A Comparison of Disc Diffusion and Microbroth Dilution Methods for the Detection of Antibiotic Resistant Subpopulations in Gram Negative Bacilli

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Abstract

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Two of the different types of antibiotic susceptibility tests available are the solid media based Kirby Bauer method and the liquid media based microbroth dilution method. The microbroth dilution method has become more widely used due to its ability to become automated, however, the Kirby Bauer method may be able to detect subpopulations of resistant bacteria that would be visualized as inner colonies in the zone of diffusion.

Using Gram negative rod isolated collected from the UWMC clinical microbiology laboratory, the presence of inner colonies was screened and both methods were compared to determine if the microbroth dilution method was able to detect the additional resistance of the subpopulations. The data went to show that the microbroth dilution method was not detecting the additional resistance in about 68% of the isolates tested. It was unable to be determined if the results were due a limitation of the microbroth dilution method or whether it is an artifact of the testing methods, but it does open up the possibilities of further testing to determine the cause of the disparity in results.

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Introduction

Since the discovery of penicillin in 1928 by Alexander Fleming, antibiotics have been used extensively to treat many bacterial illnesses and have saved millions of lives. For the last 80 years, antibiotics have evolved from simple naturally derived substances like penicillin, which has a relatively narrow spectrum of activity to more complicated synthetic antibiotics that target a multitude of bacteria [14,22]. Unfortunately bacteria have also evolved alongside the antibiotics that target them. Just as there are a variety of different mechanisms of antimicrobial action, bacteria have developed different mechanisms of resistance. These range from a simple change in the target site to developing enzymes that deactivate or destroy the antimicrobial agent itself. Antibiotic susceptibility testing (AST) is therefore one of the most important and valued services provided by clinical microbiology laboratories.

The determination of antimicrobial susceptibility can be performed in either a quantitative or qualitative fashion. The former involves determination of the minimum inhibitory concentration (MIC) of the organism being tested. The MIC is defined as the lowest concentration of antibiotic required to inhibit growth of the organism [14,16,22]. A series of doubling dilutions of the antibiotic to be tested are made in nutritive broth and the organism in question is inoculated into each dilution. The lowest dilution that exhibits visible inhibition of growth represents the MIC. The meaning of MIC value itself is interpreted against published standards yielding a qualitative result of resistant, intermediate, or sensitive as well as giving the treating physician quantitative data on the degree of resistance or sensitivity allowing them to optimize therapy. In contrast, the disc diffusion method is a strictly qualitative method where a lawn of bacteria of a specific concentration is inoculated onto an agar plate and paper discs impregnated with antibiotics are placed onto the surface. As the antibiotics diffuse out of the disc and into the agar, a diffusion gradient is formed [16,22]. The Clinical and Laboratory Standards

Institute (CLSI) is an organization that promotes the development and the use of these voluntary consensus standards. In the case of antibiotic susceptibility testing, the CLSI establishes a breakpoint that incorporates pharmacokinetic modeling, outcome studies, animal models, and MIC distribution data from wild type organisms. The antibiotic breakpoints are used to determine which MIC values correlate with whether an organism is sensitive or resistant to a given antibiotic.

Each method has its associated advantages and disadvantages. As the disc diffusion method offers the ability to view growth on the plate rather than growth in a tube or well, inner colonies can be visualized within the zones of inhibition. These inner colonies are believed to be subpopulations of the original strain that exhibit increased antibiotic resistance thus allowing them to grow closer to the disc i.e. where the antibiotic concentrations are higher [16,22]. In addition, the disc diffusion test is also relatively easy to setup and inexpensive. However, the disc diffusion method must be visually read and does not provide quantitative data. For quantitative data, tests like the micro and macrobroth dilution methods are available as well as agar based MIC methods such as the agar dilution and the E-test methods.

Mechanisms of Action for Major Antibiotic Classes

Different antibiotics classes target different mechanisms in bacteria and interfere with critical biochemical pathways. These include cell wall synthesis, protein synthesis, and DNA replication. Beginning with the development of penicillin for medicinal use in the 1940's, antibiotics have been used to treat a multitude of bacterial infections. The development of a wide variety of different antibiotic classes has enabled physicians to increase the effectiveness of treatment and successfully treat infections with organisms resistant to other classes of antibiotics

β-lactams antibiotics

β-lactam antibiotics prevent peptide crosslinking in the peptidoglycan layer, inhibiting synthesis of the bacterial cell wall. Reduction of the peptidoglycan layer destroys the integrity of the structure making the cell susceptible to lysis. Enzymes called penicillin-binding proteins (PBPs) catalyze the pentapeptide crosslinking [22,30]. Penicillins and other β-lactams are designed to bind to the penicillin-binding proteins and inactivate them, thus preventing completion of cell wall synthesis. Because these antibiotics work on cell wall synthesis, actively replicating cells are affected more than non-replicating cells.

β-lactam antibiotics belong to a large class comprised of multiple subclasses that share the β-lactam ring in their chemical structure. However these subclasses have different spectra of activity with regards to the range of bacteria that they are active against [30]. β-lactams include penicillins, monobactams, cephalosporins, and carbapenems. Penicillins are the oldest sub-class of the β-lactams and were initially used to treat a wide variety of bacterial infections in the early years following their development. As time passed, chemical variants of penicillins were

developed, increasing the effectiveness of treating certain types of infections for which penicillin showed less efficacy. For example, infections caused by certain gram negative bacilli like *Pseudomonas aeruginosa* do not respond to treatment by penicillins because of intrinsic resistance. Over many years of use, bacteria have developed resistance to penicillin and other variants of penicillins by developing penicillinases. These are inactivating enzymes that degrade penicillins upon entering the cell by cleaving the β-lactam ring. To counteract such resistance, antibiotics were subsequently developed that included penicillinase inhibitors. These include compounds such as clavulanic acid and sulbactam which mimic the structure of penicillins and competitively inhibit penicillinases [7].

Cephalosporins were developed in 1948 in Italy and are even less susceptible to activity by penicillinases [30]. Cephalosporins can be grouped into 5 different generations which are classified by their antimicrobial spectra. The earlier generations were the first to be developed and are generally active against a narrower spectrum of bacteria than the more broad-spectrum generations that were developed later. The later third and fourth generations (e.g. ceftriaxone, cefepime, etc.) exhibit increased activity towards gram-negative organisms that have more intrinsic resistance to earlier first and second generation cephalosporins (e.g. *Enterobacter* species). The difference in each of the generations of cephalosporin results from the differing functional groups that are attached to the base β-lactam ring. Newer generations have functional groups that effectively block the β-lactam ring from activity by certain β-lactamases making them better suited to treat gram-negative infections [28]. The sole fifth generation cephalosporin is ceftaroline, which has the increased coverage against gram-positive bacteria while retaining the gram negative coverage of the later generations of cephalosporins. Importantly, it is the only β-lactam antibiotic to have activity against methicillin-resistant *Staphlyoccus aureus* (MRSA).

Monobactams are a novel class of β-lactams. The only commercially available monobactam is an antibiotic called aztreonam. Monobactams are only effective against gram negative bacteria and have no effect on gram positive organisms or anaerobes. Aztreonam is generally used in patients who are allergic to penicillins or who cannot tolerate aminoglycosides [30]. Carbapenems are one of the most recent classes of β-lactam antibiotic to have been developed. They have the broadest spectrum of activity among the β-lactams and therefore are considered one of the antibiotics of last resort, often used to treat infections that are resistant to most other antibiotics [25,30].

Aminoglycosides

Unlike β-lactams, which target cell wall synthesis, aminoglycosides (e.g. amikacin, gentamycin) inhibit bacterial growth by acting as protein synthesis inhibitors. Aminoglycosides also can interfere with the cell wall stability due to their cationic properties [2,30]. Aminoglycosides are generally used against aerobic gram negative infections by organisms such as *Escherichia coli*, *Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*. Aminoglycosides are commonly used in a synergistic manner by combining therapy with an antibiotic that has activity against the bacterial cell wall, most commonly a β-lactam [20,17,30].

Fluoroquinolones

Fluoroquinolones (e.g. ciprofloxacin, levofloxacin) are a class of antibiotics with broad-spectrum activity. Fluoroquinolones inhibit bacterial growth by inhibiting DNA synthesis. The

targets for these antibiotics are DNA gyrase in gram negative organisms and DNA topoisomerase IV in gram positive organisms [30].

Mechanisms of Antibiotic Resistance

Just as there are different classes of antibiotics that target different functions of the bacterial cell, bacteria have developed/evolved a variety of mechanisms for resistance against antibiotics. These mechanisms range from a relatively simple target change, to producing hydrolyzing enzymes that cleave the antibiotic compounds themselves. Some types of resistance are intrinsic to specific organisms, (e.g. ampicillin resistance in *Klebsiella pneumoniae*) while others develop resistance by acquiring mutations or novel genes. Antibiotic resistance mechanisms can be inducible by selective pressure by certain antibiotics or they can be constitutively expressed conferring resistance without the need to have antibiotics present in the environment.

AmpC β-lactamases

β-lactamases are hydrolyzing enzymes that cleave the β-lactam ring rendering the compound unable to bind to their target i.e. penicillin binding proteins. The AmpC β-lactamase is located on a chromosomally encoded gene (*ampC*) found in a number genera of gram negative bacilli. The AmpC β-lactamase is a cephalosporinase that confers resistance to many β-lactams

including most of the cephalosporins, but does not have activity against the 4th generation cephalosporin cefepime. Carbapenems are also not cleaved by the AmpC β-lactamase. In a number of bacterial genera, expression of the *ampC* gene is inducible. The acronym SEA-CHIMP is used to denotate these organisms that contain an inducible *ampC* gene *(Serratia, Enterobacter, Aeromonas, Citrobacter, Halfnia*, Indole-positive *Proteus, Morganella*, and *Pseudomonas aeruginosa*) [13,16].

