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Sensitive EDTA-Based Microbiological Assays for Detection of Metallo-β-Lactamases in Nonfermentative Gram-Negative Bacteria

Patricia Marchiaro,[1] María A. Mussi,[1] Viviana Ballerini,[3] Fernando Pasteran,[4] Alejandro M. Viale,[1] Alejandro J. Vila,[2] and Adriana S. Limansky[1]*

Departamento de Microbiología[1] and Departamento de Química Biológica,[2] Instituto de Biología Molecular y Celular de Rosario, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, and Centro de Especialidades Médicas Ambulatorias de Rosario, Departamento Bioquímico Municipal, Secretaría de Salud Pública, Municipalidad de Rosario,[3] 2000 Rosario, and Servicio Antimicrobianos, Departamento Bacteriología, Instituto Nacional de Enfermedades Infecciosas—ANLIS "Dr. Carlos G. Malbrán," Ciudad Autónoma de Buenos Aires,[4] Argentina

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The worldwide spread of metallo-β-lactamase (MBL)-producing gram-negative bacilli represents a great concern nowadays. Sensitive assays for their specific detection are increasingly demanded to aid infection control and to prevent their dissemination. We have developed a novel microbiological assay employing crude bacterial extracts, designated EDTA-imipenem microbiological assay (EIM), to identify MBLs in nonfermentative gram-negative clinical strains. We also evaluated the ability of EIM to detect MBLs in comparison to those of other currently employed screening methods, such as the EDTA disk synergy test (EDS) with imipenem as a substrate and the Etest method. The sensitivities of EIM and Etest were similar (1 versus 0.92, respectively) and much higher than that of EDS (0.67). Moreover, both EIM and Etest displayed the maximum specificity. Modifications were introduced to EDS, including the simultaneous testing of three different β-lactams (imipenem, meropenem, and ceftazidime) and two different EDTA concentrations. This resulted in a sensitivity improvement (0.92), albeit at a cost to its specificity. A simple strategy to accurately detect MBL producers is proposed; this strategy combines (i) an initial screening of the isolates by the extended EDS assay to select the potential candidates and (ii) confirmation of the true presence of MBL activity by EIM.

Bacterial resistance to carbapenems mediated by acquired carbapenemases represents an important problem worldwide (10, 15). These carbapenemases can be classified into three molecular classes according to the Ambler scheme (15): A (penicillinases), B (metallo-β-lactamases [MBLs]), and D (oxacillinases). The MBL variants of the types IMP, VIM, SPM-1, and GIM-1 are thus far some of the most clinically relevant, due to their ability to confer broad-spectrum β-lactam resistance, the unavailability of clinically useful inhibitors, and their potential for rapid and generalized dissemination (23). This worrying situation prompts an early recognition of MBL producers for infection control and prevention of their generalized spread, in particular among emerging gram-negative, glucose-nonfermentative pathogens such as *Pseudomonas* spp. and *Acinetobacter baumannii* (15, 21).

The catalytic mechanisms of MBLs and nonmetallo-β-lactamases are different (2). Therefore, different strategies are needed for the detection of pathogens bearing these enzymes in any attempt aimed at their control and eradication. An accurate identification of MBLs will therefore rely on the availability of specific, sensitive, and simple assays able to differentiate MBLs from other carbapenemases.

Although different phenotypic methods had been described, CLSI (formerly NCCLS) (along with other international committees) currently does not include standardized recommendations for MBL screening, since these are genotypic procedures mostly restricted to specialized laboratories (23). Here, we describe a relatively simple and inexpensive microbiological assay for the detection of MBL producers. The proposed procedure, designated EDTA-imipenem (IPM) microbiological assay (EIM), displayed a performance comparable to that of the Etest (22) and was more reliable than the EDTA disk synergy test (EDS) (8).

MATERIALS AND METHODS

Bacterial strains. All bacterial isolates were identified by conventional techniques (4) and by use of an API 20NE system (bioMérieux, Marcy l'Etoile, France).

