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Antibiotic Resistance: Multi-Drug Profiles and Genetic Determinants.

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Antibiotic Resistance: Multi-drug Profiles and Genetic Determinants

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ABSTRACT

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Antimicrobial susceptibility profiles were assembled for isolates of *Moraxella catarrhalis* collected from the Mountain Home Veteran's Affairs Medical Center (VAMC) clinical laboratory in Johnson City, Tennessee. The goal of the study was to identify isolates for genetic characterization using comparisons of susceptibility profiles. Isolates of *Moraxella catarrhalis* collected from July 1984 through 1994 were analyzed for β-lactamase production using a Cefinase disk assay.

A multi-drug profile consisting of 11 β-lactam antibiotics was performed on the 41 *M. catarrhalis* isolates. Kirby Bauer disk assays were performed for 7 cephalosporin and 4 non-cephalosporin antibiotics.

In summary, 2 observations implicate more complex resistance determinants than the 2 known forms of the BRO β-lactamase. First, there was overlap in the ranges of inhibition zones. Second, several isolates had antibiotic-specific deviations from typical profiles. These data suggest either more variation in the *M. catarrhalis* BRO β-lactamase than described or contributions to resistance from undescribed determinants.

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CHAPTER 1

INTRODUCTION

Moraxella catarrhalis Profile

Moraxella (Branhamella) catarrhalis, a Gram-negative diplococcus previously thought to be a commensal of the upper respiratory tract, has more recently gained recognition as an emerging pathogen (Enright and McKenzie 1997). *Moraxella (Branhamella) catarrhalis* is the 3rd most common bacterium isolated from the middle-ear fluid of children with otitis media and it is frequently found in the sputum of adults with acute exacerbations of chronic obstructive pulmonary disease (Bootsma et al. 2000). A striking feature of *M. catarrhalis* is the rapid worldwide and local increase in β-lactamase producing strains (Bootsma et al. 2000; Walker et al. 2000) (Fig.1). This dramatic rise probably represents the fastest increase in prevalence of any known β-lactamase within a bacterial species (Wallace et al. 1989).

Resistance to β-lactam antibiotics has emerged in a number of pathogens over the past years, including *M. catarrhalis* (Jacoby 1994). The 1st reports of β-lactamase production in *M. catarrhalis* appeared in 1977 (Malmvall et al.1977; Percival et al. 1977), and a rapid increase in the frequency of β-lactamase producing strains was reported from different localities shortly thereafter (Doern et al. 1980; Doern and Jones 1988; Wallace et al. 1989). Currently, greater than 90% of *M. catarrhalis* strains are clinically resistant to β-lactam antibiotics such as penicillin, ampicillin, and amoxicillin (Doern et al. 1996; Walker et al. 2000). Several classification schemes of β-lactamases have been proposed based on the enzyme hydrolytic spectrum, susceptibility to inhibitors, genetic localization (plasmid or chromosome), DNA gene or amino acid protein sequence (Thornsberry 1991).

β-lactam antibiotics belong to a family of antibiotics characterized by a β-lactam ring, the presence of which aids the antibiotic in exerting its bactericidal activity. Penicillins, cephalosporins, clavams (or oxapenams), cephamycins, and carbapenems are members of the β-lactam family of antibiotics. The antibiotic activity results in the inactivation of a set of transpeptidases that catalyze the final cross-linking reactions of peptidoglycan synthesis (Yao and Moellering 1991).

The production of β-lactamases is the most common mechanism of β-lactam resistance and, as these enzymes are frequently plasmid encoded (Jacoby 1994), resistance can be easily transferred between bacteria. Probably the most clinically important characteristic of a β-lactamase is its ability to hydrolyze β-lactam antibiotics (Bush and Sykes 1986). However, an alternate mechanism of antibiotic resistance has emerged in several species (Spratt 1994). This type of resistance is mediated by target alterations, or the development of altered penicillin-binding proteins (PBPs) (Dowson et al. 1994; Maiden 1998). Uptake and recombination of DNA by naturally competent bacteria may result in mosaic genes, the products of which have decreased affinity for β-lactam antibiotics (Dowson et al. 1994; Maiden 1998). Although, the β-lactamase encoding *bla* locus of *M. catarrhalis* does not appear to be a mosaic gene, its dissemination mediated by transformation and recombination is reminiscent of this process (Bootsma et al. 2000).

BRO-1 and BRO-2 Alleles

Moraxella catarrhalis strains may produce either BRO-1 or BRO-2 β-lactamase, which can be distinguished on the basis of differences in their isoelectric focusing pattern (Wallace et al. 1989). BRO-1 strains represent the majority of β-lactamase producing *M. catarrhalis* and express higher levels of resistance to ampicillin (Bootsma et al. 2000). BRO-1 and BRO-2 were

shown to be alleles of the same chromosomal locus and they were also almost identical in DNA sequence, differing in only 5 base pairs, of which 1 resulted in an amino acid substitution (Bootsma et al. 1996). The β-lactamase gene of *M. catarrhalis* has been shown to be expressed as a 33-kDa lipoprotein associated with the outer membrane (Bootsma et al. 1999). A 33-kDa lipoprotein thus far has been described only for β-lactamases of Gram-positive species, suggesting that the BRO β-lactamase was derived from a Gram-positive bacterium. A significant difference was observed in the promoter region of the 2 BRO alleles, possibly explaining the lower expression level of BRO-2 compared with BRO-1. Also, the distinct G+C content of the *bla* locus compared to those of other *M. catarrhalis* genes is strong evidence for a relatively recent acquisition. The present data suggest that BRO β-lactamase originated from a Gram-positive bacterium and that its lipidation is a remnant of its origin (Bootsma et al. 1999).

β-lactam Antibiotics

Penicillins comprise a group of natural and semisynthetic antibiotics consisting of a β-lactam ring fused to a thiazolidine ring (Yao and Moellering 1991). The antibiotic actions of penicillins are based on their ability to inhibit a number of bacterial enzymes, known as penicillin binding proteins (PBP), that are essential for peptidoglycan synthesis (Yao and Moellering 1991). Cephalosporins comprise a group of antibiotics that are derivatives of the fermentation products from the fungus *Cephalosporium* (Yao and Moellering 1991). The structure is composed of a β-lactam ring fused to a dihydrothiazine ring (Yao and Moellering 1991). Cephalosporins bind to PBPs, thereby inhibiting synthesis of peptidoglycan for the bacterial cell wall. Cephalosporins are often classified based on general features of their antibacterial activity. First generation cephalosporins have strong Gram-positive activity and modest Gram-negative activity (Yao and Moellering 1991). Second-generation cephalosporins

act against certain β-lactamases found in Gram-negative organisms (Yao and Moellering 1991). Third generation cephalosporins are generally less effective against Gram-positive cocci, but more effective against the *Enterobacteriaceae* (Yao and Moellering 1991). Aztreonam is a monobactam antibiotic that binds to PBP-3 of Gram-negative aerobes. It is often given intravenously and its activity is limited to Gram-negative bacilli (Yao and Moellering 1991). Imipenem is a semisynthetic derivative of thienamycin, which is produced by *Streptomyces* spp. (Yao and Moellering 1991). Imipenem binds to PBP-1 and PBP-2 of Gram-negative and Gram-positive bacteria leading to cell elongation and lysis (Yao and Moellering 1991).

