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Detection of extended-spectrum β-lactamases producing *Enterobacteriaceae* using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry based MBT STAR-BL software module with β-lactamase inhibition assay depends on the bacterial strains

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ABSTRACT

Rapid and sensitive detection of extended-spectrum β-lactamases (ESBLs) is essential for infection control and antimicrobial treatment. Recently, a matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)-based MBT STAR-BL software module has been used for detecting β-lactamase activity; however, this system cannot differentiate ESBL producing bacteria from other third-generation cephalosporin-resistant strains. In this study, we utilized a MALDI-TOF MS-based MBT STAR-BL method to identify ESBL activity with β-lactamase inhibitors. A cefotaxime (CTX) hydrolysis assay, β-lactamase inhibition, clavulanic acid (CVA), and sulbactam (SBT) were used for detecting ESBL producers with the MBT STAR-BL software module. This software module automatically calculated the logRQ values in each assay. logRQ is the logarithm of the ratio of the summed hydrolyzed peak intensities to the summed non-hydrolyzed peak intensities and measured the efficiency of antibiotic hydrolysis. We divided the logRQ level of the β-lactamase inhibition assay by the logRQ value in the CTX hydrolysis assay, and we used this logRQ ratio as a measure of β-lactamase inhibition efficiency. We assessed the logRQ ratio calculated by this novel method for detecting ESBL producers in 132 *Enterobacteriaceae*. We performed the MALDI-TOF MS-based MBT STAR-BL approach with β-lactamase inhibitors for detecting ESBL producers and showed that the results of the inhibition assay with β-lactamase inhibitors depended on types of bacterial species. Furthermore, we improved elapsed times and accuracy in MBT STAR-BL methods by using proper β-lactamase inhibitors against specific bacterial strains to compare with the conventional standard lab method. The results suggest that the target bacterial species and β-lactamase inhibitors used were important for the utility of the MALDI-TOF MS-based MBT STAR-BL software module.

1. Introduction

Extended-spectrum β-lactamases (ESBLs) are enzymes found exclusively in *Enterobacteriaceae* that confer resistance to most β-lactam antibiotics (Paterson and Bonomo, 2005). ESBL production represents a serious clinical problem associated with a high mortality rate, increased length of stay, delay in appropriate therapy, and higher costs (Schwaber et al., 2006; Tumbarello et al., 2006). In addition, the prevalence of ESBL-producing microbes has been increasing because the plasmid encoding the ESBL gene can be transferred between bacteria via conjugation (Paterson and Bonomo, 2005; Thaden et al., 2016). Therefore, rapid and sensitive detection of ESBL-producing bacteria is essential for both patient care and infection control in clinical practice. Most clinical laboratories use culture-based phenotypic methods to discriminate between ESBL-producing bacteria and other third-generation cephalosporin-resistant strains with chromosomal AmpC-type β-lactamase and

Abbreviations: AUC, area under the curve; CTX, cefotaxime; CVA, clavulanic acid; ESBL, extended spectrum β-lactamase; $logRQ_{CTX+CVA/CTX}$, the inhibition rate of $logRQ_{CTX}$ with CVA; $logRQ_{CTX+SBT/CTX}$, the inhibition rate of $logRQ_{CTX}$ with SBT; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic; SBT, sulbactam

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K1-type β-lactamase because ESBL-producing but not chromosomal type β-lactamase strains are resistant to fourth-generation cephalosporins (Livermore, 1995; Paterson and Bonomo, 2005). Of note, chromosomal ampC-type β-lactamase hydrolyzes third-generation cephalosporins and is not inhibited by β-lactamase inhibitors such as clavulanic acid (CVA), while hyper-production of chromosomal K1-type β-lactamase is inhibited by CVA (Potz et al., 2004), but not by sulbactam (SBT) (Kimura et al., 1996). Although these phenotypic testing methods can detect ESBL producers, these methods require >16 h of incubation in routine laboratory use (Wayne, 2015). Therefore, rapid diagnostic tools to detect ESBL-producing bacteria, and especially to discriminate between ESBL and other chromosomal β-lactamases, are needed in clinical laboratories.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is routinely used to identify bacterial species by matching the mass spectrum generated from the analyte with spectra in a database (Wieser et al., 2012). This assay can rapidly identify pathogenic microbes, but predictions of drug susceptibility patterns based on bacterial identification alone have become inaccurate. Recently, Bruker Daltonics developed the automated and standardized MBT STAR-BL software module (Bremen, Germany) to detect β-lactamase activity by monitoring the mass peaks of the hydrolysis products of antibiotics such as ampicillin, piperacillin, cefotaxime (CTX), ceftriaxone, cefepime, ertapenem and meropenem (Doern and Butler-Wu, 2016). This software provides high sensitivity and specificity for detecting β-lactamase activity and is significantly faster than conventional methods (Doern and Butler-Wu, 2016). However, since it indirectly identifies the presence of at least one enzyme it cannot identify the activity of specific β-lactamases including ESBLs. Therefore, it is necessary to improve this MALDI-TOF MS-based MBT STAR-BL method to detect ESBL activity by establishing a method which excludes the possibility of chromosomal ampC-type and K1-type β-lactamases. Previous study has reported that this method using CVA is useful to rule out isolates producing ampC-type β-lactamase (Oviaño et al., 2014), while little has been reported on the method with other β-lactamase inhibitor to discriminate bacteria producing other β-lactamase types such as K1-type β-lactamase.

