JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2006, p. 3139–3144 Vol. 44, No. 9
0095-1137/06/$08.00+0 doi:10.1128/JCM.00879-06

Phenotypic Detection of Carbapenem-Susceptible Metallo-β-Lactamase-Producing Gram-Negative Bacilli in the Clinical Laboratory

Clare Franklin, Lisa Liolios, and Anton Y. Peleg*

Microbiology Unit, Alfred Pathology Service, Alfred Hospital, Commercial Road, Melbourne, Victoria 3004, Australia

Received 26 April 2006/Returned for modification 6 June 2006/Accepted 27 June 2006

Rapid detection of metallo-β-lactamase (MBL)-producing gram-negative pathogens is critical to prevent their widespread dissemination. Thus far, no standardized phenotypic method is available, and previously reported techniques have poor sensitivity for detecting carbapenem-susceptible MBL-carrying isolates, an increasingly described phenomenon. We developed a phenotypic detection method using both a double-disk synergy test and a combined-disk test with imipenem and 292 μg EDTA on one agar plate. Genotypic confirmation was used for validation. Of the 134 clinical isolates, 84 were confirmed to carry an MBL. Of these, 51 (61%) were susceptible to at least one carbapenem, and 22 (26%) were isolated from blood. The phenotypic method correctly differentiated all MBL-producing isolates (sensitivity, 100%). Fifty-one of the 52 MBL-negative isolates were correctly differentiated (specificity, 98%). This study reports the validation of a simple and accurate MBL detection method that can be easily incorporated into the daily routine of a clinical laboratory. Early detection of MBL-carrying organisms, including those with susceptibility to carbapenems, is of paramount clinical importance, as it allows rapid initiation of strict infection control practices as well as therapeutic guidance for confirmed infection.

The clinical utility of carbapenems is under threat with the emergence of acquired carbapenemases, particularly Ambler class B metallo-β-lactamases (MBLs). Such enzymes have emerged in many geographical locations (23) and often confer high-level resistance to all β-lactams except aztreonam. Five enzyme types have been identified (IMP, VIM, SPM, GIM, and SIM types), involving various host organisms, most commonly *Pseudomonas* spp., *Acinetobacter* spp., and *Enterobacteriaceae*. A particular concern is that acquired MBL genes are located on integron structures that reside on mobile genetic elements such as plasmids or transposons (23), thus enabling widespread dissemination. Clinical infections with such organisms pose serious therapeutic challenges, with increasing reports of poor patient outcomes and death (5, 8, 14).

With the worldwide increase in the occurrence, types, and rate of dissemination of MBLs, early detection is critical. The benefits of such include timely implementation of strict infection control practices as well as clinical guidance regarding the potential risks for therapeutic failure. As seen with extended-spectrum β-lactamases (ESBL) and AmpC-type β-lactamases with cephalosporins (11, 12), MBL-carrying organisms can appear susceptible to carbapenems using current Clinical and Laboratory Standards Institute or British Society for Antimicrobial Chemotherapy breakpoints (1, 3). For example, at our institution, over 30% of MBL-carrying isolates, predominately *Enterobacteriaceae*, were found to be susceptible to imipenem (IPM; MIC ≤ 4 μg/ml) (14). Yan and colleagues reported an outbreak of *Klebsiella pneumoniae* isolates carrying $bla_{\text{IMP-8}}$ and showed that 88% (35/40) were susceptible to carbapenems (24). More recently, clinical isolates of $bla_{\text{VIM-1}}$-producing *Escherichia coli* from Greece were shown to be below the breakpoint for carbapenem resistance (17). Similar findings have been reported by others (7, 20). As a result of being difficult to detect, such organisms pose significant risks, particularly due to their role in unnoticed spread within institutions and their ability to participate in horizontal MBL gene transfer with other pathogenic hospital-related organisms (14). Also, the clinical outcome of patients infected with organisms carrying these so-called "hidden" MBL genes and treated with carbapenems is unknown. However, given the poor outcomes, such as death, persistent fevers, and infection relapse, seen with cephalosporin treatment for infections caused by organisms with hidden ESBL genes (12), laboratory detection of carbapenem-susceptible MBL-carrying organisms should be considered to be of paramount clinical importance.

Currently, no standardized method for MBL detection has been proposed, and despite PCR being highly accurate and reliable, its accessibility is often limited to reference laboratories. Several nonmolecular techniques have been studied, all taking advantage of the enzyme's zinc dependence by using chelating agents, such as EDTA or 2-mercaptopropionic acid, to inhibit its activity. The commercially available MBL Etest (AB Biodisk, Solna, Sweden), which incorporates an imipenem strip on one end and an imipenem strip with EDTA on the other, is simple to perform but is highly insensitive at detecting carbapenem-susceptible MBL-carrying organisms (MIC ≤ 4 μg/ml) and is costly (23, 25). Also, poor specificity has been described with carbapenem-resistant *Acinetobacter baumannii* carrying $bla_{\text{OXA-23}}$ (18). A double-disk synergy test (DDST) using imipenem (IPM) and 0.5 M EDTA (9, 10) and a combined-disk test using either two IPM disks or two meropenem disks, one containing 930 μg (15) or 750 μg (26) of EDTA, have both been reported as reliable methods for the detection

