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Evaluation of a New Etest for Detecting Metallo-β-Lactamases in Routine Clinical Testing

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Several Etest (AB BIODISK, Solna, Sweden) gradient formats were developed for detection of metallo-β-lactamases based on the reduction of imipenem (IP) or ceftazidime (TZ) MICs in the presence of EDTA or 2-mercaptopropionic acid (MPA). The Etest metallo-β-lactamase (Etest MBL) strips consisted of a double-sided seven-dilution range of IP or TZ (4 to 256 μg/ml) and IP or TZ (1 to 64 μg/ml) overlaid with a constant concentration of EDTA or MPA. The prototype strips were evaluated on several agar media (brain heart infusion agar, Isosensitest agar, nutrient agar, and Mueller-Hinton agar for aerobes and brucella blood agar for anaerobes) with 138 challenge strains: *Acinetobacter* spp. (*n* = 9), *Aeromonas* spp. (*n* = 8), *Chryseobacterium* spp. (*n* = 28), *Escherichia coli* (*n* = 1), *Klebsiella pneumoniae* (*n* = 4), *Pseudomonas aeruginosa* (*n* = 14), *Proteus mirabilis* (*n* = 3), *Serratia* spp. (*n* = 10), *Stenotrophomonas maltophilia* (*n* = 43), *Sphingobacterium* spp. (*n* = 3), and *Bacteroides fragilis* group (*n* = 15). PCR analysis using specific primers for IMP-1, L1, CcrA, and *bla*$_{B/C}$ confirmed the presence of the metallo-β-lactamase genes. Enzyme assays were also performed with IP as an indicator substrate followed by EDTA inhibition profiles. EDTA was found to be a better inhibitor of metallo-β-lactamases, especially for anaerobes. IP was a better than TZ. Mueller-Hinton agar was the preferred medium, particularly when compared to Isosensitest agar, which frequently produced falsely low MICs for IP. Etest IP plus IP-EDTA with Mueller-Hinton agar had a sensitivity of 94% (79 of 84) and specificity of 95% (124 of 130). The Etest MBL strip appears to be an acceptable diagnostic reagent to detect metallo-β-lactamase phenotypes in the clinical microbiology laboratory.

The introduction of carbapenems into clinical practice represented a great advance for the treatment of serious bacterial infections caused by β-lactam-resistant bacteria (17). Due to their broad spectrum of activity and stability to hydrolysis by most β-lactamases, the carbapenems have been the drugs of choice for treatment of infections caused by penicillin- or cephalosporin-resistant gram-negative bacteria (6, 13, 17, 22). However, carbapenem resistance has been observed more commonly in nonfermenter species such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. The common form of resistance is through lack of drug penetration (i.e., porin mutations and efflux pumps) and/or carbapenem-hydrolyzing β-lactamases. Based on molecular studies, two classes of carbapenem-hydrolyzing enzymes have been described: serine enzymes, possessing a serine moiety at the active site, and metallo-β-lactamases (class B), requiring divalent cations as cofactors for enzyme activity (1, 7, 25, 36).

Chromosomally encoded metallo-β-lactamase enzymes from several bacteria, including *Bacillus cereus* (28), *Stenotrophomonas maltophilia* (29), *Aeromonas* spp. (31), and *Chryseobacterium* spp. (4, 5, 27), have been characterized. Metallo-β-lactamases from *Bacteroides* spp., carried on the mobile transposon Tn*1296*, have also been reported, but so far has been confined to the *Bacteroides* genus. However, in 1991, a report of a new plasmid-mediated metallo-β-lactamase, IMP-1, in a *P. aeruginosa* isolate caused great concern due to the potential risk of IMP being disseminated widely to other bacterial species (35). A survey of IMP-1-producing gram-negative bacteria in 1996 and 1997 in Japan showed that 144 (4.4%) of 3,222 *Serratia marcescens* strains produced IMP-1 through acquisition of plasmids carrying the *imp* gene (19). For many years, the detection of IMP-1-producing isolates was restricted to Japan (30), but recently the appearance of other IMP-type enzymes in Hong Kong (8) and Singapore (18) has been reported. A *bla*$_{IMP}$ allelic variant, which encodes IMP-2, was detected in an *Acinetobacter baumannii* strain isolated in Italy, the first European example (10). A novel family of class B metallo-β-lactamases, the VIM family (VIM-1 to VIM-3 enzymes), was described for *P. aeruginosa* and *Acinetobacter* isolates in Europe (20, 23, 32). The *vim* gene, like the *imp* gene, is carried on mobile gene cassettes inserted into class 1 integrons like *imp*.