The different MBL-producing bacterial strains employed in this work and their sources are described in detail in Table 1. This study also included 54 strains that did not produce MBLs (50 of *Pseudomonas aeruginosa* and 4 of *A. baumannii*) resistant to both IPM and meropenem (MEM), as determined by Kirby-Bauer methodology (14). These strains were collected between August 2002 and February 2005 from hospitalized patients in Hospital Centenario, a public institution affiliated to the National University of Rosario, Argentina. Also included in this study were *P. aeruginosa* 5200, a GES-2 serine-dependent carbapenemase-producing strain (16); the type strain *P. aeruginosa* ATCC 27853; and the *oprD*-deficient *P. aeruginosa* strain PASE1 (7).

* Corresponding author. Mailing address: Instituto de Biología Molecular y Celular de Rosario, Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina. Phone: 54-341-4350661. Fax: 54-341-4390465. E-mail: alimansky@infovia.com.ar.

TABLE 1. Carbapenemase producer strains used in this study

Microorganism	MBL	MIC (μg/ml) for IPM[a]	Reference or source
P. aeruginosa 5109[b]	VIM-11a	512	17
P. aeruginosa 5182[b]	VIM-11a	512	F. Pasteran, unpublished data
P. fluorescens 5092	VIM-2	512	F. Pasteran, unpublished data
P. aeruginosa COL-1	VIM-2	512	18
P. aeruginosa 105663	IMP-7	256	3
P. aeruginosa 4220	IMP-13	4	Clinical isolate, Malbrán collection
P. aeruginosa 48-1997	SPM-1	256	21
S. maltophilia 488	L1-like	512	Clinical isolate, laboratory collection
S. maltophilia 204	L1-like	256	Clinical isolate, laboratory collection
C. meningosepticum 202	GOB-18	32	Clinical isolate, laboratory collection[c]
C. indologenes	IND-like	256	Clinical isolate, laboratory collection
Bacillus cereus 569 H	BcII	512	5

[a] MIC value was determined by macrodilution broth, as described in reference 13.
[b] These strains also produce GES-1, OXA-2, and TEM-1.
[c] GenBank accession number DQ004496.

EIM microbiological assay. The procedure described here for the detection of MBL-producing bacteria was based on a modification of the disk assay originally developed by Masuda et al. (11). One of two procedures was used for bacterial disruption: sonic treatment or freeze-thawing. In the first, bacteria were grown overnight at 37°C in 20 ml of Luria-Bertani (LB) broth, harvested by centrifugation at 5,000 rpm for 10 min, washed once with 3 ml of 50 mM Tris-HCl (pH 8) at 4°C, and finally resuspended in 1 ml of the same buffer. Crude extracts were prepared by subjecting the cell suspension to seven 10-second bursts in a Vibra-Cell ultrasonic processor (Sonics & Materials, Inc.). The cell extracts were clarified by centrifugation at 15,000 rpm for 15 min at 4°C, and the presence of MBL activity was tested in the supernatants.

Alternatively, cell disruption was induced by freeze-thawing (20). In this case, the bacteria were carefully collected from the surface of a fresh overnight-grown Mueller-Hinton agar culture and transferred to a preweighed microcentrifuge tube until the equivalent of 100 mg of bacterial wet weight was obtained. The cells were then suspended in 1 ml of 50 mM Tris-HCl (pH 8) and collected by centrifugation at 5,000 rpm for 10 min. The pellet was frozen at −20°C for 15 min and thawed at 37°C for 10 min. After repeating this procedure 10 times, the obtained extract was centrifuged at 10,000 rpm for 10 min, and the supernatant was assayed as described below. This procedure produced results in the subsequent detection of MBL activity (see below) similar or identical to those of the bacterial sonic disruption described above.

For the detection of MBL activity in the extracts, a Mueller-Hinton agar plate (Difco) was inoculated with a liquid culture of an indicator strain, *Escherichia coli* ATCC 25922, adjusted to a turbidity of a 0.5 McFarland standard. A 10-μg IPM disk (BBL) was placed on top of the agar surface, and four filter disks were applied at its periphery within the expected zone of sensitivity (Fig. 1). One of the disks, designated S, received 20 μl of the crude extract obtained by either of the two procedures described above. A second disk (S/Zn) was loaded with the same volume of extract previously supplemented with 0.1 mM $ZnSO_4$ to stimulate the activity of any putative MBL present. This Zn^{2+} concentration was found optimal to improve enzyme detection (not shown). A third disk (S/E) also received the same volume of extract previously supplemented with 20 mM of EDTA (pH 8) to inhibit any putative MBL present. Finally, the fourth disk (B) was loaded with only 20 μl of 50 mM Tris-HCl (pH 8). The plates were incubated overnight at 37°C, and the growth patterns of the indicator *E. coli* strain were evaluated. The presence in the extract of any β-lactamase that could hydrolyze imipenem was revealed by the growth of the indicator *E. coli* cells around the S and S/Zn disks. In turn, MBLs are distinguished from nonmetalloenzymes due to the inhibition of the indicator strain around the chelator-containing S/E disk (Fig. 1).

