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Practical Agar-Based Disk Diffusion Tests Using Sulfamoyl Heteroarylcarboxylic Acids for Identification of Subclass B1 Metallo-β-Lactamase-Producing *Enterobacterales*

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ABSTRACT The worldwide distribution of carbapenemase-producing *Enterobacterales* (CPE) is a serious public health concern as they exhibit carbapenem resistance, thus limiting the choice of antimicrobials for treating CPE infections. Combination treatment with a β-lactam and one of the newly approved β-lactamase inhibitors, such as avibactam, relebactam, or vaborbactam, provides a valuable tool to cope with CPE; however, these inhibitors are active only against serine-type carbapenemases and not against metallo-β-lactamases (MβLs). Therefore, it is important to readily differentiate carbapenemases produced by CPE by using simple and reliable methods in order to choose an appropriate treatment. Here, we developed three practical agar-based disk diffusion tests (double-disk synergy test [DDST], disk potentiation test, and modified carbapenem inactivation method [mCIM]) to discriminate the production of subclass B1 MβLs, such as IMP-, NDM-, and VIM-type MβLs, from the other carbapenemases, especially serine-type carbapenemases. This was accomplished using B1 MβL-specific sulfamoyl heteroarylcarboxylic acid inhibitors, 2,5-dimethyl-4-sulfamoylfuran-3-carboxylic acid (SFC) and 2,5-diethyl-1-methyl-4-sulfamoylpyrrole-3-carboxylic acid (SPC), originally developed by us. The DDST and mCIM using SFC and SPC revealed high sensitivity (95.3%) and specificity (100%) in detecting B1 MβL-producing *Enterobacterales*. In the disk potentiation test, the sensitivities using SFC and SPC were 89.1% and 93.8%, respectively, whereas the specificities for both were 100%. These methods are simple and inexpensive and have a high accuracy rate. These methods would therefore be of immense assistance in the specific detection and discrimination of B1 MβL-producing *Enterobacterales* in clinical microbiology laboratories and would lead to better prevention against infection with such multidrug-resistant bacteria in clinical settings.

KEYWORDS carbapenemase-producing *Enterobacterales*, CPE, DDST, disk potentiation test, MβL, mCIM, subclass B1 metallo-β-lactamase, sulfamoyl heteroarylcarboxylic acid

Carbapenemase-producing *Enterobacterales* (CPE) are a serious public health concern because they show consistent carbapenem resistance, which limits the choice of antimicrobial agents in treating CPE infections (1). The production of carbapenemases, serine-type carbapenemases and/or metallo-β-lactamases (MβLs), is the prime cause of carbapenem resistance exhibited by CPE (2, 3). Detection of carbapenemase production is generally performed by using the CarbaNP test (4) and the modified carbapenem inactivation method (mCIM) (5) in clinical microbiology laboratories. Discrimination of carbapenemases produced by CPE is clinically important because some newly approved

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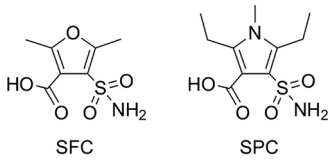

FIG 1 Chemical structures of 2,5-dimethyl-4-sulfamoylfuran-3-carboxylic acid (SFC) and 2,5-diethyl-1-methyl-4-sulfamoylpyrrole-3-carboxylic acid (SPC).

β-lactamase inhibitors, such as avibactam, relebactam, and vaborbactam, are effective only against serine-type carbapenemases (e.g., KPC) and not against MβLs (6).

