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COMMENTARY

Extended-Spectrum-β-Lactamase, AmpC, and Carbapenemase Issues[▽]

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Optimal use of microbiology laboratories is essential to combat the spread of multiply antibiotic-resistant pathogens. This is vital for patient care, as advocated by Jarvis in stressing the importance of active detection and isolation to control methicillin-resistant *Staphylococcus aureus* and other resistant hospital-acquired pathogens (17). It is also vital for hospital accreditation in the United States, where the Joint Commission has set requirements for the control of acquisition and transmission of multidrug-resistant organisms (http://www.jointcommission.org/NR/rdonlyres/31666E86-E7F4-423E-9BE8-F05BD1CB0AA8/0/HAP_NPSG.pdf-NPSG.07.03.01). These requirements cannot be met without excellence in diagnostic microbiology. The absence of new, effective anti-gram-negative antibiotics makes infection control the most important countermeasure against multidrug-resistant gram-negative pathogens. Infection control can prevent additional infections and the spread of resistant pathogens and thereby reduce the need to use antibiotics. Infection control is most effective when directed by rapid, accurate laboratory results. In short, excellence in diagnostic microbiology is critical to quality initiatives in hospitals.

Some resistant pathogens may not be recognized because they are falsely susceptible in routine tests. This can lead to patients receiving ineffective antibiotics and contribute to the spread of the pathogens. Because the detection of such "hidden" resistance is so critical, this Commentary focuses on its detection in gram-negative pathogens. Because susceptibility tests may be unreliable, special tests are required to detect the resistance mechanisms involved. The mechanisms include extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases, and carbapenemases of molecular classes A and B.

ESBLs

ESBLs are typically inhibitor-susceptible β-lactamases that hydrolyze penicillins, cephalosporins, and aztreonam and are encoded by mobile genes. The most frequently encountered ESBLs belong to the CTX-M, SHV, and TEM families. ESBL producers are usually multiply drug resistant (5, 30), but their cephalosporin and aztreonam resistance is not reliably detected by susceptibility tests (33). Many labs have adopted CLSI recommendations and only attempted to detect ESBLs in *Escherichia coli*, *Klebsiella pneumoniae*, *K. oxytoca*, and *Proteus mirabilis* (8). Since ESBL genes are transmissible, it is important that ESBLs be tested for in other organisms in hospital and long-term care facility patient populations where ESBLs are encountered. This may be unnecessary for community isolates, which at this time appear to be predominantly CTX-M-producing *E. coli*.

The need for ESBL detection is under challenge on the supposition that it is possible to set breakpoints for injectable cephalosporins and aztreonam that accurately discriminate which ESBL-producing isolates can and cannot be reliably treated with these drugs. This approach is controversial (19) but has been adopted in slightly different forms by the European Committee on Antimicrobial Susceptibility Testing and the CLSI. It is based on limited therapeutic outcome data (3, 31), pharmacokinetic/pharmacodynamic data (3), and the concept that the lower the cephalosporin MIC the greater the likelihood of successful therapy (3, 19, 31). At variance with this approach, there are reports of therapeutic failures with cefepime associated with MICs of ≤2 μg/ml (31) and 4 μg/ml in a pediatric patient (3) and with a cefotaxime MIC of 0.75 μg/ml (20). There is also the concern that instead of the simplicity of cephalosporin and aztreonam susceptibility results being automatically changed to resistant for positive isolates, labs face the impossible task of having to overcome the inherent variability of testing ESBL-labile drugs to provide precise and accurate results. The problem is that the usual twofold error of the MIC test can be greatly amplified in tests with ESBL producers (48). This introduces enormous potential for inaccurate reporting of susceptibility results. For some, the paucity and inconsistency of current human data create an impression that infected patients will become experimental guinea pigs to prove or refute a hypothesis and the care of patients should continue to be based on the proven approach of ESBL detection and editing of susceptibility results. The following is based on the assumption that ESBL detection is clinically warranted.

Most ESBL detection tests are growth based, with confirmatory tests based on a β-lactamase inhibitor potentiating (enhancing) the activity of a cephalosporin or aztreonam in the presence of an ESBL. ESBL tests not requiring a β-lactamase inhibitor, such as the three-dimensional test (49) and molecular tests (25, 35), are unsuited to the work flow of most laboratories and will not be addressed.

