Evaluation of the modified sodium mercaptoacetic acid double disk synergy test for detecting the metallo-β-lactamase producing Enterobacteriaceae

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Background: Sodium mercaptoacetic acid double disk synergy test (SMA-DDSTs) is frequently used in Japan to detect metallo-β-lactamase (MBL) producer easily. In this study, we evaluated SMA-DDST at the points of the antimicrobial disk, and the distance between SMA and an antimicrobial disk.

Methods: Forty-one clinical isolates ceftazidime (CAZ)-resistant Enterobacteriaceae and 12 reference strains were tested. Those isolates included 17, 19, 4 and 13 of only IMP-, IMP with other classes of β-lactamases-, New Delhi MBL with other classes of β-lactamases- and non-MBL-producers, respectively. SMA-DDSTs were performed with CAZ, imipenem (IPM) and meropenem (MEPM)-containing disks with the distance between SMA and antimicrobial disk at 11 mm or 16 mm (center-to-center).

Results: The sensitivities were 27/40 (68%), 17/40 (43%), 35/40 (88%), 29/40 (73%), 25/40 (63%), and 39/40 (98%) for 16 mm CAZ-SMA-DDST, 16 mm IPM-SMA-DDST, 16 mm MEPM-SMA-DDST, 11 mm CAZ-SMA-DDST, 11 mm IPM-SMA-DDST, and 11 mm MEPM-SMA-DDST, respectively. CAZ and MEPM disk showed the highest sensitivity for only MBL-producing isolates and MBL with coproducing other classes of β-lactamases-producing isolates, respectively. Among any of antimicrobials used in this study, 11 mm SMA-DDSTs showed higher sensitivity than those 16 mm. The specificity was 100% for all SMA-DDSTs.

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However, the ambiguous false-expansion of growth inhibition zone was induced in 11 mm CAZ-SMA-DDST for non-MBL-producing isolates.

Conclusion: This study suggested that the combination of 16 mm CAZ-SMA-DDST and 11 mm MEPM-SMA-DDST is suitable for screening MBL-producing isolates.

Introduction

In recent years, an increasing number of carbapenemase producers, which hydrolyze and are resistant to carbapenem antimicrobials, a last resort for the treatment of infections, have been of great concern in the world[1)]. In Japan, metallo-β-lactamase (MBL) producers, especially IMP MBL producers, are most frequently detected[2)]. Tests for MBL producers include genetic testing such as polymerase chain reaction (PCR) assay and loop-mediated isothermal amplification assay, phenotyping based on inhibition of MBL activity by EDTA, thiol compounds, etc., and double disk synergy test (DDST)[3)].

In Japan, sodium mercaptoacetic acid (SMA)-containing disks for DDSTs (SMA-DDSTs), which are commercially available and require no special device or operation, are widely used in clinical laboratory testing because of their high economic efficiency and convenience. According to the package inserts of metallo-β-lactamase SMA Eiken (SMA Eiken)(SMA 3 mg/disk, Eiken Chemical, Tokyo, Japan), the test can perform at a center-to-center distance of 15 to 20 mm between the SMA Eiken and ceftazidime (CAZ) or imipenem (IPM) disks with CAZ-resistant bacteria as target.

More recently, SMA-DDST were reported to show false-negative results for NDM and VIM MBL producers, leading to further validation of optimal conditions for SMA-DDST[4, 5)]. At present, Japan Nosocomial Infection Surveillance and some studies have reported the modified SMA-DDST that detection sensitivity can be enhanced[6, 7, 8, 9)].

The main changes compared to original were at using meropenem (MEPM) disk together with CAZ disk, and a shorter center-to-center distance of 11 mm between the SMA and antimicrobial disks. As for specificity, to the best of our knowledge, including the above-mentioned report, no false-positive case has been reported regardless of the distance between the disks and an antimicrobial. However, we occasionally had observed ambiguous expansion of growth inhibition zone in modified SMA-DDST for non-MBL producers. Thus, we believed that a reevaluation of the SMA-DDST is essential. Therefore, this study aimed to determine the best condition for the detection of MBL-producing Enterobacteriaceae among SMA-DDSTs.

