Method

Single-Tube Multiplex Polymerase Chain Reaction for the Detection of Genes Encoding Enterobacteriaceae Carbapenemase

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SUMMARY: A multiplex PCR assay in a single tube was developed for the detection of the carbapenemase genes of Enterobacteriaceae. Primers were designed to amplify the following six carbapenemase genes: *bla*_{KPC}, *bla*_{IMP}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{OXA-48-like}, and *bla*_{GES}. Of 70 *bla*_{IMP} variants, 67 subtypes were simulated to be PCR-positive based on in silico simulation and the primer-design strategy. After determining the optimal PCR conditions and performing in vitro assays, the performance of the PCR assay was evaluated using 51 and 91 clinical isolates with and without carbapenemase genes, respectively. In conclusion, the combination of multiplex PCR primers and QIAGEN Multiplex PCR Plus Kit was used to determine the best performance for the rapid and efficient screening of carbapenemase genes in Enterobacteriaceae. The assay had an overall sensitivity and specificity of 100%. This PCR assay compensates for the limitations of phenotypic testing, such as antimicrobial susceptibility testing and the modified carbapenem inactivation method, in clinical and public health settings.

INTRODUCTION

Enterobacteriaceae are a large family of Gram-negative bacteria of non-spore forming facultative anaerobes that ferment sugars to produce lactic acid. Many of these family members are commonly found in the human gut flora, some are found in water or soil, and others as parasites in a variety of different animals and plants (1). Some of these bacteria are sources of community-acquired and nosocomial infections, with a propensity to spread easily between humans and to acquire genetic material through horizontal gene transfer, mediated by plasmids and transposons. β -lactam drugs

are often considered the primary therapeutic agent for common infectious diseases; carbapenem antibiotics, in particular, are often considered as the last resort for treatment. In recent years, instances of carbapenem-resistant Enterobacteriaceae (CRE) as causative agents of nosocomial infections have been increasing, creating difficulties for the treatment of disease. Therefore, the emergence and spread of CRE is a major concern in both clinical and public health settings (2,3).

The central mechanism for carbapenem resistance is the production of β -lactamases, known as carbapenemases, which degrade carbapenem. Carbapenem-resistant bacteria that produce these enzymes are referred to as carbapenemase-producing Enterobacteriaceae (CPE). In general, β -lactamase is classified into four classes, from A to D, based on Ambler's classification (4). Among these, three classes show carbapenemase activity. Class A carbapenemases include KPC, SME, IMI, NMC-A, SFC, BIC, and several GES enzymes and are mainly produced by members of Enterobacteriaceae. Class B enzymes are metallo- β -lactamases (MBLs) that efficiently hydrolyze carbapenems and are inhibited by chelating agents, such as EDTA. They include the IMP, VIM, NDM, IND, DIM, GIM, CPH-A, KHM-

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1, and SMB enzymes. Important enzymes detected in *Enterobacteriaceae* include the IMP, VIM, and NDM families. Class D enzymes, known as oxacillinases with carbapenemase activity, are comprised of OXA-23-like, OXA-24/40-like, OXA-48-like, OXA-51-like, and OXA-58-like families. Among these, the OXA-48-like enzymes with carbapenemase activity are encountered frequently in *Enterobacteriaceae*, whereas the others are mainly detected in glucose non-fermentative bacteria, such as *Acinetobacter* species (5). Such CPE often harbors plasmids encoding enzyme genes; the plasmid diffuses and spreads across bacterial species of *Enterobacteriaceae* very easily (5). Therefore, CPE are a significant concern in clinical and public health settings.

CRE that do not produce carbapenemase are also common. Such CREs include extended-spectrum β -lactamase (ESBL)- or AmpC β -lactamase-producing *Enterobacteriaceae* with plasmid-mediated (6) or chromosome-encoded (7) genes with porin mutations (8), resulting in a carbapenem-resistant phenotype. Such resistance to carbapenem is thought to be a low risk to public health because these carbapenem-resistant phenotypes do not transfer horizontally; however occasionally, outbreaks in hospital settings with the same strain may occur, such that it is necessary to monitor them in clinical settings (9).

