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Development of TaqMan Probe-Based Real-Time PCR Method for *erm*(A), *erm*(B), and *erm*(C), Rapid Detection of Macrolide–Lincosamide–Streptogramin B Resistance Genes, from Clinical Isolates

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To achieve more accurate and rapid detection of macrolide–lincosamide–streptogramin B resistance genes, *erm*(A), *erm*(B), and *erm*(C), we developed a TaqMan probe-based real-time PCR (Q-PCR) method and compared it with conventional PCR (C-PCR), which is the most widely using *erm* gene identification method. The detection limit of Q-PCR was 5 fg of genomic DNA or 5–8 CFU of bacterial cells of *Staphylococcus aureus*. The utilization of Q-PCR might shorten the time to *erm* detection from 3–4 h to about 50 min. These data indicated that Q-PCR assay appears to be not only highly sensitive and specific, but also the most rapid diagnostic method. Therefore, the appropriate application of the Q-PCR assay will permit rapid and accurate identification of *erm* genes from clinical and other samples.

Keywords: TaqMan probe-based real-time PCR, *erm* gene, conventional PCR, *Staphylococcus aureus*

Gram-positive bacteria are the main cause of nosocomial respiratory infections and sepsis [24], and the choice of the effective antibiotics is the key to success for treatment of the Gram-positive bacterial infection. Generally, β-lactam antibiotics are the primary choice for the treatment of Gram-positive bacterial infection, but β-lactam antibiotics have limits owing to allergy and resistance [2, 6]. On that account, macrolide–lincosamide–streptogramin B (MLS_B) antibiotics are widely used as the secondary choice for the Gram-positive infectious disease [18, 22], but they also have the problem of antibiotic resistance. Therefore, it is necessary to monitor the resistance, and investigation of the pattern of antibiotic resistance should be carried out for appropriate treatment. Such efforts are helpful to treat patients effectively and reduce medical expenses.

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In order to determine the pattern of MLS_B resistance, the minimum inhibitory concentration (MIC), and resistance phenotype and genotype were determined. In particular, the resistance genes have been identified by the conventional PCR (C-PCR) [1, 3, 14]. For C-PCR, a little amount of genomic DNA is required as template for the reaction, and amplified DNA fragments should be observed by electrophoresis after the reaction. Thus, it takes a long time, near 3 to 4 h. Moreover, despite high sensitivity, false-positive determination may occur because the size of amplicons is the only basis to determine what the amplicon is.

In recent times, the real-time PCR method has been widely used to bypass the defects of C-PCR. For example, amplified DNA fragments can be observed within 1 h, and its accuracy can be confirmed by Tm values calculated with GC contents of amplified DNA fragments [26]. The real-time PCR has been widely used to identify specific clinical isolates [10, 20]. For the development, application of probes to the real-time PCR is another reliable method to ascertain the amplified DNA fragments. In fact, fluorescein-conjugated TaqMan probe (hydrolysis probe) has been applied to quantitative real-time PCR (Q-PCR), and it has been reported that detection of 1 to 5 fg of DNA or 1 to 4.5 CFU of bacteria was achieved [4, 19, 27].

Regarding the coding genes of MLS_B resistance, it was reported that the SYBR Green I-based real-time PCR method was successfully applied for the detection [7, 15, 21, 25]. This study was performed to develop a method to detect genes of *erm*(A), *erm*(B), and *erm*(C) using TaqMan probe-based Q-PCR for more rapid and accurate detection of MLS_B resistance.

Materials and Methods

DNAs and Clinical Isolates

All the clinical isolates used in this study were collected from Severance Hospital at Yonsei University, Wonju Christian Hospital, and Kosin Christian Hospital in Korea between 2005 and 2006.

Table 1. List of DNAs and bacterial strains used in this study.

DNAs and strains	Genotype	Phenotype
Tn*554*	*erm*(A)	
Tn*917*	*erm*(B)	
pE194	*erm*(C)	
Staphylococcus aureus U2441	*erm*(A)	iMLS
Staphylococcus aureus U255	*erm*(A)	iMLS
Staphylococcus aureus P652	*erm*(A)	iMLS
Staphylococcus aureus B4462	*erm*(A)	cMLS
Staphylococcus aureus T274	*erm*(A)	cMLS
Streptococcus pyogenes 06-3-R1422	*erm*(B)	iMLS
Streptococcus pyogenes 1643	*erm*(B)	iMLS
Streptococcus pyogenes 05-10-R209	*erm*(B)	iMLS
Enterococcus faecalis 21447	*erm*(B)	cMLS
Enterococcus faecalis 21806	*erm*(B)	cMLS
Staphylococcus aureus C496	*erm*(C)	iMLS
Coagulase-negative staphylococci 3099B	*erm*(C)	iMLS
Coagulase-negative staphylococci 3546B	*erm*(C)	iMLS
Staphylococcus aureus R1731	*erm*(C)	cMLS
Coagulase-negative staphylococci 21333	*erm*(C)	cMLS
Staphylococcus aureus ATCC 6538P	Erythromycin sensitive (negative control)	

