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ORIGINAL RESEARCH ARTICLE

High-Speed Quenching Probe-Polymerase Chain Reaction Assay for the Rapid Detection of Carbapenemase-Producing Gene Using GENECUBE: A Fully Automatic Gene Analyzer

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Abstract

Background The prevalence of carbapenemase-producing organisms (CPOs) globally poses a public health threat; however, detecting carbapenemases is a challenge because of their variety.

Methods GENECUBE, a fully automated gene analyzer, detects a target gene in a short time and simultaneously detects its single nucleotide polymorphism. We used this property to develop for the first time a rapid assay for detecting CPOs from cultured bacteria using GENECUBE. The original primer-probe sets were used to detect bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, and $bla_{OXA\text{-}48\text{-}like}$ from 149 CPOs (nine types) and 61 non-CPOs.

Results The sensitivity, specificity, and positive and negative predictions of the GENECUBE assay were 100%. This assay detected carbapenemase single-producers and carbapenemase co-producers with 100% accuracy. The time required for detects of four types of carbapenemase at one run was about 30 min, but it took about 1 h to detect all five types. In addition, this assay performed the rapid detection and classification of $bla_{OXA\text{-}48}$, $bla_{OXA\text{-}181}$, $bla_{OXA\text{-}232}$, and $bla_{OXA\text{-}244}$ simultaneously.

Conclusions The GENECUBE assay is a promising tool for controlling the spread of CPOs and helping to select accurate and rapid antibiotic therapies.

Key Points

GENECUBE quickly and accurately detects the five major carbapenemase-encoding genes in full automatically.

This assay can classify subtypes by recognizing single nucleotide polymorphisms in the target genes.

Users are free to design quenching probes, allowing them to accommodate new genes and/or subtypes.

1 Introduction

Carbapenems are the last-line of defense against *Enterobacterales* infection. However, carbapenem-resistant organisms (CROs) have become more commonly seen since their introduction. CRO infections are associated with higher mortality

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compared to infections caused by carbapenem-susceptible organisms [1]. Resistance to carbapenems occurs via two major mechanisms: carbapenemase production and combined β-lactamase activity and structural mutation [2]. *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM), imipenemase (IMP), and oxacillinase (OXA)-48-like, the most common carbapenemases, are known as the ''big five'' and have spread worldwide. Carbapenemases are the antibiotic resistance mechanisms that have received the most attention because they can be easily spread by plasmids and cause extensive carbapenem resistance [3]. Therefore, the rapid and accurate identification of carbapenemase-producing organisms (CPOs) and types of carbapenemases is the key to controlling their spread and treating infection.

The detection of carbapenemases will be helpful in guiding empirical and specific antibiotic therapy and improving the therapeutic efficacy of antibiotics. Moreover, the rapid identification of carbapenemases could be of significant value to public health officials for infection control and epidemiological assays [4]. Various methods have been developed for the detection of carbapenemases that depend on their phenotypic, biochemical, electrochemical, colorimetric, and immunochromatographic characteristics [5–9]. The modified carbapenem inactivation method (mCIM) is a CPO detection assay that can be performed without using special equipment, and easily implemented in many medical institutions, including those in developing countries [10]. However, although mCIM and its improved methods can detect carbapenem hydrolysis activity and classify β-lactamase according to the Ambler classification, it cannot detect the encoding genes involved [11]. However, mCIM can detect carbapenem hydrolysis activity and not the gene involved. The introduction of rapid detection equipment for specific genes has significantly reduced the time required for infectious disease detection. However, these devices detect major target genes and cannot detect non-target or novel genes [7, 9]. Among the carbapenemases, OXA-48-like and IMP have different carbapenem hydrolysis abilities depending on their subtype [4, 12]. Although the minimum inhibitory concentration (MIC) value of this type of carbapenemase shows susceptibility at a glance, the minimum bactericidal concentration value is much higher, so that a sufficient therapeutic effect is often not obtained. Therefore, the detection of such subtypes will improve the accuracy of treatment of CPO infections.