In contrast to serine β-lactamases, metallo-β-lactamases can be experimentally inhibited with metal chelators such as EDTA and thiol-based compounds. The use of β-lactams such as ceftazidime (TZ) and imipenem (IP) in combination with EDTA or 2-mercaptopropionic acid (MPA) with proximal disk diffusion techniques has been described for the detection of metallo-β-lactamases (2). However, these studies have involved a limited number of strains and dealt solely with the detection of IMP-1. A modified Hodge and EDTA-disk synergy method has also been developed to detect metallo-β-lactamases, but again, this study was confined to IMP-1 and VIM enzymes in *P. aeruginosa* and *Acinetobacter* (21). Therefore, a new Etest (AB BIODISK, Solna, Sweden) strip containing either an IP or a TZ gradient combined with a fixed concentration of EDTA or MPA was formulated. We report the results of a study to evaluate the performance of the Etest metallo-β-lactamase (Etest MBL) strips for detection of a di-

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verse range of metallo-β-lactamases, both plasmid and chromosomally mediated, in a variety of bacterial species.

MATERIALS AND METHODS

Bacterial strains and molecular identification. A set of 138 challenge strains was used, which included organisms with various susceptibilities to IP and producing different types and levels of β-lactamases, including extended-spectrum β-lactamases (ESBLs), AmpC enzymes, and metallo-β-lactamases. The bacteria included the following: *Acinetobacter* spp. (n = 9), *Aeromonas* spp. (n = 8), *Chryseobacterium* spp. (n = 28), *Escherichia coli* (n = 1), *Klebsiella pneumoniae* (n = 4), *Pseudomonas aeruginosa* (n = 14), *Proteus mirabilis* (n = 3), *Serratia* spp. (n = 5), *S. marcescens* (n = 5), *S. maltophilia* (n = 43), *Sphingobacterium* spp. (n = 3), *Bacteroides fragilis* (n = 12), *Bacteroides ovatus* (n = 1), *Bacteroides distastonis* (n = 1), and *Bacteroides. thetaiotaomicron* (n = 1). Bacterial strains were identified by 16S RNA sequencing of amplicons with primers based on the conserved regions of 16S RNA as previously described (15): forward, 5′-TCAG ATTTGAACGCTGGCGGCA-3′; and reverse, 5′-CGTATTACCGCGGCTGC TGCCAC-3′. Sequences from the 16S RNA amplicons were compared using the LASERGENE suite of programs (DNASTAR Inc., Madison, Wis.).

Etest MBL procedure. Several variations of the Etest MBL strip were produced in a configuration similar to that for Etest strips for the detection of ESBLs (9). The following formulations were used: IP (4 to 256 μg/ml) with IP (1 to 64 μg/ml)-EDTA (IPE), IP (4 to 256 μg/ml) with IP (1 to 64 μg/ml)-MPA (IPM), and TZ (0.016 to 256 μg/ml) with TZ-EDTA (TZE). The final concentrations of EDTA and MPA used in the strip corresponded to 320 and 200 μg/ml, respectively.

Media. The following agar media were used for testing the Etest MBL strips: aerobe Mueller-Hinton agar (MH) (BD Microbiology Systems [BDMS], Cockeysville, Md.), Isosensitest agar (ISO) (Oxoid, Basingstoke, United Kingdom) nutrient agar (NA) (Oxoid, Basingstoke, United Kingdom), brain heart infusion agar (BDMS), and PDM antibiotic sensitivity medium (AB BIODISK). For anaerobes, brucella agar (BMS) was supplemented with 5% horse blood plus vitamin K (1 mg/liter) and 1% hemin (BBL).