Their MLS_B resistances were determined by agar dilution methods, and their contents of *erm* genes were identified by PCR [28]. For the positive control genes of *erm*, Tn*554* for *erm*(A) [11], Tn*917* for *erm*(B) [17], and pE194 for *erm*(C) [5] were used (Table 1). The clinical strains were cultured at 37°C in a brain heart infusion (BHI) medium (Difco, Detroit, MI, U.S.A.).

Design of Primer and Probe Sets for Real-Time PCR

Primer and probe sets to detect *erm* structural genes were designed using the software supplied by Sigma Proligo (The Woodlands, TX, U.S.A.) and synthesized from the same company. The sequences of oligonucleotide used in this study are listed in Table 2.

Extraction of Genomic DNA

In order to define the detection limit of clinical isolates in a matter of number of CFU by C-PCR and Q-PCR, 15 clinical isolates harboring *erm* genes were preincubated at 37°C for 18 h. Then, seed culture was inoculated to new medium to about 0.1 of OD_{600} and incubated until the OD_{600} was 0.6. Bacterial suspension was 10-fold serially diluted, 0.1 ml of appropriate dilutions was spread onto agar to count the number of CFU, and 0.1 ml of the same dilutions was used for the extraction of genomic DNA. DNA was extracted with a DNA IQ system (Promega, Madison, WI, U.S.A.) as described in the manual, with minor modification. For efficient lysis of bacterial cell wall, lysozyme (50 mg/ml) and lysostaphin (for *S. aureus*, at a concentration of 100 μg/ml) were used. Genomic DNA of *S. aureus* ATCC 6538P was used as a negative control.

Determination of Concentration-Dependent Detection Limit with Standard *erm* Genes

As a template DNA, Tn*554* for *erm*(A) [11], Tn*917* for *erm*(B) [17], and pE194 for *erm*(C) [5] were used for PCR reaction. Template DNA (1 μg, 1 ng, 100 pg, 10 pg, 1 pg, and 100 fg) was mixed with AccuPower PCR PreMix (Bioneer, Taejeon, Korea), and 10 pmole of each primer, and the volume was adjusted to 20 μl with DNase-free H_2O. C-PCR conditions were as follows; preincubation at 94°C for 5 min, and 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 58°C for *erm*(A) and *erm*(C) or at 55°C for *erm*(B), and

Table 2. Sequences of oligonucleotides used in this study.

	Gene	Sequences (5'→3')	Length (bases)	Tm (°C)
erm(A)	Sense primer	TCAGTTACTGCTATAGAAATTGATGGAG	28	58.7
	Antisense primer	ATACAGAGTCTACACTTGGCTTAGG	25	58.8
	TaqMan probe	6FAM-AGTGACTAAAGAAGCGGTAAACCCCTCTGA-BHQ1	30	66.8
erm(B)	Sense primer	TTGGATATTCACCGAACACTAGGG	24	59.1
	Antisense primer	ATAGACAATACTTGCTCATAAGTAACGG	28	59
	TaqMan probe	6FAM-TGCTCTTGCACACTCAAGTCTCGATTCAGC-BHQ1	30	68.6
erm(C)	Sense primer	GACAATTATAAGATTAAATGAACATGATAATATC	34	56.2
	Antisense primer	AAACAATTTTGCGTATTATATCCGTAC	27	56.8
	TaqMan probe	6FAM-AAATCGGCTCAGGAAAAGGGCATTTTACCC-BHQ1	30	67.5