CRE infectious diseases are increasing in the US and Europe, as many CRE harbor β -lactamase genes, such as *bla*_{KPC}, *bla*_{OXA-48-like}, and *bla*_{NDM}. These carbapenemases have already spread internationally, but significant regional differences exist from a global perspective (2). Most CPEs detected in Japan produce IMPs, and in recent years, due to the globalization of human movement, pathogens have also spread globally in a short time. Therefore, as the number of visitors to Japan has increased, there are concerns that problematic resistant bacteria from overseas could infect the Japanese population. As a result, in 2014, infectious diseases caused by CRE were classified as notifiable diseases under infection control laws to strengthen infection surveillance in Japan. Furthermore, demands for the examination and testing of CRE infections have increased in recent years.

The purpose of this study was to develop a PCR-based tool for the easy and efficient detection of carbapenemase genes in *Enterobacteriaceae*. Multiplex PCR schemes have been previously reported by several research laboratories (10-15), and although interesting, they did not always match the needs of the clinical and public health settings in Japan, where IMP carbapenemase is the most prevalent. Carbapenemases are highly diverse and have significant regional differences across the global. Therefore, a tool needs to be developed for the efficient diagnosis of carbapenemase genes. Here, we developed a multiplex PCR assay to detect carbapenemase genes in a single-tube reaction for rapid and efficient screening.

MATERIALS AND METHODS

Bacterial strains: Six isolates with carbapenemase genes were used to prepare positive control DNA templates, stored at the Antimicrobial Resistance Research Center, National Institute of Infectious

Diseases in Japan; *Klebsiella pneumoniae* with *bla*_{KPC-2}, *Pseudomonas aeruginosa* with *bla*_{IMP-1}, *Escherichia coli* with *bla*_{NDM-1}, *Pseudomonas putida* with *bla*_{VIM-2}, *Klebsiella pneumoniae* with *bla*_{OXA-181}, and *Klebsiella pneumoniae* with *bla*_{GES-24}. To evaluate the multiplex PCR assay, 51 CRE isolates with carbapenemase genes, and 91 non-CPE isolates from clinical specimens were used. These isolates are listed in Table 1 and were selected and used by each co-author from eight regional laboratories in Japan, as follows: Akita, Yokohama city, Toyama, Osaka, Hiroshima, Kagawa, Ehime, and Kumamoto. The subtypes of the carbapenemase genes were determined mainly using PCR and Sanger sequencing with primers annealed near the dual ends of the coding sequences (16-20). The subtyping of the GES-type gene was performed according to previous reports (21). The subtyping of IMP-1-type carbapenemase genes, however, was performed using the primer pair with IMP-1a1IF: 5'-ATGAGCAAGTTATCTGTATTC-3' and IMP-1a1IR: 5'-CATCAAAACCAAGCAACTAA-3', respectively. The gene types of ESBL and AmpC β -lactamase were determined according to previous reports (22, 23).

Design of primers for PCR: The sequences of genes that encode carbapenemases were downloaded from NCBI databases (24). Target genes were selected from six groups of carbapenem-hydrolyzing β -lactamases previously reported in Japan: *bla*_{KPC}, *bla*_{IMP}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{OXA-48-like}, and *bla*_{GES} (25). The genes downloaded were as follows: 24 genes from KPC-(1 to 25), 66 from IMP-(1 to 75), 15 from NDM-(1 to 15), 37 from VIM-(1 to 39), 21 from OXA-48-like-(48 to 505), and 31 from GES-(1 to 31), respectively. The sequences were aligned using Clustal Omega (26), and highly homologous regions were selected for primer design using BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). The suitability of the primers was confirmed using the OligoEvaluator web service (<http://www.oligoevaluator.com/LoginServlet>).

