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Performance Evaluation of MBT STAR-Carba / MBT STAR-Cepha Kit by MALDI Biotyper for Carbapenemase or Extended Spectrum β-Lactamase Producing Gram Negative Bacilli

Tsubasa Yokoyama[1], Kentaro Kawahara[1], Yumiko Funashima[1,2], Takahiro Yaguchi[1,2], Osamu Ueda[3], Kentaro Wakamatsu[4], Zenzo Nagasawa[1,2]

[1]Department of Medical Laboratory Science, Graduate school of Health and Welfare Sciences, International University of Health and Welfare Graduate School

[2]Department of Medical Technology and Science, Faculty of Fukuoka Health Care, International University of Health and Welfare

[3]Division of Microbiology, Department of Pathology & Microbiology, Faculty of Medicine, Saga University

[4]Department of Respiratory Medical, National Hospital Organization Omuta National Hospital

***Corresponding author:** Takahiro Yaguchi, Department of Medical Laboratory Science, Graduate school of Health and Welfare Sciences, Japan

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ABSTRACT

Rapid and accurate detection of drug-resistant bacteria is important for AMR control, but conventional detection methods have problems in accuracy and rapidity. The MBT STAR-Carba and MBT STAR-Cepha kits have the advantage of being reported to the clinical side one to two days earlier than phenotypic confirmation testing. In this study, we evaluated the concordance between the two kits and genotypes of 80 carbapenemase-producing, 63 ESBL-producing, 41 carbapenemase-non-producing, and 62 ESBL-non-producing strains with previously confirmed resistance genes. The sensitivity and specificity of the Carba kit for detecting carbapenemase production were good at 97.5% and 90.2%, respectively, and stealth-type strains that do not meet the criteria for notification of CRE for Class 5 infections in Japan could also be detected. The Cepha kit had a good detection sensitivity of 100% for ESBL-producing bacteria, but about 30% of ESBL-nonproducing bacteria gave false-positive or withholding results. Some ESBL-nonproducing strains may be false-positive or withholding because they carry the chromosomal AmpC-type β-lactamase gene. Since the measurement time for both kits is approximately 2 hours and the method is simple, it is possible to promptly report resistant bacteria by paying attention to the retest conditions, which is expected to contribute to the proper use of antimicrobial agents.

Keywords: Carbapenemase-producing Enterobacteriaceae; Extended Spectrum β-Lactamase (ESBL); producing Enterobacteriaceae; MALDI-TOF MS; MBT STAR-Carba kit; MBT STAR-Cepha kit

Abbreviations: ESBL: Extended Spectrum β-Lactamase; CP-GNR: Carbapenemase Producing Gram Negative Rods; AMR: Antimicrobial Resistance; CLSI: Clinical and Laboratory Standards Institute; MCIM: Modified Carbapenem Inactivation Method, MRSA: Methicillin-Resistant Staphylococcus Aureus; MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry; KPC: Klebsiella Pneumoniae; IMP: Imipenemase Metallo-B-Lactamase; NDM: New Delhi Metallo-β-Lactamase; CRE: Carbapenemase-Resistant Enterobacterales; CMZ :Cefmetazole; LMOX :Latamoxef; PIPC/TAZ :Piperacillin/Tazobactam

Introduction

The most important drug resistance mechanism in Gram-negative bacteria, including Enterobacteriaceae, is the production of β-lactamases that inactivate β-lactam antibiotics [1]. Among β-lactamase-producing bacteria, carbapenemase producing Gram negative rods (CP-GNR) and Extended Spectrum β-Lactamase (ESBL) producing bacteria in particular are resistant to most β-lactam antibiotics, causing intractable infections [2,3]. In addition, these β-Lactamase-producing genes are encoded on plasmids, which can easily spread drug resistance between different bacterial species through zygotic transmission, resulting in healthcare-associated infections due to outbreaks [4,5]. Furthermore, drug-resistant bacterial infections are not only a cause of prolonged hospital stays and increased mortality, but also affect healthcare urgency, so Antimicrobial Resistance (AMR) measures are being implemented worldwide [6]. Therefore, when practicing AMR countermeasures, it is necessary to establish a microbiological testing environment that can quickly and accurately detect drug-resistant bacteria and estimate drug-resistance genes. Currently, the detection of these β-lactamase-producing Gram-negative rods is defined by the Clinical and Laboratory Standards Institute (CLSI) standard methods, which are global standards [7]. For carbapenemase-producing bacteria, the CarbaNP, modified carbapenem inactivation method [8] (mCIM), and genetic testing [9] for carbapenemase-producing bacteria, and micro-liquid dilution method and disk method [10] for ESBL bacteria.

In Japan, the β-lactamase-producing bacteria identification medium [11] and Cica beta Test (Kanto Chemical Co., Ltd.) [12] are commercially available as simple rapid test reagents and are used at many facilities. However, phenotypic testing methods such as CarbaNP, mCIM, and the Cica beta test may not detect some types of drug resistance genes. In addition, the mCIM, which requires a culture test after the identification of the bacterial species and the results of the drug susceptibility test are known, requires another day to confirm β-lactamase production, which presents a problem in terms of speed. On the other hand, detection of β-lactamase-producing bacteria by genetic testing is a highly sensitive and rapid test method because it can detect β-lactamase-producing bacteria directly from the test material. However, it is necessary to keep in mind the problems unique to genetic testing, such as nonspecific reactions and the effects of foreign substances [13], as well as the fact that known primers do not react with new drug-resistant genes that have emerged in recent reports [14]. Recently, mass spectrometers based on the principle of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) have been installed in many clinical microbiology laboratories as a rapid identification method for microorganisms. The introduction of MALDI-TOF MS has dramatically changed the conventional identification test flow, and once a single colony is obtained, it is possible to differentiate the species with the same level of accuracy as 16S rRNA gene analysis in only 10 minutes.

