Quantitative detection of metallo-β-lactamase of *blaIMP*-clusters -producing *Pseudomonas aeruginosa* by real-time PCR with melting curve analysis for rapid diagnosis and treatment of nosocomial infection

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Abstract

In this study, we established the rapid quantitative detection of metallo-beta-lactamase-producing *P. aeruginosa* in clinical isolates and samples using real-time PCR targeting gyrB (identification of *P. aeruginosa*) and blaIMP.
The relative sensitivities and specificities of this real-time PCR assay were as follows: 100.0% and 100.0% for clinical isolates, 100.0% and 98.4% for clinical specimens, respectively. The relative sensitivities and specificities of blaIMP-PCR were 100.0% in both clinical isolates and clinical specimens. The present PCR assay was easily and quickly performed, and accurately detected Pseudomonas aeruginosa and metallo-beta-lactamase.

Pseudomonas aeruginosa has emerged as one of the most problematic GNR(gram negative rod). Recently, metallo-β-lactamase-producing *P. aeruginosa* was found to be important in nosocomial infection. Carbapenem resistance in GNR caused by metallo-β-lactamase is of particular importance because it is plasmid mediated and is transferable to other GNR (Arakawa et al.,2000; Hirakata et al,.1998).

There is thus a need for sensitive and specific tests that are more rapid than culture. In this study, we applied the *gyrB*-PCR, the method of high specificity and accuracy for detection of *P. aeruginosa* (Motoshima et al., 2007)to the rapid detection of the *blaIMP* gene from clinical isolates and samples.

A total of 275 isolates of gram-negative bacilli randomly selected from our stock libraries isolated between Jan. 2005 to Oct. 2006 at the clinical microbiology laboratory of Nagasaki University Hospital were in for the present study (Table 1). All isolates were identified according to standard biochemical identification methods using standard culture media and a commercial Vitek2 system (bioMerieux, Hazelwood, MO, USA). After strains were cultured on blood-agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) overnight, bacterial DNA was extracted from a 500 μl of 0.5 McFarland bacterial solution by boiling at 100℃ for 10 minutes and chilling on ice. We also studied 88 clinical specimens that were known to be positive for *P. aeruginosa* and 64 that were negative for *P. aeruginosa*. All 152 specimens were isolated between Nov. 2004 and Nov. 2006, and included 106

sputum samples, 9 urine samples and 37 feces samples. DNA extraction from practical clinical specimens was performed using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, and 5-μl aliquots of extracted DNA solution were used as PCR templates.

Serial 10-fold dilutions were prepared from an overnight *P. aeruginosa* culture in order to confirm the lower detection limit and the dynamic range of PCR-based methods, and to compare the results of PCR with those of culture. Dilutions were performed using physiological water. Dilutions were inoculated onto Mullar-Hinton agar plates (Becton Dickinson Co. Franklin Lakes, NJ, USA). Agar plates were incubated overnight at 37°C, and the colonies were counted in order to estimate the number of CFU per dilution tube. A sample was taken from each dilution tube as a template for PCR-based methods. Standards ranging from 10 to 10^{10} CFU/ml were assayed.

MICs were determined by broth microdilution assay according to CLSI (Clinical and Laboratory Standard Institute, formerly NCCLS) reference methods. The antimicrobial agents were acquired by the respective manufactures. The phenotypic metallo-β-lactamase detection was used SMA (sodium mercaptoacetic acid; Eiken Chemical Co.) (Arakawa et al.,2000, Shigetaka,2006). The method was established based on collaborative research at the National Institute of Infectious Diseases (Tokyo, Japan) and Eiken Chemical Co.

In order to amplify the *P. aeruginosa gyrB* and *blaIMP*-clusters gene in the same run, a LightCycler (Roche Applied Science, Mannheim, Germany) equipped with the MCA analysis program was employed using 2 primer sets. The PCR assay targeted the *P. aeruginosa*-specific *gyrB* gene with primers (Qin et al.,2003), the metallo-β-lactamase gene (*blaIMP-1* gene) with primers *blaIMP*-S (acg ggt ggg gcg ttg ttc ct; *blaIMP-1*-131) and *bla-IMP*-AS (tgt gtc ccg ggc ctg gat aaa aa; *blaIMP-1*–472) corresponding to nucleotides 131-472 of the gene (accession No. AY168635). Methods and reaction conditions were the same as previously reported for *gyrB*-PCR (Motoshima et al., 2007). We used 2 capillary tubes for each sample, and the 2 gene amplifications were separated.

