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Original Article

A novel disk-based detection method with superior sensitivity for β-lactamase production in third-generation cephalosporin-resistant *Enterobacteriaceae*☆

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ABSTRACT

Objective: Current phenotypic methods for extended-spectrum β-lactamase (ESBL), AmpC β-lactamase (AmpC), and carbapenemases fail to detect isolates that co-produce other classes of β-lactamases. In this study, we have developed a novel assay (Applied Multiplex Disk Method: AMU-DM) for the phenotypic detection and identification of β-lactamases produced by *Enterobacteriaceae*.

Methods: We evaluated the performance of the method by comparison with PCR results for 78 *Enterobacteriaceae* clinical isolates that were positive by the ESBL screening test and negative by the ESBL confirmation test. Additionally, one NCTC strain and four ATCC strains were also included in the test population for the study as reference.

Results: For 79/83 (95%) isolates tested, the AMU-DM results matched those obtained by PCR. The concordance rates were 31/31 (100%), 11/11 (100%), 3/3 (100%), 0/1 (0%), 15/15 (100%), 16/19 (84%), and 3/3 (100%) for AmpC, ESBL and AmpC co-production, *Klebsiella pneumoniae* carbapenemase (KPC), KPC and ESBL co-production, metallo β-lactamase (MBL), MBL and ESBL co-production, and MBL and AmpC co-production, respectively.

Conclusion: The AMU-DM is convenient to perform, economical, and highly sensitive in identifying ESBLs, AmpCs, and carbapenemases. Our method may be useful in clinical settings for the implementation of relevant infection control measures and for surveillance purposes.

1. Introduction

Rising β-lactam resistance in *Enterobacteriaceae* is rapidly evolving into a major medical crisis [1]. Both the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have lowered the minimum inhibitory concentration (MIC) breakpoints for broad-spectrum cephalosporins and carbapenems, thereby removing the requirement of routine β-lactamase confirmation-testing before reporting results [2,3]. However, these tests are still important for adopting relevant infection control measures and for surveillance purposes.

Automated antimicrobial susceptibility testing instruments used in Japan primarily follow CLSI guidelines. Hence, CLSI-recommended extended-spectrum β-lactamase (ESBL) screening and confirmation tests are commonly used for identifying ESBL among *Enterobacteriaceae* in the laboratory. However, the confirmatory test for phenotypic detection does not identify all ESBLs because some organisms containing β-lactamases that are capable of masking ESBL production in the phenotypic test give false-negative results [4]. Such β-lactamases include AmpC β-lactamases (AmpCs) and carbapenemases such as metallo-β-lactamase (MBL) or *Klebsiella pneumoniae* carbapenemase (KPC). CLSI has described tests for suspected carbapenemase production, such as the Carba NP or the modified carbapenem inactivation method

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(mCIM) [2]; however, tests that specifically detect the production of different types of carbapenemases are lacking. Furthermore, no methods for screening and phenotypic confirmatory testing of AmpCs have been recommended by CLSI.

Despite the improvement in the performance of PCR-based detection techniques, these techniques are not widely applied in clinical laboratory testing due to cost and labor considerations [5,6]. Although less prevalent than ESBL, the increase in AmpC or carbapenemase production has shown an alarming trend worldwide [1]. Therefore, we believe that the development of an efficient and reliable phenotypic test for detecting various β-lactamases is a major challenge. In this study, we developed and evaluated a novel phenotypic test (Applied Multiplex Disk Method: AMU-DM) for identification of various β-lactamases produced by third-generation cephalosporin-resistant *Enterobacteriaceae* tested negative in the ESBL confirmation test.

2. Materials and methods

2.1. Bacterial strains

A total of 83 *Enterobacteriaceae* were tested. The test population included 78 *Enterobacteriaceae* clinical isolates (40 non-CPE and 38 CPE), and five reference strains from NCTC or ATCC. The clinical isolates included *Escherichia coli* (n = 37), *Klebsiella pneumoniae* (n = 31), and Klebsiella oxytoca (n = 10) collected from 10 acute care hospitals in Aichi between 2008 and 2017 that were positive by CLSI-ESBL screening test and negative by CLSI-ESBL confirmation test [2]. Isolates were identified using a MALDI Biotyper microbial identification system (Bruker Daltonics, Billerica, MA, USA). Phenotypic characterization, CLSI-ESBL screening, CLSI-ESBL confirmation, and carbapenem-resistant *Enterobacteriaceae* (CRE) identification (imipenem and/or meropenem non-susceptibility) were performed by broth microdilution based on CLSI criteria, and the mCIM test was also performed by following CLSI procedures [2]. In this study, the carbapenemase-gene was detected by multiplexed PCR for all isolates that were positive by mCIM.