ESBL DETECTION IN *E. COLI*, *K. PNEUMONIAE*, *K. OXYTOCA*, AND *PROTEUS MIRABILIS*

ESBL detection comprises either doing confirmatory tests of screen-positive isolates or doing confirmatory tests without prior screening. The screening approach is not ideal because it

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delays initiation of confirmatory testing, creates the possibility of human error if positive screens are ignored, and has less than 100% sensitivity (26, 50). It is better to incorporate confirmatory testing in the routine susceptibility test.

MANUAL CONFIRMATORY TESTS

The double-disk test (16) and the CLSI confirmatory tests (8) have been widely used. Optimal disk spacing is necessary with the double-disk test to avoid falsely negative or inconclusive results and sometimes necessitates repeated testing. Even so, this test is versatile and very effective when interpreted by an experienced microbiologist. The CLSI confirmatory tests are quantitative, which eliminates subjectivity in interpretation. Inconclusive off-scale results may occur with highly resistant isolates, necessitating the use of other methodologies. Inconclusive cefotaxime- and ceftazidime-based confirmatory tests should be reported as such (and not as ESBL negative) to avoid the risk of reporting false susceptibility to a more potent cephalosporin such as cefepime. Etest confirmatory strips are convenient but expensive and yield more inconclusive results than CLSI tests due to a more restricted concentration range (11, 40). Until proven otherwise, confirmed ESBL-producing isolates should be reported as resistant to all penicillins, cephalosporins, and aztreonam (8) to avoid therapy with antibiotics that may be clinically ineffective (6, 20, 32, 38). This recommendation should apply to all ESBL-producing isolates irrespective of species. Because carbapenems are often used to treat ESBL-associated infections, it is important that reduced carbapenem susceptibility of ESBL-positive isolates is not ignored, as it may indicate carbapenemase production, which contraindicates carbapenem therapy.

Confirmatory tests based on ceftazidime and cefotaxime tested alone and in combination with clavulanate are useful but have limitations in that they can yield falsely positive results with KPCs and hyperproduced K1 β-lactamases and falsely negative results with isolates that produce a high level of AmpC (46). KPC and AmpC issues should not be a problem for labs that can detect these enzymes. The K1 issue is specific to some strains of *K. oxytoca* with a ceftazidime MIC of ≤2 μg/ml (a higher MIC is indicative of ESBL production). If *K. oxytoca* is ESBL confirmatory test positive and has a ceftazidime MIC of ≤2 μg/ml, high K1 and ESBL production can be discriminated by comparing cefotaxime and ceftriaxone MICs. K1 hyperproduction is responsible for the positive confirmatory test result if the ceftriaxone MIC is ≥8-fold higher than the cefotaxime MIC. The MICs of ceftriaxone and cefotaxime are the same or similar if the isolate is an ESBL producer.

ESBL DETECTION IN ORGANISMS OTHER THAN *E. COLI*, *K. PNEUMONIAE*, *K. OXYTOCA*, AND *PROTEUS MIRABILIS*

Manual methods are best for ESBL detection in organisms other than *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *Proteus mirabilis* because the automated systems are not sufficiently accurate. Providing that an AmpC β-lactamase has not been acquired, CLSI or equivalent testing should be adequate for *Salmonella*, *Shigella*, and *Citrobacter koseri*. If these organisms produce an AmpC β-lactamase, the below approaches for AmpC-producing isolates can be used.

ESBL DETECTION IN AmpC-PRODUCING ISOLATES

Isolates that coproduce both an ESBL and a high level of AmpC are becoming more common (24). With such pathogens, a positive CLSI (or equivalent) ESBL confirmatory test can usually be accepted as accurate, except for *Acinetobacter* spp. (often falsely positive). If the test is negative or inconclusive and expanded-spectrum cephalosporin or aztreonam susceptibility is reduced (MIC, ≥4 μg/ml), the isolate should be retested by a method that is unaffected by AmpC β-lactamases. This can be done by including an AmpC inhibitor such as cloxacillin in the culture medium (Fig. 1), by including the AmpC inhibitor boronic acid in either MIC or disk tests (18), or by testing a cephalosporin that is not hydrolyzed by AmpC (e.g., cefepime) alone and in combination with clavulanate (14, 26).

