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Rapid Identification of Penicillin and Macrolide Resistance Genes and Simultaneous Quantification of *Streptococcus pneumoniae* in Purulent Sputum Samples Using a Novel Real-time Multiplex PCR Assay

Running title-

Rapid identification of drug-resistant *S. pneumoniae*

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ABSTRACT

We evaluated the RQ-mPCR (real-time quantitative PCR combined with multiplex PCR) assay, for quantification of *Streptococcus pneumoniae* and simultaneous detection of drug resistant genes by gel-based PCR, using purulent sputum samples. This assay correctly quantified *S. pneumoniae* and identified their penicillin- and erythromycin- susceptibilities, directly from samples within 3 h.

Streptococcus pneumoniae is a crucial pathogen that causes community-acquired pneumonia (CAP). CAP in adults is often treated with a combination of β-lactam antibiotics and macrolides (18, 22). However, alarmingly high frequencies of penicillin- and macrolide-resistant pneumococci have reported, especially in several Asian countries including Japan (4, 24, 30). The resistance of *S. pneumoniae* to penicillin has been shown to be closely associated with mosaic mutations in the *pbp1a*, *pbp2b*, and *pbp2x* genes (12, 35). Macrolide resistance is generally mediated by two mechanisms: 23S rRNA methylation encoded by the *erm(B)* gene or macrolide efflux via the *mef(A)* gene (23, 32). Detection of drug-resistant *S. pneumonaie* by classical techniques usually takes several days (17, 27). Recently, the utility of real-time PCR method in the detection and quantification of *S. pneumoniae* have examined using lower respiratory tract samples (2, 5, 15, 20, 36). Their results suggest that clinical infection correlates with increased pneumococcal load, and they also mentioned the capacity of quantitative real-time PCR to distinguish between colonization and true infection (15, 36). Many investigators have evaluated the accuracy of multiplex PCR methods, which are used in the screening of *S. pneumoniae* strains possessing penicillin and macrolide resistance (13, 14, 21, 33, 34). Most of these assays, however, require separate tubes, are only able to detect two or three gene fragments, and have not been evaluated for clinical respiratory tract samples. In the current studies, we developed a simultaneous single-tube real-time quantitative PCR combined with multiplex PCR (RQ-mPCR) assay that rapidly quantifies *S. pneumoniae*,

identifies alterations in *pbp1a*, *pbp2b*, and *pbp2x* genes, and detects the presence of *erm(B)* and *mef(A)* genes. We first verified this method using clinical *S. pneumoniae* strains and then evaluated the effectiveness of the method using purulent sputum samples.

We used 200 clinical isolates of *S. pneumoniae* screened by optochin susceptibility (susceptible) and bile solubility (soluble) that were collected from April 2004 to March 2006 by a laboratory at the Nagasaki University Hospital. Strains were propagated on 5% sheep blood agar (Nissui Co., Ltd., Tokyo, Japan) at 37°C with 5% CO_2. A mixture of 24 bacterial species from ATCC (see Table S1 in the supplemental material) were selected from species commonly isolated from respiratory tract and from species that are genetically related to *S. pneumoniae* (3). In addition, 17 clinical strains of *Streptococcus mitis* and 12 clinical strains of *Streptococcus oralis*, which isolated from respiratory tract samples, were collected for cross-reactivity studies.

The minimum inhibitory concentrations (MICs) were determined using broth microdilution techniques as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines (7, 8). *S. pneumoniae* ATCC 49619 was used for quality control.

A total of 200 purulent sputum samples, which were collected from April 2004 to March 2005, and from June 2007 to August 2007 by a laboratory at the Nagasaki University Hospital, were used. Only good-quality sputum samples (P2 and P3 according to the classification of Miller and Jones (19)) 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). The diluted samples were spread on 5% sheep blood agar plates with a DS500 spiral plater (InterScience Inc., Ontario, Canada) and then incubated in at 37°C containing 5% CO_2. Optochin sensitivity and bile solubility were used to identify *S. pneumoniae*. Nucleic acids were isolated from clinical strains and sputum samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

The *lytA*, *pbp1a*, *pbp2b*, *pbp2x*, *ermB*, and *mefA* genes were amplified by PCR. Primers *LytA*-F and *LytA*-R and probes *LytA*-DCR and *LytA*-ACR were designed to target a 173-bp fragment of the single copy autolysin (*lytA*) gene of *S. pneumoniae* and were gleaned from published sequence (26). The primers for amplification of the *pbp1a, pbp2b, and pbp2x* genes were newly designed as follows: *pbp1a* (353 bp), 5'-$_{1709}$AGTATATCAAGAACACTGGCTACG$_{1732}$ and 5'-$_{2061}$GCTTGGAGTGGTTGAGCTA$_{2079}$-3'; pbp2b (442 bp), 5'-$_{1291}$AAATTGGCATATGGATCTTTTC$_{1312}$-3' and 5'-$_{1732}$TATTCATCTCTGTCGGTTGC$_{1751}$-3'; pbp2x (339 bp), 5'-$_{990}$AAGTAACTATGAACCAGGATCAG$_{1012}$-3' and 5'-$_{1388}$CGAAGCATTTGTGTTTGTGT$_{1407}$-3'. The resistance *pbp1a* primers were designed to target four amino acid substitutions (Thr-574→Asn, Ser-575→Thr, Gln-576→Gly, and Phe-577→Thr) that are common to all penicillin G-intermediate and -resistant isolates (29).

