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Imipenem 비감수성 그람음성막대균에서 IMP-1 및 VIM-2형 Metallo-β-Lactamase의 검출에 Dipicolinic Acid에 기초한 Mueller Hinton Agar Biplate의 평가

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Evaluation of Dipicolinic Acid-Based Mueller Hinton Agar Biplate for Detection of IMP-1 and VIM-2 type Metallo-β-Lactamase in Imipenem Non-susceptible Gram Negative Bacilli

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Background : Since metallo-β-lactamase (MBL)-producing isolates can hydrolyze carbapenem and also easily transfer the resistance genes to other bacteria, a rapid and accurate detection of MBL has become very important. We evaluated the utility of Mueller Hinton agar (MHA) biplate containing dipicolinic acid (DPA) as a screening method to detect IMP-1 and VIM-2 type MBL-producing isolates.

Methods : Based on our preliminary tests using various concentrations of DPA, 200 and 300 μg/mL concentration of DPA were chosen for further study. Bacterial lawns were grown on MHA biplate, one half of which contained DPA while the other did not. The inhibition zone around the imipenem (IPM) disk on both sides of this plate was compared. The stability of DPA in the stored DPA-MHA biplate was also evaluated during three months using two MBL- and one non-MBL-producing isolates.

Results : When the criterion of a ≥7 mm increase of inhibition zone around the IPM disk on the MHA containing DPA compared to MHA without DPA was used, the sensitivities and specificities were 94.7% and 97.6% for 200 μg/mL DPA-MHA biplate, and 98.2% and 97.6% for 300 μg/mL DPA-MHA biplate, respectively. The activity of the DPA in this biplate was stable for three months.

Conclusions : Assays using DPA 300-MHA biplate were highly sensitive and specific for the detection of IMP-1 and VIM-2 type MBL-producing bacteria. In addition, it is easy to perform; so, it may be useful to apply this method for detection of IMP-1 and VIM-2 type MBL in clinical laboratories. *(Korean J Lab Med 2009;29:204-11)*

Key Words : *Dipicolinic acid, DPA-MHA biplate, Metallo-β-lactamase*

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INTRODUCTION

The increasing prevalence of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases in gram-negative bacilli has led to the increased need of carbapenems, which have good stability against β-lactamases. However, class B β-lactamases as defined by Ambler [1], termed metallo-β-lactamases (MBLs), can hydrolyze all β-lactams except aztreonam [2]. Since the IMP-1 enzyme was initially described from *Pseudomonas aeruginosa* in 1991 in Japan, various kinds of MBLs including IMP and VIM types have increasingly been reported in gram-negative bacilli around the world [3-6]. Thereafter, SPM-1, GIM-1, and SIM-1 type were also consecutively found in Latin America, Germany and South Korea [7-9]. The rapid and accurate detection of MBL-producing microorganisms is very important for the timely control of infections and to prevent further dissemination of these organisms. However, current CLSI (formerly NCCLS) documents have not included a standard phenotypic test to detect MBL producers.

Several screening methods by using various chelating agents such as 2-mercaptopropionic acid and EDTA have been introduced [10, 11]. These chelating agents have some inherent bacterial growth-inhibiting capability or hydrolytic effect on some antibiotics [12-14]. However, dipicolinic acid (DPA) has an extremely strong chelating activity for MBL, and it neither inhibits growth of bacteria nor hydrolyzes the antibiotics [15]. In addition, it was reported that the DPA-based microdilution by Kimura et al. [13], and the DPA-based disk methods by Shin et al. [16] revealed the good results in detecting MBL. However, the microdilution method may be impractical and the disk methods may be technically less comfortable for some practitioners, making routine application of these techniques for the detection of MBL in the clinical laboratory less likely.

In this study, we slightly modified the MBL detection method previously reported by Kimura et al. [13] to conveniently perform the procedures by using Mueller Hinton agar (MHA) biplate containing DPA, and evaluated the utility of this test for detection of IMP-1 and VIM-2 type MBL-producers.

