

Introduction

Welcome to MicroFocus, the information resource brought to you by the Dade Behring MicroScan team. The MicroFocus series is dedicated to spotlighting emerging issues in our complex, constantly changing field in order to help you keep abreast of the latest thinking and trends.

Many readers have requested more articles on gram-negative resistance topics, so in this issue we continue to explore resistance mechanisms, specifically those that impact the *Enterobacteriaceae*. As newly recognized forms of bacterial resistance become more prevalent, laboratorians will continue to face testing challenges. An understanding of key factors and attributes associated with antimicrobial resistance will support better detection, optimal reporting, and in turn will provide crucial information to those determining treatment regimens.

We received many requests for CLSI updates as well. Inserted into this issue is a checklist summarizing the 2007 CLSI Antimicrobial Susceptibility Testing recommendations. You can use this tool as a concise way to document review of the latest AST guidelines.

You have the opportunity to obtain one hour of continuing education credit by completing the evaluation and self-assessment test for the article *Carbapenemase Activity in the Enterobacteriaceae*. The evaluation/self-assessment may also be photocopied and returned to the same address on the reply card, providing everyone in your lab the opportunity to earn credit. Answers will be printed in the next issue and will also be available on www.dadebehring.com under Services→ Education→ Microbiology six months from the publication date. To enable us to better meet your needs, please take advantage of the additional space provided on the reply card to tell us about other topics you would like covered in upcoming issues of MicroFocus. E-mail me directly if you would like to become an author for an upcoming publication. We hope you will find this issue of MicroFocus both interesting and useful.

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Carbapenemase Activity in the *Enterobacteriaceae*

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The carbapenems are a class of antibiotic that inhibit cell wall synthesis. Because they bind to penicillin binding proteins 1 or 2, their extensive range of activity consists of many gram-positive bacteria including streptococci, staphylococci (methicillin-susceptible) and *Enterococcus faecalis*, a wide range of gram-negative bacilli and most anaerobic bacteria. They are stable to most beta-lactamase enzymes including extended spectrum beta-lactamases (ESBLs). Consequently, they have played an important role in treatment of infections caused by ESBL- and AmpC-producing members of the *Enterobacteriaceae*. However, with the increase in ESBL-producing organisms and the subsequent increase in the use of carbapenems for treatment of serious infections, the antibiotic pressure to develop resistance

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Spotlight on Bacterial Resistance Mechanisms: Beta-lactam Antibiotic Resistance Among *Enterobacteriaceae*

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As microbiologists and technologists working in clinical microbiology, we have an appreciation for the rapid replication and high number of bacteria present in infections or just colonizing patients. We also recognize the variety of strains existing among the population of a single species recovered from a patient. The mutation rate and induction of genes is not higher in bacteria but, because of the rapid growth, mutations are frequently encountered. Most mutants are not observed because they are lethal to the organism or not of significance. A spontaneous mutant that results in resistance to an antimicrobial drug would not likely be detected. However, if the antimicrobial drug is added to the environment (patient or culture), then the drug becomes a selective factor and removes all the

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has resulted in the appearance of carbapenem-resistant members of the *Enterobacteriaceae*. This article will review the carbapenem antibiotics, the resistance factors currently being seen and how laboratories can be alerted to the presence of these resistance factors.

The Carbapenems

The carbapenems are antibiotics with a beta-lactam ring attached to a thienamycin molecule. This structure has an intrinsic ability to resist degradation by commonly-encountered enzymes that inactivate some other beta-lactam antibiotics such as the penicillins and cephalosporins. Their mechanism of action is to bind to penicillin-binding proteins 1 and 2, causing cell elongation and lysis. The carbapenems currently available in the United States are imipenem, meropenem and ertapenem. All three are parenteral antibiotics (IV or IM administration) used primarily in hospitalized patients for treatment of serious infections. Two newer carbapenem antibiotics are doripenem and faropenem. These antibiotics are currently under preclinical evaluation and have not been approved by the FDA for distribution in the United States. Doripenem is a parenteral antibiotic with a spectrum of activity most similar to meropenem, although its anti-*Pseudomonas* activity may be the best in the carbapenem class. Faropenem is the first orally administered carbapenem and is being developed for the treatment of community-acquired pneumonia, sinusitis, acute exacerbation of chronic bronchitis and skin and soft tissue infections.

Imipenem was developed in 1985 and was the first carbapenem to be granted FDA approval. Imipenem is rapidly degraded by the renal enzyme dehydropeptidase when administered alone, so it is always co-administered with cilastatin to prevent degradation. Imipenem has activity against most gram-positive aerobic and anaerobic bacteria with the exception of methicillin-resistant staphylococci and *Enterococcus faecium*. It also has excellent activity against most gram-negative bacteria including *Pseudomonas*, *Acinetobacter* and the *Enterobacteriaceae*. Imipenem has good penetration into the central nervous system (CNS).

Meropenem is similar in coverage to imipenem. It is slightly more active against *Pseudomonas* but has less activity against gram-positive bacteria. It has the advantage of not being susceptible to breakdown by dehydropeptidase. Meropenem penetrates into inflamed meninges and may be used as a second line agent for

treatment of meningitis. Because of the similarity in spectrum of activity for the gram-negative bacteria between imipenem and meropenem, historically laboratories have been able to predict the susceptibility or resistance of one based on the testing of the other. However, in 2007 CLSI removed the "or" between these two carbapenems.¹

Ertapenem is the most recent newcomer in the carbapenem class and has a similar spectrum of activity to imipenem and meropenem – with the notable exception of having no activity against *Pseudomonas* and *Enterococcus*. Ertapenem has a longer half-life in serum than imipenem and meropenem, which allows for once-daily dosing. However, ertapenem is highly protein-bound and does not penetrate into the CNS. Ertapenem has recently become available on MicroScan® panels.

Carbapenem Resistance in Gram-negative Bacteria

Besides the inherent resistance to the carbapenems observed in *Stenotrophomonas*, carbapenem resistance in gram-negative bacteria has mostly been observed in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains (Table 1).