Antibiotic Resistance

Antibiotic resistance among many pathogenic microbes has been increasing during the last decade. It is mostly associated with: a) overuse of antibiotics in outpatient settings; b) unwarranted use of very broad spectrum antibiotics; c) poor standards for bacterial identification and patient monitoring; d) ineffective hospital infection control over nosocomial transmission of resistant strains.

Resistance to antibiotics can be intrinsic or acquired. Intrinsic resistance dictates the spectrum of activity of the antibacterial and it is always present (Thornsberry 1991). For example, Gram-negative bacteria are intrinsically resistant to cloxacillin and vancomycin due to the Gram-negative cell wall being multi-layered with a lipoprotein-lipopolysaccharide-phospholipid outer membrane external to the relatively thin peptidoglycan layer that protects the cell wall from many antibiotics and enzymes (lysozyme) (Thornsberry 1991). Of increased clinical significance is acquired resistance, in which bacteria that were previously sensitive to antibiotics become resistant. Bacteria can acquire resistance through chromosomal mutations or acquisition of genetic material (e.g., plasmids, transposons), which confers resistance to

antibiotics (Thornsberry 1991). Transfer of these plasmids from 1 organism to another can lead to widespread resistance. Of great concern is the potential for 1 species of bacteria (e.g., *Enterococcus*) to transfer plasmids to a different species of bacteria (e.g., *Staphylococcus*) (Thornsberry 1991). Changes in resistance patterns can occur after years of exposure to an antibiotic (e.g., penicillin-resistant pneumococcus) or can develop during the course of therapy for an infection (e.g., extended-spectrum β-lactamases that are seen in certain Gram-negative bacilli).

The mechanism by which bacteria become resistant to antibiotics often reflects the mechanisms by which antibiotics kill bacteria. After an antibiotic penetrates the cell wall or membrane of the bacteria, it targets a specific bacterial enzyme (e.g., penicillin-binding protein, DNA gyrase) or ribosome, thereby interfering with bacterial protein synthesis or replication. The mechanisms of resistance to different antibiotics, therefore, include the following: decreased penetration through the bacterial cell membrane, enzymatic degradation or inactivation of the antibiotic, alteration of the target site and active efflux of the antibiotic out of the bacteria. Resistance to a given class of antibiotics can occur by several mechanisms. Furthermore, as drugs of a similar class have the same mechanism of action, cross-resistance between drugs within the same class is often expected.

The most common mechanism of antimicrobial resistance is the production of enzymes that inactivate or modify the antibiotic (Medeiros 1997). Examples include the production of β-lactamases by many Gram-positive and Gram-negative organisms as well as aminoglycoside-modifying enzymes in Gram-negative pathogens (Livermore et al. 2001). Within the Gram-negative bacteria, many different β-lactamases have been identified. While some classes of β-lactamases may cause degradation of an entire class of β-lactam antibiotic (e.g., penicillinase,

cephalosporinase, carbapenemase), others are more specific to a smaller group of antibiotics (e.g., development of resistance to 3rd generation cephalosporins in certain *Klebsiella* species) (Livermore et al. 2001). Apart from protecting the producing bacteria against β-lactam antibiotics, the β-lactamase of *M. catarrhalis* can also have indirect pathogenic effects by blocking antibiotic therapy of concomitant infections with more dangerous respiratory pathogens such as pneumococci, as suggested by Wardle (1986) and as experimentally confirmed by Hol et al. (1994).

An alteration in the target site is another common mechanism through which bacteria become resistant to antibiotics. β-lactam antibiotics bind to PBPs, enzymes involved in cell wall synthesis of bacteria. By binding to PBPs, the antibiotic interferes with cell wall synthesis, resulting in inhibition of bacterial cell division. Changes in PBPs have resulted in the development of penicillin-resistant *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus.* Similarly, an alteration in DNA gyrase, the target site of quinol1 activity, is responsible for resistance in Gram-negative bacteria.

Antibiotic Resistance Testing

Of the various tests for the detection of β-lactamases, a direct test is feasible in species where few enzyme types occur and where enzyme production has clear implications for therapy (Livermore and Brown 2001). For example, the nitrocefin test is a chromogenic cephalosporin that changes from yellow to red upon hydrolysis (Livermore and Brown 2001). It is the most sensitive test for most β-lactamases.

The clinical goal of antimicrobial susceptibility testing is to predict the *in vivo* success or failure of antibiotic therapy. Tests are designed to measure the growth response of an isolated organism to a particular drug or drugs under standardized conditions. The results of antimicrobial

susceptibility testing should be combined with clinical information and experience when selecting the most appropriate antibiotic (Thornsberry 1991). The disk-diffusion method (Kirby-Bauer disk assay) is more suitable for routine testing in a clinical laboratory where a large number of isolates are tested for susceptibility to numerous antibiotics (Thornsberry 1991). An agar plate is uniformly inoculated with the test organism and a paper disk impregnated with a fixed concentration of an antibiotic is placed on the agar surface (Thornsberry 1991). Growth of the organism and diffusion of the antibiotic commence simultaneously resulting in a circular zone of inhibition in which the amount of antibiotic exceeds inhibitory concentrations (Thornsberry 1991). The diameter of the inhibition zone is a function of the amount of drug in the disk and susceptibility of the microorganism (Thornsberry 1991). This test must be rigorously standardized because zone size is also dependent on inoculum size, medium composition, temperature of incubation, excess moisture and thickness of the agar (Thornsberry 1991). If these conditions are uniform, reproducible tests can be obtained and zone diameter is a function of the susceptibility of the test organism. Zone diameter can be correlated with susceptibility as measured by the dilution method. Further correlations using zone diameters allow for the designation of an organism as clinically "susceptible", "intermediate", or "resistant" to concentrations of an antibiotic which can be attained in the blood or other body fluids of patients requiring chemotherapy (Livermore et al. 2001).

The susceptibility category implies that an infection may be appropriately treated with the usual dosage of the antimicrobial agent recommended for the type of infection present clinically. The resistant category predicts possible failure of the antimicrobial agent (Thornsberry 1991). Resistant strains are not inhibited by the usually achievable systemic concentrations of the agent with normal dosage schedules and/or fall in the range where specific microbial resistance

mechanisms are likely and/or where clinical efficacy has not been reliable in treatment studies (Thornsberry 1991). The intermediate category provides a buffer zone between the susceptible and resistant categories. It is intended to avoid major discrepancies in interpretation due to small, uncontrolled technical factors in testing (Thornsberry 1991). It should also be noted that susceptibility and resistance is a continuous scale, and that some organisms fall in a "gray zone" which is difficult to categorize at 1 end of the spectrum. Organisms in this category may or may not respond to therapy with the tested agent, depending on many factors, which include the site of the infection and the ability to increase the dose of the agent (Thornsberry 1991).