To discriminate carbapenemase genes harbored by CPE, PCR detection using specific primers is the standard technique, but its availability is limited to highly advanced institutions, and this technology has not been employed in ordinary clinical laboratories with limited resources. To compensate for this limitation in carbapenemase gene discrimination, simple phenotypic detection methods using commercially available β-lactam disks in combination with potent carbapenemase inhibitors on agar plates have been developed. For example, Arakawa et al. first developed the double-disk synergy test (DDST) for detecting MβL-producing *Enterobacterales* and *Pseudomonas aeruginosa* using the MβL inhibitor mercaptoacetic acid (7). Doi et al. described a disk potentiation test to detect the production of KPC-type β-lactamase in *Enterobacterales* using 3-aminophenyl boronic acid (8). Furthermore, Yamada et al. (9) and Sfeir et al. (10) reported the use of the sodium mercaptoacetate mCIM (SMA-mCIM) and the EDTA-mCIM (eCIM), respectively, to detect MβL-producing *Enterobacterales*. Such agar-based detection techniques are practical tools for detecting CPE with high reliability in clinical microbiology laboratories and thus would be of immense help in managing CPE in clinical settings.

In a previous study, we developed potent MβL-specific inhibitors, sulfamoyl heteroarylcarboxylic acids (SHCs), such as 2,5-dimethyl-4-sulfamoylfuran-3-carboxylic acid (SFC) and 2,5-diethyl-1-methyl-4-sulfamoylpyrrole-3-carboxylic acid (SPC) (Fig. 1) (11). Among the subclass B1, B2, and B3 MβLs, SHCs specifically inhibited the activity of B1 MβL with low K_i values but did not inhibit B2 or B3 MβL or serine-type β-lactamase (SβL) activities. The specificities of SHCs toward B1 MβLs could be due to the binding of sulfamoyl and carboxyl groups in SHCs to the two zinc ions and one basic residue (Lys224 or Arg228) in the active site, which are common in B1 MβLs but are not conserved in B2 and B3 MβLs. In antimicrobial susceptibility testing based on microdilution methods, the addition of low concentrations of SHCs reduced MIC values of carbapenem for various B1 MβL-producing *Enterobacterales*, indicating that SHC inhibitors could be applicable for specifically detecting B1 MβL-producing bacteria. The aim of the current study was to develop simple and specific methods for detecting B1 MβL-producing *Enterobacterales* using feasible agar-based disk diffusion techniques in combination with SHCs.

MATERIALS AND METHODS

Bacterial strains. In this study, we used recombinant *Escherichia coli* clones producing various carbapenemases (11, 12). In addition, a total of 119 CPE, 65 MβL-producing *Enterobacterales* and 54 serine-carbapenemase-producing *Enterobacterales*, were obtained from Japanese clinical settings, the American Type Culture Collection, and the CDC and FDA Antibiotic Resistance (AR) Isolate Bank, respectively (see Table S1 in the supplemental material). Nine non-carbapenemase-producing carbapenem-resistant *Enterobacterales* (non-CP CRE) were also used for the assays (Table S1) (11, 12).

DDST. The strains for testing were adjusted to a turbidity of a 0.5 McFarland standard using Mueller-Hinton (MH) II broth (BD Biosciences, Sparks, MD, USA). Carbapenemase-producing *E. coli* DH5α clones were inoculated onto Luria-Bertani (LB) agar plates. *E. coli* BL21(DE3) clones were spread on LB agar plates containing 0.5 mM isopropyl β-D-1-thiogalactopyranoside. CPE and non-CP CRE strains were inoculated onto MH II agar plates (BD Biosciences). β-Lactam disks (meropenem [MPM] at 10 μg, ceftazidime [CAZ] at 30 μg, cephalothin at 30 μg, and piperacillin at 100 μg) were placed onto the agar plates. Next, a disk containing an MβL inhibitor was set close to one β-lactam disk (around 16 mm center to center) (Fig. S1) (7, 13). β-Lactam disks were purchased from Eiken Chemical (Tokyo, Japan) and BD

Biosciences. Disks containing MβL inhibitors were prepared as follows. Sodium mercaptoacetate (SMA) disks (3 mg/disk) were purchased from Eiken Chemical. SFC (Enamine, Kyiv, Ukraine) and SPC (synthesized by Sundia MediTech, Shanghai, China) were dissolved in dimethyl sulfoxide, and their concentration was adjusted to 100 mg/ml (Fig. 1). Thirty microliters of the SFC or SPC solution was spotted onto blank paper disks using micropipettes. Agar plates were incubated for 18 h at 35°C, and the shapes of the growth-inhibitory zones between β-lactam disks and inhibitor-containing disks were recorded.