Materials and Methods

Strains used and antimicrobial susceptibility testing

The test collection included 53 well-characterized Enterobacteriaceae isolates meeting the specified in the package inserts of SMA Eiken (i.e., CAZ-resistant). Forty-one of them were collected from 10 hospitals in Aichi prefecture Japan, during 2011 to 2016, while the other 12 were ATCC or NCTC reference strains. Identification of isolates was performed using MALDI Biotyper (Bruker Daltonics). The β-lactamase productions were confirmed by previously published PCR analysis methods[10, 11, 12]. The minimum inhibitory concentration (MIC) was measured based on CLSI criteria by broth microdilution method[13] (Table 1).

SMA-DDSTs

SMA-DDSTs were performed as follows: After each test strain adjusted to McFarland standard No. 0.5 was applied with a sterile cotton swab to the overall surface of Mueller Hinton agar (Becton, Dickinson and Company, Tokyo, Japan) in three directions and the surface became dry, metallo-β-lactamase SMA Eiken (SMA 3 mg/disk, Eiken Chemical, Tokyo, Japan) and antimicrobial disks (CAZ 30 μg/disk or IPM 10 μg/disk or MEPM 10 μg/disk) (Eiken Chemical, Tokyo, Japan) were placed as shown in Fig. 1. The center-to-center distance between SMA and antimicrobial disk was kept at 16 mm (16 mm SMA-DDST) or 11 mm (11 mm SMA-DDST). Differences in inhibition zones were measured after an 18-h incubation at 35°C. When the no inhibition zone was observed around an antimicrobial disk, the inhibition zone diameter was measured as 6 mm that was the disk diameter. According to the package insert for SMA Eiken, the test strain was considered MBL-positive when the clear growth inhibition zone of the antimicrobial disk placed close to the SMA was at least 5 mm greater than that of the antimicrobial disk alone.

Inter-facility validation using *K. pneumoniae* ATCC 700603

At 4 facilities (A, B, C, and D) preserving *Klebsiella pneumoniae* ATCC 700603 (SHV-18 producer) in Aichi prefecture, 15 clinical microbiology technicians (a, b, c, d, e, f, g, h, i, j, k, l, m, n, and o) who had performed SMA-DDSTs in daily practice were asked to carry out CAZ-SMA-DDSTs at inter-disk distances of 11 and 16 mm (center-to-center), blinded to the origin and drug susceptibility of the test bacteria, and inhibition ring measurement and MBL production assessment were individually investigated.

Results

SMA-DDSTs

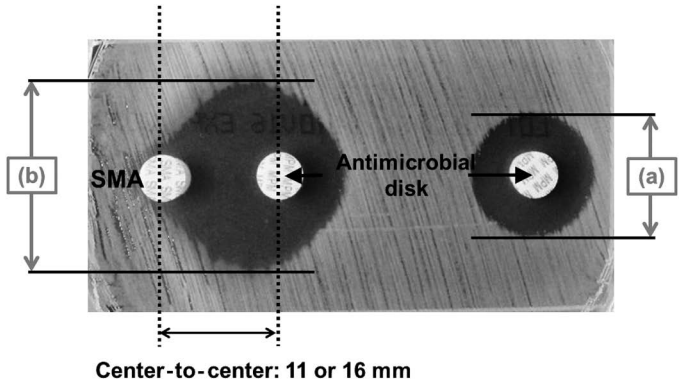
Table 2 shows the performance of SMA-DDSTs. The sensitivities were 29/40 (73%), 25/40

Table 1. Distribution of antimicrobial MICs for the metallo-*β*-lactamase (MBL)-producing and non-producing isolates in this study