GENECUBE (TOYOBO Co., Ltd., Osaka, Japan) is a fully automated testing system that can perform nucleic acid extraction, gene amplification, and gene detection in a short time (within 1 h). The system allows genetic testing to be performed with the simple preparation of setting samples, reagents, and consumables according to the instructions displayed on the touch panel. In addition, all processes are automatically performed to eliminate the complexity of operation and the risk of contamination. The novelty of this system is that it uses thermostable DNA polymerase derived from the *Thermococcus kodakarensis* KOD1 strain (KOD DNA polymerase) for a polymerase chain reaction (PCR) and a quenching probe (QProbe) to detect the amplification product, enabling high-speed amplification with a specificity of detecting a single nucleotide polymorphism [13, 14]. QProbe is easy to use because the design of the sequence is simple [15–19].

In this study, we developed a rapid detection assay for carbapenemase-encoding genes by GENECUBE using 7 types of CPOs, including 16 subtypes. To our knowledge, this the first report confirming the identification and differentiation of five major carbapenemases by the GENECUBE system with high accuracy.

2 Materials and Methods

2.1 Bacterial Isolates

An international collection of 181 *Enterobacterales* and 29 *Pseudomonadales* isolated from Japan, Bangladesh, and Egypt were evaluated in this study (Table 1). Of these isolates, 178 were clinical isolates from human blood, urine, sputum, and pus, and 32 were animal isolates from the nasal discharge of cat, dog, and sheep. These were all consecutive isolates eliminating patient duplication. These isolates were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and molecularly characterized by whole genome sequencing or sequencing of different carbapenemase-encoding genes by PCR prior to this study. The isolates collection consisted of 61 non-CPO isolates and 149 CPO isolates encoding a total of 155 carbapenemases, including KPC ($n = 22$), GES ($n = 1$), IMP ($n = 35$), VIM ($n = 16$), NDM ($n = 40$), OXA-48-like ($n = 30$), and OXA-23-like ($n = 11$) (Table 2). Of these, six included VIM and NDM co-producers ($n = 1$) and NDM and OXA-48-like co-producers ($n = 5$). The MICs of carbapenems were measured through the microdilution method (https://clsi.org/standards/products/free-resources/access-our-free-resources/). For evaluating the test results, a fresh bacterial culture yielded on Mueller–Hinton agar (MHA) plates was used.

2.2 High-Speed Quenching Probe-PCR Assay

Carbapenemase detection was evaluated using the GENECUBE system, GENECUBE test basic kit (TOYOBO), and primer-probe (PP) sets (Table 3). Briefly, bacterial isolates were grown on MHA plates overnight at 37 °C and the yielded growth was adjusted to McFarland No.

Table 1 Details of bacterial strains used

	No. of isolates	Carbapenemase-production types									
		KPC	GES	IMP	VIM	VIM + NDM	NDM	NDM + OXA-48-like	OXA-48-like	OXA-23-like	Negative
Bacterial strains											
Escherichia coli	60	9	–	9	–	–	13	1	7	–	21
Klebsiella pneumoniae	57	9	–	10	–	–	10	3	16	–	9
Klebsiella aerogenes	6	–	–	–	–	–	–	1	1	–	4
Klebsiella oxytoca	2	–	1	–	–	–	–	–	–	–	1
Enterobacter cloacae	37	3	–	16	–	–	6	–	–	–	12
Enterobacter hormaechei	7	–	–	–	5	–	–	–	–	–	2
Enterobacter kobei	1	–	–	–	–	–	1	–	–	–	–
Citrobacter freundii	5	1	–	–	1	1	1	–	–	–	1
Proteus mirabilis	5	–	–	–	–	–	1	–	1	–	3
Serratia plymuthica	1	–	–	–	–	–	–	–	–	–	1
Pseudomonas aeruginosa	13	–	–	–	4	–	2	–	–	–	7
Pseudomonas putida	2	–	–	–	2	–	–	–	–	–	–
Pseudomonas mendocina	2	–	–	–	2	–	–	–	–	–	–
Pseudomonas alcaligenes	1	–	–	–	1	–	–	–	–	–	–
Acinetobacter baumannii	11	–	–	–	–	–	–	–	–	11	–
Isolation country											
Japan	105	22	1	35	8	1	12	–	9	–	17
Bangladesh	73	–	–	–	2	–	20	5	13	11	22
Egypt	32	–	–	–	5	–	2	–	3	–	22
Total isolates	210	22	1	35	15	1	34	5	25	11	61