Disk potentiation test. The strains to be tested were spread onto agar plates, as described above for the DDST. SMA, SFC, and SPC solutions were dropped to a final amount of 3 mg/disk onto MPM disks, which were set in advance on bacterial lawn plates. The plates were incubated for 18 h at 35°C, and the diameters of the growth-inhibitory zones were measured.

SHC-mCIM. The mCIM was conducted according to methods described in previous studies (5, 9, 10, 14). In brief, the strains for testing were inoculated into sterile plastic tubes containing 2 ml broth, and MPM disks were added to the tubes. Inhibitors (SMA, SFC, and SPC) were further added at 1.5 mg/ml and incubated for 4 h at 35°C. MH II agar plates, streaked with *E. coli* ATCC 25922, were prepared, and MPM disks removed from the culture tubes were placed onto the plates. The diameters of the growth-inhibitory zones around the disks were measured after incubation at 35°C for 18 h.

Susceptibility testing. Antimicrobial susceptibility testing based on the broth microdilution method was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (14).

Statistical analysis. Receiver operating characteristic (ROC) curves were drawn using JMP version 15 software (1989 to 2021; SAS Institute Inc., Cary, NC) for zone expansion values to determine the optimal cutoff values for disk potentiation tests.

RESULTS AND DISCUSSION

In our previous study, SFC and SPC clearly showed *in vitro* inhibitory activities against subclass B1 MβLs, IMP-1, NDM-1, and VIM-2, with K_i values in the 0.02 to 9.82 μM range, whereas inhibition of the activities of SFH-1 (B2), L1 (B3), and SMB-1 (B3) was not evident (12). We found that the sulfamoyl and carboxyl groups of SFC and SPC bind to the two central zinc ions and one positively charged amino acid, Lys224 or Arg228 (Fig. 1), which is generally conserved among B1 MβL architectures (12). These results indicated the possibility that the specific inhibitory activities of SFC and SPC against the subclass B1 MβLs, regardless of subtype, could be applied to develop methods for specifically detecting B1 MβL-producing bacteria.

Before evaluation of clinically isolated CPE, recombinant *E. coli* clones producing various carbapenemases (MβLs [subclasses B1, B2, and B3] and SβLs [KPC-2 and OXA-48]) were prepared and subjected to DDST using β-lactam disks in combination with SFC- or SPC-containing disks to evaluate the inhibitory behavior of SFC and SPC against live bacteria on agar plates. Compared with B1 MβL- and serine-type carbapenemase-producing *Enterobacterales*, B2 and B3 MβL-producing *Enterobacterales* are rarely isolated; we therefore had no choice but to use *E. coli* clones to confirm inhibitor specificities toward β-lactamases produced by live bacterial cells in this study. The results are shown in Fig. S1 and S2 in the supplemental material. The criteria for positive results were as follows: (i) an enlargement of 5 mm or larger of the growth-inhibitory zone around the β-lactam disk close to the inhibitor disk compared to that around the β-lactam disk only or (ii) an expanded distortion of growth-inhibitory zones between β-lactam- and inhibitor-containing disks. For IMP-, NDM-, VIM-, KHM-, SIM-, and TMB-producing *E. coli* clones, an enlargement of the growth-inhibitory zone was observed owing to the influence of inhibitors (Fig. S1). For DIM, GIM, and SPM producers, an expanded distortion of growth-inhibitory zones was observed (Fig. S1). On the contrary, for B2 MβL (SFH-1), B3 MβL (L1, SMB-1, and AIM-1), and SβL (KPC-2 and OXA-48) producers, responses corresponding to positive results were not observed (Fig. S2). For some B1 MβLs (e.g., NDM-1), the extent of expansion of the growth-inhibitory zone in the presence of SPC was greater than that in the presence of SFC (Fig. S1). This difference was attributed to the superior inhibitory activities of SPC compared to those of SFC, in the case of such MβLs (12). Nevertheless, both SFC and SPC exhibited specific inhibitory activities on B1 MβL-producing *E. coli* clones on agar plates. Therefore, the DDST using SFC and SPC could potentially be useful for discriminating clinical isolates of subclass B1 MβL-producing *Enterobacterales* in daily testing performed in ordinary clinical microbiology laboratories.