To maximize screening efficiency, group-specific primer pairs for the *bla*_{OXA-48-like} and *bla*_{GES} groups were detected in all variants, including non-carbapenemase. Two sets of primer pairs for the detection of several *bla*_{IMP} variants were designed to detect the same size of each amplicon. This design strategy, aimed at the detection of many *bla*_{IMP} variants, was comprised of only one mixed base in the primer sequence; an A/G or C/T type mismatch at one position was allowed. The T_m value was set to 60°C or higher.

The in silico evaluation of the designed PCR primers was performed using MFEprimer-3.0 (27).

DNA extraction and multiplex PCR: DNA was prepared by suspending several colonies of each bacterial strain in 100 μ l of distilled water, boiling at 98°C for 10 min, and centrifuging the cell extract for 5 min at 15,000 $\times$ g at 4°C. The supernatant was used as the template DNA for PCR.

For PCR, the QIAGEN Multiplex PCR Plus Kit was used with 2 $\times$ PCR MasterMix (final concentration: 1 $\times$), 0.2 μ M of each primer, 2.5 μ l of Q-Solution, 2.5 μ l of CoralLoad Dye, and 1 μ l of template DNA at a total volume of 25 μ l. Amplification was carried out in a standard PCR thermal cycler as follows: 5 min at 95°C (initial activation step), followed by 25 cycles of

Table 1. Clinical gram-negative rod-shaped bacteria used in this study

	Bacterial strains	Type of β -lactamase gene detected	Analysis method ¹⁾	No. of strains tested	
				Subtotal	Total
Carbapenemase gene -positive	<i>Citrobacter freundii</i>	<i>bla</i> _{IMP-1}	1	3	51
	<i>Enterobacter cloacae</i>	<i>bla</i> _{NDM-5}	1 (16)	1	
	<i>Enterobacter cloacae</i>	<i>bla</i> _{IMP-1}	1	12	
	<i>Enterobacter asburiae</i>	<i>bla</i> _{IMP-1}	1	1	
	<i>Escherichia coli</i>	<i>bla</i> _{OXA-181}	1	2	
	<i>Escherichia coli</i>	<i>bla</i> _{IMP-6}	1, 2 (21)	2	
	<i>Escherichia coli</i>	<i>bla</i> _{IMP-1}	1	2	
	<i>Escherichia coli</i>	<i>bla</i> _{NDM-5}	1 (16)	1	
	<i>Klebsiella oxytoca</i>	<i>bla</i> _{IMP-1}	1	8	
	<i>Klebsiella oxytoca</i>	<i>bla</i> _{IMP-10}	1	2	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{IMP-1}	1	4	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{GES-24}	1 (18)	2	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{IMP-11}	1 (19)	2	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{IMP-6}	1, 2 (21)	2	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{GES-24, bla} _{IMP-11}	1 (18, 19)	1	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{KPC-2}	1 (20)	2	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{OXA-48}	1 (17)	1	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{NDM-1, bla} _{OXA-232}	1 (16, 17)	1	
	<i>Kluyvera cryocrescens</i>	<i>bla</i> _{IMP-1}	1	1	
	<i>Serratia marcescens</i>	<i>bla</i> _{GES-5}	1 (18)	1	
Carbapenemase gene -negative ²⁾	<i>Citrobacter freundii</i>	N.D. ³⁾	2	3	91
	<i>Enterobacter cloacae</i>	N.D.	2	15	
	<i>Enterobacter cloacae</i>	<i>bla</i> _{TEM, bla} _{CTX-M-1-group}	2	1	
	<i>Enterobacter cloacae</i>	EBC family ⁴⁾	2	10	
	<i>Enterobacter asburiae</i>	EBC family	2	4	
	<i>Escherichia coli</i>	CIT family ⁴⁾	2	1	
	<i>Escherichia coli</i>	<i>bla</i> _{TEM, bla} _{CTX-M-2-group}	2	3	
	<i>Escherichia coli</i>	<i>bla</i> _{CTX-M-9-group}	2	5	
	<i>Klebsiella aerogenes</i> ⁵⁾	N.D.	2	14	
	<i>Klebsiella aerogenes</i>	EBC family	2	13	
	<i>Klebsiella oxytoca</i>	N.D.	2	1	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{SHV, bla} _{CTX-M-2-group}	2	4	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{CTX-M-1-group}	2	2	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{SHV}	2	1	
	<i>Klebsiella pneumoniae</i>	<i>bla</i> _{SHV, bla} _{CTX-M-9-group}	2	1	
	<i>Klebsiella pneumoniae</i>	N.D.	2	6	
	<i>Proteus mirabilis</i>	<i>bla</i> _{CTX-M-2-group}	2	2	
	<i>Providencia stuartii</i>	N.D.	2	1	
	<i>Serratia marcescens</i>	N.D.	2	4	