MATERIALS AND METHODS

1. Bacterial strains and detection of MBL genes

All of the bacterial strains were isolated from the clinical specimens of the two university hospitals in South Korea between 2004 and 2007. The bacterial species were identified by conventional techniques or by the Vitek system (bioMérieux Inc., Durham, NC, USA). A susceptibility test for imipenem (IPM) was performed by the disk diffusion method using 10 μg IPM disks (BBL, Cockeysville, MD, USA) on MHA (Cheongmeak Inc., Cheongwon, Korea) according to the CLSI guidelines [17].

The 57 MBL-producing isolates used in this study were composed of 25 *P. aeruginosa* (8 isolates of IMP-1 type and 17 isolates of VIM-2 type), 25 *Acinetobacter* spp. (12 isolates of IMP-1 type and 13 isolates of VIM-2 type), 3 *Pseudomonas fluorescens* (VIM-2 type), 3 *Achromobacter xylosoxidans* (VIM-2) [18] and *Providencia rettgeri* (VIM-2) [19]. Forty one isolates of IPM non-susceptible but non-MBL-producing *P. aeruginosa* (21) and *Acinetobacter spp.* (20) were included for the evaluation of the method's specificity.

PCR assay for bla_{IMP-1} and bla_{VIM-2} were initially performed on all of the isolates [20, 21]. For bla_{IMP-1} or bla_{VIM-2} negative isolates, PCR assay for bla_{VIM-1} and bla_{SIM-1} were performed [9, 20]. PCR assay were performed with 2 μL of heat extracted DNA templates, 20 pmol of each primers and Hot-Start PCR premix (Bioneer Inc., Cheongwon, Korea) containing 1 U of Taq DNA polymerase in a total volume of 20 μL. A thermocycler (GeneAmp® PCR System 9700, Applied Biosystems, Foster City, CA, USA) was used with the following reaction conditions: 95℃ for 15 min, 25 cycles of 94℃ for 45 sec, 51℃ for 30 sec (56℃ for bla_{SIM-1}), 72℃ for 1 min (45 sec for bla_{SIM-1}), and finally, 72℃ for 10 min. The amplicons were loaded on agarose gels (2%) with appropriate DNA markers.

2. Susceptibility tests for detection of MBL in DPA-MH agar biplate

Based on our preliminary tests, the 200 and 300 μg/mL concentrations of DPA were therefore chosen for this study.

DPA stock solution (1 M) was prepared by dissolving 167.1 g of 2,6-pyridinedicarboxylic acid (Sigma-Aldrich Inc., St. Louis, MO, USA) in 1,000 mL of distilled water. DPA was initially dissolved in alkaline pH (≥pH 11), and then the solution was later adjusted to pH 8.0-8.5 with sodium hydroxide tablets. This solution was diluted to the appropriate concentrations for the study and was stored at 4℃. DPA-MHA biplates (Cheongmeak Inc., Cheongwon, Korea) were made with one side having no additive and the other containing either 200 or 300 μg/mL DPA. Two 10 μg IPM disks were placed on both sides of each DPA-MHA biplate which was inoculated with 0.5 McFarland of test organism. The inhibition zone around the IPM disk on both sides of the DPA-MHA biplates were compared with each other after 16 to 18h of incubation in ambient air at 35℃. VIM-2 *A. xylosoxidans* (accession number, AY686225) [18] and *P. aeruginosa* ATCC 27853 were used as positive and negative control, respectively, for MBL-production.

3. Stability of DPA in DPA-MHA biplate

To evaluate the stability of DPA, two MBL-producing isolates and *P. aeruginosa* (ATCC 27853) were periodically tested every two weeks on stored DPA-MHA biplate for a total of three months. Strains were stored in a deep freezer (-70℃) with the aliquots in glycerol broth tubes for testing periods. The DPA-MHA biplates were stored at 4℃.

