MicroFocus

A Macro Look at Micro Issues

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Introduction

Welcome to the Fall 2009 issue of Siemens MicroFocus. In this issue we give attention to a unique method for detection and differentiation of various beta-lactamase enzymes seen in Enterobacteriaceae that has been implemented by Dr. Paul Schreckenberger at Loyola Medical Center. The presence of extended-spectrum beta-lactamase-producing organisms in hospitals throughout the world has been chronicled in countless scientific papers. An increasing frequency of gram-negative organisms demonstrating multiple beta-lactamases causing multidrug-resistance has also been documented.[1, 2]

Detection and appropriate reporting of gram-negative organisms harboring beta-lactamases is critical for the support of both therapeutic management, and as an aid to implementation of infection-control measures. While characterization of some species of ESBL- and KPC-producing organisms is well documented, there are no standardized recommendations for detection of other beta-lactamases like AmpC and K1, or ESßLs in species other than *E. coli*, *Klebsiella sp.* and *P. mirabilis*.

As the complexities of enzyme profiles increase in gram-negative pathogens due to production of multiple beta-lactamases, so does the need to augment standard testing regimens in facilities where strains are likely to be present. Dr. Schreckenberger's discussion and 10-disk procedure provide a straightforward solution for your consideration.

To round out this issue, we've included an update from the Clinical and Laboratory Standards Institute (CLSI) on implementation of streamlined quality control for multi-substrate identification systems. Even though the CLSI M50-A document was published in 2008, laboratories have been reticent to implement the new guidance until there are revised laboratory and surveyor guidelines that allow an exception to the Clinical Laboratory Improvement Amendments (CLIA) of 1988. There is good news on that front, so please review the latest status described in this issue.

With over 30 years of proven performance, MicroScan Microbiology Systems is proud to continue providing bacterial identification, antimicrobial susceptibility testing products, and informatics solutions for your laboratory. We are also pleased to continue offering the MicroFocus publication. Please let us know about topics of interest or other ways we can continue to make this a relevant and

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Detection of ESBL, AmpC, K1 and KPC beta-lactamases in members of the Enterobacteriaceae – Introduction to the 10-disk Test

Paul C. Schreckenberger, PhD, D(ABMM), F(AAM)

To detect isolates with ESBL, AmpC, K1 and KPC enzymes, it is desirable to test all Enterobacteriaceae with a combination of antibiotics that will allow detection of these resistance mechanisms.

Background

ESBL-mediated resistance to cephalosporins is not always obvious in disk or dilution tests because the MICs of cephalosporins for ESBL-producers are often low (0.5-2 g/ml) and inhibition zones of disks are correspondingly large. Nevertheless, such ESBLs have been associated with clinical failure in patients, therefore, reliable detection is imperative.[1,2] Many laboratories detect and report ESBLs only in *E. coli*, *Klebsiella* species and *P. mirabilis*; however, ESBLs are known to occur in most species of Enterobacteriaceae. Furthermore, not all cephalosporin resistance in *E. coli* and *Klebsiella* species is due to ESBL production, and detection of isolates with other potent beta-lactamase types presents further challenges to the laboratory. This review highlights the types of beta-lactamases that occur in various members of the Enterobacteriaceae and describes a disk screening procedure for detection of beta-lactamase mediated resistance that can be applied to all species of Enterobacteriaceae.

ESBLs

Beta-lactamases are bacterial enzymes that inactivate beta-lactam antibiotics. Beta-lactamases that inactivate all the penicillins and cephalosporins including the extended spectrum cephalosporins are termed **Extended Spectrum Beta-Lactamases**, abbreviated as ESBLs. There are approximately 500 different ESBLs described, all of which are mutations of the classical broad-spectrum beta-lactamase enzymes that were initially named TEM and SHV (TEM-1, TEM-2, SHV-1). ESBLs are named TEM-3, -4 etc., SHV-2, -3 etc., CTX-M-1, -2 etc., OXA-1, -2 etc. ESBLs hydrolyze penicillins, cephalosporins and the monobactam, aztreonam, conferring resistance to all of these drug classes.