On *gyrB*-PCR, 124 of 275 isolates were positive, and all 124 isolates were identified as *P. aeruginosa* by Vitek2. The results of *gyrB*-PCR thus corresponded the results of phenetic identification (Table2).

By using a clinical isolate, it was demonstrated that *gyrB*-PCR produced a linear quantitative detection range of 7 logs with a lower detection limit (Figure 1). A comparison of *gyrB*-PCR with culture in clinical specimens is shown Figure 2.

In cultures, metallo-β-lactamase was detected with SMA/CAZ at 8-fold greater sensitivity than with CAZ in 52 of the 275 isolates; 29 *P. aeruginosa*, 11 *P. putida*, 6 GNF gram-negative rods, 4 *Achromobacter xylosoxidans*, 1

Serratia marcescens, and 1 *Acinetobacter baumannii*. Most those isolates were CAZ-resistant and IMP-resistant. Once we detected *blaIMP-1* gene using bla_{IMP-1} primer sets (Shibata et al., 2003) from 10 *P. aeruginosa* randomly selected from 29 *P. aeruginosa* for positive controls. From 12 of 164 clinical specimens, gram-negative rods producing metallo-β-lactamase were detected in culture. The sensitivities and specificities of *blaIMP*-PCR from clinical isolates and clinical specimens were determined, and these results were compared with those of conventional culture tests (Table 2).

As shown previously, the *gyrB*-PCR-positive samples constantly showed a sharp peak at approximately 88°C, but no peaks were seen in negative controls (Motoshima et al., 2007). In the present study, we observed an additional peak at 85°C, which represented a positive result for *blaIMP*. The results of PCR were analyzed by melting curve analysis. Each one of the possible result obtained by the assay are illustrated in Table.3. Table 3 shows the results of classification based on melting curve analysis for *gyrB* and *blaIMP*, as compared with culture data.

In this study, we attempted to establish a method of rapid detection for metallo-β-lactamase of *blaIMP*-clusters -producing *P. aeruginosa* using *gyrB*-PCR, as the entire run time of *gyrB*-PCR is approximately 3.5 hours(2.5 for pre-analytical DNA processing (clinical specimens), and 0.5 hours for PCR and MCA analysis) and we have previously demonstrated the

high accuracy of *gyrB*-PCR for *P. aeruginosa*.

The *gyrB*-PCR method has a lower detection limit of 127 copies/5 μl per reaction, which is equivalent to about 0.39 CFU/5 μl. This means what is detected if there is 1 cell in 5 μl. This represents a large detection range in clinical microbiology laboratories. By culture, resident flora sometimes inhibit identification of pathogens in clinical samples, such as sputum or feces. In this study, one clinical sample that was negative on culture was found to be positive on *gyrB*-PCR (Table 2). It is likely that this sample was below the detection limit of culture, or that it included dead cells or hidden cells among resident flora, thus preventing recovery by plating. This sample identified a low level of *P. aeruginosa* in the patient, which indicates that low levels of *P. aeruginosa* in clinical samples can be rapidly detected.

In our study, we designed a *blaIMP*-PCR primer set targeting the *bla IMP-1* gene. The primer set did not only amplify the *blaIMP-1* gene, as *blaIMP* genes (types 1~22) are highly-homologous. Our primer sets, *blaIMP*-S matched *blaIMP*-1,4,10 and *blaIMP*-AS matched *blaIMP*-1,4,6,10, with probability of 95-100%. And in this study, it was proved that 1 metallo-β-lactamase-producing Acinetobacter baumannii had *blaIMP-2* gene by sequencing analysis. Thus, a broad range of *blaIMP* genes can be detected. The specificity of the *blaIMP* primer set was confirmed by alignment of culture and PCR results. The data indicated that the primer sets accurately detected all of metallo-β-lactamase-producing *P. aeruginosa* in our laboratory, as all of these were *blaIMP* types. This corresponds with current reports that *blaIMP* types are the most common

metallo-β-lactamases in Japan; among metallo-β-lactamase-producing GNR in Japan, 84.45% are *blaIMP-1* or *blaIMP-2* (Shibata et al.,2003).