¹⁾: Decision methods of carbapenemase gene subtypes: 1, PCR and Sanger sequencing; 2, PCR. Numbers in parenthesis were the reference numbers.

²⁾: including CRE-suspected strains. PCR were performed according to reference 22 and 23.

³⁾: N.D., not detected.

⁴⁾: Notated according to reference 23.

⁵⁾: Formerly, *Enterobacter aerogenes*.

95°C for 30 s, 60°C for 90 s, and 72°C for 60 s. The last extension step was performed at 68°C for 10 min. A few aliquots of the PCR products were used for electrophoresis on a 3% NuSieve 3:1 agarose gel stained with ethidium bromide or GelRed (Biotium, Hayward, CA, USA) to confirm the amplicon DNA (Fig. 1). To assess the robustness of PCR, the standard PCR thermal cyclers used in this study were as follows: ProFlex PCR System, SimpliAmp thermal cycler, Veriti 96-well thermal cycler, GeneAmp PCR System 9700 (Thermo Fisher Scientific, Inc. MA, USA), S1000 thermal cycler (Bio-Rad Laboratories Inc., Tokyo, Japan), and PCR

Thermal Cycler Dice (TaKaRa Bio Inc. Shiga, Japan).

In addition to the QIAGEN Multiplex PCR plus Kit, other PCR enzymes used were as follows: Illustra Hot Start Mix RTG (GE Healthcare UK Ltd, England), puRe Taq Ready-To-Go PCR Beads (GE Healthcare UK Ltd, England), GoTaq Hot Start Green MasterMix (Promega KK, Tokyo, Japan), Multiplex PCR Assay Kit (Takara Bio Inc.), Takara *Ex Taq* Hot Start Version (Takara Bio Inc.).

RESULTS

Fig. 1. Detection of PCR products by multiplex PCR for six carbapenemase genes. PCR products were separated on a 3% agarose gel in $1 \times$ TAE buffer. Lane M, 100-bp DNA ladder; lane 1, KPC-type enzyme producing strain; lane 2, IMP-type; lane 3, NDM-type; lane 4, VIM-type; lane 5, OXA-48-like-type; lane 6, GES-type; lane 7, PCR products obtained using a mixture of the six template DNA from each carbapenemase-positive strain; lane 8, PCR product of the negative control (used distilled water instead of template DNA solution). The size in base pairs is shown on the left.

Design of group-specific primers for multiplex PCR in a single-tube assay: A single-tube multiplex PCR assay was designed in this study. All the primer sequences used are listed in Table 2.