ESBLs do not hydrolyze the cephamycin antibiotics (e.g., cefoxitin and cefotetan), which are close relatives to the cephalosporins. ESBLs are also inhibited by beta-lactamase inhibitors such as clavulanate, sulbactam and tazobactam. ESBLs are generally inactive against the carbapenem antibiotics (imipenem, meropenem, ertapenem).[3-6]

The Clinical and Laboratory Standards Institute (CLSI) has published guidelines for performing an ESBL confirmatory test that involves testing cefotaxime and ceftazidime alone and in combination with clavulanate.[7] Clavulanate inhibits the activity of the ESBL enzyme and makes the organisms appear more sensitive to drug plus clavulanate combinations. This "greater sensitivity" with clavulanate can be demonstrated when a disk containing drug + clavulanate has a zone diameter that is ≥ 5 mm larger than the zone diameter of the drug tested alone, or when a zone of enhanced sensitivity is observed when a cephalosporin antibiotic is placed in close proximity to a clavulanate containing disk. This is commonly referred to as the "keyhole phenomenon" (see photo on page 4).

Currently, the CLSI ESBL confirmatory test is recommend for testing *E. coli*, *Klebsiella* species and *Proteus mirabilis*.[7] There are currently no CLSI recommendations for detection and reporting of ESBLs in other members of the Enterobacteriaceae.

AmpCs

AmpC beta-lactamases differ from ESBLs in that they are cephalosporinases and are resistant to beta-lactamase inhibitors. They hydrolyze the cephamycins, but not the 4th generation cephalosporin (i.e., cefepime). AmpC is normally produced in low levels by many organisms and is not associated with resistance, but it can be

Table 1

Abbreviated Table of beta-lactam antimicrobial classes

Beta-lactam Antimicrobial Class	Basic Sub-class Information	Key Antimicrobial Agents
Penicillins	Penicillinase-labile Hydrolyzed by staphylococcal penicillinase	penicillin amoxicillin ampicillin mezlocillin piperacillin ticarcillin
	Penicillinase-stable Not hydrolyzed by staphylococcal penicillinase	methicillin nafcillin oxacillin
ß-lactam/ ß-lactamase inhibitor combinations		amoxicillin-clavulanic acid ampicillin-sulbactam piperacillin-tazobactam ticarcillin-clavulanic acid
Cephems	Cephalosporin – 1st generation	cefazolin cephalothin cefaclor cephalexin
	Cephalosporin – 2nd generation	cefonicid cefuroxime
	Cephalosporin – 3rd generation	cefotaxime ceftazidime ceftriaxone
	Cephalosporin – 4th generation	cefepime
	Cephamycin	cefotetan cefoxitin
Monobactam		aztreonam
Penems	Carbapenem	doripenem ertapenem imipenem meropenem

For all confirmed ESBL-producing strains, the test interpretation should be reported as resistant for this antimicrobial class of agents. (CLSI M100-S19)

Keyhole Formation
Seen with ESBL-producing strains

Plasmid: An extra-chromosomal DNA molecule that can replicate independently of the main chromosome of a cell. Plasmids are important in certain bacteria since they code for proteins, especially enzymes, which can confer resistance to multiple antibiotic classes.

produced at high levels and cause resistance. High-level production of AmpC usually causes resistance to all beta-lactams, except carbapenems and 4th generation cephalosporins.