Etest MBL interpretative criteria. For the aerobes, individual colonies were picked from 18-h plates and suspended in 0.85% saline to a turbidity of a 0.5 McFarland standard. The *Bacteroides* cultures were grown for 24 to 72 h, and individual colonies were picked and suspended in brucella broth to a turbidity of a 1 McFarland standard. Cotton swabs were used to transfer the inoculum to the plates, which were thoroughly swabbed and dried before the Etest MBL strips were applied. Plates for the aerobes were incubated for 16 to 20 h at 35°C. The anaerobic cultures were incubated in an anaerobic jar and incubated at 35°C for 20 to 24 h. The MIC end points were read where the inhibition ellipses intersected the strip. A reduction of IP or TZ MICs by ≥3 twofold dilutions in the presence of EDTA or MPA was interpreted as being suggestive of metallo-β-lactamase production. Equally, the presence of a "phantom" zone between the two gradient sections or deformation of the IP or TZ ellipses was also indicative of metallo-β-lactamase.

Metallo-β-lactamase assays. Crude lysates of bacterial cells were prepared as previously described (3). Bacteria were grown overnight in nutrient broth at 35°C, and the cells were harvested by centrifugation, washed in cacodylate buffer (30 mM, pH 7.0) supplemented with 100 mM zinc, and ribolysed (3). The ribolysed cells were centrifuged at 12,000 × g to remove cellular debris. The lysates were analyzed for the ability to hydrolyze IP (loss of substrate at 299 nm) with and without 10 mM EDTA (incubated at 25°C for 20 min). The activities of the enzymes were converted to specific activities (micromolar substrate hydrolyzed per minute per milligram of protein), using −7,000 as the extinction coefficient for IP as previously described (3).

Detection of metallo-β-lactamase genes. PCR analysis was performed to confirm the presence of the metallo-β-lactamase with a standard cycle program (95°C for 1 min, 52°C for 1 min, and 72°C for 1 min) for 30 cycles. For amplification of the metallo-β-lactamase genes, the following primers, based on conserved regions of the gene, were used: L1 forward (5′-ACCATGCGTTCTACC CTGCTCGCCTTCGCC-3′), L1 reverse (5′-TCAGCGGGCCCCGGCCGTTT CCTTGGCCAG-3′), IND-1 forward (5′-CAGCTTTGATGATGTCAATG-3′), IND-1 reverse (5′-CCAGAAGATCCAGAGTATCG-3′), BlaB forward (5′-CT TGCTCTTGGACTTACAGG-3′), BlaB reverse (5′-CTGATTAAATCCAGTG TATG-3′), IMP-1 forward (5′-CTACATGACCGCGTCTGTCA-3′), IMP-1 reverse (5′-TGACAACGTTCGCTGTTGC-3′), CfiA forward (5′-GTACACTTA TGTATCCCTCG-3′), and CfiA reverse (5′-GCCATAGTCGCCATGTCCATG TCCGG-3′) (5, 16, 24, 30, 33, 34). Amplicons were analyzed on agarose gels (0.8%) with appropriate DNA markers.

TABLE 1. Sensitivities of IP and TZ as substrates on MH

Metallo-β-lactamase producers (n)	Metallo-β-lactamase type[a]	Sensitivity[b] (%)	
		IP-IPE	TZ-TZE
Acinetobacter spp. (3)	IMP-1	100	0
P. aeruginosa (1)	IMP-1	100	100
Serratia spp. (8)	IMP-1	100	100
S. maltophilia (4)	L1	100	0
B. fragilis group (10)	CcrA	100	70
Total		100	54

[a] Metallo-β-lactamase producers as defined by genotypic methods.
[b] Based on the number of metallo-β-lactamase producers that were correctly identified.

Specificity and sensitivity. The performance of the Etest MBL strips in detecting metallo-β-lactamase-producing strains was evaluated by comparison with reference biochemical and genetic methods. The specificity is based on the number of correct negative results, i.e., the true number of non-metallo-β-lactamase producers that were correctly identified (14). The sensitivity is based on the number of metallo-β-lactamase producers that were correctly identified (14).

RESULTS

Comparison of substrates and inhibitors in detecting metallo-β-lactamases. The specificities and sensitivities of the two substrates, IP and TZ, were determined using a subset (n = 26) of the metallo-β-lactamase producers (as indicated by the presence of the enzyme). IP (100% sensitivity) was found to be a much better substrate than TZ (54% sensitivity) (Table 1); the latter was unable to detect the presence of metallo-β-lactamase in *S. maltophilia* (n = 4) and *Acinetobacter* spp. (n = 3) and had low sensitivity for anaerobes (70%) (Fig. 1). The sensitivities for EDTA and MPA against a subset (n = 83) of metallo-β-lactamase producers are listed in Table 2. Also, in contrast to the results of Arakawa et al. (2), EDTA was found to be more sensitive (97%) than MPA (52%) for detecting metallo-β-lactamases. MPA did not detect IMP-1 in *P. aeruginosa* or the CcrA enzymes in *B. fragilis*. Furthermore, MPA had low sensitivities for detecting the *Chryseobacterium* (60%) and *S. maltophilia* (63%) metallo-β-lactamases. The clearer inhibition ellipses of IP-IPE were easier to interpret than those of IP-IPM, which often had colonies within the inhibition zones.

