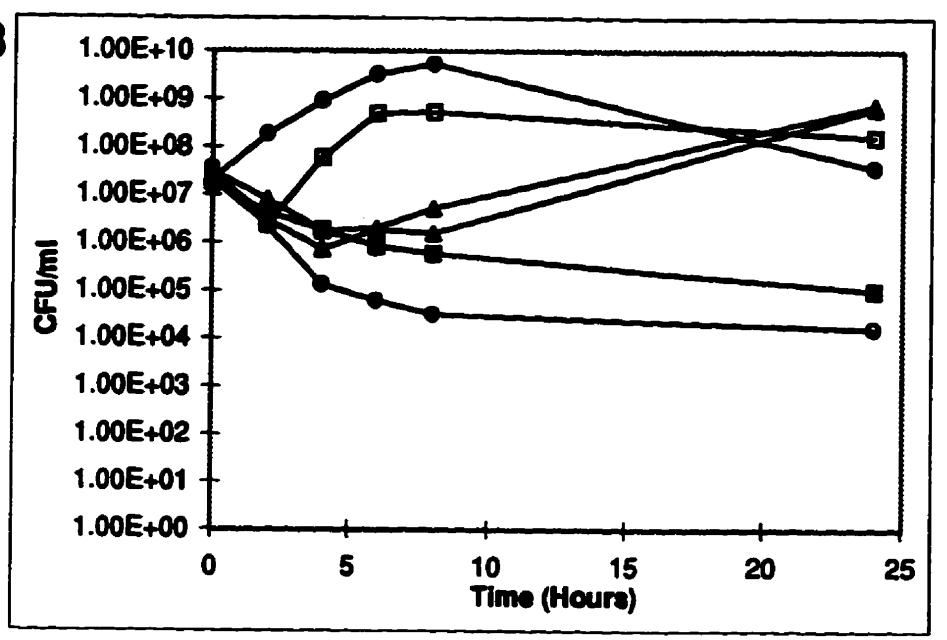
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CFU/ml
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20
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Time (Hours)

Figure 12: Representative ß-lactam kill-curves for ß-lactamase positive (ROB$^+$) *H. influenzae* at 4x MIC with low ($5x10^5$ CFU/ml) and high ($1x10^7$ CFU/ml) initial inocula: A, ROB+ strain 408 with a low initial inoculum; B, ROB+ strain 408 with a high initial inoculum; Growth control (•), cefuroxime (■), loracarbef (▲), cefaclor (Δ), amoxicillin/clavulanate (○) and ampicillin (□).

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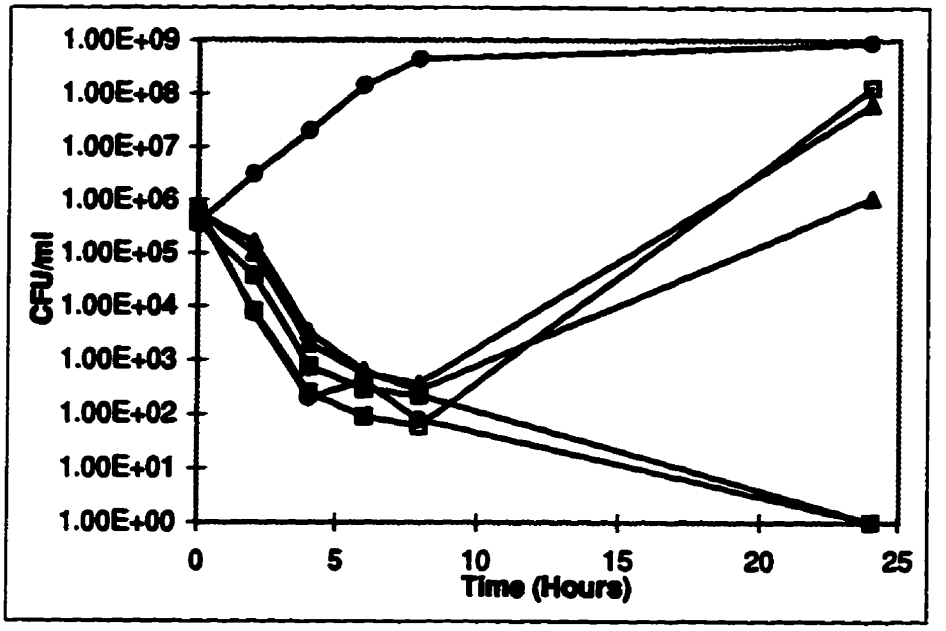
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Time (Hours)

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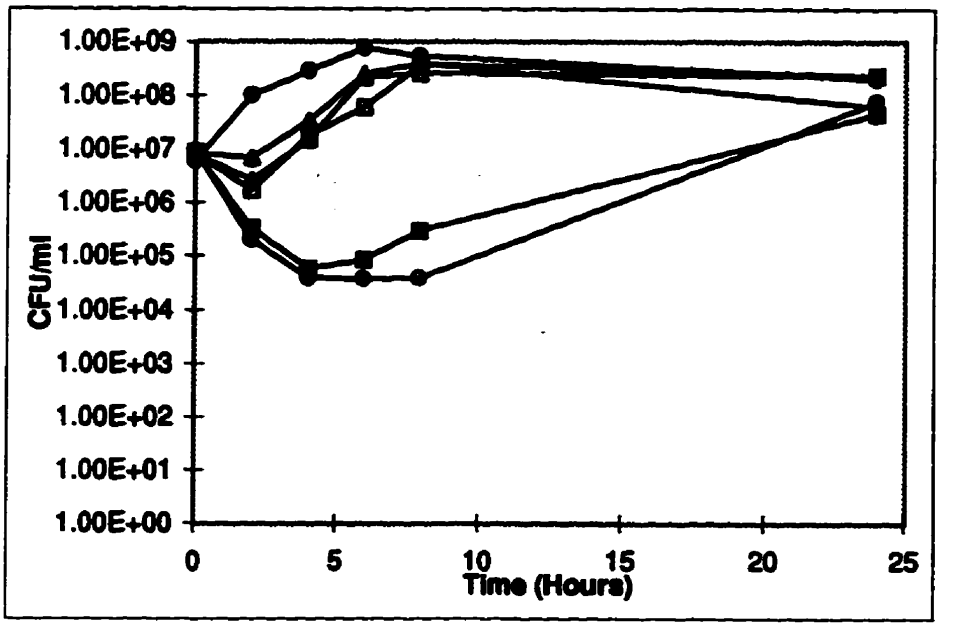
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CFU/ml
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Time (Hours)