This study was conducted in order to assess the susceptibility of *Moraxella catarrhalis* isolates from the Johnson City VAMC. The susceptibility information was used to create a profile of the isolates for further study. Profiles were analyzed statistically to uncover isolates that fall out of the normal range of susceptibility. Those isolates were classified as deviant and require further analysis, emphasizing the purpose of the study and the question. The question I sought to answer was, what is the magnitude of phenotypic variation in antibiotic profiles within a bacteria population?

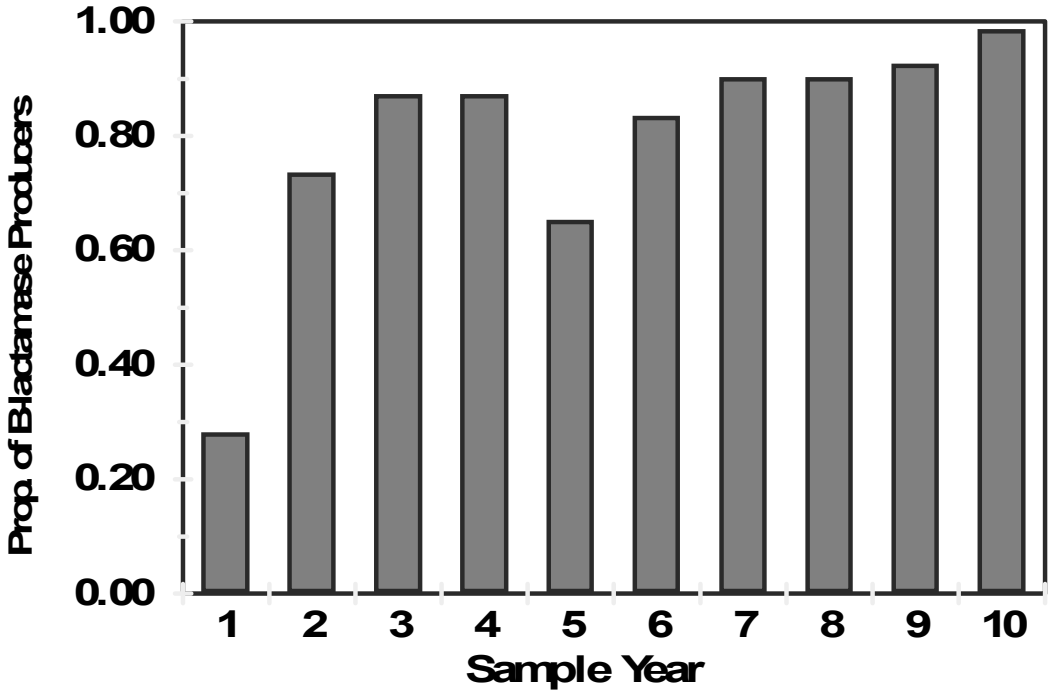

Figure 1. Proportion of β-lactamase Producers Among the VAMC Population

Figure Legend: Sample years correspond to collection years. 1= 1984-1985, 2= 1985-1986, 3= 1986-1987, 4= 1987-1988, 5= 1988-1989, 6= 1989-1990, 7= 1990-1991, 8= 1991-1992, 9= 1992-1993, 10= 1993-1994.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Cultures

The vast majority of *M. catarrhalis* strains in the James H. Quillen Veterans Affairs Medical Center (VAMC) collection were isolated from sputum samples of patients (Walker et al. 2000). The collection includes over 1000 isolates that were obtained during a 10-year time period (1984-1994). An additional 40 isolates from previous years (1983-1984) and 40 isolates from subsequent years (1994-1998) were available for testing. Isolates from the 10-year period have been intensely studied (Walker et al. 1998; Walker et al. 2000; Walker and Levy 2001), while the pre and post-dated isolates were not subjected to Kirby Bauer disk assay antibiotic testing.

Strains in the collection were frozen in skim milk to prevent desiccation and stored at -70°C. Aliquots of cells from frozen culture were used to inoculate Todd Hewitt (TH) agar plates and incubated overnight at 35°C.

Cefinase Disk Assay for β-lactamase Activity

Nitrocefin disks, ("Cefinase"; Becton Dickson, Sparks, MD) were used to assay β-lactamase activity. Cefinase disks were labeled with strain number corresponding to VAMC collection number, placed on sterile aluminum foil, and moistened with 1 drop of sterile dH_2O. An inoculating loopful of cells was then spread directly from a plate onto a Cefinase disk and the reaction was allowed to proceed for a maximum of 15 minutes. A positive reaction, observed as a color change from yellow to red, was interpreted as indicating β-lactamase production (Fig. 2).

Kirby Bauer Disk Assay for β-lactamase Sensitivity Tests

Each of 40 strains from the 10-year VAMC collection of *M. catarrhalis* was tested for susceptibility to 11 different β–lactam antibiotics, 7 of which were cephalosporins and 4 were non-cephalosporins. The following cephalosporins were tested: ceftazidime (Becton Dickinson, Cockeysville, MD), cefaclor (Becton Dickinson, Cockeysville, MD), cefixime (Becton Dickinson, Sparks, MD), ceftriaxone (Difco, Detroit, MI), cefotaxime (Difco, Detroit, MI), cefuroxime (Becton Dickinson, Cockeysville, MD), and cefamandole (Becton Dickinson, Cockeysville, MD). Non-cephalosporins included: penicillin G (Becton Dickinson, Sparks, MD), amoxicillin/clavulanic acid (Becton Dickinson, Sparks, MD), aztreonam (Becton Dickinson, Sparks, MD), and imipenim (Difco, Detroit, MI).

The Kirby Bauer disk assay was used in all antibiotic testing according to NCCLS guidelines (NCCLS, 1997). Using a sterile inoculating loop, *M. catarrhalis* colonies were transferred from plates into sterile 10 ml tubes and mixed by vortexing for 15-20 seconds. To standardize the number of cells in each antibiotic assay, turbidity of the suspension was visually adjusted with sterile 0.85% NaCl to approximately that of a 0.5 McFarland turbidity standard (1.5×10^8 CFU/ml).

Within 15 minutes of adjusting the inoculum to a McFarland 0.5 turbidity standard, sterile cotton swabs were dipped into the suspension and rotated against the wall of the tube above the liquid to remove excess suspension. Cotton swabs were used to inoculate agar plates by swabbing each plate 3 times, rotating the plate approximately 60° between swabs to ensure even distribution. The sides of the petri plate were avoided and care was taken to prevent aerosols. The inoculum was allowed to be absorbed for at least 3 minutes but no longer than 15 minutes before applying Kirby Bauer disks (Fig. 3).

Kirby Bauer disks were applied to the agar surface by using a dispenser and applying gentle pressure with sterile forceps to ensure complete contact of disk with agar. Disks impregnated with different antibiotics were separated by a minimum of 24 mm from center to center and no more than 5 disks were placed on a 100 mm plate.