Species (no.)	PCR-confirmed β-lactamase type				MIC (μg/mL) and no. of isolates									
	Carbapenemase type	ESBL		AmpC	CAZ		IPM				MEPM			
		SHV/TEM	CTX-M-group		16	≥32	≤1	2	4	≥8	≤1	2	4	≥8
Escherichia coli (1) [a]	IMP					1		1				1		
Klebsiella oxytoca (7)	IMP				2	5	5	2			2	2	1	2
Klebsiella pneumoniae (9)	IMP					9	6	1		2		2	4	3
Enterobacter cloacae (3)	IMP			EBC-group		3	3					3		
Klebsiella pneumoniae (1)	IMP			DHA-group		1	1					1		
Escherichia coli (4)	IMP		CTX-M-2			4	2	1	1				1	3
Klebsiella oxytoca (1)	IMP		CTX-M-2			1	1							1
Klebsiella pneumoniae (2)	IMP		CTX-M-1			2	2					2		
Klebsiella pneumoniae (8)	IMP		CTX-M-2			8	5	2	1				2	6
Klebsiella pneumoniae (1)	NDM		CTX-M-1			1				1				1
Escherichia coli (1)	NDM		CTX-M-1			1				1				1
Citrobacter braakii (1)	NDM			DHA-group		1				1				1
Klebsiella pneumoniae (1) [b]	NDM		CTX-M-1	CMY-2		1				1				1
Escherichia coli (1) [c]		SHV-4				1	1				1			
Klebsiella oxytoca (1) [d]		SHV-5				1	1				1			
Klebsiella pneumoniae (1) [e]		SHV-18				1	1				1			
Escherichia coli (1) [f]		TEM-3				1	1				1			
Escherichia coli (1) [g]		TEM-26				1	1				1			
Klebsiella pneumoniae (1) [h]		TEM-12/10				1	1				1			
Escherichia coli (1)			CTX-M-1			1	1				1			
Escherichia coli (2)			CTX-M-9			2	2				2			
Klebsiella pneumoniae (2) [i]	KPC					2			1	1				2
Klebsiella pneumoniae (1) [j]				DHA-1		1	1				1			
Escherichia coli (1) [k]			CTX-M-9	CMY-2		1	1				1			

(a); NCTC 13476, (b); ATCC BAA-2146, (c); ATCC BAA-200, (d); ATCC 51983, (e); ATCC 700603, (f); ATCC BAA-201, (g); ATCC BAA-198, (h); ATCC 51503, (i); ATCC BAA-1705 and BAA-1900, (j); ATCC BAA-1144, (k); ATCC BAA-2355.

Fig. 1. Disk placement position and interpretation criteria of sodium mercaptoacetic acid (SMA)-double disk synergy test in this study



The test strain was considered as metallo-β-lactamase positive when (b) – (a) ≥ 5 mm.

Table 2. The performance of sodium mercaptoacetic acid (SMA)-double disk synergy test (DDST) with the center-to-center distance between SMA and antimicrobial disk at 16 mm or 11 mm