The resistance *pbp2x* primers were designed to target amino acid substitutions in the 337STMK motif, and the resistance *pbp2b* primers were designed to target amino acid substitutions close to the 448SSN motif (25, 28). The primers for amplification of the *ermB* (224bp) and *mefA* (294bp) genes were gleaned from a published sequence (21). All of the primers used for RQ-mPCR had almost identical annealing temperatures (range from 59.0 °C to 62.5°C), which reduces the occurrence of unwanted bands originating from nonspecific amplification. The PCR product amplified from *S. pneumoniae* ATCC49619 using the *LytA*-F and *LytA*-R primer set was ligated into the pTAC-1 plasmid vector (BioDynamics, Tokyo, Japan) using the TA PCR cloning technique. Plasmid standards containing 2.9×10^6 to 2.9×10^0 copies/μL were prepared by diluting the plasmid extracts in water. The standard curve was generated and exported using LightCycler Software (v3.5). PCR was performed on a LightCycler instrument. The final 20-μl single-tube reaction mixture contained 2× LightCycler FastStart DNA Master HybProbe (Roche Diagnostics, Basal, Switzerland), 5 mM $MgCl_2$, 0.5 μM of each primer **(***LytA*-F, *LytA*-R, *pbp1a*-F, *pbp1a*-R, *pbp2b*-F, *pbp2b*-R, *pbp2x*-F, *pbp2x*-R, *mef*-F, *mef*-R, *erm*-F, and *erm*-R), 0.2 μM of each hybridization probe (*LytA*-DCR and *LytA*-ACR), and 2 μl of DNA template. The cycling conditions were as follows: 95ºC for 10 min, followed by 40 cycles of 95ºC for 5 s, 60ºC for 10 s, and 72ºC for 15 s. All runs included a negative control of water and a calibrator/positive control of 2.9×10^6 copies/μl of the plasmid used for the standard curve. Data was analyzed using LightCycler

Software (v3.5) in the F2/F1 mode with a fit point calculation method. Following amplification, 10 μl of PCR product was separated by electrophoresis on a 3% agarose gel (Cambrex Bio Science Rockland, Inc. Rockland, ME, USA) for 30 min at 100 V. The positions of DNA fragments are shown in Figure 1A (Lane M, 100 Base-Pair Ladder (Amersham Biosciences)). Figure 1B shows the presence of the amplified products after agarose gel electrophoresis when DNA was extracted from representative PCG- and EM-resistant *S. pneumoniae* strains (lane 1, ATCC 49619 (MIC of PCG 0.25 μg/mL and EM <0.5 μg/mL); lane 2, clinical strain 4808/S (MIC of PCG <0.015 μg/mL and EM <0.5 μg/mL); lane 3, clinical strain 1512/F (MIC of PCG 0.03 μg/mL and EM 2 μg/mL); lane 4, clinical strain 1565/F (MIC of PCG 0.03 μg/mL and EM 32 μg/mL); lane 5, clinical strain 8315/F (MIC of PCG 0.12 μg/mL and EM 0.5 μg/mL); lane 6, clinical strain 4827/F (MIC of PCG 0.12 μg/mL and EM 32 μg/mL); lane 7, clinical strain 8605/Z (MIC of PCG 8 μg/mL and EM <0.5 μg/mL); lane 8, clinical strain 8729/Z (MIC of PCG 4 μg/mL and EM 8 μg/mL); lane 9, clinical strain 1824/F (MIC of PCG 2 μg/mL and EM 16 μg/mL); lanes M, 100 Base-Pair Ladder).

Six *S. pneumoniae* ATCC strains were all positive for the *lytA* gene. None of the DNA extracts (≥10 ng/μl) from 47 non-pneumococcal organisms (including 17 clinical isolates of *S. mitis* and 12 clinical isolates of *S. oralis*) crossreact with the primer/probe set, showed the *lytA* primer/probe set was 100% specific for detecting *S. pneumoniae* strains. We used *S.*