Molecular characterization by multiplexed PCR-based screening of β-lactamase-encoding genes for ESBL (bla_{SHV}, bla_{TEM}, and bla_{CTX-M}), plasmid-mediated AmpC (bla_{ACC}, bla_{CIT}, bla_{DHA}, bla_{EBC} bla_{FOX}, and bla_{MOX}), and carbapenemase (bla_{IMP}, bla_{VIM}, bla_{NDM} bla_{SPM}, bla_{AIM}, bla_{DIM}, bla_{GIM}, bla_{SIM}, bla_{KPC}, bla_{BIC}, and bla_{OXA-48}) was performed as described previously [7–9]. The CTX-M gene group was confirmed when bla_{CTX-M} was detected by PCR [10]. For bla_{SHV}, bla_{TEM}, bla_{DHA}, bla_{CMY}, bla_{IMP}, bla_{NDM} and bla_{KPC}, specific PCR and direct sequencing were performed following previously described

Table 1
Bacterial strains.

PCR confirmed strain group (no.)	Species (no.)	No. Of CRE	Source (no.)
non-CPE clinical isolates (40)		**1**	
AmpC (29)			
bla_{CMY-2} (15)	*E. coli* (15)	1	urine (11), blood (2), bile (1), stool (1)
bla_{DHA-1} (14)	*E. coli* (8)	0	urine (7), pus (1)
	K. pneumoniae (5)	0	sputum (2), urine (2), bile (1)
	K. oxytoca (1)	0	urine (1)
ESBL and AmpC (11)			
bla_{CMY-2} + bla_{DHA-1} (1)	*E. coli* (1)	0	genital secretion (1)
bla_{SHV-12} + bla_{DHA-1} (2)	*K. pneumoniae* (2)	0	blood (1), stool (1)
$bla_{CTX-M-1-family}$ + bla_{DHA-1} (3)	*E. coli* (3)	0	urine (2), blood (1)
$bla_{CTX-M-1-family}$ + bla_{CMY-2} (1)	*E. coli* (1)	0	urine (1)
$bla_{CTX-M-9-family}$ + bla_{DHA-1} (2)	*E. coli* (1)	0	urine (1)
	K. pneumoniae (1)	0	stool (1)
$bla_{CTX-M-9-family}$ + bla_{CMY-2} (2)	*E. coli* (2)	0	urine (2)
CPE (38)		**35**	
KPC (1)			
bla_{KPC-2} (1)	*K. pneumoniae* (1)	1	unknown (1)
KPC and ESBL (1)			
bla_{KPC-2} + bla_{SHV-12} + $bla_{CTX-M-1-family}$ (1)	*K. pneumoniae* (1)	1	pharyngeal swab (1)
MBL (15)			
$bla_{IMP-unsequenced}$ (1)	*E. coli* (1)	1	NCTC 13476 (1)
bla_{IMP-1} (14)	*K. pneumoniae* (7)	6	sputum (3), urine (2), blood (1), unknown (1)
	K. oxytoca (7)	5	urine (3), bile (1), blood (1), stool (1), sputum (1)
MBL and ESBL (19)			
bla_{IMP-1} + $bla_{CTX-M-1-family}$ (1)	K. oxytoca (1)	1	urine (1)
bla_{IMP-1} + $bla_{CTX-M-9-family}$ (1)	*K. pneumoniae* (1)	1	sputum (1)
bla_{IMP-1} + $bla_{CTX-M-2-family}$ (15)	*E. coli* (5)	5	urine (2), blood (1), genital secretion (1), stool (1)
	K. pneumoniae (9)	9	urine (3), stool (2), sputum (3), central vein catheter (1)
	K. oxytoca (1)	1	sputum (1)
bla_{NDM-1} + bla_{SHV-28} + $bla_{TEM-104}$ + $bla_{CTX-M-1-family}$ (1)	*K. pneumoniae* (1)	1	blood (1)
bla_{NDM-5} + $bla_{TEM-104}$ + $bla_{CTX-M-1-family}$ (1)	*E. coli* (1)	1	rectal swab (1)
MBL and AmpC (3)			
bla_{IMP-1} +bla_{DHA-1} (3)	*K. pneumoniae* (3)	3	sputum (2), urine (1)
Reference strains (5)			
AmpC (1)			
bla_{DHA-1} (1)	*K. pneumoniae* (1)	N/A	ATCC BAA-1144
ESBL and AmpC (1)			
$bla_{CTX-M-9-family}$ + bla_{CMY-2} (1)	*E. coli* (1)	N/A	ATCC BAA-2355
KPC (2)			
bla_{KPC-2} (1)	*K. pneumoniae* (1)	N/A	ATCC BAA-1705
bla_{KPC-3} (1)	*K. pneumoniae* (1)	N/A	ATCC BAA-1900
MBL (1)			
$bla_{IMP-unsequenced}$ (1)	*E. coli* (1)	N/A	NCTC 13476

