Original Article

Analysis of Clinical Isolates of Extended-Spectrum β -Lactamase-Producing Bacteria with Primer and Probe Sets Developed to Detect *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} Using a Fully Automated Gene Detection System

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SUMMARY: In this study, we evaluated extended-spectrum β -lactamase (ESBL)-producing bacteria with the newly developed primer and probe sets to detect *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} using BD MAXTM, a fully automated multiplex polymerase chain reaction assay system. In 36 isolates confirmed by whole-genome sequencing to have *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{SHV}, the developed primer and probe sets accurately detected each gene without being influenced by the presence of other β -lactamase genes. In nine control strains that do not harbor either *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{SHV} no cross-reaction was observed. In 191 strains phenotypically determined to be ESBL-producers by conventional antimicrobial susceptibility tests, 189 strains were *bla*_{CTX-M}⁺, *bla*_{TEM}⁺, or *bla*_{SHV}⁺ as assessed by BD MAXTM using the developed primer and probe sets, and two strains were negative for these genes. Whole-genome sequencing revealed that these two strains were phenotypically false-positive ESBL-producers. The accuracy of the primer and probe sets seems to be satisfactory, and they may be applicable to detect CTX-M-type ESBL-producing bacteria.

INTRODUCTION

The number of extended-spectrum β -lactamase (ESBL)-producing organisms is increasing and causes a significant social burden owing to a longer duration of hospitalization, selection of broad antibiotics, and nosocomial infection outbreaks (1,2). Several genes related to ESBL can be easily transferred among members of the family Enterobacteriaceae, because these genes are normally located in plasmids. Therefore, early detection and screening of ESBL-producing organisms are important to initiate adequate infection control measures in medical facilities (3–5). Usually, in a clinical laboratory, ESBL-producing organisms are identified by antimicrobial susceptibility tests.

BD MAXTM (Nippon Becton Dickinson, Tokyo, Japan) can extract, purify, amplify, and detect nucleotides in 24 different bacterial specimens at a time and perform fully automated data analysis within approximately 3 h. This machine can detect multiple target genes within a single sample using multiplex polymerase chain reaction (PCR)

technology (6–8). Assay systems based on BD MAXTM are used for various purposes, including tests for MRSA (9), *Clostridioides difficile* (10), norovirus (11), measles (12), and enteric or vaginal infective pathogens (10,11,13).

In this study, we developed a new primer and probe sets to detect *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} genes with BD MAXTM. The accuracy of the products was evaluated by testing isolates confirmed by whole-genome sequencing to have or not *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} and isolates phenotypically determined as ESBL-producing bacteria.

MATERIALS AND METHODS

Bacterial strains: Forty-five strains that were confirmed to have more than one ESBL-related gene by whole-genome sequencing were tested to evaluate the accuracy of the developed primer and probe sets. These strains were obtained from the Department of Microbiology and Infectious Diseases, Toho University School of Medicine (Tokyo, Japan). Of these, 14 strains harbored the *bla*_{CTX-M} gene only, nine strains harbored *bla*_{TEM} only, one strain harbored *bla*_{SHV} only, two strains harbored *bla*_{CTX-M} with other β -lactamase genes, four strains harbored *bla*_{TEM} with other β -lactamase genes, and six strains harbored *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{CTX-M}, *bla*_{SHV} or *bla*_{TEM}, and *bla*_{SHV} with other β -lactamase genes. Nine strains harbored β -lactamase genes other than *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{SHV}.

Phenotypical ESBL-producers were tested to evaluate

Received November 6, 2018. Accepted June 4, 2019.

J-STAGE Advance Publication June 28, 2019.

DOI: 10.7883/yoken.JJID.2018.466

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the characteristics of the ESBL-producing strains with the developed primer and probe sets. ESBL phenotypes were determined by a BD Phoenix™ automated identification and susceptibility testing system (Nippon Becton Dickinson). These strains were isolated from urine or blood specimens in the clinical microbiological laboratories of Fukushima Medical University Hospital (Fukushima, Japan) and Kimitsu Chuo Hospital (Chiba, Japan). In total, 191 isolates were collected from January 2012 to December 2015.