* Corresponding author. Present address: Division of Infectious Diseases, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. Phone: (617) 667-0185. Fax: (617) 975-5235. E-mail: apeleg@bidmc.harvard.edu.

of MBLs in carbapenem-resistant *Pseudomonas* and *Acinetobacter* strains. When the latter method was studied using carbapenem-susceptible isolates, the sensitivity was poor, ranging from 10% to 86% (25). Thus far, no method has been reported to show adequate sensitivity and specificity for the detection of both carbapenem-susceptible and carbapanem-resistant MBL-positive isolates.

MBL-producing gram-negative organisms have recently been described in Australia (13, 14). Like others, significant proportions of the organisms are carbapenem susceptible. This prompted our laboratory to design a simple and cost-effective phenotypic MBL detection method capable of identifying such organisms. We report the validation of this method using a broad range of bacterial species.

MATERIALS AND METHODS

Bacterial strains and clinical isolates. Eighty-four MBL-producing and 52 non-MBL-producing gram-negative isolates were included in the current study (Table 1). All isolates were nonduplicates, and except for three isolates, all were taken from clinical specimens collected at the Alfred Hospital, Melbourne, Australia, from January 2004 through December 2005. All MBL-producing isolates were from consecutive patients. Only one isolate of a given genus from each patient was included. Two isolates, one carrying bla_{IMP-1} and one carrying bla_{VIM-2}, were provided by Jan Bell from the Women's and Children's Hospital, Adelaide, Australia, and one bla_{VIM-4}-carrying isolate was provided by Peter Wiese from the Royal Melbourne Hospital, Melbourne, Australia (13). Non-MBL-producing isolates were randomly selected from isolates at our institution that were considered to be resistant to third-generation cephalosporins (MIC > 8 μg/ml) or carbapenems (MIC > 4 μg/ml) and negative for bla_{IMP} or bla_{VIM} during the same time period. Organisms were identified using an automated system (VITEK; bioMérieux Vitek Systems Inc., Hazelwood, MO) and by use of an API 20NE system (bioMérieux, Marcy l'Etoile, France). Organisms known to inherently carry an MBL gene, such as *Stenotrophomonas maltophilia*, *Aeromonas hydrophilia*, and *Flavobacterium* species, were excluded.

TABLE 1. Results of the phenotypic method in the detection of MBL-producing gram-negative bacilli

Organism	No. of isolates	No. (%) of isolates positive on phenotypic plate	MBL gene type
MBL-positive isolates	84		
Serratia marcescens	37	37 (100)	IMP-4
P. aeruginosa	19	19 (100)	IMP-4
P. aeruginosa	1	1 (100)	VIM-4
Pseudomonas stutzeri	1	1 (100)	VIM-2
Klebsiella pneumoniae	10	10 (100)	IMP-4
Klebsiella oxytoca	1	1 (100)	IMP-4
Escherichia coli	6	6 (100)	IMP-4
Enterobacter cloacae	6	6 (100)	IMP-4
Citrobacter koseri	1	1 (100)	IMP-4
Acinetobacter junii	1	1 (100)	IMP-4
Acinetobacter baumannii	1	1 (100)	IMP-1
MBL-negative isolates	52		
P. aeruginosa	15	1 (7)	ND[a]
Pseudomonas mendocina	1	0 (0)	ND
A. baumannii	10	0 (0)	ND
E. cloacae	5	0 (0)	ND
S. marcescens	4	0 (0)	ND
E. coli	4	0 (0)	ND
K. pneumoniae	3	0 (0)	ND
K. oxytoca	3	0 (0)	ND
Citrobacter braakii	1	0 (0)	ND
Achromobacter xylosoxidans	3	0 (0)	ND
Burkholderia cepacia	2	0 (0)	ND
Ralstonia pickettii	1	0 (0)	ND

[a] ND, not detected.

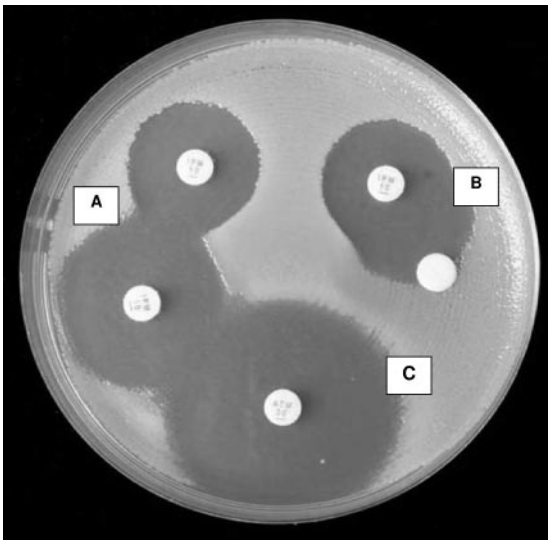

FIG. 1. The phenotypic appearance of an MBL-producing *Serratia marcescens* isolate carrying the bla_{IMP-4} gene. (A) Combined-disk test, using two imipenem (10 μg) disks, one with 292 μg EDTA, showing an increase in zone inhibition of >4 mm around the disk with EDTA. (B) Double-disk synergy test, using an IPM (10 μg) disk placed 20 mm (center to center) from a blank filter disk containing 292 μg EDTA. (C) Aztreonam (30 μg) disk with a >30-mm zone of inhibition.