The potential of the PCR of all the primers was determined via *in silico* simulation for all applicable carbapenemase gene sequences. The *in silico* PCR results were combined with the results of MFEprimer-3.0. The primers paired with stable duplex formation for target sequences (indicated the lower of ΔG value) were determined as PCR-positive. At least one of the paired primers without a stable duplex was considered to be PCR-negative. All members of the *bla*_{KPC} and *bla*_{NDM} groups were estimated as detectable. However, some genes of the *bla*_{IMP}, *bla*_{VIM}, and *bla*_{OXA-48-like} groups were not detectable. There were nine undetectable genes of carbapenemase as follows: three genes of *bla*_{IMP}-31, 35, and 58, three of *bla*_{VIM}-27, 37, and 49, and three of *bla*_{OXA}-54, 436, and 535 (Table 2). Sixteen genes of IMP-type carbapenemases in Table 2 were estimated to be PCR-positive from the results, with the option of allowing one mismatch of the MFEprimer3.0 command line program. In addition, some genes of the *bla*_{OXA-48-like} and *bla*_{GES} groups without carbapenemase activity were also estimated to be

Table 2. Sequences of primers used for multiplex PCR to detect genes encoding carbapenemases in Enterobacteriaceae, and *in silico* simulations of PCR performances

Targeted Gene groups	Primer Name	Sequence (5' to 3' Direction) ¹⁾	Length (bases)	T _m (°C)	Amplicon Size (bp)	Subtype numbers by <i>in silico</i> PCR simulations of group-specific primers for detecting carbapenemase genes ²⁾			
						Subtypes of detecting carbapenemase genes	Subtypes of detecting non-carbapenemase genes	Subtypes of carbapenemase genes detectable by transition-typed primers	Subtypes of not detecting carbapenemase genes ³⁾
<i>bla</i> _{KPC} ⁴⁾	mc-kpc-f	CGGAACCATTCGCTAACTCG	21	67.5	322	(29) ⁵⁾ 1-22, 24-30			
	mc-kpc-r	AACAAATTGGCGGCGCGCT	19	73.5					
<i>bla</i> _{IMP} ⁴⁾	mc-imp-f1	TCTCRATCTATCCCCACGTATGC	23	65.8	269	(51) 1-10, 13, 15-20, 23-26, 28, 30, 33, 37, 38, 40, 42-45, 47, 49, 51-53, 55, 56, 59, 60-62, 66, 69-75		(16) ⁶⁾ 11, 12, 14, 14a, 21, 22, 27, 29, 32, 41, 48, 54, 63, 64, 67, 68	(3) 31, 35, 58
	mc-imp-f2	TCTCAATCTATTCCAACATATGCATCTG	28	66.5					
	mc-imp-r1	GCGGACTTTGGCCAAGCTTCTA	22	70.2					
	mc-imp-r2	GCMGAATGTGGCCACGCTTCAA	22	73.3					
<i>bla</i> _{NDM}	mc-ndm-f	CGGTTTGGCGATCTGGTTTT	20	68.0	207	(24) 1-24			
	mc-ndm-r	GACCGGCAGGTGATCTCC	19	67.0					
<i>bla</i> _{VIM} ⁴⁾	mc-vim-f	GTTTGGTCGCATATCGCAAC	20	65.2	155	(49) 1-20, 23-26, 28-36, 38-48, 50-54			(3) 27, 37, 49
	mc-vim-r	CCAATTGCTTYTCAATCTCCG	22	66.3					
<i>bla</i> _{OXA-48-like}	mc-oxa48-f	GCTCTGGAATGAGAATAAGCAGCA	24	67.4	125	(12) 48, 48b, 162, 181, 199, 204, 232, 244, 245, 247, 484, 517	(16) ⁷⁾ 163, 252, 370, 405, 416, 438, 439, 505, 514, 515, 519, 546, 547, 566, 567		(3) 54, 436, 535
	mc-oxa48-r	TAACCACGCCCAAATCGAG	19	65.8					
<i>bla</i> _{GES} ⁸⁾	mc-ges-f	CTGTGGCTAAAGTCCTCTATGGCG	24	68.8	94	(10) 4-6, 14-16, 18, 20, 21, 24	(24) ⁹⁾ 1-3, 7-13, 17, 19, 22, 25-35		
	mc-ges-r	GTCGCGTCTCCCGTTGGTT	20	70.5					

¹⁾ For degenerate primers: R = A or G; Y = C or T; M = A or C

²⁾ All designed primers were not tested because of difficulties in preparing all the positive control templates in this study. *in silico* PCR simulations were calculated using MFEprimer-3.0 (27).