0.5 bacterial suspension by saline. DNA was extracted by diluting this bacterial suspension 100-fold by sample solution (TOYOBO). The PP set was prepared by mixing the PP diluent (PPD Mix; TOYOBO), forward primer, reverse primer, QProbe, and the internal control reagent (IC Mix; TOYOBO). The DNA solution, PP set, and enzyme reagent (KOD DNA polymerase mixture, KOD Mix; TOYOBO) were placed in GENECUBE and automatic detection was performed using the following program. For the IMP set, the PCR included 60 cycles of denaturation at 97 °C for 1 s, annealing at 54 °C for 3 s, and extension at 63 °C for 5 s. For the other sets, PCR included 50 cycles of denaturation at 97 °C for 1 s, annealing at 58 °C for 3 s, and extension at 63 °C for 5 s. The use of asymmetric fast PCR reduces the occurrence of non-specific amplification during annealing and elongation. The fluorescence (FL) intensity was subjected to a melting curve analysis and the peak value and temperature of the amount of change was determined.

2.3 Statistical Analysis

The results from the molecular characterization of the CPO by PCR and sequencing served as the standard and were used to calculate the accuracy, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and 95% confidence intervals (CIs) of the high-speed Quenching Probe-PCR (QP-PCR) assay [20]. Accuracy is the proportion of true positive and true negative results of the evaluated assays and indicates the overall probability that a gene was correctly identified.

3 Results

3.1 Detection of Carbapenemases

The amplification curve of FL intensity for each PP set is shown in Fig. 1. The peak temperatures (average ± 2 standard deviations) of the positive samples in the KPC, IMP, VIM, and NDM sets were 65.7 ± 0.8 °C, 60.0 ± 1.6 °C, 59.8 ± 0.5 °C, and 64.8 ± 0.7 °C, respectively. Interestingly, when the SNP was present in the sequence corresponding to the QProbe, the peak temperature was lower than in a perfect match. In the VIMm set, the peak temperature for $bla_{\text{VIM-4}}$ with a perfect match was 58.6 ± 0.2 °C, whereas that for $bla_{\text{VIM-2}}$ with a single mismatch was 52.9 ± 1.2 °C.

Table 2 Profile of isolates used

Carbapenemases	Subtypes	No. of isolates	MIC90 (MIC range)		
			IPM	MEPM	DRPM
KPC	KPC-2	20	32 (4–32)	32 (4–32)	32 (2 to 32)
	KPC-19	2	8 (4–8)	8 (4–8)	4 (2 to 4)
GES	GES-20	1	1 (1)	0.25 (0.25)	0.25 (0.25)
IMP	IMP-1	24	16 (0.5–16)	16 (0.25–16)	16 (0.25 to 16)
	IMP-6	11	0.5 (0.25–0.5)	16 (2–32)	16 (2 to 32)
VIM	VIM-2	10	64 (1–64)	64 (0.5–64)	32 (1 to 32)
	VIM-4	5	2 (1–2)	0.5 (0.12–0.5)	0.12 (0.12)
VIM + NDM	VIM-2 + NDM-1	1	2 (2)	2 (2)	1 (1)
NDM	NDM-1	12	16 (4–32)	32 (4–32)	32 (4 to 32)
	NDM-4	2	16 (16)	32 (32)	32 (32)
	NDM-5	10	16 (2–32)	32 (8–128)	32 (4 to 32)
	NDM-7	10	32 (8–128)	64 (8–64)	32 (16 to 64)
NDM + OXA-48-like	NDM-5 + OXA-181	3	128 (64–128)	> 128 (> 128)	>128 (128 to > 128)
	NDM-5 + OXA-232	2	> 128 (> 128)	>128 (> 128)	> 128 (> 128)
OXA-48-like	OXA-48	7	2 (1–4)	4 (0.25–16)	1 (0.25 to 8)
	OXA-181	11	16 (1–16)	32 (0.12–32)	32 (0.12 to 64)
	OXA-232	5	4 (0.5–4)	16 (0.12–16)	8 (0.25 to 8)
	OXA-244	2	0.5 (0.5)	0.12 (0.12)	0.25 (0.12 to 0.25)
OXA-23-like	OXA-23	11	32 (8–32)	32 (16–32)	32 (8 to 32)
Negative	–	61	16 (≤ 0.03–32)	16 (≤ 0.03–16)	4 (≤ 0.03 to 16)

