New and Sensitive Assay for Determining Pseudomonas aeruginosa Metallo-Beta-Lactamase Resistance to Imipenem†‡

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We present an imipenem lysate metallo-beta-lactamase (MBL) functional assay. This assay eliminates false-positive results due to the bactericidal effects of EDTA, can be performed with inexpensive reagents available in most laboratories, and is as accurate as the MBL Etest. It is appropriate for both high-accuracy screens and laboratories in developing countries with limited resources.

The widespread dissemination of metallo-beta-lactamase (MBL) resistance to carbapenem antibiotics, such as imipenem (IMP), among nonfermentative gram-negative pathogens has become a global concern (17). MBLs confer wide-spectrum resistance to all beta-lactams except for monobactams, and their catalytic activities are generally not inhibited by non-MBL inhibitors, such as clavulanic acid and tazobactam. Fortunately, MBLs are sensitive to metal chelators, such as EDTA and thiol-based compounds (1, 17), and these inhibitors serve as an important criterion for MBL functional screens. Currently, the most widely accepted standardized MBL functional screen is the MBL Etest (AB BioDisk, Solna, Sweden). However, due to the high cost and/or unavailability of the Etest strips, many clinical microbiology laboratories use alternative screening methods, such as the modified Hodge test, the double-disk synergy test (DDST or DDS), and the IMP-EDTA disk method (6, 7, 12, 15, 18). The modified Hodge test discriminates MBLs from other resistance mechanisms based on carbapenemase activity, but it does not confirm the metal dependence of the carbapenemase in the test strain and is often used for preliminary screens in combination with other methods (6, 7). The DDST and IMP-EDTA disk method diagnose metal dependence by using chelating agents and the standard disk agar diffusion susceptibility test (7, 12, 15, 18) but suffer from the potential issue of EDTA having a direct bactericidal effect on the test strain, which has the potential of confounding the test results, leading to a significant proportion of false-positive results (2).

We propose a new IMP-lysate MBL assay that provides a simple, inexpensive, and reproducible functional screen for MBL-producing Pseudomonas aeruginosa. The logic behind this assay rests upon the sensitivity of MBLs to the chelator EDTA. A zone of clearing or inhibition is created when an IMP-impregnated disk is placed on a lawn of sensitive bacteria. When the cell lysate from an IMP-resistant bacterial strain is spotted onto the IMP disk, the MBL activity of that strain effectively lowers the concentration of IMP diffusing from the disk into the agar, thereby reducing the diameter of the zone of inhibition. Mixing the IMP-resistant cell lysate with EDTA prior to spotting it on the IMP disk inhibits MBL activity and thereby restores the zone of inhibition to the diameter seen with the IMP disk alone. Since neither efflux pumps nor cell impermeability is influenced by EDTA, the addition of EDTA to these cell lysates will not change the zone of inhibition. Throughout this report, we use the notations “I” to indicate the IMP test disk, “L” to indicate the cell lysate, and “E” to indicate the addition of EDTA.

We tested 34 P. aeruginosa strains collected from clinical settings in North and South America and Europe. Multilocus sequence typing (P. W. Wang and D. S. Guttman, unpublished data) indicates that they span much of the diversity in the P. aeruginosa species complex. All of these strains were previously shown to be IMP resistant (see Table S1 in the supplemental material), and IMP resistance was again confirmed by disk diffusion assays with 10-μg IMP disks (BD Sensi-Disc; VWR, West Chester, AZ) on Mueller-Hinton II agar (Sigma, St. Louis, MO), following Clinical and Laboratory Standards Institute (CLSI) standards for performance and interpretation for disk diffusion susceptibility tests (10, 11). All tests were performed in duplicate (data not shown). To ensure that CLSI conditions were met, the diameters of the zones of inhibition of standard quality control strains (i.e., P. aeruginosa ATCC 27853 and Escherichia coli ATCC 25922) were also measured in parallel. While this assay can determine if strains are resistant to IMP, it is not capable of distinguishing the mechanism of IMP resistance, which can be due to the presence of an MBL, a serine-based carbapenemase gene such as blaOXA-eflux pumps, or impermeability of the cell.