elongation at 72°C for 45 s, and the final extension at 72°C for 10 min. The PCR product was electrophoresed on a 1% agarose gel containing ethidium bromide. As a preliminary experiment, the designed primer sets were tested for their reactivity with SYBR Green I. LightCycler FastStart DNA mastermix for SYBR Green I (Roche Diagnostic) and Roche LightCycler (version 1.5) were used. Thermal cycling conditions were as follows: preincubation at 95°C for 10 min and the 45-cycle amplification program consisted of 20°C/s to 95°C with 10 s hold, cooling at 20°C/s to 58°C for *erm*(A) and *erm*(C) or at 55°C for *erm*(B) with 10 s hold, and heating at 20°C/s to 72°C with an 18 s hold. The one-cycle melting curve analysis program consisted of heating at 20°C/s to 95°C with 0 s hold, cooling at 20°C/s to 65°C with 15 s hold, and heating at 0.1°C/s to 95°C with 0 s hold. Finally, the experiment ended with one cycle of cooling at 20°C/s to 40°C with 30 s hold. For the Q-PCR, Q-PCR reactant was prepared in a 20-μl volume containing 4 μl of LightCycler TaqMan Master (Roche Diagnostic), 0.5 μM of each primer, 0.2 μM of TaqMan probe, template DNA, and DNase-free H_2O. Roche LightCycler (version 1.5) was used, and the thermal cycling was as follows: preincubation at 95°C for 10 min and 45 cycles of denaturation at 95°C for 10 s, annealing for 40 s at 58°C for *erm*(A) and *erm*(C) or at 55°C for *erm*(B) and extension at 72°C for 1 s, and one cycle of cooling at 40°C for 30 s. After the Q-PCR, Ct values were analyzed by quantification mode (LightCycler Ver.4.1).

Determination of Detection Limit in the Count of Viable Cells with Clinical Isolates

Genomic DNAs extracted from the serially diluted bacterial suspension, corresponding to the appropriate number of CFU, were used as a template for amplification. For C-PCR reaction, template DNA corresponding to appropriate CFU was mixed with AccuPower PCR PreMix (Bioneer, Taejeon, Korea), and the composition and the reactive condition were the same as mentioned above. The Q-PCR assay was performed in the same manner mentioned above and the amplification condition was the same as mentioned above. After the Q-PCR, Ct values were also analyzed by quantification mode (LightCycler Ver.4.1).

Results

Specificities of Primers and Probes

Table 2 presents the sequences of the primers and TaqMan probes designed for the *erm*(A), *erm*(B), and *erm*(C) genes. To verify the specificity of the primer–probe sets, all three target genes were tested with two other primer–probe sets. All three sets of primers and probes were found to be highly specific and did not cross-amplify the other two *erm* genes (data not shown).

Determination of Concentration-Dependent Detection Limit with Standard *erm* Genes

In order to define the detection limit of *erm* genes according to the concentration of genomic DNA, C-PCR and Q-PCR were performed with standard *erm*(A), *erm*(B), and *erm*(C) at various concentrations. In C-PCR, *erm*(A), *erm*(B), and *erm*(C) were amplified normally at a size of 358 bp, 299 bp, and 278 bp, respectively. The detection limit was 10 pg and the DNA band was indistinctly formed at the concentration (Fig. 1). Likewise, real-time PCR was performed using SYBR Green I in order to ascertain whether the primer, designed for this study, reacts normally. In the result, the three genes were amplified normally at the specific Tm values. In the case of *erm*(A), the Tm value was at 80±0.2°C, the Tm value of *erm*(B) was at 82.53±0.25°C, and *erm*(C) was at 79.01±0.12°C. Tm values due to primer dimer were 78.36±0.23°C for *erm*(A), 79.52±0.35°C for *erm*(B), and 75.56±0.45°C for *erm*(C). With serially diluted DNA template, each of the three genes was successfully amplified until Ct values were ≤38 with expected Tm value. However, when Ct values were ≥40, this sample did not show the expected Tm value and the sequencing result was a primer dimer. Thus, we adopted a Ct value of ≤38 as positive and ≥39 as a negative result. As a result of Q-PCR with 3 repeated experiments, a standard curve that shows concentration-dependent Ct value was obtained (Fig. 2A; r^2=0.9633).

As shown in Fig. 2B, in the case of Q-PCR, the Ct value indicated about 36 even when 5 to 10 fg (<10 fg) of DNA were used as templates; namely, DNA was clearly amplified. Accordingly, it was proven that the Q-PCR method, to which the primer and the probe were applied, is highly sensitive and accurate.