pneumoniae ATCC 49619 (positive for *lytA* and *pbp1a*), *S. pneumoniae* clinical strain 4808/S (positive for *lytA* only), and *S. pneumoniae* clinical strain 1824/F (positive for *lytA*, *erm(B)*, *mef(A)*, *pbp1a*, *pbp2x*, and *pbp2b*) to examine the analytical sensitivity of *S. pneumoniae* quantification by RQ-mPCR (see Figure S1-A in the supplemental material). The detection limit of *lytA* quantification was 20 copies/assay (10 copies/μl), which correspond to 5×10^2 colony forming units (CFU)/mL. To verify the analytical sensitivities of drug resistance genes in RQ-mPCR, we used *S. pneumoniae* clinical strain 1824/F and assessed the appearance of PCR products by gel electrophoresis (see Figure S1-B in the supplemental material). The detection limits of the five drug resistant genes were between 5.8×10^1 and 5.8×10^2 copies/assay (between 1×10^3 and 1×10^4 CFU/mL). To validate the RQ-mPCR technique, all of the 200 *S. pneumoniae* strains that were tested by RQ-mPCR were also screened for the presence of individual resistance genes by single PCR using the above PCR conditions. The results of the two methods were in full agreement (data not shown), suggesting that the multiplex PCR primer sets are reliable. The RQ-mPCR results of 200 *S. pneumoniae* strains and the MIC distribution of penicillin G and erythromycin are shown in Table 1. All of the 200 strains were positive for the *lytA* gene. The multiplex PCR correctly identified penicillin susceptibility (MIC ≤0.06 μg/mL) or non-susceptibility (MIC ≥0.12 μg/mL) in 189 (94.5%) out of 200 isolates evaluated. The sensitivity, specificity, positive predictive values and negative predictive values of our assay were 98.1% (103/105), 90.5% (86/95), 91.9%

(103/112), and 97.7% (86/88), respectively. Our assay also correctly identified erythromycin susceptibility (MIC ≤0.5 μg/mL) or resistant (MIC ≥1 μg/mL) in 200 (100%) out of 200 isolates evaluated. All of these isolates yielded 100% sensitivity, 100% specificity, 100% positive predictive values and 100% negative predictive values.

Of 200 purulent sputum samples, 56 samples were *S. pneumoniae*-positive, and the remaining 144 samples were *S. pneumoniae*-negative as determined by the conventional culture method. All 56 *S. pneumoniae*-positive samples were also positive for the *lytA* gene, and 143 of the 144 *S. pneumoniae*-negative samples were negative for the *lytA* gene. Therefore, the sensitivity and specificity for identification of *S. pneumoniae* using purulent sputum samples in RQ-mPCR compared to the conventional culture method was 100% (56/56) and 99.3% (143/144). The correlation between the conventional culture counts and the level of *lytA* gene expression by RQ-mPCR using the 56 pneumococcal culture-positive sputum samples is shown in Figure S2 in the supplemental material.

The penicillin- and macrolide-resistant genes detected by RQ-mPCR in purulent sputum samples are shown in Table 2. We compared these results to those in isolated *S. pneumoniae* from the same sputum samples. Of 56 pneumococcal culture-positive sputum samples, all were positive for *lytA* gene, and for 51 samples, the detected genes were in complete agreement with isolated *S. pneumoniae*. For the remaining five samples, the genes were not in complete agreement with isolated *S. pneumoniae*: two samples were false positives for

erm(B); one sample was false positive for *mef(A)*, *pbp1a*, *pbp2x*, and *pbp2b*; one sample was false positive for *pbp2x* and *pbp2b*; and one sample was false positive for *mef(A)* and a false negative for *pbp1a*. The sensitivity and specificity of this assay for detecting genes directly from sputum samples relative to isolated *S. pneumoniae* were 100% and 93.9% for *erm(B)*, 100% and 94.8% for *mef(A)*, 94.4% and 97.5% for *pbp1a*, 100% and 94.1% for *pbp2x*, and 100% and 95.6% for *pbp2b*. With regard to the 144 pneumococcal culture-negative sputum samples, the detection rates of drug resistant genes were 0% for *pbp1a*, 2.7% (4/144) for *pbp2x*, 1.4% (2/144) for *pbp2b*, 20.8% (30/144) for *erm(B)*, and 11.1% (16/144) for *mef(A)*.

Microorganisms closely related to *S. mitis* and harboring *lytA* gene, which are classically associated with *S. pneumoniae*, have been reported previously (37). However, positive results were not obtained from the mitis group of Streptococci (including our collected clinical strains of 17 *S. mitis* and 12 *S. oralis*) that were tested for cross-reactivity in this study. Compared to past reports (13, 14, 21, 34, 35), we obtained lower *pbp2b* detection rates in PCG-intermediate *S. pneumoniae* strains. This may have been due to a difference in the regions targeted by the *pbp2b* primers. The PCR results of macrolide resistant genes in this study matched previous report using the same primers (21), and they were also consistent with reports using other primers (11, 31). Although the analytical sensitivities of resistance genes on gel electrophoresis was lower than that of *lytA* quantification, the detection limit of 1×10^3 to 1×10^4 CFU/mL in resistant strains is within the permissible range for use with

clinical samples, including bronchoalveolar fluid, which requires a diagnostic sensitivity for pathogens in excess of 10^4 CFU/mL (1, 9). With regard to one sputum sample which showed a false positive for the *lytA* gene, the RQ-mPCR assay (confirmed repeatedly) showed the presence of 1×10^4 CFU/mL of *S. pneumoniae*, and this specimen grew to 2×10^7 CFU/mL of *Staphylococcus aureus* by culture method. This discrepancy may have resulted from a failure to detect *S. pneumoniae* that was surrounded by *S. aureus*, and/or have resulted from the detection of atypical *S. mitis* or *S. oralis*, both of which harbor the *lytA* gene (37).