Comparison of EIM with currently employed phenotypic procedures. The procedure proposed here was compared with two methods widely employed for the screening of MBL-producing bacteria: the EDTA disk synergy test (8) and the Etest MBL strip test (22).

The Etest MBL test was done according to the recommendations of the manufacturers (AB BIODISK, Solna, Sweden). A reduction of the MIC by three or more twofold dilutions in the presence of EDTA was interpreted as indicative of MBL production (22).

The EDS was conducted essentially as described previously (8). In short, an overnight LB liquid culture of the tested isolate was diluted to a turbidity of a 0.5 McFarland standard and spread on the surface of a Mueller-Hinton agar plate. A disk containing 10 μg of IPM was placed on the surface, and a second disk containing 10 μl of 0.5 M EDTA was placed 15 mm (edge-to-edge distance) apart from the first disk. After incubating overnight at 37°C, the presence of an expanded growth inhibition zone between the two disks was interpreted as positive for synergy.

Modifications introduced to increase the sensitivity of the EDS (Fig. 2) included the following: (i) the incorporation of MEM and ceftazidime (CAZ) to the original assay and (ii) a parallel test employing a disk with a lower EDTA concentration (10 μl of 0.1 M EDTA) placed 10 mm, edge to edge, from the other disk. The EDS that included both modifications was named the extended EDS (eEDS).

Detection of MBL and/or MBL-coding genes. The precise identification of MBL activity in crude extracts of the employed bacterial strains was done by a spectrophotometric assay using IPM as a substrate in media lacking or containing EDTA (12). In short, the cells were disrupted by ultrasonic treatment as described above, and carbapenemase activity was measured in the extracts at 30°C by following IPM hydrolysis at 300 nm in 10 mM HEPES (pH 7.5), 0.01 mM $ZnSO_4$, and 0.4 mM IPM. The presence of MBL activity was confirmed by EDTA inhibition by adding 10 mM EDTA to the crude extracts 10 min before performing the assay.

The presence of the genes $bla_{\text{VIM-like}}$, $bla_{\text{IMP-like}}$, and $bla_{\text{SPM-1}}$ (for both *Pseudomonas* spp. and *A. baumannii*) and $bla_{\text{L1-like}}$, $bla_{\text{IND-like}}$, and $bla_{\text{GOB-like}}$ (for *Stenotrophomonas maltophilia*, *Chryseobacterium indologenes*, and *Chryseobacterium meningosepticum*, respectively) was analyzed by PCR, employ-