Table 1

Mechanisms of Carbapenem Resistance	
Organism group	Resistance mechanisms
<i>Stenotrophomonas</i>	Metallo-β-lactamase
<i>Pseudomonas</i>	Metallo-β-lactamase, loss of porins, efflux
<i>Acinetobacter</i>	Metallo-β-lactamase, loss of porins, efflux
<i>Enterobacteriaceae</i>	Serine carbapenemase (KPC, SME), loss of porins

These organisms, like *Stenotrophomonas*, can carry a metallo-β-lactamase enzyme that can hydrolyze the carbapenem molecule. It is called a metallo-β-lactamase because it requires divalent cations such as zinc for expression of activity. These enzymes can inactivate all three carbapenems but are rarely observed in the enteric gram-negative rods. Another mechanism of carbapenem resistance is a change in outer membrane proteins (porins) that can block entrance of the antibiotic into the cell. Organisms that only have the loss of porins usually have an increase in the MIC of the carbapenem but may still fall within the susceptible category under the CLSI breakpoints (Tables 2, 3). However, if the loss of porins coincides with the hyperproduction of an AmpC beta-lactamase, frank

resistance may be noted. A third resistance factor noted mainly in *Pseudomonas* and *Acinetobacter* is an efflux pump mechanism that pumps the antibiotic out of the cell.

Table 2

CLSI MIC Breakpoints for the <i>Enterobacteriaceae</i>			
Antibiotic	Susceptible	Intermediate	Resistant
Ertapenem	≤2	4	≥8
Imipenem	≤4	8	≥16
Meropenem	≤4	8	≥16

Table 3

CLSI Disk Diffusion Breakpoints (in mm) for <i>Enterobacteriaceae</i>			
Antibiotic	Resistant	Intermediate	Susceptible
Ertapenem	≤15	16-18	≥19
Imipenem	≤13	14-15	≥16
Meropenem	≤13	14-15	≥16

The KPC Carbapenemases

Until recently, resistance to the carbapenems in the *Enterobacteriaceae* has been a rare event and mainly noted in rare strains of *Serratia marcescens* that carry a serine carbapenemase called SME. Because of the rarity of carbapenem resistance in enteric gram-negative rods, the most recent CLSI document continues to recommend confirmation of any *Enterobacteriaceae* resistant to a carbapenem as well as any *Stenotrophomonas* susceptible to the antibiotics (Table 4).

In a recently published survey of more than 37,557 strains of enteric gram-negative rods from 2000-2004, only 51 strains (0.001%) were found to possess a carbapenemase enzyme.² However, in 2001 a new serine carbapenemase (later called KPC-1) that was capable of hydrolyzing the carbapenems as well as the extended spectrum cephalosporins was recovered from a patient in North Carolina infected with *Klebsiella pneumoniae*.³ In 2003, a similar carbapenemase was recovered predominantly in *Klebsiella pneumoniae* strains isolated from hospitals along the East Coast of the United States.^{4,5} This resistance factor was labeled KPC-2 and differed by only one amino acid from KPC-1. Yet another carbapenemase that also

differed by only one amino acid from KPC-2 has been recovered from an isolate of *Enterobacter*.⁶ Enteric bacteria from many genera^{7,8} have now been noted to carry the KPC-resistance genes (Table 5) and the rapid spread throughout the hospitals in the New York City area caused the New York State Health Department to issue an advisory in 2005. KPC-producing strains have now been noted sporadically in many regions throughout the United States.

Table 4

CLSI Glossary III Suggestions for Verification of Antimicrobial Susceptibility Test Results and Confirmation of Organism Identification ¹	
Organism or Group	Category I ^a Phenotypes that have not been reported, are uncommon, and/or result from technical errors
<i>Enterobacteriaceae</i> (any)	Carbapenem – I or R
<i>Stenotrophomonas maltophilia</i>	Carbapenem – S

Category I^a

When results listed in this category are observed on individual patient isolates, they should be verified by one or more of the following:

1. Ensuring the unusual results are not due to transcription errors, contamination or use of a defective panel, plate or card.
2. Checking previous reports on the patient to determine if the isolate was encountered and verified earlier.
3. Confirming the identification of the isolate.
4. Repeating the susceptibility test to confirm the results. Sometimes it is helpful to use an alternative test method for the repeat test.

Table 5

Genera noted to have KPC-resistance genes ⁷
<i>Klebsiella pneumoniae</i> (KPC-1, KPC-2, KPC-3)
<i>Citrobacter freundii</i> (KPC-2, KPC-3)
<i>Enterobacter</i> spp. (KPC-3)
<i>Serratia marcescens</i> (KPC-3, SME-1, SME-2)

A major problem with the detection of these enzymes is that some automated susceptibility systems may not be able to detect carbapenem resistance.^{9,10} Another issue that causes alarm is that the KPC-resistance genes are often carried on transmissible plasmids that also have resistance genes for the fluoroquinolones, aminoglycosides and ESBLs. Thus, these isolates tend to be resistant to a broad range of antibiotics (Table 6).