Abbreviations: CPE: carbapenemase-producing *Enterobacteriaceae*, CRE: carbapenem-resistant *Enterobacteriaceae*, ESBL: extended-spectrum-β-lactamase, AmpC: AmpC-β-lactamase, KPC: *Klebsiella pneumoniae* carbapenemase, MBL: metallo-β-lactamase, N/A: not applicable.

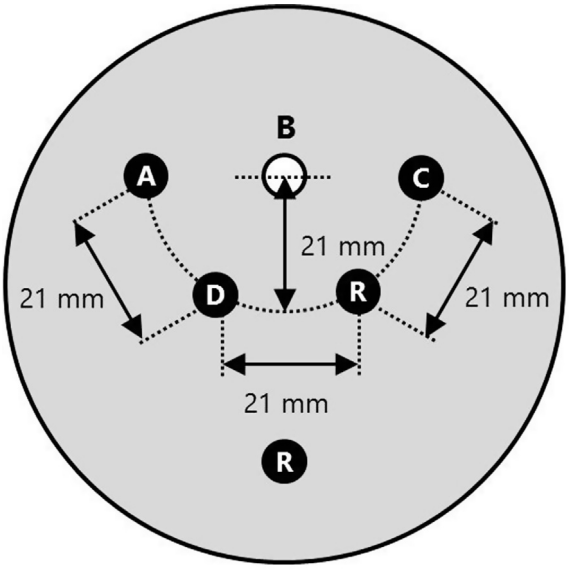

Fig. 1. Disk placement position for the Applied Multiplex Disk Method. (R): cefpodoxime (CPDX) 10 μg, (A): CPDX/cloxacillin (CLX) 750 μg, (B): sodium mercaptoacetic acid 3 mg, (C): CPDX/phenylboronic acid 600 μg, (D): CPDX/clavulanic acid 10 μg.

methods [11–13]. Direct sequencing was performed using ABI BigDye Terminator v3.1 Cycle Sequencing Kits and an ABI 3730xl sequencer (Applied Biosystems, Foster City, CA, USA). Additionally, one NCTC strain and four ATCC strains were also included in the test population for the study as reference strains. The profiles of β-lactamase production by five reference strains were cited based on reports published by ATCC (The Global BioSource Center) and Public Health England (Table 1).

2.2. Preparation of combination disks for AMU-DM

A cefpodoxime (CPDX) (10 μg) disk (Eiken, Tokyo, Japan) was supplemented with 10 μL of the following inhibitors: 60 mg/mL phenylboronic acid (PBA) (Sigma Aldrich, Tokyo, Japan) as a KPC and AmpC inhibitor and 75 mg/mL cloxacillin (CLX) (Sigma Aldrich) as an AmpC inhibitor. Hence, the final amounts of the β-lactamase inhibitor on the disks were 600 μg of PBA and 750 μg of CLX. PBA and CLX were dissolved in dimethyl sulfoxide (Sigma Aldrich) and sterile water, respectively. The amount of the inhibitors was decided based on previously reported values [14]. A CPDX/CVA disk (Eiken) containing CPDX with clavulanic acid (CVA) (10 μg) and an SMA disk (Eiken) containing sodium mercaptoacetic acid (SMA) (3 mg) were also used to inhibit ESBL and MBL, respectively. Additionally, an ethylenediaminetetraacetic acid (EDTA) disk, a BD BBL Taxo blank discs (Becton-Dickinson, Tokyo, Japan) supplemented with 10 μL of the 0.5 M EDTA (Sigma Aldrich) (i.e., 1460 μg/disk), was prepared as an alternative MBL inhibitor [15].