Plates were incubated for 16-18 hours at 35°C in an ambient-air incubator. Susceptibility was measured only if a lawn of bacteria was present. To score susceptibility, plates were rested lid down on a black non-reflecting surface and the diameter of the inhibition zone was measured to the nearest whole millimeter by holding a caliper micrometer against the back of the plates. Plates were examined visually for isolated colonies within the inhibition zone that may have represented resistance. Because plates contained bacterial cells from a single strain of *M. catarrhalis*, multi-drug profiles were easily assembled.

Statistical Analysis

The multi-drug profile was used as a method for inferring genetic variation and highlighting isolates for further sequence analysis and determining possible variation among and within antibiotics. A strain was considered significantly different from others in its susceptibility if its inhibition zone was greater than 2 standard deviations from the mean. To assess concordance of multi-drug profiles, strains showing significant deviation from means were evaluated in a non-quantitative manner. For example, if 2 strains showed significant deviation from means in regard to the same antibiotic, then the remainder of the profile was examined to determine if the 2 strains showed similar susceptibilities to the remaining antibiotics. To compare β-lactamase producers with β-lactamase non-producers, 2-sample t-tests, and confidence intervals were computed using Minitab (Minitab, Inc. 1993). Grubbs outlier test was also

performed in order to assess if there existed any isolates detected as deviants other than those identified by 2 standard deviations (Graphpad, Inc.).

Haplotype Analysis

Haplotype profiles were previously studied for the VAMC isolates tested. Of those some of the isolates selected for analysis had identical multi-locus genotypes. Each of 8 genotypes was represented by 2 or 3 isolates. Isolates representing 1 genotype were all β-lactamase non-producers, isolates representing 6 genotypes were all β-lactamase producers (Table 1). Genotype 109CC was represented by 2 β-lactamase non-producers (isolates #604; #907) and 1 β-lactamase producer (isolate #830) (Table 1).

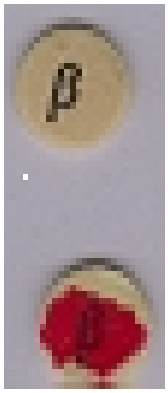

Figure 2. Nitrocefin Disk Assay for β-lactamase Activity. A red color change indicates that cleavage has occurred and that a β-lactamase producer is present.

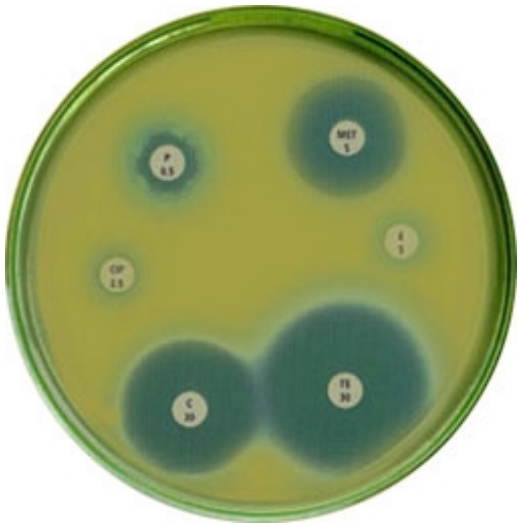

Figure 3. Kirby Bauer Disk Assay. Antibiotic susceptibility plates showing antibiotic disks placed on an agar plate incubated with a lawn of bacteria. The "halo" around the disk indicates the susceptibility of the bacteria to that particular antibiotic. A small zone indicates resistance and a large zone indicates susceptibility.

CHAPTER 3

RESULTS

Multi-Antibiotic Profiles

A total of 14 separate antibiotic profiles were found among the 41 isolates tested. Many of the isolate profiles were typical which means that inhibition zones produced in response to any of the antibiotics did not deviate from the mean by more than 2 standard deviations. Among the β-lactamase producers, there were 8 different profiles consisting of 1 typical and 7 deviant profiles (Table 1). The deviant profiles included antibiotic-specific reductions in susceptibility, including: a cefuroxime specialist (#250); isolates with low susceptibility to amoxicillin/ clavulanic acid (#830 & 785); a cefixime specialist (#417); an isolate especially susceptible to ceftazidime (#691); and an aztreonam and cefamandole specialist that was also sensitive to cefotaxime (#566). The profile also included multi-specialists such as isolate #359 which was highly sensitive to aztreonam, ceftazidime, ceftriaxone, and cefotaxime, and isolate #813 which was highly sensitive to cefaclor, cefixime, ceftriaxone, cefotaxime, cefuroxime, cefamandole, and penicillin G.

Among the β-lactamase non-producers there were 5 unique profiles and 1 typical profile (Table 1). Isolate #604 had high resistance to cefuroxime, while isolate #123 showed significantly lower susceptibility to ceftazidime, and isolate #444 was more sensitive to aztreonam. Isolate # 347 appears to have been a multi-specialist with increased sensitivity to both cefaclor and cefamandole. Isolate #474 appeared unique in showing resistance to amoxicillin/clavulanic acid, with sensitivity to ceftriaxone and imipenem.

Comparisons of β-lactamase Producers and Non-Producers

β-lactamase producers were significantly less susceptible than non-producers to 9 of the 11 antibiotics (Table 2). Only ceftazidime and cefotaxime had similar sized inhibition zones in producers and non-producers (Table 2). Among the non-cephalosporin antibiotics, only the response to aztreonam was not significantly different between the β-lactamase producers and β-lactamase non-producers (Table 2).

Outlier Test

Grubbs' outlier test identified isolates that were outliers relative to the means. Isolates with large z values are considered outliers. Among the non-producers, isolates #474, #123, and #347 were identified as outliers (Table 3). Among the producers, isolates #691, #056, #359, and #813 were identified as deviant isolates by the Grubbs' test (Table 3). Isolates with large deviant patterns include isolate #813, which was an outlier for 4 out of 11 antibiotics and was identified as farthest from the mean, but not quite significantly different in susceptibility to 1 other antibiotic (Table 3).

Haplotype Comparisons

Many isolates were grouped in pairs based on identical multi-locus genotypes (Walker and Levy, 2001). Letter "A" denotes genotype #444DC, which contains 2 unique isolates, #444 and #546. Isolate #546 had a typical profile while isolate #444 showed increased susceptibility to the antibiotic aztreonam. Letter "B" denotes genotype 109CC, which consisted of 3 separate isolates, #604, #830, and #907. Isolates #907 and #604 were both β-lactamase non-producers and isolate #830 was a β-lactamase producer. Isolate #907 displayed a typical profile, while isolate #604 showed decreased susceptibility to cefuroxime. Letter "C" identified isolates #813 and #417 that were both β-lactamase producers and showed deviant susceptibility profiles. Isolate

#813 displayed a deviant profile with increased susceptibility to 7 of 11 antibiotics (cefaclor, cefamandole, cefixime, cefotaxime, ceftriaxone, cefuroxime, and penicillin G). Isolate #417 displayed increased susceptibility to only 1 antibiotic (cefixime). Letter "D" denoted haplotype 396AB, which contained isolates #566 and #785. Isolate #566 displayed decreased susceptibility to both cefamandole and aztreonam and displayed increased susceptibility to cefotaxime. In contrast, isolate #785 displayed increased resistance to amoxicillin. Letter "E", haplotype 418CC, contained isolates #944, #770, and #980, which all displayed a typical antibiotic profile. Letter "F", haplotype 709CC, represented by isolates #712 and #709 also displayed a typical antibiotic profile.