Benzylpenicillins, ampicillins, amoxicillins, and most cephalosporins are strong inducers and good substrates for AmpC β-lactamase. Cefoxitin and imipenem are good inducers but are weak substrates. Cefotaxime, ceftriaxone, ceftazidime, cefuroxime, piperacillin, and aztreonam are weak inducers as well as weak substrates [16]. In a normal cell, *ampC* β-lactamase expression is low due to negative regulation by the AmpR repressor which binds to the promoter region of the *ampC* gene. The AmpR detaches from the promoter in response to the concentration of N-acetylglucosamine-1,6-anhydro-N-acetulmuramic oligopeptides[13,15,16]. These oligopeptides are released normally by the cell wall and are transported into the cell by the AmpG transmembrane protein. Inside the cell these oligopeptides are broken down by AmpD, a cystolic amidase, and the UDP-MurNAc-pentapeptides are transported out into the cell wall where they are recycled back into the peptidoglycan layer. The AmpR regulatory protein remains in its normal configuration atop the *ampC* promoter region and expression of AmpC β-lactamase is minimal [15].

When the cell is exposed to β-lactam antibiotics, damage to the cell wall by the drug leads to a large release of the oligopeptides into the cytoplasm. The concentration of the peptides in the cell wall increases to a point where the AmpD cystolic amidase cannot recycle the peptides

quickly enough and the N-acetylglucosamine-1,6-anhydro-N-acetulmuramic oligopeptides cause AmpR to alter its configuration, opening up the promoter site of the *ampC* gene and increased production of the AmpC β-lactamase. AmpC is transported out into the cell wall where it disables the β-lactam antibiotics by cleaving the β-lactam ring. After the antibiotics are hydrolyzed and the cell wall is no longer being damaged, the amount of the oligopeptides lowers in the cell allowing AmpR to return to its normal position causing the production of the AmpC β-lactamase to return to basal levels[12,15].

In some cases, AmpC β-lactamase production can be stably derepressed leading to constitutive high level production of the enzyme. In most cases of derepressed *ampC*, a mutation that causes decreased activity in one or more of the enzymes in the pathway will lead to derepressed expression of the AmpC β-lactamase. The most common cause of AmpC overexpression is due to mutations in the *ampD* gene [16,22]. This leads to an accumulation of muropeptides in the cell regardless of β-lactam exposure leading to AmpC hyperinducibility or constitutive expression. Mutations that occur in the *ampR* gene are less commonly seen than mutations in *ampD* but will also lead to high level constitutive or hyperinducible phenotypes. The least common cause of derepression is a mutation that affects AmpG and these isolates will generally display hyperinducibility [16,22].

Unfortunately, the *ampC* β-lactamase gene has also been incorporated into mobile genetic elements. These plasmid-mediated AmpC β-lactamases confer resistance to the same spectra of β-lactams, but are constitutively expressed. There are multiple different varieties of plasmid encoded AmpCs. Some of the families are regulated by AmpR but others are not and are expressed in higher amounts [13]. There are no CLSI guidelines for the detection of AmpC

producing organisms. Like ESBL's AmpC producing organisms will have resistance to third generation cephalosporins but are susceptible to cefepime. Thus the preferred treatment for infections with AmpC producing organisms is 4th generations cephalosporins or carbapenems[26].

<u>Extended spectrum β-lactamases (ESBLs)</u>

Extended spectrum β-lactamases consist of hundreds of β-lactamases that confer resistance against certain β-lactam antibiotics, specifically against the so-called extended spectrum β-lactams such as cephalosporins. There are 4 main families of ESBL's: TEM, SHV, CTX-M, and OXA[12]. ESBL's are found mostly in organisms in the family *Enterobacteriaceae* and generally confer resistance to 1st, 3nd, and 4th generation cephalosporins [22]. Each family has different affinities towards certain cephalosporins [12]. Unlike AmpC β-lactamases which are classified as a Class C β-lactamase, most ESBL's are classified as a Class B β-lactamase which are characterized by serine in the active site.

The most common family of ESBL belongs to the TEM family which contains over 90 different variants of the enzyme [12]. However, not all β-lactamases in the TEM family display the ESBL phenotype. The different variations of TEM enzymes result from mutations in certain amino acid peptides that slightly change the substrate spectrum or the effectiveness against B-lactams [12]. SHV-1 β-lactamases are commonly found in *Klebsiella pneumoniae* isolates and are responsible for up to 20% of the plasmid mediated ampicillin resistance[12]. There are fewer derivatives of SHV β-lactamase than observed with the TEM family. However, most of the SHV derivatives are closely related and display the ESBL phenotype. Organisms such *Escherichia coli*, *Citrobacter diversus*, and *Pseudomonas aeruginosa* can harbor SHV ESBLs[12]. CTX-M

type β-lactamases are a newer family of ESBLs that have been found in *Enterobacteriaceae*. Although these display an ESBL phenotype of resistance i.e. are susceptible to second generation cephalosoprins *in vitro*, CTX-M has only about a 40% homology to TEM and SHV ESBL's[12]. CTX-M is related to an AmpC β-lactamase that is found in *Kluyvera ascorbata*[12]. Although CTX-M is an ESBL, it does not hydrolyze ceftazidime which allows organisms that produce CTX-M to be effectively treated with ceftazidime[12]. OXA type β-lactamases are a new but rapidly growing family of β-lactamases. These β-lactamases are generally found in *Pseudomonas aeruginosa* and unlike the ESBL's found in Enterobacteriaceae, OXA β-lactamases are classified as a Class D β-lactamase. OXA ESBL's confer resistance to ampicillin, cephalothin, and ceftazidime and are characterized by their high hydrolytic activity. OXA ESBL's are also not as sensitive to inhibition by clavulanic acid as other ESBLs[12].

Previously, laboratories used clavulanic acid (a β-lactamase inhibitor) in combination with a cefotaxime and ceftazidime to confirm possible ESBL producing organisms [3,12]. Currently, the CLSI no longer requires ESBL testing for isolates suspected of ESBL activity as the breakpoints for these antibiotics were lowered. Most ESBL's belong to the TEM or SHV families and one characteristic of the ESBL's in those families is that they have a relatively large active site allowing them to cleave many more types of β-lactams including newer generation cephalosporins. However this large active site also makes these enzymes susceptible to inhibition by β-lactmase inhibitors[7].

Carbapenemases

Carbapenems are often considered to be antibiotics of last resort due to their wide spectrum of activity and relative resistance to most (but not all) β-lactamases. Thus, carbapenem

resistance is relatively uncommon compared to resistance against other β-lactams. However, it is a growing concern in the United States and other parts of the world [1]. Carbapenem resistance occurs mainly through carbapenemases which like other β-lactamases, are hydrolyzing enzymes designed to cleave the β-lactam ring of the antibiotic. Organisms such as *Pseudomonas aeruginosa* can also utilize mechanisms that reduce antibiotic uptake by decreasing the concentration certain porins in the cell wall to decrease susceptibility to carbapenems.

Klebsiella pneumoniae carbapenemases (KPC) confer resistance to carbapenems as well as other β-lactam antibiotics. KPC's are classified as a β-lactamase group 2f and are notable for the fact that the gene for the enzyme is located on transferable plasmids [11,12]. *Klebsiella pneumoniae* carbapenemases have become an important public health concern along the East Coast of the United States where there have been a growing number of reports of resistance due to KPC-producing organisms. KPC were initially found in *Klebsiella pneumoniae*, but have since been found in other members of the *Enterobacteriaceae* such as *E. coli* and *Enterobacter cloacae* [17]. Of concern, carbapenem resistance in *Klebsiella pneumoniae* has risen from about 0.6% in 2004 to 5.6% in 2008 in the United States[1]. Treatment of infections involving KPC producing isolates is challenging and these infections are generally associated with to a high mortality rate. Antibiotics such as fosfomycin and older, more toxic antibiotics such as colistin have been shown to be effective in treating certain isolates of Carbapenem resistant *Enterobacteriaceae* depending on the site of infection [1,11].

Class B Metallo-β-lactamases are a class of carbapenemase characterized by the presence of a heavy metal usually Zn^{2+} in the active site of the enzyme. Metallo-β-lactamases were traditionally found in environmental pathogens such as *Bacillus cereus, Aeromonas species,* and

Stenotrophomonas maltophilia [25]. These β-lactamases are classified by their requirement for Zn^{2+} and are inhibited by EDTA. The most common metallo-β-lactamase families include the VIM, IMP, GIM, and SIM enzymes. These families are associated with certain species of bacteria and because of its location on the chromosome usually do not transfer outside the species and do not spread beyond the countries of their origin [25]. More recently, the New Delhi Metallo-β-lactamase (NDM-1) has emerged as an important mechanism of carbapenem resistance in *Enterobacteriaceae*. These β-lactamases are located on plasmids that have the ability to be transferred to other species. In addition to the NDM-1 β-lactamase, the plasmid that harbors this gene also confers resistance to many other antibiotic classes[25].

A new class of carbapenemase has been found in multi-drug resistant *Enterobacteriaceae* mainly *Klebsiella pneumoniae*. The OXA-48 β-lactamase is classified as a Class D β-lactamase. The OXA-48 β-lactamase is able to hydrolyze penicillins at a high level and carbapenems at a low level [4,24]. However, high levels of carbapenem resistance can be attained if permeability defects decrease intake of carbapenems into the cell. The gene for the OXA-48 β-lactamase is plasmid encoded and is able to be transferred to other organisms [4,24].