MALDI Biotyper (Bruker Daltonics, Inc.), which is based on MALDI-TOF MS, is equipped with a subtyping module and a STAR-BL module to detect β-lactamase-producing Gram-negative rods, and these functions are used to detect methicillin- These functions can be used to detect methicillin-resistant Staphylococcus aureus (MRSA), Bacteroides fragilis carrying the cfiA gene, and carbapenemase-producing Klebsiella pneumoniae (KPC) [15,16]. In Japan, the MBT STAR-Carba kit for the detection of carbapenemase-producing bacteria (Bruker Daltonics Co., Ltd.; Carba kit) and the MBT STAR-Cepha kit for the detection of cephalosporinase-producing bacteria (Bruker Daltonics Co., Ltd.; Cepha kit) were released commercially in February 2020. This has shortened the detection of β-lactamase to approximately 2 hours, allowing for detection in routine microbiology tests at facilities with MALDI Biotyper. Since both kits can also detect β-lactamase production from blood culture, the Carba/Cepha kit can be used to confirm the presence or absence of β-lactamase production on the same day that the species identified by MALDI Biotyper is confirmed, thereby shortening the time between isolation culture and phenotypic testing and enabling a two-day early report to the clinical side.

As for the evaluation of Carba / Cepha kit, there are reports of ESBL and imipenemase metallo-β-lactamase (IMP) type [17]., but there are no reports of New Delhi metallo-β-lactamase (NDM) type, VIM metallo-β-lactamase (VIM) type, KPC, oxacillin carbapenemase-48-like (OXA) type, or other than IMP type. Therefore, we evaluated the performance of the Carba/Cepha kit in detecting ESBL-producing bacteria and CP-GNR strains carrying drug resistance genes of the IMP, NDM, VIM, KPC, and OXA types, which are called the "big five" of CP-GNR.

Materials and Methods

Bacterial Strains

Strains isolated from clinical materials at the International University of Health and Welfare Group Hospitals were collected at the Center for Clinical Microbiology and Genetic Testing Research, Fukuoka School of Health and Medical Sciences, International University of Health and Welfare, and selected and used from stored Enterobacteriaceae and glucose non-fermentative Gram-negative rods. 69 Enterobacteriaceae strains producing carbapenemase (IMP type or NDM type or VIM type or KPC or OXA-48), 4 strains of Pseudomonas aeruginosa and 7 strains of Acinetobacter baumannii were used to evaluate Carba kit performance. As a control, 41 strains of Enterobacteriaceae were used for carbapenemase non-producing bacteria (Table 1). K. pneumoniae NCTC 13443 was used as the positive control strain in the Carba kit and Escherichia coli ATCC 25922 as the negative control. 52 strains of E. coli and 11 strains of K. pneumoniae ESBL-producing bacteria (Cefotaxime (CTX)-M type or TEM type or SHV type) were used to evaluate the performance of the Cepha kit. As a control, 62 strains of Enterobacteriaceae were used for ESBL non-producing bacteria (Table 2). The positive control strain for the Cepha kit was clinical isolate ESBL-producing (CTX-M-9 group and TEM-bearing) E. coli, and the negative control strain was E. coli ATCC 25922.

Table 1: Target strains used in carba kit.

	Bacteria species	Number of strains	Genotype (breakdown)
Carbapenemase-producing bacteria (Gram-negative fermenting bacteria)	Klebsiella pneumoniae	27	IMP type (7), NDM type (7), VIM type (1), KPC type (2), OXA-48(8), NDM type/OXA-48(2)
	Escherichia coli	20	IMP type (3; of which IMP-6(1)), NDM type (13), KPC type (2), OXA-48(1), NDM type/OXA-48(1)
	Enterobacter cloacae	19	IMP type (19)
	Other Enterobacterales*1	3	IMP type (3)
Carbapenemase-producing bacteria (Gram-negative non-fermentative bacteria)	Acinetobacter baumannii	7	OXA-48(3), NDM type/OXA-48(4)
	Pseudomonas aeruginosa	4	IMP type (2), VIM type (2)
Carbapenemase non-producing bacteria	Escherichia coli	23	
	Enterobacter cloacae	9	
	Klebsiella spp.	4	
	Other Enterobacterales*2	5	

Note:*1 Citrobactor spp. Morganii include

*2 Citrobactor spp. Morganii. Serratia marcescens include

Table 2: Target strains used in cepha kit.

	Bacteria species	Number of strains	Genotype (breakdown)
ESBL-producing bacteria	Escherichia coli	52	CTX-M-1 group(1), CTX-M-1 group & TEM(6), CTX-M-2 group(3), CTX-M-2 group & TEM(1), CTX-M-9 group(8), CTX-M-9 group & TEM(23), TEM(4), SHV(5), SHV & TEM(1)
	Klebsiella pneumoniae	11	CTX-M-1 group & SHV & TEM(1), CTX-M-2 group & SHV(1), CTX-M-9 group & SHV(8), SHV(1)
ESBL non-producing bacteria	Klebsiella spp.	25	
	Escherichia coli	19	
	Enterobacter cloacae	9	
	Other Enterobacterales*1	9	

Note: *1 Citrobactor spp. Proteus mirbilis include

Identification Test and Culture

Target species were identified using MALDI Biotyper (MBT Compass 4.1, BDAL library ver. 5.0.0.0). The cell smear method was used, and those with Score Values less than 2.0 were re-measured by the ethanol/formic acid extraction method [18]. The strains were cultured on "KBM" BTB II agar medium (Kosin Bio Co., Ltd.) under aerobic conditions at 35°C for 20-24 hours, and the single colonies grown were pure-cultured strains under the same culture conditions.