Table 3 List of primers and quenching probes used

Primer-probe set	Name	Target	Sequence (5′–3′)	Amplicon size (bp)	Genbank accession number (position)
KPC set	KPC-F	bla_{KPC}	GCGATACCACGTTCCGTCTG	185	MK940322 (464–483)
	KPC-R		GCCGGTCGTGTTTCCCTTTAGC		(648–627)
	KPC-QP		AGCTGAACTCCGCCATCCC-BODIPY-FL		(500–518)
IMP set	IMP-F	bla_{IMP}	TTACDGCTAMAGAYACTGAAAANTTAG	163	AY251052 (206–232)
	IMP-R		AGBTCATTHGTTAATWCAGANGCATAYGT		(368–340)
	IMP-QP		GAGAATTAAGCCACTCTATTCC-BODIPY-FL		(328–307)
VIM set	VIM-F	bla_{VIM}	CGCGGAGATTGARAAGCAAA	247	MK807022 (288–307)
	VIM-R		CGCAGCACCRGGATAGAARAG		(534–514)
	VIM-QP3		GGCAACGTACGCATCAC-BODIPY-FL		(393–409)
VIMm set[a]	VIM-QP4		GATTCCCACGCATTCTC-BODIPY-FL		(447–463)
NDM set	NDM-F	bla_{NDM}	TTTGGCGATCTGGTTTTCCG	101	MK834314 (136–155)
	NDM-R		ATCAAACCGTTGGAAGCGAC		(236–217)
	NDM-QP		CTGGCAGCACACTTCCTATCTC-BODIPY-FL		(174–195)
OXA48 set	OXA48-F1	$bla_{OXA-48-like}$	TGCCCGCCAAATTGG	219	MK469978 (378–392)
	OXA48-R1		GCTTCGGTCAGCATGGC		(596–580)
	OXA48-QP		GCCCGAAATGTCCTCATTACC-BODIPY-FL		(453–433)

Details of the detectable subtypes are shown in Supplementary Table 1

[a]VIMm set uses the same primers as the VIM set

In the OXA-48 set, the peak temperatures for $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-244}}$ with a perfect match were 62.9 ± 2.9 °C and 62.7 ± 2.4 °C, respectively, whereas that for $bla_{\text{OXA-181}}$ and $bla_{\text{OXA-232}}$ with three mismatches were 43.8 ± 0.9 °C and 44.0 ± 0.4 °C, respectively.

3.2 Performance Characteristics

The PP set for carbapenemases accurately detected each target gene from 149 CPO isolates. Each PP set detected the target carbapenemase with 100% sensitivity and 100% PPV and distinguished target and non-target with 100% specificity and NPV (Table 4). Although cross-reactivity is a concern in the detection of carbapenemase co-producers, the PP sets accurately classified the three types of co-producers. The maximum running time required for these detections was 30 min, and the maximum turnaround time, including DNA extraction and reagent settings, was 46 min (Table 5).

4 Discussion

The worldwide emergence and diversification of CPO is represent a major health problem for humanity. There is an increasing demand for the rapid and accurate detection of carbapenemases so that accurate diagnosis and decision-making for antibiotic therapy can be made for CPO infections [4]. The ideal CPO detection method is a test with 100% sensitivity and the highest specificity. However, accurate detection of all carbapenemases is difficult, because the diversity of carbapenemases is complex [6, 7]. In this study, we evaluated the performance characteristics of a high-speed QP-PCR assay for detecting the "big five" carbapenemases

