Multiplex Real-time PCR for Rapid Detection of β-Lactamase–negative, Ampicillin-resistant *Haemophilus influenzae* (BLNAR)

Running title: **Multiplex Real-time PCR for BLNAR Detection**

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Abstract

We evaluated a multiplex real-time quantitative PCR method for quantification of *Haemophilus influenzae* (*H. influenzae*) and rapid detection of β-lactam–resistant strains. We designed five PCR primer sets to simultaneously detect the β-lactam–resistant genes and quantify the pathogen. To demonstrate the validity of this assay, we used 191 clinical isolates, including 141 *H. influenzae* strains, and 100 purulent sputum samples, including 30 samples from which *H. influenzae* had been isolated . This assay showed 92.9% sensitivity and 91.8% specificity for detecting β-lactam resistant genes, relative to the conventional phenotypic method, and this assay correlated well with conventional quantitative culture counts. By using this assay, we could quantify *H. influenzae* and identify β-lactam susceptibility in only 3 h and with only one tube. This method will be helpful for the rapid detection of *H. influenzae* infections and the selection of appropriate antibiotics.

Introduction

Haemophilus influenzae (*H. influenzae*) is a secondary major pathogen isolated from community-acquired pneumonia patients. *H. influenzae* is also frequently isolated from other infectious sources such as acute otitis media, sinusitis, acute exacerbation of chronic obstructive pulmonary disease, and acute meningitis, mainly in the pediatrics field (Jansen et al, 2006; Mendelman et al, 1987; Reid et al, 1987). Since the first reports of ampicillin (AMP)-resistant strains of *H. influenzae* in the United States in 1974, the major mechanism for β-lactam resistance in *H. influenzae* has been considered to be related to either the TEM-1 or ROB-1 types of β-lactamase (Doern et al, 1997; Doern et al, 1986; Thomas et al, 1974). The β-lactamase–negative, AMP-resistant (BLNAR) strains continued to be isolated at low frequencies in the 1980s (Mendelman et al, 1984). However, surveillance studies from the 1990s to 2000s showed a marked increase in the frequency of BLNAR strains in Asian countries and European countries, especially Japan. (Fluit et al, 2005; Hasegawa et al, 2004). The mechanism of resistance in the BLNAR strains involves decreased affinities of penicillin-binding proteins (PBPs) for β-lactam antibiotics (Markowitz et al, 1980). Among the PBPs of *H. influenzae*, alterations in PBP3-mediated septal peptidoglycan synthesis during cell division are important for developing resistance (Ubukata et al, 2001). BLNAR strains

can be classified into three groups based on the deduced amino-acid substitutions in the *ftsI* gene, which encodes PBP3 (Ubukata et al, 2001). Group I strains contain a substitution of Arg-517 for His-517 (Arg-517-His) near the conserved Lys-Thr-Gly (KTG) motif, and group II strains contain a substitution of Arg-526 for Lys-526 (Asn-526-Lys). Group III strains contain substitutions of three amino acid residues (Met-377, Ser-385, and Leu-389) positioned near the conserved Ser-Ser-Asn (SNN) motif for Ile-377, Thr-385, and Phe-389, respectively, in addition to the Asn-526-Lys substitution (Ubukata et al, 2001; Hasegawa et al, 2004). Of the various *ftsI* gene mutations, the degree of resistance to β-lactam antibiotics largely depends on three substitutions: Arg-517-His, Asp-526-Lys, and Ser-385-Thr (Hasegawa et al, 2004). Intermediate AMP resistance is commonly found in groups I and II; however, isolates in group III are associated with a higher level of AMP resistance; groups I and II are called low BLNAR, and group III is called BLNAR in genetical sight (Ubukata et al, 2001; Hasegawa et al, 2003; Osaki et al, 2005). The conventional phenotypic method requires at least two or three days to detect the organism and determine drug susceptibility; this time loss may lead to treatment failure, resulting in a poor outcome and the development of more β-lactam resistant strains due to the selection of inappropriate antibiotics. The aim of the present study was to develop the multiplex real-time

quantitative PCR (mRQ-PCR) method for quantification of *H. influenzae* and the rapid detection of β-lactam–resistant strains.