Drug Efflux

Many organisms utilize protein pumps within their cell wall that confer resistance to certain types of antibiotics by actively transporting the drugs out of the cell. This mechanism of resistance can be found in both gram positive and gram negative organisms. Gram negative efflux pumps confer resistance to multiple classes of antibiotics and also protect the cell from certain toxins. *Pseudomonas aeruginosa* has many different types of efflux mechanisms which it uses to protect it from the effects of many antibiotic agents. One of the most common efflux

pumps found in *Pseudomonas aeruginosa* belongs to the resistance-nodulation-division (RND) family[20]. The RND family efflux pumps are composed of three parts: A periplasmic membrane fusion protein, an outer membrane factor, and a cytoplasmic membrane transporter. RND family efflux pumps are secondary transporters that do not use ATP for energy but rather use proton motive force to operate[20]. Although up to 12 different RND pumps have been detected in *Pseudomonas aeruginosa*, not all of these are used for the efflux of antibiotics. The most clinically significant efflux pump in *Pseudomonas aeruginosa* is the MexAB-OprM efflux pump which has the ability to export many classes of antibiotics from the cell including fluoroquinolones, tetracyclines, chloramphenicol, β-lactams, macrolides, novobiocin, trimethoprim, and sulfonamides [31].

The MexAB efflux pump expression is known to peak as the cell density reaches stationary phase. Two regulatory genes *mexR* and *nalD* are responsible for the negative regulation of the MexAB-OprM pathway. Mutations that occur in either of these regulatory genes have been shown to increase expression of MexAB-OprM leading to increased resistance to multiple classes of antibiotics[30]. Additional members of the RND efflux pump family can work in conjunction with the MexAB-OprM system to confer additional resistance to other antimicrobials. The MexCD-OprJ efflux pump, for example, preferentially exports 4th generation cephalosporin antibiotics (e.g Cefepime)[20]. The MexEF-OprN efflux pump exports fluoroquinolones, chloramphenicol, and trimethoprim. These efflux pumps confer additional resistance to antibiotics as knockouts of these mechanisms do not affect intrinsic resistance of *Pseudomonas* [20].

Resistance to Aminoglycosides

There are three main mechanisms that confer resistance to aminoglycosides: 1) Decreased/reduced uptake, 2) Alterations to the ribosomal binding site, or 3) Production of aminoglycoside-modifying enzymes[2]. In general, Gram negative bacteria modify the amount of certain porins and channels in their cellular membrane[20,22]. In *P. aeruginosa*, for example, a cellular membrane embedded porin, OprD, is used to transport multiple nutrients into the cell. Mutations that decrease the transcriptional expression and/or disrupt translational production of a functional porin are the main causes for OprD mediated resistance [20]. Resistance can also occur due to mutations in the ribosomal binding site that decrease antibiotic interaction with the ribosome. Resistance via this mechanism usually leads to resistance to streptomycin only as other aminoglycosides bind to multiple sites on both ribosomal subunits[2]. The most common type of resistance is due to enzymatic modification of the antibiotic itself. There are over 50 different enzymes isolated that modify the antibiotic by adding various acetyl, adenyl, and phosphate groups to the antibiotic[2]. Organisms with aminoglycoside modifying enzymes generally display high levels of aminoglycoside resistance [2].

Resistance to fluoroquinolones

Resistance to fluoroquinolones in Gram negative bacteria can result from two distinct mechanisms: 1) target-site mutation and 2) resistance due to antibiotic efflux. Low level resistance against fluoroquinolones is generally due to target site mutations in DNA gyrase [12]. If there is high level resistance observed, it is most likely due to increased efflux of fluoroquinolones[12]. In *Pseudomonas aeruginosa*, resistance to fluoroquinolones is generally the result of mutations in the *gyrA* gene which encodes the DNA gyrase enzyme which is

responsible for relieving the torsional strain of caused by DNA helicase during DNA unwinding in DNA replication. Mutations usually occur at two positions within the gene encoding amino acids 83 and 87 [12]. Mutations in *gyrB* that leads to resistance has been observed but is rarely observed. Other mutations that can lead to fluoroquinolone resistance cause resistance due to increased efflux pump expression. Treatment with fluoroquinolones can select for these mutations found in the *nfxA, nfxB,* and *nfxC* genes[12]. Mutations in these genes lead to hyper production of efflux mechanisms which not only increases resistance to fluoroquinolones but also to multiple other classes of antibiotics[12].

In addition, resistance to fluoroquinolones has also been found to be plasmid mediated. The *qnr* gene encodes for the Qnr protein which binds to and protects both DNA gyrase and topoisomerase IV from inhibition by ciprofloxacin [32]. The level of resistance conferred through the Qnr protein is very low, close to that of a first step mutation in the DNA gyrase. However, the presence of the *qnr* gene in *E. coli* facilitates the selection of additional resistance mutations to flurorquinolones[32].

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing is an essential test provided by clinical laboratories to guide clinicians in selecting the most effective antimicrobial therapy for bacterial infections. In this age of increasing antibiotic resistance, treatment is becoming more complex and options for therapy are often limited. A variety of antibiotic susceptibility testing methodologies are used in clinical microbiology laboratories. These range from simple diffusion-based methods to more

complex broth-based automated systems. Antibiotic susceptibility testing can be performed in either a quantitative or qualitative manner. Quantitative methods determine the lowest concentration of antibiotic that is required to inhibit the growth of a certain organism *in vitro*. This figure is known as the MIC or the Minimum Inhibitory Concentration or MIC and is usually reported in mcg/mL [22]. Qualitative susceptibility testing (i.e. disc diffusion) is reported as sensitive, intermediate, or resistant based on published breakpoints. Microbiological breakpoints are published annually by the CLSI and take into account the MIC distribution, pharmacokinetic and pharmacodynamic data as well as outcome studies [10].

Macrobroth and Microbroth dilution

Determination of an organism's MIC to a given antibiotic can be achieved using broth, agar, or diffusion based methods. Although both macro and micro-broth based testing can be performed, macrobroth dilution is less desirable as a result of increased hands-on time and materials required with this method. Microbroth dilution uses broth filled microtiter wells with a series of doubling dilution antibiotic concentrations to determine the MIC. This is generally performed in a 96-well format allowing up to 12-15 antibiotics to be tested simultaneously. The tests may be read manually or in an automated fashion using specialized machines[22].

Disc Diffusion method

The disc diffusion method, also commonly known as the Kirby Bauer Test, was developed in 1966 at the University of Washington and is still used in many clinical microbiology labs [16,22]. In the Kirby Bauer test, a standard concentration of an organism is plated onto Mueller Hinton agar. Afterwards, paper discs containing fixed concentrations of

antibiotics are placed onto the surface of the media. The antibiotic diffuses out from the disc into the agar resulting in a concentration gradient of antibiotic where concentrations are highest closest to the disc and decrease moving outward from the disc. Susceptibility is visualized by a zone of inhibition around the disc, and the zone sizes correlate to known MIC values. Just as with MIC values from macrobroth and microbroth dilutions, zone sizes also have CLSI-defined breakpoints that determine whether the isolate is susceptible, intermediate, or resistant[16]. The correlation made between zone sizes and MIC values can be made because of the standardized nature of the Mueller Hinton agar. Factors such as pH, agar depth, and ion concentration are standardized in Mueller Hinton agar for the Kirby Bauer test to prevent variance in zone size. Mueller-Hinton agar can be modified with different growth factors like 5% sheep's blood for *Streptococcus pneumoniae* or hemin and NAD for the growth of *Haemophilus influenzae*. This allows susceptibility testing to be performed for organisms that usually may not be able to grow in the broth used for micro and macrobroth dilution tests[4,15,22].

E-test

The E-test provides quantitative MIC results but is based on diffusion of antibiotics into solid media much like the Kirby Bauer method. Each E-test uses antibiotic infused plastic strips that are laid atop solid media with a lawn of bacterial inoculum. E-test strips are engineered to create a polar gradient that is higher at one end of the strip that starts to gradually lower on the other. The resulting growth pattern usually resembles a teardrop shape where there is a larger zone around one end of the strip that gradually shrinks to a point somewhere along the strip's length. The point where the growth intersects the E-test strip itself is the MIC value. Typically, MIC values determined by E-tests are within one doubling dilution of those determined by

conventional broth- or agar-based methods [22]. Like the Kirby Bauer Assay, E-tests are useful in determining the susceptibility of fastidious organisms because the strips can be placed atop plates that are more nutrient rich.

Automated Systems for Antibiotic Susceptibility Testing

A number of commercially available automated systems have been developed that can record and interpret results of susceptibility testing without the need for significant hands on time. Because of the ease of use, many clinical laboratories have implemented automated susceptibility testing. Automated AST systems have the ability to both read and interpret data thus minimizing labor costs and the potential for technologist error. In addition, most automated AST systems also have the ability to interface directly with the Laboratory Information System (LIS) decreasing the need for manual entry of susceptibility results. Finally automated systems have the ability to test organisms for susceptibility to a larger number of antibiotics than would otherwise be possible using agar-based method potentially, relaying more information to physicians to assist with the optimization of patient treatment [5,9].

Importantly, integration of expert systems for the interpretation of susceptibility testing results has also resulted in increased popularity of automated systems. In particular, preprogrammed interpretative rules and flagging of unusual or unexpected results is an advantage of automated susceptibility testing. This automated interpretation reduces human error or omission in the reporting, as well as reducing training time and costs by reducing the information that is needed to be taught to technologists about susceptibility patterns [30].