6. Experiments with Clavulanate

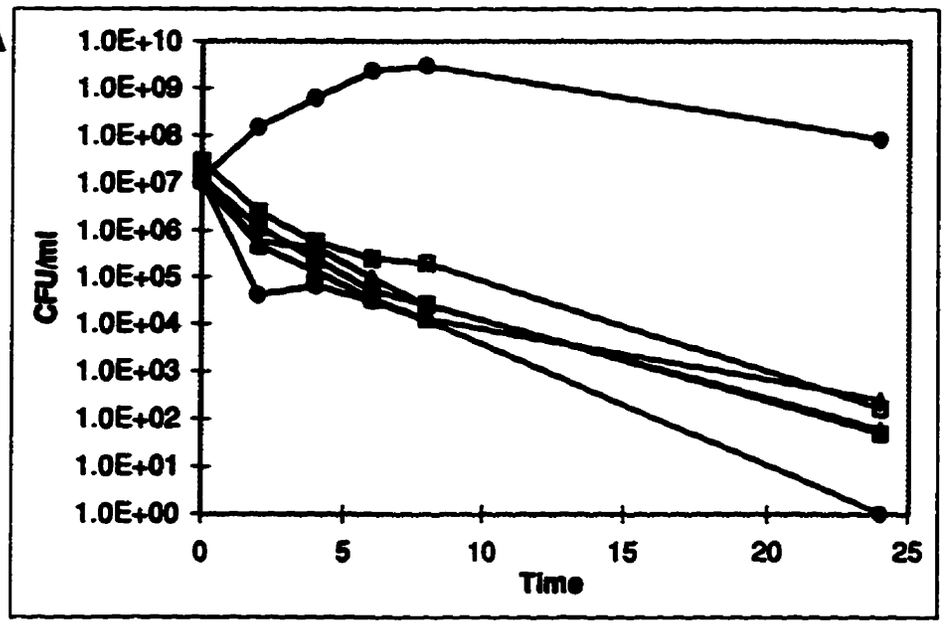
To examine the role of ß-lactamase in the inoculum effect of *H. influenzae* with ß-lactams, a ß-lactamase inhibitor was used. The influence clavulanate on the inoculum effect was tested using MIC determinations and kill-curves. Viable cell count ß-lactam/clavulanate MICs using high initial inocula were not significantly higher than ß-lactam MICs determined turbidimetrically using low inocula (Table 7). The low inocula MICs of clavulanate alone for the TEM^+ (310) and ROB^+ (408) strains used in MIC determinations were 32 µg/ml and 128 µg/ml, respectively.

Figures 13A (strain 310) and 13B (strain 408) show the effect of 4 µg/ml of clavulanate on ß-lactam killing at the high initial inocula. Figures 13A and 13B show increased ß-lactam killing compared to kill-curves in Figures 11B and 12B, respectively, which were incubated in the absence of clavulanate. ß-lactam killing in Figures 13A and 13B was similar to killing in ß-lactamase negative strains (Figure 10B). Table 8 depicts differences in bacterial kill between strains grown in the presence (Figures 13A and 13B) and absence of clavulanate (Figures 11B and 12B). The difference in bacterial killing was most pronounced at 24 hours. For the TEM^+ strain 310 and the ROB^+ strain 408, the difference in log kill between ß-lactam plus 4 µg/ml clavulanate and ß-lactam alone ranged from 3.22 to 6.76 log CFU/ml for cefuroxime, loracarbef, cefaclor, amoxicillin/clavulanate and ampicillin (Table 8). At 24 hours, cefuroxime and the TEM^+ strain 310 showed the least change in killing with the addition of clavulanate (Table 8). Loracarbef and cefaclor demonstrated the greatest changes in bacterial killing which were particularly evident with the ROB^+ strain 408 (Table 8). Significant differences in growth were not detected over

24 hours between strains incubated in the presence and absence of 4 µg/ml of clavulanate (Figure14).

Figure 13: ß-lactam kill-curves with the addition of 4 µg/ml clavulanate for ß-lactamase positive *H. influenzae* at 4x MIC with a high ($1x10^7$ CFU/ml) initial inocula: A, TEM^+ strain 310; B, ROB^+ strain 408; Growth control (•), cefuroxime (■), loracarbef (▲), cefaclor (Δ), amoxicillin/clavulanate (○) and ampicillin (□).

A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CFU/ml
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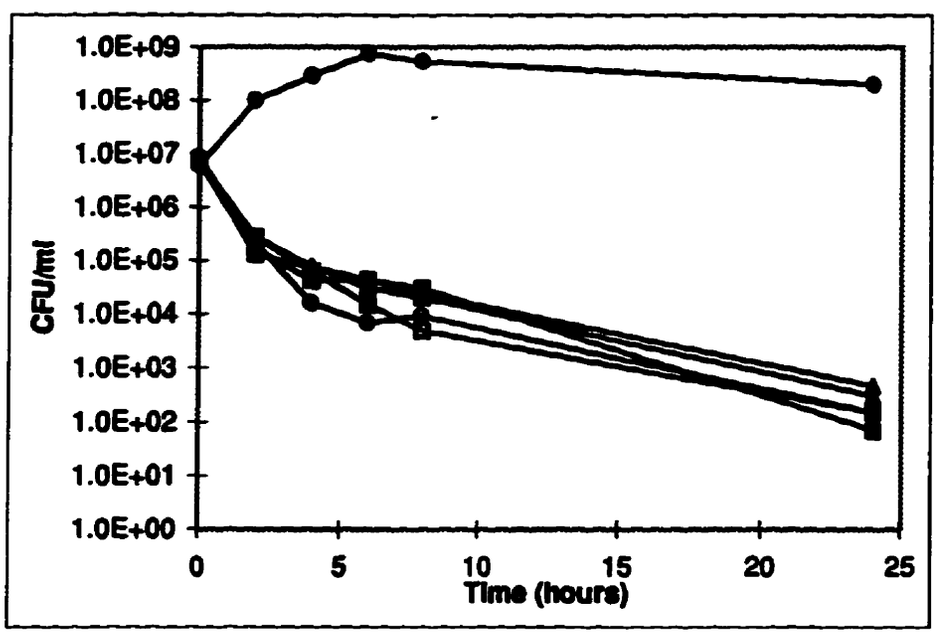
Time