2.3. AMU-DM procedure

The turbidity of each test strain was adjusted to the McFarland standard No. 0.5. The suspensions were then spread on the surface of Muller Hinton II agar plates in three directions, using a sterile cotton swab (Becton-Dickinson), till the surface of the plate was dry. CPDX disks and the prepared disks were then placed on the agar plates. The center-to-center distances between adjacent disks were kept at 21 mm for SMA, CPDX, CPDX/PBA, CPDX/CVA, and CPDX/CLX. Another CPDX disk was placed on the same plate for reference, ensuring sufficient space between the disks to allow for the formation of a clearly defined inhibition zone (Fig. 1). For accurate disk placement, the full-scale “disk placement sheet” was used. Inhibition zone differences were measured after an 18-h incubation period at 35 °C.

2.4. Interpretation of AMU-DM

Firstly, the zones of each disk were measured and compared to the that of the reference disk (combination disk test). Strains were identified as KPC-producing strains when the disk CPDX/PBA showed a zone difference ≥5 mm than the reference disk, and as AmpC-producing strains when both CPDX/PBA and CPDX/CLX disks showed significant zone differences (≥4 mm for CPDX/PBA disk and ≥5 mm for CPDX/CLX disk) compared to the reference disk.

Secondly, the multiplex-disk synergy effects between the disks were observed. The presence of β-lactamases was inferred when the inhibition zones were enhanced on the side of each inhibitor-

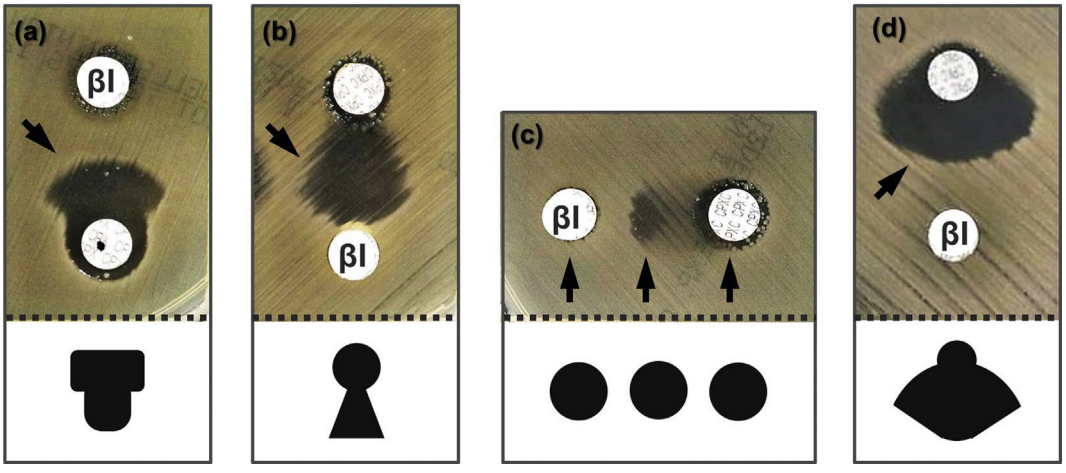

Fig. 2. An example of a positive result in cefpodoxime-β-lactamase inhibitor disk synergy tests. The presence of β-lactamases was inferred when the inhibition zones around the cefpodoxime-containing disk were enhanced on the side of β-lactamase inhibitor-containing disk (βI), such as clavulanic acid for extended-spectrum-β-lactamse, cloxacillin and phenylboronic acid for AmpC-β-lactamse or sodium mercaptoacetic acid for metallo-β-lactamse, resulting in characteristic zone shapes referred to as: (a) champagne-cork image, (b) keyhole image, (c) ellipsis image: inhibition spot was observed between the disks and a series of three points (disk-inhibition spot-disk) appeared like an ellipsis mark and (d) phantom image. Black arrows indicate each characteristic zone shape images shown in the bottoms.

Table 2
Interpretation of Applied Multiplex disk method using cefpodoxime (CPDX) 10 μg disk, clavulanic acid (CVA), cloxacillin (CLX), sodium mercaptoacetic acid 3 mg disk (SMA), and phenylboronic acid (PBA).