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Table 1. *Moraxella catarrhalis* Strains Tested for Susceptibility to 11 Antibiotics. Diameters of the inhibition zone in response to Kirby Bauer disk assays are shown in millimeters. Strain genotypes are also shown (Walker and Levy 2001). Strains are grouped by presence or absence of β-lactamase activity. Isolates are sorted beginning with those that are typical (no significant deviations for any antibiotics), followed by 6 groups of isolates with identical profiles. Note: A, B, C, D, E, and F denote 6 genotypes represented by 2-3 isolates each. Highlighted and italicized items fall beyond 2 standard deviations of either susceptibility or resistance. Red items represent isolates that test as both outliers (Grubbs', 2000) and fall greater than 2 standard deviations (sd) from the mean. Blue items represent isolates 2 standard deviations from the mean, but not detected by Grubbs' outlier test. Abbreviations: strain = *Moraxella catarrhalis* VAMC number; β-lac = β-lactamase positive or negative; Cefac = cefaclor; Cefam = cefamandole; Cefix = cefixime; Cefotax = cefotaxime; Ceftaz = ceftazidime; Ceftria = ceftriaxone; Cefuro = cefuroxime; Amox = amoxicillin /clavulanic; Aztre = aztreonam; Imipe = Imipenem; Penic = penicillin G; Geno = genotype; Id = Isolates with the same letter represent isolates with identical genotypes.

	Strain	β-lac	Cefac	Cefam	Cefix	Cefotax	Ceftaz	Ceftria	Cefuro	Amox	Aztre	Imipe	Penic	Geno	Id
Typical Isolates	#907	Neg	37.0	29.0	26.0	32.3	40.0	30.0	36.0	35.0	24.8	35.4	28.0	109CC	B
	#150	Neg	33.0	35.6	34.0	29.0	42.6	44.0	29.5	46.0	30.0	46.0	37.7	144GE	
	#337	Neg	39.0	41.0	31.0	44.0	38.0	45.0	41.0	47.0	7.5	49.4	44.5	151DC	
	#190	Neg	41.4	40.0	36.0	46.0	46.5	49.2	36.0	44.0	32.0	50.4	44.0	190GE	
	#546	Neg	30.0	30.0	22.0	36.0	42.7	38.4	28.0	46.0	31.4	42.0	39.0	444DC	A
	#587	Neg	34.0	36.0	38.0	41.0	35.0	37.6	38.0	43.0	33.0	44.5	34.0	587AB	
	#474	Neg	40.0	38.0	42.0	31.0	46.0	***63.0***	42.0	***24.0***	18.0	***70.0***	40.0	474KG	
Deviant Isolates	#444	Neg	40.0	44.0	44.0	50.0	48.0	52.7	34.0	54.0	***56.0***	52.0	48.0	444DC	A
	#347	Neg	***54.0***	***60.0***	26.0	30.0	42.0	30.0	49.0	38.0	35.0	47.5	31.0	347AB	
	#123	Neg	33.0	38.0	22.0	27.6	***24.0***	25.6	30.0	40.5	35.0	39.5	40.0	123GE	
	#604	Neg	38.0	27.0	30.0	38.4	42.0	42.0	***14.0***	40.0	22.0	42.0	34.0	109CC	B
		Mean	**38.1**	**38.1**	**31.9**	**36.8**	**40.6**	**41.6**	**34.3**	**41.6**	**29.5**	**47.2**	**38.2**		
		Sd	6.1	8.6	7.2	7.2	6.4	10.5	8.7	7.4	11.6	8.6	5.8		
		+2 Sd	50.3	55.2	46.4	51.3	53.3	62.5	51.7	56.4	52.7	64.4	49.8		
		-2 Sd	26.0	20.9	17.4	22.4	27.9	20.6	16.9	26.8	6.3	29.9	26.6		

	Strain	β̃-lac	Cefac	Cefam	Cefix	Cefotax	Ceftaz	Ceftria	Cefuro	Amox	Aztre	Imip	Penic	Geno	Id
Typical Isolates	#844	Pos	12.0	15.0	24.0	30.0	40.0	24.2	23.3	34.6	23.5	32.0	0.0	067AB	
	#685	Pos	15.0	12.0	22.0	21.5	35.0	22.0	17.6	31.6	12.0	31.5	0.0	077AB	
	#430	Pos	24.0	10.0	27.0	33.8	40.0	24.0	27.0	40.0	30.5	39.4	5.3	098CC	
	#113	Pos	18.0	15.0	20.0	34.5	39.3	33.4	14.0	38.0	22.8	36.0	18.4	113CC	
	#119	Pos	21.2	15.0	29.0	28.0	36.6	28.0	24.5	37.0	30.0	34.0	12.0	119FB	
	#130	Pos	21.0	16.0	20.0	29.0	37.4	22.0	23.0	28.0	25.0	38.0	7.0	130FA	
	#856	Pos	20.0	12.0	22.0	23.0	37.5	24.0	24.0	35.0	25.0	36.0	0.0	130FC	
	#868	Pos	25.0	17.9	29.0	33.5	41.5	39.0	26.0	38.5	26.0	43.5	9.0	151AC	
	#543	Pos	15.0	12.0	23.0	27.0	38.0	25.0	20.0	31.0	25.0	41.0	9.0	206BC	