We tested for MBL activity via the IMP-lysate MBL assay. Each P. aeruginosa strain was grown in 35 ml liquid culture to 2 × 10⁶ CFU overnight (37°C, 250 rpm) in Luria-Bertani (LB) broth (1% tryptone, 1% NaCl, and 0.5% yeast extract in distilled water). Cell lysates were prepared by centrifuging each culture for 10 min at 4,000 rpm, discarding the supernatant,
and resuspending the cells in 1 ml of 0.05 M sodium phosphate buffer (pH 7). The cells were then lysed by five repeated freeze-thaw cycles between /H11002 -20°C and room temperature (RT). Crude cell lysate was separated from solid cell debris and unlysed cells by centrifugation (10 min, RT, 3,200 /H11003 g), followed by filtration through a 0.22- /H9262 m syringe filter (Fisher Scientific, Pittsburgh, PA). The filtrate was collected in a YM30 Microcon device (Millipore, Bedford, MA) and concentrated by 8 min of centrifugation at 14,000 /H11003 g at RT. Twenty to thirty microliters of concentrated lysate was recovered each time. Nine microliters of the concentrated lysate was added to a sterile microcentrifuge tube containing 0.5 /H9262 l of 0.5 M EDTA (pH 8). Test plates were prepared by plating the indicator strain (E. coli ATCC 25922), grown overnight in LB broth to a 0.5 McFarland turbidity standard (1 × 108 CFU), onto 40-mm-thick Mueller-Hinton II agar plates by use of a sterile cotton swab following CLSI standards (10, 11). Two 10-μg IMP disks were placed onto the surface of the test plate, ~60 mm apart. Nine microliters of concentrated P. aeruginosa cell lysate was immediately added to one disk (IL), and 9.5 μl of the lysate-EDTA mixture was added to the other disk (ILE). A plain IMP disk (I) and a sterile 5-mm filter paper disk with 9.0 μl phosphate buffer and 0.5 μl EDTA (E) were used as controls on the same plate. The diameters of the inhibition zones were measured after 15 to 17 h of incubation at 37°C.

For comparison, we also performed MBL Etests following the manufacturer’s recommendations. All strains were tested by Etest except for STH_PA66, HUN_PA396, PA1006609A, PA1006609B, and PA105663, for which Etest results had been published previously (see Table S1 in the supplemental material). The IMP-sensitive strain E. coli ATCC 25922 was used as the indicator strain for the IMP-lysate MBL assay, serving as both the quality control and a reference for zone-of-inhibition diameter comparisons. This strain was consistently sensitive to the IMP-impregnated disks under our experimental conditions and displayed a very consistent zone of inhibition of 26.07 ± 1.77 (mean ± standard deviation [SD]) mm between subsequent runs.

We confirmed the IMP-lysate MBL assay by comparing the size of the zone of inhibition for the IMP disk alone (I) to those for IMP disks with lysates (IL) from the negative control strains PA1006609A and PA1006609B, which are IMP resistant via the action of an efflux pump and cell impermeability, respectively. Neither of these cell lysates influenced the diameter of the zone of inhibition (Fig. 1). We also confirmed that

FIG. 1. (A) IMP-lysate MBL assay. Mueller-Hinton II agar was inoculated with an even layer of IMP-susceptible indicator strain E. coli ATCC 25922. (Left) IMP disks spotted with P. aeruginosa concentrated cell lysates (IL) or cell lysates supplemented with 0.5 μl of 0.5 M EDTA (pH 8) (ILE) from two MBL-producing strains (PA555 [blaVIM1] and PA396 [blaVIM2]) (see Table S1 in the supplemental material). A significant decrease in the inhibition zone with the absence of EDTA indicates that the strain has an active MBL. (Center) Cell lysates (IL) from the negative control strains with IMP resistance through either efflux pumps (PA1006609A [Efflux]) or cellular impermeability (PA1006609B [Imperm]) (13) caused no reduction in inhibition zone. An IMP disk alone (I) provided a reference for inhibition zone diameter comparison, and filter paper impregnated with 0.5 μl of an EDTA and buffer mixture (E) showed no inhibition. (Right) MBL-producing strain with a distinct allele (PaVir02 [blaIMP]) and MBL-negative control strain (PHU149 [MBL -]). (B) Increase in zone of inhibition for the indicator strain E. coli ATCC 25922 when IMP disks were spotted with either cell lysates plus EDTA or cell lysates alone. Lysates from 34 strains of P. aeruginosa were tested, half of which were determined to be MBL positive and half of which were negative by the standard MBL Etest. The addition of EDTA to Etest-positive strains resulted in at least a 7-mm increase in the diameter of the zone of inhibition, while the addition of EDTA to Etest-negative strains had negligible effects. Plotted data are differences between the diameter measurements (mm) for IMP disks plus cell lysates plus EDTA (ILE) and IMP disks plus cell lysates (IL).
a paper disk carrying 0.5 μl of 0.5 M EDTA (pH 8) alone (E) was not potent enough to create any zone of inhibition with the indicator strain (Fig. 1A).