B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CFU/ml
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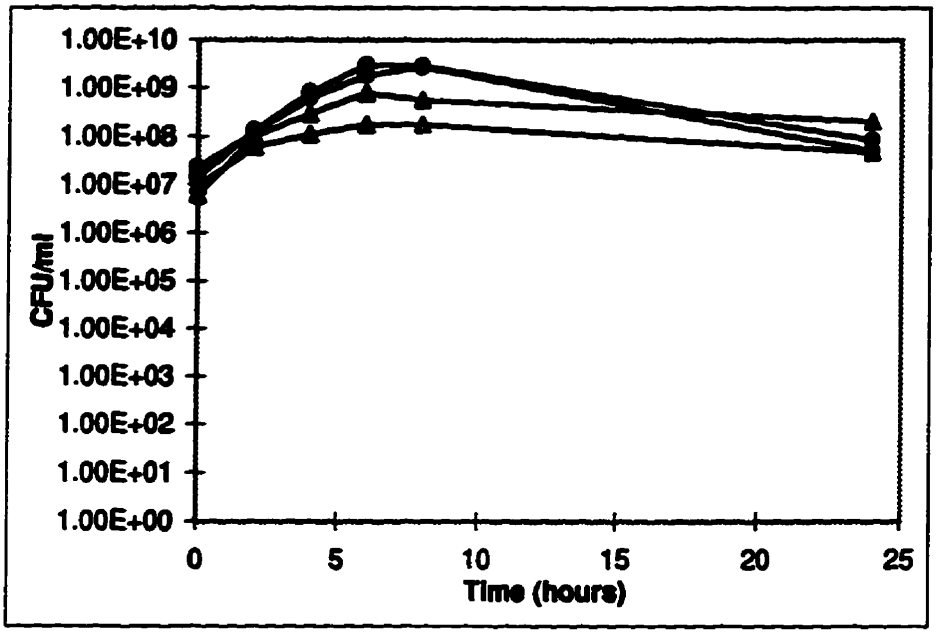
25
Time (hours)

Figure 14: The effect of 4 µg/ml of clavulanate on the growth of strains 310 and 408. 310 growth control (•), 310 + clavulanate (○), 408 growth control (▲), 408 + clavulanate (Δ).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CFU/ml
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Time (hours)

Table 7: The effect of the addition of clavulanate in a 1:1 ratio on viable cell count MICs (µg/ml) for 2 strains of ß-lactamase positive *H. influenzae* at the high inoculum.

Antibiotic	MIC (µg/ml) Low Inoculum[a]		MIC (µg/ml) High Inoculum[b]		MIC (µg/ml) High Inoculum ß-lactam/Clavulanate 1:1	
	TEM+[c]	ROB+[d]	TEM+	ROB+	TEM+	ROB+
Cefuroxime	0.5	1	0.5	4	0.5	0.25
Loracarbef	2	2	32	256	2	0.5
Cefaclor	4	4	128	128	2	0.5
Amox/Clav	1[e]	1[e]	4[e]	1[e]	1[f]	0.5[f]
Ampicillin	16	16	>256	256	0.5	0.25

a. $5x10^5$ CFU/ml initial inoculum, MICs evaluated turbimetrically
b. $1x10^7$ CFU/ml initial inoculum, MICs evaluated by viable cell counts
c. TEM+ is strain 310
d. ROB+ is strain 408
e. ratio of amoxicillin to clavulanate is 2:1
f. ratio of amoxicillin to clavulanate is 1:1

Table 8: Influence of clavulanate on ß-lactam killing of 2 strains of ß-lactamase positive *H. influenzae* with a high initial inoculum[a].

Time hours	Cefuroxime (2 µg/ml)		Loracarbef (8 µg/ml)		Cefaclor (16 µg/ml)		Amox/Clav (4 µg/ml)		Ampicillin (64 µg/ml)	
	TEM[b]	ROB[c]	TEM	ROB	TEM	ROB	TEM	ROB	TEM	ROB
2	0.34[d]	0.21	0.80	1.32	0.53	1.25	1.25	-0.41	0.11	0.72
4	0.58	0.02	0.26	2.58	0.45	2.26	-0.17	0.01	2.17	2.37
6	1.07	0.18	0.98	3.84	2.10	3.66	-0.16	0.7	3.45	3.52
8	1.21	0.9	1.96	4.22	2.91	4.00	-0.05	0.58	3.57	4.81
24	3.22	5.71	6.76	5.24	5.09	5.65	5.79	5.99	6.13	6.11

a. $1x10^7$ CFU/ml initial inoculum
b. TEM+ is strain 310
c. ROB+ is strain 408
d. Values are the difference in log kill between ß-lactam plus 4µg/ml clavulanate and ß-lactam alone. Positive values indicate greater killing with the addition of clavulanate. Negative values indicate less killing with the addition of clavulanate.

7. pLS88AR Transformation.

The TEM-1 ß-lactamase gene was transformed into *H. influenzae* Rd to observe the effect of ß-lactamase production on high inoculum MIC determinations and kill-curves. Figure 15 confirms that the plasmid isolated from a transformant able to grow on kanamycin and ampicillin contains the desired TEM-1 gene. The digested plasmid (lane 2) contains two bands, one corresponding to pLS88 (lane 3) and the other corresponding to the TEM-1 PCR product (lane 4).

8. MIC Determination with *H. influenzae* Rd and pLS88AR.

As with clinical strains, turdibimetrically evaluated MICs for *H. influenzae* Rd at the high initial inocula ($1x10^7$ CFU/ml) were greater than 256 µg/ml for all ß-lactam tested (Table 9). Corresponding with previously tested ß-lactamase negative strains (Table 5), viable count MICs for *H. influenzae* Rd did not significantly increase with the high initial inocula (Table 9).

Transformation of *H. influenzae* Rd with pLS88AR resulted in an increase in low inoculum MICs for cefaclor, amoxicillin/clavulanate and ampicillin (Table 10). However, the increase in MIC evaluated at the low initial inoculum was significant only for ampicillin where an increase in MIC from 0.25 µg/ml to 64 µg/ml was demonstrated upon transformation of the plasmid.

The MIC determinations at the high initial inocula for *H. influenzae* Rd with pLS88AR (Table 10) reflect the results obtained with TEM^+ clinical strains of *H. influenzae* (Table 6). Turbidimetrically determined ß-lactam MICs were greater than 256 µg/ml with the high initial inocula. MICs assayed by viable counts significantly increased

with an increase in inoculum for loracarbef, cefaclor, amoxicillin/clavulanate, ampicillin, but not cefuroxime (Table 10).