Antimicrobial susceptibility testing. MICs for all *Enterobacteriaceae* and *Acinetobacter* spp. were determined by use of Vitek GNS susceptibility cards. Susceptibility test results for all other nonfermentative gram-negative organisms were determined by use of agar dilution (2), disks (1), or Etest methodology.

Phenotypic detection of MBLs. An MBL phenotypic detection method was designed using a single agar plate and comprised three components (Fig. 1). (i) In the combined-disk test, two IPM disks (10 μg), one containing 10 μl of 0.1 M (292 μg) anhydrous EDTA (Sigma Chemicals, St. Louis, MO), were placed 25 mm apart (center to center). An increase in zone diameter of >4 mm around the IPM-EDTA disk compared to that of the IPM disk alone was considered positive for an MBL. This cutoff was chosen after preliminary research at our institution showed that an IPM (10 μg) disk plus 292 μg EDTA could increase the zone diameter by up to 4 mm compared to that of an IPM disk alone in 50 MBL-negative organisms, but the increase was >4 mm in six MBL-positive organisms (three *Serratia marcescens* isolates, one *Klebsiella pneumoniae* isolate, one *Escherichia coli* isolate, and one *Pseudomonas aeruginosa* isolate). Thus, a change of >4 mm was chosen for further study. Ten microliters of 0.1 M (292 μg) EDTA was chosen, as higher concentrations led to inhibitory effects with the EDTA alone (data not shown). (ii) In the DDST, an IPM (10 μg) disk was placed 20 mm (center to center) from a blank disk containing 10 μl of 0.1 M (292 μg) EDTA. Enhancement of the zone of inhibition in the area between the two disks was considered positive for an MBL (Fig. 1). (iii) The final component was an aztreonam (30 μg) disk. Given the unique sensitivity of MBLs to this antibiotic, we studied the inhibition zone sizes of all isolates to determine the utility of this component in phenotypic MBL detection. Disks were applied to the surface of the inoculated agar as shown in Fig. 1, and plates were incubated overnight at 35°C. All antibiotic disks were obtained from Oxoid.

Media and inoculum. Test organisms were inoculated onto IsoSensitest agar (Oxoid, Basingstoke, United Kingdom) using a 1-in-10 dilution of a 0.5 McFarland standard inoculum. A subset of organisms also underwent testing using a heavier inoculum (0.5 McFarland standard). During preliminary investigations, we tested MBL-producing isolates using both IsoSensitest and Mueller-Hinton agars. This included four *S. marcescens* isolates, two *K. pneumoniae* isolates, two *P. aeruginosa* isolates, one *Citrobacter koseri* isolate, and one *Enterobacter cloacae* isolate. Five of these isolates were carbapenem susceptible, and all were carrying bla_{IMP-4}. With both media, all MBL-producing isolates were detected. However, zone diameters with IPM-EDTA compared with IPM alone

TABLE 2. Summary of results for the combined-disk test and the double-disk synergy test in the detection of MBL-producing gram-negative bacilli

Phenotypic method	No. of PCR-confirmed MBL-carrying organisms (n = 84)	No. of PCR-confirmed non-MBL-carrying organisms (n = 52)	Sensitivity (%)	Specificity (%)
Combined-disk test	84 (100%)	1 (2%)	100	98
Double-disk synergy test	66 (79%)	1 (2%)	79	98

were on average 2 mm greater on IsoSensitest agar than on Mueller-Hinton agar. Also, enhancement of the inhibition zone between IPM and EDTA for the DDST was more interpretable with IsoSensitest agar than with Mueller-Hinton agar. So, despite the apparent paucity of bivalent zinc ions in IsoSensitest agar, this medium was chosen for further study. All isolates were tested on at least two batches of IsoSensitest agar to assess consistency. Importantly, IsoSensitest and Mueller-Hinton agars have both been shown to have adequate sensitivity for phenotypic MBL detection (22).