A discrepancy in RQ-mPCR specificity for samples 102/5F01 (false positive for *mef(A)*), 107/5F23 (false positive for *pbp2x* and *pbp2b*), 113/5F28 (false positive for *mef(A)*, *pbp1a*, *pbp2x*, and *pbp2b*), and 101/6S11 and 101/6D13 (false positive for *erm(B)*) was confirmed by single PCR. This discrepancy indicates the presence of similar resistance genes in other microorganisms in the sputum samples. Avoiding these problems with crossreactivity is currently difficult because the mosaic genes that encode altered, low-affinity *pbp* genes are considered products of recombination events involving horizontal transfer from closely related species (10, 16) and also because *erm(B)* and *mef(A)* macrolide resistant genes in *S. pneumoniae* are highly homologous to genes in other *Streptococcus*-related species (6). In sample 102/5F01, *pbp1a* was detected by a single PCR, suggesting that the discrepancy in sensitivity (false negative) was due to the presence of PCR inhibitors or a decline of sensitivity in this sputum sample. Although the sensitivity and specificity rates of our assay

for sputum samples were in general satisfactory, further investigations are needed to remove PCR inhibitors from samples and to increase the sensitivity of the assay. Although our RQ-mPCR assay showed high correlation between the conventional culture counts and the level of *lytA* gene expression, further data from patients with pneumonia is needed to evaluate and interpret the results of our assay.

In summary, the RQ-mPCR method developed here had high sensitivity and specificity for pneumococci and could detect drug resistance in both clinical *S. pneumoniae* strains and in sputum samples. Furthermore, the results can be obtained directly from clinical samples within 3 h (2h for DNA extraction and preparation of PCR mixture, and 1h for PCR assay and electrophoresis), and this assay requires only a single tube. This method may be helpful for the rapid screening of resistance in pneumococcal isolates, and should allow the administration of earlier, more focused and effective treatment of drug-resistant *S. pneumoniae*.

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Figure Legends

Figure 1. New multiplex PCR assay for simultaneous detection of *lytA*, penicillin resistance genes (altered *pbp1a*, pbp2x, and pbp2b), and macrolide resistance genes (*erm(B)* and *mef(A)*).

(A) Comparison of single target versus multiplex PCR using "resistant" control strain 1824/F. Lane M, 100 Base-Pair Ladder (Amersham Biosciences). (B) Agarose gel electrophoresis of PCR products amplified with RQ-mPCR using control strains. Lane 1, ATCC 49619 (MIC of 0.25 μg/mL for PCG and <0.5 μg/mL for EM); lane 2, clinical strain 4808/S (MIC of <0.015 μg/mL for PCG and <0.5 μg/mL for EM); lane 3, clinical strain 1512/F (MIC of 0.03 μg/mL for PCG and 2 μg/mL for EM); lane 4, clinical strain 1565/F (MIC of 0.03 μg/mL for PCG and 32 μg/mL for EM); lane 5, clinical strain 8315/F (MIC of 0.12 μg/mL for PCG and 0.5 μg/mL for EM); lane 6, clinical strain 4827/F (MIC of 0.12 μg/mL for PCG and 32 μg/mL for EM); lane 7, clinical strain 8605/Z (MIC of 8 μg/mL for PCG and <0.5 μg/mL for EM); lane 8, clinical strain 8729/Z (MIC of 4 μg/mL for PCG and 8 μg/mL for EM); lane 9, clinical strain 1824/F (MIC of 2 μg/mL for PCG and 16 μg/mL for EM); lanes M, 100 Base-Pair Ladder (Amersham Biosciences).

Legends of Supplemental Figures

Figure S1. Analytical sensitivity of *S. pneumoniae* quantification and identification of resistant genes by RQ-mPCR assay using *S. pneumoniae* strains.

(A) Comparison of plasmid and genomic DNA standard curves of *S. pneumoniae* strains in *lytA* amplification using RQ-mPCR. The horizontal axis represents concentrations (copies/assay) of plasmid and genomic DNA of *S. pneumoniae* strains, and the vertical axis represents the crossing point (C*p*) obtained by the RQ-mPCR method. Dark circles represent plasmid, white circles represent ATCC 49619, white triangles represent strain 4808/S, and white squares represent strain 1824/F. DNA purified from 0.5 McFarland *S. pneumoniae* strains was quantified by comparison with the plasmid standard curve and adjusted to 2.9×10^6 target copies/μL. Ten-fold of serial dilutions of plasmid and genomic *S. pneumoniae* DNA were amplified in duplicate, and standard curves were plotted for each. Amplification of plasmid DNA was linear from 10^6 to 10^1 copies/assay. Comparison of the curves gave a correlation coefficient of 0.9946, indicating that the plasmid standard curve was accurate for the genomic *S. pneumoniae* DNA. The detection limit of *lytA* quantification was 20 copies/assay (10 copies/μl). (B) Identification of sensitivities for detection of resistance genes by the RQ-mPCR method using serial 10-fold dilutions of *S. pneumoniae* strain 1824/F. Ten-fold of serial dilutions of DNA purified from 0.5 McFarland (2.9×10^6 target copies/μL) *S.*