Automated systems are not without their limitations, particularly in laboratories in low resource settings. Firstly, automated systems are considerably more expensive than their non-automated counterparts. Secondly, automated systems are generally broth-based and cannot be used for all clinically important organisms, particularly those species that are more fastidious in nature [22]. For those organisms (e.g. anaerobes), laboratories must perform susceptibility testing using alternative methods. A further disadvantage associated with the use of automated systems is the difficulty in changing the concentration of antibiotics tested in the response to changes in published breakpoints, although certain systems have potential for customization [9]. Finally, although certain automated systems offer susceptibility results in only a few hours, rather than the usual 16-24 hours, these have been shown to lack sensitivity for the detection of certain types of resistance [9]. These include the detection of β-lactamase activity in GNRs, low level glycopeptide resistance in enterococci and streptococci, and methicillin resistance in staphylococci. However modifications to the interpretative software are reported to have resolved these issues to some extent.

A number of different automated susceptibility testing systems are available, each differing in the method by which growth is detected. The BD Phoenix uses a gravity-fed 136-well cartridge that can test 16-25 antibiotics as well as a redox indicator system to detect growth in the wells [22]. The redox indicator system allows susceptibilities to be determined within 6-8 hours of incubation. The results are then interpreted by the expert system called the BD-Xpert which analyzes the MIC values and compares them to the organism ID to determine qualitative interpretations of susceptibility [30]. The MicroScan WalkAway SI is a self-contained reading and incubating unit that uses 40 or 96 test panels that can determine both antibiotic susceptibilities as well as bacterial identification [5,22]. The microdilution panels are hydrated

and inoculated by hand before being placed into the instrument which then incubates the panels for a certain period of time depending on the inoculated organism. The MicroScan WalkAway System uses a photometer to measure turbidity in the wells to determine growth. The instrument can read initial susceptibilities after 4-5 hours to see if an organism is unequivocally susceptible or highly resistant to certain antibiotics. Organisms with patterns that indicate inducible of slowly expressed resistance are automatically incubated for an extended period of time. Standard panels are read after at least 4-6 hours or overnight growth. However, customized panels that use fluorogenic substrates allow certain Gram Negative Rods to be read in 3.5 to 15 hours [22]. Just as with other automated methods, the expert system MicroScan LabPro interprets the MIC values and contains rules about the organism to further refine the susceptibilities reported [30].

Finally, the TREK Sensititre is uses a fluorometric detection system to detect bacterial growth and is based on a 96-well microtitre format. Hydrolysis of fluorogenic substances is monitored to detect growth in each of the wells [22]. MIC results can be obtained from a plate within 5 hours, but because of inaccuracies with detection of some resistance mechanisms, the FDA has only been cleared for 18 hour (Gram negative) and 24 hour (Gram Positive) incubations [22]. The Trek Sensititre system is the system is currently used at the University of Washington Medical Center Clinical Microbiology laboratory for testing of Staphylococci, Enterococci and non-fastidious Gram-Negative organisms.

Project Design

The Kirby Bauer method has many advantages as an antibiotic susceptibility testing method: It is cheap, simple, and easy to interpret. However, because of the hands-on time requirement associated with this method, automated microbroth based methods like the Trek Sensititre are replacing the Kirby Bauer as the primary mode of antibiotic susceptibility testing in many clinical laboratories.

One feature unique to diffusion-based methods such as the Kirby-Bauer is the ability to detect inner colonies that maybe indicative of more resistant subpopulations. It is unknown whether the broth-based methods such as Trek Sensititre can detect resistant sub-populations that are visually represented as inner colonies. Thus, the goal of this project was to determine whether resistance as detected by inner colonies on the Kirby Bauer assay is also detected by microbroth dilution.

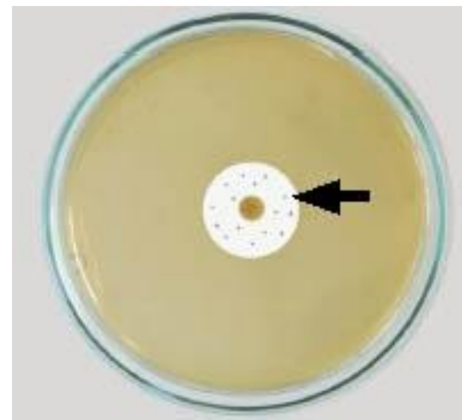

Figure 1: Kirby-Bauer plate demonstrating inner colonies within zone of diffusion. Source: http://rfdp.seafdec.org.ph/publication/manual/antibiotics/pic20.jpg

Between September 2011 and May 2012, clinical isolates of Gram-Negative bacilli that would normally be tested using the Trek Sensititre system also underwent testing by the Kirby Bauer method. We excluded Gram-Negative bacilli isolated from urine isolates to alleviate an unnecessary increase in the daily workload. Gram-Negatives were chosen as the focus of the study because of their propensity to produce inner colonies. Gram-Negative bacilli from cystic fibrosis patients were also excluded as these isolates are not generally tested using the Trek Sensititre system due to their frequent hypermucoid phenotype and resulting interference with

automated susceptibility interpretation by the ARIS system. The panel of antibiotics used in the project was the same panel used by the clinical microbiology lab for clinical testing. The panel includes 12 antibiotics which include 9 β-lactam antibiotics (ampicillin, ampicillin/sulbactam, piperacillin/tazobactam, ertapenem, meropenem, ceftazidime, ceftriaxone, cefepime, and aztreonam), 2 aminoglycosides (gentamicin and amikacin), and 1 fluoroquinolone (ciprofloxacin). These were also the antibiotics that were compared to the Trek Sensititre results.

All Kirby Bauer plates placed into a 4°C refrigerator following incubation for up to one week for batched reading. Any inner colonies present were subcultured onto blood agar and subsequently retested both the Trek Sensititre and the Kirby Bauer methods. The Trek susceptibility and Kirby Bauer results from the inner colonies were then compared to the Trek susceptibility results from the original isolate. We hypothesized that if the Trek Sensititre is indeed able to detect resistant subpopulations, the two susceptibility results should be similar. Similarly, if the Trek Sensititre cannot detect these resistant subpopulations, then the susceptibility results of the inner colonies would appear to be more resistant having a higher MIC and/or a change in interpretation.

Procedures

Kirby Bauer

A suspension of the organism following overnight growth was made to equal a 0.5 MacFarland Standard, or approximately $1.5x10^{8}$ cfu/mL. Using a sterile swab, a lawn of the inoculum was made on Mueller Hinton agar by covering the entire surface of the plate three

times. The Mueller Hinton agar was stamped with antibiotic discs within 15 minutes of the inoculated preparation and incubated in a 35°C incubator for 16-24 hours under ambient air conditions. Using calipers, the diameters of the zone of clearing were measured and the zone sizes recorded and interpreted according to CLSI procdeures. A representative inner colony from each zone (if inner colonies were seen in multiple zones) was subcultured onto a single blood agar plate.

Trek Sensititre

Testing by the Trek Sensititre was performed according to the manufacturer's instructions. The suspension of the organism was made using a cation-controlled saline mixture. Once the suspension was made, 30 µL of the suspension was added to a 10 mL vial of growth medium. The vial was placed into the AutoInoculator, which inoculated 50 µL of the suspension into each well of the 96-well microtitre plate. The Sensititre plate was sealed and scanned into the computer and put into the Automated Reader and Incubation System (ARIS2X). Gram Negative plates were incubated for 16 hours before being read.

Data Analysis

The results of testing by Kirby-Bauer and Trek Sensititre were directly compared. In the event that inner colonies were present on the original Kirby-Bauer plate, the results of the original isolates and subcultured inner colonies were also compared by both methods. Two distinct results were evaluated: 1) MIC value and 2) Interpretation. Changes in interpretations were categorized as follows: 1) Very Major Error, 2) Major Error, and 3) Minor Error according to standard CLSI criteria. A two-fold change in the MIC value was also considered significant.

A very major error is classified as a change in interpretation from sensitive o an interpretation of resistant. This is the most serious category of error as such errors are the most likely to be associated with treatment failure. Major errors are classified as a change in the interpretation from sensitive to intermediate or from intermediate to resistant. Minor errors are classified as changes from intermediate to sensitive or from resistant to intermediate or sensitive. The final category was a change in the MIC value by 2 or more doubling dilutions without a change in the interpretation. The normal error range for the MIC results from the Trek Sensititre is ±1 doubling dilutions. A change by 2 or more doubling dilutions is significant and an increase by 2 or more doubling dilutions indicates that in inner colonies are more resistant than the original isolate even if there was no change in the interpretation.

Results

Organisms included in the study

In total 308 isolates of Gram-Negative bacilli were tested by both Kirby-Bauer and Trek Sensititre methods. Of the 308 isolates tested, 54 (17.5%) were positive for the presence of inner colonies. The distribution of bacterial species included in the study is shown in Figure 1.