The AmpC gene is found on the chromosome in 100% of the following organisms: *Enterobacter sp, Hafnia alvei, Morganella morganii, Citrobacter freundii, Serratia marcescens, Providencia sp, Aeromonas sp, Pseudomonas aeruginosa.*[8] Chromosomal AmpC beta-lactamases can be produced inducibly or constitutively. Inducible expression of the AmpC gene occurs with the enzyme produced at a high level when the organism is exposed to inducing agents such as cephamycins (i.e, cefoxitin), ampicillin and carbapenems (e.g., imipenem, meropenem, ertapenem). Clavulanic acid is also an inducer of the AmpC gene and for this reason, antibiotic disks containing clavulanic acid may have zone diameters that are smaller compared to the same antibiotic tested without clavulanic acid. Induction is temporary and may be reversed when the antibiotic inducer is removed. In some organisms, mutations occur that cause the AmpC gene to become permanently expressed at high levels. These organisms are termed permanently derepressed mutants. Plasmid-mediated AmpC beta-lactamases can also be found in organisms that do not carry the chromosomal AmpC gene. Plasmid-mediated AmpCs have been detected in organisms such as *E coli, Klebsiella sp, Proteus sp* and *Salmonella sp*. There are currently no CLSI guidelines for detecting AmpC chromosomal or plasmid-mediated resistance.

K1 ß-lactamase

Klebsiella oxytoca produces a chromosomal beta-lactamase, the K1 enzyme. Hyperproduction of the K1 enzyme can occur by mutation. The K1 enzyme is predominantly a penicillinase that can also significantly hydrolyze aztreonam, cefuroxime and ceftriaxone and has weak activity against cefotaxome or ceftazidime. A distinctive feature of hyperproducers of K1 is greater activity against ceftriaxone than cefotaxime and greater activity against aztreonam than ceftazidime. Organisms producing K1 beta-lactamases may give false positive ESBL confirmatory tests with automated testing systems. There are currently no CLSI guidelines for detecting K1 beta-lactamases.

KPCs

Class A carbapenamases have recently been described that hydrolyze all beta-lactam antibiotics including the carbapenem class of antibiotics. These have been named KPCs (Klebsiella Pneumoniae Carbapenemase) because they were first found in strains of *K. pneumoniae*. They are now known to occur in other members of the Enterobacteriaceae as well. KPC resistance may not be detected by commercial susceptibility testing systems, which leads to false susceptible reporting of imipenem. Ertapenem has been shown to be a good screening agent for detecting KPC resistance. The CLSI document M100-S19, published in January 2009, provides guidelines for screening and confirmation of KPC type resistance.[7] Briefly, any organism that produces a zone diameter of <22 mm with either ertapenem or meropenem disks, or any organism that has an MIC >1 with ertapenem, meropenem or imipenem, is considered to be screen positive for KPC and should be confirmed by the modified Hodge test or molecular methods.[7]

To detect isolates with ESBL, AmpC, K1, and KPC enzymes, it is desirable to test all Enterobacteriaceae with a combination of antibiotics that will allow detection of these resistance mechanisms. In our laboratory we have expanded the ESBL confirmatory disk test to include 10 antibiotic disks for use in testing all members of the Enterobacteriaceae that have a susceptibility pattern that is suspicious for the presence of an ESBL, AmpC, K1 or KPC type resistance gene. Through the application of this test we have been able to successfully detect antibiotic resistance in many species of Enterobacteriaceae that would not have been detected using our automated susceptibility testing system.

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The 10-disk Test for Phenotypic Detection of ß-Lactamase Resistance in Gram-Negative Bacilli: Testing and Interpretation Guide

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When to Perform 10-Disk Test:

Set up 10-disk test to confirm presence of ESBL in following instances:

1. Any *E. coli* or *Klebsiella* when phenotype does not agree with ESBL confirmation test on any automated susceptibility system (e.g., ceftazidime is Intermediate (I) or Resistant (R) but ESBL confirmatory test is Neg)
2. Any Enterobacteriaceae when one of the 3rd generation cephalosporins tests I or R and no ESBL confirmatory test result is available
3. Any Enterobacteriaceae when atypical or multi-drug resistant pattern exists (e.g., *P. mirabilis* resistant to multiple drugs)
4. Any Enterobacteriaceae resistant to all drugs except imipenem

Specimen:

The specimen consists of a pure isolate of the Enterobacteriaceae, which has a susceptibility result that is consistent with an ESBL or an AmpC pattern, for example, ceftazidime is I/R, ceftriaxone is I/R, aztreonam is I/R, and requires confirmation by a disk method.