PCR amplification and DNA sequencing. PCR analysis was performed on all isolates to confirm the presence of an MBL. The primers used for the bla_{IMP} and bla_{VIM} genes were described previously (19, 21). For each target gene, PCR amplification was carried out in a 50-μl reaction volume using a Thermal Cycler 9600 instrument (Applied Biosystems, Norwalk, Conn.). The reaction mixture contained 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside triphosphate, a 0.2 μM concentration of each specific primer, and 1.35 U of AmpliTaq Gold DNA polymerase (Applied Bioystems). The master mix was spiked with template DNA from one to two colonies of pure culture, prior to amplification. For both PCRs, an initial 10-min denaturation step at 95°C was performed followed by 35 cycles of 45 s of denaturation at 94°C, 45 s of primer annealing at 56°C, and 50 s of primer extension at 72°C for bla_{IMP} detection, or 35 cycles of 30 s of denaturation at 94°C, 40 s of primer annealing at 52°C, and 50 s of primer extension at 72°C for the bla_{VIM} gene detection. Following the last cycle, an additional 7-min extension step was performed at 72°C, and the products were then held at 4°C. After agarose gel electrophoresis with ethidium bromide, the PCR products were analyzed under UV light. Nucleotide sequencing confirmed the MBL gene type as described previously (14).

Sensitivity and specificity. The performance of the phenotypic MBL detection method was evaluated using PCR as the gold standard. The sensitivity (the number of MBL-carrying organisms that were correctly differentiated) and specificity (the number of non-MBL-carrying organisms that were correctly differentiated) of the phenotypic method were calculated (6).

RESULTS

Clinical bacterial isolates. A total of 136 nonduplicate isolates across 10 different gram-negative genera were included in the study (Table 1). Of the 84 MBL-producing isolates, 34 (40%) were from respiratory tract specimens, 22 (26%) were from blood, 10 (12%) were from vascular catheter tips, 8 (10%) were from wound swabs, 5 (6%) were from urine, 4 (5%) were from operative tissue, and 1 (1%) was from wound drain fluid. Of the 52 non-MBL-producing isolates, 24 (46%) were isolated from respiratory tract specimens, 14 (27%) were from wound swabs, 7 (13%) were from urine, 3 (6%) were from blood, 2 (4%) were from vascular catheter tips, and 2 (4%) were from wound drain fluid.

Carbapenem susceptibilities. Overall, 51 (61%) MBL-carrying isolates tested sensitive to either imipenem or meropenem. Of these, 31 (37%) had an imipenem MIC of ≤4 μg/ml, including 10 *Klebsiella* spp. isolates, 9 *S. marcescens* isolates, 5 *E. coli* isolates, 5 *E. cloacae* isolates, 1 *C. koseri* isolate, and 1 *A. baumannii* isolate. Forty-eight (57%) had meropenem MICs of ≤2 μg/ml. Of the 22 MBL-carrying isolates cultured from blood, 11 (50%) were susceptible to either imipenem or meropenem. Of these carbapenem-susceptible isolates causing bloodstream infection, all were *Enterobacteriaceae*. The remaining 11 isolates from blood were carbapenem-resistant *P. aeruginosa*. All MBL-carrying organisms were resistant to ceftazidime (MIC > 8 μg/ml) and ticarcillin/clavulanate (MIC > 16 μg/ml). Of the MBL-negative isolates, 32 (62%) were susceptible to either imipenem or meropenem.

Phenotypic MBL detection method. The phenotypic MBL detection method identified all isolates that were confirmed to be carrying an MBL by PCR (100% sensitivity), including 81 bla_{IMP-4}-carrying isolates, 1 bla_{IMP-1}-carrying *A. baumannii* isolate, 1 bla_{VIM-2}-carrying *Pseudomonas* sp. isolate, and 1 bla_{VIM-4}-carrying *Pseudomonas* sp. isolate. Of the 84 MBL-carrying isolates, 66 (79%) were found to be positive in both the DDST and the combined-disk test (Table 2). Eighteen isolates (21%), all bla_{IMP-4}-carrying *P. aeruginosa* isolates, were positive in the combined-disk test only. The increase in zone

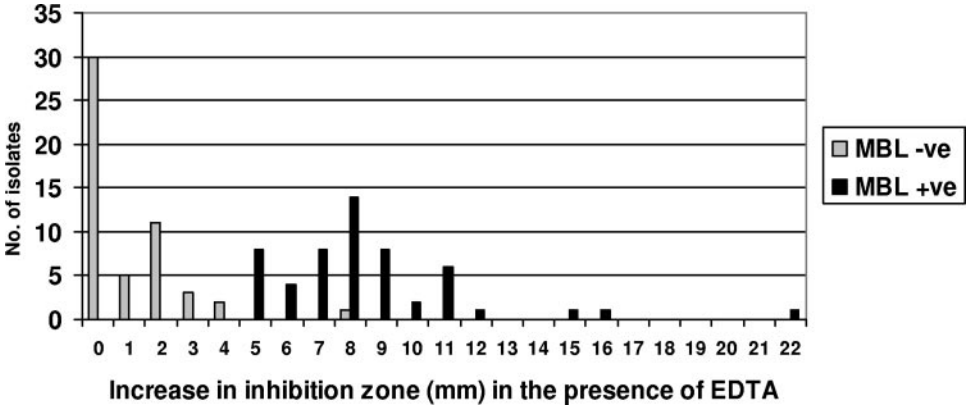

FIG. 2. Increase (in millimeters) in zone of inhibition around the imipenem-EDTA disk compared with the imipenem disk alone for 52 MBL-negative (MBL -ve) and 54 MBL-positive (MBL +ve) clinical isolates.