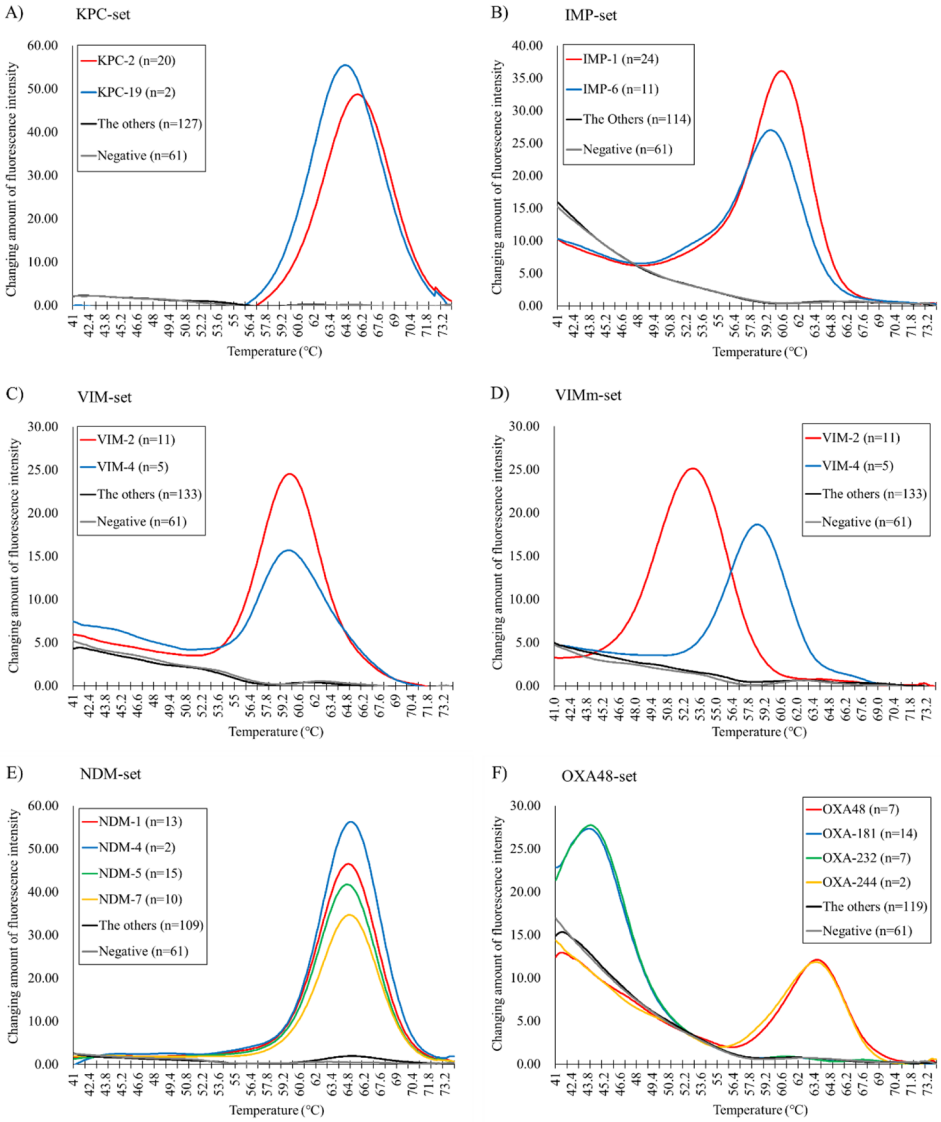

Fig. 1 Melting curve according to changing amount of fluorescence (FL) intensity

(KPC, IMP, VIM, NDM, and OXA-48-like). We examined 210 isolates, including 149 known CPOs. This is the first report on CPO detection by GENECUBE.

CPO can be detected using the disk diffusion method, PCR, and mass spectrometry accurately, rapidly, and easily [5–9]. However, no method can detect the big five carbapenemases with 100% accuracy, and it is difficult to correctly distinguish strains carrying two or more carbapenemases [21]. In this study, the carbapenemases were detected rapidly and accurately by an assay using the originally designed PP set and GENECUBE. The PP set, designed to detect many subtypes, not only detected the corresponding carbapenemase with 100% sensitivity and PPV, but also correctly distinguished multiple carbapenemases from one isolate. The NDM and OXA-48-like co-producer is more antibiotic resistant than the carbapenemase single producer (Table 2). Moreover, each PP set showed no cross-reactivity with other carbapenemases. A high PPV assay allows for fast and accurate treatment of CPE-infected patients. In addition, the NPVs of the big five carbapenemase-encoding genes were 100% in this study. The high NPV of the assay allows for the rapid and accurate identification of patients who do not carry CPOs. This may help in infection control interventions, such as patient isolation, to prevent outbreaks.