Influence of different media on the detection of metallo-β-lactamases. The choice of media used to detect metallo-β-lactamases can be extremely important. Accordingly, the specificities for the different media in tests with a subset of metallo-β-lactamase producers (n = 14) were evaluated. These strains were *P. aeruginosa* carrying IMP-1 (n = 1), *Acinetobacter* spp. carrying IMP-1 (n = 3), *Serratia* spp. carrying IMP-1 (n = 3), *S. maltophilia* carrying an active L1 metallo-β-lactamase (n = 2), *Chryseobacterium* spp. carrying an active *bla*-like metallo-β-lactamase (n = 2), and *Bacteroides* group (n = 3). Brain heart infusion agar and NA had specificities of 64 and 79%, respectively (results not shown). NA failed to detect the presence of IMP-1 in one *Serratia* strain and one *Acinetobacter* strain. MH and ISO, which are recommended standardized susceptibility media, were investigated further using a subset of 115 strains. MH was observed to have a reproducibly higher

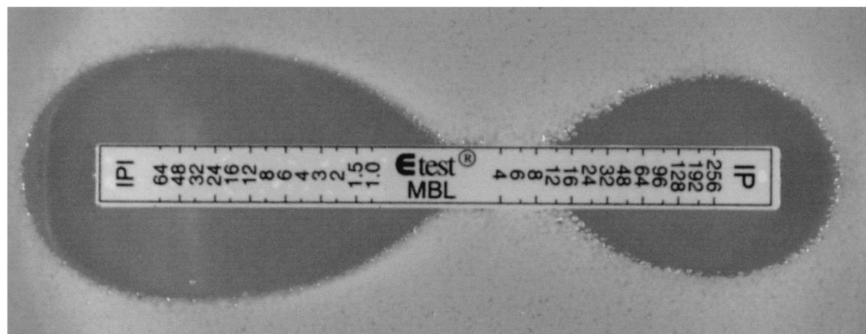

FIG. 1. Etest MBL strip tested against an *S. marcescens* strain expressing the IMP-1 metallo-β-lactamase. The IP MIC has decreased from 16 to <1 mg/liter upon exposure to a constant concentration of EDTA.

sensitivity (97%) than ISO (overall sensitivity of 93%), detecting all aerobic metallo-β-lactamase producers. In addition, MICs of IP on ISO were falsely low even for metallo-β-lactamase producers, giving 9.4% very major errors (resistant to susceptible) and 8.5% unacceptable minor errors (resistant to intermediate) (data not shown). The brand-to-brand variations of MH (from Accumedia, BDMS, Oxoid, Difco, and Remel) were also evaluated using metallo-β-lactamase-negative reference strains and confirmed metallo-β-lactamase producers. Most MH brands gave acceptable results, although the Difco MH brand gave consistently lower IP MICs for the two control strains and had a tendency to overstate the number of metallo-β-lactamase producers, because the MIC reduction in the presence of EDTA was more pronounced on Difco medium.