Table 1. Continued

Strain	β-lac	Cefac	Cefam	Cefix	Cefotax	Ceftaz	Ceftria	Cefuro	Amox	Aztre	Imip	Penic	Geno	Id
Deviant Isolates														
#339	Pos	25.0	10.0	23.0	30.0	37.0	28.5	21.7	33.0	26.0	38.3	0.0	327AB	E
#327	Pos	21.0	16.0	28.0	34.0	39.0	28.0	27.6	39.7	41.0	41.0	0.0	327AB	E
#980	Pos	20.0	15.0	24.0	21.0	36.0	23.0	12.0	33.0	20.0	37.0	0.0	418CC	E
#498	Pos	17.4	13.4	20.0	25.0	31.0	23.0	19.0	33.0	21.5	30.0	6.0	498AB	
#585	Pos	20.0	12.0	22.8	25.0	39.0	33.0	25.0	34.0	10.0	42.0	12.0	585CC	
#661	Pos	30.0	19.0	22.0	32.0	38.0	30.0	30.0	43.0	22.0	40.0	7.5	588DC	
#712	Pos	23.0	14.0	26.4	32.5	38.5	33.0	21.0	40.4	26.0	36.0	13.2	709CC	F
#709	Pos	18.5	15.0	22.0	28.8	38.0	26.8	25.0	36.0	23.6	33.0	12.0	709CC	F
#735	Pos	28.0	22.0	28.4	30.0	42.0	28.0	28.0	40.0	34.0	42.8	7.2	735DC	
#809	Pos	18.0	14.3	18.0	26.0	34.0	20.2	18.0	35.5	22.2	35.0	0.0	809EB	
#566	Pos	20.4	***5.0***	34.5	***44.0***	46.0	40.0	29.0	47.0	***7.0***	*46.5*	10.5	396AB	D
#359	Pos	22.0	20.0	22.0	***57.0***	***52.0***	***60.0***	18.0	38.0	***48.6***	*62.0*	16.0	359KG	
#691	Pos	14.0	12.4	30.0	34.0	***18.0***	33.0	25.0	38.0	24.0	42.0	10.0	206BC	
#813	Pos	***40.3***	***38.0***	***37.4***	***49.0***	38.1	***52.0***	***39.0***	24.0	32.0	50.6	***45.0***	151DC	C
#417	Pos	21.0	17.0	***36.0***	36.0	40.0	40.0	28.0	42.0	40.0	42.0	16.0	151DC	C
#830	Pos	18.7	15.7	28.0	40.0	42.6	34.0	25.0	***19.0***	27.0	39.0	17.0	109CC	B
#785	Pos	16.0	15.7	28.4	34.0	42.0	22.0	22.6	***18.0***	12.0	43.4	16.0	396AB	D
#250	Pos	10.0	12.0	23.0	26.0	24.0	22.2	***11.0***	30.0	16.0	26.0	0.0	077CC	
#056	Pos	20.0	9.0	26.0	24.0	38.3	22.0	18.0	30.0	32.6	***10.0***	0.0	056AB	
	Mean	**20.3**	**14.8**	**25.3**	**31.1**	**37.5**	**29.3**	**22.8**	**34.3**	**25.0**	**37.8**	**8.3**		
	Sd	5.7	5.5	4.8	8.1	5.9	9.3	5.6	6.4	8.8	8.5	9.2		
	+2 Sd	31.6	25.8	34.9	47.2	49.4	47.9	34.0	47.2	42.7	54.7	26.7		
	-2 Sd	8.9	3.9	15.7	15.0	25.6	10.7	11.6	21.4	7.3	20.9	-10.1		

Table 2. t-test Results Comparing Susceptibility Between β-lactamase Producers and Non-Producers in Response to 11 Antibiotics.

I. Non-Cephalosporins

Antibiotic Name	β-lactamase	Mean	N	df	t	P
Amoxicillin/Clavulanic Acid	Producer	34.28	30	1	2.79	0.014
	Non-Producer	41.59	11			
Aztreonam	Producer	25.00	30	1	1.12	0.28
	Non-Producer	29.50	11			
Imipenim	Producer	22.81	30	1	3.91	0.002
	Non-Producer	34.32	11			
Penicillin G	Producer	10.14	30	1	12.05	<0.001
	Non-Producer	38.20	11			

II. Cephalosporins

Antibiotic Name	β-lactamase	Mean	N	df	t	P
Cefaclor	Producer	20.27	30	1	8.13	<0.001
	Non-Producer	38.13	11			
Cefamandole	Producer	14.85	30	1	8.05	<0.001
	Non-Producer	38.05	11			
Cefixime	Producer	25.26	30	1	2.7	0.018
	Non-Producer	31.91	11			
Cefotaxime	Producer	31.21	30	1	2.09	0.050
	Non-Producer	41.59	11			
Ceftazidime	Producer	37.51	30	1	1.36	0.190
	Non-Producer	40.62	11			
Ceftriaxone	Producer	29.31	30	1	3.29	0.005
	Non-Producer	41.60	11			

Table 2. Continued

Cefuroxime	Producer	22.81	30	1	3.91	0.002
	Non-Producer	34.32	11			

Table 3. Isolates Showing Deviant Susceptibility Patterns Based on Grubbs' Outlier Test. Isolates are Sorted Based on β-lactamase Activity.

β-lactamase $^-$	**Genotype**	**ID #**	**Deviant Pattern**
Single Deviants	474KG	474	Imipenem susceptibility
	123GE	123	Ceftazidime resistance
Double Deviants	347AB	347	Cefaclor & Cefamandole susceptibility

β-lactamase $^+$	**Genotype**	**ID #**	**Deviant Pattern**
Single Deviants	206BC	691	Ceftazidime resistance
	056AB	056	Imipenem resistance
Double Deviants	359KG	359	Cefotaxime & Ceftriaxone susceptibility
Triple Deviants	151DC	813	Cefixime, Cefamandole, Cefaclor & Penicillin G susceptibility

CHAPTER 4

DISCUSSION

Purpose of Antibiotic Profiles

Antibiotic profiles were used to assess the susceptibility of *M. catarrhalis* isolates to a series of β-lactam antibiotics. β-lactamase is the primary antibiotic resistance factor for β-lactam antibiotics. Isolates that tested positive for β-lactamase production all displayed some degree of resistance, but there was evidence of variation in the resistance profiles among those β-lactamase positive isolates. Variation among β-lactamase producing isolates indicates that there must be a difference in the activity of the β-lactamase or other factors must influence resistance. For example, recent reports suggest that additional variation in BRO β-lactamase and/or in non-β-lactamase factors may underlie novel susceptibility patterns (Baquero1996; Berk and Kalbfleisch 1996). Other factors that may underlie antibiotic resistance include alterations in the target molecules that prevent interaction with the drug and/ or impermeability of the cell.

Livermore et al. (2001) has offered a protocol for using antibiotic susceptibilities to guide strategies to manage resistance. Livermore suggested that first susceptibility testing be performed in order to determine those isolates that are resistant. Resistant isolates should then be subjected to an extensive battery of antibiotic tests (Livermore et al. 2001). The use of indicator drugs to detect the presence of a mechanism that gives resistance not only to the indicator itself, but also to related agents is another suggestion for detecting those isolates that are antibiotic resistant mutants (Livermore et al. 2001). Livermore further suggested that the information gathered from antibiotic profiles should be used to determine resistance patterns, especially indicating that β-lactams are ideal drugs for discovering deviants.

This study used β-lactam antibiotics in a profile analysis of isolates from the VAMC. I suggest that deviant isolates may also be tested as suggested by Livermore in an effort to determine their resistance mechanism. Livermore suggested that ceftazidime can be used as an antibiotic indicator for most of the TEM and SHV-derived extended-spectrum β-lactamases (ESBL) types, while cefotaxime resistance is a better indicator for the CTX-M type enzymes in other countries (Livermore et al. 2001). From the VAMC, isolates and #691 of the β-lactamase producers showed increased resistance to ceftazidime hinting to the possibility of some variant alteration of the BRO β-lactamase, while isolate #123 of the β-lactamase non-producers showed decreased susceptibility to ceftazidime.

Possible Modes of Antibiotic Resistance

The most frequent explanation for intrinsic antimicrobial resistance is explained by decreased accumulation of the antibiotic or impermeability (Thornsberry 1991). Impermeability to some β-lactam antibiotics may also be mediated by bacterial modifying enzymes that do not inactivate the compounds but rather bind to them and alter their structures (Thornsberry 1991). Alterations in antibacterial target molecules that prevent interaction with the antibiotic represents 1 of the most important mechanisms to clinically used antibiotics (Thornsberry 1991). The cellular targets of β-lactam antibiotics are the penicillin binding proteins, and alterations in the binding sites are known to affect resistance to β-lactam antibiotics. Each of these mechanisms work together to confer a certain degree of antibiotic resistance and sometimes they work in conjunction with the β-lactamase enzyme.