³⁾ Host organisms with these genes are as follows: *Pseudomonas* spp. for three *bla*_{IMP}-(31,35 and 58) and three *bla*_{VIM}-(37 and 49); *Shewanella* spp. for two *bla*_{OXA}-(54 and 535) as glucose non-fermenters; *Klebsiella pneumoniae* for *bla*_{VIM}-27 and *Citrobacter freundii* for *bla*_{OXA}-436 as glucose fermenters. These information are obtained from the public data under accession numbers as follows: KF148593, JF816544, KU647281, JX131372, KU663374, AY500137, KX828709, HQ858608 and KT959108, respectively.

⁴⁾ Missing type numbers indicate *bla*_{KPC}-23, *bla*_{IMP}-(36, 39, 46, 50, 57, 65), and *bla*_{VIM}-(21,22,40); these were not detected in the public database.

⁵⁾ Numbers of corresponding β -lactamase genes in parenthesis.

⁶⁾ Subtypes that the duplex will probably be amplified if it is a primer with at least one nucleotide mutation by analysis of MFEprimer-3.0. *bla*_{IMP}-11 was indicated to be PCR-positive *in vitro* (Figure 2).

⁷⁾ Two of the 15 β -lactamases are ESBL; *bla*_{OXA}-(163, 405).

⁸⁾ *bla*_{GES}-23 is undetectable by the PCR primers, which is ESBL.

⁹⁾ Five of the 24 β -lactamase genes are ESBL; *bla*_{GES}-(1-3, 8, 11).

Fig. 2. PCR products by a mismatch primer for detection of a *bla*_{IMP} variant type by multiplex PCR. (a) Sequence alignments of binding regions with the forward primer, mc-imp-f1, and the reverse primer, mc-imp-r2, for *bla*_{IMP-11}. The binding regions show one mismatch base-pairing (bold face) in each primer sequence. (b) PCR products using the template DNA from *Klebsiella pneumoniae* with *bla*_{IMP-11} and *bla*_{GES-24} by multiplex PCR. (c) Singleplex PCR for *bla*_{IMP-7} gene DNA using primers for *bla*_{IMP} of the multiplex PCR. Primer sets used for singleplex PCR are as follows: f1, mc-imp-f1; f2, mc-imp-f2; r1, mc-imp-r1; r2, mc-imp-r2 (Table 2). Lane M, DNA ladder; lane S, the template DNA with *bla*_{IMP-7} gene; lane P1, the template DNA with *bla*_{IMP-1} gene as reference; lane P2, the PCR products obtained using a mixture of six template DNA from each carbapenemase-positive strains; lane N, PCR product of the negative control (distilled water).

detected as follows: 15 *bla*_{OXA}-types and 24 *bla*_{GES}-types. The primer sequences for the detection of all groups, calculated lengths of corresponding amplicons, and *in silico* information are also provided in Table 2.

PCR performances using positive DNA templates with carbapenemase genes: The multiplex primer sets were evaluated using DNA extracts from each strain with *bla*_{KPC}, *bla*_{IMP}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{OXA-48-like}, and *bla*_{GES} genes as the positive controls. Under optimized conditions of PCR using the QIAGEN Multiplex PCR Plus Kit, the mixed primers yielded amplified products of the expected size, and the same size detected when using individual primers for the six groups (Fig. 1) using six standard PCR thermal cyclers. Small amounts of non-specific amplified products were detected. Setting the annealing temperature to 60°C was critical to obtain accurate results.