Metallo-β-lactamase detection by Etest MBL compared to biochemical activity and genetic probing. Etest MBL strips were used in tests with 130 strains, 83 of which produce metallo-β-lactamases. The reference standard used in this study for the presence of a metallo-β-lactamase was based on the ability of the bacterium's crude lysate to readily hydrolyze IP (>10 μM substrate hydrolyzed/min/mg of protein) and inhibition of hydrolysis in the presence of EDTA (10 mM). For equivocal results, PCR analysis, i.e., the presence of an amplicon of the right size for the metallo-β-lactamase gene, was used as confirmation that the bacterium could potentially produce the enzyme. With the sole exception of *Aeromonas* spp. (IP MIC of ≤2 mg/liter), all species that possessed metallo-β-lactamase activity and/or carried a metallo-β-lactamase gene gave a positive Etest MBL result (Table 3) based on an IP MIC of ≥4 mg/liter and inhibition with EDTA (Fig. 1). Based on the biochemical (T. R. Walsh, unpublished results) and genetic (4, 5) criteria used, not all *Chryseobacterium* spp. produce a metallo-β-lactamase. Interestingly, two of the *S. maltophilia* strains failed to produce a metallo-β-lactamase yet showed a positive result for the presence of the gene. These isolates were deemed negative, as previous studies have demonstrated mutations rendering the enzyme incapable of readily hydrolyzing IP (3). The Etest MBL strip also performed well with anaerobes, as indicated by the results for *Bacteroides* spp. The Etest MBL results were in 100% agreement with the results from the genotypic and biochemical methods (Table 3). Typically, IP MICs for metallo-β-lactamase positive strains ranged from 4 to ≥256 μg/liter, i.e., overlap of intermediate and resistant categories. *S. maltophilia* and *P. aeruginosa* strains for which MICs were ≥16 mg/liter could be separated into two phenotypes, i.e., metallo-β-lactamase producer by Etest criteria or resistant by another, uncharacterized mechanism. Specifically, the Etest IP-IPE MBL strip had a detection sensitivity of 94% and specificity of 95% compared to the more rigorous reference methods. Other β-lactamase phenotypes (ESBL AmpC and MIR-1) remained negative (Table 3). Those strains specifically carrying ESBLs and MIR-1 are noted in Table 3, whereas those carrying an active and inducible AmpC were *P. aeruginosa* (n = 13) and *Serratia* spp. (n = 2). *P. aeruginosa* CI 9-10-6 (metallo-β-lactamase-negative and having IP MICs of 8 to 32 mg/liter) and *S. marcescens* CI 10-4-9 (metallo-β-lactamase positive with IP MICs of 16 to 64 and IPE MICs of 2 to 8 mg/liter) were useful control strains with on-scale Etest MICs. They can be used to monitor the performance of MH with respect to variations in zinc concentration, which may vary from brand to brand and batch to batch and affect the accuracy of metallo-β-lactamase detection.

TABLE 2. Sensitivities of EDTA and MPA as inhibitors of metallo-β-lactamases on MH

Metallo-β-lactamase producers (n)	Metallo-β-lactamase type	Sensitivity (%)	
		IP-IPE	IP-IPM
Acinetobacter spp. (3)	IMP-1	100	100
Chryseobacterium spp. (20)	BlaB/IND-1	85	60
P. aeruginosa (1)	IMP-1	100	0
Serratia spp. (8)	IMP-1	100	88
S. maltophilia (41)	L1	95	63
B. fragilis group (10)	CcrA	100	0
Total		97	52

DISCUSSION

The mobility of β-lactamase genes associated with class 1 integrons and being disseminated throughout bacterial populations is of great concern to microbiologists and physicians alike. This now appears to be the case with metallo-β-lactamase genes, such as those of the IMP and VIM series which are distributed intergenerically, each conferring carbapenem resistance and now having been reported in widespread geographical locations (12, 19). Typically, these enzymes have arisen in gram-negative, non-glucose-fermenting bacteria and

TABLE 3. Accuracy of Etest IP-IPE compared to biochemical and genotypic methods

Organism(s) (*n*)	No. metallo-β-lactamase:		β-Lactamase type	Specificity (%)[b]	Sensitivity (%)[c]
	Negative	Positive[a]			
Acinetobacter spp. (9)	6	3	IMP-1	100	100
Chryseomonas spp. (28)	8	20	BlaB/IND-1	86	85
E. coli (1)	1	0	TEM	100	NR[d]
K. pneumoniae (4)	4	0	TEM ESBLs	100	NR
P. aeruginosa (14)	13	1	IMP-1	100	100
P. mirabilis (3)	3	0	MIR-1	100	NR
Serratia spp. (10)	2	8	IMP-1	100	100
S. maltophilia (43)	2	41	L1	95	95
S. spiritivorum (3)	3	0	ND[e]	100	100
B. fragilis group (15)	5	10	CcrA	100	100
Total (130)	47	83		95	94

[a] Positive by enzyme assay and/or PCR.
[b] Based on the number of correct negative results, i.e., the true number of non-metallo-β-lactamase producers that were correctly identified.
[c] Based on the number of metallo-β-lactamase producers that were correctly identified.
[d] NR, not relevant (β-lactamase phenotype other than metallo-β-lactamase).
[e] ND, not determined.

where therapeutic options are severely limited (7, 25). *Bacteroides* spp. are another group of bacteria that occasionally possess mobile metallo-β-lactamases that confer carbapenem resistance and can be associated with clinical failure (11). Detection of such enzymes would be valuable from an epidemiological perspective and for guiding therapeutic choices. Therefore, detection of metallo-β-lactamases, both chromosomally and plasmid mediated, is a valuable tool for a diagnostic microbiology laboratory.