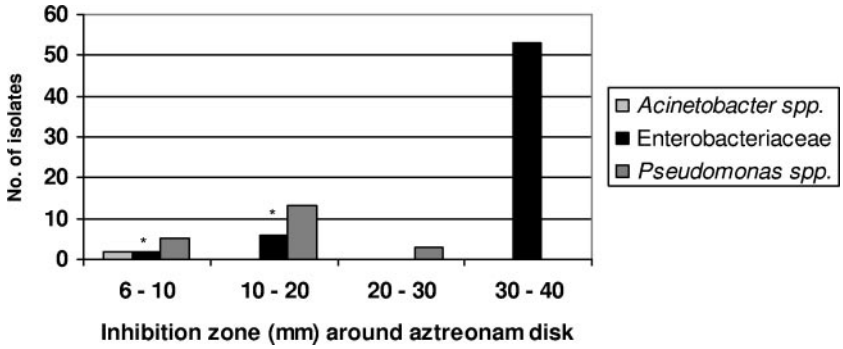

FIG. 3. Inhibition zone diameters (in millimeters) around the aztreonam disk (30 μg) for the 84 metallo-β-lactamase-carrying isolates. Asterisks indicate *Enterobacteriaceae* isolates ($n = 8$) found to produce an AmpC-type or extended-spectrum β-lactamase.

inhibition with IPM-EDTA compared with IPM alone is shown in Fig. 2. A breakpoint of >4 mm, as determined by our preliminary work, was highly effective at distinguishing between MBL and non-MBL producers. Interestingly, 12 MBL-producing isolates had an increase in zone diameter of <7 mm (≥7 mm being the cutoff used by other investigators [15, 25, 26]), 11 (92%) of which were *Enterobacteriaceae*, including 6 *K. pneumoniae* isolates, 1 *E. coli* isolate, 3 *E. cloacae* isolates, and 1 *C. koseri* isolate. No difference in zone diameter increase was observed between carbapenem-susceptible and -resistant isolates. Twelve MBL-producing organisms were also tested using a heavier inoculum (0.5 McFarland standard), including two *E. cloacae* isolates, six *S. marcescens* isolates, one *K. pneumoniae* isolate, and three *P. aeruginosa* isolates. For the combined-disk test, 11 of these isolates had a smaller difference in the zone of inhibition around the IPM-EDTA disk than around the IPM disk alone when a heavier inoculum was used. Two isolates had a zone size difference of ≤4 mm with the heavier inoculum, a result that is below our definition of phenotypic MBL production. For the DDST, one *P. aeruginosa* isolate was negative with the heavier inoculum yet positive with the lighter inoculum (1-in-10 dilution of a 0.5 McFarland standard). All other isolates gave similar results, but the synergy enhancement between the IPM and EDTA disks was more pronounced with a lighter inoculum.

A large zone of inhibition (>30 mm) around the aztreonam disk was seen in 53 (63%) MBL-carrying isolates, all *Enterobacteriaceae* (Fig. 3). The remaining 31 isolates (37%), including 21 *Pseudomonas* spp. isolates, 2 *Acinetobacter* spp. isolates, 6 *E. cloacae* isolates, and 2 *K. pneumoniae* isolates, showed a reduced zone of inhibition or none at all. The eight *Enterobacteriaceae* with reduced inhibition zones were all confirmed to have a coexisting AmpC-type β-lactamase or ESBL, as determined by a phenotypic method (16).

The phenotypic MBL detection method correctly differentiated 51 of the 52 PCR-confirmed MBL-negative isolates (98% specificity), with one *P. aeruginosa* isolate giving a false-positive result with both the DDST and the combined-disk test (Fig. 2). Overall, the sensitivity and specificity of the phenotypic MBL detection method were 100% and 98%, respectively. The above results were consistent between two batches of IsoSensitest agar.

DISCUSSION

MBL-producing gram-negative organisms have now been reported in many geographic regions (23). Their ability to rapidly disseminate within an institution and lead to poor outcomes when infection ensues is a concern (5, 8, 14), and therefore, early laboratory detection is of great clinical importance. Unfortunately, the phenotypic appearance of MBL-carrying organisms varies depending on the bacterial host, with increasing reports of carbapenem-susceptible isolates, primarily *Enterobacteriaceae* such as *Klebsiella* spp. and *E. coli* (14, 17, 24). Such organisms often carry hidden MBL genes, whereby the microbiologist and the clinician remain unaware of their presence within an institution. Such a scenario creates the potential for untoward clinical and infection control consequences and is by no means unique to MBLs. Similar scenarios have been described for ESBL and AmpC-type β-lactamases, with studies suggesting poor clinical outcomes in patients treated with extended-spectrum cephalosporins for serious infections (11, 12). Whether similar outcomes will be observed with carbapenem treatment for infections caused by carbapenem-susceptible MBL-carrying organisms (hidden MBLs) is currently unknown, but the ability of these isolates to participate in horizontal MBL gene transfer with other gram-negative pathogens and contribute significantly to MBL-related outbreaks has been described in our previous work (14). As a consequence, more sensitive means of laboratory detection of MBL-producing isolates are urgently required if we are to prevent the ongoing spread of these problematic organisms.