Examples of the DDST targeting *Enterobacterales* clinical isolates are presented in Fig. 2. The criteria for the DDST of *Enterobacterales* clinical isolates were the same as those for *E. coli* clones. An apparent expansion of growth-inhibitory zones between the MPM- and inhibitor-containing disks was observed for IMP-1- and NDM-1-producing *E.*

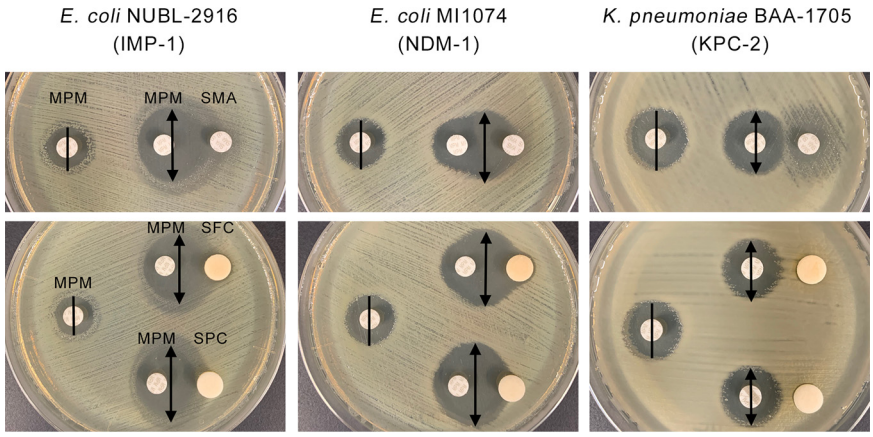

FIG 2 Double-disk synergy tests for IMP-1-producing *E. coli*, NDM-1-producing *E. coli*, and KPC-2-producing *K. pneumoniae*. SMA, sodium mercaptoacetate; SFC, 2,5-dimethyl-4-sulfamoylfuran-3-carboxylic acid; SPC, 2,5-diethyl-1-methyl-4-sulfamoylpyrrole-3-carboxylic acid. A difference of 5 mm or larger resulting from the subtraction of the diameter of the inhibition zone around the meropenem (MPM) disk (black line) from the diameter of the inhibition zone between the MPM and inhibitor disks (black lines with arrows) and an expanded distortion of the growth-inhibitory zone between the MPM and inhibitor disks (black triangles) indicate positive results. No or reduced changes in growth-inhibitory zones between the MPM and inhibitor disks indicate negative results.

coli, whereas it was not observed for KPC-2-producing *Klebsiella pneumoniae*. The results of the DDST are summarized in Table 1. SFC and SPC showed positive results for all IMP-type producers (21/21; 100%), NDM-type producers (34/34; 100%), VIM-type producers (5/5; 100%), and one NDM-VIM-type dual-MβL-producing *K. pneumoniae* isolate (1/1; 100%). In contrast, no positive response for 54 serine carbapenemase (KPC, GES, OXA, SME, and NMC-A) producers was observed in the presence of SFC or SPC (Table 1). One SMB-1 producer showed a positive result in the presence of SMA, whereas no change in zones was found in the presence of SFC and SPC; this was because SMA could behave as a potent inhibitor of B3 MβLs, including SMB-1, but SFC and SPC had no evident inhibitory activity against B3 MβLs (11, 15). Three *K. pneumoniae* isolates producing both NDM and OXA carbapenemases gave false-negative results despite producing NDM-1 (Table 1). We speculate that the production of OXA-type carbapenemase might mask the inhibitory effect on NDM-1 by SFC and SPC. This false-negative result is supported by the fact that the addition of 4 μg/ml of SFC or SPC reduced the MPM MIC values for three NDM and OXA dual-carbapenemase-producing *K.*