Determination of the Detection Limit According to the Viable Cells of Clinical Isolates

In order to define the detection limit of clinical isolates in a manner of viable cell, 15 kinds of clinical isolates containing

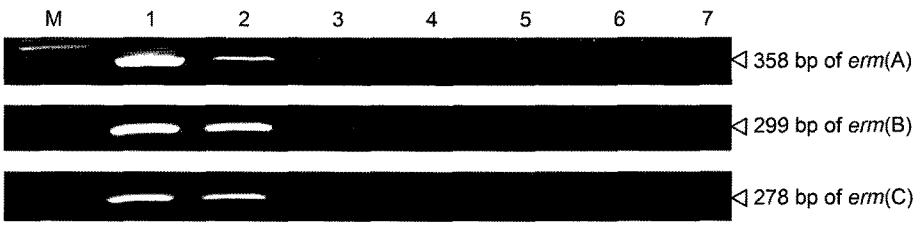

Fig. 1. Agarose gel electrophoresis of *erm*(A), *erm*(B), and *erm*(C) gene fragments amplified by C-PCR with 10-fold serial dilutions of template DNA.
Lane M, DNA ladder; lane 1, 1 μg of DNA; lane 2, 1 ng of DNA; lane 3, 100 pg of DNA; lane 4, 10 pg of DNA; lane 5, 1 pg of DNA; lane 6, 100 fg of DNA; lane 7, negative control.

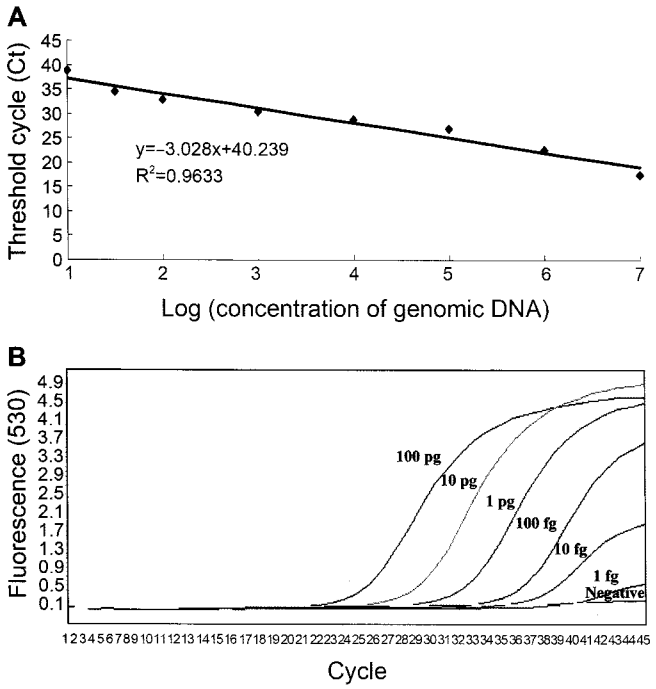

Fig. 2. Standard curve of threshold cycles in contrast to the concentration of genomic DNA.
Q-PCR was carried out three times with *S. aureus* harboring the *erm*(C) gene, and the average data were used for the curve. **A.** Standard curve; **B.** LightCycler printouts showing detection of the *erm*(C) gene by Q-PCR assay. Fluorescent signal related to cycle number on a panel of 10-fold dilutions between 100 pg and 1 fg.

erm genes were used. DNA samples showed almost identical Ct values in 3 repeated experiments and the mean value of the Ct value is shown in Table 3 with standard deviation. As shown in Table 3, in the case of C-PCR, DNA could be detected only when the viable cells was in the number of 5×10^3 CFU. In the case of Q-PCR, the *erm* genes were detected even when the number of viable cells was 5–8 CFU (Table 3).

DISCUSSION

MLS_B antibiotics have been widely used to treat Gram-positive bacterial infection, and rRNA methylation caused by *erm* genes is related to the mechanism of antibiotic resistance [5, 11, 17]. Ordinarily, *erm* genes are observed in Gram-positive bacteria, such as the genera *Staphylococcus*, *Enterococcus*, *Streptococcus*, and *Bacillus*, and antibiotic-producing bacteria *Streptomyces*. A recent study [22] reported that the MLS_B resistance of *S. aureus* isolated in Korea counted up to 55.1%. The result suggests that resistance genes in clinical Gram-positive strains need to be quickly detected for effective treatment. On that score, this study aimed at developing the method to quickly detect *erm* genes on the basis of Q-PCR. Although the C-PCR method is widely used for the detection of *erm* gene, it takes a long time, and the sensitivity of C-PCR is not good. The Q-PCR assay can give accurate results within 2 h, from genomic DNA extracts to data analysis after amplification, and it is very useful for establishing an early detection. Q-PCR assay for the detection of specific genes of Gram-negative bacteria have been actively quantified so far. *Vibrio vulnificus* [8, 9] and the genus *Salmonella* [12, 23] were quantified using the Q-PCR method, and the genes resistant to β-lactam antibiotics [4] or tetracycline [19] were also detected in *Klebsiella pneumonia* and those genes were quantified by using the Q-PCR method. In addition, the mechanism of resistance to clarithromycin [16] or azithromycin [13] was researched on *Helicobacter pylori* or *Treponema pallidium*. However, until now, there have been few data on the quantification of specific antibiotic-resistant genes from Gram-positive bacteria. In this sense, the Q-PCR method was applied to detect *erm* genes for MLS_B resistance from Gram-positive bacteria, with rapidity and accuracy. In line with the purpose, 3 types of genes such as *erm*(A), *erm*(B), and *erm*(C) were diluted by 10-fold serial dilution and C-PCR and Q-PCR were performed in order to ascertain their detection limit according to concentrations of template DNA.