Isolates that have the β-lactamase enzyme show resistance, but variation was present among the different β-lactamase positive isolates. In analyzing the susceptibility profiles to

penicillin, the only isolate that had a deviant profile was isolate #813. Isolate #813 also had a deviant profile for cefaclor, cefamandole, cefixime cefotaxime, ceftriaxone, and cefuroxime with statistically significant decreased resistance to those antibiotics. The profile of #813 suggests an additional factor that confers resistance among the β-lactamase positive isolates tested. Isolates # 813 and #359 should be subjected to sequencing of the β-lactamase gene in order to determine if alterations exist in their ß-lactamases.

Profiles in Genetically Identical Isolates

Comparisons of genetically identical isolates offer information on potential differences in β-lactamase activity. Isolates denoted by AA, BB, CC etc. in table 1 refer to groups of genetically identical isolates. There are evident differences in the susceptibility profiles of some genetically identical isolates. For example, among the β-lactamase positive isolates, there were 7 different antibiotic profiles. Within those profiles the most profound discovery involved 3 isolates that were genetically similar but differed in their β-lactamase activity. Genotype 109CC, (Table 1) included isolates #604 and #907 that were β-lactamase negative and isolate #830 that was β-lactamase positive. This genotype encompassed the entire spectrum of antibiotic resistance by phenotypes. Within genotype 109CC, isolate #604, a β-lactamase non-producer, showed relatively low susceptibility to cefuroxime but the remainder of its profile appeared similar to the genetically identical isolate #907. Isolate #907 had a profile that was typical of β-lactamase non-producers. Isolate #830 had a profile similar to other β-lactamase producers with the exception of decreased susceptibility to amoxicillin/clavulanic acid. The antibiotic specific increase in resistance suggests a mutated β-lactamase gene may be present in isolate #830.

Genotype 418CC, represented by isolates #944, #770, and #980 and genotype 709CC, represented by isolates #709 and #712, were each β-lactamase producers with typical profiles and no deviant isolates. In contrast, genotype 151DC was represented by isolates #813 and #417, which were also β-lactamase producers, but they represent a different end of the spectrum. Isolate #813 as previously mentioned, has an increased susceptibility to 7 out of the 11 antibiotics tested, while isolate #417 displays increased susceptibility only to cefixime. The differences in profiles between isolates #813 and #417 continues to suggest that alterations in target site and/or decreased permeability may also work with the β-lactamase enzyme to aid in conferring antibiotic resistance.

Alternative Hypotheses and Expectations

Alternative hypotheses for the role of β-lactamase susceptibility include the scenario of β-lactamase as the sole determinant of β-lactamase resistance, in which case the susceptibilities of β-lactamase positive and β-lactamase negative isolates are not expected to overlap (Figure 4a). The alternative hypothesis that β-lactamase has no influence on resistance predicts complete overlap of the susceptibilities (Figure 4b). A 3rd hypothesis is that if β-lactamase has some influence on the susceptibility, but it is not the sole determinant, then you would expect to see partial overlap. The susceptibility tests showed overlap in susceptibility, especially when comparing the cephalosporin antibiotics such as cefamandole with penicillin G (Fig. 5). The non-cephalosporin, amoxicillin/clavulanic acid, used clinically as Augmentin also showed overlap of its susceptibility profiles indicating a β-lactamase affect with additional determinants that aid in explaining resistance to that antibiotic (Fig. 6). Also, in 9 of the 11 the antibiotic profiles compiled from the VAMC isolates, there existed overlap between the ß-lactamase producers and

non-producers, suggesting that β-lactamase is not the only determinant of resistance in the isolates tested. If other determinants were not essential to the resistance profiles we should expect that there would not be any difference between the isolates and how they react with the antibiotics.

Suggestions for Future Projects

Those isolates determined to be different from the typical isolate (#831, #359, etc.) based on their antibiotic profile should be further analyzed. I suggest that the isolates determined to be ß-lactamase producers and that display unusual profiles should have their β-lactamase gene sequenced to determine the type of β-lactamase gene present. Based on the fact that 2 alleles are known that confer β-lactamase activity in *M. catarrhalis,* determining the type of allele present in those deviant isolates might offer important information about resistance.

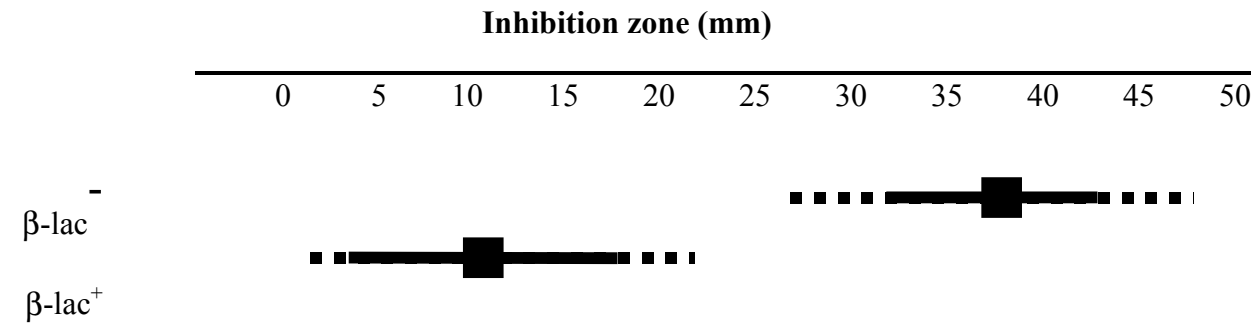
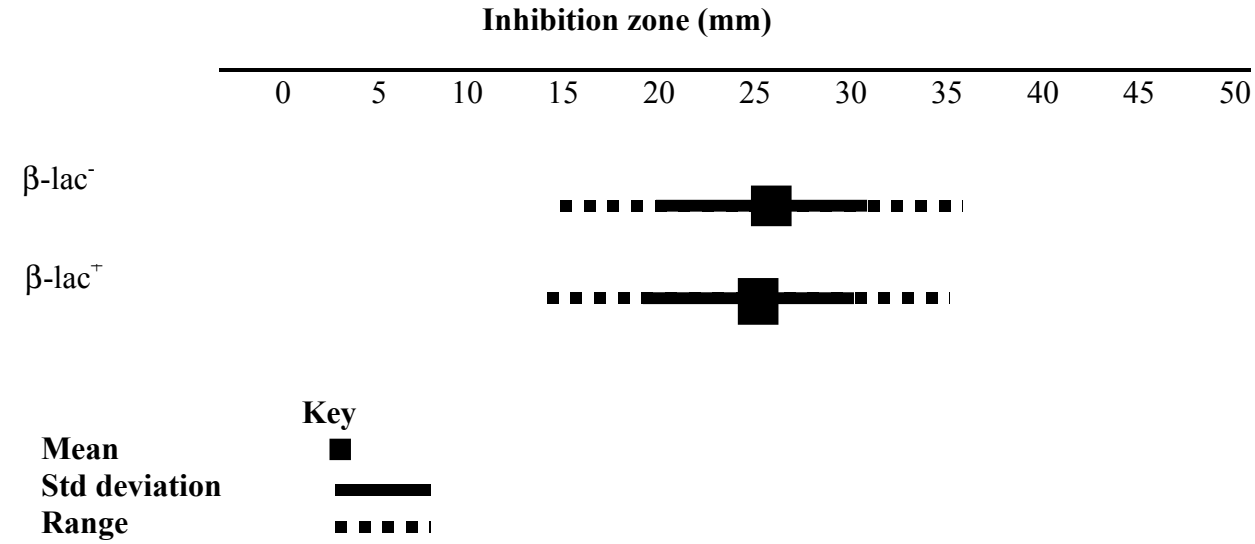