Drug Susceptibility Testing

Cultures were grown according to the method shown in the procedure for the WalkAway® 96 Plus System (Beckman Coulter Co., Ltd.). The target strains were inoculated on a [19] Microscan Neg MIC 3J panel (Beckman Coulter Inc.) after preparation of the bacterial solution by the prompt method. Cultivation was performed on a WalkAway® 96 Plus system at 35°C for 18 hours under aerobic incubation conditions. The results of the drug susceptibility test were automatically determined by LabPro System version 5.0 according to CLSI's M100-ED30 [7]. If any discrepancy was found between the drug susceptibility test results and the Carba /Cepha kit results, the Carba kit was retested using the mCIM method [8] and the Cepha kit was retested using the Microscan Neg MIC 3.31E panel.

Drug Resistance Gene Testing

CP-GNR drug resistance genes were measured with Xpert Carba-R (Beckman Coulter Co., Ltd.) reagents using the GeneXpert System GX-IV instrument (Beckman Coulter Co., Ltd.) 14). For IMP-type strains, DNA was extracted using Cica geneus® DNA extraction reagent (Kanto Chemical Co., Ltd.) to confirm the IMP-1 group or IMP-6 group, and differentiated using Cica Geneus® Carbapenemase Genotype Detection KIT 2 (Kanto Chemical Co., Ltd.).ESBL-producing bacteria were

DNA extracted with Cica geneus® DNA extraction reagent, and the type of genes possessed was confirmed with Cica geneus® ESBL Genotype Detection KIT2 (Kanto Chemical Co., Ltd.). Cica geneus® does not detect TEM or SHV other than ESBL.

Carba Kit Bacterial Solution Preparation Method

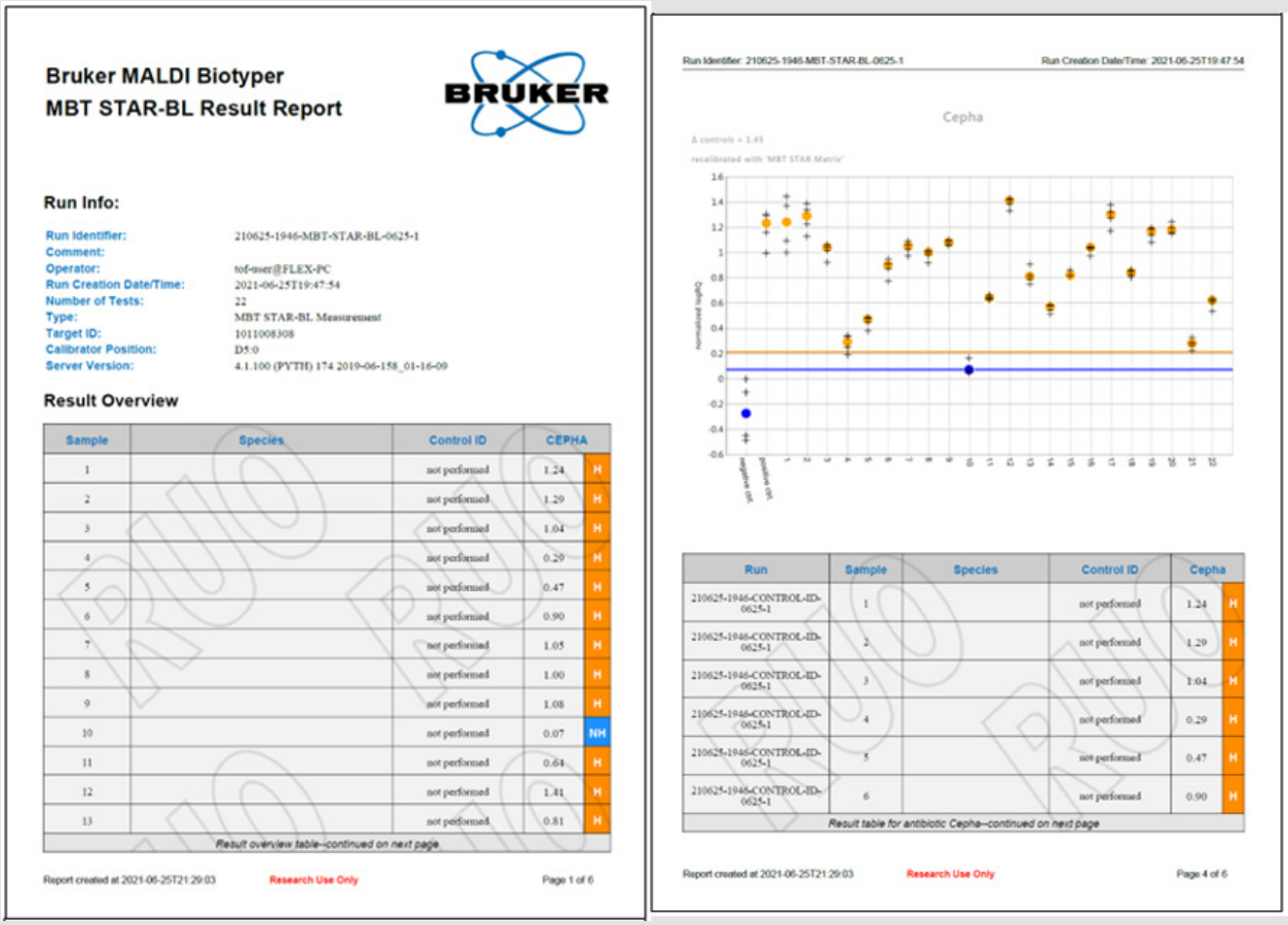
The MBT STAR -BL Assay manual was followed. For the Carba kit, 50 µL of MBT STAR Buffer was added to the MBT STAR-Carba Antibiotic Reagent tube, and then mixed with a vortex mixer for 30 seconds to dissolve the drug solid-phased in the tube. After 2 minutes of standing, the fresh isolate was mixed again with a vortex mixer for 30 seconds, and 5-10 mg of the fresh isolate was suspended in MBT STAR-Carba Antibiotic Reagent tubes.