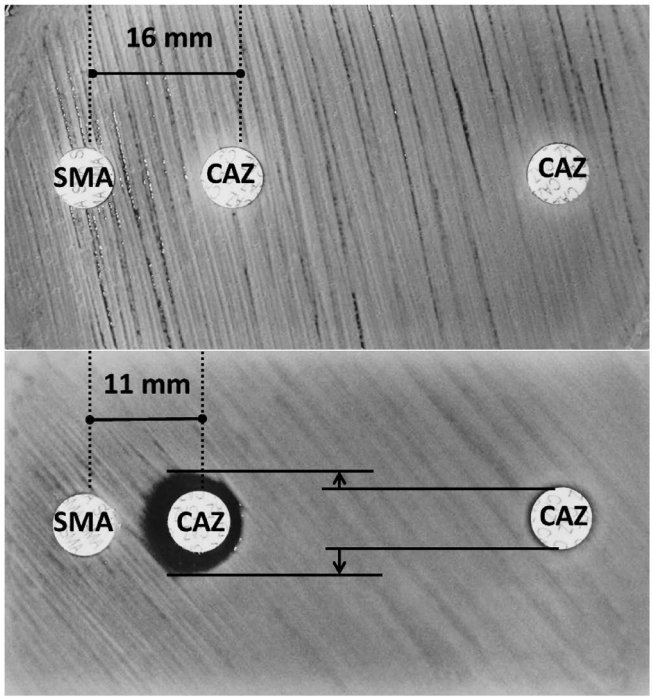
PCR-confirmed β-lactamase type (no.)	No. (%) of positive result by DDSTs with:					
	CAZ-SMA		IPM-SMA		MEPM-SMA	
	11 mm	16 mm	11 mm	16 mm	11 mm	16 mm
MBL (40)	29 (73)	27 (68)	25 (63)	17 (43)	**39 (98)**	35 (88)
IMP-type (17)	**17 (100)**	**17 (100)**	14 (82)	13 (76)	16 (94)	16 (94)
IMP-type, EBC-group (3)	2 (67)	2 (67)	1 (33)	1 (33)	**3 (100)**	**3 (100)**
IMP-type, DHA-group (1)	0	0	**1 (100)**	0	**1 (100)**	**1 (100)**
IMP-type, CTX-M-type (15)	10 (67)	8 (53)	7 (47)	2 (13)	**15 (100)**	**15 (100)**
NDM-type, DHA-group (1)	0	0	0	0	**1 (100)**	0
NDM-type, CTX-M-type (2)	0	0	1 (50)	0	**2 (100)**	0
NDM-type, CTX-M-type, CIT-group (1)	0	0	**1 (100)**	**1 (100)**	**1 (100)**	0
Non-MBL (13)	0	0	0	0	0	0

The boldface with underline indicates the highest sensitivity in each β-lactamase types.
MBL indicates metallo-β-lactamase.

(63%), 39/40 (98%), 27/40 (68%), 17/40 (43%), and 35/40 (88%) for 11 mm CAZ-SMA-DDST, 11 mm IPM-SMA-DDST, 11 mm MEPM-SMA-DDST, 16 mm CAZ-SMA-DDST, 16 mm IPM-SMA-DDST, and 16 mm MEPM-SMA-DDST, respectively. CAZ-SMA-DDSTs detected only IMP-producing isolates with high sensitivity, but the tests were inadequate for the isolates that coproducing other classes of β-lactamases (Table 2 and Fig. 2). An IMP-producing *Klebsiella*

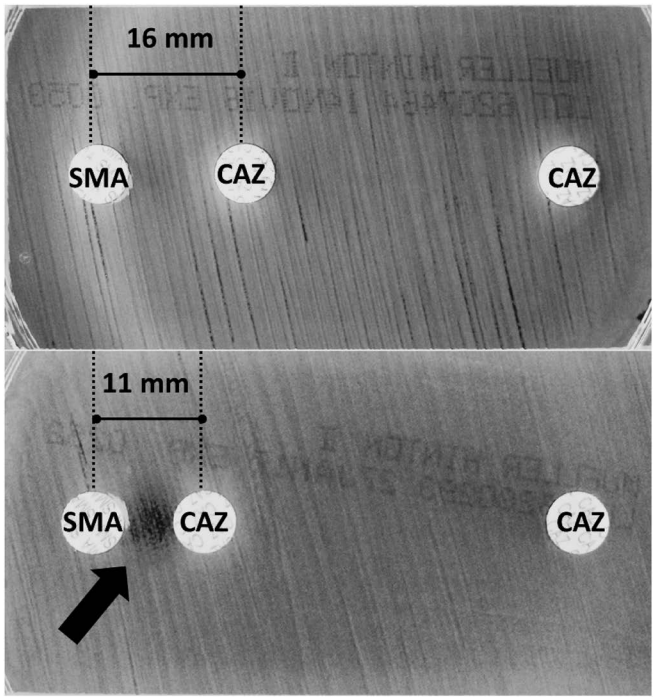
Fig. 2. Unclear expansion of inhibition zone that includes both false negative and non-specific were observed in ceftazidime (CAZ)-sodium mercaptoacetic acid (SMA)-double disk synergy test