RESULTS

1. Susceptibility tests for detection of MBL in DPA-MH agar biplate

For the 57 MBL-positive isolates, the inhibition zone around the IPM disk increased by 2 to 29 mm (mean, 13 mm) on the MHA with 200 μg/mL of DPA and 4 to 30 mm (mean, 15 mm) on the MHA with 300 μg/mL of DPA as compared to those on the MHA without DPA (Fig. 1). For the 41 MBL-negative isolates, the inhibition zone around the IPM disk increased by 0 to 7 mm (mean, 0.86 mm) and 1 to 8 mm (mean, 2.3 mm) on the MHA with 200 and 300 μg/mL DPA, respectively, as compared to those on the MHA without DPA (data not shown). When the criterion of a ≥7 mm increase of inhibition zone around the IPM disk on MHA with DPA

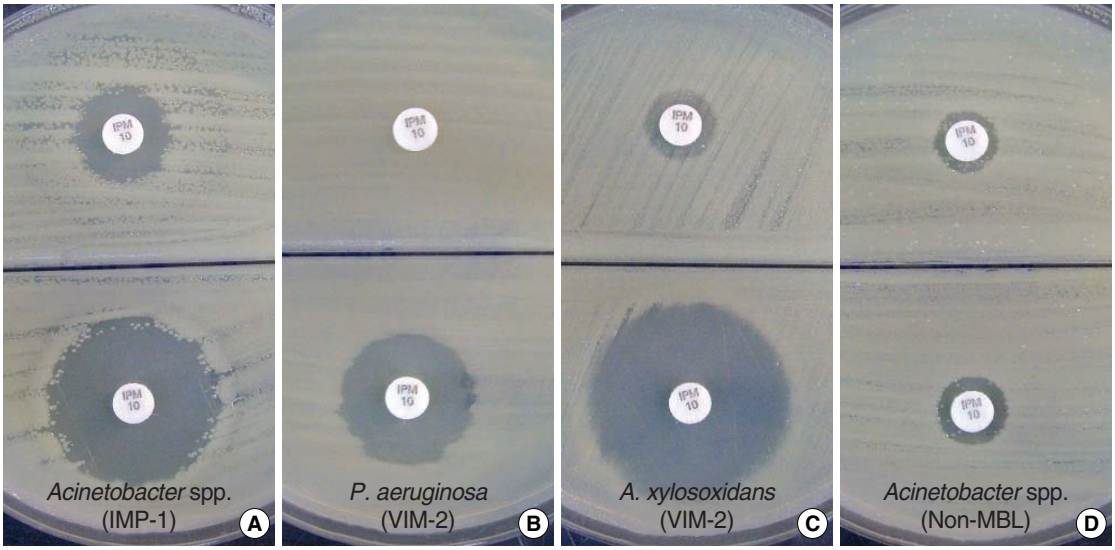

Fig. 1. DPA-based MHA biplate for the detection of MBL in IPM non-susceptible clinical isolates. (A) IMP-1 type *Acinetobacter* spp., (B) VIM-2 type *Pseudomonas aeruginosa*, (C) VIM-2 *Achromobacter xylosoxidans*, and (D) non-MBL producing *Acinetobacter* spp. The lower part of each biplates contained 300 μg/mL DPA. MBL producing isolates (A-C) showed increased inhibition zones (≥7 mm) around IPM disks in the presence of DPA as compared to disks that lacked DPA. The non-MBL-producing isolate (D) showed no significant difference in inhibition zone size.
Abbreviations: IPM, imipenem; MHA, Mueller Hinton agar; DPA, dipicolinic acid.

as compared to MHA without DPA was used, only one (VIM-2 type *Acinetobacter* spp.) out of 57 MBL- positive isolates showed a false negative result on the DPA 300 μg/mL MHA, which was VIM-2 type *Acinetobacter* spp., co-producing OXA-51 like carbapenemase (data not shown). For the 41 MBL-negative isolates, one sample (*P. aeruginosa*) showed a false positive result. Using the DPA 200 μg/mL MHA and the same criterion, 3 out of the 57 MBL-positive isolate (5.3 %) showed false negative results, which included two isolates of *Acinetobacter* spp. and one VIM-2 *P. rettgeri* co-producing ESBL, AmpC-β-lactamase and MBL [19] (Fig. 2, Table 1). The co-production of the three β-lactamases was detected by combined use of cefotaxime/clavulanic acid disk and boronic acid on DPA-MHA plate (Fig. 3). One out of the 41 MBL-negative isolates showed false positive result. Overall, DPA 300 μg/mL MHA biplates detected 56 out of the 57 MBL-producing isolates (sensitivity, 98.2%) and 40 out of the 41 non-MBL-producing isolates were correctly differentiated (specificity, 97.6%). The sensitivity and specificity was 94.7% and 97.6%, respectively, for DPA 200 μg/mL MHA biplate (Table 1).