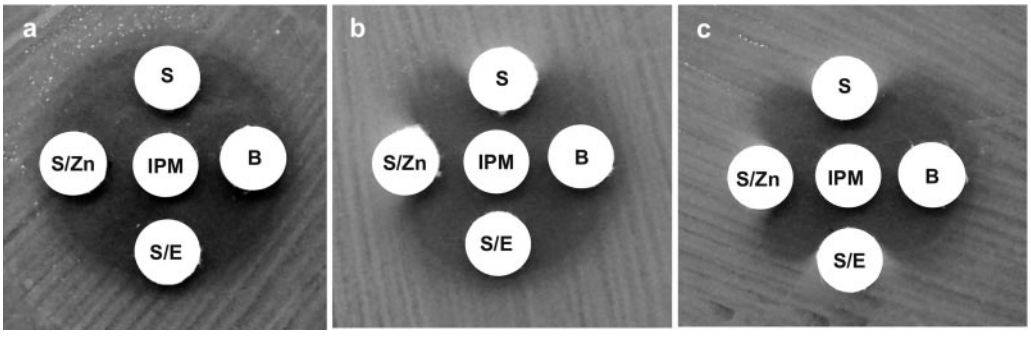

FIG. 1. Detection of MBLs in different *P. aeruginosa* strains by EIM. The presence of enzymes hydrolyzing IPM in bacterial extracts was tested by the growth of the *E. coli* indicator strain, as indicated in Materials and Methods. The different disks were as follows: S, containing bacterial extract; S/Zn, containing bacterial extract supplemented with $ZnSO_4$; S/E, containing bacterial extract supplemented with EDTA; B, containing buffer. (a) MBL-lacking *P. aeruginosa* ATCC 27853; (b) VIM-11a-producing *P. aeruginosa* 5109; (c) GES-2 (serine-dependent) carbapenemase-producing *P. aeruginosa* 5200.

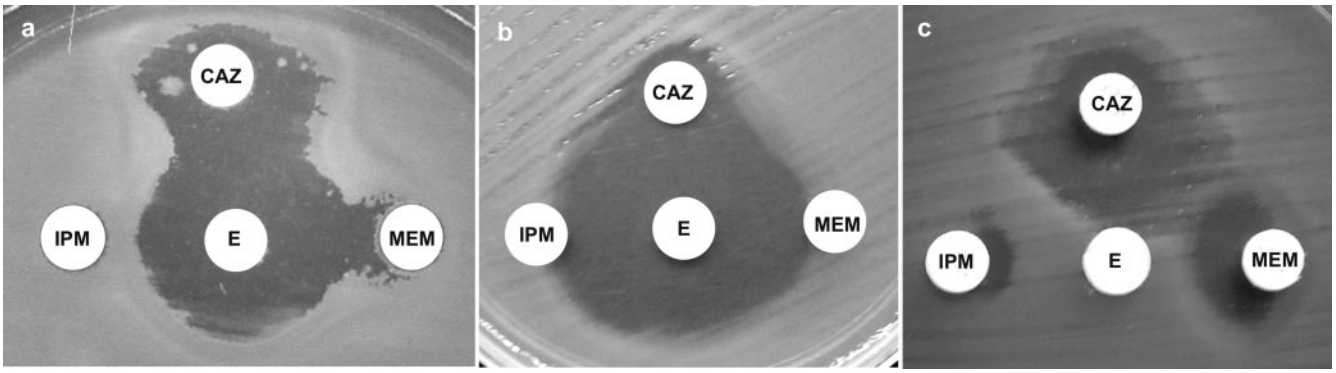

FIG. 2. The use of various β-lactam and EDTA concentrations increases EDS sensitivity. (a) The use of only IMP in the EDS may fail to detect MBL producers, as shown for this VIM-2-producing *P. fluorescens* strain. On the other hand, clear synergistic zones were observed when MEM and CAZ were incorporated to the assay. (b) The use of 10 μl of 0.5 M EDTA may result in some cases in undesirable large growth inhibition zones, making MBL detection difficult, as shown for this IND-producing *C. indologenes* strain. (c) The latter problem could be avoided by reducing the EDTA amounts employed. As shown in the figure, the use of 10 μl of 0.1 M EDTA resulted in clear synergy zones, unequivocally revealing MBL production by this strain.

ing the specific forward and reverse primers described in Table 2 on template DNA extracted by standard procedures (19). The reaction mixture contained 0.5 μM of each of the corresponding forward and reverse primers, 200 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Invitrogen, Life Technologies), 10 mM Tris-HCl (pH 8.4), and 50 mM KCl. The cycling protocol involved a 5-min denaturation at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C (or 55°C for bla_{L1} and bla_{GOB}), and 1 min at 72°C, followed finally by a 10-min incubation at 72°C.

The presence of the genes $bla_{IMP\text{-}like}$ or $bla_{VIM\text{-}like}$ was also tested in all *Pseudomonas* spp. and *A. baumannii* isolates by colony hybridization employing $bla_{IMP\text{-}7}$ (3) or $bla_{VIM\text{-}2}$ (18) probes labeled by [α-^{32}P]dATP (19).