Table 6

Antibiograms of KPC-producing clinical isolates ^{4,8}			
	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>K. pneumoniae</i>
Resistance genes	TEM-1, KPC-2	TEM-1, KPC-2	TEM-30, SHV-12, KPC-2, TEM-1
Imipenem	32	8	≥64
Meropenem	16		
Ertapenem	32		
Ceftriaxone	>32		
Ceftazidime	16	16	≥64
Cefepime	32		
Aztreonam	>256		
Piperacillin tazobactam	256	>128	≥128
Levofloxacin	32		
Ciprofloxacin		1	≥4
Gentamicin	8		
Tobramycin	32		
Amikacin	4	4	32
Trimeth/sulfa	>8/152		
Colistin	0.5		

Laboratory Detection of KPC Enzymes: Issues and Problems

The problem with detecting these KPC-resistance genes can best be described by a recent clinical report that describes a treatment failure of a patient with a *Klebsiella pneumoniae* bacteremia who was treated initially with meropenem.⁵ The laboratory tested meropenem by disk diffusion and found the isolate to be susceptible. Because it previously had been accepted that a class drug approach to susceptibility testing with the carbapenems could reliably predict susceptibility or resistance to the other members of the class, it was assumed the isolate was also susceptible to ertapenem. On the basis of the meropenem susceptibility, the patient was switched to ertapenem for convenience of dosing. One week later the patient once again became bacteremic with *Klebsiella pneumoniae*. When the laboratory tested the isolate by Etest* strips, they found the ertapenem MIC was >8 while the meropenem MIC was 3. By CLSI criteria (Table 2), the ertapenem would be considered resistant while the meropenem would be considered susceptible but at the susceptible breakpoint. The isolate was also determined to be positive for an ESBL.

This patient scenario points out the importance of being able to test for ertapenem routinely if the antibiotic has

been added to your formulary. Even if ertapenem is not on your hospital formulary, it may be beneficial to have the antibiotic on hand, since it appears that ertapenem is the most sensitive carbapenem for the detection of KPCs. Since most *K. pneumoniae* isolates exhibiting KPC-2 have also possessed ESBLs, you may want to limit testing to only those isolates in which you are confirming an ESBL.

One issue that is critical in accurately testing imipenem is the inoculum effect. It is very important to accurately adjust the concentration of organisms in the inoculum because imipenem, more so than the other carbapenems, is greatly affected by under-inoculation of MIC panels or in disk diffusion testing.⁶ Imipenem also is the least stable of the carbapenems under storage, so proper storage conditions must be maintained and quality control testing is critical to assure accurate patient results.

Different Approaches to Testing

Detection of the carbapenemase-producing organisms is difficult and continues to be sporadically encountered in most areas of the United States. Unfortunately, there is no single phenotypic test that will alert the laboratorian to the presence of KPC resistance in clinical isolates. The following are some suggestions on various approaches a laboratory can take to help detect carbapenem resistance:

* Etest is a registered trademark of AB Biodisk, Solna, Sweden.

- If you use an automated system and ertapenem has been added to your hospital formulary, it may be time to validate a new panel so you can test routinely. MicroScan currently has ertapenem available on a number of susceptibility panels. Any increase in ertapenem MIC should prompt a closer evaluation of the organism for the presence of a KPC.
- If you are testing routinely by disk diffusion or confirming ESBLs with the double disk test, adding an ertapenem disk to your ESBL confirmation plate may help you pick up KPC-producing strains. Look for ertapenem zone diameters near the breakpoint of 19 mm. In particular, look for breakthrough colonies just inside the zone of inhibition (Figure 1). These may represent carbapenemase-producing isolates.
- If you are confirming ESBLs with the Etest strips, consider adding an ertapenem Etest strip to your confirmation plate. The breakpoint for resistance is 8 mcg/ml. Any isolate with an MIC of ≥ 8 mcg/ml should be evaluated further for the presence of a KPC-enzyme. Again, look for breakthrough colonies inside the inhibitory zone (Figure 2).
- Any *Enterobacteriaceae* testing intermediate or resistant to meropenem or imipenem, or with an elevated MIC (≥ 1 mcg/ml) to these two carbapenems should be checked with ertapenem, since it seems to be most sensitive to the carbapenemase enzymes. Since only a relatively few of the MicroScan® MIC panels dilutions of imipenem or meropenem down to 1 mcg/ml, it may be difficult to identify isolates with elevated MICs that still fall within the susceptible category.

Figure 1

Disk diffusion of a KPC-2 producing strain of *Klebsiella pneumoniae*, indicating resistance to ertapenem. Breakthrough colonies appear within the zone of inhibition surrounding imipenem and meropenem.



Figure 2

Etest of the KPC-2 producing strain of *Klebsiella pneumoniae* shown in Figure 1. Ertapenem would be considered frankly resistant but with imipenem, only a fraction of the population exhibits resistance at an MIC of 4 mcg/ml.



- If you are using the MicroScan® ESBL *plus* panel to confirm ESBLs, the panel has concentrations of imipenem and meropenem as low as 0.5 mcg/ml. This should allow you to detect isolates with elevated MICs to meropenem and imipenem that may require further testing for the presence of a carbapenemase.
- If you find an isolate suspicious for a carbapenemase enzyme, you should forward the isolate to a reference laboratory, to your state health laboratory or to the CDC for confirmation. At this time, only molecular methods are capable of identifying the type of carbapenemase enzyme a resistant isolate possesses.

Treatment Options

If the KPC-producing organism carries a plasmid that also possesses resistance genes for other antibiotics such the cephalosporins, aminoglycosides and fluoroquinolones, treatment options are considerably diminished. Currently, colistin, polymyxin B and tigecycline have all shown activity against the KPC-producing bacteria. Laboratories should have the capability of testing for these antibiotics or they should forward the isolate to a reference laboratory for further testing.

Conclusion

While KPC-producing strains are still relatively rare throughout most of the country, it likely won't be long before we begin to encounter them on a more frequent basis. Think now about how to modify your approach to testing the carbapenems to optimize detection of this new resistance mechanism.

CEU Questions for "Carbapenemase Activity in the *Enterobacteriaceae*"

1. Which of the following groups of bacteria known today have the most likely potential to possess carbapenem-inactivating enzymes?
 - a. *MSSA* and *Enterobacteriaceae*
 - b. *Enterobacteriaceae* and *S. maltophilia*
 - c. *Klebsiella* spp. and *P. aeruginosa*
 - d. *Enterobacteriaceae*, *Stenotrophomonas*, *Pseudomonas* and *Acinetobacter*
2. The incidence of carbapenemase enzymes in enteric gram-negative bacilli is low but is increasing.
 - a. True
 - b. False
3. Closer evaluation for the presence of what resistance mechanism is warranted when a *Klebsiella pneumoniae* isolate shows an increase in ertapenem MIC?
 - a. Metallo- β -lactamase
 - b. AmpC
 - c. KPC
 - d. CTX-M
4. Susceptibility or resistance to a carbapenem can be predicted based on the results of testing one class representative.
 - a. True
 - b. False
5. Critical issues impacting the accuracy of imipenem susceptibility testing include:
 - a. Drug instability
 - b. pH
 - c. Low inoculum density
 - d. b and c
 - e. a and c

Answers will be published in the next issue of *MicroFocus* and online at www.dadebehring.com.