pneumoniae strain 1824/F were amplified by RQ-mPCR. Lane M, 100 Base-Pair Ladder (Amersham Biosciences). Bands were observed for *lytA* (173 bp), *erm(B)* (224 bp), *mef(A)* (294 bp), *pbp1a* (353 bp), *pbp2x* (399 bp), and *pbp2b* (442 bp). Multiplex PCR containing the *lytA* primer/probe set and primers for *erm(B), mef(A), pbp1a, pbp2x,* and *pbp2b* followed by gel electrophoresis could reliably detect 5.8×10^2 copies/assay (29 copies/μl). Six weak bands were detected using 5.8×10^1 copies/assay, but no bands were observed using 5.8×10^0 copies/assay.

Figure S2. Correlation between *lytA* quantification by RQ-mPCR and numbers of CFU by conventional culture for 56 pneumococcal culture-positive sputum samples.

Solid circles represent sputum samples (n=56). The logarithm of CFU counts determined by conventional culture is plotted on the horizontal axis, and the logarithm of the DNA copy number of *lytA* as determined by RQ-mPCR is plotted on the vertical axis. The correlation coefficient was 0.825, and the equation was y=0.838x-0.3914.

Figure 1

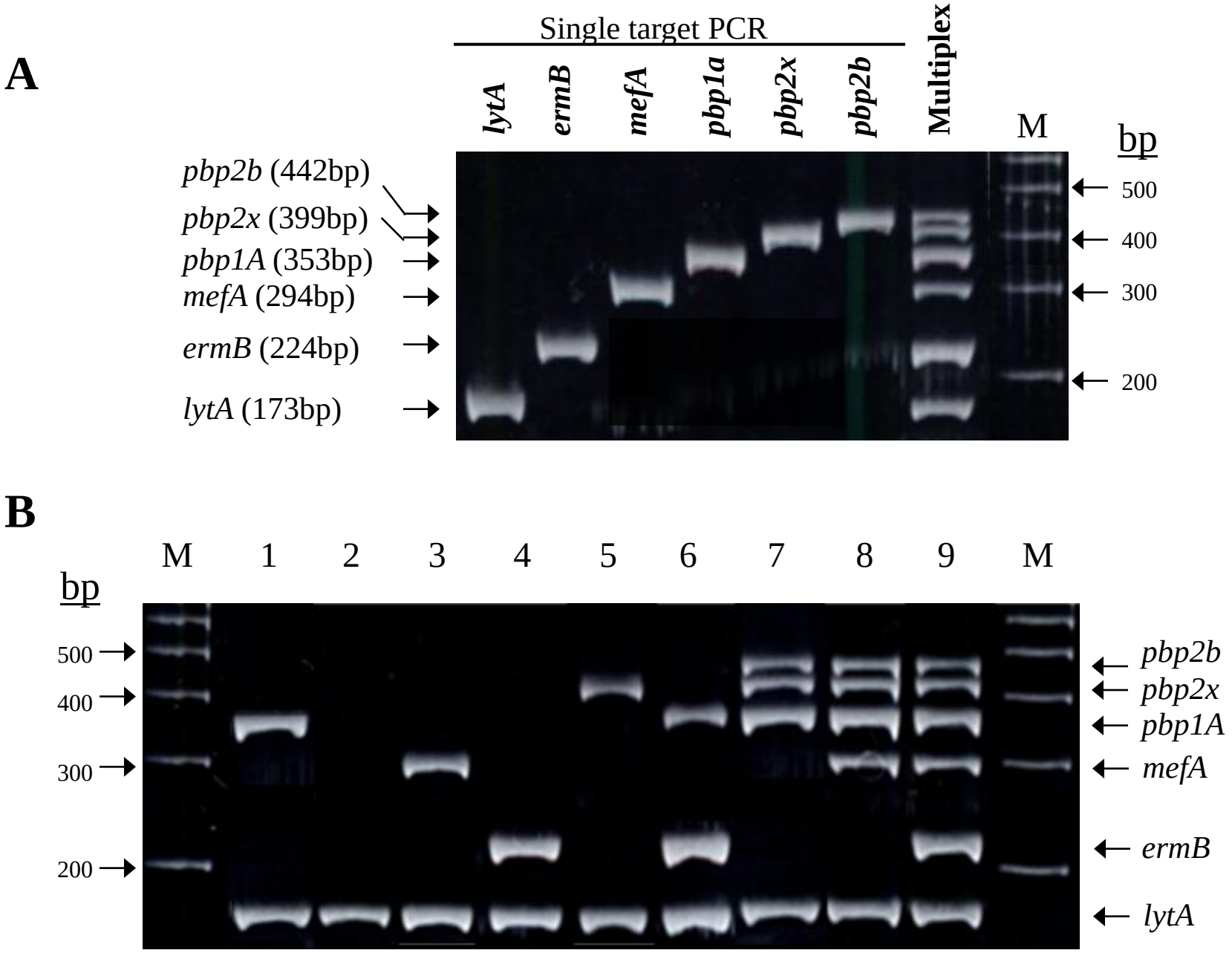
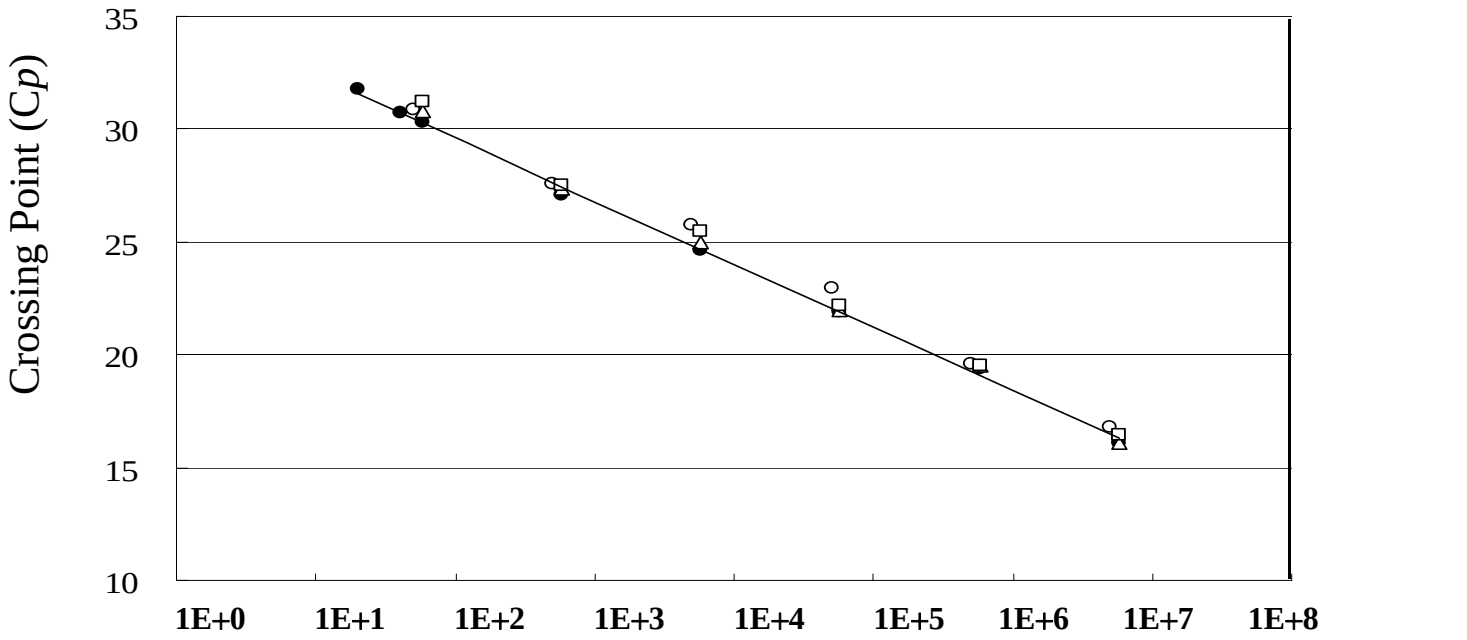