Materials and Methods

Clinical isolates and samples; We used 191 clinical isolates (including 141 clinical isolates of *H. influenzae* identified by colony morphology, Gram staining, growth on chocolate agar, and the X and V factor requirement). Strains were grown on chocolate II agar plates (Nissui Pharmaceutical Co., Tokyo, Japan) and incubated overnight at 37°C in 5% CO_2. These strains were collected from April 2003 to March 2006 by a laboratory at Nagasaki University Hospital. The *H. influenzae* Rd-transformed ROB-1 *bla* gene was kindly provided by Meiji Seika Kaisha Ltd. (Tokyo, Japan). A total of 100 purulent sputum samples including 30 samples from which *H. influenzae* had been isolated that were collected from September 2007 to June 2008 by a laboratory at Nagasaki University Hospital were used for the quantitative assay and PCR.

Susceptibility testing; Susceptibilities were tested in duplicate for each isolate by using inocula of 10^5 colony forming units (CFU) per mL and determined by a broth dilution method with *Haemophilus* test medium, according to the Clinical and Laboratory Standards Institute (CLSI) recommendation. The production of β-lactamase

was confirmed by a nitrocefin test (Showa Chemical, Tokyo, Japan). These strains were classified according to CLSI AMP susceptibility criteria: susceptible strain (AMP MIC≤1 μg/mL), intermediate strain (AMP MIC=2 μg/mL), and resistant strain (AMP MIC≥4 μg/mL).

DNA isolation; Bacterial DNA was extracted from clinical isolates and purulent sputum samples by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Only good quality sputum samples (P2 and P3, according to the classification of Miller and Jones) were used. Sputum samples were diluted 1: 100 and 1: 10,000 with 0.45% sodium chloride and treated with Sputazyme solution (Kyokuto Pharmaceutical Industries Co., Ltd., Tokyo, Japan).

Designation and composition of primers; Five sets of primers were designed for mRQ-PCR (as shown Table 1): P6 primers to amplify the *P6* gene encoding the P6 outer membrane protein specific to *H. influenzae* (Nelson et al, 1988); TEM-1 primers to amplify part of the *bla* gene encoding TEM-1 β-lactamase (Sutcliffe, 1978); ROB-1 primers to amplify a part of the *bla* gene encoding ROB-1 β-lactamase (Juteau et al, 1990); PBP3-INT primers to amplify the portion of the *ftsI* gene that contains the Lys-526 amino acid substitution corresponding to the sequences identified for

intermediate strains (Ubukata et al, 2001; Hasegawa et al, 2004); and PBP3-BLN primers to amplify the part of the *ftsI* gene that contains the Thr-385 amino acid substitution and Lys-526 substitution, which are reported as the most important positions involved in the increased MIC against β-lactam agents (Ubukata et al, 2001; Hasegawa et al, 2004; Osaki et al, 2005). When no DNA bands were detected, the strain was identified as AMP susceptible. We used the strains shown in Table 2 for positive and negative controls to qualify this assay.

Quantification assay; For the quantification assay, PCR products amplified from *H. influenzae* ATCC51907 with the P6 primers were ligated into the pTAC-1 plasmid vector (BioDynamics, Tokyo, Japan) by using the TA PCR closing technique. Plasmid standards contained 2.0×10^8 to 2.0×10^0 copies per μL. The standard curve was generated by using the LightCycler Software (v3.5) (Roche Diagnostics, Basel, Switzerland). PCR was performed on a LightCycler instrument. The diluted sputum samples were also spread on chocolate II agar plates with a DS500 spiral plater (InterScience Inc., Ontario, Canada) and then incubated overnight at 37°C in 5% CO_2 to perform the quantitative culture method. We then compared the results of quantitative real-time PCR with the results from the quantitative culture method.

Multiplex PCR assay; Multiplex PCR was performed in a total volume of 20 μL containing 2 μL of DNA template, 4 μL of LightCycler TaqmanMaster mixture (Roche Diagnostics, Basel, Switzerland), a 0.1 μM concentration of Taqman probe, and a 0.5 μM concentration of each primer. Thermal cycling was performed with an initial hold for 10 min at 95°C, followed by 30 cycles of 10 s at 95°C and 10 s at 64°C. Data was analyzed with the LightCycler Software (v3.5) in the F1 mode with a fit point calculation method. Following PCR, 10 μL of the PCR product was separated by electrophoresis on a 2% agarose gel (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) for 30 min at 100 V.