References:

1. Clinical Laboratory Standards Institute. 2007. Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement. CLSI Document M100-S17. Clinical Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.
2. Deshpande, L.M., Jones, R.N., Fritsche, T.R., Sader, H.S. 2006. Occurrence and characterization of carbapenemase-producing *Enterobacteriaceae*: Report from the SENTRY antimicrobial surveillance program (2000-2004). *Microb. Drug Resist.* 12:223-230.
3. Yigit, H., Queenan, A.M., Anderson, G.J., Sanchez-Sanchez, A.D., Biddle, J.W., Stewart, C.D., et al. 2001. Novel carbapenemase-hydrolyzing β -lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* 45:1151-1161.
4. Bradford, P.A., Bratu, S., Urban, C., Visalli, M., Mariano, N., Landman, D., Rahal, J.J., Brooks, S., Cebular, S., Quale, J. 2004. Emergence of carbapenem-resistant *Klebsiella* species possessing the class A carbapenem-hydrolyzing KPC-2 and inhibitor-resistant TEM-30 β -lactamases in New York City. *Clin. Infect. Dis.* 39: 55-60.
5. Lomaestro, B.M., Tobin, E.H., Shang, W., Gootz, T. 2006. The spread of *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* to upstate New York. *Clin. Infect. Dis.* 43:e26-28.
6. Bratu, S., Landman, D., Alam, M., Tolentino, E., Quale, J. 2005. Detection of KPC carbapenem-hydrolyzing enzymes in *Enterobacter* spp. from Brooklyn, New York. *Antimicrob. Agents Chemother.* 49:776-778.
7. Deshpande, L.M., Rhomberg, P.R., Sader, H.S., Jones, R.N. 2006. Emergence of serine carbapenemases (KPC and SME) among clinical strains of *Enterobacteriaceae* isolated in the United States Medical Centers: Report from the MYSTIC Program (1999-2005). *Diag. Microbiol. Infect. Dis.* 56:367-372.
8. Bratu, S., Brooks, S., Burney, S., Kochar, S., Gupta, J., Landman, D., Quale, J. 2007. Detection and spread of *Escherichia coli* possessing the plasmid-borne carbapenemase KPC-2 in Brooklyn, New York. *Clin. Infect. Dis.* 44:972-975.
9. Tenover, F.C., Kalsi, R.K., Williams, P.P., Carey, R.B., Stocker, S., Lonsway, D., et al. Carbapenem resistance in *Klebsiella pneumoniae* not detected by automated susceptibility testing. *Emerg. Infect. Dis.* [serial on the Internet] 2006 Aug. Available from <http://www.cdc.gov/ncidod/EID/vol12no08/06-0291.htm>.
10. Whittier, S., Wu, F., Della-Latta, P. 2007. Comparison of antimicrobial susceptibility tests for detection of KPC-positive *Klebsiella pneumoniae*. *ECCMID Abstract P-1629*.

Spotlight on Bacterial Resistance Mechanisms: Beta-lactam Antibiotic Resistance Among *Enterobacteriaceae*

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susceptible bacteria. The environment now favors the resistant mutant bacterium. We realize that, within a short period of time, all the bacteria will be progeny of the resistant bacterium and all the isolates will be resistant to the antimicrobial drug. Every time antimicrobial drugs are used, there is the possibility of selecting out resistant strains.

A second more common mechanism for bacteria becoming resistant to antimicrobial agents is by the acquisition of

genetic material from other bacteria that already have resistant genes and spread the genetic information to previously susceptible strains. These organisms now become resistant with the newly acquired resistance genes. Several mechanisms of genetic transfer between bacteria have been described. The transfer of extra-chromosomal nucleic acid in the form of plasmids appears to be the predominant method. The plasmids carrying resistance genes are numerous and of different sizes and content.

Trying to predict the resistance among bacteria such as *Enterobacteriaceae* is dangerous without performing antimicrobial susceptibility testing on the isolate.

Resistance to all classes of antibiotics exists, and the genetic information for the resistance mechanisms is continually spreading among bacteria. A frequently asked question is: "Where did the resistance mechanisms originate?" Other than the spontaneous mutations, which are then shared among bacteria, some of the original genes are from the organisms that produce the antimicrobial compounds and have evolved resistance mechanisms to keep from killing themselves. Or, the resistance may have evolved from other organisms living in the same environment as the antibiotic-producing strains. The emergence of new antimicrobial drugs is always followed by emergence of clinically significant resistant organisms that acquire resistance as described above (mutation or acquisition of resistance genes). Entire books have been written on antimicrobial resistance mechanisms. The evolving resistance mechanisms and continuing spread among clinically significant organisms increases the need for clinical microbiologists and technologists to keep current and have greater understanding of antimicrobial drug resistance. Two of the goals of MicroFocus are to assist you in keeping current and help you in understanding bacterial antimicrobial resistance. There is continuing pressure on the microbiology laboratory to detect resistance and report rapidly to physicians, infection control, pharmacy and other health care personnel. In this issue, we are concentrating on beta-lactam resistance in *Enterobacteriaceae*. The antimicrobial resistance topic is too large an area to be covered in one or two MicroFocus articles, so we will continue to address additional aspects and newer developments in future issues.