We then tested the lysates from the 34 IMP-resistant P. aeruginosa strains. The inhibition zones around the ILE disks significantly increased in size for 17 strains in response to the EDTA treatment (Fig. 1B). In all cases, Etest-positive strains exhibited an increase in the size of the inhibition zone of at least 7 mm (average = 15.11 mm), while the Etest-negative strains exhibited an increase of no more than 1 mm (average = 0.06 mm; P ≪ 0.0001 by two-tailed, homoscedastic, unpaired t test), indicating that the results from the IMP-lysate MBL assay are completely concordant with those of the Etests. The data from multiple trials indicate that the observation of a 4-mm increase in the zone of inhibition in the presence of EDTA, which splits the lower bound of the MBL-positive strains and the upper bound of the MBL-negative strains, is a reasonable cutoff for establishing if a strain is MBL positive by this assay. It is important to recognize that in all cases the addition of EDTA to the cell lysate (ILE) effectively restored the zone of inhibition back to the level observed with the IMP disk alone (I) by inhibiting the action of the MBL. The mean zone of inhibition for the IMP disk alone (I) for a single trial was 26.38 ± 2.05 (mean ± SD) mm, while the mean for the IMP disk plus cell lysate and EDTA (ILE) was 26.15 ± 1.99 (mean ± SD) mm (P = 0.63 for two-tailed, homoscedastic, unpaired t test).

In general, we had strong agreement between independent trials, with a Pearson correlation coefficient (r) of 0.892 (P < 0.0001) (see Fig. S1 in the supplemental material). While some strains showed substantial differences between independent assays, none of these differences were great enough to influence the call of MBL positive or negative. One possible explanation for inconsistency between assays may be variation in the cell lysate volumes following concentration using the Microcon concentration device, which led to different MBL concentrations. When filtration was not performed, unlysed P. aeruginosa cells always caused growth around the IMP disk (IL), making interpretation difficult. Also, when an MBL-carrying lysate was not concentrated via a Microcon concentration device, it failed to show positive carbapenemase activity against the IMP disk (data not shown). Therefore, we concluded that these two steps cannot be eliminated from the protocol.

As with most functional MBL detection methods, the IMP-lysate MBL assay relies on two main criteria, namely, carbapenemase activity and its metal dependence. EDTA sensitivity provides a robust and easily scored functional diagnosis that distinguishes MBLs from serine-based carbapenemases such as that encoded by blaOXA (7, 12, 15, 18). The DDST and IMP-EDTA disk method both rely on increased carbapenem inhibition in the test strain when EDTA is added to the agar medium or the disk. However, these methods require quantities of EDTA that may directly inhibit bacterial cell growth (7, 12, 18), thereby confounding MBL diagnosis. A major advantage of the IMP-lysate MBL test is that by allowing EDTA to interact with the carbapenemase in vitro, its concentration can be reduced to one that has a minimal effect on the indicator strain’s cell growth. Therefore, EDTA sensitivity of the test strain cell lysate becomes the single factor in inhibition zone diameter changes with the indicator strain, making the detection based on metal dependence much more reliable.

In conclusion, the IMP-lysate MBL assay is entirely consistent with the widely used MBL Etest. An increase in the inhibition zone of ≥4 mm in the presence of EDTA is a very robust predictor of the presence of an MBL. We propose that the IMP-lysate MBL assay is an inexpensive alternative that should be considered when the scale of a screen is moderate and when a high level of confidence is required. While this assay takes more time to perform than the Etest, it is as rapid as other IMP resistance assays, such as the modified Hodge test. Additionally, many assays can be performed simultaneously, thereby reducing the per-assay cost incurred for technical support. We propose that the IMP-lysate MBL assay may be particularly useful for clinical microbiology laboratories in developing countries, where highly sensitive MBL assays are required but access to expensive reagents is cost prohibitive or logistically challenging.

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