The Etest MBL strip, based on a combination of a β-lactam substrate and a β-lactam/metallo-β-lactamase inhibitor, was specifically designed to detect as many clinically relevant metallo-β-lactamases as possible. Of the various configurations evaluated, the best combination was IP-IPE. This is in marked contrast to the results of Arakawa et al. (2), who found TZ and MPA to be the best combination. However, that study used a limited number of strains and did not evaluate the detection of metallo-β-lactamase, either chromosomally or plasmid mediated, produced by nonfermenting gram-negative bacteria. The combination of IP and IPE was shown to work equally well for anaerobes and aerobes, whereas IP-MPA did not perform well, especially with the anaerobes.

The choice of media to be used with the Etest MBL strip was very important in order to maximize sensitivity. When the metallo-β-lactamase-positive subset of strains were tested with NA and brain heart infusion agar, the results showed very low specificities, and ISO was also limited in its ability to detect metallo-β-lactamase producers, possibly due to the lower levels of zinc compared to those in MH. Disturbingly, this was shown to be the case for the IMP-1 metallo-β-lactamase as well as some of the chromosomally mediated metallo-β-lactamases. Furthermore, tests on ISO underestimate the IP MIC, giving unacceptably high levels of very major errors and minor errors. The quality performances of various brands of MH tested with recommended control strains and metallo-β-lactamase producers were acceptable (except for Difco MH).

The results from this study also demonstrated that some metallo-β-lactamase producers were inhibited at relatively low IP MICs and would be deemed susceptible or intermediate (MIC, 4 to 8 mg/liter) according to the current NCCLS breakpoints. For *Aeromonas* spp. it has long been established that although they possess a very effective metallo-β-lactamase that readily hydrolyzes the carbapenems, under standardized in vitro testing conditions, they are usually categorized as carbapenem susceptible (MIC of ≤4 mg/liter) (26). Not surprisingly, therefore, the Etest MBL did not detect the presence of metallo-β-lactamases in these strains despite the fact that they tested positive by the biochemical and genotypic methods. In contrast, other strains (*P. aeruginosa* [expressing IMP-1] and *S. maltophilia* [expressing L1]) did test positive with the Etest MBL yet were not resistant according to the NCCLS breakpoint (≥16 mg/liter). Paradoxically, some *S. maltophilia* strains lack the L1 enzyme, and this must cast doubt on identification methods based on this species always being resistant to carbapenems (3). The detection of metallo-β-lactamases in *Chryseobacterium* strains was found to be problematic, as the enzyme cannot always be biochemically detected and the heterogeneity of the gene makes PCR analysis difficult (4, 5).

Although the true clinical or therapeutic impact of metallo-β-lactamases remains unknown, given the awesome spread of TEM, SHV, CTX-M, and OXA β-lactamase genes throughout gram-negative aerobic bacteria, it is very worrisome that metallo-β-lactamase genes appear to be spreading in a similar manner. Furthermore, some of the plasmid-mediated-enzyme producers do not appear to be accurately detected using current resistance breakpoint criteria, and therefore the emergence and spread of this resistance mechanism will not be fully exposed by using standardized susceptibility testing methods. The implementation of a simple laboratory metallo-β-lactamase detection method that is quick, specific, sensitive, and reproducible is attractive, particularly where carbapenem and other β-lactam therapeutic regimens are indicated or preferred. The Etest MBL strip (IP-IPE) has the ability to detect metallo-β-lactamases, both chromosomally and plasmid mediated, in aerobic and anaerobic bacteria. This novel method could be used by clinical laboratories to monitor the emergence of metallo-β-lactamases in a range of clinically signifi-

cant bacteria and by surveillance networks to establish the spread of the enzyme.

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