In this study, we detected ESBL-producing microbes using the MALDI-TOF MS-based MBT STAR-BL approach and β-lactamase inhibitors. To discriminate between ESBL-producing bacteria and other third-generation cephalosporin-resistant strains with ampC-type or K1-type β-lactamases, we used the logRQ level in each assay to calculate the logRQ ratio as a β-lactamase inhibition parameter. We then evaluated the use of the calculated logRQ ratio to distinguish ESBL from other third-generation cephalosporinases in *Enterobacteriaceae*.

2. Materials and methods

2.1. Bacterial isolates

We analyzed 132 *Enterobacteriaceae* strains isolated from 63 blood, 23 urine, 13 stool, 12 sputum, and 21 other samples at the Hamamatsu University School of Medicine, Shizuoka, Japan (Tables 1 and 2). These isolates were identified using the Bruker Microflex LT system and MALDI Biotyper Compass software 4.1.70 with the V7.0.0.0 spectra library (Bruker Daltonics, Bremen, Germany). We determined the susceptibility of all isolates with a conventional broth microdilution assay. Each clinical isolate was plated on blood agar and incubated at 36 °C overnight. The 96-well MicroScan panels (Beckmann Coulter, Krefeld, Germany) were inoculated with each isolate to yield an appropriate density of 10^5 CFU/mL and incubated for 16–20 h at 35 °C in ambient air. The minimum inhibitory concentrations were determined by the MicroScan WalkAway® 96 Plus system (Beckmann Coulter). The susceptible, intermediate, and resistance categories were based on Clinical and Laboratory Standards Institute document M100-S25 (Wayne, 2015). *Escherichia coli* ATCC 25922 was used as a β-lactamase-negative control strain.

Table 1
The distribution of identified species, ESBL-encoding genes, and the logRQ values in CTX resistant isolates.

Species identification	PCR detection of ESBL-encoding genes	logRQ values		
		CTX	CTX + CVA	CTX + SBT
Escherichia coli	CTX-M-2	0.81	0	ND[a]
Escherichia coli	TEM, CTX-M-9	0.41	0.09	ND
Escherichia coli	TEM	0.57	0.18	ND
Escherichia coli	TEM	0.27	0.04	ND
Escherichia coli	CTX-M-1, CTX-M-9	0.72	0.11	ND
Escherichia coli	TEM, CTX-M-9	0.98	0.08	ND
Escherichia coli	CTX-M-2	0.69	−0.1	ND
Escherichia coli	OXA-1, CTX-M-1	0.45	0.24	ND
Escherichia coli	CTX-M-9	0.96	0.02	ND
Escherichia coli	CTX-M-9	1.15	0.58	ND
Escherichia coli	CTX-M-9	0.77	0.12	ND
Escherichia coli	CTX-M-9	0.55	0.25	ND
Escherichia coli	TEM, CTX-M-9	0.59	0.28	ND
Escherichia coli	CTX-M-9	0.9	0.19	ND
Escherichia coli	CTX-M-9	1.46	0.45	ND
Escherichia coli	TEM, CTX-M-9	1.66	0.42	ND
Escherichia coli	TEM	0.19	0.08	ND
Escherichia coli	TEM	0.06	0.23	ND
Escherichia coli	CTX-M-9	0.7	0.05	ND
Escherichia coli	SHV	0.08	−0.19	ND
Escherichia coli	SHV	0.04	−0.06	ND
Proteus mirabilis	CTX-M-2	2.13	0.15	ND
Proteus mirabilis	CTX-M-2	2.19	0.41	ND
Proteus mirabilis	CTX-M-2	1.15	−0.04	ND
Proteus mirabilis	CTX-M-2	0.5	0.14	ND
Proteus mirabilis	TEM	1.34	0.04	ND
Proteus mirabilis	CTX-M-9	1.2	0.24	ND
Proteus mirabilis	CTX-M-2	1.18	0.13	ND
Proteus mirabilis	CTX-M-2	0.53	0.18	ND
Proteus mirabilis	CTX-M-2	1.34	0.13	ND
Proteus mirabilis	TEM	1.31	−0.1	ND
Proteus hauseri	CTX-M-2	1.09	0.02	ND
Enterobacter aerogenes	CTX-M-9	0.42	0.1	ND
Enterobacter asburiae	TEM, CTX-M-1	0.42	0.1	ND
Enterobacter asburiae	TEM, CTX-M-1	0.5	0.16	ND
Enterobacter asburiae	TEM, CTX-M-2	1.01	0.35	ND
Enterobacter cloacae	TEM, SHV	0.41	0.14	ND
Citrobacter koseri	TEM, CTX-M-1	0.68	0.2	ND
Citrobacter koseri	CTX-M-1	0.46	0.01	ND
Citrobacter koseri	CTX-M-1	1.53	0.4	ND
Raoultella ornithinolytica	SHV, CTX-M-1	0.6	−0.08	ND
Klebsiella pneumoniae	SHV, CTX-M-1	0.89	0.12	0.37
Klebsiella pneumoniae	SHV, CTX-M-9	0.88	0.01	0.3
Klebsiella pneumoniae	SHV, CTX-M-1	0.91	0.12	0.38
Klebsiella pneumoniae	TEM, SHV, OXA, CTX-M-1	0.66	0.04	0.23
Klebsiella pneumoniae	SHV, CTX-M-1	0.41	−0.2	−0.07
Klebsiella pneumoniae	SHV	0.27	−0.05	−0.1
Klebsiella pneumoniae	TEM, CTX-M-1	1.17	0.56	0.66
Klebsiella pneumoniae	SHV, CTX-M-9	0.55	0.21	0.07
Klebsiella pneumoniae	TEM, CTX-M-1	0.81	−0.31	0.09
Klebsiella pneumoniae	TEM, CTX-M-1	0.72	0.16	0.2
Klebsiella pneumoniae	TEM, CTX-M-1	0.42	−0.02	0.03
Klebsiella pneumoniae	TEM, SHV, CTX-M-1	0.45	0.18	0.17
Klebsiella pneumoniae	SHV	0.42	−0.05	0.12
Klebsiella pneumoniae	TEM, CTX-M-1	1.18	0.13	0.25
Klebsiella pneumoniae	TEM, CTX-M-1	1.17	0.03	0.01
Klebsiella pneumoniae	SHV	0.14	−0.04	−0.03
Klebsiella pneumoniae	SHV	−0.21	−0.22	0.13
Klebsiella pneumoniae	TEM, SHV, CTX-M-1	0.45	0.14	0.22
Klebsiella pneumoniae	OXA, CTX-M-9	1.02	−0.1	0.31
Enterobacter aerogenes	–	0.67	0.61	ND
Enterobacter aerogenes	–	0.7	0.69	ND
Enterobacter cloacae	–	0.77	0.82	ND
Enterobacter aerogenes	–	−0.26	−0.37	ND
Enterobacter aerogenes	–	1.08	1.03	ND
Enterobacter aerogenes	–	0.47	0.55	ND
Enterobacter cloacae	–	0.53	0.59	ND
Enterobacter cloacae	–	0.58	0.5	ND
Enterobacter cloacae	–	−0.2	−0.18	ND
Enterobacter aerogenes	–	0.73	0.72	ND