Cepha Kit Bacterial Solution Preparation Method

The MBT STAR -BL Assay manual was followed. To the wells of the MBT STAR-Cepha Antibiotic Reagent microtiter plate supplied with the Cepha kit, 75 µL of MBT STAR Buffer was added to dissolve the drug solid-phased in the wells. After 2 minutes of standing, 50 µL of dissolved MBT STAR Buffer was inoculated into 1.5 mL microtubes, and 5-10 mg of fresh isolate was suspended in this solution.

Measurement Method with MALDI-TOF MS

The prepared MBT STAR-Carba Antibiotic Reagent tubes and the prepared 1.5-mL microtubes were placed in a thermomixer and incubated at 35°C, 900 rpm for 30 minutes with shaking. However, when measuring Acinetobacter spp with the Carba kit, the culture was incubated at 35°C for 60 minutes with shaking at 900 rpm. Each tube was then centrifuged at 13,000 rpm for 2 minutes, and 1 µL of the supernatant was dropped onto the target plate, two spots per strain. After the target plates were dried, 1 µL of MBT STAR Matrix was dropped on each spot, dried again, and measured with a MALDI biotipers. For the measurement conditions, the MBT STAR-BL module was set up in MBT Compass version 4.1 and the MBT_STAR_BL per Method was selected in flexControl version 4.0.



Bruker MALDI Biotyper
MBT STAR-BL Result Report

BRUKER

Run Info:

Run Identifier:	210625-1946-MBT-STAR-BL-0625-1
Comment:	
Operator:	tof-user@FLEX-PC
Run Creation Date/Time:	2021-06-25T19:47:54
Number of Tests:	22
Type:	MBT STAR-BL Measurement
Target ID:	1011008308
Calibrator Position:	D5:0
Server Version:	4.1.100 (PYTH) 174 2019-06-158_01-16-09

Result Overview

Sample	Species	Control ID	CEPHA	
1		not performed	1.24	H
2		not performed	1.29	H
3		not performed	1.04	H
4		not performed	0.29	H
5		not performed	0.47	H
6		not performed	0.90	H
7		not performed	1.05	H
8		not performed	1.00	H
9		not performed	1.08	H
10		not performed	0.07	NH
11		not performed	0.64	H
12		not performed	1.41	H
13		not performed	0.81	H

Result overview table--continued on next page.

Report created at 2021-06-25T21:29:03 Research Use Only Page 1 of 6

Run Identifier: 210625-1946-MBT-STAR-BL-0625-1 Run Creation Date/Time: 2021-06-25T19:47:54

Cepha

Run	Sample	Species	Control ID	Cepha	
210625-1946-CONTROL-ID-0625-1	1		not performed	1.24	H
210625-1946-CONTROL-ID-0625-1	2		not performed	1.29	H
210625-1946-CONTROL-ID-0625-1	3		not performed	1.04	H
210625-1946-CONTROL-ID-0625-1	4		not performed	0.29	H
210625-1946-CONTROL-ID-0625-1	5		not performed	0.47	H
210625-1946-CONTROL-ID-0625-1	6		not performed	0.90	H

Result table for antibiotic Cepha--continued on next page

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Figure 1: Example of MBT STAR-BL module measurement result report.

Carba / Cepha Kit Result Interpretation and Evaluation Method

The results of the Carba/Cepha kit are displayed with logRQ values and "H", "NH", "? (Figure 1). The logRQ value is determined by setting the difference between the positive and negative controls as 1 and quantifying the degradation activity of the target strain relative to the positive control. With regard to the Carba kit's determination thresholds, a logRQ value of 0.40 or higher is "H": if the enzyme producing carbapenemase is found, a logRQ value of 0.19 or lower is "NH": if no enzyme production is found, and a logRQ value of 0.20 to 0.39 is "?: Judgment is judged to be withheld. In the Cepha kit, a logRQ value of 0.22 or higher is "H": if the enzyme producing ESBL is found, a logRQ value of 0.07 or lower is "NH": if no enzyme production is found, a logRQ value in the range of 0.08 to 0.21 is "?: Judgment is judged to be withheld. The performance of the Carba/Cepha kit was evaluated by calculating the sensitivity and specificity of detection of carbapenemase-producing bacteria or ESBL-producing bacteria, positive predictive value, and negative predictive value. The performance was evaluated including "? (pending judgment) were included in the evaluation of the performance.