AUTOMATED ESBL DETECTION

Automated ESBL tests offer the potential for rapid ESBL detection. Unfortunately, many published evaluations are suboptimal, lacking an accurate reference method and/or sufficiently challenging isolates to assess accuracy (47). When such studies were performed, an ESBL confirmatory test was found to be essential because the expert systems alone were not sufficiently accurate. The Vitek 2 and Phoenix confirmatory tests in combination with expert systems exhibited high sensitivity and specificity in studies that included many ESBL types and detected some ESBLs that are not reliably detected with the CLSI methodology (44, 45, 47). Both systems could have been improved by modifications to their expert system software (47). Automated microdilution-based tests, such as MicroScan and Sensititre, can have the same level of accuracy as the CLSI microdilution confirmatory tests if the same concentration ranges are tested and, augmented by a good expert system, have the potential for greater accuracy. If abbreviated concentration ranges are tested, they will detect fewer ESBLs in some patient populations.

PLASMID-MEDIATED AmpC β-LACTAMASES

AmpC β-lactamases preferentially hydrolyze narrow-, broad-, and expanded-spectrum cephalosporins and cephamycins and resist inhibition by clavulanate, sulbactam, and tazobactam. Many gram-negative bacilli produce a chromosomally mediated AmpC which, when hyperproduced, may cause resistance to penicillins, aztreonam, cephamycins, and narrow-, broad-, and expanded-spectrum cephalosporins. Because several terminologies are in use, for simplicity, transmissible AmpC β-lactamases are referred to here as plasmid-mediated AmpC β-lactamases. These enzymes have been detected in some isolates of *Klebsiella* spp., *Salmonella* spp., *C. freundii*, *E. aerogenes*, *P. mirabilis*, and *E. coli* (15) and are typically associated with multidrug resistance. The most commonly encountered plasmid-mediated AmpC β-lactamases belong to the CMY, FOX, and DHA families. Accurate