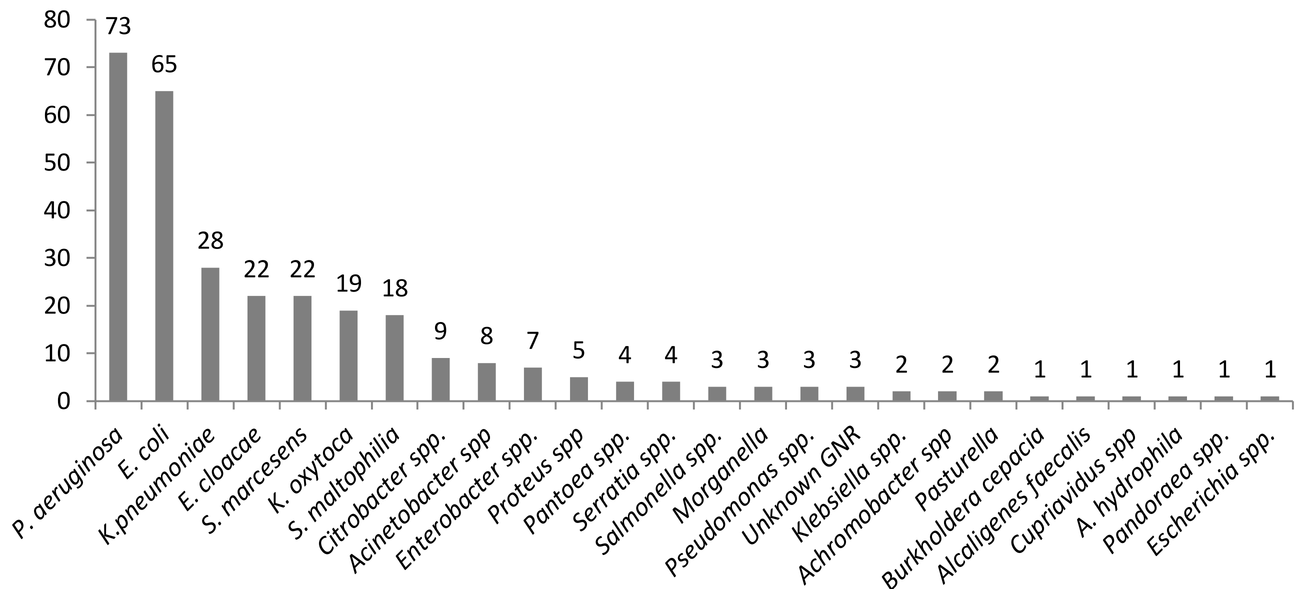

Figure 2. *Distribution of Gram-Negative bacilli screened*

The ten most commonly recovered organisms were: *Pseudomonas aeruginosa (73), Escherichia coli (65), Klebsiella pneumoniae (28), Enterobacter cloacae (22), Serratia marcescens (22), Klebsiella oxytoca (19), Stenotrophomonas maltophilia (18), Citrobacter* spp *(9), Acinetobacter* spp *(8), and Enterobacter* spp *(7). Pseudomonas aeruginosa* and *Escherichia coli* isolates accounted for 45% of the total number of isolates included in the study. The remaining 55% of the isolates comprised both fermenting and non-fermenting organisms, with six of the ten most frequently encountered organisms being members of the *Enterobacteriaceae* family. The frequency with which isolates screened positive for inner colonies by Kirby Bauer is shown in Figure 3 below.

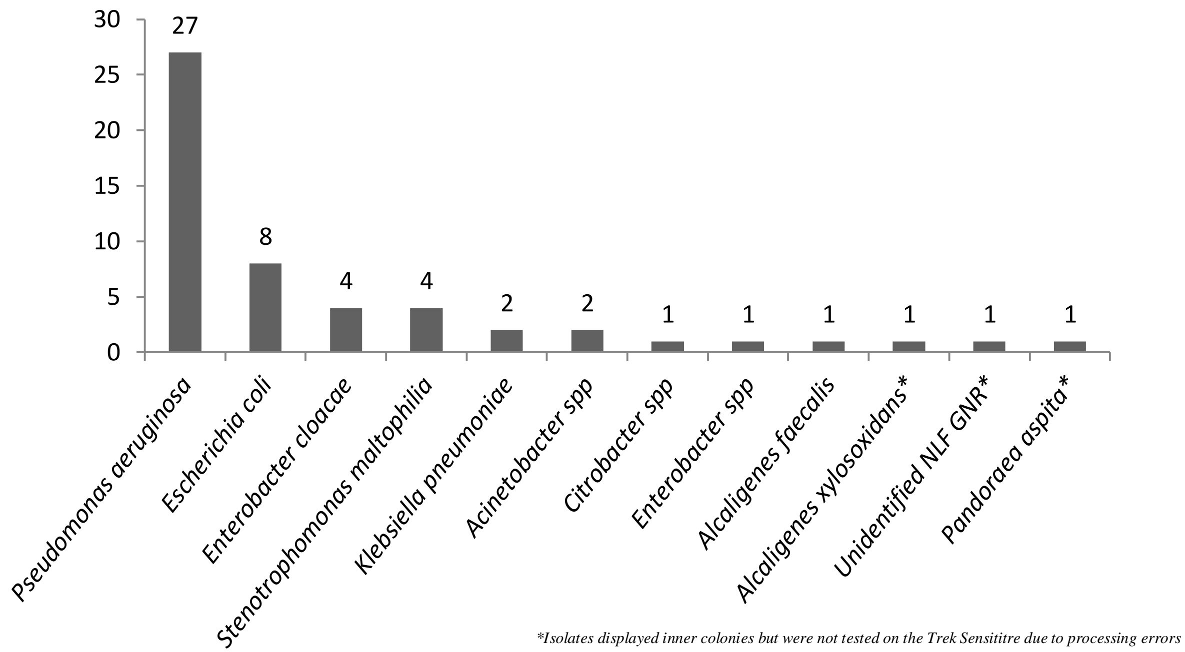

Figure 3. Frequency of isolates that screened positive for presence of inner colonies from Kirby bauer

Organism	Number of total isolates Screened	Number of isolates positive for inner colonies on Kirby Bauer	Percentage
Pseudomonas aeruginosa	73	27	37%
Escherichia coli	65	8	12%
Klebsiella pneumoniae	28	2	7%
Enterobacter cloacae	22	4	18%
Stenotrophomonas maltophilia	18	4	22%
Citrobacter spp	9	1	11%
Acinetobacter spp	8	2	13%
Enterobacter spp	7	1	14%
Alcaligines faecalis	1	1	100%

Table 1: Percentage and count of isolates that produced inner colonies. The 50 isolates that were tested via the Trek Sensititre and not excluded from the study are represented above.

Of the 308 total organisms screened for inner colonies, a total of 53 isolates displayed inner colonies. Only 50 isolates that displayed inner colonies were tested on the Trek Sensititre due to processing errors. Most of the inner colonies were seen in *Pseudomonas aeruginosa* isolates (27), followed by *E. coli* (8) and *E. cloacae* (4) and *S. maltophilia* (4).

Organism	Total number of Isolates with inner colonies	Number of isolates with significant change in MIC/interpretation	Percentage
Pseudomonas aeruginosa	27	21	78%
Escherichia coli	8	5	63%
Stenotrophomonas maltophilia	4	3	75%
Enterobacter cloacae	4	0	0%
Klebsiella pneumoniae	2	1	50%
Acinetobacter lwoffii	2	1	50%
Citrobacter freundii	1	1	100%
Enterobacter asburiae	1	1	100%
Alcaligines faecalis	1	1	100%

Table 2: Percentage of inner colonies that showed a significant change in the MIC or interpretation in at least one of the 12 antibiotics tested

The inner colonies from the 50 isolates were then tested using the Trek Sensisitre and the Kirby Bauer method. The antibiotic susceptibility results were compared to those of the original isolate. Table 2 above shows the total number of isolates tested and the amount of isolates that displayed a significant change in the MIC or the interpretation in at least one of the 12 antibiotics tested.

Organism	Number of isolates with inner colonies	Number of isolates that demonstrated a significant change in the MIC or interpretation	Total number of isolates that displayed at least one Very Major Error (VME)	Total number of very major errors (VME) observed in total	Total number of isolates that displayed at least one major error (ME)	Total number of major errors (ME) observed in total	Total number of isolates that displayed at least one minor error (MiE)	Total number of minor errors (MiE) observed in total	Total number of isolates that displayed at least one significant increase in MIC without a change in interpretation	Total number of changes of MIC without change in interpretation observed in total
Pseudomonas aeruginosa	27	21	7	10	12	18	4	5	10	15
Escherichia coli	8	5	4	8	4	5	1	1	0	0
Stenotrophomonas maltophilia	4	3	3	4	1	1	0	0	0	0
Enterobacter cloacae	4	0	0	0	0	0	0	0	0	0
Klebsiella pneumonia	2	1	0	0	0	0	1	1	0	0
Acinetobacter lwoffii	2	1	0	0	1	1	0	0	0	0
Citrobacter freundii	1	1	1	4	1	1	0	0	0	0
Enterobacter asburiae	1	1	1	2	0	0	0	0	0	0
Alcaligines faecalis	1	1	0	0	0	0	0	0	1	2
Total	**50**	**34**	**16**	**28**	**19**	**26**	**6**	**6**	**11**	**17**

Table 3: Distribution of significant changes in the interpretation or MIC. Only organisms that produced inner colonies where a significant change was observed are listed in the table. Because there were 12 antibiotics tested per isolate, a single isolate has the chance to create multiple errors observed per isolate.

The change in the MIC of the inner colonies was tabulated and sorted based on the organism tested. The data were sorted by the category of MIC change. Because there were 12 antibiotics tested per isolate, the data was split into two categories: 1) The number of isolates that demonstrated a change in the MIC and 2) The total number of significant changes of MIC observed. This helped to highlight the number of isolates that showed a change in the MIC interpretations vs. the extent of the change seen between the original isolate and the inner colony.