Result

Identification Results of Bacterial Strains and Drug Resistance Genes

(Table 1) shows the identification results of CP-GNR by MALDI Biotyper and genotypes by GeneXpert System GX-IV, and (Table 2) shows the identification results of ESBL-producing bacteria by MALDI Biotyper and genotypes by Cica geneus® ESBL Genotype Detection KIT2. All strains used in this study had a MALDI Biotyper score of 2.0 or higher.

Drug Susceptibility Testing

Among 80 CP-GNR strains, KPC-type E. coli 1 showed imipenem (IPM) MIC ≤ 1 μg/mL and meropenem (MEPM) MIC ≤ 0.12 μg/mL, OXA-48-like type E. coli 1 showed IPM MIC ≤ 1 μg/mL and MEPM MIC 1 μg/mL, IMP-1 type 1 Enterobacter cloacae showed IPM MIC ≤ 1 μg/mL and MEPM MIC 1 μg/mL, and these strains were stealth types that did not fall under the Japanese category 5 infection, Carbapenemase-resistant Enterobacterales (CRE). Of the 41 carbapenemase-nonproducing strains, 13 were determined to be CRE, meeting the criteria for category 5 infection. All 63 ESBL-producing strains tested positive according to CLSI's ESBL screening criteria. Of the 62 ESBL non-producing strains, 35 E. coli and K. pneumoniae and Proteus mirabilis strains that met CLSI's ESBL screening criteria were determined to be negative for ESBL screening. The remaining 27 ESBL non-producing strains were excluded from the ESBL screening decision because they were non-target species for ESBL screening.

Performance Evaluation of Carba Kit

Of the 80 carbapenemase-producing strains in the Carba kit, 78 tested positive for carbapenemase and 2 tested negative for carbapenemase. Among the 41 carbapenemase non-producing strains, 2 strains tested positive for carbapenemase, 37 strains tested negative for carbapenemase, and 2 strains withheld judgment. These results showed that the Carba kit had a sensitivity of 97.5% (78/80), specificity of 90.2% (37/41), positive predictive value of 97.5% (78/80), and negative predictive value of 94.8% (37/39) (Table 3). The mean LogRQ value for positive carbapenemase was 0.99 (range: 0.58-1.18). The two carbapenemase-producing strains that gave false-negative results were NDM E. coli and E. coli harboring NDM and OXA-48-like types, which showed IPM and MEPM MIC values of ≥16 μg/mL. The LogRQ values were as low as 0.14 and 0.20, respectively (Table 4). Mass spectral waveforms were detected at approximately 300 m/z and 489 m/z, the masses of carbapenem antibacterial agents, in the mass spectra of the strains that showed false negative results, as well as in the carbapenemase nonproducing strains (Figure 2A,2B,2C,2D). The three stealth-type strains with low MIC values for carbapenems showed LogRQ values above 0.8 and all tested positive for carbapenemase. The two carbapenemase non-producing strains that showed false positives were E. coli and E. cloacae, with LogRQ values of 0.57 and 0.59. The mass spectral waveforms of these two strains were similar to those of carbapenemase-nonproducing bacteria, with no loss of antimicrobial mass due to hydrolysis of carbapenems, and spectral waveforms at approximately 300 m/z and 489 m/z with lower ion intensities than those of carbapenemase-nonproducing bacteria (Figure 2D). The two strains that showed withholding of judgment were E. coli and E. cloacae (Table 3). The two E. coli strains with false positives and withheld determinations had IPM MIC ≤ 1 μg/mL and MEPM MIC ≤ 0.12 μg/mL for drug susceptibility testing, while the two E. cloacae strains with false positives and withheld determinations had IPM MIC 2 μg/mL, cefmetazole (CMZ) MIC ≥ 64 μg/mL, and MEPM MIC 1 μg/mL and were determined to be CRE. Of the 13 carbapenemase non-producing CRE strains, the remaining 11 were 1 E. coli, 6 E. cloacae, 1 Citrobacter koseri, 1 Morganella morganii, and 2 Serratia marcescens, all of which tested negative for carbapenemase All were determined to be carbapenemase-negative. The false-positive or false-negative and pending determination strains were tested for confirmation of carbapenemase production by the phenotypic mCIM method. The false-negative strains of carbapenemase-producing bacteria were determined to be positive by the mCIM method, while the false-positive and pending determination strains of carbapenemase non-producing bacteria were determined to be negative by the mCIM method.