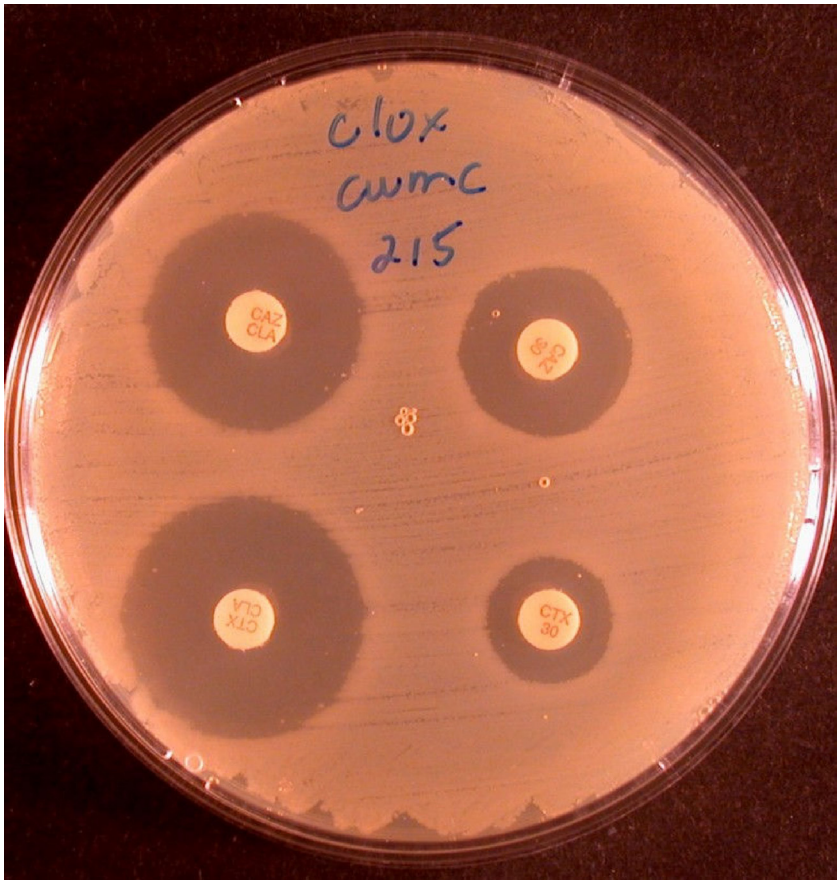

FIG. 1. *E. coli* that produces an ESBL and a high level of AmpC. Shown is a CLSI ESBL confirmatory test on Mueller-Hinton agar supplemented with 200 μg/ml cloxacillin. No inhibition zones were obtained on unsupplemented Mueller-Hinton agar. The disks, from left to right, are as follows: upper, ceftazidime-clavulanate and ceftazidime; lower, cefotaxime-clavulanate and cefotaxime.

prevalence data are scarce due to lack of testing, but they appear to be less common than ESBLs (15).

Laboratories should be able to detect AmpC β-lactamases because they have been associated with false cephalosporin susceptibility and also to recognize isolates for which there is the potential to falsely report isolates as ESBL negative (27, 29). It is unnecessary to detect AmpC production in organisms that produce an inducible chromosomal AmpC β-lactamase because the organism identification is indicative of AmpC production; i.e., 100% of *E. cloacae*, *E. aerogenes*, *C. freundii*, *S. marcescens*, *Providencia* sp., *Morganella morganii*, *Hafnia alvei*, *Aeromonas* sp., and *P. aeruginosa* isolates can be assumed to be AmpC producers. These organisms have the potential to readily mutate to develop resistance during therapy with β-lactam antibiotics other than the carbapenems, penems, or zwitterionic (sometimes referred to as fourth-generation) cephalosporins (e.g., cefepime).

The CLSI ESBL screen can be used to screen for plasmid-mediated AmpC β-lactamases (28). Alternatively, cefoxitin insusceptibility (intermediate or resistant) is a useful screen for *Klebsiella* spp., *Salmonella* spp., *C. koseri*, *P. mirabilis*, and *E. coli* in areas where the ACC-1 and ACC-4 enzymes are not encountered (so far not detected in the United States). Because phenotypic tests do not differentiate between chromosomal and plasmid-mediated AmpC β-lactamases, plasmid-mediated AmpC β-lactamases are most accurately detected with the multiplex AmpC PCR test of Pérez-Pérez and Hanson (34). If molecular testing is not available, screen-positive isolates should be tested with a phenotypic confirmatory test that will distinguish AmpC production from other resistance mechanisms.

Phenotypic confirmatory tests based on the detection of cephamycin hydrolysis or AmpC inhibition will distinguish AmpC β-lactamases from ESBLs and porin mutations. Confirmatory tests that detect cephamycin hydrolysis include the AmpC disk test (4), the Gots test (13) (recently known as the modified Hodge test or MHT, the name that will be used herein [58]), and the three-dimensional test (49). These are performed separately from the routine susceptibility test. The MHT is occasionally falsely negative with AmpC producers that test positive with the AmpC disk test (27). This is probably because Tris/EDTA is used in the AmpC disk test to permeabilize gram-negative cells and release β-lactamases to participate in the test whereas the MHT uses intact cells, which may not release β-lactamases as efficiently. A positive AmpC disk test is shown in Fig. 2.

Boronic acid and cloxacillin are the most commonly used AmpC inhibitors. By inhibiting AmpC β-lactamases, they potentiate the activity of cephems (usually cefotetan and cefoxitin). Boronic acid-based tests are sometimes less sensitive than the AmpC disk test for detecting the plasmid-mediated DHA-1 enzyme (27). Boronic acid also inhibits KPC enzymes and sometimes certain ESBLs and OXA-12 and inhibits some bacterial