Definition of positive test result	β-lactamase production:						
	AmpC	KPC	MBL	ESBL and AmpC	ESBL and MBL	KPC and MBL	MBL and AmpC
Increase in inhibition zone							
CPDX ± PBA 600 μg ≥ 5 mm	+	+	−	−	−	−	−
CPDX ± CVA 10 μg ≥ 5 mm	−	−	−	−	−	−	−
CPDX ± CLX 750 μg ≥ 4 mm	+	−	−	−	−	−	−
Enhanced a characteristically shaped inhibition zone between							
SMA and CPDX	−	−	+	−	−	−	−
SMA and CPDX/PBA	−	−	+	−	−	+	+
SMA and CPDX/CVA	−	−	+	−	+	−	−
SMA and CPDX/CLX	−	−	+	−	−	−	+
CPDX/CVA and CPDX/CLX	−	−	−	+	−	−	−

Abbreviations: extended-spectrum-β-lactamase, AmpC: AmpC-β-lactamase, KPC: *Klebsiella pneumoniae* carbapenemase. MBL: metallo-β-lactamase.
"+" and "−" indicate "positive" and "negative", respectively. a: like as a "champagne-cork," "keyhole," "ellipsis," or "phantom image".

containing disk, resulting in characteristic zone shapes referred to as "champagne-cork," "keyhole," "ellipsis," or "phantom image" (Fig. 2). MBL-producing strains were identified when the zone between SMA and all adjacent disks (CPDX, CPDX/PBA, CPDX/CVA, and CPDX/CLX) displayed the inhibition effect; ESBL and AmpC-co-producing strains were identified when only the zone between the disk CPDX/CVA and CPDX/CLX showed an inhibition effect; MBL and ESBL co-producing strains were identified when only the zone between CPDX/CVA and SMA disks showed inhibition; and finally, if the zone between SMA and CPDX/PBA and also that between SMA and CPDX/CLX showed the inhibition effects, the strains were identified as MBL and AmpC-producing strains.

The interpretation of AMU-DM results and representative inhibitory effects are summarized in Table 2. Cases that did not meet the criteria described in the table were considered uninterpretable.

2.5. Alternative MBL inhibitor

As an additional test, the SMA disk was replaced with the EDTA disk (eAMU-DM) for MBL-producing (including co-producing other β-lactamases) isolates that were uninterpretable by AMU-DM. Additionally, an MBL reference strain (NCTC 13476) was also tested.

2.6. Evaluation of AMU-DM

The performance of AMU-DM for the detection and identification of carbapenemase-, ESBL- and/or AmpC-producing *Enterobacteriaceae* was evaluated by a blind-study (to remove bias), comparing the results with those from PCR (considered gold standard) experiments.

Table 3
Results obtained using the Applied Multiplex Disk Method (AMU-DM).

PCR confirmed strain group (no.)	Number (%) of AMU-DM result		
	concordant	discordant	uninterpretable
AmpC (31)	31 (100)	0	0
ESBL + AmpC (11)	11 (100)	0	0
KPC (3)	3 (100)	0	0
KPC + ESBL (1)	0	0	1 (100) [a]
MBL (15)	15 (100)	0	0
MBL + ESBL (19)	16 (84)	0	3 (16) [b]
MBL + AmpC (3)	3 (100)	0	0
Total	79 (95)	0	4 (5)

Abbreviations: ESBL: extended-spectrum-β-lactamase, AmpC: AmpC-β-lactamase, KPC: *Klebsiella pneumoniae* carbapenemase. MBL: metallo-β-lactamase.
[a] *Klebsiella pneumoniae* producing KPC-2, SHV-12 and CTX-M-9 family.
[b] one *K. pneumoniae* co-producing IMP-6 and CTX-M-2 family, one *K. pneumoniae* co-producing NDM-1, SHV-28, TEM-104 and CTX-M-1 family, and one *Escherichia coli* co-producing NDM-5, TEM-104 and CTX-M-1 family.

3. Results

Table 3 and Fig. 3 show the performance data of AMU-DM and the representative inhibitory effects, respectively. Overall, AMU-DM results for 79/83 (95%) isolates were similar to those obtained for the isolates by PCR. The concordance rates were as follows: 31/31 (100%) for AmpCs; 11/11 (100%) for ESBL and AmpC co-production; 3/3 (100%) for KPC; 0/1 (0%) for KPC and ESBL co-production; 15/15 (100%) for MBLs; 16/19 (84%) for MBL and ESBL co-production; and 3/3 (100%) for MBL and AmpC co-production. Four isolates did not demonstrate a significant inhibition zone: one *K. pneumoniae* isolate co-producing KPC-2, SHV-12, and CTX-M-1 families, one *K. pneumoniae* isolate co-producing IMP-6 and CTX-M-2 families, one *K. pneumoniae* isolate co-producing NDM-1, SHV-28, TEM-104, and CTX-M-1 families, and an *E. coli* isolate co-producing NDM-5, TEM-104, and CTX-M-1 families.