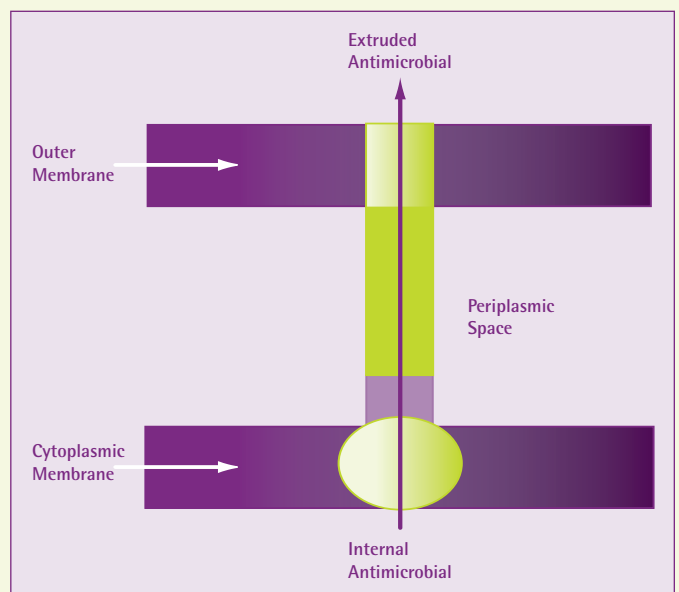
Antimicrobial resistance in *Enterobacteriaceae* involves several mechanisms: efflux, absorption, binding site changes and enzyme inactivation. The significance of each will vary with the drug class and species of bacteria, but all have been described among *Enterobacteriaceae*.

EFFLUX: This resistance mechanism involves the bacterium pumping the drug out of the organism. Several efflux systems exist in *Enterobacteriaceae* and form a tripartite complex that spans both membranes and the periplasmic space (Figure 1). Many classes of drugs are removed from bacteria via efflux pumps (e.g., aminoglycosides, macrolides, tetracyclines), but it is not a major mechanism for beta-lactam resistance among *Enterobacteriaceae*.

ABSORPTION: This is a major factor among Gram-negative bacterial resistance, because they possess an outer membrane in addition to the cytoplasmic membrane. (Gram-positive

bacteria only contain the cytoplasmic membrane.) The outer membrane plays several roles, and one important role is as a barrier to large molecules gaining access to the bacterium. The main reason Gram-negative bacteria are inherently resistant to glycopeptides (e.g., vancomycin) is that the glycopeptide molecules are too large to pass through the porins and thus are excluded from Gram-negative bacteria. The membrane is composed of lipo-polysaccharide. Interspersed within the membrane are large proteins called porins (these can be thought of as pores).

Figure 1



Bacteria contain several different porin proteins within their outer membrane. The individual porin structure controls which molecules can gain entrance through the outer membrane. Mutations resulting in a change of amino acid(s) in the porin protein can result in porin molecular changes and the permeability of the porin changes. This can result in prevention of certain antibiotics from gaining entrance to the periplasmic space (between the outer and inner membranes), and the bacteria become resistant to the extruded antibiotics. This has been the main mechanism of carbapenem resistance in *Pseudomonas* previously seen in the United States. Sometimes production of a porin protein is totally lost by the bacteria and, therefore, any molecules entering exclusively through that particular porin are prevented from gaining access. This could be one or several different antibiotics to which the bacterium will become resistant. Porin location is depicted in Figure 2.

BINDING SITE CHANGES: Beta-lactam antimicrobial drugs derive their antibacterial action by binding to the

enzymes necessary for the synthesis of bacterial cell wall formation. A series of these enzymes are referred to as "penicillin-binding proteins" (PBP). The PBPs are membrane-bound and catalyze the transglycosylation and/or transpeptidation reactions that cross link the peptidoglycan of the bacterial cell wall. Binding of the beta-lactam antibiotics to the PBPs blocks the PBPs from performing their enzymatic function; cell wall is no longer formed, and the bacterium eventually ruptures and dies.

Changes in the molecular structure of the PBP may block the ability of the beta-lactam antibiotic to bind to the PBP, but it still functions as an enzyme to take part in cell wall formation. This results in resistance to the beta-lactam antibiotics. Some well-known examples of PBP structural changes and loss of affinity for beta-lactam drugs are seen in penicillin-resistant *Streptococcus pneumoniae* and methicillin- (oxacillin-) resistant *Staphylococcus aureus*. The PBP structural change mechanism is less prominent among Gram-negative bacteria, but it has been described^{1,2}; for example, low affinity PBPs in penicillin-resistant strains of *Haemophilus influenzae* and *Neisseria gonorrhoeae*.