Sensitivity, specificity, and predictive values. Calculations were done essentially following reference 6. The sensitivity is based on the ratio $a/(a + c)$, where a represents the number of strains that were correctly identified as MBL producers by the tested assay and c represents the number of true MBL producers incorrectly identified as nonproducing strains. The specificity is based on the ratio $d/(b + d)$, where d is the true number of strains not producing MBLs correctly identified by the tested assay and b is the number of strains that were incorrectly identified as MBL producers. The positive and negative predictive values are represented by $a/(a + b)$ and $d/(c + d)$, respectively.

RESULTS AND DISCUSSION

EIM assay. A microbiological assay was developed in order to detect metalloenzymes in clinical isolates with cellular extracts of the studied bacteria. The presence of carbapenemases in these extracts can be readily detected, and metalloenzymes can be distinguished from serine-carbapenemases by evaluating the effect of EDTA on the growth of a reference *E. coli* strain. The growth of the indicator *E. coli* cells around the bacterial extract with and without $ZnSO_4$ indicated the presence of imipenemase activity. Moreover, MBLs can be differentiated from nonmetalloenzymes by indicator strain growth inhibition around a bacterial extract supplemented with EDTA. Typical results observed of the proposed assay are shown in Fig. 1. The patterns generated by bacterial strains in which no imipenemase activity was spectrophotometrically detected are shown in Fig. 1a. A clear growth inhibition zone of the indicator *E. coli* strain is observed in these strains around the IPM disk. In these cases, the inhibitory zone embraces the S (bacterial extract), S/Zn (bacterial extract with $ZnSO_4$ added), and S/E (bacterial extract plus EDTA) disks as well as the control buffer (B) disk. The same result was observed when extracts from an *oprD*-deficient *P. aeruginosa* strain (PASE1 [7]) were used in the assay (not shown). In contrast, for a VIM-11a MBL producer strain (*P. aeruginosa* 5109; Table 1),

TABLE 2. Primers for PCR detection of MBL genes

Target gene	Primer pair (5′ to 3′)[a]	Expected PCR product (bp)	Source or reference
$bla_{VIM\text{-}like}$	VIMF ATTGGTCTATTTGACCGCGT VIMR CTACTCAACGACTGAGCGAT	778	This study
$bla_{IMP\text{-}like}$	IMP1F GAGCAAGTTATYTGTATTCT IMP1R ACCAGTTTTGCCTTACYATA	630	This study
	IMP2F TGTTTTATGTGTATGCTTCC IMP2R AGTTACTTGGCTGTGATGGT	708	This study
$bla_{SPM\text{-}I}$	SPMF GGCGCGTTTTGTTTGTTG SPMR CTACAGTCTCATTTCGCCAACG	770	This study
$bla_{L1\text{-}like}$	L1F ACCATGCGTTCTACCCTGCTCGCCTTCGCC L1R TCAGCGGGCCCCGGCCGTTTCCTTGGCCAG	880	This study
$bla_{GOB\text{-}like}$	GOBF GCTATGAGAAATTTTGCTACACTG GOBR TCATACTTATTTATCTTGGG	880	1
$bla_{IND\text{-}like}$	INDF GCVCAGGTHAAAGAYTTTGT INDR ACATGGCCDCCRCCTTTCCA	629	This study

[a] V: A, C, G; H: A, T, C; Y: C, T; D: A, T, G; R: A, G.

TABLE 3. Comparison of different methods for the identification of MBL presence in bacterial strains employed in this study

MBL present or assay characteristic[a]	Strain (no. of isolates tested in each case)	No. of isolates for which positive MBL activity was detected by or value for assay characteristic of:			
		EIM	Etest	EDS	eEDS
MBL					
VIM-like	*P. aeruginosa* (3)	3	3	3	3
	P. fluorescens (1)	1	1	0	1
IMP-like	*P. aeruginosa* (2)	2	1	2	2
SPM-1	*P. aeruginosa* (1)	1	1	0	0
L1-like	*S. maltophilia* (2)	2	2	0	2
GOB-like	*C. meningosepticum* (1)	1	1	1	1
IND-like	*C. indologenes* (1)	1	1	1	1
BcII	*B. cereus* (1)	1	1	1	1
Total	(12)	12	11	8	11
None	*P. aeruginosa* (50)	0	0	16	32
	P. aeruginosa ATCC 27853	0	0	0	0
	P. aeruginosa 5200	0	0	0	0
	A. baumannii (4)	0	0	1	1
Total	(56)	0	0	17	33
Assay characteristic					
Sensitivity		1.00	0.92	0.67	0.92
Specificity		1.00	1.00	0.70	0.41
Positive predicted value		1.00	1.00	0.32	0.25
Negative predicted value		1.00	0.98	0.91	0.96