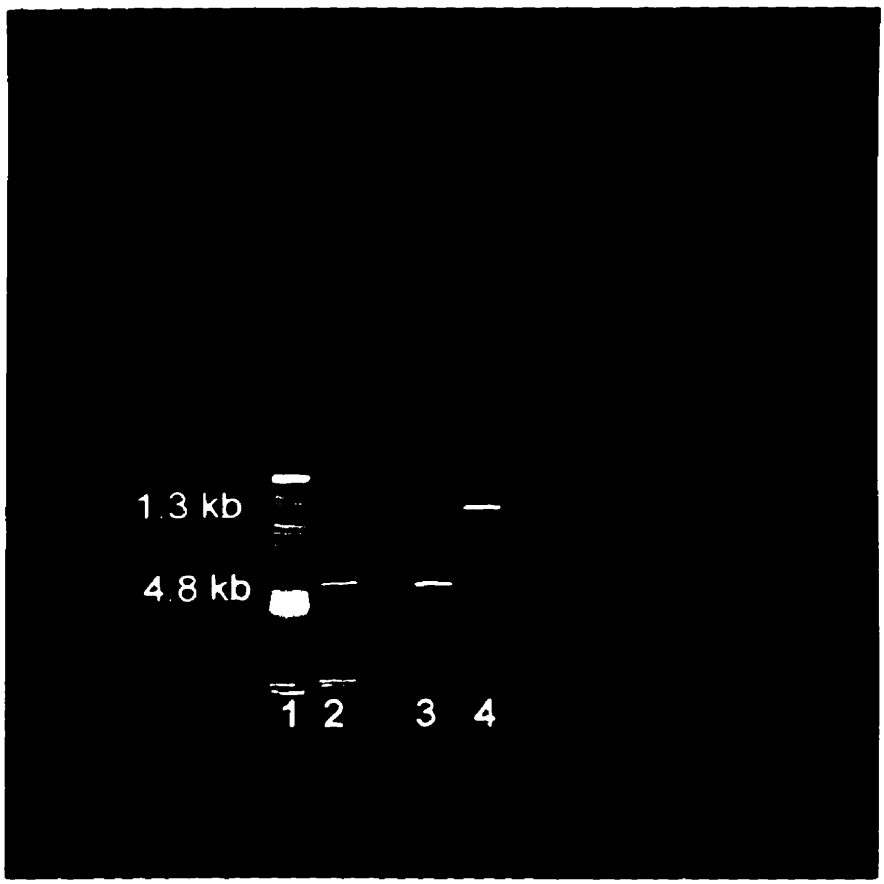

Figure 15: pLSSAR *Eco*R1 digest. Lane1, 1 kb ladder; lane 2, pLSSAR digested with *Eco*R1; lane 3, pLS88 digested with *Eco*R1; lane 4, *TEM-1* PCR product.

Table 9: ß-lactam MICs (µg/ml) for *H. influenzae* Rd at low and high initial inocula.

Antibiotic	MIC (µg/ml) Low Inoculum[a]	MIC (µg/ml) High Inoculum[b] Visually Determined	MIC (µg/ml) High Inoculum Viable Cell Counts
Cefuroxime	1	>256	1
Loracarbef	2	>256	2
Cefaclor	2	>256	2
Amox/Clav[c]	0.25	>256	0.25
Ampicillin	0.25	>256	0.25

a. $5x10^5$ CFU/ml
b. $1x10^7$ CFU/ml
c. Amoxicillin/clavulanate (2:1)

Table 10: ß-lactam MICs (µg/ml) for *H. influenzae* Rd with pLS88AR at low and high initial inocula.

Antibiotic	MIC (µg/ml) Low Inoculum[a]	MIC (µg/ml) High Inoculum[b] Visually Determined	MIC (µg/ml) High Inoculum Viable Cell Counts
Cefuroxime	1	>256	1
Loracarbef	2	>256	32
Cefaclor	4	>256	64
Amox/Clav[c]	0.5	>256	4
Ampicillin	64	>512	>512

a. $5x10^5$ CFU/ml
b. $1x10^7$ CFU/ml
c. Amoxicillin/clavulanate (2:1)

9. Kill-Curves with *H. influenzae* Rd and pLS88AR.

Kill-curves were performed with *H. influenzae* Rd with and without pLS88AR to determine the effect of transformation a ß-lactamase gene on the inoculum effect. Figures 16A and 16B present the kill-curves for *H. influenzae* Rd and the low and high initial inocula. The rates of ß-lactam killing are similar for both the low (Figure 16A) and high (Figure 16B) inocula. Loracarbef, amoxicillin/clavulanate and ampicillin produce a greater than 3 log reduction in bacterial concentration after 24 hours of incubation regardless of the initial inoculum. Incubation with cefuroxime or cefaclor results in slightly less ß-lactam killing at both low and high initial inocula (Figure 16A and 16B).

Kill-curves for *H. influenzae* Rd with pLS88AR at low and high initial inocula are shown in Figures 17A and 17B. Transformation with pLS88AR results in differences between low and high inoculum kill-curves with *H. influenzae* Rd. Table 11 presents the difference in log kill between the low and high inocula for *H. influenzae* Rd with pLS88AR. With the exception of cefuroxime, the rates of ß-lactam killing are not similar with the low and high initial inocula kill-curves (Figure 17A and 17B). The inoculum effect demonstrated by low and high inocula kill-curves of *H. influenzae* Rd with pLS88AR is reminiscent of the kill-curves produced by the TEM^+ clinical strains (Figure 11A and 11B).

Figure 16: ß-lactam kill-curves for *H. influenzae* Rd at 4x MIC with low ($5x10^5$ CFU/ml) and high ($1x10^7$ CFU/ml) initial inocula: A, low initial inoculum; B, high initial inoculum; Growth control (•), cefuroxime (■), loracarbef (▲), cefaclor (Δ), amoxicillin/clavulanate (○) and ampicillin (□).