TABLE 1 Summary of the results of double-disk synergy test, disk potentiation test, and mCIM for CPE

			No. of isolates with a positive result (% of positive results)								
			DDST			Disk potentiation test			mCIM		
Class(es)	β-Lactamase(s)	No. of isolates	SMA	SFC	SPC	SMA	SFC	SPC	SMA	SFC	SPC
B1 MβL	IMP	21	21 (100)	21 (100)	21 (100)	15 (71.4)	21 (100)	21 (100)	21 (100)	21 (100)	21 (100)
	NDM	34	31 (91.2)	34 (100)	34 (100)	1 (2.9)	33 (97.1)	33 (97.1)	20 (58.8)	34 (100)	34 (100)
	VIM	5	5 (100)	5 (100)	5 (100)	0 (0)	2 (40)	5 (100)	1 (20)	5 (100)	5 (100)
	NDM + VIM	1	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)
B1 MβL + SβL	NDM + OXA	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
B3 MβL	SMB	1	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
SβL	KPC	26	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	GES	7	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	OXA	11	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	SME	8	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	NMC-A	2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

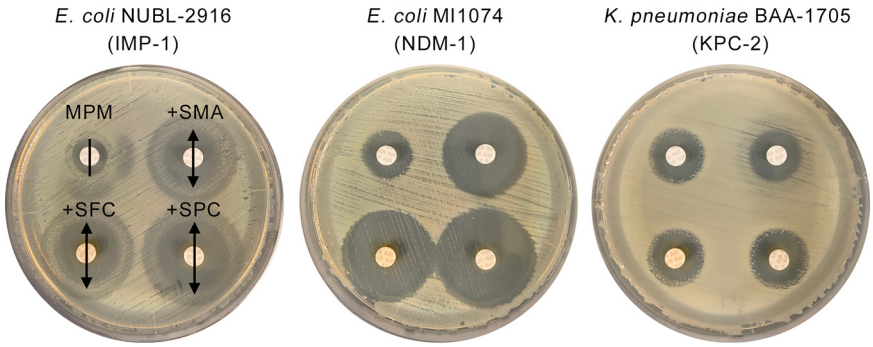

FIG 3 Disk potentiation tests for IMP-1-producing *E. coli*, NDM-1-producing *E. coli*, and KPC-2-producing *K. pneumoniae*. SMA, sodium mercaptoacetate; SFC, 2,5-dimethyl-4-sulfamoylfuran-3-carboxylic acid; SPC, 2,5-diethyl-1-methyl-4-sulfamoylpyrrole-3-carboxylic acid. A difference of 7 mm or larger resulting from the subtraction of the diameter of the inhibition zone around the meropenem (MPM) disk (black line) from the diameter of the inhibition zone around the disks with inhibitors (black lines with arrows) indicates a positive result, whereas a difference of less than 6 mm indicates a negative result.

pneumoniae isolates in antimicrobial susceptibility testing by no or a 2-fold reduction (Table S1). Overall, the DDST using SFC and SPC could detect B1 MβL-producing *Enterobacterales* with high sensitivity (61/64; 95.3% [95% confidence interval {CI}, 86.9% to 99.0%]) and specificity (55/55; 100% [95% CI, 93.5% to 100.0%]). Non-CP CRE showed negative results in all DDSTs (Table S1).