It is necessary, however, to investigate the detection of other metallo-β-lactamases in the future, as other metallo-β-lactamase genes, particularly *VIM* types, are increasing and exhibit a worldwide distribution. Their genetic determinants are carried on mobile gene cassettes inserted into chromosome- or plasmid-borne integrons and can rapidly disseminate in the clinical settings via the integron system and associated mobile DNA elements (Lagatolla et al.,2006).

'Rapid detection' is naturally expected and a greater variety of metallo-β-lactamase genes will be identified in the future. We attempted to devise a new method for the rapid extraction of DNA from a variety of clinical samples, and the detection of metallo-β-lactamase genes and integrons using *gyrB*-PCR. We did not describe in this study, but we succeeded in the detection of *VIM-2* gene using positive controls. It is hoped that multiple color detection will open the door to multiplex real-time detection PCR (Pirnay et al.,2000).

The present results indicate that *gyrB*-PCR and *blaIMP*-PCR have the potential for rapid quantitative application in clinical laboratories, and are valuable in the diagnosis and treatment of nosocomial infection.

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Table.1. Bacterial strain and Number of strains

Bacterial strain	Number of strains
Citrobacter braakii	4
Citrobacter koseri	1
Citrobacter freundii	6
Enterobacter aerogenes	1
Enterobacter asburiae	1
Enterobacter cloacae	7
Escherichia coli	32
Klebsiella oxytoca	4
Klebsiella pneumoniae	14
Serratia marcescens	3
Morganella morganii	4
Providencia rettgeri	1
Proteus mirabilis	1
Achromobacter xylosoxidans	5
Acinetobacter baumannii	11
Chryseobacterium meningosepticum	1
Chryseobacterium indologenes	1
Burkholderia cepacia [1)]	4
Burkholderia pickettii [1)]	3
Pseudomonas fluorescens	2
Pseudomonas putida	17
Pseudomonas stutzeri	1
Pseudomonas testosteroni	3
Sphingomonas paucimobilis[1)]	1
Stenotrophomonas maltophilia [1)]	8
Genus Moraxella	2
Genus Haemophilus	7
GNF-gram negative rod	6
Pseudomonas aeruginosa	124
Total	275

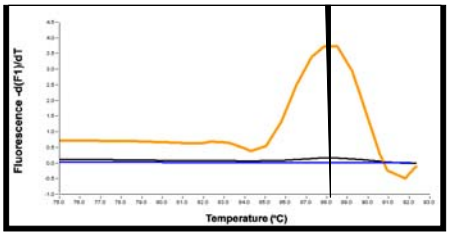
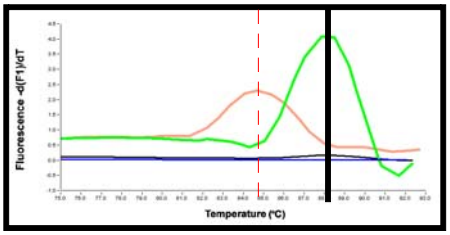
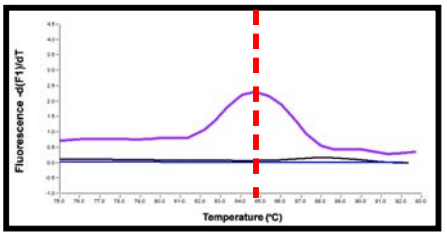
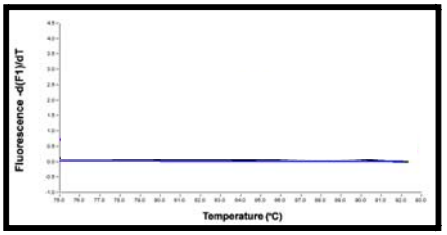
1) *Burkholderia cepacia*, *Burkholderia pickettii* and *Sphingomonas paucimobili* were before classified into Genus *Pseudomonas*.