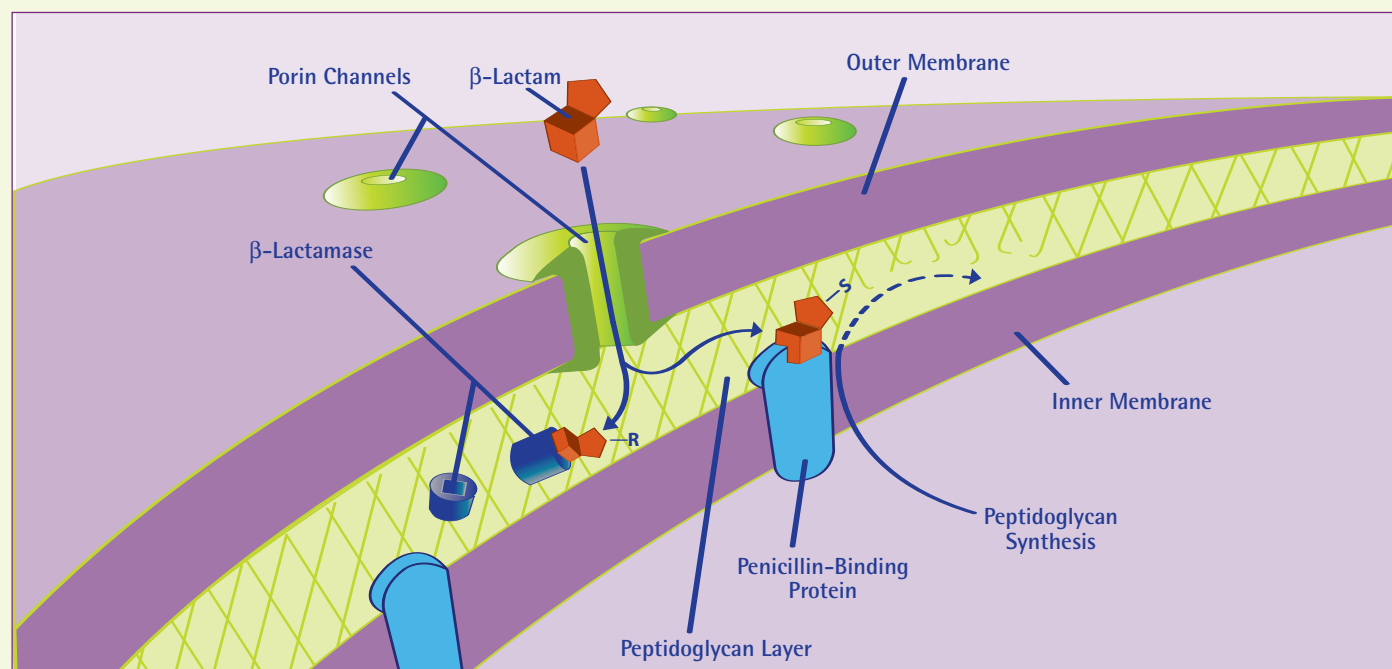
ENZYMATIC INACTIVATION: The most frequently observed beta-lactam resistance mechanism among *Enterobacteriaceae* is the enzymatic inactivation of the beta-lactam antibiotics. The enzymes are collectively referred to as "beta-lactamases," and hundreds have been individually described. Attempts to categorize the enzymes

have resulted in two classification schemes: molecular and functional. The molecular system uses molecular structure as the basis of dividing the enzymes into four classes: A, B, C and D. The functional, or phenotypic system, divides the beta-lactamases based on the properties of the enzymes (such as inactivation by clavulanic acid, etc.) into four groups: 1, 2, 3 and 2d (see Table 1).

The **Molecular Class A or Functional Group 2** contains the largest number of beta-lactamases. The genes for the enzymes are usually on plasmids and are, therefore, readily spread among members of the same species or even among different genera. The best known representatives of Class A are the TEM-1, SHV-1 and mutations of these enzymes known as ESBLs. The TEM, SHV and CTX-M ESBLs have been discussed in the previous issue of MicroFocus (Fall 2006, Hindler). Recently, a new Class A beta-lactamase group with carbapenemase activity has emerged in the eastern United States. They are referred to as KPC and have created a major stir. We seem to be where we were with ESBLs ten years ago or VRE 20 years ago. The more frequent use of carbapenems to treat serious ESBL-producing bacterial infections can only hasten the spread of KPC and other carbapenemase-producing *Enterobacteriaceae* across the United States and other countries.

The **Molecular Class B or Functional Group 3** encompasses the metallo-beta-lactamases. These are extremely potent

Figure 2



enzymes and are able to inactivate our last line of beta-lactam antibiotics, namely, carbapenems (imipenem, meropenem and ertapenem). Metallo-beta-lactamase-producing bacteria are resistant to almost all beta-lactam antibiotics (except aztreonam) and frequently carry genes for resistance to other classes of antibiotics. This results in "pan-resistant" bacteria which present significant therapeutic challenges.

Carbapenem hydrolyzing enzymes can be found in all four classes of beta-lactamases:

Class A – KPC enzymes (discussed by Dr. Fader in this issue of MicroFocus)

Class B – The metallo-beta-lactamases, e.g., IMP

Class C – ACC-1 enzyme in conjunction with deletion of porin

Class D – OXA-23, etc., beta-lactamases

The **Molecular Class C or Functional Group 1** is common to many *Enterobacteriaceae*, and low levels of the enzyme may even have a role in microbial replication. However, induction to high level production in species with regulatory genes results in resistance to many beta-lactam antibiotics. The best-known example of Class C beta-lactamase is AmpC. Laboratory detection of organisms with regulatory gene control for high-level induction of AmpC is a challenge.

We have all had the experience of isolating bacteria that appear susceptible to third-generation cephalosporins (cefotaxime or ceftazidime), and the patient is treated with one of these antibiotics. Initially, the patient responds but then relapses with the same organism. Upon re-isolation and testing, the isolate demonstrates resistance. Re-testing of the first isolate shows it is still susceptible, but the second isolate is resistant. We now understand that certain species with gene control mechanisms of AmpC production can be induced to high-level production of AmpC by the third-generation beta-lactams or may even mutate to high-level production and become clinically and laboratory resistant.

Both the laboratory and the physician need to be aware of such organisms capable of "derepressed" AmpC beta-lactamase production. This is the reason physicians will not treat serious infections solely with a third-generation cephalosporin when the infection is by a species with inducible regulatory AmpC production, as seen in *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens*, etc. This is a case where phenotypic susceptibility testing (disk or MIC) may be misleading and why organism identification as well as susceptibility results are important. Having just the antimicrobial testing results without the bacterial identification can be dangerous.

The **Molecular Class D or Functional Group 2d** beta-lactamases are heterogeneous and less well understood. Some of the oxacillinase group may have carbapenemase

Table 1 Classification of Beta-lactamases^{3,4}

Molecular Class	Functional Group	Activities	Example
A	2	<ul style="list-style-type: none"> ■ Preferentially penicillinases ■ Serine at active site ■ Most inhibited by clavulanic acid 	TEM, SHV, CTX-M ESBLs (mutants of TEM, SHV)
B	3	<ul style="list-style-type: none"> ■ Carbapenemases ■ Metallo-b-lactamases 	IMP, VIM
C	1	<ul style="list-style-type: none"> ■ Cephalosporinases ■ Serine at active site ■ Not inactivated by clavulanic acid 	AmpC
D	2d	<ul style="list-style-type: none"> ■ Carbapenem hydrolyzing ■ Oxacillin hydrolyzing 	OXA-23

hydrolyzing activity. The number and frequency of these beta-lactamases seem to be increasing in both number and clinical significance. They will be discussed in a future issue.