(*continued on next page*)

Table 1 (*continued*)

Species identification	PCR detection of ESBL-encoding genes	logRQ values		
		CTX	CTX + CVA	CTX + SBT
Enterobacter cloacae	–	0.41	0.47	ND
Enterobacter aerogenes	–	0.11	−0.09	ND
Enterobacter aerogenes	–	0.58	0.45	ND
Enterobacter aerogenes	–	0.76	0.58	ND
Enterobacter aerogenes	–	0.04	−0.25	ND
Citrobacter farmeri	–	0.37	0.22	ND
Citrobacter freundii	–	0.64	0.63	ND
Klebsiella oxytoca	–	1.11	0.23	0.82
Klebsiella oxytoca	–	1.07	0.08	1.04
Klebsiella oxytoca	–	0.46	0.19	0.26
Klebsiella oxytoca	–	1.11	−0.04	0.6
Klebsiella oxytoca	–	0.51	0.21	0.57
Klebsiella oxytoca	–	1.1	0.03	0.96
Klebsiella oxytoca	–	0.97	0.33	0.58
Klebsiella oxytoca	–	0.87	0.13	0.87
Klebsiella oxytoca	–	0.75	0.13	0.73
Klebsiella oxytoca	–	1.06	−0.34	0.98
Klebsiella oxytoca	–	3.2	0.67	1.35

[a] ND: No data.

2.2. Genotype characterization by PCR

To confirm the presence of ESBL genes in CTX-resistant isolates and define ESBL-producing microbes, we performed PCR detection for *bla* genes in CTX-resistant *Enterobacteriaceae* using specific primers for bla_{SHV}/bla_{TEM}/bla_{OXA-1}-like, and bla_{CTX-M} genes including phylogenetic groups 1, 2, and 9, as reported previously (Dallenne et al., 2010). DNA extraction and PCR amplification were performed with the Cica geneus® DNA extraction reagent (Kanto Chemical Co. Inc., Tokyo, Japan) and the TaKaRa Ex Taq® (Takara Bio, Shiga, Japan), respectively, according to the manufacturer's instructions. The primer sequences are shown in Table 3.

2.3. CTX hydrolysis assay with the MALDI-TOF MS-based MBT STAR-BL software module

We performed a CTX hydrolysis assay using the MALDI-TOF MS-based MBT STAR-BL software module. Briefly, solutions of CTX (0.5 mg/mL) were prepared in incubation buffer (10 mM ammonium bicarbonate, 10 μg/mL zinc chloride, pH 8–9). Bacterial colonies were mixed with the CTX solution and vortexed for 5 s. The negative control (*E. coli* ATCC 25922), positive control (CTX-resistant *E. coli* expressing $bla_{CTX-M\ group\ 9}$ isolated from clinical specimen), and samples were incubated at 36 °C with shaking for 2 h, followed by centrifugation at 13,000 × *g* for 2min. One microliter of the supernatant was applied on the MSP 96 target (Bruker Daltonics), and the dried spots were overlaid with 1 μL of MBT STAR-BL Matrix (10 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid) (Bruker Daltonics). After drying, automated mass spectrometric analysis was performed with MALDI-TOF MS. For instrument calibration, 1 μL of MBT STAR ACS standard (Bruker Daltonics) was measured in parallel with the samples in each run. We used the MBT STAR-BL software module to detect ESBL producers, which automatically calculated the normalized $logRQ_{CTX}$. A $logRQ_{CTX} > 0.40$ indicated positive β-lactamase activity according to the manufacturer's instructions, whereas $logRQ_{CTX} \leq 0.40$ was defined as negative in this study.