Conducting the BD Phoenix™ automated identification and susceptibility tests: The BD Phoenix™ automated identification and susceptibility tests were conducted using the NMIC/ID 208 BD Phoenix GN Combo panels and the GN-30 BD Phoenix panels (Nippon Becton Dickinson). These panels were inoculated and incubated according to the manufacturer's instructions. The BD Phoenix ESBL test utilized the growth response to cefotaxime, ceftazidime, cefpodoxime, and ceftriaxone, with or without clavulanic acid, to detect the production of ESBL. The results were analyzed using the BD Xpert system (Nippon Becton Dickinson) (14). Antibiotic susceptibilities were determined according to CLSI M100-S25.

Developed primer and probe sets: The primers and fluorescent hydrolysis probes, named as ESBL PRIMERS/PROBES (Nippon Becton Dickinson), were designed for the detection of *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} through a proprietary process developed by G&G Science (Fukushima, Japan). Primers and probes were designed around specific motifs to detect genes encoding *bla*_{CTX-M-1, 2, 8, 9, 25} type (*bla*_{CTX-M} type), *bla*_{TEM}, and *bla*_{SHV}. Subgroups of *bla*_{CTX-M} were not determined by the product. The probes for *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} were conjugated to FAM, HEX, and Cy5, respectively. The primers and probes were premixed in the same tube for multiplex-PCR. All primers and probes were used at final concentrations of 500 nM and 250 nM, respectively. The primers and probes were mixed into a volume of 12.5 µL/sample by mixing the primer and probe solution (8.25 µL) with the TNA primer and probe diluent (4.25 µL) included in the BD MAX™ TNA MMK (SPC) (Nippon Becton Dickinson).

Operation of the BD MAX™ system: The following components were set up in the BD MAX™ instrument according to the manufacturer's instructions, and the BD MAX assay was started: unitized reagent strip, DNA extraction reagent contained in the BD MAX™ ExK TNA-2 kit (Nippon Becton Dickinson), sample buffer tube, primer and probe solution, master mix, and BD MAX™ cartridge (Nippon Becton Dickinson). DNA extraction was conducted in the unitized reagent strip, and amplification and detection of nucleotides were conducted in the BD MAX™ cartridge. The BD MAX assay system was operated using the following PCR cycling protocol: one cycle at 85°C for 300 s (hot-start activation) and at 60°C for 900 s (reverse transcription for specimen processing control), followed by 40 cycles at 95°C for 15 s and at 59°C for 61.8 s. Fluorescence gains and thresholds were set for FAM at 50 and 200, HEX at 50 and 300, and Cy5 at 50 and 300, respectively. The cut-off C_T value was 35.0. Bacterial strains were cultivated using trypticase soy agar with 5% sheep blood (Nippon Becton Dickinson) in an

aerobic atmosphere, overnight at 37°C. After cultivation, colonies were suspended in BD Phoenix ID Broth™ (Nippon Becton Dickinson) adjusted to 0.5 McFarland with a BD Phoenix Spec Nephelometer™ (Nippon Becton Dickinson). Then, the 0.5 McFarland bacterial suspension was diluted 40-fold in saline; 200 µL of the suspension was used for the experiment in accordance with the manufacturer's instructions.

Whole-genome sequencing: The sequencing library was prepared with the Nextera XT DNA Sample Prep Kit (Illumina, San Diego, CA, USA). Sequencing was performed using the Miseq (Illumina) next generation sequencer. The obtained sequence data were analyzed using the CLC Genomics Workbench (Qiagen). Acquired antimicrobial resistance genes were identified using the ResFinder, version 2.1 database <<https://cge.cbs.dtu.dk/services/ResFinder/>>. All whole-genome sequencing data used in this study were deposited in the NCBI database under the BioProject number PRJDB6851.

Statistical analysis: Fisher's exact test was used to analyze the performance of the BD MAX assay system in detecting *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV}. Statistical analyses were carried out using GraphPad Prism7 (GraphPad Software, San Diego, CA, USA).

Ethical statement: This research protocol was approved by the Fukushima Medical University ethics committee (Reference number: 2092).