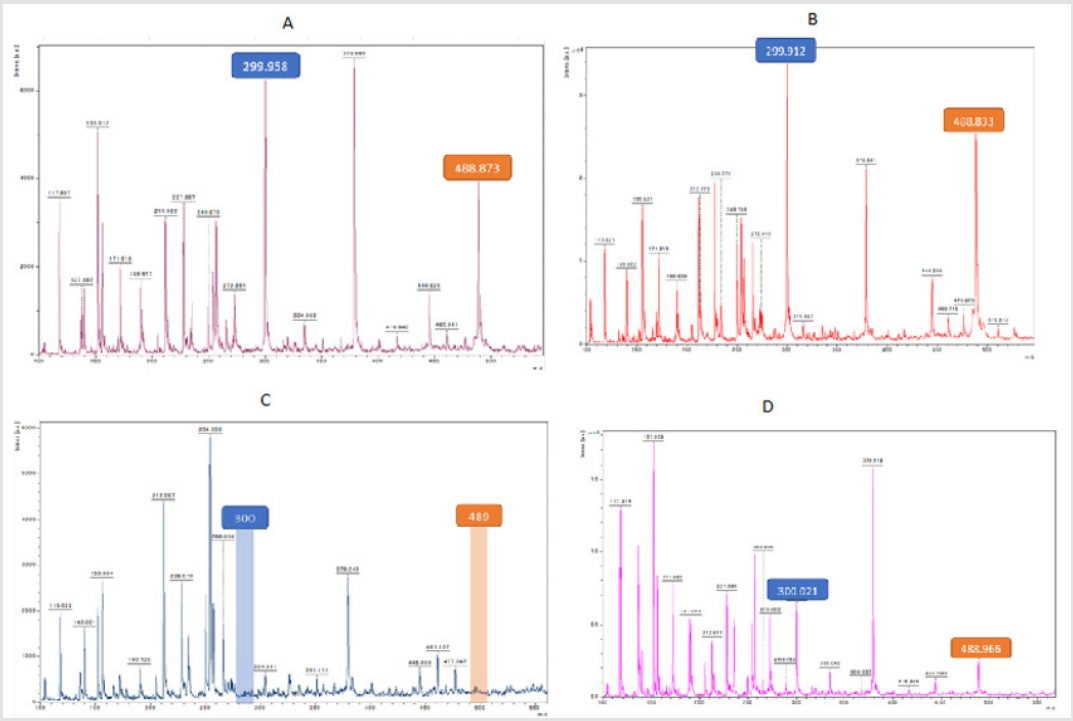

Figure 2:
A. Spectrum of carbapenemase-nonproducing bacteria
300 m/z: Antibacterial drug + proton addition
489 m/z: Antibacterial drug + matrix molecule
The above two antimicrobial drug related masses are detected.
B. False-negative spectrum of carbapenemase-producing bacteria Detection of antimicrobial-associated masses as well as spectra of carbapenemase-nonproducing bacteria.
C. Spectra of carbapenemase-producing bacteria: Antimicrobial-associated masses disappear Antimicrobial-associated mass disappears.
D. False positive spectrum of carbapenemase non-producing bacteria: The Detect antimicrobial-associated masses with weak ionic strength.

Table 3: Results of STAR-BL Carba kit.

	Positives (H)	Negative (NH)	Suspension of judgment (?)	Total
Carbapenemase-producing bacteria	78	2	0	80
Carbapenemase non-producing bacteria	2	37	2	41
Total	80	39	2	121

Table 4: False Positives, False Negative and Suspension Determination in the carba Kit.

	Bacteria species	Judgment value	logRQ	GeneXpert	MIC	(ug/m)	mCIM
					IPM	MEPM	
False Negative	Escherichia coli	NH	0.14	NDM	≧16	≧16	Positives
	Escherichia coli	NH	0.20	NDMtype/OXA-48	≧16	≧16	Positives
False Positives	Escherichia coli	H	0.57		≦1	≦0.12	Negative
	Enterobacter cloacae	H	0.59		2	1	Negative
Suspension of judgment	Escherichia coli	?	0.25		≦1	≦0.12	Negative
	Enterobacter cloacae	?	0.31		2	1	Negative

Performance Evaluation of Cepha Kit

In the evaluation of the Cepha kit for detection of ESBL-producing bacteria, all 63 ESBL-producing strains were determined to be positive for cephalosporinase. Among the 62 ESBL non-producing strains, 7 strains tested positive for cephalosporinase, 41 strains tested negative for cephalosporinase, and 14 strains withheld judgment. These results showed that the detection performance with the Cepha kit was 100% sensitivity (63/63), 66.1% specificity (41/63), 90.0% positive predictive value (63/70), and 100% negative predictive value (41/41) (Table 5). The mean LogRQ value for cephalosporinase-positive was 1.07 (range: 0.28-1.42). The seven ESBL-nonproducing strains that tested false positive were one E. coli, one K. pneumoniae, two Klebsiella oxytoca, two E. cloacae, and one Citrobacter koseri, all with LogRQ values less than 0.50 (mean LogRQ value: 0.32, range range: 0.23-0.40) (Table 6). The 14 strains that showed withholding of judgment were 2 E. coli, 3 K. pneumoniae, 1 K. oxytoca, 2 Klebsiella aerogenes, and 6 E. cloacae. Of the 7 isolates that gave false positive results and 14 isolates that showed a withholding of judgment, E. coli and K. pneumoniae and K. oxytoca, which are ESBL target organisms for the CLSI standard method, all had negative ESBL screening tests, and the Microscan Neg MIC 3.31E panel was used to The ESBL confirmation test also confirmed that the bacteria were ESBL non-producers.

Table 5: Results of STAR-BL Cepha kit.

	Positives (H)	Negative (NH)	Suspension of judgment (?)	Total
ESBL-producing bacteria	63	0	0	63
ESBL non-producing bacteria	7	41	14	62
Total	70	41	14	125

Table 6: False positives, False Negatives and pending determinations in the cepha kit.