(a)



Klebsiella pneumoniae producing IMP with CTX-M (clinical isolate)

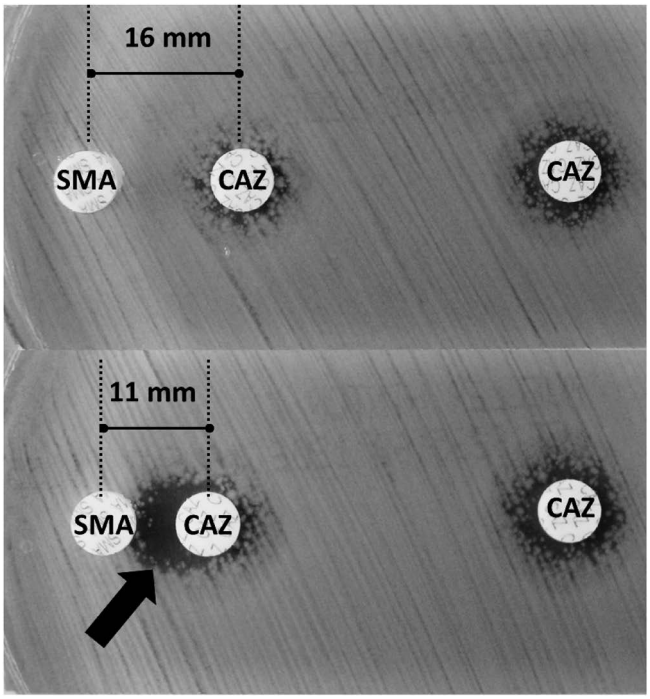
(b)



Citrobacter braakii producing NDM with CTX-M (clinical isolate)

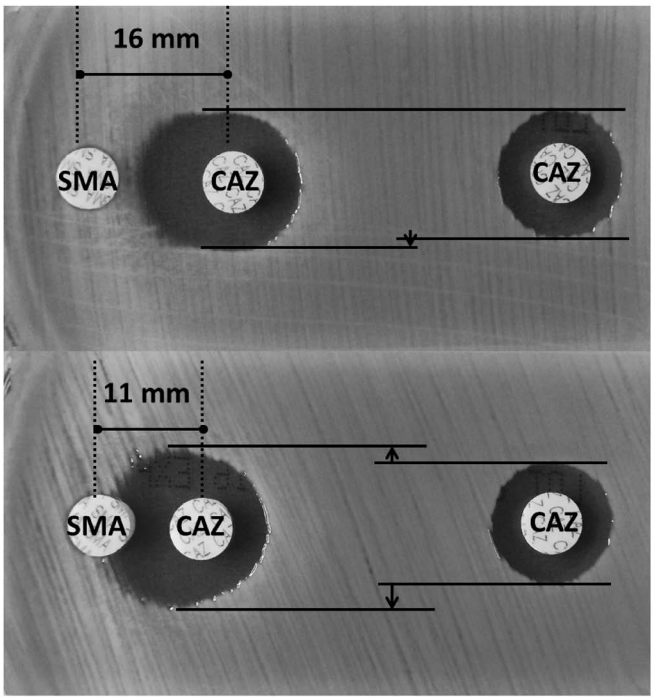
Fig. 2. Continued

(c)



Escherichia coli producing SHV-4 (ATCC BAA-200)