Materials:

1. Antibiotic disks are placed in 12 cartridge dispenser, kept in the refrigerator (2-8°C), until use (Table 2)
2. Mueller Hinton (MH) agar plate, 150 mm, kept in refrigerator (2-8°C), until use
3. Sterile saline or tryptic soy broth (TSB)
4. Sterile swabs
5. 0.5 McFarland barium turbidity standard / photometer (colorimeter)

Table 2

Antibiotic disks

Antibiotic	Concentration µg/mL
Aztreonam	30
Ceftazidime	30
Ceftazidime + clavulanate	30/10
Cefotaxime	30
Cefotaxime + clavulanate	30/10
Cefoxitin	30
Ceftriaxone	30
Cefepime	30
Ertapenem	10
Imipenem	10

Method:

1. Allow the MH agar plate and disk dispenser to come to room temperature before use.
2. Prepare a 0.5 McFarland standard of the organism to be tested in sterile saline or TSB. Standardize the inoculum using the colorimeter.
3. Streak the bacterial suspension evenly in 3 planes onto the surface of the MH agar plate, using a cotton swab. Rim the edge of the plate.
4. Place the disk dispenser over the MH agar plate and depress the knob. This will allow the antibiotic disks to dispense and automatically "tamp" the disk into place.
5. All of the disks must be placed on the same MH agar plate in a specified order (See MicroFocus insert template).
6. Incubate the MH agar plate overnight in a non-CO_2 incubator at 35°C.
7. The following day, read and record all zones of inhibition (see MicroFocus insert interpretive worksheet).

Results:

1. **Detection of ESBLs** (Ceftazidime and cefotaxime disks with and without clavulanic acid are used to detect ESBLs, Figures 1 and 2.)

   A. If the zone size increases 5 mm or more when clavulanate is added compared to the drug alone the isolate is considered an ESBL. Only one antibiotic must be "reversed" by the clavulanate to be an ESBL. For example: The CAZ/CLA zone is 22 mm, while the zone for CAZ alone is 11 mm.

   B. If an "enhancement" or extension of the zone of inhibition is seen between any of the cephalosporin antibiotics and the clavulanate containing disks, the presence of an ESBL can be predicted. This phenomenon is often referred to as the "KEYHOLE" effect, or "CLAVULANIC" effect and is indicative of ESBL production.

2. **Detection of AmpC beta-lactamases** (Cefepime and cefoxitin disks are used to detect ampC beta-lactamases, Figures 3 and 4.)

   A. AmpC strains are resistant to the cephamycins (e.g., cefoxitin and cefotetan).

   B. AmpC strains are susceptible to cefepime.

   C. High level AmpC producers cause resistance to all 1st, 2nd and 3rd generation cephalosporins, the beta-lactam-inhibitor drugs and the monobactams (i.e., aztreonam).