In summary, the frequent use of antimicrobial drugs creates a constant pressure for the selection and spread of antimicrobial resistance. Bacteria are able to transmit their genetic resistance information to other genera and species and present continuing challenges to microbiologists and physicians. As laboratorians, we have a responsibility to keep abreast of emerging resistance and newer laboratory

procedures for resistance detection. In this issue, we have discussed some mechanisms Gram negative bacteria utilize against antimicrobial agents, especially the enzymatic inactivation of beta-lactam antibiotics. The list of beta-lactam-inactivating enzymes (beta-lactamases) is continually evolving and spreading. The dynamic nature of resistance mechanisms evolving among bacteria will continue to keep the field of clinical microbiology both exciting and challenging.

References:

1. Dougherty, T. J. 1986. Genetic analysis and penicillin-binding protein alterations in *Neisseria gonorrhoeae* with chromosomally mediated resistance. *Antimicrob Agents Chemother.* 30:649-652. 2. Mendelman, P. M., Chaffin, D. O. and Kalaitzoglou G. 1990. Penicillin-binding proteins and ampicillin resistance in *Haemophilus influenzae*. *J Antimicrob Chemother.* 25:525-534. 3. Ambler, R. P. 1980. The structure of b-lactamases. *Philos Trans R Soc Lond (Biol).* 289:321-331. 4. Bush, K., Jacoby, G. A. and, Madeiros A. A. 1995. A functional classification scheme for b-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother.* 39:1211-1233.

Answers to "Spotlight on ESBLs: A Continuing Laboratory Challenge" Self-Assessment Questions published in the Fall 2006 issue.

1. Extended-spectrum β-lactamases are most commonly found in:

b. *E. coli*, *Klebsiella* spp., and *P. mirabilis*

2. According to CLSI rules, which of the following are NOT edited to "R" for ESBL producers?

e. Piperacillin-tazobactam

3. What is the function of clavulanic acid in the ESBL confirmatory test?

c. It blocks the ESBL and prevents it from hydrolyzing cefotaxime or ceftazidime.

4. Which of the following is NOT a characteristic associated with ESBL producing bacteria?

e. They are easily detected with routine disk diffusion or MIC tests for cefotaxime and ceftazidime.

5. Which of the following would be considered ESBL positive?

	MIC (µg/ml)			
	Cefotaxime	Cefotaxime/CA	Ceftazidime	Ceftazidime/CA
c.	32	≤0.5/4	16	≤0.25/4

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Tuesday, May 22, 9:00 AM: Antimicrobial Susceptibility Testing Methods – Gram-Positive Non-Molecular

1. Abstract C-004. **MICroSTREP plus/MICroFAST Panels: Evaluation of the WalkAway System Automated Read Methods for *S. pneumoniae* and other *Streptococcus* spp.** Hindler, J.¹, D. Bruckner¹, S. Mirrett², L.B. Reller³, P. Schreckenberger³, J. Tjho³, J. Borrelli⁴, S. Connell⁴, B.L. Zimmer⁴. ¹UCLA Medical Center, Los Angeles, CA; ²Duke University Medical Center, Durham, NC; ³Loyola Medical Center, Maywood, IL; ⁴Dade Behring Inc., West Sacramento, CA. **Study Conclusion:** Results from the WalkAway automated reading method for the MicroScan MICroSTREP plus/MICroFAST panels correlate well with those obtained from the CLSI reference broth microdilution method for susceptibility testing of *S. pneumoniae* and other *Streptococcus* spp.
2. Abstract C-007. **MIC Results for Streptococci Using CLSI Broth Microdilution Reference Panels and Inocula Prepared With or Without a Wetting Agent.** Tjho, J.¹, P. Schreckenberger¹, Hindler, J.², K. Ward², O. Louie³, S. Shinn³, S. Connell³, B.L. Zimmer³. ¹Loyola Medical Center, Maywood, IL; ²UCLA Medical Center, Los Angeles, CA; ³Dade Behring Inc., West Sacramento, CA. **Study Conclusion:** MICs obtained when testing streptococci with frozen broth microdilution panels prepared and tested according to CLSI recommendations are not affected by the addition of 0.1% Pluronic to the water inoculum diluent.
3. Abstract C-011. **Multicenter Evaluation of Vancomycin in a Synergies plus Dried Panel with Staphylococci and Enterococci.** Schreckenberger, P.¹, J. Tjho¹, J. Hindler², D. Bruckner², S. Mirrett³, L. Reller³, J. O'Connor⁴, L. Mann⁴, H. Boyd⁴, J. Johnston⁴. ¹Loyola Medical Center, Maywood, IL; ²UCLA Medical Center, Los Angeles, CA; ³Duke University Medical Center, Durham, NC; ⁴Dade Behring Inc., West Sacramento, CA. **Study Conclusion:** This study showed that the MicroScan Synergies plus panel gives excellent correlation with the CLSI reference broth microdilution method for vancomycin susceptibility testing.