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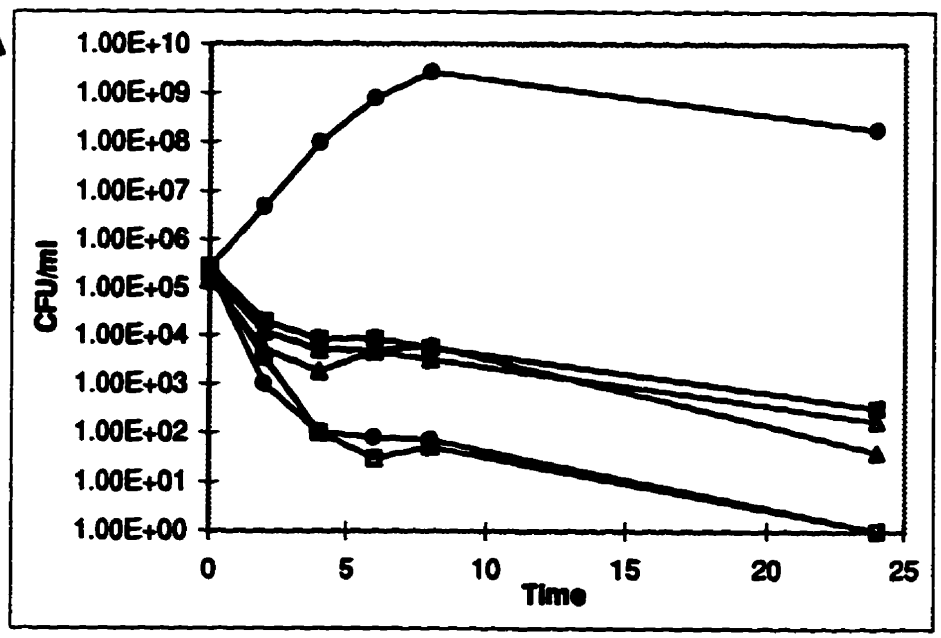
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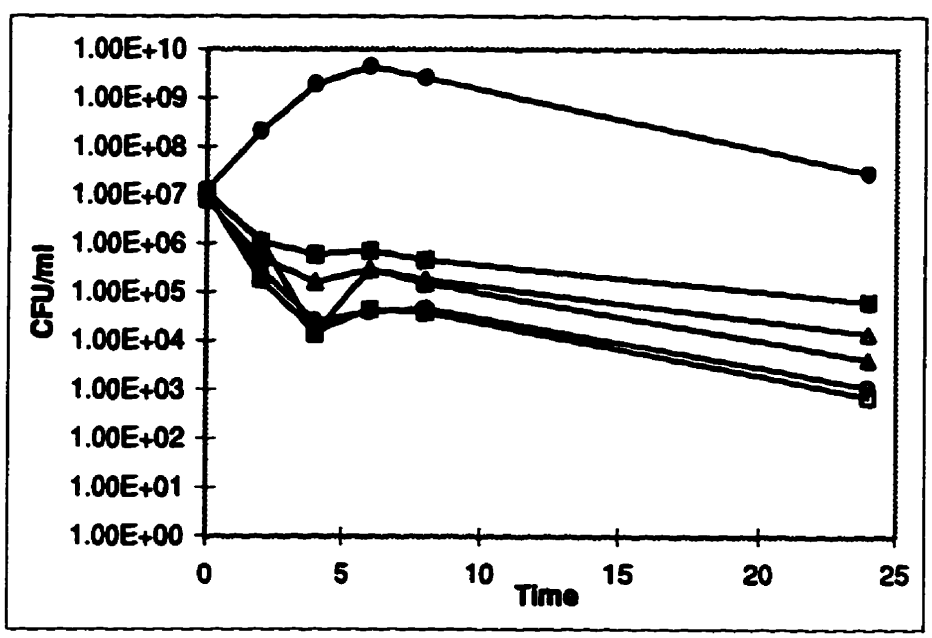
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CFU/ml
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Time

Figure 17: ß-lactam kill-curves for *H. influenzae* Rd with pLS88AR at 4x MIC with low ($5x10^5$ CFU/ml) and high ($1x10^7$ CFU/ml) initial inocula: A, low initial inoculum; B, high initial inoculum; Growth control (●), cefuroxime (■), loracarbef (▲), cefaclor (Δ), amoxicillin/clavulanate (○) and ampicillin (□).

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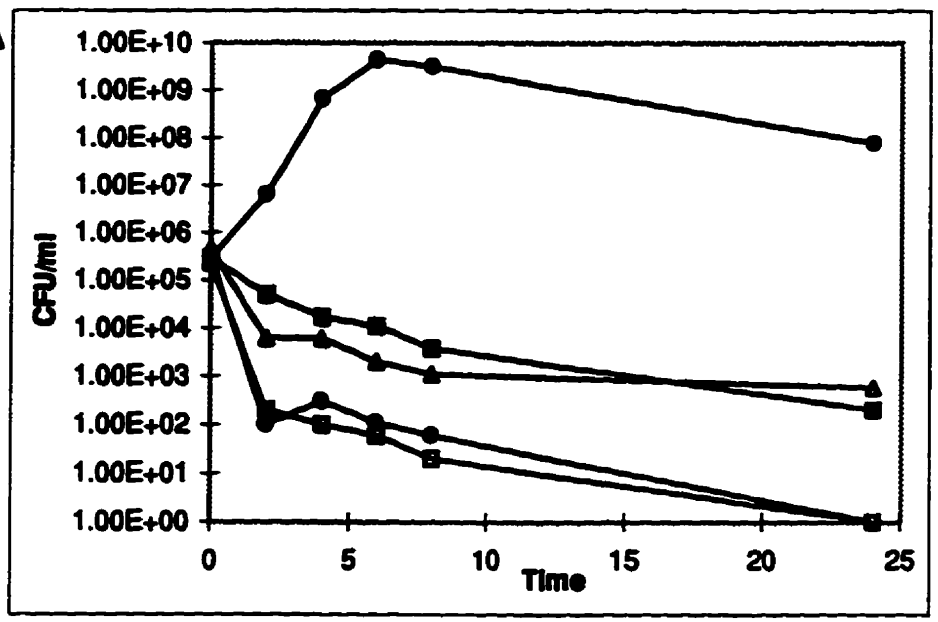
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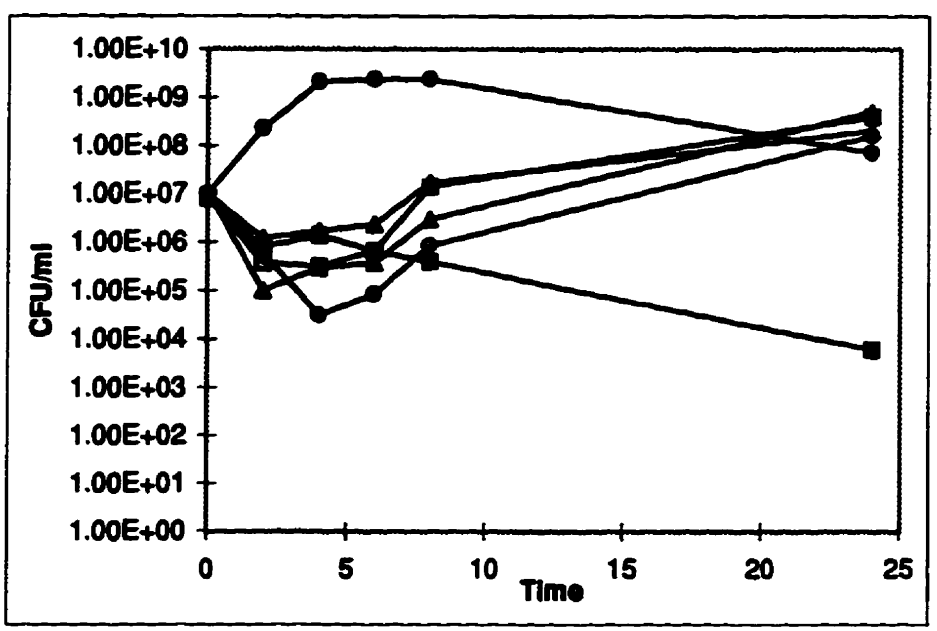
Time

B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1.00E+02
1.00E+01
1.00E+00
CFU/ml
0
5
10
15
20
25
Time

Table 11: Log kill[a] of *H. influenzae* Rd with pLS88AR at the low ($5x10^5$ CFU/ml) and high ($1x10^7$ CFU/ml) initial inocula.