Results

The detection and quantification of *H. influenzae*; Two *H. influenzae* ATCC strains (51907 and 49247) were positive for the *p6* gene. None of the DNA extracts from 50 clinical isolates of non-*H. influenzae* organisms cross-reacted with the P6 primers; therefore, the P6 primers were specific for detecting *H. influenzae* (Table 3). The detection limit of the *p6* quantification was 20 copies per assay, which corresponds to 5×10^2 CFU per mL. The 141 *H. influenzae* strains were all positive for the *p6* genes. Of 100 purulent sputum samples, 30 samples were *H. influenzae* positive and the remaining

70 samples were *H. influenzae* negative, as determined by the conventional culture method. Twenty-nine of the *H. influenzae*–positive samples were positive for the *p6* gene, and 65 of 70 *H. influenzae*–negative samples were negative for the *p6* gene (sensitivity and specificity of 96.7% and 92.9%, respectively).

Amplified DNA profiles; Figure 1A shows single PCR-amplified DNA profiles. The143-bp band in lane 1 is a segment of the *p6* gene used for detecting *H. influenzae*. The 465-bp band in lane 2 is the amplified segment of the mutated *ftsI* gene containing the Thr-385 and Lys-526 substitutions. The 554-bp band in lane 3 is the amplified segment of the mutated *ftsI* gene containing the Lys-526 substitution. Lane 4 contains the 302-bp band amplified from the TEM-1–type β-lactamase gene, and lane 5 contains the 192-bp band amplified from the ROB-1–type β-lactamase gene. Figure 1B shows the multiplex PCR-amplified DNA profiles of clinical strains. The strains with the 143-bp band only (lane 1 and lane 4) are AMP-susceptible strains. The strain with the 465-bp band and the 143-bp band is β-lactamase–negative, AMP-resistant *H. influenzae* (lane 2). The strain with the 554-bp band and the 143-bp band is β-lactamase–negative, AMP-intermediate *H. influenzae* (lane 5). The strain with bands of 465 bp, 302 bp, and 143 bp is *H. influenzae* that is positive for both TEM-1–type β-lactamase and the PBP3 substitution (lane 6).

Sensitivity and specificity of multiplex PCR; To validate the mRQ-PCR method, the 141 *H. influenzae* isolates and 30 purulent sputum samples that were tested by mRQ-PCR were also screened for the presence of individual resistance genes by single PCR. The results of the two methods were in full agreement, suggesting that the multiplex PCR primer sets are reliable. The sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of the primers used in this study compared to the conventional phenotypic method are shown in Table 4. The primers used to detect the AMP-resistant intermediate strain (MIC≥4 μg/mL) had 92.9% sensitivity and 91.8% specificity, and the TEM-1 primers had 100% sensitivity and 100% specificity. In the detection of resistant genes, the sensitivity and specificity when this assay was performed directly on purulent sputum samples showed the same tendency as when it was performed on isolated strains (data not shown). None of the clinical strains producing the ROB-1 type of β-lactamase were isolated in this study.

Relationship between resistant gene and the MIC distribution of β-lactam agents; The mRQ-PCR results of 141 isolated *H. influenzae* strains and the MIC distribution for intravenous β-lactam agents are shown in Table 5. The results of multiplex PCR could reflect the β-lactam susceptibility, especially cephalosporin. Piperacillin, ceftazidime, and meropenem were lower in MIC_{50} and MIC_{90} values against the PBP-BLN positive

strain. There were only two strains which were both PBP-BLN and TEM-1 positive, so they were not studied.

Quantification assay by using clinical samples; We investigated the correlation between the conventional culture counts and the level of the *p6* gene expression determined by mRQ-PCR. Thirty *H. influenzae* culture-positive sputum samples were tested for three times. The *p6* gene was not detected in one sample; this sample was hemosputum, and we suspect that it contained PCR inhibitors. The coefficient of correlation between the conventional culture counts and the level of the *p6* gene expression is 0.73. This data indicates that the results of mRQ-PCR correlate well with conventional culture counts. So it may be a useful tool to determine if *H. influenzae* is the causative pathogen for lower respiratory infections.