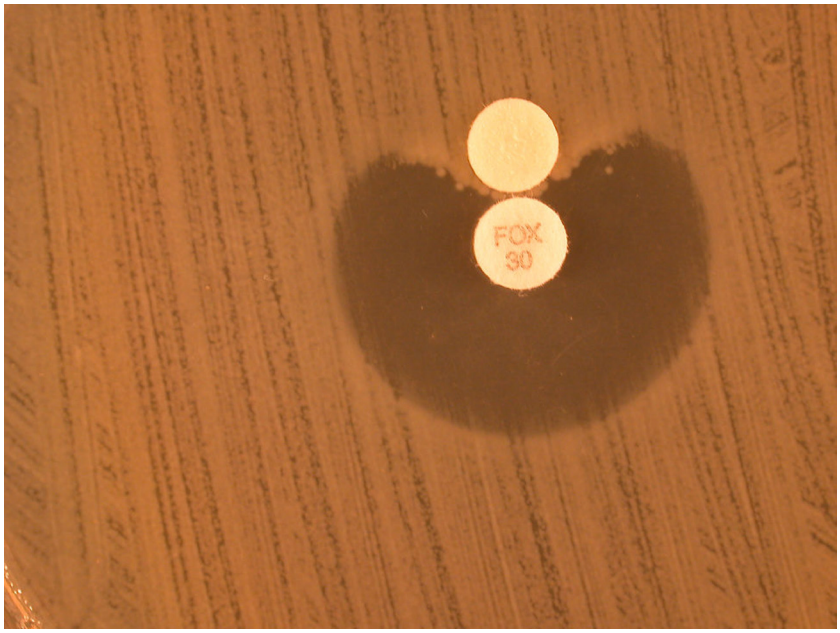

FIG. 2. AmpC disk test with the upper Tris/EDTA disk inoculated with the test isolate according to the method of Black et al. (4) and placed adjacent to a cefoxitin (FOX) disk on a lawn of cefoxitin-susceptible *E. coli*. The indented zone margin is evidence of hydrolysis of cefoxitin by the AmpC enzymes that diffused out of the permeabilized cells on the Tris/EDTA disk.

strains, making it necessary to interpret tests with care. If a disk or well containing boronic acid alone is included as a control, boronic acid-based tests may be conveniently included in routine susceptibility tests (18). The above issues have not been reported for cloxacillin-based AmpC detection tests, the most convenient of which seems to be a double-disk test (similar to that used for ESBL detection but utilizing a cloxacillin disk instead of a clavulanate-containing disk) (27, 42). As with any type of double-disk test, care must be taken to ensure that disk spacing is optimal.

Detection of an AmpC β-lactamase in *Klebsiella* spp., *Salmonella* spp., *C. koseri*, or *P. mirabilis* is confirmatory for plasmid-mediated AmpC production because these organisms lack a chromosomal AmpC β-lactamase. Based on a report by Pai et al. (29), it would be prudent to report the isolate resistant to narrow-, broad-, and expanded-spectrum cephalosporins and to notify infection control. Inducible plasmid-mediated AmpC β-lactamases such as DHA-1, DHA-2, ACT-1, CFE-1, and CMY-13 may confer a greater risk of a poor therapeutic outcome than those produced constitutively (26, 29). The AmpC enzyme can be investigated for inducibility by the cefoxitin disk approximation test (43). Phenotypic detection of AmpC in *E. coli* does not indicate if the enzyme is chromosomal or plasmid mediated, but as a crude guide, lack of multiple drug resistance is suggestive of a chromosomal AmpC whereas multiple drug resistance is consistent with either plasmid-mediated or chromosomal AmpC production. Molecular testing is necessary if the laboratory is to definitively determine if an *E. coli* isolate is carrying a plasmid-mediated AmpC.

CARBAPENEMASES

Carbapenemases are diverse enzymes that vary in the ability to hydrolyze carbapenems and other β-lactams. Detection is a crucial infection control issue because (i) they are often associated with extensive, sometimes total, antibiotic resistance and (ii) more-resistant organisms such as strains of *Pseudomonas* and *Acinetobacter* spp. that have acquired a carbapenemase can be vectors responsible for carbapenemase transmission to members of the family *Enterobacteriaceae* in which the resistance mechanism is not recognized. The major concern is with transmissible and not chromosomal carbapenemases. The transmissible enzymes can be acquired unpredictably by important pathogens such as *P. aeruginosa*, *A. baumannii*, and members of the family *Enterobacteriaceae*. The chromosomal enzymes occur predictably in less common pathogens such as *S. maltophilia*, *Aeromonas* spp., *Chryseobacterium* spp., and others.