Time	Cefuroxime		Loracabef		Cefaclor		Amox/Clav		Ampicillin	
hours	low[b]	high[c]	low	high	low	high	low	high	low	high
2	0.78	1.00	1.42	0.91	1.88	2.04	3.51	1.21	3.06	1.36
4	1.25	0.76	1.15	0.76	1.88	1.58	3.03	2.46	3.36	1.44
6	1.44	1.09	1.44	0.63	2.36	1.47	3.46	2.03	3.58	1.10
8	1.91	1.30	2.00	-0.24	2.62	0.56	3.73	1.02	4.06	-0.22
24	3.18	3.12	3.85	-1.31	2.91	-1.66	5.51	-1.25	5.36	-1.66

a. Log kill is the log (initial inoculum) minus log (CFU/ml at the time point). Negative values indicate regrowth.
b. low initial inoculum
c. high initial inoculum

10. Experiments with Clavulanate and *H. influenzae* Rd and pLS88AR.

The influence of clavulanate on the inoculum effect exhibited by *H. influenzae* Rd with pLS88AR was tested using MIC determinations and kill-curves. Viable cell count ß-lactam/clavulanate MICs at the high initial inocula were not significantly higher than low inoculum ß-lactam MICs (Table 12). The inoculum effect demonstrated by *H. influenzae* Rd with pLS88AR and loracarbef, cefaclor, amoxicillin/clavulanate and ampicillin was reversed by the addition of clavulanate.

Figure 18 shows the effect of 4 µg/ml of clavulanate on ß-lactam killing at the high initial inocula. Figure 18 shows increased ß-lactam killing compared to the kill-curves in Figure 17B, which were incubated in the absence of clavulanate. Table 13 depicts differences in bacterial kill between strains grown in the presence (Figure 18) and absence of clavulanate (Figure 17B). The difference is most pronounced at 24 hours with cefuroxime showing the least change in killing with the addition of clavulanate and ampicillin displaying the greatest change (Table 13).

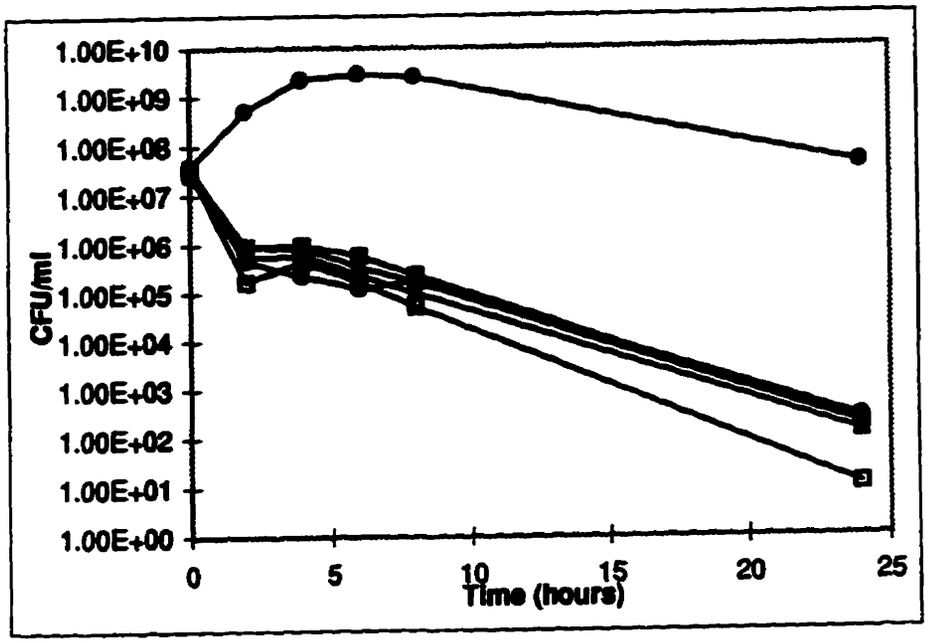
Table 12: The effect of the addition of clavulanate in a 1:1 ratio on viable cell count MICs (µg/ml) for *H. influenzae* with pLS88AR at the high inocula.

Antibiotic	MIC (µg/ml) Low Inoculum[a]	MIC (µg/ml) High Inoculum[b]	MIC (µg/ml) High Inoculum ß-lactam/Clavulanate 1:1
Cefuroxime	1	1	1
Loracarbef	2	32	2
Cefaclor	4	64	2
Amox/Clav	0.5[c]	4[c]	0.5[d]
Ampicillin	64	>512	0.5

a. $5x10^5$ CFU/ml initial inoculum, MICs evaluated turbimetrically
b. $1x10^7$ CFU/ml initial inoculum, MICs evaluated by viable cell counts
c. ratio of amoxicillin to clavulanate is 2:1
d. ratio of amoxicillin to clavulanate is 1:1

Figure 18: ß-lactam kill-curves for *H. influenzae* Rd with pLS88AR at 4x MIC with a high ($1x10^7$ CFU/ml) initial inocula: A, with 4 µg/ml clavulanate; B, without clavulanate. Growth control (•), cefuroxime (■), loracarbef (▲), cefaclor (Δ), amoxicillin/clavulanate (○) and ampicillin (□).

A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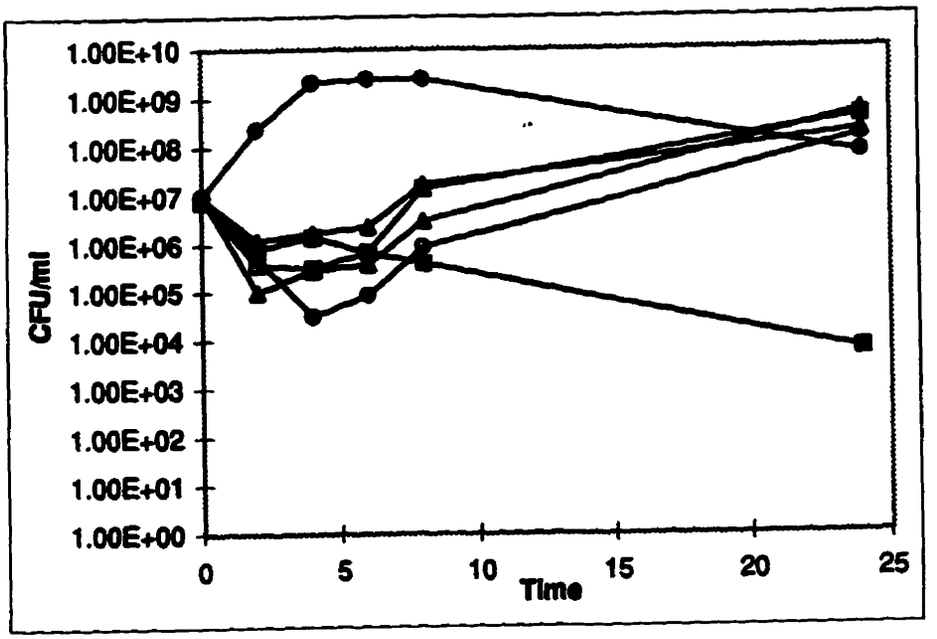
1.00E+03
1.00E+02
1.00E+01
1.00E+00
CFU/ml
0
5
10
15
20
25
Time (hours)

B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1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
CFU/ml
0
5
10
15
20
25
Time

Table 13: Influence of clavulanate on ß-lactam killing of *H. influenzae* Rd with pLS88AR and a high initial inocula[a].