Fig. 4 shows the comparison of inhibitory effect between AMU-DM and eAMU-DM for MBL reference strain and for three isolates co-producing MBL and ESBL that were uninterpretable in AMU-DM. The MBL reference strain was not identified by eAMU-DM, whereas AMU-DM identified this strain. In contrast, one *K. pneumoniae* isolate co-producing NDM-1, SHV-28, TEM-104, and CTX-M-1 families was identified by eAMU-DM but not by AMU-DM. One *K. pneumoniae* isolate co-producing IMP-6 and CTX-M-2 families and an *E. coli* isolate co-producing NDM-5, TEM-104, and CTX-M-1 families were not identified by either AMU-DM or eAMU-DM.

4. Discussion

Here we have developed and evaluated a novel assay (AMU-DM) to detect β-lactamase production using a single agar plate. In AMU-DM, combination disk tests using CLX and PBA were performed to detect the AmpCs and KPC [16–18]. In these tests, we used a CPDX disc, whereas the previous report used other antimicrobial discs [14,16–18]. However, in the present study, both CPDX/CLX and CPDX/PBA and only CPDX/PBA showed a concordant rate of 100% for AmpC-only- and KPC-only-producers, respectively.

Double-disk synergy tests (DDST), based on the inhibition of MBL activity by inhibitors, are the most commonly used phenotypic tests for the detection of MBLs [15,19,20]. Although previous studies have evaluated ceftazidime-, imipenem-, or meropenem-SAM-DDST in *Enterobacteriaceae*, to the best of our knowledge, CPDX based SMA-DDST were yet to be evaluated [21,22]. In this study, all MBL-only producers were correctly identified by CPDX-