RESULTS

First, we applied the developed primer and probe sets to the 45 strains that were confirmed to have more than one β-lactamase gene, including *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{SHV}, by whole-genomic sequencing (WGS). As shown in Table 1, 14 strains with *bla*_{CTX-M} only, nine strains with *bla*_{TEM} only, and one strain with *bla*_{SHV} only were accurately determined to have *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV}, respectively. Twelve strains that were positive for either one of *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{SHV} with other β-lactamase genes were also detected correctly. No false-positive detection of *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{SHV} occurred in the nine strains that did not have these genes (Table 1). Therefore, the sensitivity and specificity were uniformly 100% (95% confidence interval: sensitivity 90.4%–100.0%; specificity 70.1%–100.0%) for the developed primer and probe sets in the 45 strains for which WGS was performed.

Next, we applied the developed primer and probe sets to phenotypically determined ESBL-producers, by conventional antimicrobial susceptibility tests, using the BD Phoenix™ system. A total of 191 clinical ESBL-producers were enumerated from two facilities in Chiba and Fukushima prefectures. Among them, 170 were *Escherichia coli*, 14 were *Klebsiella pneumoniae*, and seven were *Proteus mirabilis*. The assay results obtained using the developed primer and probe sets indicated that 114 (59.7%) strains harbored *bla*_{CTX-M}, 58 (30.4%) strains harbored *bla*_{CTX-M}+*bla*_{TEM}, six (3.1%) strains harbored *bla*_{CTX-M}+*bla*_{SHV}, and five (2.6%) strains harbored *bla*_{CTX-M}+*bla*_{TEM}+*bla*_{SHV}. Hence, *bla*_{CTX-M}-possessing strains accounted for 183 of the 191 (95.8%) strains examined. In two strains, we were unable to detect *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{SHV} (Fig.1). We did, however, detect antimicrobial

Analysis of ESBL Bacteria by a Fully Automated PCR

Table 1. Analysis of whole-genome sequencing-determined strains using the developed primer and probe sets