Time hours	Cefuroxime (4 µg/ml)	Loracarbef (8 µg/ml)	Cefaclor (16 µg/ml)	Amox/Clav (2 µg/ml)	Ampicillin (64 µg/ml)
2	0.58[b]	0.64	-0.32	0.72	0.95
4	0.80	0.84	0.09	-0.21	0.47
6	0.68	1.34	0.60	0.48	1.21
8	0.87	2.51	1.87	1.32	3.05
24	2.13	6.56	6.98	6.44	8.16

a. $1x10^7$ CFU/ml initial inoculum
b. Values are the difference in log kill between ß-lactam plus 4µg/ml clavulanate and ß-lactam alone. Positive values indicate greater killing with the addition of clavulanate. Negative values indicate less killing with the addition of clavulanate.

D. DISCUSSION

In summary, MIC determinations and kill-curves demonstrated that the inoculum effect was present only in ß-lactamase producing clinical strains of *H. influenzae*. The addition of the ß-lactamase inhibitor, clavulanate, eliminated the inoculum effect with all strains tested. Transformation of the *TEM-1* ß-lactamase gene into *H. influenzae* Rd, a ß-lactamase negative strain, resulted in the production of an inoculum effect. The results suggest that ß-lactamase is responsible for the inoculum effect of *H. influenzae* with ß-lactams.

1. The Turbidimetrically Evaluated Inoculum Effect with *H. influenzae* and ß-lactams is not due to Viable Cells.

Previous studies have consistently reported that ß-lactamase positive strains of *H. influenzae* demonstrate an inoculum effect with ß-lactams (4, 9, 15, 20, 26, 28, 29, 41, 43, 44, 75, 76, 81, 87). Disagreement, however, exists concerning the existence of a similar inoculum effect with ß-lactamase negative strains of *H. influenzae* (4, 9, 20, 26, 28, 43, 44, 87). The discrepancy between these two groups appears to be method and inocula based and therefore difficult to critically compare.

The results obtained using turbidimetrically determined high inocula MICs demonstrated an inoculum effect with ß-lactamase negative strains as well as ß-lactamase positive strains of *H. influenzae* (Tables 5 and 6). The high inocula, turbidimetrically determined MICs were shown to be misleading as turbid test tubes did not necessarily indicate growth of the bacteria. In some cases, turbid tubes contained less than 100 viable bacteria per milliliter suggesting that the concentration of antibiotic was sufficient for

inhibition of growth but not prevention of turbidity. The contents of these tubes were Gram-stained and the turbidity appeared to be due to cellular debris, large aggregates, but not intact bacteria. This observation suggests that a method of MIC evaluation that is independent of visual interpretation may provide more accurate results when high initial inocula are used in susceptibility testing.

The observation of large aggregates in high inoculum MIC tubes has been reported previously (10, 15, 20, 43, 63, 81). In 1974, Roberts and colleagues reported ampicillin induced L forms of *H. influenzae* which were deemed responsible for errors in MIC interpretations when using high initial inocula (10^7 CFU/ml) (63). Other investigators have also observed large spherical bodies in turbid broth containing ampicillin (10). The reasons for the occurrence of these large aggregates and their clinical significance remain unknown. Nonetheless, their existence can skew visually determined MICs when the inoculum is as little as one-half a log higher than $5x10^5$ CFU/ml (63). Therefore all strains of *H. influenzae* may demonstrate an inoculum effect regardless of ß-lactamase production if high inocula MICs are determined turbidimetrically.

2. An Inoculum Effect is Present with ß-lactamase Positive but not ß-lactamase Negative *H. influenzae*.

A viable cell count method was used to determine MICs when the high initial inocula was tested. MICs were determined from the concentration of live bacteria following incubation with a ß-lactam. When high initial inocula MICs were determined using viable cell counts, ß-lactamase negative strains of *H. influenzae* did not demonstrate an inoculum effect (Table 6). ß-lactam kill-curves of ß-lactamase negative strains of *H.*

influenzae (Figures 10A and 10B) also did not show an inoculum effect. Significant differences were not shown (P=0.9673) in the rates of bacterial killing over time between low and high initial inocula for ß-lactamase negative strains of *H. influenzae* for all ß-lactams tested.

ß-lactamase positive strains of *H. influenzae* were shown to demonstrate an inoculum effect with the ß-lactams tested with the exception of TEM+ strains with cefuroxime (Table 6). The results from the viable cell count MIC data correlated well with the observations made from kill-curves for ß-lactamase positive strains (Table 6). Kill-curves for ß-lactamase positive strains were significantly effected by increased inocula (P=0.0010) (Figures 11A, 11B, 12A and 12B). The kill-curves for ampicillin, loracarbef and cefaclor appear to be more greatly affected by an increase in initial inoculum than those for cefuroxime and amoxicillin/clavulanate.(Figures 11A, 11B, 12A and 12B).

Kill-curves at both the low and high initial inocula for the ß-lactamase positive strains (Figures 11A, 11B, 12A and 12B) suggested that both loracarbef and cefaclor were less active against ROB$^+$ strains as compared to the TEM$^+$ strains. Similar results were demonstrated by all TEM$^+$ and ROB$^+$ strains tested with loracarbef and cefaclor. The results from viable cell count MIC demonstrated a greater inoculum effect with ROB$^+$ than TEM$^+$ strains with loracarbef and cefaclor (Table 6). These observations suggest that loracarbef and cefaclor may be less stable with ROB$^+$ strains than with TEM$^+$ strains, however, an insufficient number of strains were tested to be conclusive (Table 6). Future experiments involving the introduction of the *ROB-1* ß-lactamase gene into *H. influenzae* Rd may provide more conclusive comparisons of the two ß-lactamases.

High inoculum MICs and kill-curves with cefuroxime were least affected by an increase in bacterial inoculum. An inoculum effect with cefuroxime was not demonstrated by the TEM^+ clinical strains or pLS88AR in *H. influenzae* Rd as evaluated by viable cell counts (Table 6 and Table 10). Only two out of four ROB^+ strains displayed increased viable cell count MICs at the high inoculum and this increase was not more than four fold (Table 6). Compared with the other ß-lactams, high inoculum cefuroxime kill-curves were least affected by the addition of clavulanate (Table 8 and Table 13). These results suggest that cefuroxime may be more stable to ß-lactamase hydrolysis with respect to the other ß-lactams tested.