In the current study, we report a phenotypic MBL detection system that is highly sensitive (100%) and specific (98%) at detecting both carbapenem-susceptible (61% of isolates) and carbapenem-resistant MBL-carrying isolates across a wide range of gram-negative genera from clinically important specimens. The method is simple to perform, and the materials used are cheap, nontoxic, and easily accessible, making it highly applicable to routine clinical laboratories. With the emergence of carbapenem-susceptible MBL-carrying organisms, the issue of which isolates to select for phenotypic MBL detection is now more challenging. Clearly, screening only carbapenem-resistant organisms, as is most often performed, is suboptimal. On the other hand, selecting all isolates creates unnecessary work with a lower yield. All MBL-carrying isolates

tested in the current study (seven different gram-negative genera), including the carbapenem-susceptible isolates, were resistant to ceftazidime and ticarcillin/clavulanate, and we feel that such a phenotype is an appropriate initial threshold from which to pursue MBL production, especially in *Enterobacteriaceae*. This threshold may be adjusted according to the prevalence of MBLs at an institution.

Yan and colleagues reported on the laboratory detection of carbapenem-susceptible MBL-carrying organisms and compared three methods (25). The first was a DDST, using a plain filter disk with 2-mercaptopropionic acid and four antibiotic disks (ceftazidime, ceftazidime-clavulanate, cefepime, and cefepime-clavulanate). In order to achieve an adequate level of sensitivity, the investigators needed to combine the DDST results of all four antibiotic disks. In doing so, the specificity was reduced, with 10 of 30 (33.3%) *A. baumannii* isolates testing falsely positive. The second method was a combined-disk test using imipenem and 10 μl of 0.5 M EDTA, with an increase in zone diameter cutoff of ≥7 mm with IPM-EDTA compared with IPM alone. The sensitivity varied depending on the bacterial host but was lowest for carbapenem-susceptible isolates (10% for *K. pneumoniae*). In an attempt to improve sensitivity, the investigators combined the four antibiotic disks mentioned above with EDTA. Highly varied results were achieved, depending on the genera involved and the antibiotic used. No single combination provided adequate results for all organisms tested. The final method was the MBL Etest, and as reported by others (23), this method is not applicable to carbapenem-susceptible MBL-carrying organisms (MIC ≤ 4 μg/ml). Thus, no method, apart from the DDST using four different antibiotic disks, which is cumbersome and more costly and has greater risk for subjective errors, was adequate for the detection of carbapenem-susceptible and -resistant MBL-carrying organisms.

In the current study, we utilized several unique methodological techniques in an attempt to maximize the detection of such challenging organisms. Firstly, both a DDST and a combined-disk test were performed concurrently on a single agar plate. As described above, no single method provided ideal results for MBL detection, and therefore we employed both techniques. Despite this, our results showed that the combined-disk test had a sensitivity of 100% and a specificity of 98%, whereas the DDST had a sensitivity of 79% and a specificity of 98% (Table 2). Overall, the combined-disk test is the preferred method due to its objective interpretation. Secondly, we used IsoSensitest agar, as our preliminary investigations showed greater increases in zone diameters for the combined-disk test and greater clarity with the DDST compared with tests using Mueller-Hinton agar. Walsh and colleagues, while evaluating the MBL Etest, found that both media had excellent sensitivity (97% for Mueller-Hinton agar, 93% for IsoSensitest agar) (22). Also, Mueller-Hinton agar is known to contain variable concentrations of zinc, and the medium can vary from brand to brand and batch to batch, affecting the potential accuracy of MBL detection (22). Thirdly, for the combined-disk test, we used a lower cutoff (>4 mm) than others (≥7 mm) (15, 25, 26) for the increase in zone diameter with IPM-EDTA as opposed to IPM alone. This value provided excellent discriminatory power for detecting MBL-producing isolates (Fig. 2). Interestingly, 12 of our MBL-producing isolates had increases in zone diameter of <7 mm, 11 (92%) of which were *Enterobacteriaceae*, a group often missed in other investigations (25). Other unique methodological techniques used in the present study were a lighter inoculum (1-in-10 dilution of a 0.5 McFarland standard) and a lower concentration of EDTA (292 μg). We believe that such modifications were helpful for our ability to detect all MBL-producing isolates. Interestingly, we had one false-positive result, a *P. aeruginosa* isolate from a wound swab. False positives have been reported with EDTA-based methods and may be explained by the effects of EDTA on a zinc-dependent OprD pump responsible for carbapenem entry in *P. aeruginosa* (4). Also, the isolate may have been carrying an MBL gene type that was not tested in the current study.