Discussion

Several issues should be considered when choosing the appropriate therapy for infections caused by *H. influenzae*. For example, it is unclear whether *H. influenzae* isolates are sufficiently pathogenic to cause respiratory tract infection and whether the causative *H. influenzae* is a drug-resistant strain. In the present study, we assayed isolated strains as well as purulent sputum samples that were presumably from the respiratory tract of patients with respiratory infection. Although quantitative culture is

useful for the identification of the causative pathogen (Bartlett et al, 1978), traditional laboratory diagnostic methods for the identification of causative pathogens and drug susceptibility take up to 48 h or more. The administration of appropriate antibiotics within 4 h can prevent deaths in the medicare population and offers cost savings for hospitals (Houck et al, 2004); early detection of the pathogen and its drug susceptibility is important to improve the patient's outcome. Some authors have reported the feasibility of quantitative PCR for the etiologic diagnosis of bacterial infection (Johansson et al, 2008; Kee et al, 2008). In the present study, we demonstrated that quantitative PCR can be successfully used to rapidly detect *H. influenzae*. The results of quantitative PCR were well correlated with conventional culture counts. All isolated strains were positive for the *p6* gene. All sputum samples from which *H. influenzae* was cultured were positive for the *p6* gene, with the exception of one hemosputum sample. Among the sputum samples from which *H. influenzae* was not cultured, there were five false-positive results, suggesting that the amount of the pathogens was very small or that the pathogens were dead. However, our data implies that quantitative PCR can rapidly identify the causative pathogen from lower respiratory infections.

Severe cases of *H. influenzae* infection (Mendelman et al, 1990; Dworkin et al, 2007) and the failure of antibiotic treatment due to drug resistance have been reported (van der

Ploeg et al, 2008; Rubin et al, 1981). Our multiplex PCR assay rapidly and simultaneously detects *H. influenzae* and the β-lactam resistant genes with only a single tube, and the assay can be directly performed on both isolated strains and sputum samples. Recent surveillance studies in Asian countries and European countries showed a marked increase in the frequency of BLNAR strains. BLNAR strains, for which the increases in the MICs of cephalosporin agents are greater, have increased rapidly in both pediatric and adult patients. The increase in the number of BLNAR strains is apparently related to the clinical use of low doses of oral cephalosporin agents, mainly by pediatric patients. In the present study, we established the mRQ-PCR assay to rapidly detect the β-lactam resistant genes; the sensitivity of the primers to detect BLNAR strains (MIC≥4 μg/mL) was 92.9%, and the corresponding specificity was 91.8%. The sensitivity of the primers to detect the intermediate strain (MIC = 2 mg/L) was low (66.7%), but the specificity remained high (94.4%). There were 11 strains that had a false-negative result for the PBP3-INT band; the PBP3-BLN band was positive in eight strains, and no band was detected in three strains. In the PBP3-BLN band false-positive strains, the discrepancy between genotype and phenotype in the intermediate strains indicates that these strains may have the potential to develop into highly resistant strains in the near future. For the three intermediate strains for which no band was detected, we

suspect that the discrepancy occurred because there are several other amino acid substitutions correlated with intermediate strains. Amino acid substitutions have been described in association with β-lactam resistance for 24 different positions in the transpeptidase domain of PBP3 (Straker et al, 2003; Kubota et al, 2006; Dabernat et al, 2002).

In conclusion, this assay has high sensitivity and specificity for *H. influenzae* and can detect β-lactam–resistant strains from clinical isolates and purulent sputum. Moreover, the assay can be finished directly from clinical samples within 3 h (2 h for DNA extraction and preparation of the PCR mixture, and 1h for PCR and electrophoresis), and it requires only a single tube. This method may be a useful tool for the rapid screening of β-lactam susceptibility and should allow the administration of earlier and more effective treatment of β-lactam–resistant *H. influenzae*.