Carbapenemases belong to molecular classes A, B, and D. The class A enzymes (Bush group 2f) are inhibited to various degrees by clavulanate and usually hydrolyze penicillins or cephalosporins more efficiently than carbapenems (7). For this reason, some, such as KPC enzymes, lack potent carbapenemase activity and may be considered ESBLs that also hydrolyze carbapenems. Class A carbapenemases include the KPC, IMI, and SME families, NMC-A, and some GES enzymes. They are most commonly produced by members of the family *Enterobacteriaceae* but have been recently detected in isolates of *P. aeruginosa* in Colombia (53) and Puerto Rico (41) and *A. baumannii* in Puerto Rico (41). Class B enzymes (Bush group 3) are metallo-β-lactamases (MBLs) which typically hydrolyze carbapenems efficiently but not aztreonam and resist currently available β-lactamase inhibitors but are inhibited by chelating agents such as EDTA. The most important include the VIM and IMP families and SPM-1, which have been detected in strains of *P. aeruginosa*, members of the family *Enterobacteriaceae*, and *A. baumannii*. Most class D carbapenemases

hydrolyze carbapenems weakly and are inhibited poorly by clavulanate. They belong to the OXA family and are most commonly produced by *Acinetobacter* spp. but have also been reported in some *P. aeruginosa*, *K. pneumoniae*, and *E. coli* strains.

There are many gaps in our understanding because carbapenemase issues have only recently drawn widespread attention. Detection tests are still evolving, hindered by the heterogeneity of both enzymes and hosts, which confers different levels of carbapenem susceptibility. For example, carbapenemase-producing members of the family *Enterobacteriaceae* may have imipenem MICs as low as 0.125 μg/ml (36). *Acinetobacter* spp. that produce OXA or KPC carbapenemases are usually carbapenem resistant, but MBL producers may be carbapenem susceptible (55). Carbapenemase-producing *P. aeruginosa* strains more consistently exhibit reduced susceptibility, having imipenem MICs of ≥8 μg/ml (37). This variability means that a single carbapenem screening criterion cannot be set to identify all isolates requiring confirmatory testing. Because of this, different carbapenem screens may be necessary for different organism/enzyme groups. Another approach, yet to be systematically explored, is to use other drugs for screening. For example, cephalosporins have been recommended as alternative or additional screens (8, 55) but can be falsely positive if other resistance mechanisms such as ESBLs or AmpC β-lactamases are present and falsely negative for some carbapenemase producers. Caution is necessary when recommending dual drugs in a screen; e.g., in 2009, the CLSI made resistance to certain expanded-spectrum cephalosporins an essential component of the carbapenemase screen for members of the family *Enterobacteriaceae* (8), but SME producers and some KPC producers are screen negative because they do not meet this criterion. If ESBL or high-level AmpC production is not present in an organism, aztreonam susceptibility in combination with reduced carbapenem susceptibility is suggestive of MBL production. Confirmatory testing is also problematic, with controversy about which tests are best for each type of carbapenemase and how to report positive carbapenemase tests to the clinician. The reporting of carbapenem susceptibility for carbapenemase producers is a controversial issue due to mixed therapeutic outcomes with carbapenems (9, 56), making it important to obtain guidance from an infectious disease clinician. Overall, there is much scope for improved screening, confirmatory testing, and reporting. Carbapenemase detection is an important, but neglected, diagnostic challenge which urgently needs input from observant, skilled microbiologists.

Given the currently unresolved, complex testing issues, the CLSI considered it better not to detect carbapenemases routinely but instead to reduce carbapenem breakpoints so that a susceptible test predicts effective therapy and the resistance mechanism is irrelevant. This approach could be dangerous to patients because there are not convincing therapeutic outcome data to justify reporting carbapenemase producers as carbapenem susceptible. Also, the current KPC epidemic demonstrates the widespread adverse consequences that can ensue when labs fail to rapidly detect carbapenemase producers (12). Since susceptibility tests alone will not detect all carbapenemase producers, prevention and control must rely on labs performing detection tests. This means that although there is room for improvement, the best current methods must be used.