[a] MBLs were determined by PCR analysis. The lack of MBLs was determined by both spectrophotometric and genotypic analyses. Assay characteristics are defined in Materials and Methods.

the inactivation of IPM is revealed by the growth of the indicator *E. coli* strain around the S and S/Zn disks (Fig. 1b). The absence of growth around the S/E disk indicated enzyme inhibition by EDTA and confirmed the presence of a metalloenzyme. Finally, Fig. 1c displays the results obtained with an imipenemase-producing, but not MBL-producing, *P. aeruginosa* clinical strain (*P. aeruginosa* 5200; see Materials and Methods). As in Fig. 1b, IPM hydrolysis was indicated by the growth of the indicator strain around both the S and S/Zn disks. In this case, however, growth is also observed around the S/E disk, revealing the lack of EDTA inhibition due to the presence of a nonmetalloenzyme (Fig. 1c).

Comparison of EIM with other phenotypic methodologies in the detection of different MBL types. The sensitivity of the above-proposed methodology for the identification of bacteria bearing VIM-like, IMP-like, SPM-1, L1-like, GOB-like, IND-like, or BcII was compared to those of the Etest and the original EDS (Table 3). As seen in Table 3, all producers of the above-indicated MBLs (12/12) were detected by the EIM procedure. The Etest showed a slightly lower sensitivity (0.92), identifying 11/12 strains as MBL producers. Remarkably, the strain not identified by the Etest was *P. aeruginosa* 4220, an IMP-13 producer showing a MIC of <4 μg/ml (Table 1). This reinforces the observation made by other authors (9, 23) concerning the poor sensitivity of this test for the detection of low-expression MBL producers.

The EDS showed the lowest sensitivity (0.67) among the analyzed procedures (Table 3). However, the incorporation of MEM and CAZ substantially increased the ability of this assay to detect MBL producers, as exemplified in Fig. 2a. As seen for a VIM-2-producing *Pseudomonas fluorescens* strain, although a clear result could not be observed in this case when IPM was tested, unambiguous EDTA synergistic inhibition zones were clearly generated for both MEM and CAZ.

A second modification aimed to increase the sensitivity of the EDS was simultaneous testing of two concentrations of the chelating agent (Fig. 2b and c). In some cases, the higher amounts of chelating agent employed resulted in bacterial growth inhibition zones, giving rise to confusing results (Fig. 2b). This undesirable effect could be mostly avoided by incorporating into the assay a second disk with a lower EDTA concentration (Fig. 2c). As seen in the figure, for an IND-producing *C. indologenes* strain, unequivocal synergistic inhibition zones were observed for the three β-lactams tested at the lowest EDTA concentration assayed (Fig. 2c).

The two modifications described above increased the sensitivity of the EDS from 0.67 to 0.92 (Table 3). However, as seen in Table 3, this also resulted in a lowered specificity level (from 0.70 to 0.41). On the contrary, both EIM and the Etest performed similarly in terms of specificity, being able to identify all tested strains not producing MBL (Table 3).

Concluding remarks. EIM, a convenient procedure for the detection of MBL producers among nonfermentative gram-negative bacteria of clinical origin, is presented here. Both specificity and sensitivity of EIM were either higher than or identical to those of phenotypic procedures currently in use and comparable to those of genotypic or enzyme assays considered as gold standards. The detection of MBL activity in isolates displaying very low MICs for IPM probably results from the use of cell extracts rather than intact cells, a situation

that avoids the presence of potential permeability barriers that may interfere with the access of the enzyme to the IPM substrate. Thus, the EIM represents an acceptable and inexpensive methodology for the diagnosis of MBL-producing species and for the control of the spread of the resistance.

Finally, the simplicity of an expanded-range EDS (see above) provides a convenient assay for the initial screening of potential MBL producers in the clinical setting. The combined use of the eEDS and EIM methods may result in an appealing low-cost approach to implement in clinical settings.

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