Table 1. Results of dipicolinic acid-containing Mueller Hinton agar biplate for detection of metallo-β-lactamase in imipenem non-susceptible gram-negative bacilli

Species and allele gene of MBL genes (N of isolates)	DPA 200-MHA biplate		DPA 300-MHA biplate	
	Positive†	Negative	Positive	Negative
Pseudomonas aeruginosa (46)				
IMP-1 type MBL (8)	8	0	8	0
VIM-2 type MBL (17)	17	0	17	0
N-MBL (21)	1	20	1	20
Acinetobacter spp. (45)				
IMP-1 type MBL (13)	12	1	13	0
VIM-2 type MBL (12)	11	1‡	11	1‡
N-MBL (20)	0	20	0	20
Other isolates* (7)				
VIM-2 type MBL (7)	6	1	7	0
Total isolates (98)				
MBL (57)	54	3	56	1‡
N-MBL (41)	1	40	1	40
Sensitivity (%)	94.7		98.2	
Specificity (%)	97.6		97.6	

**Pseudomonas fluorescens* (3), *Achromobacter xylosoxidans* (3) and *Providencia rettgerii* (1); †The criterion of a ≥7 mm increase in inhibition zone size around the imipenem disk in DPA-containing MHA compared to MHA without DPA was used as a positive indication of MBL; ‡Isolates co-producing MBL and OXA-51 type carbapenemase.
Abbreviations: DPA, dipicolinic acid; MHA, Mueller Hinton agar; MBL, metallo-β-lactamase; N-MBL, non-MBL.