WGS accession no.	Species	β -lactamase encoding genes confirmed by WGS	Assay result by the developed primer and probe		
			CTX-M	TEM	SHV
TUM2750	<i>E. coli</i>	<i>bla</i> _{CTX-M-3}	+	–	–
TUM9505	<i>E. coli</i>	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{OXA-1}	+	–	–
TUM11481	<i>E. coli</i>	<i>bla</i> _{CTX-M-27}	+	–	–
TUM14675	<i>E. coli</i>	<i>bla</i> _{CTX-M-15}	+	–	–
TUM15926	<i>E. coli</i>	<i>bla</i> _{CTX-M-8}	+	–	–
TUM9589	<i>E. coli</i>	<i>bla</i> _{CTX-M-24}	+	–	–
TUM9875	<i>E. coli</i>	<i>bla</i> _{CTX-M-9}	+	–	–
TUM13620	<i>E. coli</i>	<i>bla</i> _{CTX-M-14}	+	–	–
TUM14672	<i>E. coli</i>	<i>bla</i> _{CTX-M-14}	+	–	–
TUM11567	<i>P. mirabilis</i>	<i>bla</i> _{CTX-M-2}	+	–	–
TUM11568	<i>P. mirabilis</i>	<i>bla</i> _{CTX-M-2}	+	–	–
TUM11569	<i>P. mirabilis</i>	<i>bla</i> _{CTX-M-2}	+	–	–
TUM11570	<i>P. mirabilis</i>	<i>bla</i> _{CTX-M-2}	+	–	–
TUM11571	<i>P. mirabilis</i>	<i>bla</i> _{CTX-M-2}	+	–	–
TUM9991	<i>E. cloacae</i>	<i>bla</i> _{CTX-M-3} , <i>bla</i> _{TEM-1A-like} , <i>bla</i> _{ACT-7}	+	–	–
TUM9994	<i>P. rettgeri</i>	<i>bla</i> _{CTX-M-1}	+	–	–
TUM1134	<i>E. coli</i>	<i>bla</i> _{TEM-48}	–	+	–
TUM1135	<i>E. coli</i>	<i>bla</i> _{TEM-10}	–	+	–
TUM1136	<i>E. coli</i>	<i>bla</i> _{TEM-6} , <i>bla</i> _{OXA-4}	–	+	–
TUM1137	<i>E. coli</i>	<i>bla</i> _{TEM-12-like}	–	+	–
TUM1138	<i>E. coli</i>	<i>bla</i> _{TEM-10-like}	–	+	–
TUM1521	<i>E. coli</i>	<i>bla</i> _{TEM-189-like}	–	+	–
TUM1650	<i>E. coli</i>	<i>bla</i> _{TEM-25}	–	+	–
TUM1809	<i>E. coli</i>	<i>bla</i> _{TEM-10}	–	+	–
TUM1813	<i>E. coli</i>	<i>bla</i> _{TEM-8}	–	+	–
TUM1825	<i>E. coli</i>	<i>bla</i> _{TEM-2}	–	+	–
TUM1808	<i>K. pneumoniae</i>	<i>bla</i> _{SHV-11}	–	–	+
TUM3433	<i>E. coli</i>	<i>bla</i> _{CMY-24} , <i>bla</i> _{TEM-176}	–	+	–
TUM3434	<i>E. coli</i>	<i>bla</i> _{TEM-1B} , <i>bla</i> _{DHA-1}	–	+	–
TUM1382	<i>K. pneumoniae</i>	<i>bla</i> _{TEM-1B} , <i>bla</i> _{LEN25} , <i>bla</i> _{MOX-1}	–	+	–
TUM3435	<i>K. pneumoniae</i>	<i>bla</i> _{TEM-1B} , <i>bla</i> _{SHV-12} , <i>bla</i> _{DHA-1} , <i>bla</i> _{OXA-1}	–	+	+
TUM13502	<i>K. pneumoniae</i>	<i>bla</i> _{GES-4} , <i>bla</i> _{TEM-1B} , <i>bla</i> _{SHV-11}	–	+	+
TUM15668	<i>E. cloacae</i>	<i>bla</i> _{KPC-2} , <i>bla</i> _{CTX-M-15} , <i>bla</i> _{TEM-1B} , <i>bla</i> _{ACT-7}	+	+	–
TUM15679	<i>E. cloacae</i>	<i>bla</i> _{OXA-48} , <i>bla</i> _{CTX-M-15} , <i>bla</i> _{TEM-1B} , <i>bla</i> _{ACT-7}	+	+	–
TUM1469	<i>K. pneumoniae</i>	<i>bla</i> _{CMY-8} , <i>bla</i> _{CTX-M-3} , <i>bla</i> _{SHV-11}	+	–	+
TUM13867	<i>E. coli</i>	<i>bla</i> _{IMP-6} , <i>bla</i> _{CTX-M-2} , <i>bla</i> _{TEM-1B}	+	+	–
TUM4670	<i>E. coli</i>	<i>bla</i> _{CMY-2}	–	–	–
TUM4660	<i>P. mirabilis</i>	<i>bla</i> _{CMY-2}	–	–	–
TUM10	<i>E. cloacae</i>	<i>bla</i> _{ACT-15}	–	–	–
TUM1144	<i>E. cloacae</i>	<i>bla</i> _{ACT-7-like}	–	–	–
TUM1506	<i>E. cloacae</i>	<i>bla</i> _{ACT-15-like}	–	–	–
TUM5376	<i>E. cloacae</i>	<i>bla</i> _{ACT-15-like}	–	–	–
TUM1507	<i>C. freundii</i>	<i>bla</i> _{CMY-78}	–	–	–
TUM2748	<i>M. morgani</i>	<i>bla</i> _{MOR-2-like}	–	–	–
TUM12213	<i>S. enterica</i>	<i>bla</i> _{CMY-2}	–	–	–

WGS, whole-genome sequencing; *E. cloacae*, *Enterobacter cloacae*; *P. rettgeri*, *Providencia rettgeri*; +, detected; –, not detected.

Fig. 1. Analysis of the phenotypic ESBL-producing strains using the developed primer and probe sets.

resistance-related genes, such as *aadB*, *aadA5*, *aadA1*, *mph(A)*, *cmlA1*, *sul1*, and *dfrA17*, in these strains. No β -lactamase-related genes were detected by WGS. Consequently, there were no discrepancies between the results obtained using the developed primer and probe sets and the WGS analysis. All isolates were reasonably susceptible to cefmetazole, latamoxef, and imipenem. Compared with *K. pneumoniae*, *E. coli* and *P. mirabilis* were more susceptible to piperacillin/tazobactam (PIPC/TAZ) but less susceptible to levofloxacin (LVFX). However, although *K. pneumoniae* isolates were less susceptible to PIPC/TAZ, they were more susceptible to LVFX than *E. coli* (Table 2).