As a result of C-PCR, the lowest detectible concentration of template DNA was 10 pg. In the case of Q-PCR, Ct values were in inverse proportion to concentrations (Fig. 2A). In the case of the PCR method to which TaqMan Probe was applied, the Ct value indicated 34 to 36 even at 5 fg of DNA, and it was proved that DNA can be effectively detected. Detection limit was also determined according to the number of CFU. In the result, the Ct value was in inverse proportion to the number of CFU (data not shown). The detection limit of C-PCR was 5×10^3 CFU, but that of Q-PCR indicated 5 to 8 CFU. In our study, when the Ct value was more than 38 (≥39–40), sequencing of PCR products revealed them to be a primer dimer, not the *erm* gene. Therefore, we adopted a Ct value of ≥39 as a negative result. According to previous reports, the detection limit of Gram-negative bacteria [4, 8, 9] was 10^3 CFU for C-PCR and between 1 and 10 CFU for Q-PCR. In this study, Gram-positive bacteria, of which staphylococci, enterococci, and streptococci are the representative organisms, were used. Any significant differences were not observed in the detection limit. In consideration of the fact that most *erm* genes are distributed in Gram-positive bacteria, the sensitivity on Gram-positive bacteria is the most considerable point for development of an *erm* gene identification method. It was proven that the primer and the probe designed for this study were extremely good in detecting *erm* genes. In conclusion, a Q-PCR method to detect *erm* genes is not only the most sensitive and accurate, but also the most rapid method. Therefore, this Q-PCR method is expected to be applied to the direct detection of genes from clinical samples.

Table 3. Comparison of C-PCR and Q-PCR assays for the detection of *erm* genes in 15 clinical isolates.

Strains	No. of CFU	Result by indicated assay		
		C-PCR	Q-PCR	
			Ct value[a]	Interpretation
erm(A)				
Staphylococcus aureus U2441	3.8×10^3	+	27.45±0.23	+
	8	-	34.35±0.35	+
Staphylococcus aureus U255	4.3×10^3	+	27.26±0.18	+
	7	-	35.24±0.32	+
Staphylococcus aureus P652	5.2×10^3	+	27.12±0.12	+
	5	-	35.67±0.34	+
Staphylococcus aureus B4462	4.3×10^3	+	27.32±0.28	+
	7	-	35.21±0.43	+
Staphylococcus aureus T274	4.5×10^3	+	27.21±0.29	+
	5	-	36.45±0.38	+
erm(B)				
Streptococcus pyogenes 06-3-R1422	5.1×10^3	+	27.05±0.25	+
	7	-	35.34±0.46	+
Streptococcus pyogenes 1643	4.7×10^3	+	27.89±0.32	+
	6	-	36.67±0.52	+
Streptococcus pyogenes 05-10-R209	4.6×10^3	+	28.23±0.36	+
	5	-	36.67±0.48	+
Enterococcus faecalis 21447	5.2×10^3	+	27.18±0.19	+
	6	-	35.12±0.36	+
Enterococcus faecalis 21806	5.1×10^3	+	27.12±0.22	+
	6	-	35.45±0.33	+
erm(C)				
Staphylococcus aureus C496	4.9×10^3	+	28.55±0.23	+
	7	-	35.89±0.39	+
Coagulase-negative staphylococci 3099B	4.8×10^3	+	28.23±0.31	+
	5	-	35.45±0.51	+
Coagulase-negative staphylococci 3546B	5.3×10^3	+	27.12±0.23	+
	8	-	36.56±0.61	+
Staphylococcus aureus R1731	4.7×10^3	+	28.52±0.30	+
	6	-	35.02±0.38	+
Coagulase-negative staphylococci 21333	5.2×10^3	+	27.32±0.32	+
	5	-	36.34±0.52	+

[a]The values are means±standard deviations for 3 independent experiments.

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