2.4. β-lactamase inhibition assay with the MALDI-TOF MS-based MBT STAR-BL software module

Next, we selected isolates which had a $logRQ_{CTX} > 0.40$, and performed a β-lactamase inhibition assay. MALDI-TOF MS analysis was performed in the presence or absence of 0.05 mg/mL CVA (Sigma-Aldrich, Tokyo, Japan) to distinguish ESBL-producing bacteria from AmpC-type β-lactamase producers with equivalent bacterial concentrations. $logRQ_{CTX+CVA}$ was automatically calculated by the MBT STAR-BL software module in the CVA assay. Negative $logRQ_{CTX+CVA}$

Table 2
The distribution of identified species and the logRQ values in CTX sensitive isolates.

Species identification	logRQ values
	CTX
Escherichia coli	−0.05
Escherichia coli	−0.04
Escherichia coli	−0.1
Escherichia coli	−0.06
Escherichia coli	0.09
Escherichia coli	−0.08
Escherichia coli	−0.12
Escherichia coli	−0.13
Proteus mirabilis	−0.14
Proteus mirabilis	−0.2
Proteus hauseri	0.05
Enterobacter aerogenes	−0.13
Enterobacter cloacae	−0.14
Enterobacter cloacae	−0.15
Enterobacter cloacae	−0.13
Enterobacter aerogenes	−0.11
Enterobacter aerogenes	0.26
Enterobacter ludwigii	0.14
Enterobacter asburiae	0.09
Enterobacter aerogenes	0.06
Enterobacter asburiae	−0.03
Enterobacter cloacae	−0.18
Citrobacter freundii	−0.01
Citrobacter freundii	−0.05
Citrobacter koseri	−0.11
Citrobacter koseri	0.16
Citrobacter koseri	0.18
Citrobacter freundii	0.1
Raoultella ornithinolytica	−0.08
Raoultella ornithinolytica	−0.13
Klebsiella pneumoniae	−0.13
Klebsiella pneumoniae	−0.23
Klebsiella pneumoniae	−0.17
Klebsiella pneumoniae	−0.3
Klebsiella pneumoniae	−0.2
Klebsiella pneumoniae	−0.22
Klebsiella pneumoniae	−0.09
Klebsiella pneumoniae	−0.11
Klebsiella pneumoniae	−0.06
Klebsiella oxytoca	0.09
Klebsiella oxytoca	0.12
Klebsiella oxytoca	0.24
Klebsiella oxytoca	0.29
Klebsiella oxytoca	−0.02

Table 3
The primers used for ESBL genotype characterization by PCR.

Primer name	Sequence (5′–3′)	Reference
MultiTSO-T_for	CATTTCCGTGTCGCCCTTATTC	Dallenne et al. (2010)
MultiTSO-T_rev	CGTTCATCCATAGTTGCCTGAC	
MultiTSO-S_for	AGCCGCTTGAGCAAATTAAAC	
MultiTSO-S_rev	ATCCCGCAGATAAATCACCAC	
MultiTSO-O_for	GGCACCAGATTCAACTTTCAAG	
MultiTSO-O_rev	GACCCCAAGTTTCCTGTAAGTG	
MultiCTXMGp1_for	TTAGGAARTGTGCCGCTGYA	
MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT	
MultiCTXMGp2_for	CGTTAACGGCACGATGAC	
MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT	
MultiCTXMGp9_for	TCAAGCCTGCCGATCTGGT	
MultiCTXMGp9_rev	TGATTCTCGCCGCTGAAG	
CTX-Mg8/25_for	AACRCRCAGACGCTCTAC	
CTX-Mg8/25_rev	TCGAGCCGGAASGTGTYAT	

values were defined as zero. We calculated $logRQ_{CTX+CVA/CTX}$ as the inhibition ratio by CVA, that is the $logRQ_{CTX+CVA}$ value divided by the $logRQ_{CTX}$ value. In addition, we also performed inhibition analysis of *Klebsiella* spp. with 0.01 mg/mL SBT (Sigma-Aldrich) to rule out the presence of K1-type β-lactamases. Similarly, we calculated the $logRQ_{CTX+SBT/CTX}$ as the inhibition ratio with SBT. We evaluated the sensitivity and specificity of this system for detecting ESBL producers with β-lactamase inhibitors compared to PCR amplification.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism ver. 7.03 (GraphPad Software, San Diego, CA) and JMP 13.2.0 (SAS Institute Inc., Cary, NC). We performed CTX hydrolysis assay and β-lactamase inhibition assay with CVA or SBT in a single measurement, and we calculated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for detecting ESBL producers in each assay and a combination of CVA and SBT inhibition assay. We defined the optimal cutoff value and area under the curve (AUC) for detecting ESBL producers using receiver operating characteristic (ROC) curve analysis.