A
Single target PCR
lytA
ermB
mefA
pbp1a
pbp2x
pbp2b
Multiplex
M
bp
pbp2b (442bp)
pbp2x (399bp)
pbp1A (353bp)
mefA (294bp)
ermB (224bp)
lytA (173bp)
500
400
300
200
B
M 1 2 3 4 5 6 7 8 9 M
bp
500
400
300
200
pbp2b
pbp2x
pbp1A
mefA
ermB
lytA

Figure S1

A


Crossing Point (Cp)
35
30
25
20
15
10
1E+0
1E+1
1E+2
1E+3
1E+4
1E+5
1E+6
1E+7
1E+8
concentration (copies/assay)

B

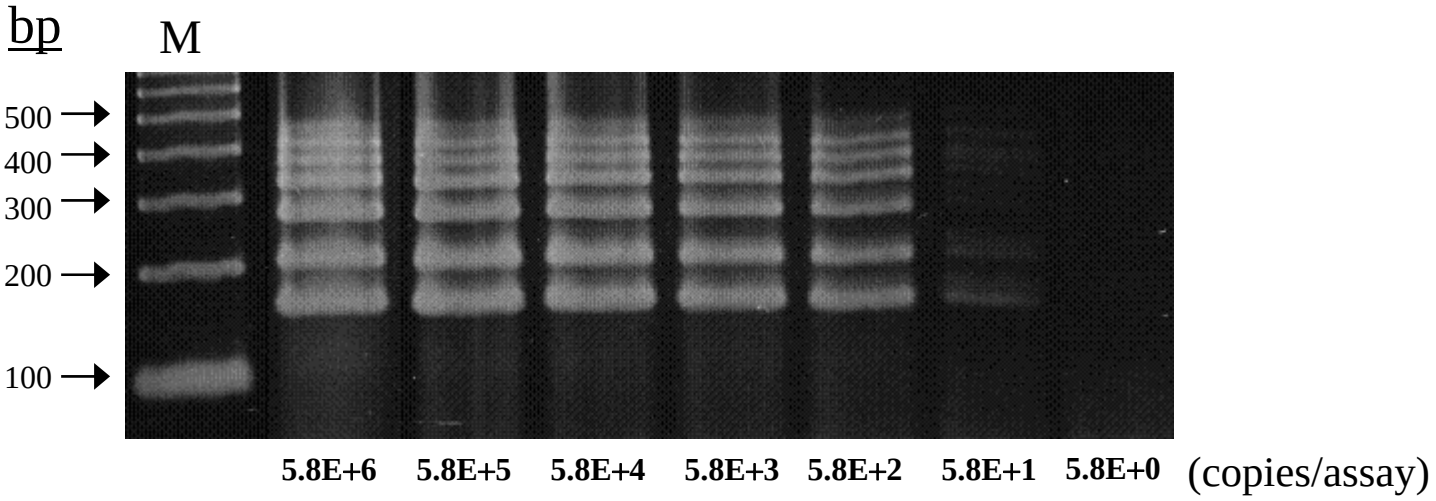

bp
M
500
400
300
200
100
5.8E+6
5.8E+5
5.8E+4
5.8E+3
5.8E+2
5.8E+1
5.8E+0
(copies/assay)