Evaluation of the primer mixture for multiplex PCR in a single tube using clinical CRE isolates: To confirm the PCR performance of the multiplex PCR primers, template DNA extracted from 51 clinical isolates of six genera previously identified as CRE with carbapenemase genes, and 91 clinical isolates of seven genera without carbapenemase genes (Table 1), were used. PCR was performed at eight laboratories using several thermal cyclers. Non-specific amplified products

were observed in part using the DNA of the CRE strains with the carbapenemase gene (e.g. a ~1000-bp PCR product in lane P2 of Fig. 2b). However, when compared with the amplified products using positive controls, their sizes were always different, and the amount of amplification was low.

Further, with PCR using the template DNA of CRE strains without the carbapenemase genes, the amplified product expected as carbapenemase gene was not detected. From the above results, it was possible to determine whether the amplified product of the expected size targeting the carbapenemase gene could be obtained, even when a non-specific amplified product was detected. As a result, it was confirmed that the combination of the QIAGEN Multiplex PCR Plus Kit and our multiplex primer sets had the best performance. The assay had an overall sensitivity and specificity of 100%.

In addition, commercially available kits or enzymes other than the QIAGEN Multiplex PCR Plus Kit were used to perform and test the PCR assay. Depending on the kit, specific or non-specific amplified products were detected. Using puRe Taq Ready-to-Go PCR Beads, GoTaq Hot Start Green MasterMix, and Ex Taq HS, small amounts of non-specific amplification products were detected, whereas non-specific amplification was

observed using the Multiplex PCR Assay Kit.

Evaluation of PCR products using a mismatch primer for the detection of a *bla*_{IMP} variant type in multiplex PCR: In the multiplex primer mixture for detecting *bla*_{IMP}, several *bla*_{IMP} variants were expected to be detected using MFEprimer-3.0 (27). In this study, 16 out of 70 *bla*_{IMP} genes were expected to be amplified *in silico*, with at least one primer having a mismatched primer. In this study, the IMP types used for evaluating the PCR assay were IMP-1, 6, 10, and 11 for the CPE strains (Table 1). Among them, the IMP-11 type was presumed to be PCR-positive using the primer set with each transition-type mismatch base using MFEprimer-3.0 (Fig. 2a). In multiplex PCR using the template DNA from the *Klebsiella pneumoniae* strain with *bla*_{IMP-11} and *bla*_{GES-24} (Table 1), both carbapenemase genes were PCR-positive (Fig. 2b). Four singleplex PCR assays used a combination of two forward primers, and two reverse primers were then performed to confirm the actual primer pairs in this PCR. The PCR product was only detected using the primer pair of mc-imp-f1 and mc-imp-r2 (Fig. 2c). This demonstrated that one of the four sets of PCR primers for the *bla*_{IMP}-group functioned as a specific amplification one-transition-type mismatch mode.

DISCUSSION

In addition to carbapenems, CPE often acquires resistance to other antimicrobials used in clinical settings (26) and are difficult to treat if infectious diseases are caused. It is, therefore, necessary to be vigilant about infection when resistance genes are transformed with a plasmid into a bacterial species and then propagated. The first laboratory examinations included “MIC value of the assigned antibiotic” or “the inhibition zone diameter of disk” in Enterobacteriaceae isolates. However, some CPEs showed susceptibility to certain carbapenem antibiotics (5). Therefore, it is difficult to distinguish CPE by phenotypic examination using conventional antimicrobial susceptibility tests alone. Several methods for detecting CPE have been reported. The method for detecting carbapenemase by immunochromatography is quick (15 min).

Moreover, although the disc method and selective agar medium of the CRE screening method for the detection of carbapenemase are commercially available, they require around 18–24 h to determine the result, based on the culture time of the bacteria. Although these methods are already commercially available and easy to use, some detection performances for determining CPE are insufficient (29). Meanwhile, the detection of the carbapenemase gene is considered a reliable detection method. In addition, the rapid and accurate detection of foreign CRE is required to prevent nosocomial infections. These tests detect carbapenemase genes and require molecular epidemiological information. Therefore, the multiplex PCR method is the best screening tool, as it can rapidly determine multiple genotypes. For example, the method of Poirel *et al.* (11) targeted several carbapenemase genes; however, this reaction required three tubes and may require further optimization for use in Japan.