3. Results

3.1. Strain characterization

We determined the bacterial species, CTX susceptibility and ESBL-encoding genes. These isolates included 44 *Klebsiella* spp., 31 *Enterobacter* spp., 29 *Escherichia* spp., 14 *Proteus* spp., 11 *Citrobacter* spp. and 3 *Raoultella* spp. strains. The CTX susceptibility of these *Enterobacteriaceae* was classified by the broth microdilution test. There were 88 CTX-resistant (Table 1) and 44 CTX-sensitive *Enterobacteriaceae* (Table 2). We also detected ESBL-encoding genes in CTX-resistant *Enterobacteriaceae* with a PCR detection assay. Sixty of the 88 CTX-resistant isolates (68.2%) had at least one ESBL gene; 22 were CTX-M-type, 14 were CTX-M- and TEM-type, 6 were TEM-type, 6 were SHV-type, 6 were CTX-M- and SHV-type, and 6 were other-type ESBLs. However, these genes could not be detected in the remaining 28 CTX-resistant isolates (Table 1).

3.2. CTX hydrolysis assay of Enterobacteriaceae with the MALDI-TOF MS-based MBT STAR-BL software module

We used a CTX hydrolysis assay to detect ESBL producers in all strains with the MALDI-TOF MS-based MBT STAR-BL software module. All CTX-sensitive strains had a $logRQ_{CTX} \leq 0.40$ (Table 2, Fig. 1A). In the 88 CTX-resistant strains, 52 of the 60 ESBL producers (86.7%) and 23 of the 28 ESBL non-producers (82.1%) had a $logRQ_{CTX} > 0.40$ that showed positive β-lactamase activity (Table 1, Fig. 1A). The median $logRQ_{CTX}$ values in ESBL producers and non-producers were 0.70 (range: −0.21 to 3.20) and 0.69 (range: −0.26 to 1.11), respectively. This assay for the detection of ESBL producers in all strains had sensitivity 86.7% (52/60), specificity 68.1% (49/72), PPV 69.3% (52/75), and NPV 86.0% (49/57), respectively. The area under the curve (AUC) for detecting ESBL producers in all strains was 0.80 (Fig. 1B). This CTX hydrolysis assay to detect ESBL producers in all samples with the MALDI-TOF MS-based MBT STAR-BL software module didn't have high accuracy and needs to be improved to discriminate between ESBL producers and non-producers in CTX-resistant isolates with β-lactamase activity indicated by a $logRQ_{CTX} > 0.40$.

3.3. MBT STAR-BL software module based β-lactamase inhibition assay with CVA in CTX-resistant isolates

Next, we used a β-lactamase inhibition assay with CVA on the CTX-resistant isolates with β-lactamase activity indicated $logRQ_{CTX} > 0.40$ to separate ESBL producers from non-producers. However, the β-lactamase inhibition assay with CVA was unable to discriminate between the ESBL producers and non-producers for all isolates in this study (Fig. 2A). The median $logRQ_{CTX+CVA/CTX}$ ratios of the ESBL producers and non-producers were 0.16 (range: 0–0.53) and 0.82 (range: 0–1.17), respectively (Fig. 2A). The AUC for detecting ESBL producers in CTX-resistant isolates with β-lactamase activity indicated by a $logRQ_{CTX} > 0.40$ was 0.77 (Fig. 2B). In the ROC curve analysis, the optimal cutoff value of the $logRQ_{CTX+CVA/CTX}$ ratio was 0.76. This inhibition assay with addition of CVA for the detection of ESBL producers had sensitivity 86.7% (52/60), specificity 84.7% (61/72), PPV 82.5% (52/63), and NPV 88.4% (61/69), respectively. Nevertheless, we found that ESBL producers and non-producers were perfectly distinguished in CTX-resistant non-*Klebsiella* spp. such as *Enterobacter* spp. with this CVA assay. In CTX-resistant non-*Klebsiella* spp., the median of $logRQ_{CTX+CVA/CTX}$ ratios of the ESBL producers and non-producers were 0.21 (range: 0–0.53) and 0.99 (range: 0.76–1.17), respectively. Subsequently, we investigated CTX-resistant *Klebsiella* spp. and changed the β-lactamase inhibition assay to use SBT to discriminate between ESBL producers and non-producers in those strains.

3.4. MBT STAR-BL software module based β-lactamase inhibition assay with SBT on CTX-resistant Klebsiella spp.

We then used another inhibition approach using SBT with *Klebsiella* spp. isolates. The median $logRQ_{CTX+SBT/CTX}$ ratio of ESBL-producing *Klebsiella* spp. was <0.30 (range: 0–0.56), while it was 0.87 (range: 0.42–1.12) in ESBL non-producing *Klebsiella* spp. (Fig. 3A). The AUC for the detection of ESBL producers in CTX-resistant *Klebsiella* spp. was 0.98 (Fig. 3B). ROC curve analysis showed that the optimal cutoff value for $logRQ_{CTX+SBT/CTX}$ was 0.54. This assay for the detection of ESBL producers in CTX-resistant *Klebsiella* spp. had sensitivity 93.8% (15/16), specificity 90.9% (10/11), PPV 93.8% (15/16), and NPV 90.9% (10/11), respectively. As the result, the MBT STAR-BL software module based β-lactamase inhibition assay with β-lactamase inhibitors, that used properly depending on the bacterial strains, had sensitivity 85.0% (51/60), specificity 98.6% (71/72), PPV 98.1% (51/52), NPV 88.8% (71/80), and the AUC improved 0.99 for detecting ESBL producers in all isolates.