Pseudomonas aeruginosa

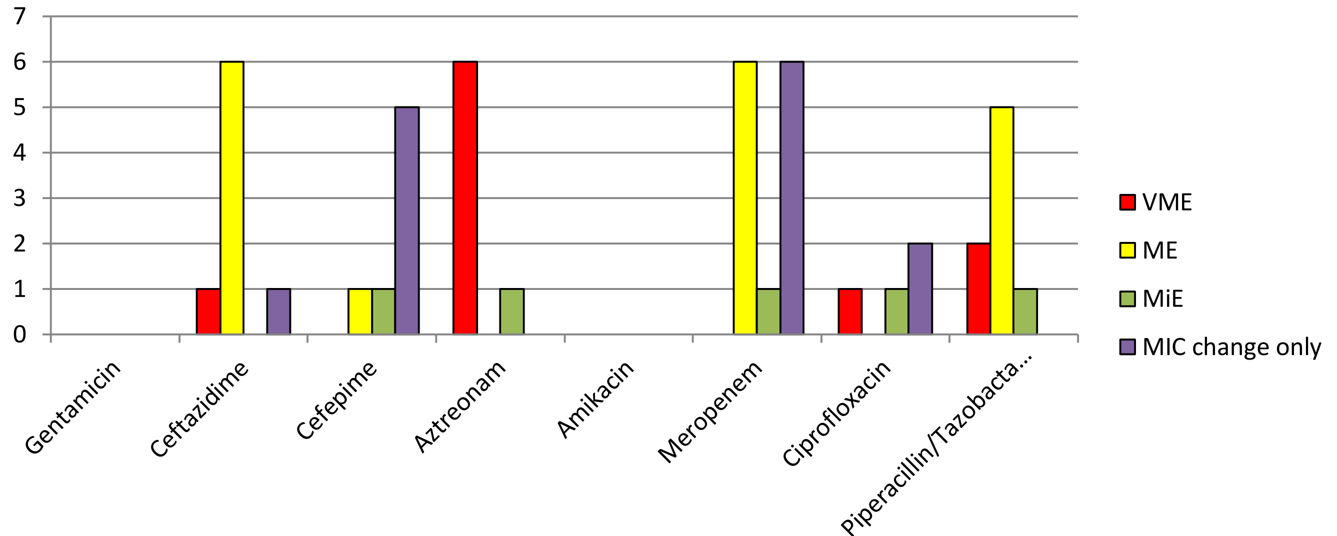

Figure 4: Distribution of antibiotics where errors were observed in Pseudomonas aeruginosa. *Antibiotics presented are those which have CLSI-approved breakpoints and interpretations. Ertapenem, Ceftriaxone, Ampicillin, and Ampicillin/Sulbactam were omitted from the chart above as there are no CLSI interpretations of those antibiotics to* Pseudomonas aeruginosa. *VME = Very Major Error, ME = Major Error, MiE = Minor Error, MIC change only = increase of 2 doubling dilutions without interpretation change*.

Pseudomonas aeruginosa was the organism with the highest instance of inner colonies (27 out of 73 isolates tested) and also an organism known to frequently possess multiple mechanisms of antibiotic resistance. In total 21 of the 27 *Pseudomonas* aeruginosa isolates tested displayed a change in the MIC interpretation. Figure 4 above depicts the distribution of errors sorted by the type of error and the antibiotic where the discrepancy occurred. The errors were seen in six of the eight antibiotics tested with CLSI breakpoints and interpretations.

Categorical error rates for *Pseudomonas aeruginosa* isolates were 25.9% very major errors, 44.4% major errors, 14.8% minor errors, and 37.0% significant increase in MIC without change in interpretation. Very major errors were seen mainly in aztreonam. Major errors were seen mainly in three different antibiotics ceftazidime (6), meropenem (6), and piperacillin/tazobactam (5). Minor errors were seen once in cefepime, aztreonam, meropenem,

and piperacillin/tazobactam. Increases in MIC without a change in the interpretation was seen in four antibiotics with a majority of the changes seen in meropenem (6) and cefepime(5).

Escherichia coli

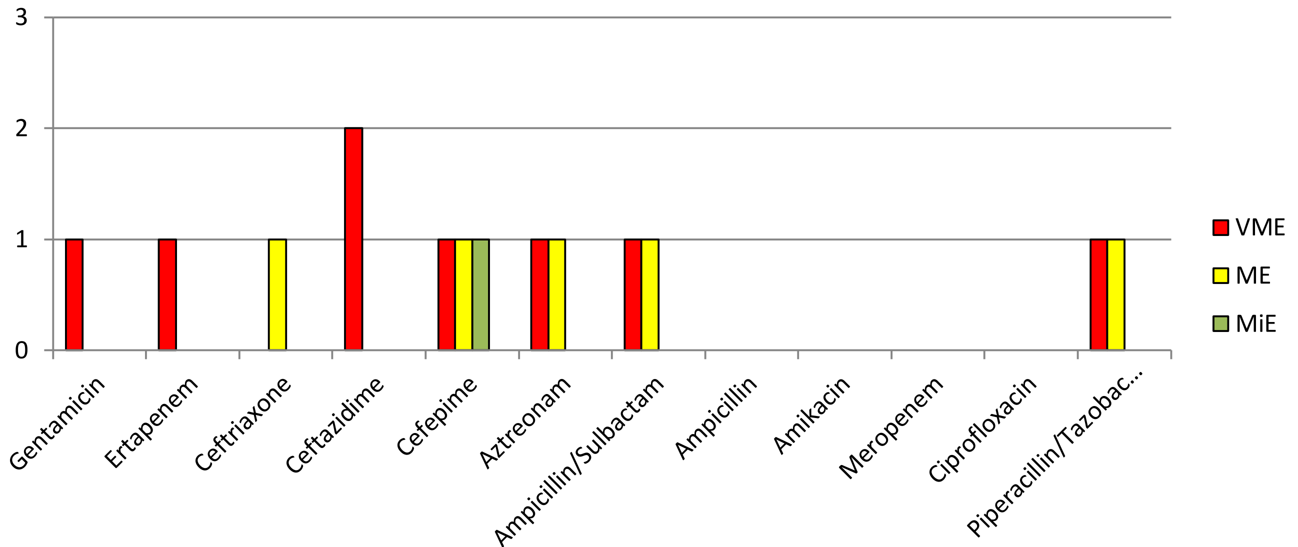

Figure 5: Distribution of antibiotics where errors were observed in Escherichia coli *isolates. VME = Very Major Error, ME = Major Error, MiE = Minor Error, MIC change only = increase of 2 doubling dilutions without interpretation change.*

The second most common isolate to display inner colonies was *Escherichia coli*. Of the 65 total *E. coli* isolates that were screened for the presence of inner colonies, only 8 isolates were positive. Categorical error rates for the *Escherichia coli* isolates were 80% for very major errors, 80% major errors, and 20% minor errors. Unlike the results of the *Pseudomonas aeruginosa* isolates, the errors seen in the *E. coli* were widely observed in many more antibiotics with very major errors seen in seven of the twelve antibiotics tested and major errors observed in five of the twelve antibiotics tested. Minor errors were only observed in one antibiotic in one isolate.

Other isolates tested (S. maltophilia, K. pneumoniae, A. lwoffii, C. freundii, and A. faecalis)

Organism	GM	ETP	CRO	CAZ	CEF	ATM	SAM	AM	AN	MEM	CIP	TZP
S. maltophilia												
S. maltophilia												
S. maltophilia												
K. pneumoniae												
Acinetobacter. lwoffi												
Citrobacter freundii												
E. asburiae												
Alcaligenes faecalis												

Table 4: Errors observed in the remainder of bacterial isolates tested grouped by antibiotic and classification of error. Cells shaded in grey indicate that the antibiotic lacks CLSI interpretations and cannot be evaluated for changes in interpretation. Cells highlighted in red indicate the presence of a Very Major Error (VME). Yellow highlighted cells indicate a Major Error (ME). Green cells indicate a Minor Error (MiE). Cells highlighted in purple indicate a significant change in the MIC without a change in interpretation.

In total, 32 non-*Enterobacteriaceae* were screened for inner colonies of which 6 isolates screened positive for inner colonies. There were three species total that produced inner colonies, *Stenotrophomonas maltophilia, Acinetobacter lwoffii, and Alcaligenes faecalis*. Three of the four *Stenotrophomonas maltophilia* isolates with inner colonies observed displayed a change in the MIC interpretation. Categorical error rates for *Stenotrophomonas maltophilia* were 100% very major errors and 25.0% major errors. One of the two *Acinetobacter lwoffii* isolates tested displayed errors. The isolate displayed a major error to one antibiotic. The one *Alcaligines faecalis* isolate tested did not display a change in the MIC interpretation but a significant increase in the MIC was seen in two antibiotics.

Two *Klebsiella pneumoniae* isolates were tested and one of those isolates tested displayed a change in the MIC interpretation. The categorical rate for *Klebsiella pneumoniae* was 100% minor errors. The two other members of the *Enterobacteriaceae* that displayed inner colonies were *Citrobacter freundii* and *Enterobacter asburiae.* Both isolates displayed changes in the MIC interpretation. The *Citrobacter freundii* displayed both very major and major errors

and the *Enterobacter asburiae* did not display any errors in MIC interpretation but did display increases in the MIC of two antibiotics without a change in the MIC interpretation.

Reproducibility Studies

In order to demonstrate the reproducibility of the results, we conducted three additional studies to make sure that results were not skewed by outside variables. The three studies were conducted at the conclusion of the original study using isolates that were screened from the original study. The isolates used in the study were taken from frozen stocks from clinical specimens.