		Judgment		MIC	(ug/mL)			
	Bacteria species	Value	logR RQ	CTX	CAZ	CPDX	CTRX	AZT
False Positives	Escherichia coli	H	0.23	≦1	≦1	≦2	≦1	≦4
	Klebsiella pneumoniae	H	0.35	≦1	≦1	≦2	≦1	≦4
	Klebsiella oxytoca	H	0.38	≦1	≦1	≦2	≦1	≦4
	Klebsiella oxytoca	H	0.40	≦1	≦1	≦2	≦1	≦4
	Enterobacter cloacae	H	0.25	≦1	≦1	≦2	≦1	≦4
	Enterobacter cloacae	H	0.39	≦1	≦1	4	≦1	≦4
	Citrobacter koseri	H	0.33	≦1	≦1	4	≦1	≦4
	Escherichia coli	?	0.15	≦1	≦1	≦2	≦1	≦4
	Escherichia coli	?	0.11	≦1	≦1	≦2	≦1	≦4
	Klebsiella pneumoniae	?	0.10	≦1	≦1	≦2	≦1	≦4
	Klebsiella pneumoniae	?	0.09	≦1	≦1	≦2	≦1	≦4
	Klebsiella pneumoniae	?	0.12	≦1	≦1	≦2	≦1	≦4
	Klebsiella oxytoca	?	0.19	≦1	≦1	≦2	≦1	≦4
	Klebsiella aerogenes	?	0.15	≦1	≦1	≦2	≦1	≦4
	Klebsiella aerogenes	?	0.18	≦1	≦1	≦2	≦1	≦4
	Enterobacter cloacae	?	0.21	≦1	≦1	≦2	2	≦4
	Enterobacter cloacae	?	0.22	≦1	≦1	≦2	≦1	≦4

	Enterobacter cloacae	?	0.10	≦1	≦1	≦2	≦1	≦4
	Enterobacter cloacae	?	0.18	≦1	≦1	≧8	≦1	≦4
	Enterobacter cloacae	?	0.10	≦1	≦1	≧8	≦1	≦4
	Enterobacter cloacae	?	0.12	≦1	≦1	≦2	≦1	≦4

Discussion

To evaluate the performance of the Carba and Cepha kits, we tested CP-GNR, which carries a carbapenemase-producing drug resistance gene, and ESBL-producing bacteria, which are Gram-negative rods and frequently detect drug-resistant bacteria. The Carba/Cepha kit is based on the principle of detecting the mass change that occurs when CP-GNR- or ESBL-producing bacteria hydrolyze the antimicrobials contained in each reagent kit. Therefore, even new drug-resistant gene-carrying bacteria with no set primers that are easily missed by genetic testing can be determined by the presence or absence of enzyme activity, which has the advantage of being able to determine the presence or absence of enzyme activity. In particular, among CP-GNRs, the stealth type has been pointed out as an issue that cannot be detected by drug susceptibility testing due to the low MIC values of carbapenems. When screening CP-GNRs with drug susceptibility testing, care should be taken to avoid overlooking stealth forms that are sensitive to carbapenems. When screening stealth CP-GNRs in drug susceptibility testing, the characteristics of resistance to latamoxef (LMOX), which detects class B of the Ambler classification well, and piperacillin/tazobactam (PIPC/TAZ), which has increased MIC values in class D can be used [20,21]. However, the KPC-type E. coli 1 strain showed low MIC values in the sensitive region even with LMOX and PIPC/TAZ, making it a strain that was overlooked in the results of drug susceptibility testing. Therefore, it is difficult to detect such stealth-type strains in drug susceptibility testing [3].

The Carba kit was able to detect carbapenemases well against two IMP-6 strains with weak IPM degrading activity, four KPC and 12 OXA strains with relatively low MIC values for carbapenems, and three stelless strains. This is due to the fact that even if the carbapenem antibacterial agents have weak degradation activity and the strains show MIC values in the sensitive range for IPM or MEPM, the high or low MIC values do not affect the Carba kit's judgment because of the mass change caused by hydrolysis of the antibacterial agents in the Carba kit (Figure 2A-2D). Validation of the Carba kit reported by Dortet [22] et al. showed a sensitivity of 100% and specificity of 98.2%, and also detected carbapenem low-susceptibility strains. Ota [17] et al. also validated IPM-type carbapenemase-producing bacteria and reported 100% detection sensitivity for carbapenemase-producing bacteria, including 11 strains of IMP-6 type, one of the stealth types. Therefore, the results suggest that the detection performance of the kit is not affected by resistant genotypes or strains with low MIC values for carbapenems, which are difficult to detect in routine testing. The two strains that were false-negative by CP-GNR in this study were both IPM; MIC ≥ 16 μg/mL and MEPM; MIC ≥ 16 μg /mL and were strains with high MIC values for carbapenems. If carbapenem MICs and Carba kit results diverge, misinformation to the clinic can be avoided by confirming the presence of carbapenemase production by other means, such as genetic testing.

The two strains that gave false-negative results had high MIC values for carbapenems, suggesting that they are highly active in degrading carbapenems, but they did not completely hydrolyze the antimicrobials in the Carba kit as carbapenemase-producing bacteria do (Figure 2B). The Carba kit accurately tested negative for most carbapenemase-nonproducing CRE. The four strains that were false positive or withheld in this validation were two carbapenemase-non-producing CRE strains and two carbapenemase-non-producing non-CRE strains. Although the withheld strains can be retested by the mCIM method or genetic testing, one of the two false positive strains is a CRE and falls under the notification criteria of the Infectious Disease Control Law, which may lead to false reports. The two strains that tested false positive with the Carba kit had LogRQ values less than 0.6, with IPM MIC values ≤ 2 μg /mL and MEPM MIC values ≤ 1 μg /mL, while the CP-GNR showed higher MIC values for carbapenems, and stealth-type strains also showed a trend toward higher LogRQ values of 0.8 or higher. Unfortunately, no strains were identified in this study that met both conditions: MIC values of ≤2 μg /mL for carbapenems and LogRQ values of 0.8 or higher for detectable stealth forms. Based on the above results, to accurately test for carbapenemase-producing bacteria using the Carba kit in the MALDI Biotyper, we recommend the following:

1) When the carbapenem MIC value is ≤ 2 μg /mL and the LogRQ value is 0.4-0.8,

2) When the judgment is reserved,

3) When the carbapenem MIC value is ≥ 16 μg /mL and the Carba kit result is negative.