4. Discussion

In the present study, we found that the detection of ESBL-producing *Enterobacteriaceae* using a MALDI-TOF MS-based MBT STAR-BL software module and β-lactamase inhibition. *Enterobacteriaceae* is one of the most important bacteria that produce ESBLs and other β-lactamase enzymes in clinical field. Therefore, we selected various kinds of *Enterobacteriaceae* isolated in our hospital. To evaluate a MALDI-TOF MS-based MBT STAR-BL method, we confirmed characterization of those strains used in this study by the broth microdilution test and the PCR amplification assay. This MALDI-TOF MS-based MBT STAR-BL method is much faster than the conventional growth-based assays, yielding results within 3 h (Table 4) and has recently gained attention. In addition, we found that the results of the inhibition assay with a β-lactamase inhibitor depended on the bacterial species. We were able to properly discriminate between ESBL producers and CTX-resistant non-*Klebsiella* isolates using a MALDI-TOF MS-based MBT STAR-BL software module-based β-lactamase inhibition assay with CVA and from CTX-resistant *Klebsiella* spp. using SBT. As the result, we improved elapsed times and accuracy in MBT STAR-BL methods by using proper β-lactamase inhibitors against specific bacterial strains to compare with the conventional method (Table 4).

Jung et al. (2014) reported that a MALDI-TOF MS-based β-lactamase assay enabled them to rapidly predict resistance. The sensitivity and specificity for detecting resistance to third-generation cephalosporins in *Enterobacteriaceae* were 100% and 91.5%, respectively. On the

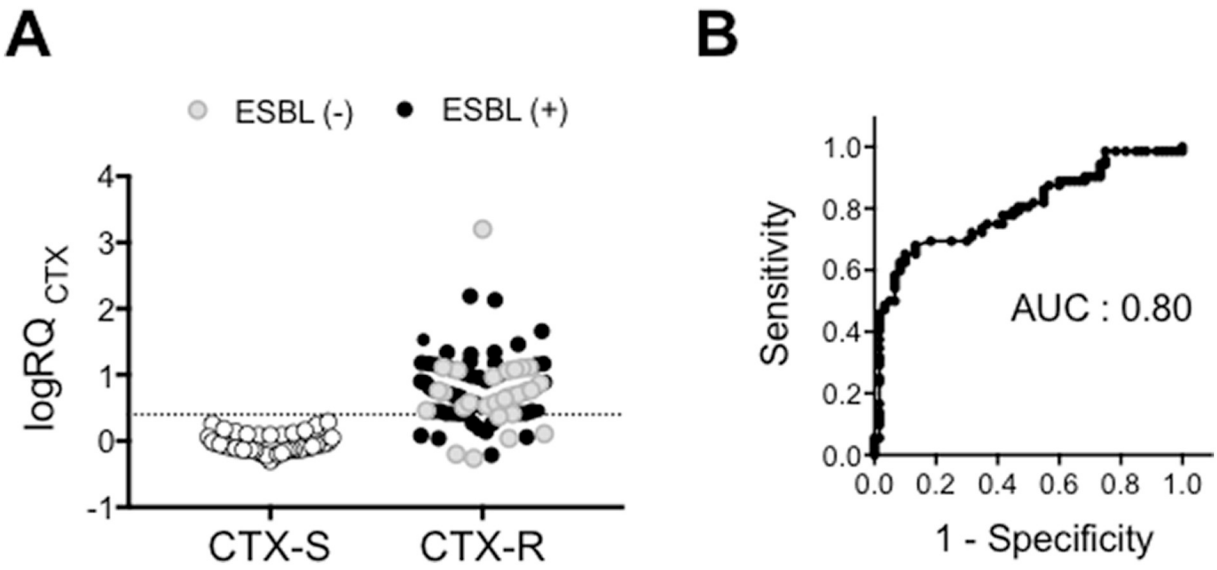

Fig. 1. Forty-four CTX-sensitive isolates (white dots), 60 CTX-resistant ESBL-producing isolates (black dots), and 28 CTX-resistant ESBL non-producing isolates (gray dots) were used to calculate normalized $logRQ_{CTX}$ to detect *Enterobacteriaceae* ESBL producers with the MALDI-TOF MS-based MBT STAR-BL software module. The $logRQ_{CTX}$ values of CTX sensitive isolates compared with those of ESBL producers and non-producers in CTX resistant isolates (A). Receiver operating characteristic curve for this assay using the MALDI-TOF MS-based MBT STAR-BL software module obtained when the cutoff for detecting ESBL-producers was set to the logRQ >0.40 (B).