Table 2. Sencitivities and specificities of *gyrB*-PCR and *bla-IMP*-PCR compared with culture

result of PCR		result by culture * positive	result by culture * negative	total
result of *gyrB*-PCR				
isolates	positive	124 (100%)	0 (0%)	124
	negative	0 (0%)	151 (100%)	151
	total	124	151	275
specimens	positive	88 (100%)	1 (1.6%)	89
	negative	0 (0%)	63 (98.4%)	63
	total	88	64	152
result of *bla-IMP*-PCR				
isolates	positive	52 (100%)	0 (0%)	52
	negative	0 (0%)	223 (100%)	223
	total	52	223	275
specimens	positive	12 (100%)	0 (0%)	12
	negative	0 (0%)	140 (100%)	140
	total	12	140	152

*CAZ/SMA that was 8 times or more lower sensitivity than CAZ was determined metallo-beta-lactamase positive.

Table 3. Result of classification based on the figure of melting curve analysis of *gyrB* and *bla-IMP* compared with culture

figure of melting curve analysis		no. of samples or isolates		sensitivities
		PCR	culture	
 Type1: non-metallo-ß-lactamase producing *P.aeruginosa*.	clinical specimens :	78	77	100%
	clical isolates :	97	97	100%
 Type2 : metallo-ß-lactamase producing *P.aeruginosa*.	clinical specimens :	10	10	100%
	clical isolates :	27	27	100%
 Type3 : metallo-ß-lactamase producing non-*P.aeruginosa*.	clinical specimens :	2	2	100%
	clical isolates :	25	25	100%
 Type4: non-metallo-ß-lactamase producing non-*P.aeruginosa* or nothig identified.	clinical specimens :	62	63	98.4%
	clical isolates :	126	126	100%
	Total of clinical specimens :		152	
	Total of clinical isolates :		275	

Melting peak patterns for *P. aeruginosa* and metallo-ß-lactamase. Types 1-4 were classified based on combinations of the two melting peak patterns. The black line indicates 88℃, and the red dotted line indicates 85℃.

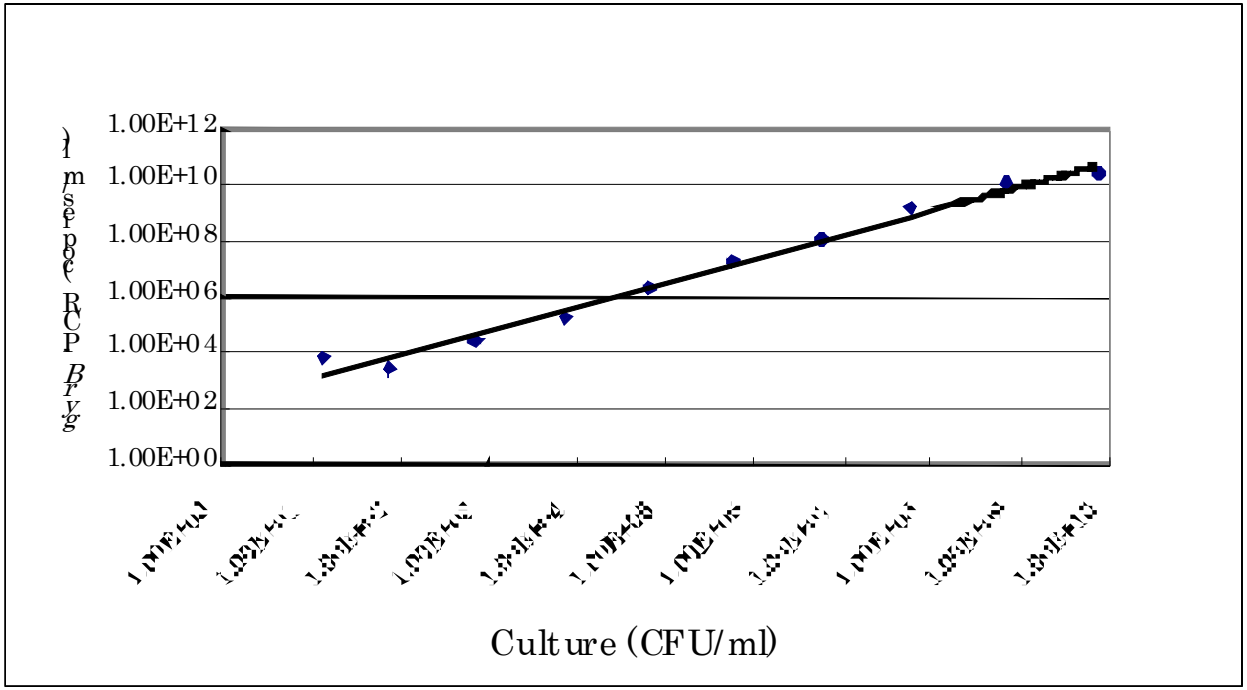

1.00E+12
1.00E+10
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
Culture (CFU/ml)