DISCUSSION

Through the two series of assays conducted in this study, we evaluated the accuracy of the developed primer and probe sets using the BD MAXTM system to test the presence of *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{SHV} in bacterial strains. Among the 45 strains that were analyzed using WGS, we detected no false-positive or false-negative identifications of *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} genes using the product. Moreover, the sensitivity and specificity were uniformly 100% (Table 1). In our analysis of 191 strains that were phenotypically determined to be ESBL-producers (Fig.1), we identified 189 strains as positive for the *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{SHV} genes using this assay system (Table 2). Although negative identification occurred in the case of two phenotypic ESBL-strains, WGS confirmed that these strains do not harbor ESBL-related genes. Therefore, these two strains were false-positive phenotypically determined ESBL-strains, and the product showed significant accuracy in determining

the presence of the *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} genes.

In 2015, Ogutu *et al.* reported a multiplex PCR system for the detection of *bla*_{CTX-M-1,9}, *bla*_{TEM}, *bla*_{SHV}, and *bla*_{OXA-1}. They examined 203 clinical isolates of *K. pneumoniae* and *E. coli* by using group-specific PCR primers in singleplex reactions followed by optimization to multiplex reactions. Their test results indicated 100% sensitivity and specificity in determining β -lactamase subtypes, which were confirmed by sequencing the PCR products (15). In terms of sensitivity and specificity, the results obtained in the present study are comparable with those of Ogutu *et al.* In addition to their accuracy, the developed primer and probe sets can be applied to the BD MAXTM system, which can analyze 24 colony specimens at a time within 3 h, based on a fully automated PCR system. This may confer greater utility for the detection of the *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} genes in clinical laboratories.

The frequency of ESBL-producing bacteria in Japan has been increasing since around 2000 (4). A study conducted in 2012, in which the authors examined the proportions of ESBL-producing bacteria throughout Japan, revealed that 14.0% of *E. coli*, 3.3% of *K. pneumoniae*, 3.1% of *K. oxytoca*, and 15.9% of *P. mirabilis* were ESBL-producers. All these isolates belonged to CTX-M groups. Neither SHV- nor TEM-type were detected in this study (16). In a further study conducted in Japan, of the 107 *E. coli* strains examined, 36 (33.6%) were found to carry CTX-M-type genes solely, whereas 62 (57.9%) carried both CTX-M- and TEM- and/or SHV-type genes. Only nine strains (8.4%) carried TEM- and/or SHV-type genes without CTX-M-type genes (17). Consistent with these findings, in the present study, we detected CTX-M-type genes in 95.8%

Analysis of ESBL Bacteria by a Fully Automated PCR

Table 2. Susceptibility patterns of the phenotypic ESBL-producing strains assayed using the broth microdilution test