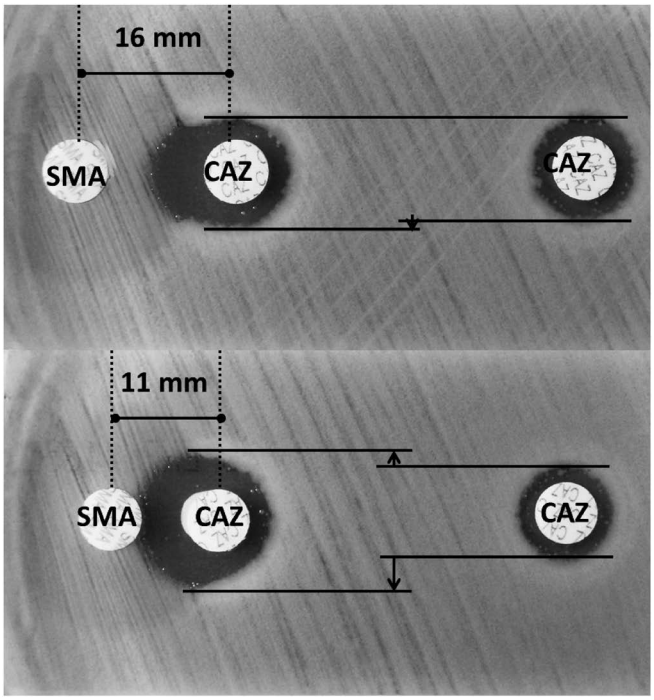
(d)



K. pneumoniae producing SHV-18 (ATCC 700603)

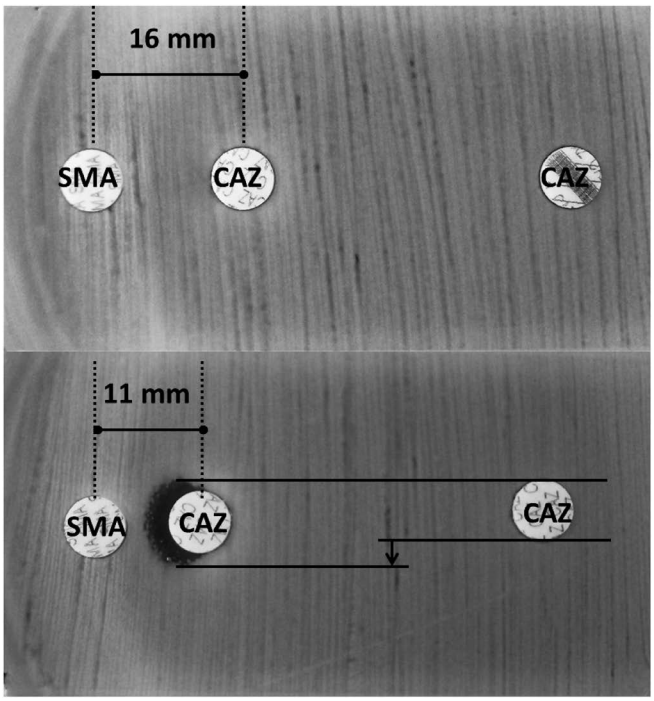
Fig. 2. Continued

(e)



K. pneumoniae producing KPC-2 (ATCC BAA-1705)

(f)



K. pneumoniae producing KPC-3 (ATCC BAA-1900).

Expansion of inhibition zone by the effect of SMA is indicated arrows.

oxytoca that showed low-level carbapenem MIC (both IPM and MEPM MIC; ≤1 μg/mL) was detected only CAZ-SMA-DDSTs. The 16 mm MEPM-SMA-DDST detected IMP-producing isolates with high sensitivity, but the test was inadequate for NDM-producing isolates. The 11 mm MEPM-SMA-DDST improved this point and showed the best sensitivity. Among any of antimicrobials used in this study, 11 mm SMA-DDSTs showed higher sensitivity than those 16 mm. The specificity was 100% for all SMA-DDST. However, ambiguous expansion of growth inhibition zone was observed for *Escherichia coli* ATCC BAA-200 (SHV-4 producer), *K. pneumoniae* ATCC 700603 (SHV-18 producer), *K. pneumoniae* ATCC BAA-1705 (KPC-2 producer) and *K. pneumoniae* ATCC BAA-1900 (KPC-3 producer) in 11 mm CAZ-SMA-DDST (Fig. 2).