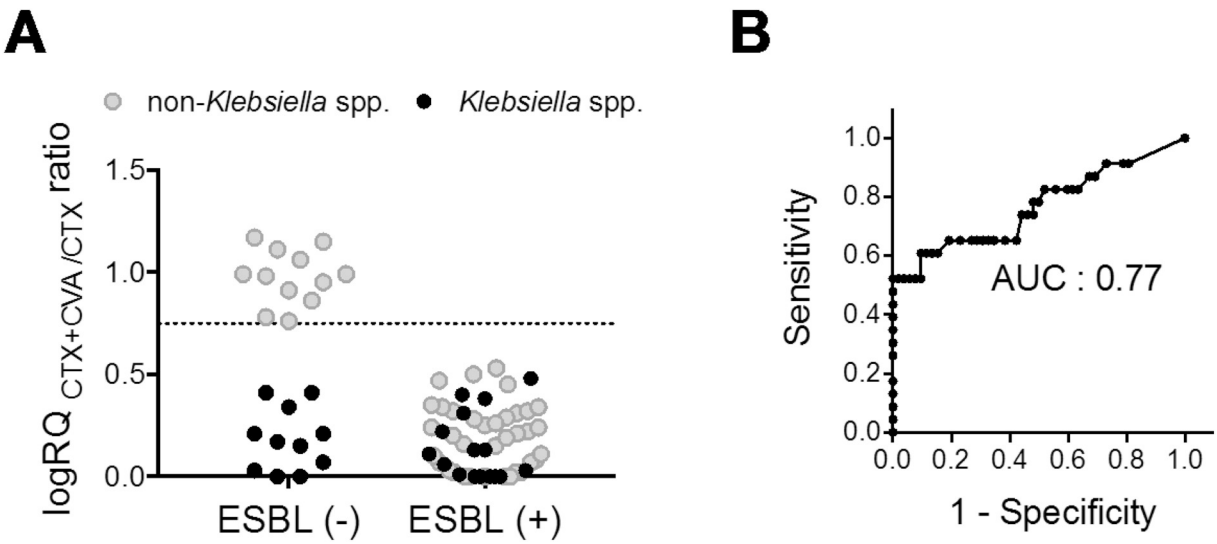

Fig. 2. The MBT STAR-BL software module and CVA based β-lactamase inhibition assay for detecting ESBL producers in $logRQ_{CTX}$ positive isolates. The inhibition ratio of $logRQ_{CTX}$ with CVA ($logRQ_{CTX+CVA/CTX}$) was measured in 48 non-*Klebsiella* spp. (gray dots) and 27 *Klebsiella* spp. (black dots). The $logRQ_{CTX+CVA/CTX}$ ratio of ESBL non-producers compared with that of ESBL producers (A). The ROC curve for detecting ESBL producers obtained when the $logRQ_{CTX+CVA/CTX}$ ratio of optimal cutoff for detection was set to 0.76 (B).

contrary, Kawamoto et al. (2019) found a sensitivity and specificity of 100% and 94.7%, respectively, for the detection of ESBL producers in *Escherichia coli* and *Klebsiella pneumoniae* with a MALDI-TOF MS-based MBT STAR-BL software module. Though they divided the strains into ESBL producers having positive β-lactamase activity and non-producers with the CTX hydrolysis analysis using the MBT STAR-BL software module, the assay could not distinguish ESBL from other third-generation cephalosporinases including AmpC or K1-type β-lactamases. Therefore, we attempted to develop a new MBT STAR-BL approach to distinguish between these isolates. Oviaño et al. (2014) reported that MALDI-TOF MS analysis with CVA is useful to rule out isolates producing AmpC-type β-lactamases such as FOX-4, CMY-2, DHA-1, −6, and − 7. Similarly, we could mostly differentiate ESBL producers from non-producers by the β-lactamase inhibition assay using CVA. On the contrary, this assay could not distinguish between ESBL producers and non-producers in *Klebsiella* spp. In particular, *Klebsiella* strains hyper-producing K1-type β-lactamases were false-positives in the culture-based ESBL detection method because CVA inhibited hyper-production of K1-type β-lactamases such as ESBL (Potz et al., 2004). Thus, we used SBT in addition to CVA inhibition to distinguish between ESBL and K1-type β-lactamase producing *Klebsiella* isolates. We defined CTX-resistant *Klebsiella* spp. without ESBL, which had a $logRQ_{CTX+CVA/CTX}$ ratio <0.76 and were ceftazidime-sensitive and CTX, aztreonam, and ampicillin/SBT-resistant (Thomson, 2010; Livermore, 1995; Kimura et al., 1996), similar to the K1-type β-lactamase producers in this study. The additional SBT inhibition assay was effective at differentiating ESBL strains from K1-type β-lactamase producers in *Klebsiella* spp.