Species and detected genes		A/S	P/T	CMZ	CPDX	CTX	CAZ	LMOX	AZT	LVFX	IPM	GM
<i>E. coli</i> <i>bla</i> _{CTX-M} (n = 106)	S	29.6%	54.0%	56.1%	0.0%	0.0%	33.3%	56.1%	19.6%	10.6%	56.1%	52.4%
	I	13.8%	1.1%	0.0%	0.0%	0.0%	9.0%	0.0%	14.8%	0.5%	0.0%	0.0%
	R	12.7%	1.1%	0.0%	56.1%	56.1%	13.8%	0.0%	21.7%	45.0%	0.0%	3.7%
<i>E. coli</i> <i>bla</i> _{TEM} (n = 2)	S	0.0%	1.1%	1.1%	0.0%	0.5%	0.5%	1.1%	1.1%	0.0%	1.1%	0.5%
	I	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	R	1.1%	0.0%	0.0%	1.1%	0.5%	0.5%	0.0%	0.0%	1.1%	0.0%	0.5%
<i>E. coli</i> <i>bla</i> _{SHV} (n = 1)	S	0.5%	0.5%	0.5%	0.0%	0.0%	0.5%	0.5%	0.5%	0.5%	0.5%	0.0%
	I	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	R	0.0%	0.0%	0.0%	0.5%	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.5%
<i>E. coli</i> <i>bla</i> _{CTX-M+TEM} (n = 57)	S	2.1%	26.5%	30.2%	0.0%	0.5%	20.1%	30.2%	11.1%	11.1%	30.2%	18.5%
	I	9.5%	1.6%	0.0%	0.0%	0.5%	1.6%	0.0%	4.8%	0.5%	0.0%	0.0%
	R	18.5%	2.1%	0.0%	30.2%	29.1%	8.5%	0.0%	14.3%	18.5%	0.0%	11.6%
<i>E. coli</i> <i>bla</i> _{TEM+SHV} (n = 2)	S	0.0%	1.1%	1.1%	0.0%	0.0%	0.0%	1.1%	0.0%	1.1%	1.1%	1.1%
	I	1.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	R	0.0%	0.0%	0.0%	1.1%	1.1%	1.1%	0.0%	1.1%	0.0%	0.0%	0.0%
<i>K. pneumoniae</i> <i>bla</i> _{CTX-M} (n = 1)	S	0.0%	0.5%	0.5%	0.0%	0.0%	0.0%	0.5%	0.0%	0.5%	0.5%	0.0%
	I	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	R	0.5%	0.0%	0.0%	0.5%	0.5%	0.5%	0.0%	0.5%	0.0%	0.0%	0.5%
<i>K. pneumoniae</i> <i>bla</i> _{SHV} (n = 1)	S	0.0%	0.5%	0.5%	0.0%	0.0%	0.0%	0.5%	0.5%	0.5%	0.5%	0.5%
	I	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	R	0.0%	0.0%	0.0%	0.5%	0.5%	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%
<i>K. pneumoniae</i> <i>bla</i> _{CTX-M+TEM} (n = 1)	S	0.0%	0.5%	0.5%	0.0%	0.0%	0.5%	0.5%	0.5%	0.5%	0.5%	0.0%
	I	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	R	0.0%	0.0%	0.0%	0.5%	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.5%
<i>K. pneumoniae</i> <i>bla</i> _{CTX-M+SHV} (n = 6)	S	0.5%	2.6%	3.2%	0.5%	0.5%	2.6%	3.2%	1.1%	2.6%	3.2%	1.6%
	I	1.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.5%	0.0%	0.0%	0.0%
	R	1.6%	0.5%	0.0%	2.6%	2.6%	0.5%	0.0%	1.6%	0.5%	0.0%	1.6%
<i>K. pneumoniae</i> <i>bla</i> _{CTX-M+TEM+SHV} (n = 5)	S	0.0%	1.6%	2.6%	0.0%	0.0%	0.5%	2.6%	0.0%	1.6%	2.6%	2.1%
	I	0.5%	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	R	2.1%	0.5%	0.0%	2.6%	2.6%	2.1%	0.0%	2.6%	1.1%	0.0%	0.5%
<i>P. mirabilis</i> <i>bla</i> _{CTX-M} (n = 7)	S	0.5%	3.7%	3.7%	0.0%	0.0%	3.7%	3.7%	3.2%	0.5%	0.5%	2.6%
	I	3.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.1%	1.1%	0.0%
	R	0.0%	0.0%	0.0%	3.7%	3.7%	0.0%	0.0%	0.5%	2.1%	2.1%	1.1%

S, susceptible; I, intermediate; R, resistant; A/S, ampicillin/sulbactam; P/T, piperacillin/tazobactam; CMZ, cefmetazole; CPDX, cefpodoxime; CTX, cefotaxime; CAZ, ceftazidime; LMOX, latamoxef; AZT, aztreonam; IPM, imipenem; LVFX, levofloxacin; GM, gentamicin.

of the 191 phenotypically determined ESBL producers examined. Furthermore, in a study conducted in India, the *bla*_{TEM} gene was found to be predominant (96.42%), followed by *bla*_{CTX-M} (75%) and *bla*_{SHV} (17.85%) (18). Similarly, *bla*_{TEM} was the gene predominantly isolated (83.33%) in a study conducted in Iran, followed by *bla*_{TEM} (31.48%) and *bla*_{CTX-M} (20.37%) (19). Moreover, in North Central Nigeria, 61.5%, 53.8%, and 38.5% of the isolates examined harbored *bla*_{SHV}, *bla*_{CTX-M}, and *bla*_{TEM} genes, respectively (20). Therefore, the developed primer and probe sets used in the present study would seem to be more applicable in areas where CTX-M-type ESBL-producing strains are dominant and TEM-/

SHV-type ESBL-producers are relatively scarce, such as Japan (21,22).