If the Carba kit result is negative, we recommend retesting using a different method. We believe that this type of condition setting would prevent misreporting to clinicians when tests are performed with the Carba kit. The Cepha kit was able to detect all CTX-M, TEM, and SHV types of ESBL-producing bacteria, and no false negative strains were identified. From this study, the detection performance of the Cepha kit for ESBL-producing bacteria is considered to be very good, but the

sensitivity of the Cepha kit for SHV-type ESBL-producing bacteria was 13% (2/15) in the study reported by Ota et al. 17), which is different from our report. In the initial phase of our validation, we selected strains with weak degrading activity of the antimicrobials contained in the Cepha kit as positive controls for the Cepha kit. As a result, we confirmed that ESBL strains with good degradation activity tended to have higher LogRQ values of 2 to 3 or more. The LogRQ value of the ESBL-producing bacteria used in this study was 1.07, suggesting that the positive control strains had general or strong degrading activity against Cepha kit-containing antimicrobial agents. Although the protocol is similar to that of Ota et al.'s study, the clinical isolates used in the positive control are different, and the ESBL-producing bacteria used by Ota et al. had a relatively high LogRQ value of 2 or higher, suggesting that the strain used in the positive control may affect detection performance. It is important to understand that the Cepha kit is designed to detect cephalosporinase-producing bacteria, not ESBL-producing bacteria.

Thus, ESBL-producing bacteria will also be positive with the Cepha kit, but AmpC-type β-lactamase-producing bacteria will also be positive with the Cepha kit. Non-ESBL-producing strains included strains other than those subject to ESBL by the CLSI standard method (E. coli, K. pneumoniae, K. oxytoca, and P. mirabilis), and false positives or reservations were reported by 8 strains of E. cloacae, 2 strains of K. aerogenes, and 1 strain of C. koseri. The false positive or withheld strains were 8 E. cloacae, 2 K. aerogenes, and 1 C. koseri. It should be noted that these strains are chromosomal AmpC-type β-lactamase producing and may be positive in the Cepha kit depending on the amount of enzyme produced. Four of the seven false-positive strains and six of the 14 strains with reservations of judgment were ESBL target strains, but all were negative in the ESBL screening test using the CLSI standard method. LogRQ values obtained from this study showed that false-positive strains had a LogRQ value of less than 0.5 (mean LogRQ value: 0.32, range: 0.23-0.40), which is lower than the mean LogRQ value of 1.07 (range: 0.28-1.42) for ESBL-producing bacteria. The mean LogRQ value was 1.07 (range: 0.28-1.42). Therefore, when the Cepha kit is used, it should be limited to ESBL target species and reported to the clinician along with the ESBL screening test results for drug susceptibility testing. In the case of using the Cepha kit directly from the culture medium of a positive blood culture bottle, we believe that false reports can be avoided by retesting and reporting a LogRQ value of less than 0.5.

For the Carba kit, K. pneumoniae ATCC BAA-1705 is recommended as positive control and K. pneumoniae ATCC 700603 as negative control; for the Cepha kit, E. coli CCUG 62975 as positive control and E. coli ATCC 25922 is recommended as a negative control. However, if these strains cannot be prepared, clinical strains with good enzyme activity may be used as control strains. As a precaution, K. pneumoniae ATCC 700603, which is used as a positive control for the ESBL confirmation test of the CLSI standard method, is annotated as not to be used in the Cepha kit due to its tendency to change enzyme activity. The measurement time of the Carba /Cepha kit is approximately 2 hours for a single measurement, allowing for reporting one day earlier than conventional detection methods. In this verification, performance was evaluated using colonies grown on agar medium, but from the viewpoint of early treatment, it would be useful if carbapenemase- and ESBL-producing bacteria could be detected directly from blood culture bottle solutions using both kits. In particular, direct rapid identification testing from positive blood culture bottles by MALDI-TOF MS enables clinical reporting of bacterial species about 2 days earlier. At the same time, the use of the Carba/Cepha kit with MALDI-TOF MS enables reporting of carbapenemase or ESBL production about 3 days earlier than with conventional culture testing methods.

By introducing this type of test operation into clinical microbiology laboratories, reporting of drug-resistant bacteria will be realized within the same day of a positive blood culture bottle, which is expected to contribute to clinical practice through appropriate use of antimicrobial agents.

Conclusion

Performance evaluation of the Carba/Cepha kit showed that both sensitivity and specificity of detection by the Carba kit were good, and even stealth-type carbapanemase-producing bacteria with low MIC values could be detected. The Cepha kit showed good detection sensitivity of 100% for ESBL-producing bacteria, but about 30% of the non-ESBL-producing bacteria showed false positives and withholding of judgment. However, this suggests the usefulness of ESBL detection when evaluating ESBL target strains by the CLSI standard method when judged in conjunction with screening criteria by drug susceptibility testing, since about half of the strains that gave false positive results contained strains that harbor chromosomal AmpC. Especially in blood culture testing, which requires appropriate antimicrobial treatment at an early stage, incorporating both of these kits into routine testing is expected to realize high quality testing.

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Declaration of Competing Interest

The authors declare no conflict of interests.

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