The carbapenem-degrading ability varies depending on the type and subtype of the carbapenemase. Some carbapenemases, such as IMP-1/-6/-34 and OXA-48/-181/-232/-244, cannot decompose carbapenem efficiently and show false susceptibility to carbapenem [4, 12, 22]. These carbapenemase-producing carbapenem-susceptible organisms often become resistant after carbapenem administration and cause treatment failure, and should be detected before starting treatment. The most important feature of the QP-PCR assay is that one PP set can simultaneously detect and classify multiple variants. Mutations in the target gene can be easily detected by slightly changing the position of the QProbe. When there is a mismatch between the target gene amplification product and the QProbe nucleotide sequence, the QProbe dissociation temperature decreases. Since the decrease in the dissociation temperature correlates with the number of mismatches, the presence or absence of gene polymorphism and mutation can be confirmed by the difference in peak temperature. Using this function, VIM-2 and VIM-4, which could not be classified using the VIM set, can be detected as one mismatch using the VIMm set (Fig. 1c, d). In addition, the OXA-48 set detected the peak with three mismatches at 20 °C below a perfect match and accurately classified OXA-48/-244 and OXA-181/-232 (Fig. 1f). The time required for the entire assay was approximately 30 min (Table 5). This assay took less time and detected more genes than other molecular methods, such as BC-GN panel and Eazyplex® SuperBug complete A [23, 24]. For drug resistance gene testing, multiple genetic testing techniques such as PCR analysis and real-time PCR can be used within one institution. GENECUBE can be used as a tool for unifying multiple gene analyses, thus saving time and labor.

Our study has some limitations. Firstly, the DNA was manually extracted from the culture suspension. It is important to

Table 4 Quenching probe assay for detecting carbapenemase-producing genes

Results of GENECUBE	Carbapenemase-production		Accuracy (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
	Positive	Negative					
KPC set							
Positive	22	0	100% (98.3–100)	100% (91.7–100)	100% (99.0–100)	100% (91.7–100)	100% (99.0–100)
Negative	0	188					
IMP set							
Positive	35	0	100% (98.2–100)	100% (94.7–100)	100% (98.9–100)	100% (94.7–100)	100% (98.9–100)
Negative	0	175					
VIM and VIMm set							
Positive	16	0	100% (98.3–100)	100% (88.8–100)	100% (99.1–100)	100% (88.8–100)	100% (99.1–100)
Negative	0	194					
NDM set							
Positive	40	0	100% (98.2–100)	100% (95.3–100)	100% (98.9–100)	100% (95.3–100)	100% (98.9–100)
Negative	0	170					
OXA48 set							
Positive	30	0	100% (98.2–100)	100% (93.8–100)	100% (99.0–100)	100% (93.8–100)	100% (99.0–100)
Negative	0	180					

CI confidence interval

Table 5 Assay characteristics

	GENECUBE
Detection principles	QP-PCR assay
Target genes	bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, $bla_{OXA48\text{-}like}$
Sample	DNA solution
DNA extraction	Bacterial colony: manually (2 min/1 sample) Specimen: automatic (10 min/8 samples)
No. of samples	Max 8 samples
No. of test items	Max 4 items
No. of processing tests	Max 16 tests
Hands-on time	1 min (setting) + DNA extraction
Running time	30 min/16 tests
Turnaround time	46 min/16 tests (bacterial colony); 40 min/16 tests (specimen)
Limitations	PP-sets with different PCR conditions cannot be measured simultaneously (only the IMP-set is different in this study)

be able to detect CPO directly from the sample in tests aimed at screening. Although this study did not consider direct detection from specimens, the GENECUBE system can also automatically extract DNA from specimens and/or bacterial colonies in 10 min. Therefore, the high-speed QP-PCR assay using GENECUBE gives results in less than 1 h. Validation with DNA extracted from clinical specimens is required, but this assay would help in starting the appropriate antimicrobial therapy with which to treat the patient. Next, our PP set for CPO detection has the disadvantage that each is a singleplex-PCR reagent. Reaction with one out of the five PP sets will be delayed because a maximum of four can be used in GENECUBE simultaneously. Multiplex-PCR of PP sets should be considered. However, singleplex-PCR with freely customizable combinations may involve a lower cost than other assays if the type of carbapenemase (metallo or serine) has been estimated by phenotypic testing. In addition, we can detect newly emerging resistance genes by creating an original PP set. This suggests that a surveillance system for new antimicrobial-resistant organisms will be established faster in the clinical setting.

5 Conclusion

GENECUBE-based, high-speed QP-PCR assay detected bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, and $bla_{OXA48\text{-}like}$ quickly and accurately. This automated and rapid assay is a promising tool to help control the spread of CPOs.

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Declarations

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Conflict of interest No conflicts of interest to declare.

Code availability Not applicable.

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Informed consent Not applicable

Availability of data and material The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

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