The present study does, however, have several limitations. First, *bla*_{TEM-1}, *bla*_{TEM-2}, and *bla*_{SHV-1} are classified into group 2 of the Bush–Jacoby classification and are non-ESBL-encoding genes (23). The current primer and probe sets might generate false-positive results for non-ESBL *bla*_{TEM-} or *bla*_{SHV}-containing strains, particularly in areas where these strains are highly prevalent. Second, the most prevalent ESBL-related genes are known to be *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} (3). However, genes such as *bla*_{VEB-1}, *bla*_{PER-1}, and *bla*_{BEL-1} are ESBL-related genes (24,25). An outbreak

of ESBL-producing *P. mirabilis* carrying VEB-1 has been reported at a neonatal intensive care unit in India (26). The utility of the developed primer and probe sets would, thus, be limited in these areas. Third, using the current assay system, it is not possible to perform direct detection of *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{SHV} from clinical specimens such as urine or sputum. Although gene detection from isolated colonies using a fully automated PCR system will confer greater utility to clinical laboratory staff, it is more desirable to detect target genes directly from specimens.

In conclusion, the newly developed primer and probe sets used in this study showed a satisfactory accuracy in the detection of the *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} genes. Moreover, we observed no discrepancy between the results obtained by whole-genome sequencing and the current assay system. This product applied in conjunction with a fully automated PCR system may facilitate the detection of *bla*_{CTX-M}, *bla*_{TEM}, or *bla*_{SHV} in clinical laboratories. The primer and probe sets may be optimally effective in the screening of ESBL-producing bacteria in regions or countries where CTX-M-type ESBL are highly prevalent.

Conflict of interest This study was conducted in collaboration with Nippon Becton Dickinson. Kaichi Ohta is an employee of Nippon Becton Dickinson. Haruka Kuse and Hideki Okada are employees of G&G Science.