MBLs are characterized by their ability to hydrolyze all β-lactams except for aztreonam (23), and therefore we studied the utility of an aztreonam (30 μg) disk in phenotypic MBL detection. Unfortunately, the results were varied and are best explainned by the presence of other mechanisms for aztreonam resistance, most importantly ESBL or AmpC-type β-lactamases. The utility of this component was greatest with *Enterobacteriaceae*, with 87% (53/61) having a large zone of inhibition (>30 mm) (Fig. 3). Of the eight *Enterobacteriaceae* isolates that showed reduced inhibition zones, all were found to have a coexisting AmpC-type β-lactamase or ESBL. Thus, a large inhibition zone around the aztreonam disk (>30 mm) was supportive for an MBL but was not necessary. Interestingly, an amoxicillin/clavulanate disk placed 25 mm (center to center) from the aztreonam disk can be utilized to assist in the phenotypic detection of a coexisting ESBL.

The majority of our MBL-producing isolates were carrying the $bla_{\text{IMP-4}}$ gene, but isolates carrying other MBL types, such as $bla_{\text{IMP-1}}$, $bla_{\text{VIM-2}}$, and $bla_{\text{VIM-4}}$, were also detected. Given the small number of the latter types of isolates, our results cannot be extrapolated to other MBL types, and further evaluation using different MBLs, including SPM, GIM, and SIM types, is required.

In conclusion, our study validates a simple and highly sensitive phenotypic method for the detection of MBL production in a broad range of host organisms. Most importantly, the method is highly sensitive at detecting carbapenem-susceptible MBL-producing isolates, a concerning phenotype that is being described with increased frequency. Such organisms, carrying hidden MBL genes, are a threat to infection control efforts and may result in poor clinical outcomes when carbapenems are used for treatment in those with serious infections. Such a hypothesis needs further evaluation. Thus, all diagnostic laboratories must now be on high alert for MBL-producing organisms as early detection is critical to prevent the consequences of this worrying resistance mechanism.

ACKNOWLEDGMENTS

We thank Jan Bell from the Women's and Children's Hospital, Adelaide, Australia, for providing the $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-2}}$-carrying strains and Peter Wiese from the Royal Melbourne Hospital, Melbourne, Australia, for providing the $bla_{\text{VIM-4}}$-carrying strain. We also thank David L. Paterson, Denis W. Spelman, and Peter Ward for their review of the manuscript.

REFERENCES

1. **Andrews, J. M.** 2005. BSAC standardized disc susceptibility testing method (version 4). J. Antimicrob. Chemother. **56:**60–76.