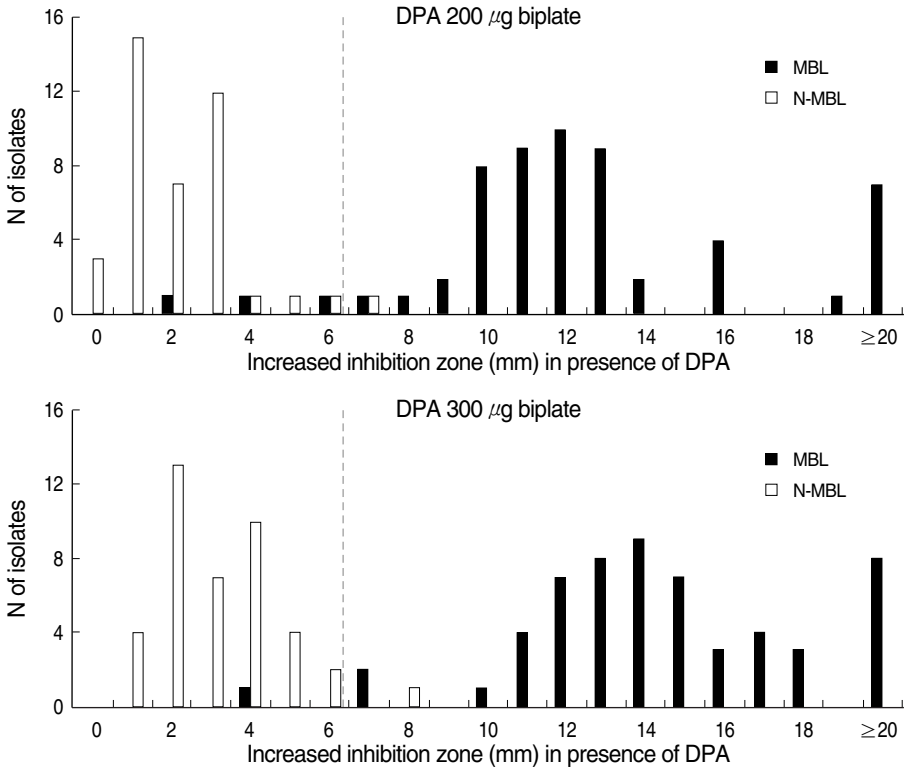

Fig. 2. Increase of inhibition zone size around the imipenem disk in MHA with DPA as compared with inhibition zones produced by imipenem disk in MHA without DPA for 57 MBL positive and 41 MBL negative clinical isolates.
Abbreviations: DPA, dipicolinic acid; MHA, Mueller Hinton agar; MBL, metallo-β-lactamase; N-MBL, non-MBL.

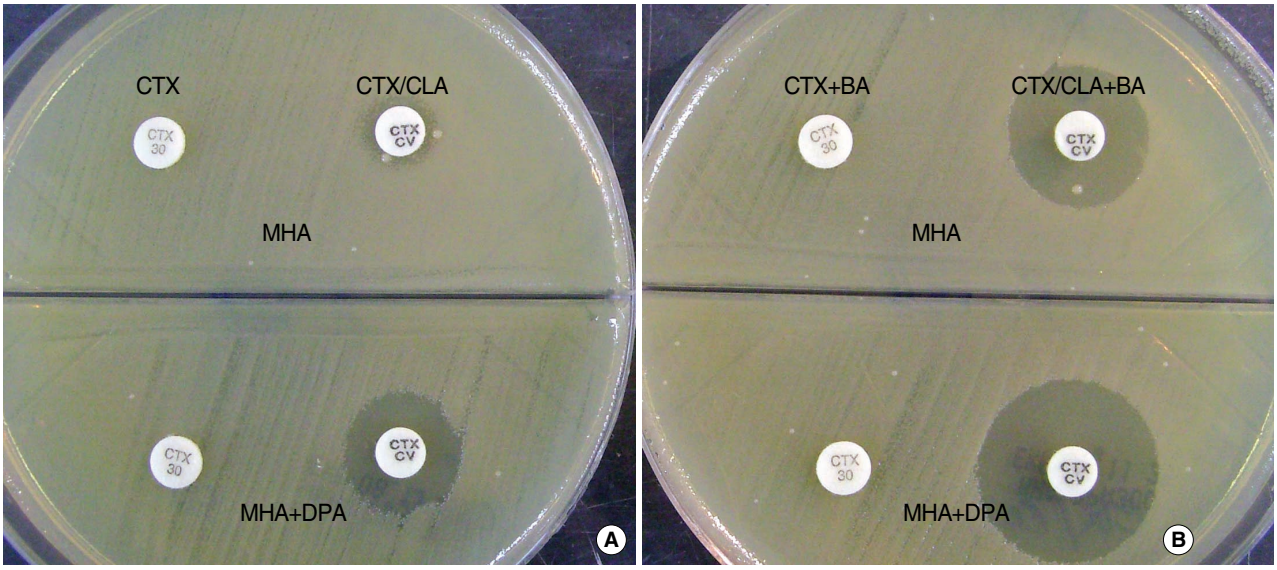

Fig. 3. DPA-based MHA biplate for the detection of three type β-lactamase from *Providencia rettgerii* carrying ESBL, AmpC β-lactamase and MBL. When boronic acid (BA) was add to cefotaxime (CTX) and cefotaxime/clavulanic acid (CTX/CLA) disk, a ≥5 mm increase of inhibition zone around CTX/CLA disk in comparison to CTX disk suggested the co-production of ESBL and AmpC β-lactamase (A→B), and the increase of inhibition zone around CTX/CLA disks on DPA-containing MHA relative to MHA at both A and B suggested the concurrent production of MBL (upper→lower).
Abbreviations: DPA, dipicolinic acid; MHA, Mueller Hinton agar; ESBL, extended-spectrum β-lactamase; MBL, metallo-β-lactamase.