Figure 1

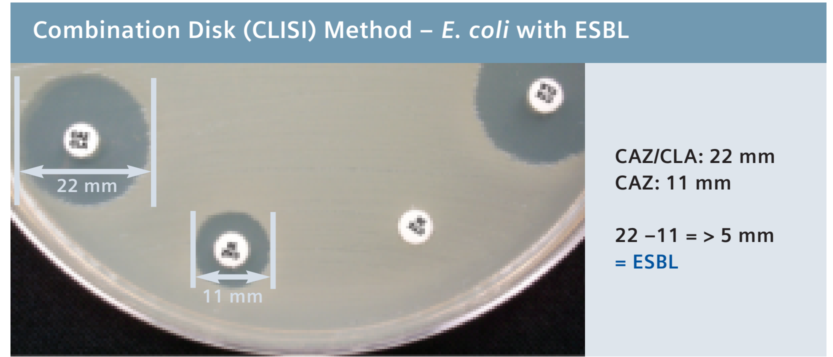


Figure 2

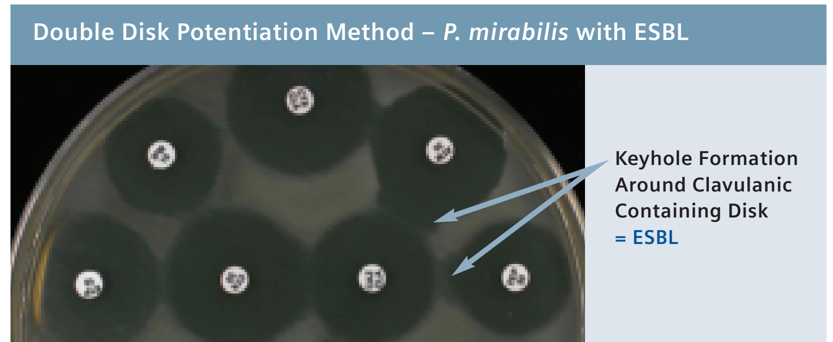


Figure 3

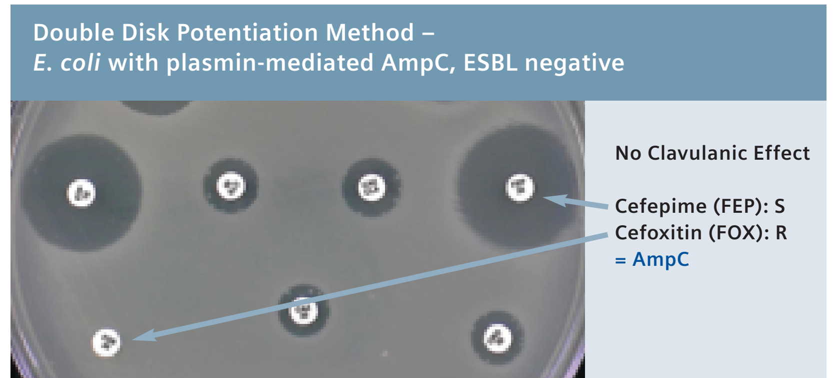


Figure 4

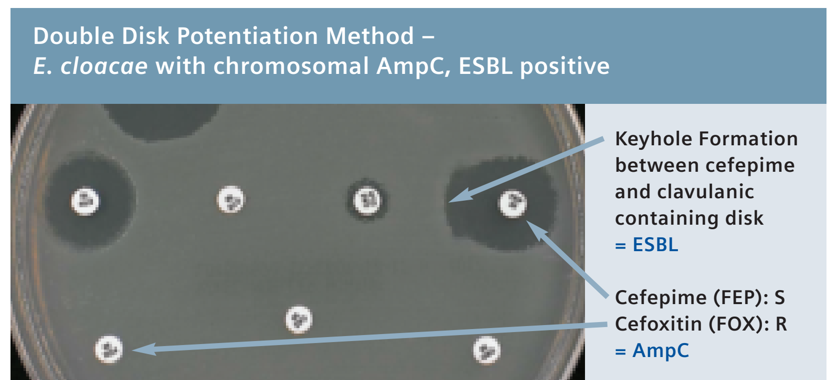
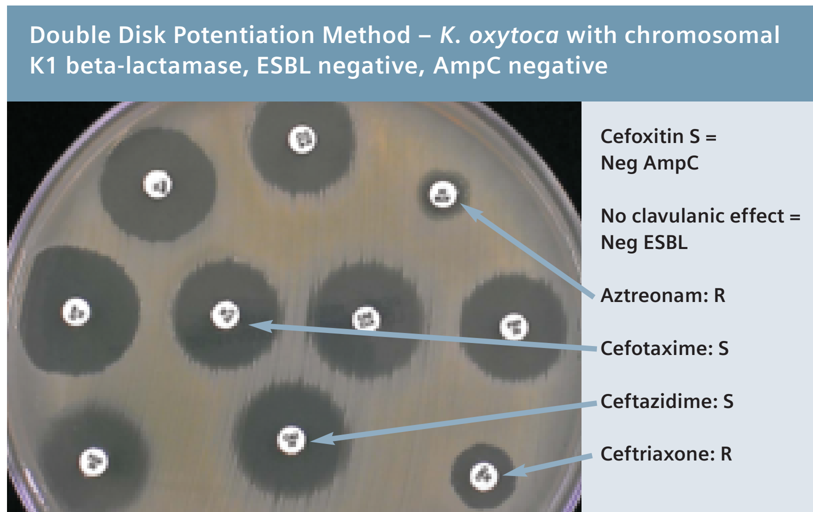
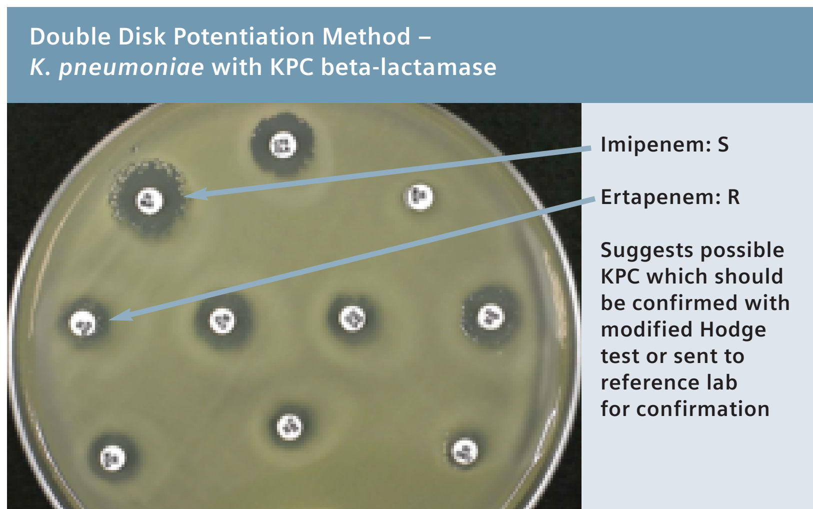


Figure 5



Double Disk Potentiation Method – *K. oxytoca* with chromosomal K1 beta-lactamase, ESBL negative, AmpC negative

Figure 6



Double Disk Potentiation Method – *K. pneumoniae* with KPC beta-lactamase

3. **Detection of K1 beta-lactamases** (Aztreonam, ceftazidime, cefotaxime and ceftriaxone disks are used to detect K1 beta-lactamases, Figure 5.)

4. **Detection of Carbapenemase** (Ertapenem and imipenem disks are used to screen for carbapenemase resistance, Figure 6.)

5. Record all disk diffusion mm zone size readings in the culture work up.

6. If ESBL is confirmed, change/override any previous susceptibility result to resistant, if the antibiotic is a penicillin, cephalosporin, or monobactam regardless of how the drug tests, following CLSI interpretive guidelines for ESBL. Refer to CLSI document M100-S19. Report cephamycins (e.g., cefoxitin) and beta-lactam inhibitor drugs as they test (in other words report as susceptible if they test susceptible, do not override).

7. If ESBL is not confirmed then report drugs as they test. For example, if organism is shown to be an AmpC or K1, report drugs as they test, do not override and make resistant.

8. If ESBL is present along with AmpC or K1 then apply the ESBL reporting rules and report all penicillins, cephalosporins and monobactams as resistant.

9. If KPC is confirmed then report all beta-lactam drugs as resistant regardless of how they test.

QUALITY CONTROL:

Disk diffusion testing is performed weekly with ATCC# 700603 *Klebsiella pneumoniae* and *E. coli* ATCC 25922 following CLSI guidelines. If correct quality control results are not obtained, the test is invalid and patient results cannot be reported.

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Streamlined Quality Control in Commercial Microbial Identification Systems: Have You Implemented CLSI Document M50-A in Your Laboratory?