Reproducibility Studies: Trek Sensititre

We selected five isolates for further study that did not produce inner colonies by Kirby Bauer testing. Each isolate was subjected to testing by the TREK Sensititre in biological duplicate and the results were compared to the original test isolate. The isolates tested were *Pseudomonas aeruginosa (n=2)*, *Klebsiella pneumoniae (n=1)*, *Enterobacter cloacae (n=1)*, and *Escherichia coli* (n=1). We observed only one instance where a change in the MIC result was noted: for the *Enterobacter cloacae* isolate, the result of the Ampicillin was listed as <=8 (S) when the original result was >=8 (R). With the exception of this isolate, there was full agreement with the results obtained from the original isolate. Since the change observed was only one doubling dilution apart, the change in the MIC may be attributed to the normal ±1 doubling dilution error rate of the Trek Sensititre. In addition, *Enterobacter* species are known to be considered intrinsically resistant to Ampicillin.

		Ceftriaxone	Ciprofloxacin	Ertapenem	Ampicillin	Amikacin	Amp/Sulbactam	Aztreonam	Cefepime	Ceftazidime	Pip/Tazo	Gentamicin	Meropenem
K. pneumoniae	original	<=0.25 S	<=0.06 S	<=0.12 S	>16 R	<=16 S	<=8 S	<=1 S	<=0.25 S	<=0.5 S	<=8 S	<=4 S	<=1 S
	test 1	<=0.25 S	<=0.06 S	<=0.12 S	>16 R	<=16 S	<=8 S	<=1 S	<=0.25 S	<=0.5 S	<=8 S	<=4 S	<=1 S
	test 2	<=0.25 S	<=0.06 S	<=0.12 S	>16 R	<=16 S	<=8 S	<=1 S	<=0.25 S	<=0.5 S	<=8 S	<=4 S	<=1 S
E. coli	original	<=0.25 S	<=0.06 S	<=0.12 S	<=8 S	<=16 S	<=8 S	<=1 S	<=0.25 S	<0.5 S	<=8 S	<=4 S	<=1 S
	test 1	<=0.25 S	<=0.06 S	<=0.12 S	<=8 S	<=16 S	<=8 S	<=1 S	<=0.25 S	<0.5 S	<=8 S	<=4 S	<=1 S
	test 2	<=0.25 S	<=0.06 S	<=0.12 S	<=8	<=16 S	<=8 S	<=1 S	<=0.25 S	<=0.5 S	<=8 S	<=4 S	<=1 S
P. aeruginosa	original	>2 NI	2 I	>1 NI	>16 NI	<=16 S	>16 NI	2 S	1 S	1 S	<=8 S	<=4 S	<=1 S
	test 1	>2 NI	2 I	>1 NI	>16 NI	<=16 S	>16 NI	2 S	1 S	1 S	<=8 S	<=4 S	<=1 S
	test 2	>2 NI	2 I	>1 NI	>16 NI	<=16 S	>16 NI	2 S	1 S	1 S	<=8 S	<=4 S	<=1 S
E. cloacae	original	<=0.25 S	<=0.06 S	<=0.12 S	>8 R	<=16 S	<=8 S	<=1 S	<=0.25 S	<=0.5 S	<=8 S	<=4 S	<=1 S
	test 1	<=0.25 S	<=0.06 S	<=0.12 S	>8 R	<=16 S	<=8 S	<=1 S	<=0.25 S	<=0.5 S	<=8 S	<=4 S	<=1 S
	test 2	<=0.25 S	<=0.06 S	<=0.12 S	<=8 S	<=16 S	<=8 S	<=1 S	<=0.25 S	<=0.5 S	<=8 S	<=4 S	<=1 S
P. aeruginosa	original	>2 NI	2 I	1 NI	>16 NI	<=16 S	>16 NI	<=1 S	4 SC	<=0.5 S	<=8 S	<=4 S	<=1 S
	test 1	>2 NI	2 I	1 NI	>16 NI	<=16 S	>16 NI	<=1 S	4 SC	<=0.5 S	<=8 S	<=4 S	<=1 S
	test 2	>2 NI	2 I	1 NI	>16 NI	<=16 S	>16 NI	<=1 S	4 SC	<=0.5 S	<=8 S	<=4 S	<=1 S

Table 5: MIC data from the Trek Sensititre reproducibility study. Repeated Trek runs were recorded with original susceptibility results recorded in the patients' records. Noted are the MIC values and MIC interpretation

Reproducibility Studies: Inner colonies

As mentioned previously, the presence of inner colonies is generally thought to represent either resistant subpopulations or hetero-resistance. In order to determine the reproducibility of inner colonies using the Kirby Bauer method, we selected five isolates that were positive for inner colonies for further analysis. These isolates were recovered from frozen stocks and were tested by the Kirby Bauer assay in biological duplicate. The results obtained were compared to the original Kirby Bauer results. The isolates tested include *Pseudomonas aeruginosa* (n=3), *Escherichia coli* (n=1), and *Enterobacter cloacae* (n=1).

Organism		Ceftriaxone	Ciprofloxacin	Ertapenem	Ampicillin	Amikacin	Amp/Sulbactam	Aztreonam	Cefepime	Ceftazidime	Pip/Tazo	Gentamicin	Meropemen
P. aeruginosa	original	NI	S	NI	NI	S	NI	S	R	S	S	I	S
	test 1	NI	S	NI	NI	S	NI	S	S	S	S	I	S
	test 2	NI	S	NI	NI	S	NI	S	S	S	S	I	S
E. coli	original	R	S	R	R	S	R	R	R	R	S	S	S
	test 1	R	S	R	R	S	R	R	S	R	S	S	S
	test 2	R	S	S	R	S	R	R	S	R	S	S	S
E. cloacae	original	S	R	S	R	S	R	S	S	R	S	S	S
	test 1	S	R	S	R	S	R	S	S	R	S	S	S
	test 2	S	R	S	R	S	R	S	S	R	S	S	S
P. aeruginosa	original	NI	R	NI	NI	S	NI	S	R	R	R	R	R
	test 1	NI	R	NI	NI	S	NI	S	R	S	R	R	R
	test 2	NI	R	NI	NI	S	NI	S	R	R	R	R	R
P. aeruginosa	original	NI	S	NI	NI	S	NI	S	S	S	S	I	S
	test 1	NI	S	NI	NI	S	NI	S	S	S	S	I	S
	test 2	NI	S	NI	NI	S	NI	S	S	S	S	I	S

Table 6: Results of the inner colony reproducibility study. Shaded cells indicate antibiotics that displayed inner colonies when tested via the Kirby Bauer Method. Green shaded cells indicate the inner colonies from the original isolate while gray zones indicate inner colonies in the repeat tests.

Four out of the five isolates tested were positive for inner colonies when tested with the Kirby Bauer Assay. Interestingly, although inner colonies were observed, the results did not match those observed previously. In some cases more inner colonies were observed as seen in isolates 2 and 5 from the table above and in other cases fewer inner colonies were observed as seen in isolate 1 above. The discrepancy may be due to the fact that the isolates came from frozen stocks compared but was freshly isolates when the original testing was performed. The inner colonies seen in the two test runs are more similar to the inner colonies from the original isolate. The freezing process may have altered the organism and changed the isolate’s susceptibility pattern. It is important to note that in many of the cases, the antibiotics where inner colonies were observed were either already resistant or did not have any CLSI interpretations.

Reproducibility studies: Kirby Bauer Testing

We selected five isolates that did not produce inner colonies when initially tested by the Kirby Bauer Assay. In order to determine whether these isolates would continue to screen negative for inner colonies, we retested the isolates in biological duplicate. The isolates tested were the same isolates that were tested in the Trek Sensititre Reproducibility Study. Because there was no zone size data from the original testing to compare against, the isolate was run in triplicate compared to the other studies where the isolates were run in duplicate. None of the 5 isolates tested produced inner colonies for any of the antibiotics tested. This is consistent with an absence of a resistant subpopulation.

Discussion

The Trek Sensititre is FDA-cleared for antibiotic susceptibility testing and has been shown to be comparable to both broth-based and and the disc diffusion for routine clinical testing. Gram-Negative isolates tested on the Trek Sensititre and Microscan walkaway showed a 93.5% essential agreement with each other demonstrating that the Trek Sensititre can accurately determine antibiotic susceptibility[5]. We hypothesized that the Trek Sensititre would be able to detect resistant subpopulations that are visualized as inner colonies using the Kirby Bauer method but are missed by microbroth dilution methods. When both methods were compared, the Trek Sensititre had a lower detection rate for antibiotic resistant subpopulations compared with Kirby Bauer as previously hypothesized. For the most part the MIC interpretations from the Trek Sensititre were in agreement to those of the Kirby Bauer. However, when inner colonies were

present on the Kirby Bauer plates, the Trek Sensititre MIC results from those inner colonies did not always correlate to the Trek Sensititre MIC results from the original isolate. These data indicate that to some degree, the Trek Sensititre does not appear to be as sensitive at detecting resistance. The degree to which the Trek Sensititre detects (or does not detect) resistant subpopulations varied depending on the organism tested.

Pseudomonas aeruginosa

Pseudomonas aeruginosa infections are arguably one of the most complex to treat due to the multitude of different resistance mechanisms potentially present, most notably drug efflux pumps and chromosomal and plasmid encoded antibiotic mechanisms that can be activated from induction by antibiotics or simply by mutation. The ability to detect antibiotic resistant subpopulations is therefore likely to be important for the effective care and treatment of *Pseudomonas aeruginosa* infections. It is therefore not surprising that *Pseudomonas aeruginosa* produced inner colonies at the highest rate. It is also important to note that of the 27 total isolates that produced inner colonies, 21 isolates (78%) displayed a significant change in MIC or interpretation.