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Figure legends

Figure 1. (A) DNA fragments amplified by single PCR. Lane 1, clinical strain (AMP-susceptible), the P6 primers were used. ; lane 2, clinical strain (β-lactamase–negative, AMP-resistant), the PBP-BLN primers were used.; lane 3, clinical strain (β-lactamase–negative, AMP-intermediate), the PBP-INT primers were used.; lane 4, clinical strain (TEM-1–type β-lactamase–positive, AMP-resistant), the TEM-1 primers were used.; lane 5, *H. influenzae* Rd-transformed ROB-1 *bla* gene (ROB-1–type β-lactamase–positive, AMP-resistant), the ROB-1 primers were used.; lane M, (100 base-pair ladder). (B) DNA fragments amplified by multiplex PCR. Lane 1, clinical strain (AMP-susceptible); lane 2, clinical strain (β-lactamase–negative, AMP-resistant); lane 3, clinical strain (β-lactamase–negative, AMP-intermediate); lane 4, clinical strain (AMP-susceptible); lane 5, clinical strain (β-lactamase–negative, AMP-intermediate); lane 6, clinical strain (positive for both TEM-1 type β-lactamase and PBP3 substitution); lane M, (100 base-pair ladder).

Table 1. Oligonucleotide primers and probe

Primer name (gene)	*Primer*	*Sequence (5'to3')*	*Primer length*	*Position*	*Amplicon length(bp)*
P6[a] (*P6)*	Forward	tggcggatactctgttgct	20	175-194	143
	Reverse	gcgcatctaagatttgaacg	20	316-335	
TEM-1[b] (*bla*)	Forward	ttgccgggaagctagagtaa	20	1062-1081	302
	Reverse	cgccgcatacactattctca	20	1363-1382	
ROB-1[c] (*bla*)	Forward	ctaatccgcagcctgctagt	20	475-494	192
	Reverse	acaacgccttgaaagtggac	20	705-686	
PBP3-BLN[d] (*ftsI*)	Forward	gtcacaccacggttacttgaa	21	1134-1154	465
	Reverse	cccgcagtaaatgccacatatttc	24	1598-1578	
PBP3-INT[e] (*ftsI*)	Forward	gatactacgtcctttaaattaagcg	25	1048-1072	554
	Reverse	cccgcagtaaatgccacatatttc	21	1598-1578	
P6 –TaqMan probe		5'-FAM-atacgttcaaatcttagatgcgcacgc-TAMRA-3'			

[a]P6 outer membrane protein gene (GenBank Accession No. M19391); to amplify the *P6* gene encoding P6 outer membrane protein specific to *H. influenzae*.

[b]TEM-1-type *bla* gene (GenBank Accession No. J01749); to amplify a part of the *bla* gene encoding TEM-1 β-lactamase.

[c]ROB-1-type *bla* gene (GenBank Accession No. AF022114); to amplify a part of the *bla* gene encoding ROB-1 β-lactamase.

[d]PBP3-BLN (*ftsI*) gene (GenBank Accession No. U32793); to amplify a part of *ftsI* gene having Thr-385 amino acid substitution and Lys-526 substitution.

[e]PBP3-INT (*ftsI*) gene (GenBank Accession No. U32793); to amplify a portion of *ftsI* gene containing the Lys-526 amino acid substitution .

Table 2. Control strains used in evaluating multiplex PCR

Primers	Isolates		Species	MIC to ampicillin (μg/mL)
P6	Positive	ATCC51907	*H. influenzae*	0.005
	Negative	ATCC49619	*S. pneumoniae*	-
PBP-BLN	Positive	NU5466	*H. influenzae*	8
	Negative	ATCC51907	*H. influenzae*	0.005
PBP-INT	Positive	NU6912	*H. influenzae*	1
	Negative	ATCC51907	*H. influenzae*	0.005
TEM-1	Positive	NU5024	*H. influenzae*	64
	Negative	ATCC51907	*H. influenzae*	0.005
ROB-1	Positive	MS1	**H. influenzae*	32
	Negative	ATCC51907	*H. influenzae*	0.005

**H. influenzae* Rd-transformed ROB-1 *bla* gene

Table 3. Organisms used to investigate the cross-reacting of the P6 primers

isolated bacteria (n)	
Haemophilus parainfluenzae (3)	*Genus Candida* (2)
Pseudomonas aeruginosa (3)	*Enterococcus faecalis* (2)
Burkholderia cepatia (2)	*Enterococcus fecium* (2)
Maraxella catarrhalis (2)	*Escherichia coli* (3)
Klebsiella pneumoniae (3)	*Acinetobacter baumannii* (2)
Staphyrococcus aureus (3)	*Stenotrophomonas maltophilia* (2)
Streptococcus pneumoniae (4)	*Legionella pneumophila* (1)
Streptococcus agalactiae (2)	*Other commensal bacteria (14)

*Other commensal bacteria included non pathogenic *Streptococcus*, *Neisseria*, *Haemophilus*, coaglase negative staphylococcus and Gram positive rod.