Table 2. Stability of dipicolinic acid activity in DPA 300-Mueller Hinton agar biplate during 12 weeks

Storage (weeks)	Inhibition zone around IPM 10 μg disk (mm)					
	IMP-1 *Acinetobacter* spp.		VIM-2 *P. aeruginosa*		*P. aeruginosa* ATCC 27853	
	MHA	DPA 300 μg	MHA	DPA 300 μg	MHA	DPA 300 μg
Immediate	13	28 (15)*	7	32 (25)	23	27 (4)
1	13	28 (15)	9	33 (24)	23	26 (3)
2	14	28 (14)	9	33 (24)	23	27 (4)
4	15	28 (13)	9	34 (25)	25	29 (4)
6	15	28 (13)	9	34 (25)	23	26 (3)
8	15	28 (13)	9	34 (25)	25	28 (3)
10	14	28 (14)	8	32 (24)	25	28 (3)
12	14	28 (14)	9	33 (24)	24	27 (3)

*The difference of inhibition zone size around IPM 10 μg disks between 300 μg/mL DPA-containing MH agar and MH agar without DPA.
Abbreviations: IPM, imipenem; MHA, Mueller Hinton agar; DPA, dipicolinic acid.

2. Stability of DPA in DPA-MHA biplate

Two MBL-producing isolates, VIM-2 *P. aeruginosa* and IMP-1 *Acinetobacter* spp. displayed a significant (≥7 mm) increase in inhibition zone size on the DPA containing MHA compared with that on MHA not containing DPA. There were no significant differences with respect to the increases in the size of inhibition zones around the IPM disks on both sides of the biplates with or without DPA during the three months that DPA stability was tested. *P. aeruginosa* (ATCC 27853) revealed both no significant increases in the inhibition zone and no significant differences with regard to increases in range on both sides during the same testing (Table 2).

DISCUSSION

To prevent the dissemination of MBL producing isolates among gram-negative bacilli, a simple and accurate detection method for these enzyme producing isolates is essential [22]. In a biochemical study by Siemann et al. [15], it was reported that DPA is the most potent irreversible inhibitor of IMP-1 among the 6-types of chelating agents, including EDTA which is approximately 50 times less effective. In this study, we modified the disk diffusion method using DPA for detection of MBL-producing organism previously reported [13], and assessed the modified method (i.e. DPA-MHA biplate) to detect IMP-1 and VIM-2 type MBL-producer by

using a greater number of clinical isolates of MBL than in the previous report and by using various concentration of DPA. Our preliminary study evaluated the performance of the MHA with the addition of either 50 μg/mL, 500 μg/mL or 835 μg/mL of DPA. An IPM disk on MHA with 50 μg/mL of DPA increased the mean inhibition zone of seven MBL-positive isolates by only 2.14 mm (0-6 mm) compared with MHA without DPA. An IPM disk on MHA with 835 μg/mL of DPA increased the mean inhibition zones of four out of five IPM-resistant MBL-negative isolates by 7-10 mm. An IPM disk on MHA with 500 μg/mL of DPA increased the mean inhibition zones of three out of five IPM-resistant MBL-negative isolates by 6-8 mm compared to those on MHA without DPA (data not shown). Based on these results, MHA containing 200 and 300 μg/mL of DPA was chosen for further studies. When the criterion of a ≥7 mm increase of inhibition zone around the IPM disk on MHA containing DPA as compared to MHA without DPA was used, this method detected 94.7% and 98.2% on 200 μg/mL and 300 μg/mL DPA containing MHA, respectively. One undetectable isolate on both the 200 and 300 μg/mL DPA containing MHA was a VIM-2 type *Acinetobacter* spp. co-producing an OXA type carbapenemase (OXA-51, data not shown), which is a currently-prevalent resistance mechanism for *Acinetobacter* spp. worldwide [23]. This type of β-lactamase was not inhibited by DPA and thus isolates with co-expression of MBL could cause false negative results as in this case. Since sodium chloride can inhibit OXA enzymes, the use of sodium chloride with the DPA-MHA is probably able to detect MBL in OXA enzyme co-producing isolates [24]. When 400 mM sodium chloride was added to the IMP disk which were then placed on both sides of the DPA-MHA biplate (DPA 300 μg/mL), the inhibition zone size was increased from 4 mm to 7.5 mm (data not shown), leading to a positive result for MBL based on the criterion chosen for this study (≥7 mm). Other undetectable isolates on the 200 μg/mL DPA MHA, which were detected on the 300 μg/mL DPA MHA, were one isolates each of IMP-1 type *Acinetobacter* spp. and VIM-2 *P. rettgeri*. The *P. rettgeri* isolate was co-carrying an ESBL, AmpC β-lactamase and MBL [19], which were successfully detected by the combined use of clavulanic acid or boronic acid with the DPA-MHA biplate (Fig. 3). All of the 41 non-MBL-producing isolates except for one *P. aeruginosa* isolate were correctly differentiated from MBL-producers by the two DPA containing MHA (specificity, 97.6%). This specificity would be comparable or superior to the other methods of detecting MBL [16, 22, 25, 26].