2. **Andrews, J. M.** 2001. Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. **48**(Suppl. 1)**:**5–16.
3. **Clinical and Laboratory Standards Institute/NCCLS.** 2005. Performance standards for antimicrobial susceptibility testing; 15th informational supplement. CLSI/NCCLS M100-S15. Clinical and Laboratory Standards Institute, Wayne, Pa.
4. **Conejo, M. C., I. Garcia, L. Martinez-Martinez, L. Picabea, and A. Pascual.** 2003. Zinc eluted from siliconized latex urinary catheters decreases OprD expression, causing carbapenem resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **47:**2313–2315.
5. **Hirakata, Y., T. Yamaguchi, M. Nakano, K. Izumikawa, M. Mine, S. Aoki, A. Kondoh, J. Matsuda, M. Hirayama, K. Yanagihara, Y. Miyazaki, K. Tomono, Y. Yamada, S. Kamihira, and S. Kohno.** 2003. Clinical and bacteriological characteristics of IMP-type metallo-beta-lactamase-producing *Pseudomonas aeruginosa*. Clin. Infect. Dis. **37:**26–32.
6. **Ilstrup, D. M.** 1990. Statistical methods in microbiology. Clin. Microbiol. Rev. **3:**219–226.
7. **Ito, H., Y. Arakawa, S. Ohsuka, R. Wacharotayankun, N. Kato, and M. Ohta.** 1995. Plasmid-mediated dissemination of the metallo-β-lactamase gene bla_{IMP} among clinically isolated strains of *Serratia marcescens*. Antimicrob. Agents Chemother. **39:**824–829.
8. **Laupland, K. B., M. D. Parkins, D. L. Church, D. B. Gregson, T. J. Louie, J. M. Conly, S. Elsayed, and J. D. Pitout.** 2005. Population-based epidemiological study of infections caused by carbapenem-resistant *Pseudomonas aeruginosa* in the Calgary Health Region: importance of metallo-beta-lactamase (MBL)-producing strains. J. Infect. Dis. **192:**1606–1612.
9. **Lee, K., Y. Chong, H. B. Shin, Y. A. Kim, D. Yong, and J. H. Yum.** 2001. Modified Hodge and EDTA-disk synergy tests to screen metallo-beta-lactamase-producing strains of *Pseudomonas* and *Acinetobacter* species. Clin. Microbiol. Infect. **7:**88–91.
10. **Lee, K., Y. S. Lim, D. Yong, J. H. Yum, and Y. Chong.** 2003. Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-β-lactamase-producing isolates of *Pseudomonas* spp. and *Acinetobacter*. spp. J. Clin. Microbiol. **41:**4623–4629.
11. **Pai, H., C. I. Kang, J. H. Byeon, K. D. Lee, W. B. Park, H. B. Kim, E. C. Kim, M. D. Oh, and K. W. Choe.** 2004. Epidemiology and clinical features of bloodstream infections caused by AmpC-type-β-lactamase-producing *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **48:**3720–3728.
12. **Paterson, D. L., W. C. Ko, A. Von Gottberg, J. M. Casellas, L. Mulazimoglu, K. P. Klugman, R. A. Bonomo, L. B. Rice, J. G. McCormack, and V. L. Yu.** 2001. Outcome of cephalosporin treatment for serious infections due to apparently susceptible organisms producing extended-spectrum β-lactamases: implications for the clinical microbiology laboratory. J. Clin. Microbiol. **39:**2206–2212.
13. **Peleg, A. Y., J. M. Bell, A. Hofmeyr, and P. Wiese.** 2006. Inter-country transfer of gram-negative organisms carrying the VIM-4 and OXA-58 carbapenem-hydrolysing enzymes. J. Antimicrob. Chemother. **57:**794–795.
14. **Peleg, A. Y., C. Franklin, J. M. Bell, and D. W. Spelman.** 2005. Dissemination of the metallo-beta-lactamase gene bla_{IMP-4} among gram-negative pathogens in a clinical setting in Australia. Clin. Infect. Dis. **41:**1549–1556.
15. **Pitout, J. D. D., D. B. Gregson, L. Poirel, J.-A. McClure, P. Le, and D. L. Church.** 2005. Detection of *Pseudomonas aeruginosa* producing metallo-β-lactamases in a large centralized laboratory. J. Clin. Microbiol. **43:**3129–3135.
16. **Pitout, J. D. D., M. D. Reisbig, E. C. Venter, D. L. Church, and N. D. Hanson.** 2003. Modification of the double-disk test for detection of *Enterobacteriaceae* producing extended-spectrum and AmpC β-lactamases. J. Clin. Microbiol. **41:**3933–3935.
17. **Scoulica, E. V., I. K. Neonakis, A. I. Gikas, and Y. J. Tselentis.** 2004. Spread of bla_{VIM-1}-producing *E. coli* in a university hospital in Greece. Genetic analysis of the integron carrying the bla_{VIM-1} metallo-beta-lactamase gene. Diagn. Microbiol. Infect. Dis. **48:**167–172.
18. **Segal, H., and B. G. Elisha.** 2005. Use of Etest MBL strips for the detection of carbapenemases in *Acinetobacter baumannii*. J. Antimicrob. Chemother. **56:**598.
19. **Senda, K., Y. Arakawa, S. Ichiyama, K. Nakashima, H. Ito, S. Ohsuka, K. Shimokata, N. Kato, and M. Ohta.** 1996. PCR detection of metallo-β-lactamase gene (bla_{IMP}) in gram-negative rods resistant to broad-spectrum β-lactams. J. Clin. Microbiol. **34:**2909–2913.
20. **Senda, K., Y. Arakawa, K. Nakashima, H. Ito, S. Ichiyama, K. Shimokata, N. Kato, and M. Ohta.** 1996. Multifocal outbreaks of metallo-β-lactamase-producing *Pseudomonas aeruginosa* resistant to broad-spectrum beta-lactams, including carbapenems. Antimicrob. Agents Chemother. **40:**349–353.
21. **Tsakris, A., S. Pournaras, N. Woodford, M. F. Palepou, G. S. Babini, J. Douboyas, and D. M. Livermore.** 2000. Outbreak of infections caused by *Pseudomonas aeruginosa* producing VIM-1 carbapenemase in Greece. J. Clin. Microbiol. **38:**1290–1292.
22. **Walsh, T. R., A. Bolmström, A. Qwärnström, and A. Gales.** 2002. Evaluation of a new Etest for detecting metallo-β-lactamases in routine clinical testing. J. Clin. Microbiol. **40:**2755–2759.
23. **Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann.** 2005. Metallo-β-lactamases: the quiet before the storm? Clin. Microbiol. Rev. **18:**306–325.
24. **Yan, J. J., W. C. Ko, S. H. Tsai, H. M. Wu, and J. J. Wu.** 2001. Outbreak of infection with multidrug-resistant *Klebsiella pneumoniae* carrying bla_{IMP-8} in a university medical center in Taiwan. J. Clin. Microbiol. **39:**4433–4439.
25. **Yan, J. J., J. J. Wu, S. H. Tsai, and C. L. Chuang.** 2004. Comparison of the double-disk, combined disk, and Etest methods for detecting metallo-beta-lactamases in gram-negative bacilli. Diagn. Microbiol. Infect. Dis. **49:**5–11.
26. **Yong, D., K. Lee, J. H. Yum, H. B. Shin, G. M. Rossolini, and Y. Chong.** 2002. Imipenem-EDTA disk method for differentiation of metallo-β-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter*. spp. J. Clin. Microbiol. **40:**3798–3801.