Figure 4: Diagrammatic Depiction of Expected Patterns in Susceptibility under Alternative Hypothesis for Role of β-lactamase in Resistance

a. Hypothesis 1: β-lactamase as Sole Determinant



b. Hypothesis 2: b-lactamase has No Effect

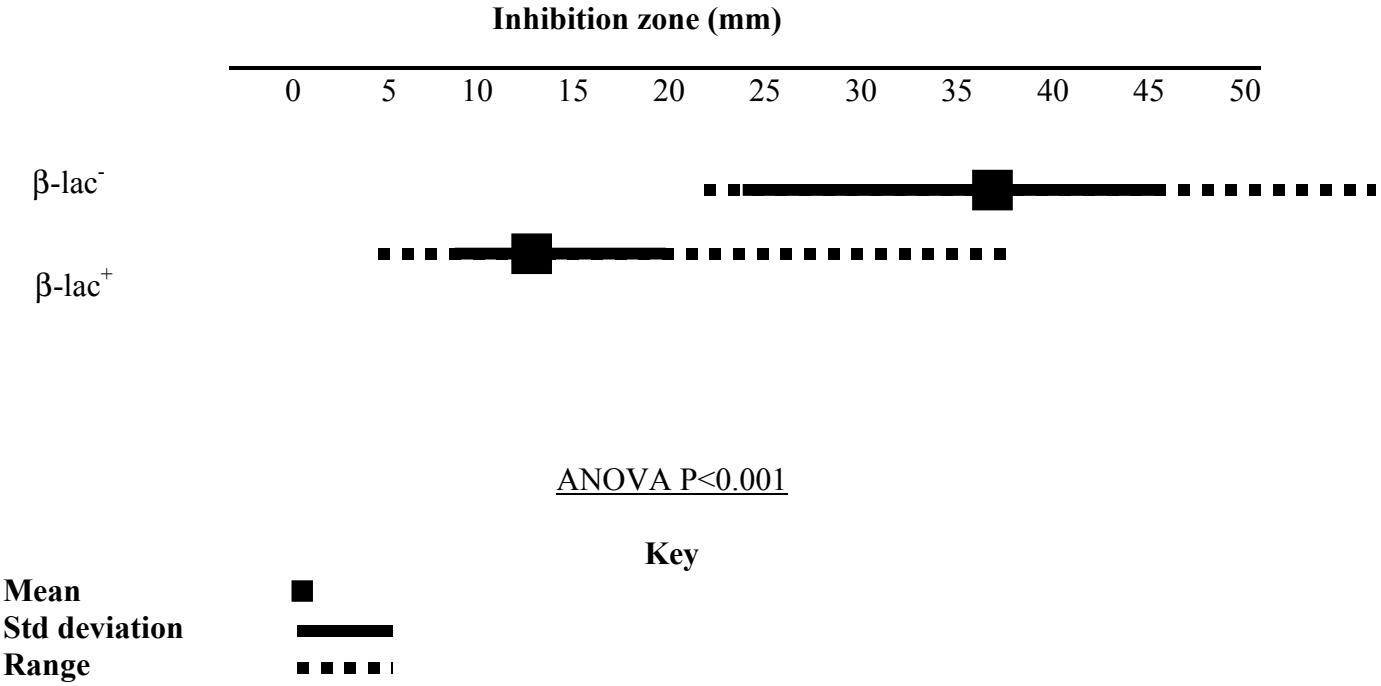


Figure 5. Cefamandole Antibiotic Susceptibility Profile.Profile indicates a major β-lactamase effect between the producers and non-producers.

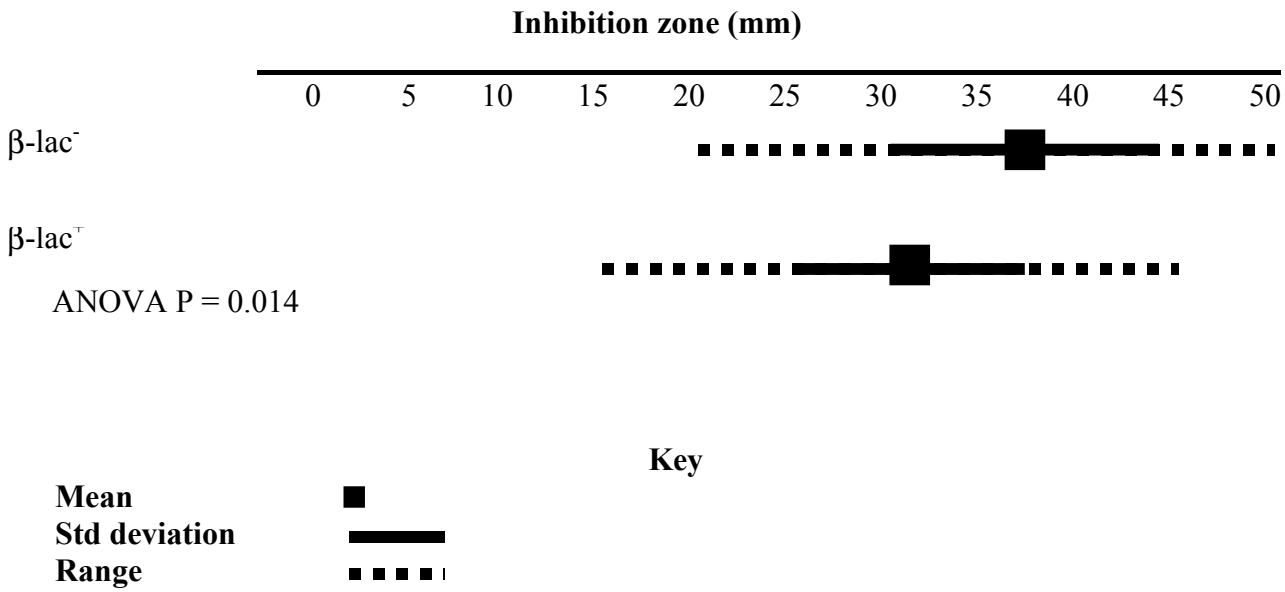


Figure 6. Amoxicillin/clavulanic Acid Antibiotic Response Shows overlap in inhibition zones of β-lactamase producers and β-lactamase non-producers

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