In Japanese clinical microbiology laboratories, the DDST, using CAZ and MPM disks in combination with commercially available SMA disks that specifically inhibit MβLs, is performed routinely to identify MβL-producing *Enterobacterales* (13, 16, 17). SMA shows superior *in vitro* inhibitory activity against IMP-1 (50% inhibitory concentration [IC_{50}], 0.24 μM) compared with its activity against NDM-1 (IC_{50}, >500 μM) (12). The low inhibitory activity of SMA toward NDM-1 leads to some false-negative results in the DDST, as shown in Table 1 (13). Because SPC, rather than SFC, shows comparable inhibition against NDM- and IMP-type MβLs, with low K_i values (11), the DDST using SPC is superior to that using SMA, in terms of sensitivity, for detecting *Enterobacterales* producing any of the B1 MβLs. Nevertheless, SMA has some merits that are not seen in the case of SFC and SPC: SMA is active against B3 MβL in addition to B1 MβL and is able to target not only *Enterobacterales* but also *P. aeruginosa* and *Acinetobacter* spp. (16, 17).

Next, SFC and SPC were used to develop a disk potentiation test. Examples of these tests are shown in Fig. 3. The addition of SMA, SFC, or SPC could expand the growth-inhibitory zone of B1 MβL-producing *E. coli*, which was not observed for KPC-2-producing *K. pneumoniae*. Although the toxicity of SFC or SPC alone against live bacterial cells is not fully understood, a maximum of a 4-mm expansion of the inhibitory zone around disks containing 3 mg SFC or SPC alone was observed for several strains. In addition, inhibitory zone expansions of up to 6 mm were observed for serine-type carbapenemase producers (Table S1). ROC curves were generated using the diameters of inhibitory zone expansion under the influence of SFC and SPC to estimate the optimal cutoff values, and it was found that those with a diameter of 7 or 8 mm had a good balance between sensitivity and specificity. Therefore, the tentative cutoff value for the disk potentiation test in this study was set at ≥7 mm. The results of disk potentiation tests are summarized in Table 1. The addition of 3 mg SMA led to the detection of only 16/64 (25.0% [95% CI, 15.0% to 37.4%]) B1 MβL-producing *Enterobacterales* because of the lack of sensitivity toward NDM-type and/or VIM-type MβL producers (1/43; 2.3%). In contrast, SPC, rather than SFC, showed very high sensitivity (60/64; 93.8% [95% CI, 84.8% to 98.3%]), with an inhibitory zone expansion of 8 to 27 mm and 100% specificity (55/55) (95% CI, 93.5% to 100.0%). Not surprisingly, non-CP CRE showed negative results in the disk potentiation tests using SFC and SPC (Table S1). Although the disk potentiation test, as well as the DDST, could detect *Enterobacterales* producing B1 MβL

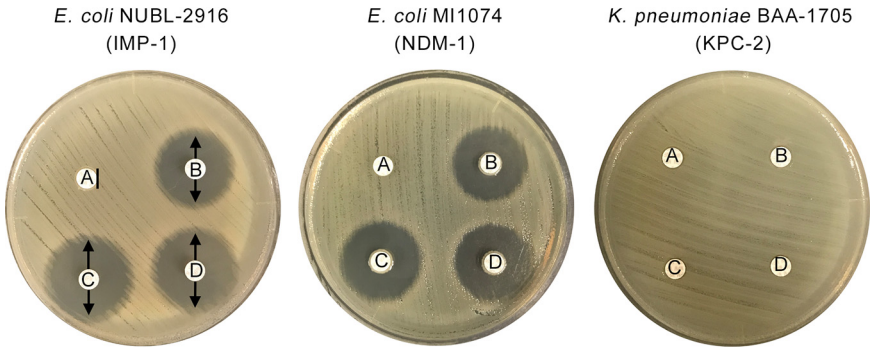

FIG 4 SHC-mCIM for IMP-1-producing *E. coli*, NDM-1-producing *E. coli*, and KPC-2-producing *K. pneumoniae*. (A) No inhibitor. (B) SMA at 1.5 mg/ml. (C) SFC at 1.5 mg/ml. (D) SPC at 1.5 mg/ml. SMA, sodium mercaptoacetate; SFC, 2,5-dimethyl-4-sulfamoylfuran-3-carboxylic acid; SPC, 2,5-diethyl-1-methyl-4-sulfamoylpyrrole-3-carboxylic acid. A difference of 5 mm or larger resulting from the subtraction of the diameter of the inhibition zone around the meropenem (MPM) disk (black line) (A) from the diameter of the inhibition zone around the disks with inhibitors (black lines with arrows) (B through D) indicates a positive result, whereas a difference of less than 5 mm indicates a negative result.