Very major errors were mainly seen in aztreonam (n=6). This poses a potential problem to patient care as treatment with aztreonam or any of the other antibiotics that displayed very major errors could potentially lead to treatment failure. Aztreonam is used to treat gram negative infections, including *Pseudomonas aeruginosa* and is used in patients who are allergic to penicillin or cannot tolerate aminoglycosides. Aztreonam resistance can arise in a number of different ways: 1) decreased uptake, 2) destruction by β-lactamases, and 3) altered penicillin binding protein. Four of the six isolates that displayed very major errors to aztreonam also

showed errors to ceftazidime and piperacillin/tazobactam. Two of the six isolates that displayed errors to aztreonam also showed an increase in meropenem MIC. Major errors were observed in relatively equal numbers in Ceftazidime(6), Meropenem(6), and Piperacillin/Tazobactam(5). Significant increases in MIC without an interpretation change were disproportionately observed in *Pseudomonas aeruginosa* isolates. Of the 11 isolates that displayed an instance of MIC change without interpretation change, 10 of those were seen in *P. aeruginosa* isolates. Not only that but those changes were observed mainly in two very important antibiotics used for treatment, cefepime and meropenem.

One interesting pattern that was observed in *Pseudomonas aeruginosa* isolates was that many of the inner colonies were observed inside the zones of clearing around the ertapenem and meropenem discs. It should be noted that ertapenem is an inappropriate antibiotic to treat *Pseudomonas aeruginosa* infections and is therefore not reported by our laboratory. However, it does seem that inner colonies seen in ertapenem show increased MIC's to meropenem. As seen above in Figure 3, the antibiotic where the most number of total errors were observed was meropenem and given that inner colonies to carbapenems were the most common it shows that these inner colonies were the ones responsible for the changes in the antibiotic susceptibility patterns observed.

Other Non Enterobacteriaceae

Stenotrophomonas maltophilia is one of the most clinically significant organisms of the non-*Enterobacteriaceae* tested. *S. maltophilia* is an environmental pathogen that is a rare cause of infections in immunocompromised individuals. Infections that are associated with this organism are have been associated with high morbidity and mortality in immunocompromised

patients. Treatment of *S. maltophilia* is very challenging due to many mechanisms for antibiotic resistance that the organism naturally habors. Furthermore, conventional methods of susceptibility testing have proven to be unreliable for accurately determining the MIC in *Stenotrophomonas maltophilia* isolates for most antibiotics. Only 2 of the 12 antibiotics that were observed in the study have CLSI breakpoints and interpretations for *S. maltophilia*. At the time of the study, susceptibility testing for *Stenotrophomonas maltophilia* was done through the Trek Sensititre in the UWMC Clinical Microbiology Laboratory. However, due to the unreliability of the results via conventional methods, *Stenotrophomonas maltophilia* isolates are now tested via E-tests.

Three of the four *S. maltophilia* isolates tested displayed a difference between the resistance patterns of the original isolate compared to that of the inner colonies. Very major errors were observed in both of the reportable antibiotics (ceftazidime and ciprofloxacin) as well as a major error in ceftazidime. The results from the original study show that the inner colonies displayed by *S. maltophilia* isolates were not detected by the Trek Sensititre system. However, because of the shift from microbroth dilution to other methods of testing and the unreliability of conventional methods, isolates of *S. maltophilia* may not be appropriate for determining whether inner colonies can be detected by the Trek Sensititre method.

Alcaligenes faecalis is an opportunistic pathogen that is rarely implicated in human infection. In the study only one isolate produced inner colonies. Those inner colonies did not end up creating any change in MIC interpretation but it did show increased resistance to ceftazidime and cefepime. *Acinetobacter lwoffii* is the last of the non *Enterobacteriaceae* tested. *Acinteobacter* species are environmental organisms that are a common pathogen seen in

hospitals. These organisms are intrinsically resistant to some classes of antibiotics such as penicillin and aminoglycosides. Eight total *Acinetobacter* isolates were tested of which inner colonies were observed in two of the isolates. Only one isolate, an *Acinetobacter lwoffii*, had a change in the MIC. Only one major error was observed in ampicillin.

Enterobacteriaceae

The family *Enterobacteriaceae* includes many different species of Gram-Negative bacilli, all of which can have different patterns of antimicrobial resistance. In particular, there is a growing global concern about resistance to β-lactam antibiotics due to the production of carbapenemases.

In the study, over two thirds of the isolates that were screened for errors were in the family *Enterobacteriaceae*. Of those 203 isolates tested, only 17 screened positive for inner colonies which shows that spontaneous mutations and the chance of resistant subpopulations in members of the *Enterobacteriaceae* is rare. Of those 17 isolates where inner colonies were observed, 8 isolates (47%) displayed a significant change in MIC or interpretation when retested using the Trek Sensititre system. Inner colonies of *Escherichia coli* isolates were observed more frequently than other *Enterobacteriaceae*. Five isolates of *E. coli* demonstrated a change in MIC or interpretation. Unlike the patterns observed in *Pseudomonas aeruginosa* isolates, changes in the MIC and interpretation were spread across multiple antibiotics and did not tend to cluster around a few antibiotics. Changes in the MIC/interpretation were observed only in the β-lactam antibiotics.

Very major and major errors occurred in *E. coli* isolates at a higher rate than in *Pseudomonas aeruginosa* isolates. Of the five isolates where a change in the MIC was observed, four isolates displayed at least one very major error or major error and one isolate tested displayed a minor error. The error rate seen in *Escherichia coli* isolates is therefore much higher than the error rate seen in isolates of *Pseudomonas aeruginosa*. This pattern may be due to the fact that resistance in *Pseudomonas aeruginosa* is most likely due to efflux mechanisms or decreased solubility which leads to small increases in antibiotic resistance as can be seen in the major errors and increases in MIC without an interpretation change. The resistance seen in *E. coli* isolates consists mainly of very major and major errors which seems like the inner colonies gained more of a complete resistance. This complete resistance is most likely due to the effect of β-lactamases like AmpC β-lactamases or Extended Spectrum β-lactamases(ESBLs).

Another member of the *Enterobacteriaceae* that displayed interesting results was *Citrobacter freundii*. Significant changes in the MIC were only observed in one of the nine isolates that were tested, but the one isolate displayed major errors in 4 antibiotics (Ceftriaxone, Ceftazidime, Aztreonam, and Ampicillin/Sulbactam) and a major error in 1 antibiotic (Piperacillin/Tazobactam). Similarly to what was observed in *E. coli* isolates, the changes in interpretation in *C. freundii* were observed in the β-lactam antibiotics. This could indicate that a β-lactamase may be the cause of the resistance in the subpopulation. A hyper-induced or derepressed AmpC β-lactamase may be the cause due to the increased resistance many β-lactams except in Cefepime, a 4th generation cephalosporin.

The remaining two isolates tested that belong to the family *Enterobacteriaceae* are *Klebsiella pneumoniae* and *Enterobacter asburiae*. The *K. pneumoniae* isolate only had one

minor error seen in Cefepime. This change in the interpretation can most likely be contributed to the normal ±1 doubling dilution error rate attributed to the Trek Sensititre system. The one *Enterobacter asburiae* isolate displayed very major errors to Ampicillin and Ampicillin/Sulbactam. The mechanism causing this resistance may be a penicillinase which explains resistance to only those two antibiotics.

Conclusions

Automated microbroth dilution-based testing methods are increasingly used by many clinical microbiology laboratories to determinate antimicrobial susceptibility. Thus, it is vital that these methods are able to accurately determine the correct antibiotic susceptibility patterns of microorganisms being tested. In our laboratory, we use the Trek Sensititre system. However, if this system cannot accurately detect resistance visualized as inner colonies on the Kirby Bauer Assay, then the clinical importance of the Trek Sensititre may be called into question.

Our study showed that the Trek Sensititre was unable to detect the additional resistance of inner colonies that are detected using the Kirby Bauer Assay. Nevertheless, there are a number of important limitations associated with the study: Firstly, the inner colonies used in the study were pooled from all inner colonies from the Kirby Bauer plate. Because the colonies were pooled, in some cases from different antibiotic zones, it is possible that a mixed population of resistant organisms that were selected for by the different antibiotics were tested. This possible mixture of multiple different subpopulations may make the results difficult to interpret. Secondly, some inner colonies were selected from antibiotics that do not have any CLSI-

approved interpretations and would be considered inappropriate to report. For example, some *Pseudomonas aeruginosa* isolates displayed inner colonies to ertapenem, an antibiotic that has no CLSI interpretations and a drug that would not be used clinically. The Trek Sensititre results from those inner colonies showed additional issues with multiple different antibiotics. Clinically, the patient would not have been treated with that antibiotic, so that this resistant set of subpopulations may not have been selected for. Thus the results from the study may not be clinically significant but it does open up questions to the possibility that the Trek Sensititre may not detect clinically significant resistant subpopulations.

Given that the Trek Sensititre can miss resistant subpopulations in Gram negative bacilli, it is important to establish the clinical relevance of this finding. If the inner colonies selected for by each of the antibiotic discs was sub-cultured individually instead of pooled together and then run on the Trek Sensititre, then the following results may possibly differ from the pooled inner colonies. An important future direction would be to determine whether the unreported resistance from the organisms in the study had an effect on patient care however, this was beyond the scope of this thesis study. Thus, the clinical significance of this study's findings require future study. Nevertheless, laboratories should be aware of the potential for missing sub-populations of antimicrobial resistant organisms using broth-based microdilution methods.

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