Karen Appold, Editorial Consultant, Royersford, PA

Microbial identification systems (MISs) are test systems that use multiple substrates or reagents to identify aerobic or anaerobic bacteria, yeasts, molds, or yeast-like algae grown from culture. As MISs have become increasingly complex, they have incorporated more reagents and substrates, which has resulted in the need for a greater number of quality control (QC) organisms to check positive and negative reactivity for all system components.

In 2005, the American Society for Microbiology (ASM), at the suggestion of the Clinical Laboratory Improvement Advisory Committee (CLIAC), conducted a microbiology laboratory survey to determine the QC failure rates of commercial MISs in a random selection of laboratories that perform bacterial and fungal identification from culture. The data showed a failure rate of less than 0.1% for all commercial MISs surveyed. The ASM presented the QC survey data to CLIAC and recommended that the Clinical and Laboratory Standards Institute (CLSI) use its consensus process to analyze the data and develop guidelines to address appropriate QC for MISs.[1]

As a result of the ASM's survey, CLSI developed the document *Quality Control for Commercial Microbial Identification Systems; Approved Guideline* (M50-A) published in August 2008. This document includes guidelines for a modified QC approach to follow when using an MIS of proven reliability. The guidelines enable reduced QC rather than meeting all QC requirements included in the Clinical Laboratory Improvement Amendments (CLIA) of 1988. Additionally, it specifies the QC responsibilities of manufacturers, distributors, and users, and identifies conditions under which an MIS with proven reliability can qualify for streamlined QC testing.

All MIS users have knowledge of the large number of QC strains required to meet the CLIA guidelines. Products with 35 to 40 biochemical substrates can require testing of up to 10 different QC strains to fully qualify all substrates. There is no doubt that this requirement creates a significant strain on budgets and staffing resources, particularly in cases in which the test is performed only periodically.

Based on the ASM survey, 9.2% of the QC failures evaluated resulted from organism issues, whereas less than 0.1% were caused by substrate or reagent failures attributable to the MIS device.[2] M50-A enables a laboratory to test only the key indicator microorganism — substrate combinations. Once implemented, this new practice will reduce unnecessary material and labor costs spent on excessive QC testing for the clinical laboratory.

M50-A discusses, step by step, how a laboratory can qualify or initiate and continue performing streamlined QC. In general, a laboratory is required to have documentation from manufacturers of the MIS devices in use so that those MISs meet certain requirements for quality. The laboratory must either perform a verification study for each MIS, demonstrating its performance is comparable to the manufacturer, or conduct an acceptable historical review of QC performance for three consecutive lot numbers of the MIS, including positive and negative controls for each substrate/reagent. Documentation must be available and maintained for either of these options. To continue performing streamlined QC, the laboratory must

test the key indicator strains specified by the manufacturer with each new batch, lot number, and shipment; perform testing following manufacturer's instructions; monitor and document QC performance; and investigate, resolve, and report QC failures.

In October of 2008, the Centers for Medicare & Medicaid Services (CMS) distributed a letter to all State Survey Agency Directors with regard to the revised CLIA policy on QC for commercially available MISs.

This letter revises the analytical system information for microbial information systems found in the CMS' Interpretative Guidelines (IG) for Laboratories and Laboratory Surveyors.

- It incorporates the information on streamlined QC of commercial microbial identification systems (MISs provided in the CLSI document *Quality Control for Commercial Microbial Identification Systems; Approved Guideline*, M50-A, into the IG for Laboratories and Laboratory Surveyors.
- The process for streamlined QC of commercial MISs is a four-step process that includes quality assessment program requirements, general requirements, specific requirements to initiate the performance of streamlined QC, and requirements to continue to qualify for streamlined QC.

The College of American Pathologists (CAP) released a new checklist edition in June: Microbiology Checklist – MIC 06152009.

For additional details within the Microbiology Checklist, see the reference section MIC 21626.

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MicroScan users can access Technical Support Bulletin 168 on the Siemens website for a complete description of how to implement streamlined quality control for microbial identification on our Dried Conventional and Synergies *plus*® Panels.

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useful tool. If you have interest in becoming an author, please e-mail me directly.

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