Figure S2

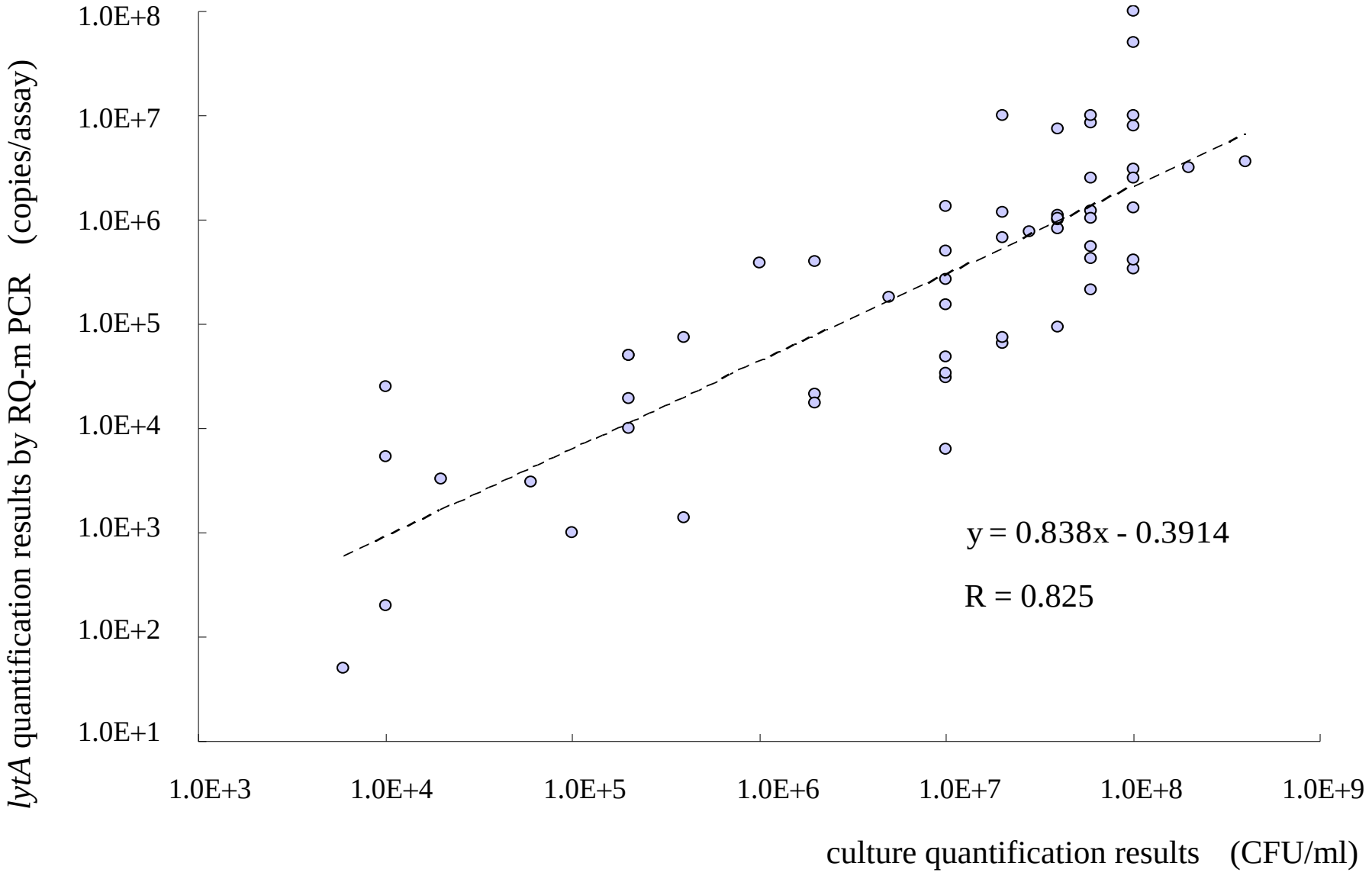

1.0E+8
1.0E+7
1.0E+6
1.0E+5
1.0E+4
1.0E+3
1.0E+2
1.0E+1
lytA quantification results by RQ-m PCR (copies/assay)
y = 0.838x - 0.3914
R = 0.825
1.0E+3
1.0E+4
1.0E+5
1.0E+6
1.0E+7
1.0E+8
1.0E+9
culture quantification results (CFU/ml)

Table 1. Organisms used for *lytA* specificity studies

Organism	Strain	*lytA* 173bp PCR product
Streptococcus pneumoniae	ATCC 49619	positive
Streptococcus pneumoniae	ATCC 6303	positive
Streptococcus pneumoniae	ATCC 6305	positive
Streptococcus pneumoniae	ATCC 27336	positive
Streptococcus pneumoniae	ATCC 49136	positive
Streptococcus pneumoniae	ATCC 49150	positive
Stapylococcus aureus	ATCC 6538	negative
Streptococcus bovis	ATCC 9809	negative
Streptococcus saliverius	ATCC 13419	negative
Streptococcus gordonii	ATCC 35557	negative
Streptococcus mitis	ATCC 9811	negative
Streptococcus oralis	ATCC 9811	negative
Streptococcus mutans	ATCC 35668	negative
Streptococcus agalactiae	ATCC 13813	negative
Streptococcus equimilis	ATCC 35666	negative
Streptococcus sanguinis	ATCC 10556	negative
Streptococcus pyogenes	ATCC 19615	negative
Enterococcus faecaris	ATCC 19433	negative
Echerichia coli	ATCC 4157	negative
Pseudomonas aeruginosa	ATCC 9027	negative
Haemophilus influenzae	ATCC 49247	negative
Moraxella catarrhalis	clinical strain	negative
Klebsiella pneumoniae	clinical strain	negative
Candida albicans	ATCC 14053	negative
Streptococcus mitis	clinical strains (n=17)	negative
Streptococcus oralis	clinical strains (n=12)	negative

Gram positive and negative bacteria and *C. albicans* were tested as a neat extract of a 0.5 Mcfarland.

Table 2. RQ-mPCR results and MICs of penicillin G and erythromycin in 200 pneumococcal isolates