Inter-facility validation using *K. pneumoniae* ATCC 700603

Measurement results are shown in **Table 3**. At a CAZ-SMA distance of 16 mm, expansion of growth inhibition zone ranged from 0 to 2 mm with a mean of 0.6 mm, showing MBL-negative results for all operators. At a CAZ-SMA distance of 11 mm, on the other hand, expansion of growth inhibition zone ranged from 1 to 6 mm with a mean of 2.9 mm, showing a false MBL-positive result for one operator (g).

Discussion

In this study, we reevaluated SMA-DDST at the points of the antimicrobial disk, and the distance between SMA and an antimicrobial disk.

At first, the antimicrobial disk, our result demonstrated that CAZ and MEPM disk showed the highest sensitivity for only MBL-producing isolates and coproducing other classes of β-lactamases, respectively. Recently in Japan, IMP-6-producing isolates has increased[2]. They are mostly acquired high activity against MEPM but not at IPM, and coproducing extended-spectrum β-lactamases (ESBLs)[14]. This fact suggested that CAZ- and IPM-SMA-DDST reduce their performance, and MEPM-SMA-DDST increase. Presently, the package insert of metallo-β-lactamase SMA Eiken recommends that use with CAZ or IPM disk. However, we strongly considered that using MEPM disk in SMA-DDST is necessary.

Second, the distance between SMA and an antimicrobial disk, among any of antimicrobials used in this study, 11 mm SMA-DDSTs showed higher sensitivity than those 16 mm. These results were supports the findings of a previous evaluation[8, 9]. Furthermore, our result obtained as a new knowledge that the ambiguous false-expansion of growth inhibition zone was induced in 11 mm CAZ-SMA-DDST for SHV- or KPC-producing isolates. As stated above, CAZ-SMA-DDST was suitable for only IMP-producing isolates, and in particular for IMP-producing isolates that often showed low-level carbapenem resistance. In our results, both 11 mm and 16 mm CAZ-SMA-DDSTs showed 100% sensitivity for only IMP-producing isolates. Therefore, we considered that

Table 3. Results of CAZ-SMA-DDSTs for *Klebsiella pneumoniae* ATCC 700603 among 15 operators in 4 facilities

Facility	Operator	inhibitory zone diameter and expantion (mm)			Result of CAZ-SMA-DDST
		CAZ	CAZ-SMA DDST		
			distance between two disks		
			11 mm	16 mm	
A	a	13	+2	+1	-
	b	15	+3	0	-
	c	13	+4	+1	-
	d	13	+2	0	-
	e	13	+2	0	-
	f	13	+3	0	-
	g	**12**	**+6**	+1	+
B	h	13	+4	0	-
C	i	10	+3	+2	-
	j	11	+4	0	-
	k	12	+2	+2	-
	l	11	+4	+2	-
	m	14	+1	0	-
D	n	13	+1	0	-
	o	12	+3	0	-
Range		10 - 15	1 - 6	0 - 2	
Average		12.5	2.9	0.6	

CAZ-SMA-DDSTs not necessary to shorten the distance between SMA and CAZ.

In conclusion, our result demonstrated that the combination of 16 mm CAZ-SMA-DDST and 11 mm MEPM-SMA-DDST is suitable for screening MBL-producing isolates. One limitation of this study is that the MBL-producing isolates except IMP or NDM have not been studied. Thus, our result may not be applicable to MBL-producing isolates from other types than IMP or NDM. However, we strongly believed that this method was useful in IMP-endemic areas such as our country, Japan.