Experiments in which clavulanate was used in combination with cefuroxime, loracarbef, cefaclor and ampicillin did not demonstrate an inoculum effect (Table 7). The elimination of the inoculum effect with clavulanate was due to the inhibition of ß-lactamase as concentrations of clavulanate were sub-inhibitory (≤ 4 µg/ml). Increasing the concentration of clavulanate in the amoxicillin/clavulanate combination resulted in the elimination of an inoculum effect. This suggests that at a high inoculum, increased clavulanate concentrations are required to compensate for increased ß-lactamase concentrations. The results from clavulanate experiments suggest that combinations of loracarbef or cefaclor with clavulanate may be of value in clinical situations, however further investigation is required. As well, experiments comparing ß-lactam to ß-lactam/clavulanate combinations may be another useful tool in the evaluation of ß-lactam stability.

3. ß-lactamase is Responsible for the Inoculum Effect with ß-lactamase Positive *H. influenzae*.

To test whether ß-lactamase is responsible for the inoculum effect of *H. influenzae* with ß-lactams, the ß-lactamase gene *TEM-1* was introduced into a ß-lactamase negative strain, *H. influenzae* Rd. Prior to transformation with pLS88AR, *H. influenzae* Rd did not exhibit an inoculum effect as evaluated by viable cell count MICs and kill-curves at the low and high initial inocula (Table 9, Figures 16A and 16B). *H. influenzae* Rd containing pLS88AR demonstrated an inoculum effect with loracarbef, cefaclor, amoxicillin/clavulanate and ampicillin according to MIC determinations and kill-curve experiments (Table 10, Figures 17A and 17B). This inoculum effect was effectively reversed by the addition of clavulanate (Table 12, Figure 18) and further demonstrated the dependence of the inoculum effect on ß-lactamase.

The results obtained with pLS88AR in *H. influenzae* Rd are similar to those of the TEM^+ clinical strains. Viable cell count MICs increased with loracarbef, cefaclor, amoxicillin/clavulanate and ampicillin, but not cefuroxime for both the TEM^+ clinical strains and the strain containing pLS88AR (Tables 6 and 10). The inoculum effect displayed by kill-curves as well as experiments with clavulanate were also similar. Since pLS88AR encodes for TEM-1, these similarities are not surprising. However, the similarities confirm that the laboratory constructed strain behaves like clinical isolates and provides further evidence that the ß-lactamase is responsible for the inoculum effect.

The ability of ß-lactam antibiotics to bind to their target penicillin binding proteins (PBPs) is dependent upon their concentration in the periplasmic space of Gram-negative bacteria such as *H. influenzae* (14). The outer membrane of *H. influenzae* is very

permeable to ß-lactams and provides little barrier against the free diffusion of penicillins and cephalosporins into the periplasmic space (83). For ß-lactamase positive organisms, the likelihood of ß-lactams reaching their targets is dependent upon two factors, extracellular antibiotic concentration and the rate of hydrolysis by ß-lactamase (14). The number of ß-lactamase molecules is amplified with increases in bacterial inocula. Therefore, there is greater hydrolysis of ß-lactam molecules with higher bacterial inocula which results in an inoculum effect (14). With higher initial inocula, the MICs for ß-lactams increase because more ß-lactam hydrolysis occurs. The addition of clavulanate inhibits ß-lactamase action and therefore the inoculum effect. The data presented in this thesis which demonstrated an inoculum effect with ß-lactamase positive strains of *H. influenzae*, but not ß-lactamase negative strains, supports this explanation.

The results suggest that an inoculum effect is present with amoxicillin/clavulanate but not cefuroxime with TEM^+ strains. Cefuroxime is resistant to ß-lactamase hydrolysis by the TEM enzyme, increasing the concentration of ß-lactamase by increasing the inoculum does not affect the stability of this ß-lactam. With the combination of amoxicillin/clavulanate, increases in inoculum increase the number of ß-lactamase molecules, the targets for clavulanate. If the concentration of ß-lactamase is greater than that of clavulanate, uninhibited ß-lactamase molecules are free to hydrolyze amoxicillin resulting in an inoculum effect.

4. Clinical Significance of the Inoculum Effect.

Does the inoculum effect of *H. influenzae* with ß-lactams possess clinical significance ? An increase in MIC, with a high inocula, may be 4 fold or greater but not

result in MICs exceeding NCCLS recommended breakpoints. When resistance breakpoints are considered, none of the ROB+ strains were classified as resistant to cefuroxime (MIC $\geq$ 16µg/ml) (56) using high initial inocula. Two of the four TEM$^+$ strains would be classified as resistant as would 3 of the 4 strains with cefaclor (MIC $\geq$ 32 µg/ml) (56). All of the ROB$^+$ strains exceeded the MIC breakpoint for both loracarbef ($\geq$ 32 µg/ml) (56) and cefaclor ($\geq$ 32 µg/ml) (56) with the high initial inoculum. For amoxicillin/clavulanate, 3 of 4 TEM+ strains could be considered resistant (MIC $\geq$ 8/4) (56) according to viable cell count MICs, while only one of the ROB+ strains had increased MICs high enough for the resistant classification. In instances where increases in MICs do not result in a resistant classification, the inoculum effect may still be significant. Increases in MIC may decrease the extent of ß-lactam killing which may negatively affect clinical outcome.

Differences in the clinical significance of the two ß-lactamases, TEM-1 and ROB-1, is unknown. The results presented in this thesis suggest that the inoculum effect is greater in strains of *H. influenzae* that produce the ROB-1 ß-lactamase, especially for loracarbef and cefaclor. Inoculum effect data indicates that ROB-1 may have weak hydrolyzing activity against cefuroxime at the high inoculum, whereas cefuroxime is stable with the TEM-1 enzyme. However, further studies are required to determine the significance of these findings.

The concentration of *H. influenzae* at sites of infection, such as in otitis media have not been determined. If concentrations of *H. influenzae* approach $1x10^7$ CFU/ml, the inoculum effect may play a role in the clinical outcome for patients treated with loracarbef, cefaclor or amoxicillin/clavulanate. The data presented in this thesis suggests

that of the ß-lactams tested cefuroxime would be the least affected by high concentrations of *H. influenzae*.

5. Conclusions

In conclusion, the inoculum effect with ß-lactams was only present in ß-lactamase positive strains of *H. influenzae* and was effectively reversed by the addition of the ß-lactamase inhibitor, clavulanate. This data suggests that the inoculum effect of ß-lactams with *H. influenzae* was dependent upon the activity of ß-lactamase. Introduction of a ß-lactamase gene into a ß-lactamase negative strain of *H. influenzae* resulted in the production of an inoculum effect. These results confirm that ß-lactamase is responsible for the inoculum effect of *H. influenzae* with ß-lactams.

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