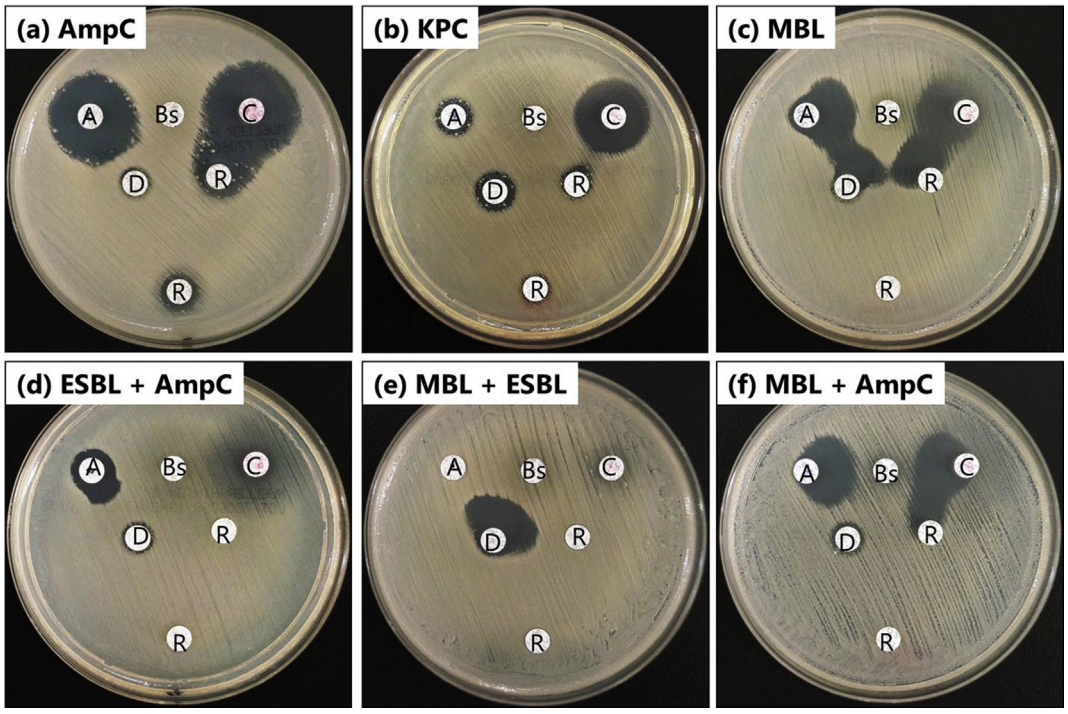

Fig. 3. Representative inhibitory effects for the Applied Multiplex Disk Method.(a): *Klebsiella pneumoniae* producing DHA-1 (ATCC BAA-1144), (b): *K. pneumoniae* producing KPC-3 (ATCC BAA-1900), (c): *Escherichia coli* producing IMP-unsequenced (NCTC 13476), (d): *E. coli* producing CTX-M-9 and CMY-2, (ATCC BAA-2355), (e): K. oxytoca producing IMP-1 and CTX-M-1 family (clinical isolated), and (f): *K. pneumoniae* producing IMP-1 and DHA-1 (clinical isolated). (R): cefpodoxime (CPDX) 10 μg, (A): CPDX/cloxacillin (CLX) 750 μg, (Bs): sodium mercaptoacetic acid 3 mg, (C): CPDX/phenylboronic acid 600 μg, (D): CPDX/clavulanic acid 10 μg.

based SMA-DDST by the AMU-DM interpretation. Thus, AMU-DM can successfully detect AmpC-, KPC-, and MBL-only producers with full concordant rates.

Currently, MASTDISC ID ESBL & AmpC ID set (MAST, Liverpool, United Kingdom), SMA disk, and MASTDISCS Carba plus (MAST) are available in Japan for phenotypic identification of ESBL and AmpC, MBL, and carbapenemases (KPC, MBL, and OXA-48), respectively. Target β-lactamases of these commercial assay kits are different requiring the storage of multiple kits in our laboratory. According to the above results, AMU-DM can integrate multiple phenotypic β-lactamase detection tests. Thereby, we believe that most of other phenotypic tests for β-lactamase detection may be discontinued. However, our method had limited detection abilities of GES- or OXA-48-type carbapenemases, and carbapenemase tests, such as mCIM or carba NP, along with the AMU-DM to identify *Enterobacteriaceae* isolates producing carbapenemases must be attempted [2].

The presence of a certain β-lactamase may be masked by other β-lactamases [22]. In such cases, existing phenotypic methods might reduce their performance and we had to perform the molecular methods for β-lactamases detection. For the isolates that co-produce multiple classes of β-lactamases, we combined multiple inhibitors for multiplex-disk synergy test in AMU-DM. Hence, theoretically our method is more advantageous in β-lactamase detection i.e. AMU-DM supports the detection of co-producers of ESBL and AmpC, MBL and ESBL, MBL and AmpC, and KPC and MBL. These multiplex disk synergy tests could be used to successfully detect ESBL and AmpC co-producers as well as MBL and AmpC co-producers with full concordant rates.

However, three ESBL and MBL co-producers (including two NDM-producers and one IMP-producer) showed inconclusive results. Consistent with our results, previous studies have reported that SMA-DDST cannot detect NDM production by ESBL and MBL co-producers [23–25]. Thus, in this study, we tested an alternative inhibitor EDTA (eAMU-DM) for the detection of MBL against these isolates. As a result, the eAMU-DM method could identify one NDM-1 and ESBL co-producer, but not an NDM-5 and ESBLs co-producer, one IMP-6 and CTX-M-2 family co-producer and IMP-producing reference strain. Additionally, the EDTA disk showed an inhibition effect by itself against the tested isolates. Hence, we recommend using SMA disk on AMU-DM in areas endemic for IMP-harboring bacterial strains. The isolates that were interpreted uninterpretable by both AMU-DM and eAMU-DM, one IMP-6 and CTX-M-2 families co-producing *K. pneumoniae* isolate and an *E. coli* isolate co-producing NDM-5 and ESBLs, may have had elevated CPDX MIC based on the production of MBL and ESBL together with the other mechanisms, such as porin changes or hyperproduction of chromosomal AmpC (in *E. coli*) [26]. For these isolates, further evaluation is necessary using other antimicrobial agents in future studies.

In this study, one KPC and ESBL co-producer also showed inconclusive results. Our disk placement position would theoretically not allow for the correct detection of some β-lactamase co-producers. The requirement of CVA and PBA synergy effect for the detection of KPC and ESBL co-producers makes our disk placement position unadaptable. Another discordant case may be considered for both KPC and AmpC co-producers. These isolates may be shown the inhibition zone in PBA only and interpreted as KPC singular-producers. We hypothesize that optimal disk placement may vary among regions according to differences in the epidemiology of β-lactamase producers.