In the lab, a carbapenem-intermediate or -resistant result should always raise the suspicion of possible carbapenemase production, as should reduced carbapenem susceptibility within the susceptible range in isolates of members of the family *Enterobacteriaceae* and *Acinetobacter* spp. In the case of *P. aeruginosa*, an intermediate or resistant result can often be due to non-carbapenemase resistance mechanisms and additional information is needed if the specificity of the screen is to be increased. The best carbapenem for screening is unknown. Ertapenem has been recommended as the best screening agent for KPC detection because KPC producers are usually insusceptible but may remain susceptible to other carbapenems (1). This does not, however, preclude having KPC or other carbapenemase screening criteria within the susceptible ranges of carbapenems.

The MHT has been used for carbapenemase confirmation. It does not distinguish between carbapenemase types and lacks sensitivity for MBL detection unless performed according to the "true" MHT methods on MacConkey agar (57) or with zinc added to the carbapenem disk (22). Falsely positive results can occur with high-level AmpC producers, more likely with an imipenem disk than with other carbapenems (27). As with screening tests, it is unclear which is the optimal carbapenem disk for confirmatory tests. Imipenem is the least specific agent but is the most sensitive for the detection of OXA carbapenemases (27).

Specific MBL confirmatory tests involve chelating agents as inhibitors, e.g., EDTA, 2-mercaptopropionic acid, and sodium mercaptoacetic acid. These may be used alone or as a combination of chelators to potentiate the activity of a carbapenem or ceftazidime (2, 21, 23, 54). It is necessary also to test the chelator alone to determine that it does not cause false-positive results by inhibiting the test isolate (39).

Although inhibited by clavulanate, class A carbapenemases are not reliably detected by clavulanate-based methods. More-sensitive methods include the MHT and Tris/EDTA-based and boronic acid-based tests (1, 10, 27, 51). The MHT and Tris/EDTA-based methods are useful but sometimes yield falsely positive results with strains that hyperproduce AmpC, especially the MHT (Creighton University data). Boronic acid-based testing is reported to be specific for KPC detection in *K. pneumoniae* if performed with imipenem or meropenem but not if performed with ertapenem if the isolate produces a plasmid-mediated AmpC β-lactamase (10, 52). Specificity data are not yet available for this test for species that hyperproduce a chromosomal AmpC β-lactamase.

For future phenotypic detection of carbapenemases, the ideal goal is to include carbapenemase detection in the routine susceptibility test, perhaps by including a broad confirmatory test that will detect all types of carbapenemases and which can be followed up with more specific testing if necessary. With regard to specific testing, molecular methodologies have the potential to provide a high level of specificity. If such tests are convenient, reliable, and available, there is considerable potential for their utilization in outbreak situations.

CONCLUSION

Microbiology laboratories must be able to detect resistant pathogens in a timely manner, especially those that are falsely

susceptible *in vitro* to drugs that may be considered for therapy of infected patients. Microbiological excellence is needed more than ever, and it is critical that ESBLs, AmpC β-lactamases, and carbapenemases be promptly and accurately detected. In recent years, there has been a trend toward the deskilling of microbiology laboratories to minimize or eliminate the need for scientific judgment and interpretation. This is a very dangerous approach, as the challenge of increasingly complex antibiotic-resistant pathogens creates an increasing need for trained, expert scientists. Laboratories that are adequately funded and staffed and properly equipped and which keep abreast of new developments will provide a high return on investment by making better patient outcomes possible, facilitating effective infection control, reducing the escalation of resistance, and helping hospitals to meet accreditation standards. If microbiology laboratories are incapable of providing a standard of excellence, many patients will suffer, resistant pathogens will spread, and disaster in the form of uncontrolled antibiotic resistance is inevitable.

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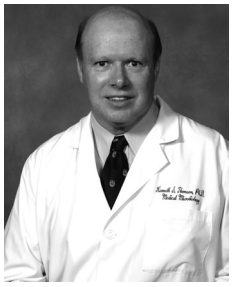

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