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Conflict of interest

Hiroshige Mikamo received Research funding from Sumitomo Dainippon Pharma Co., Ltd., Taisho Toyama Pharmaceutical Co., Ltd., Daiichi Sankyo Co., Ltd., Pfizer Co., Ltd., Astellas Pharma Inc., MSD K.K., Toyama Chemical Co., Ltd., Takeda Pharmaceutical Co., Ltd., Meiji Seika Pharma Co., Ltd., Shionogi & Co., Ltd., Boehringer Ingelheim Co., Ltd., Taisho Pharmaceutical Co., Ltd., KYORIN Pharmaceutical Co., Ltd., Bayer Yakuhin Ltd.; Consulting fee/honorarium from Sumitomo Dainippon Pharma Co., Ltd., Taisho Toyama Pharmaceutical Co., Ltd., Daiichi Sankyo Co., Ltd., Pfizer Co., Ltd., MSD K.K., Astellas Pharma Inc., Meiji Seika Pharma Co., Ltd., MIYARISAN Pharmaceutical Co., Ltd.; Leadership position/Advisory role from Toyama Chemical Co., Ltd. The other authors declare that they have no conflict of interest.

References

1) Nordmann P, Naas T, Poirel L: Global spread of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis. 2011; 17: 1791–8.
2) Yano H, Ogawa M, Endo S, *et al.*: High frequency of IMP-6 among clinical isolates of metallo-*β*-lactamase-producing *Escherichia coli* in Japan. Antimicrob Agents Chemother. 2012; 56: 4554–5.
3) Arakawa Y, Shibata N, Shibayama K, *et al.*: Convenient test for screening metallo-*β*-lactamase-producing Gram-negative bacteria by using thiol compounds. J Clin Microbiol. 2000; 38: 40–3.
4) Miki K, Takegawa H, Etoh M, *et al.*: First outbreak report of VIM-1 metallo-*β*-lactamase producing *Pseudomonas aeruginosa* in Japan. J Jpn Assoc Infect Dis. 2010; 84: 721–6.
5) Ishii Y: Characteristics of New Delhi metallo-*β*-lactamase (NDM-1), a novel carbapenem resistance factor. Jpn J Chemother. 2010; 58: 639–43.
6) Japan Nosocomial Infection Surveillance: http://www.nih-janis.jp/material/material/NDM-1%20SMA20101007.pdf (accessed 31 January 2017).
7) Japan Nosocomial Infection Surveillance: http://www.nih-janis.jp/material/material/modified%20SMA-disk%20method.pdf (accessed 31 January 2017).
8) Hattori T, Kawamura K, Arakawa Y: Comparison of test methods for detecting metallo-*β*-lactamase-producing Gram-negative bacteria. Jpn J Infect Dis. 2013; 66: 512–8.
9) Kitanaka H, Sasano M, Yokoyama S, *et al.*: Invasive infection caused by carbapenem-resistant *Acinetobacter soli*, Japan. Emerg Infect Dis. 2014; 20: 1574.
10) Monstein HJ, Östholm-Balkhed Å, Nilsson MV, *et al.*: Multiplex PCR amplification assay for the detection of *bla*SHV, *bla*TEM and *bla*CTX-M genes in Enterobacteriaceae. Acta Pathol Microbiol Immunol Scand. 2007; 115: 1400–8.
11) Pérez-Pérez FJ, Hanson ND: Detection of plasmid-mediated AmpC *β*-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol. 2002; 40: 2153–62.
12) Sakanashi D, Miyazaki N, Suzuki T, *et al.*: Screening multiplex PCR for six important plasmid-mediated carbapenemase genes. Jpn J Med Technol. 2015; 64: 460–7.
13) Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Twenty-fourth informational supplement. CLSI: 2014; M100–S24.
14) Yano H, Kuga A, Okamoto R, *et al.*: Plasmid-encoded metallo-*β*-lactamase (IMP-6) conferring resistance to carbapenems, especially meropenem. Antimicrob Agents Chemother. 2001; 45: 1343–8.