Our study had some limitations. Firstly, this method was evaluated only for ESBL-confirmation test-negative isolates. Therefore, the conclusion of this study should be only for these isolates. Further performance evaluation for the species that were not covered by the CLSI ESBL tests will be required. Secondly, because both KPC and MBL co-producers and NDM- or VIM-type MBL producers are rare and IMP-type MBL producers are frequent in our

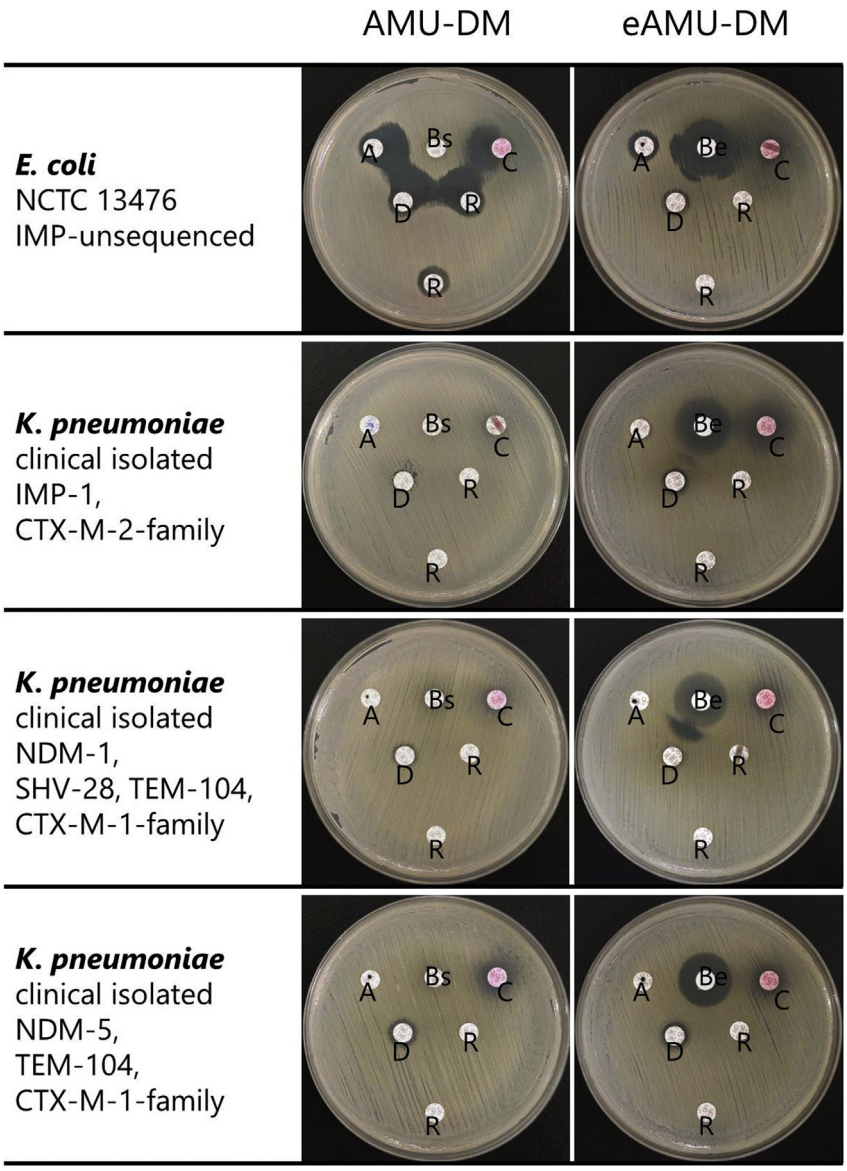

Fig. 4. Comparison of inhibitory effect for using sodium mercaptoacetic acid (SMA) disk and ethylenediaminetetraacetic acid (EDTA) disk on Applied Multiplex Disk Method (AMU-DM) to detect MBL. (R): cefpodoxime (CPDX) 10 μg, (A): CPDX/cloxacillin 750 μg, (Bs): SMA 3 mg, (Be): EDTA 1460 μg, (C): CPDX/phenylboronic acid 600 μg, (D): CPDX/clavulanic acid 10 μg.

geographic region, our study had a key limitation in the inclusion of β-lactamase varieties of the isolates. Finally, we did not perform promotor analysis of constitutive hyperproduction of the chromosomal AmpC for *E. coli*. Hence, the effect of the chromosomal AmpC in five *E. coli* isolates co-producing MBL and ESBL was not evaluated.

In conclusion, we believe that this method would be able to bridge the gap between the ESBL and carbapenemase tests and be useful in the implementation of infection-control measures and for surveillance purposes. We recommend AMU-DM for all isolates that were positive by CLSI-ESBL screening test and negative by CLSI-ESBL confirmation test.

Conflicts of interest

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

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