In contrast, the method of Sakanashi *et al.* (14)

is suitable for use in the laboratories of medical facilities since it is a one-tube reaction and targets the carbapenemase genes specific to Japan. However, for this method, the primers need to be prepared in-house. Since a specific enzyme and PCR thermal cyclers are required, optimization may be necessary. The Cica Geneus® Carbapenemase Genotype Detection KIT 2 (Kanto Chemical Co., Inc.) is a commercially available product that not only targets the six major carbapenemase genes detected in Japan but can also detect carbapenemase-type GES genes. However, this assay is performed in a two-tube reaction, which is expensive. Therefore, our single-tube PCR method seems to be superior in terms of both operationality and cost. Furthermore, it is important to deal with the emergence of new CRE subtypes, as well as new carbapenemase genes. The PCR assay presented here will maintain its performance in the public health research community in Japan.

In the multiplex PCR assay, the *T_m* values of all the primer sequences were designed to be 60°C or higher to maintain their specificity. Therefore, setting the annealing temperature to 60°C was critical to obtain accurate results in actual PCR. A major feature of the multiplex PCR method is the primers used for detecting IMP-type carbapenemases. Since there are multiple genotypes for the IMP type, a primer that allows for transition-type mismatch was designed to detect the IMP type. The primers with transition-type mismatch were expected to function as PCR primers despite a mismatch, as there is no change in the purine or pyrimidine bases. The primers designed in this study were subjected to PCR quality control using MFEprimer-3.0 (27). This software analyzes the specific reaction of primer and its target DNA duplex formation by searching for thermodynamic stability using the *k*-mer algorithm on the template DNAs. The number of *bla*_{IMP} detectable by the program as transition-typed primers was 16 (Table 2). The *bla*_{IMP} types used for evaluating the PCR method were IMP-1, 6, 10, and 11 for the CPE strains (Table 1). Among them, *bla*_{IMP-11} was PCR-positive with both a mismatch and a primer pair (Fig. 2a). The results suggest that PCR with one transition-type mismatched primer has a functioning specificity (Fig. 2).

Non-detectable carbapenemase genes were evaluated *in silico*, including three genes of *bla*_{IMP}, three genes of *bla*_{VIM}, and three genes of *bla*_{OXA48-like} groups (Table 2). Among these nine genes, the host organisms with seven genes were glucose non-fermentative bacteria (e.g., *Pseudomonas* or *Shewanella* spp.), which did not belong to Enterobacteriaceae (Footnote c in Table 2). Therefore, it is not believed that these results reduce the value of our multiplex PCR assay for CRE testing in this study.

In the PCR-positive cases of the *bla*_{OXA48-like} and *bla*_{GES} groups, the selection of the CPE isolate is not possible due to the inability to distinguish carbapenemase from ESBL and/or others. Among the 15 subtypes of the *bla*_{OXA48-like} group with carbapenemase activity, 12 subtypes were detectable *in silico*; however, 16 without carbapenemase activity were also detected. In the *bla*_{GES} group, among 34 detectable subtypes, ten subtypes had carbapenemase activity. Therefore, DNA sequencing following PCR amplification using other primer sets is needed to confirm the carbapenemase-type for

*bla*_{OXA-48-like} (17) and *bla*_{GES}. Alternatively, a PCR assay for the identification of carbapenemase-type *bla*_{GES} may be useful (30).

In conclusion, the multiplex PCR primers tested *in silico* and *in vitro* may be useful for the rapid and efficient screening of the carbapenemase genes of *Enterobacteriaceae* in both clinical and public health settings. This PCR assay will compensate for the shortfall of phenotypic testing, e.g., drug susceptibility testing and modified carbapenem inactivation method (31). The rapid and accurate diagnosis of CPE will contribute to future attempts at controlling and managing CRE infections.

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Conflict of interest None to declare.

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