REFERENCES

- Bradford PA. Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev*. 2001;14:933-51.
- Lautenbach E, Patel JB, Bilker WB, et al. Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*: risk factors for infection and impact of resistance on outcomes. *Clin Infect Dis*. 2001;32:1162-71.
- Pitout JD, Laupland KB. Extended-spectrum β -lactamase-producing Enterobacteriaceae: an emerging public-health concern. *Lancet Infect Dis*. 2008;8:159-66.
- Nakamura T, Komatsu M, Yamasaki K, et al. Epidemiology of *Escherichia coli*, *Klebsiella* species, and *Proteus mirabilis* strains producing extended-spectrum β -lactamases from clinical samples in the Kinki Region of Japan. *Am J Clin Pathol*. 2012;137:620-6.
- Hara T, Sato T, Horiyama T, et al. Prevalence and molecular characterization of CTX-M extended-spectrum β -lactamase-producing *Escherichia coli* from 2000 to 2010 in Japan. *Jpn J Antibiot*. 2015;68:75-84.
- Dalpke AH, Hofko M, Zimmermann S. Comparison of the BD Max methicillin-resistant *Staphylococcus aureus* (MRSA) assay and the BD GeneOhm MRSA a chromopeptidase assay with direct- and enriched-culture techniques using clinical specimens for detection of MRSA. *J Clin Microbiol*. 2012;50:3365-7.
- Engel T, Slotboom BJ, van Maarseveen N, et al. A multi-centre prospective evaluation of the Check-Direct ESBL Screen for BD MAX as a rapid molecular screening method for extended-spectrum beta-lactamase-producing Enterobacteriaceae rectal carriage. *J Hosp Infect*. 2017;97:247-53.
- Dalpke AH, Hofko M, Zimmermann S. Development and evaluation of a real-time PCR assay for detection of *Pneumocystis jirovecii* on the fully automated BD MAX platform. *J Clin Microbiol*. 2013;51:2337-43.
- Silbert S, Gostnell A, Kubasek C, et al. Evaluation of the BD Max StaphSR assay for detecting Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Methicillin-Susceptible *S. aureus* (MSSA) in ESwab-collected wound samples. *J Clin Microbiol*. 2017;55:2865-7.
- Putsathit P, Maneerattanaporn M, Piewngam P, et al. Prevalence and molecular epidemiology of *Clostridium difficile* infection in Thailand. *New Microbes New Infect*. 2016;15:27-32.
- McHugh MP, Guerendiain D, Hardie A, et al. Detection of norovirus by BD MAXTM, Xpert[®] Norovirus, and xTAG[®] Gastrointestinal Pathogen Panel in stool and vomit samples. *J Clin Virol*. 2018;105:72-6.
- Thapa K, Ellem JA, Basile K, et al. Simultaneous co-detection of wild-type and vaccine strain measles virus using the BD MAX system. *Pathology*. 2018;50:450-4.
- Van Der Pol B, Daniel G, Kodsi S, et al. Molecular-based testing for sexually transmitted infections using samples previously collected for vaginitis diagnosis. *Clin Infect Dis*. 2019;68:375-81.
- Färber J, Moder KA, Layer F, et al. Extended-spectrum beta-lactamase detection with different panels for automated susceptibility testing and with a chromogenic medium. *J Clin Microbiol*. 2008;46:3721-7.
- Ogutu JO, Zhang Q, Huang Y, et al. Development of a multiplex PCR system and its application in detection of *bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M-1}, *bla*_{CTX-M-9} and *bla*_{OXA-1} group genes in clinical *Klebsiella pneumoniae* and *Escherichia coli* strains. *J Antibiot*. 2015; 68:725-33.
- Shibasaki M, Komatsu M, Sueyoshi N, et al. Community spread of extended-spectrum β -lactamase-producing bacteria detected in social insurance hospitals throughout Japan. *J Infect Chemother*. 2016;22:395-9.
- Chong Y, Yakushiji H, Ito Y, et al. Clinical and molecular epidemiology of extended-spectrum β -lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in a long-term study from Japan. *Eur J Clin Microbiol Infect Dis*. 2011;30:83-7.
- Jena J, Debata NK, Sahoo RK, et al. Molecular characterization of extended spectrum β -lactamase-producing Enterobacteriaceae strains isolated from a tertiary care hospital. *Microb Pathog*. 2018;115:112-6.
- Ghorbani-Dalini S, Kargar M, Doosti A, et al. Molecular epidemiology of ESBL genes and multi-drug resistance in diarrheagenic *Escherichia Coli* strains isolated from adults in Iran. *Iran J Pharm Res*. 2015;14:1257-62.
- Olowo-Okere A, Ibrahim YKE, Olayinka BO. Molecular characterisation of extended-spectrum β -lactamase-producing Gram-negative bacterial isolates from surgical wounds of patients at a hospital in North Central Nigeria. *J Glob Antimicrob Resist*. 2018;14:85-9.
- Paterson DL, Bonomo RA. Extended-spectrum β -lactamases: a clinical update. *Clin Microbiol Rev*. 2005;18:657-86.
- Arakawa Y, Ike Y, Nagasawa M, et al. Trends in antimicrobial-drug resistance in Japan. *Emerg Infect Dis*. 2000;6:572-5.
- Bush K, Jacoby GA. Updated functional classification of β -lactamases. *Antimicrob Agents Chemother*. 2010;54:969-76.
- Naas T, Bogaerts P, Bauraing C, et al. Emergence of PER and VEB extended-spectrum β -lactamases in *Acinetobacter baumannii* in Belgium. *J Antimicrob Chemother*. 2006;58:178-82.
- Poiriel L, Brinas L, Verlinde A, et al. BEL-1, a novel clavulanic acid-inhibited extended-spectrum β -lactamase, and the class 1 integron In120 in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2005;49:3743-8.
- Jain S, Gaiand R, Kothari C, et al. VEB-1 extended-spectrum β -lactamase-producing multidrug-resistant *Proteus mirabilis* sepsis outbreak in a neonatal intensive care unit in India: clinical and diagnostic implications. *JMM Case Rep*. 2016;3:e005056.