RQ-mPCR results	MIC (μg/ml) distribution of PCG									
	≤0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8
none	50	26	10	2						
only *pbp2x*		1	6	9	4					
only *pbp1a*			2	4	7	4				
pbp1a + 2x				4	1	3	2			
pbp1a + 2x + 2b					4	6	16	20	18	1

RQ-mPCR results	MIC (μg/ml) distribution of EM						
	<0.5	1	2	4	8	16	32≤
none	56						
only *mef(A)*		4	15	17	9	1	
only *erm(B)*				3	7	10	67
mef(A) + erm(B)					1	2	8

Abbreviations: PCG, penicillin-G; EM, erythromycin. The CLSI MIC breakpoints for *S. pneumoniae* are as follows: PCG, susceptible ≤0.06μg/ml, intermediate 0.12-1μg/ml, resistant ≥2μg/ml; EM, susceptible ≤0.25μg/ml, intermediate 0.5μg/ml, resistant ≥1μg/ml [7].

Table 3. Comparison of RQ-mPCR results between in pneumococcal positive sputum samples and in isolated *S. pneumoniae*.

no. of pneumococcal culture positive samples (n=56)		no. of gene positive sputum samples by RQ-mPCR					
		lytA	*erm(B)*	*mef(A)*	*pbp1a*	*pbp2x*	*pbp2b*
samples with complete agreement	[a] 51 / 56	[b] 51	27	19	15	24	12
samples with disagreement (n=5)	102/5F01	+	−	FP	FN	+	+
	107/5F23	+	−	+	−	FP	FP
	113/5F28	+	+	FP	FP	FP	FP
	101/6S11	+	FP	+	+	+	+
	101/6D13	+	FP	+	+	+	+
		[c](0 / 0)	(2 / 0)	(2 / 0)	(1 / 1)	(2 / 0)	(2 / 0)
sensitivity		100 %	100 %	100 %	94.4 %	100 %	100 %
specificity		99.3 %	93.9 %	94.8 %	97.5 %	94.1 %	95.6 %

Abbreviations: FP, false-positive; FN, false-negative.

[a] no. of samples with complete agreement to RQ-mPCR results in isolated *S. pneumoniae* / no. of all samples.

[b] no. of samples with gene positive in 45 samples. [c] (no. of false positive / no. of false negative).

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Streptococcus agalactiae	ATCC 13813	negative
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