alone, with a carefully adjusted cutoff value (7 mm in this study), it is of concern to note that the toxicity due to high doses (3 mg) of SFC or SPC results in the expansion of the growth-inhibitory zone, thereby producing false-positive results with the disk potentiation test. This may be a limitation of this test, and the disk potentiation test using high doses of SFC and SPC (3 mg) would not be preferable for implementation in clinical microbiology laboratories when detecting B1 MβL producers despite showing high sensitivity with carefully adjusted cutoff values.

Finally, we evaluated the SHC-mCIM using SFC and SPC. Representative SHC-mCIM results are shown in Fig. 4. The addition of SMA, SFC, or SPC to culture tubes prevented MPM from hydrolyzing via B1 MβLs (IMP-1 and NDM-1) produced by *E. coli*, resulting in larger growth-inhibitory zones around MPM disks (Fig. 4, disks B to D) on agar plates than that around disk A. On the contrary, the addition of SMA, SFC, or SPC, as well as the condition without an inhibitor (disk A), did not inhibit KPC-2 activity, resulting in no formation of the inhibitory zone (disks B to D). The results of the SHC-mCIM are summarized in Table 1. Most of the CPE tested (112/119; 94.1%) gave positive results with the mCIM ($\leq$15 mm) according to CLSI guidelines (Table S1), whereas the results of one SMB producer and six GES producers were classified as intermediate or negative ($\geq$16 mm) (14). The false-negative results with the mCIM for GES producers are known to be partly due to the low catalytic activity of GES-type β-lactamase toward carbapenems (10, 18). The SHC-mCIM, using SFC or SPC, could clearly discriminate the production of B1 MβLs in CPE with high sensitivity (61/64; 95.3% [95% CI, 86.9% to 99.0%]) and specificity (55/55; 100% [95% CI, 93.5% to 100.0%]), with 5-mm cutoff values, which are the same as those of other mCIMs (9, 10), whereas the SMA-mCIM, as well as the DDST and disk potentiation test, failed to detect some NDM and VIM producers (Table 1). As expected, non-CP CRE gave negative results in all the mCIMs (Table S1).

A variety of mCIMs for detecting carbapenemases have been developed (9, 10, 19–21), among which some are considered gold-standard methods (10, 14). The EDTA-mCIM and SMA-mCIM could detect MβL production but could not classify the subclasses of MβLs. On the contrary, the SHC-mCIM developed in this study could specifically identify B1 MβLs. Therefore, the SHC-mCIM, together with the EDTA-mCIM and SMA-mCIM, made it possible to roughly classify MβLs produced by *Enterobacterales* in the clinical microbiology field, although these methods are still laborious for use in clinical laboratory settings.

A limitation of the DDST, disk potentiation test, and SHC-mCIM developed in this study is the inability of these tests to discriminate the production of both MβL and serine-type carbapenemases in a single bacterial strain (Table 1). The same limitation was

TABLE 2 Interpretation of the double-disk synergy test, disk potentiation test, and SHC-mCIM results