The emergence of ESBLs has a significant impact on the mortality

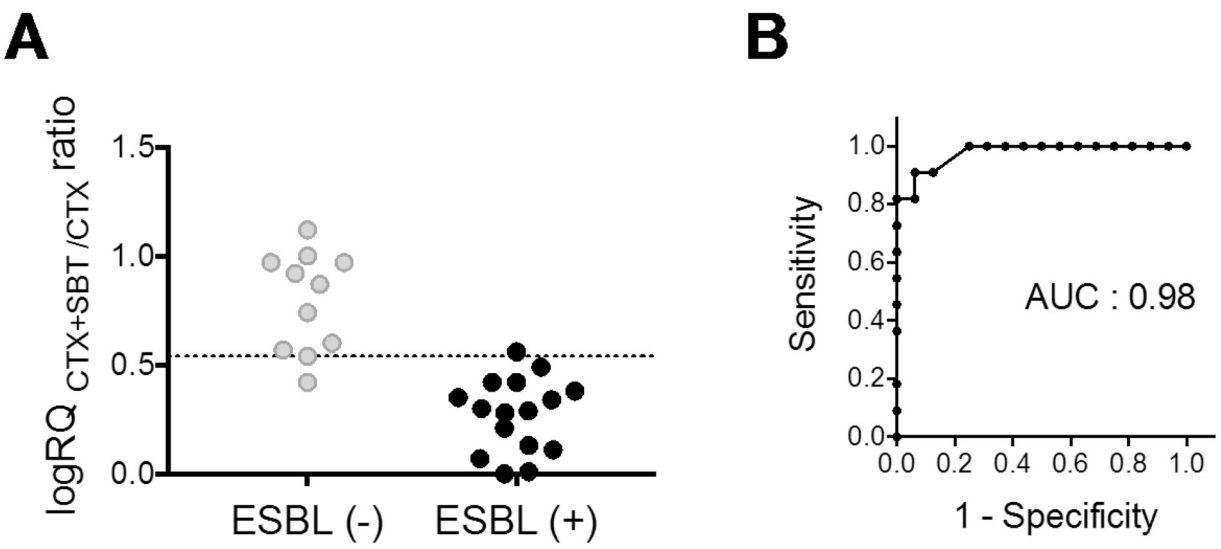

Fig. 3. The MBT STAR-BL software module and SBT based β-lactamase inhibition assay for detecting ESBL producers in $logRQ_{CTX}$ positive *Klebsiella* isolates. The inhibition ratio of $logRQ_{CTX}$ with SBT ($logRQ_{CTX+SBT/CTX}$) was measured in 17 ESBL producers (black dots) and 11 ESBL non-producers (gray dots). The $logRQ_{CTX+SBT/CTX}$ ratio of ESBL non-producers compared with ESBL producers (A). The ROC curve for detecting ESBL producers obtained when the $logRQ_{CTX+SBT/CTX}$ ratio of optimal cutoff for detection was set to 0.54 (B).

rates and hospital costs due to infectious diseases (Schwaber et al., 2006; Tumbarello et al., 2006). The ESBL genotypes predominantly derived from the CTX-M, TEM, and SHV-type groups. TEM and/or SHV-type genes occurred in 0–16.1% of the ESBLs producing *Enterobacteriaceae* such as *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* (Chong et al., 2013). In this study, all 47 CTX-M-type ESBL-producing *Enterobacteriaceae* were positive for β-lactamase activity in the CTX hydrolysis analysis with the MALDI-TOF MS-based MBT STAR-BL software module. On the contrary, three of the six TEM-type and one of the six SHV-type ESBL producers exhibited positive β-lactamase activity. Similarly, Oviaño et al. (2017) described that isolates carrying TEM-type ESBL genes did not hydrolyze CTX or have positive β-lactamase activity in the MALDI-TOF MS-based MBT STAR-BL approach. Another previous report has shown that CTX-M-type ESBLs have better hydrolyzing activity against CTX than TEM or SHV-type ESBLs (Rossolini et al., 2008). In fact, we found that the $logRQ_{CTX}$ of SHV-type strains was significantly lower than the $logRQ_{CTX}$ of CTX-M-type strains. Therefore, the results suggested that the genotype of ESBLs might affect $logRQ_{CTX}$ in the MALDI-TOF MS-based MBT STAR-BL approach.

The present study has several limitations. First, we only used CTX hydrolysis to detect ESBL producers. However, the current MBT STAR-BL software module in our country can analyze CTX or ceftazidime hydrolysis to detect third-generation cephalosporinases. Furthermore, the applicability of this software has been demonstrated for different β-lactam antibiotics such as ampicillin and meropenem. Thus, further experiments would be required to detect a greater variety of β-lactamase in parallel. In addition, the sample size of each bacterial species and kinds of *Enterobacteriaceae* tested is small. In the clinical laboratory, it will be necessary to confirm the validity of this assay with a greater number of *Enterobacteriaceae* species or other bacterial strains.

In conclusion, we performed the MALDI-TOF MS-based MBT STAR-BL approach with β-lactamase inhibitors for detecting ESBL producers and showed that the results of the inhibition assay with β-lactamase inhibitors depended on types of bacterial species. Furthermore, we improved elapsed times and accuracy in MBT STAR-BL methods by using proper β-lactamase inhibitors against specific bacterial strains to compare with the conventional standard lab method. The results suggested that the target bacterial species and β-lactamase inhibitors used were important for the utility of the MALDI-TOF MS-based MBT STAR-BL approach.

Table 4
The comparison between culture-based phenotypic method and MBT STAR-BL software module methods.

Method	Sensitivity (%)	Specificity (%)	Positive predict value (%)	Negative predict value (%)	Incubation time (min)	Preprocessing and measurement time (min)	Total elapsed time (min)
Combination disc method	85[a]	89[a]	No data	No data	960[b]	5	965
$logRQ_{CTX}$	86.7	68.1	69.3	86	120	20	140
$logRQ_{CTX}$ with CVA	86.7	85	82.5	88.4	120	25	145
$logRQ_{CTX}$ with CVA and SBT	85	98.6	98.1	88.8	120	30	150

[a] Garrec et al. (2011).
[b] Wayne (2015).

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Declaration of competing interest

None of the authors has any financial relationships with commercial entities that have an interest in the subject of this manuscript.

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