Fig.1

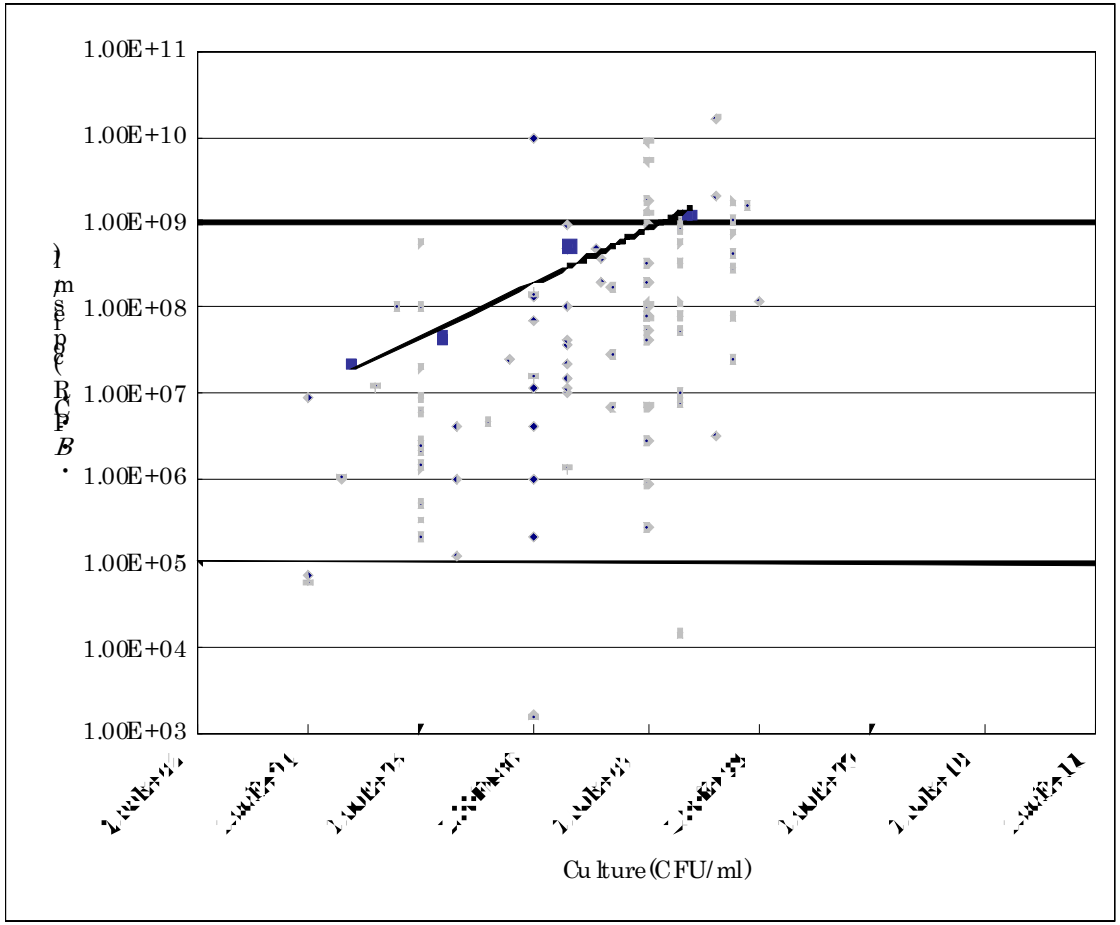

1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
Culture (CFU/ml)

Fig. 2

FIGURE LEGENDS

Fig. 1. Correlation between quantitative *gyrB*-PCR and culture of serial 10-fold dilutions of *P. aeruginosa.*
y = 168x0.858 , r=1

Fig. 2. Correlation between quantitative *gyrB*-PCR and culture in clinical specimens (n=152). Large points represent average amounts of *gyrB*-PCR per 1.00E+04-1.00E+05, 1.00E+05-1.00E+06, 1.00E+06-1.00E+07, 1.00E+07-1.00E+08 on culture results. Linearity was y = 33112x0.6266 , r=0.944.