When we assessed the stability of DPA in MHA, there were no significant differences in the quality of results obtained on MHA with or without DPA when tested over three months. Therefore, DPA-containing MHA is considerably stable for the recommended period of MHA storage.

In conclusion, the IPM-DPA with MHA (300 μg/mL) method was highly specific in differentiating non-MBL-producing isolates as well as very sensitive in detecting IMP-1 and VIM-2 type MBL-producing gram-negative bacilli, including *Pseudomonas* spp. and *Acinetobacter* spp. Moreover, if this DPA agar were to be commercially produced, this procedure would become an appropriate screening method to detect MBL in a routine clinical laboratory due to the simple procedure involved and the greater advantage in terms of quality control. However, we used only IMP-1 and VIM-2 type MBL to evaluate the DPA-MHA biplate screening method in detecting MBL. Therefore, further tests with other types of MBL, such as SIM, SPM and GIM types, are required to determine if this screening method is more widely applicable.

요 약

배경 : Metallo-β-lactamae (MBL) 생성 균주는 carbapenem 항생제를 가수분해할 수 있으며 이들 내성유전자를 다른 세균으로 쉽게 전달할 수 있으므로 MBL의 빠르고 정확한 검출은 매우 중요하게 되었다. 저자들은 IMP-1 및 VIM-2형 MBL 생성균주의 검출에 이용되는 선별검사로 dipicolinic acid (DPA)를 포함하는 Mueller Hinton agar (MHA)의 유용성을 평가하였다.

재료 및 방법 : 다양한 DPA 농도를 이용한 예비 실험에서 200 μg/mL 및 300 μg/mL DPA 농도가 본 실험을 위하여 선택되었다. 한쪽은 DPA가 포함되고 반대쪽에는 DPA가 포함되지 않은 DPA-MHA biplate에 균을 접종한 후, plate의 양쪽에서 imipenem (IPM) 디스크 주위의 억제대 직경의 차이를 비교

하였다. 보관된 DPA-MHA biplate에서 DPA 안정성을 평가하기 위해 2개의 MBL 생성주와 하나의 non-MBL 생성주를 대상으로 3개월 동안 평가하였다.

결과 : DPA-MHA biplate 양쪽에서 IPM 디스크 주위에서 생성된 억제대 증가의 차이가 7 mm 이상일 경우를 MBL 생성주의 기준으로 정하였을 때, DPA 200 μg/mL 배지와 300 μg/mL 배지의 예민도 및 특이도는 각각 94.7%, 97.6% 및 98.2%와 97.6%이었다. DPA-MHA biplate에서 DPA 활성도는 3개월 동안 안정하였다.

결론 : MP-1 및 VIM-2형 MBL의 검출에 300 μg/mL DPA-MHA biplate는 높은 예민도와 특이도를 보였다. 게다가 이들 방법은 사용 절차가 쉽고 간편하므로, 임상미생물검사실에서 IMP-1 및 VIM-2형 MBL을 검출방법으로 사용하기에 유용할 것으로 사료된다.

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