Test method	Criterion	Interpretation
DDST	Difference of 5 mm or larger resulting from subtraction of the diam of the inhibition zone around the β-lactam disk from the diam of the inhibition zone between the β-lactam and inhibitor disks	Positive
	Expanded distortion of growth-inhibitory zones between the inhibitor and β-lactam disks compared with those around the β-lactam disks alone	
	No or narrowed distortion of growth-inhibitory zones between the inhibitor and β-lactam disks compared with those around the β-lactam disks alone	Negative
Disk potentiation test	≥7-mm zone expansion around the MPM/inhibitor disk compared with the MPM disk zone size	Positive
	≤6-mm zone expansion around the MPM/inhibitor disk compared with the MPM disk zone size	Negative
SHC-mCIM	≥5-mm expansion around the MPM disk from inhibitor-containing tubes compared with that without an inhibitor	Positive
	≤4-mm expansion around the MPM disk from inhibitor-containing tubes compared with that without an inhibitor	Negative

pointed out in a previous study (10) and may be a problem for our methods in association with the increase of such MβL and serine-type dual-carbapenemase producers, although the prevalence of such bacteria is still below 3% among meropenem-nonsusceptible isolates collected as part of a global surveillance program (22). Generally, the classification of dually produced β-lactamases can be managed by applying specific inhibitors. For example, dually produced, extended-spectrum β-lactamase and AmpC β-lactamase could be discriminated using specific inhibitors such as clavulanic acid and 3-aminophenyl boronic acid (or cloxacillin), respectively (16, 23). Therefore, to discriminate dually produced NDM and OXA carbapenemases in a single strain, as shown in Table 1, the development of a specific OXA-type carbapenemase inhibitor, in addition to an MβL inhibitor, is desired.

Another issue with our methods that needs to be investigated is their versatility: whether they can be used for non-*Enterobacterales*, such as *Pseudomonas* spp. and *Acinetobacter* spp. The *in vitro* inhibitory activities of SHCs, based on susceptibility tests, against *Acinetobacter* spp. were almost the same as those against *Enterobacterales*, whereas they were lower against *P. aeruginosa* (11). Our methods could therefore cover *Acinetobacter* spp., although there may be specific issues with glucose-nonfermentative Gram-negative bacteria in the disk diffusion tests, such as the methodology of carbapenemase extraction in the mCIM, which was previously reported (19, 24). In addition, considering the lower inhibitory activity of SHCs against *P. aeruginosa*, adjustments like attaining high doses of inhibitors in each test are necessary when targeting this organism. Nonetheless, it would be worthwhile to evaluate the versatility of our methods against *P. aeruginosa* and *Acinetobacter* spp., and we will do this in the near future.

In conclusion, we developed and evaluated three agar-based disk diffusion test methods, the DDST, disk potentiation test, and SHC-mCIM, for detecting B1 MβL-producing *Enterobacterales*. Based on the results of our evaluation, the interpretation of the results of the three tests is summarized in Table 2. Although many disk-based techniques have been developed to identify carbapenemase production (5, 7, 8, 25), to the best of our knowledge, our methods are the first practical disk-based methods that can specifically discriminate B1 MβLs from a variety of carbapenemases, without depending on nucleic acid analyses requiring expensive reagents. However, our methods have one disadvantage: they are time-consuming compared to molecular methods.

In the present study, the evaluation of B2 and B3 MβL-producing *Enterobacterales* could not be fully performed because they are rarely found in clinical settings (26–28). Nevertheless, our techniques make it possible to discriminate the major B1 MβLs, the IMP, NDM, and VIM types, from the dominant serine-type carbapenemases (KPC and OXA) in *Enterobacterales*. All three tests using SHCs developed in the present study showed high sensitivity and specificity for detecting B1 MβL producers. However, the

DDST and SHC-mCIM, rather than the disk potentiation test, are preferred because the possibility of false-positive results could not be excluded due to the toxicity of SHCs at high doses, which provide a measurable expansion of growth-inhibitory zones, in the disk potentiation test. Nevertheless, our methods, especially the DDST and SHC-mCIM, will be useful to detect B1 MβL producers in clinical microbiology laboratories as they are simple, highly reliable, and inexpensive. More importantly, the results obtained from such methods can subsequently be used to regulate antimicrobial-resistant bacteria in clinical settings.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 9.2 MB.

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