Tuesday, May 22, 9:00 AM: Antimicrobial Susceptibility Testing Methods – Gram-Positive Molecular and Non-Molecular

4. Abstract C-029. **Preliminary Feasibility Study of an Inducible Clindamycin Resistance Test for Staphylococci Using an Investigational Use Only MicroScan Dried Overnight Panel.** Sei, K., M. Bacsafra, H. Bains, L. Mann, R. Williams, B.L. Zimmer. Dade Behring Inc., West Sacramento, CA. **Study Conclusion:** This preliminary feasibility study shows that the MicroScan IUO Dried Overnight panels Inducible Clindamycin Resistance Test gives excellent correlation with the CLSI D-zone disk approximation test for detection of inducible clindamycin resistance in staphylococci.
5. Abstract C-030. **Cefoxitin Preliminary Feasibility MIC Results for Staphylococci Obtained Using an Investigational Use Only MicroScan Dried Overnight Panel.** Sei, K., H. Bains, M. Bacsafra, L.G. Van Pelt, B.L. Zimmer. Dade Behring Inc., West Sacramento, CA. **Study Conclusions:** This preliminary feasibility study shows the MicroScan IUO Dried Overnight panel correlates well with a CLSI reference panel, cefoxitin disk diffusion testing, and *meaA* testing for detection of methicillin resistance in staphylococci. Reference panel results for *S. aureus* gave 100% correlation with *meaA* results; reference results for CNS were slightly less specific.
6. Abstract C-031. **Multicenter Evaluation of Four Automated ID/AST Systems for Antimicrobial Susceptibility Testing of Commonly Isolated *Enterococcus* spp. and *Staphylococcus* spp.** Bankert, D.A.¹, A.E. Crist, Jr.¹, W. George², A. Han³, A. Horning³, R.L. Sautter⁴, S. Strauss⁵, M.T. Wilson⁶, B.L. Zimmer⁷. ¹York Hospital, York, PA; ²Lancaster General Hospital, Lancaster, PA; ³American Hospital, Dubai, United Arab Emirates; ⁴Carolinas Pathology Group, Charlotte, NC; ⁵Reading Hospital and Medical Center, Reading, PA; ⁶Geisinger Medical Center, Danville, PA; ⁷Dade Behring Inc., West Sacramento, CA. **Study Conclusion:** All systems tested provided an accurate method for antimicrobial susceptibility testing of challenge strains of *Enterococcus* spp. and *Staphylococcus* spp.

Tuesday, May 22, 9:00 AM: Antimicrobial Susceptibility Testing Methods – Gram-Negative Molecular and Non-Molecular

7. Abstract C-048. **Comparison of Performance of the MicroScan, BD Phoenix, and VITEK-2 Systems for Identification and Susceptibility Testing of Gram-Negative and Gram-Positive Clinical Isolates.** Wang, Y.F.^{1,2}, A. Suantio¹, M. Shapiro², B. Brooks², X. Huang², R. Burton², C. Williams². ¹Emory University School of Medicine, Atlanta, GA; ²Grady Memorial Hospital, Atlanta, GA. **Study Conclusion:** Over 95% of correct identification and over 95% of agreement of AST results were achieved when three automated systems (MicroScan, Phoenix, and Vitek-2) were evaluated at the same time. The MicroScan Synergies plus and the MicroScan Overnight panels produced slightly better performance.
8. Abstract C-049. **Comparison of ESBL Confirmation Susceptibility Results Obtained on CLSI Reference Microdilution Broth Panels Inoculated With or Without a Wetting Agent.** Mendoza, G., M. Bacsafra, A.M. Chipman, J. O'Connor, B.L. Zimmer. Dade Behring Inc., West Sacramento, CA. **Study Conclusion:** Individual reference antimicrobial agent MICs as well as full dilution and streamlined dilution ESBL confirmation results are not affected by the use of Pluronic in the inoculum diluent when testing these organisms.
9. Abstract C-050. **ESBL Confirmation: Multicenter Evaluation of MicroScan Dried Overnight Panels with a Streamlined ESBL Confirmation Test.** S. Mirrett¹, L. Reller¹, M.J. Ferraro², P. Schreckenberger³, J. Tjho³, J. Hindler⁴, D. Bruckner⁴, K. Ward⁴, J. Borrelli⁵, A.M. Chipman⁵, B.L. Zimmer⁵. ¹Duke University Medical Center, Durham, NC; ²Massachusetts General Hospital, Boston, MA; ³Loyola Medical Center, Maywood, IL; ⁴UCLA Medical Center, Los Angeles, CA; ⁵Dade Behring Inc., West Sacramento, CA. **Study Conclusion:** The Streamlined ESBL Confirmation Test on selected MicroScan panels demonstrated excellent agreement when compared with results obtained from molecular characterization of a challenge set of ESBL-POS and -NEG strains. The Streamlined ESBL Confirmation Test results were highly reproducible as demonstrated with either inoculation method and both manual and instrument (WA and AS4) readings.

Wednesday, May 23, 9:00 AM: Diagnostic Bacteriology Identification – Gram-Positive Non-Molecular

10. Abstract C-142. **Verification Testing of the New MicroScan Rapid Pos ID 2 (Synergies plus) Panel with Clinical Isolates of Gram-positive Bacteria.** L. Mann, C. Beck, T. Tallent, S. Clark, J. Bobolis, J. Skinner, D. Nothhaft, L. Smoot. Dade Behring Inc., West Sacramento, CA. **Study Conclusion:** The results of this evaluation with fresh clinical isolates show that the new MicroScan Rapid Pos ID2 (Synergies plus) Panel will provide rapid, accurate identification results for clinically important gram-positive bacteria in two hours.

Thursday, May 24, 1:00 PM: Molecular Typing, Epidemiology and Surveillance – Gram-Negative

11. Abstract C-302. **Use of MicroScan LabPro Export Functions to Prepare Multicenter Annual Antibiogram Reports According to CLSI M39-A2 Guidelines.** Chipman, A.M.¹, R. Pond¹, D. Welch², L. Stevens², B.L. Zimmer¹. ¹Dade Behring Inc., West Sacramento, CA; ²Medical Microbiology Consulting, LLC, Dallas, TX; ³Medical City Dallas Hospital, Dallas, TX. **Study Conclusions:** The use of the Export feature in the MicroScan LabPro System, in conjunction with the developed template antibiogram file, offers an approach to compare sites, standardize reporting, and reduce data entry errors in the creation of a multicenter antibiogram report.

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