Table 4. Sensitivity and specificity of multiplex PCR by using isolated colony samples (compared to the conventional phenotypic method)

Primer name	Positive number		Sensitivity (%)	Specificity (%)	* PPV (%)	** NPV (%)
	conventional method	PCR				
P6	141	141	100	100	100	100
PBP3-BLN	42	39	92.9	91.8	88.6	94.7
PBP3-INT	33	22	66.7	94.4	78.6	90.3
TEM-1	7	7	100	100	100	100
No band detected	59	54	91.5	91.5	88.5	93.8

*PPV: positive predictive value

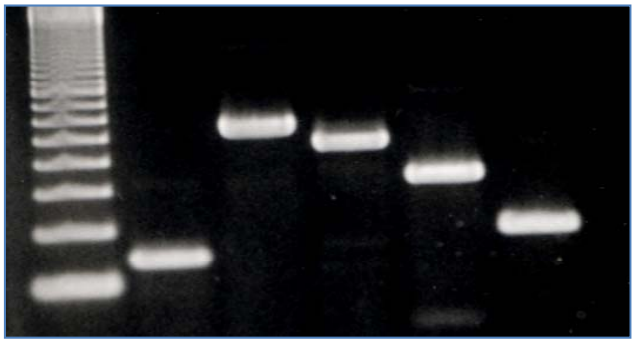
**NPV: negative predictive value

Table 5. MIC distributions and resistant genes of *H. influenzae* isolated in Nagasaki University Hospital

Antimicrobial agent	*primer* (number)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Range MIC (μg/mL)
Ampicillin	No band detectd (n=54)	≦0.5	1	≦ 0.5-2
	PBP-INT(n=22)	1	2	0.5-2
	PBP-BLN (n=39)	4	16	1- ≧ 32
	TEM-1 (n=7)	8	≧ 32	8- ≧ 32
Piperacillin	No band detectd	≦ 0.5	<=0.5	≦ 0.5
	PBP-INT	≦ 0.5	<0.5	≦ 0.5
	PBP-BLN	≦ 0.5	0.5	≦ 0.5 - 4
	TEM-1	16	≧ 32	16- ≧ 32
Cefotiam	No band detectd	1	4	1-8
	PBP-INT	4	≧ 32	1- ≧ 32
	PBP-BLN	2	≧ 32	2- ≧ 32
	TEM-1	≧32	≧ 32	≧ 32
Cefotaxime	No band detectd	≦ 0.5	≦ 0.5	≦ 0.5- 1
	PBP-INT	1	2	≦ 0.5-4
	PBP-BLN	1	4	1- 16
	TEM-1	1	4	1- 16
Ceftazidime	No band detectd	≦ 0.5	≦ 0.5	≦ 0.5
	PBP-INT	<0.5	≦ 0.5	≦ 0.5
	PBP-BLN	<0.5	2	≦ 0.5- 4
	TEM-1	2	4	2-4
Cefepime	No band detectd	≦ 0.5	≦ 0.5	≦ 0.5
	PBP-INT	≦ 0.5	1	≦ 0.5- 4
	PBP-BLN	2	4	1- 4
	TEM-1	1	4	≦ 0.5- 4
Imipenem	No band detectd	≦ 0.5	≦ 0.5	≦ 0.5
	PBP-INT	≦ 0.5	≦ 0.5	≦ 0.5-1
	PBP-BLN	2	8	≦ 0.5- 8
	TEM-1	2	4	2-4
Meropenem	No band detectd	≦ 0.5	≦ 0.5	≦ 0.5
	PBP-INT	≦ 0.5	≦ 0.5	≦ 0.5
	PBP-BLN	≦ 0.5	≦ 0.5	≦ 0.5- 1
	TEM-1	≦ 0.5	≦ 0.5	≦ 0.5

*The strains which is both PBP-BLN and TEM-1 positive were excluded because the sample size was small (n=2).

(A)



M 1 2 3 4 5

(B)

M 1 2 3 4 5 6

Figure 1.