Detection of *tet*(K) and *tet*(M) in *Staphylococcus aureus* of Asian Countries by the Polymerase Chain Reaction

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This study describes the use of the polymerase chain reaction (PCR) to detect the *tet*(K) and *tet*(M) tetracycline resistance genes in *Staphylococcus aureus*. Primers based on the DNA sequence of the *tet*(K) and *tet*(M) genes from *S. aureus* were used as primers in the PCR assay to detect the presence of genes for resistance to tetracycline and minocycline. Two-hundred and fifteen isolates of *S. aureus* from Asian countries as Japan, Indonesia, China, Korea and Thailand were examined, and the results confirm that *tet*(K) specifies resistance to tetracycline but not to minocycline and *tet*(M) specifies resistance to both tetracycline and minocycline. We observed two different types of clinical isolates of *S. aureus* strains resistant to minocycline and tetracycline: the first carried only the *tet*(M) gene, while the second carried both the *tet*(M) and the *tet*(K) genes. Almost all of the clinical isolates of *S. aureus* resistant to minocycline and tetracycline from Indonesia, China and Thailand carried both *tet*(M) and *tet*(K) genes, while most of clinical isolates of *S. aureuss* from Japan and Korea carried only *tet*(M) gene.

During the 1950s and 1960s, tetracycline was one of the most used antibiotic in the world. Recently, the usage of tetracycline and its various derivatives have limited use in treatment of clinical infections because tetracycline resistance has appeared in many groups of medically important bacteria[1]. In our previous epidemiology study in *Staphylococcus aureus* from Indonesia during 1986 to 1993, we found that the isolation frequencies of tetracycline resistant strains among clinical isolates was more than 60% and resistant to minocycline was 30%, and almost all methicillin-resistant *S. aureus* (MRSA) strains were resistant to minocycline and tetracycline[2]. In contrast, INOUE, M. *et al.* reported, that among *S. aureus* isolated in 1986 and 1989, were resistant to minocycline and tetracycline at frequencies of 2.6% and 6.5%, respectively[3].

Bacteria can become resistant to tetracycline by several approaches: limit the access of tetracycline to the ribosome by efflux: the ribosome may be altered to prevent the effective binding of tetracycline, or enzymes may be produced that inactivate tetracycline[4]. The tetracycline resistance (TC^r) determinants in staphylococci were assigned to the classes K, L, M, and O. In *S. aureus*, TC^r determinants of the classes K and M had been studied in detail. The *tet*(M) determinant is known to protect ribosomes from inhibitory effects of tetracycline, where as *tet*(K) and *tet*(L) determinants specify membrane-associated efflux systems. The *tet*(M) gene is chromosomally or plasmid borne, and its gene product mediates resistance to tetracycline and minocycline[5,6]. The *tet*(K) gene is in plasmid borne and mediates inducible resistance only to tetracycline but not to minocycline[7,8].

BLANCHARD, A. *et al.*, ROBERTS, M. C. *et al.* and PANG, Y. *et al.* have used the polymerase chain reaction (PCR) to determine tetracycline resistance genes in Gram-negative and -positive microorganisms[9~11]. The objective of this study was to define the pattern of tetracycline resistance determinant genes *tet*(K) and *tet*(M) in clinical isolates of *S. aureus* from Asian countries.

Materials and Methods

Bacterial Strains

A total of 215 clinical isolates of *S. aureus* isolated in 1993 to 1995 from Asian countries were evaluated: 136 strains from Japan, 60 strains from Indonesia, 6 strains from China, 5 strains from Korea and 8 strains from Thailand. Isolates were stored in 50% glycerol at −80°C.

Table 1. Control laboratory strains used in this study.

Strains	Sources	MIC μg/ml		Reference
		Tc	Mn	
S. aureus MS 353	Clinical isolate	0.39	0.025	12, 13
S. aureus KU 1896	Transduction pMS7	100	0.39	12, 13
S. aureus KU 2035	Transduction *tet* M	100	12.5	This study

Tc: Tetracycline, Mn: minocycline.

S. aureus was identified by conventional laboratory methods including morphology of the colony, Gram stain, mannitol fermentation, and catalase and coagulase production. As control laboratory positive strains, we used *S. aureus* KU1896 and KU2038. *S. aureus* KU1896 and KU2038 are transductants that transduced the tetracycline-resistance *tet*(K) gene of plasmid pMS7[12] and a minocycline-resistance *tet*(M) gene from a *S. aureus* clinical isolates, respectively. A clinical isolates of tetracycline susceptible strain of *S. aureus* MS353 was also used for negative control of *tet*(K) and *tet*(M)[12] (Table 1).

Antibiotic Susceptibility Testing

Used in susceptibility testing were tetracycline hydrochloride (Wako Pure Chemical Industries. Ltd.) and minocycline (Lederle, Tokyo, Japan). Susceptibility testing and minimal inhibitory concentration (MIC) were determined in Sensitivity Test agar (Nissui, Tokyo) by the agar dilution method with an inoculum of 3×10^4 cfu/spot that was delivered by a Microplanter inoculator (Sakuma Seisaku, Tokyo, Japan)[3].

Oligonucleotide Primers

Two 18-mer oligonucleotides primers were synthesized based on the DNA sequence of pT181, a tetracycline-resistance plasmid *tet*(K) from *S. aureus*. Primer K1, 5′-CAGCAGATCCTACTCCTT-3′, corresponded to nucleotides 531 to 549 numbering of KHAN *et al.*[7,8], and primer K2, 5′-TCGATAGGAACAGCAGTA-3′, was complementary to nucleotides 682 to 700. These primers, located within *tet*(K), were separated by 168 bp.

Two 20-mer oligonucleotides primers for *tet*(M) were synthesized based on the *tet*(M) sequence from *S. aureus* 101: primer M1, including nucleotides 562 to 582 numbering of MIRJANA NESIN *et al.*[6] with the sequence 5′-GTGGACAAAGGTACAACGAG-3′, and primer M2 which was complementary to nucleotides 948 to 968 with the sequence 5′-CGGTAAAGTTCGTCACACAC-3′. The *tet*(M) primers produced a predicted fragment of 405 bp. These primers were prepared at Takara Shuzo Co., Ltd. Kyoto, Japan, and designed for use together as a combination of primers in this PCR assay.

DNA Extraction

A bacterial colony was harvested from agar plates, and suspended in 100 μl of 20 mM Tris-HCl, 140 mM NaCl, 5 mM EDTA (pH 8.0). One μl of lysostaphin solution (2.5 mg/ml in distilled water; Sigma, Chemical Co., St. Louis, Mo.) was added to 100 μl of the bacterial suspension and incubated at 37°C for 20 minutes, then placed on ice for 5 minutes. A volume of 200 μl of cold water was added. The suspention was kept on ice for 10 minutes. After incubation at 65°C for 5 minutes, 300 μl of phenol (TE saturated) was added to the bacterial suspension and mixed gently with a rotator, after being centrifuged at $12,000 \times g$ for 10 minutes. A volume of 150 μl of supernatant containing DNA was placed in a new Eppendorf tube and 150 μl of chloroform - isoamyl alcohol (24 : 1) was added and gently mixed. After being centrifuged for 5 minutes ($12,000 \times g$), 100 μl of supernatant was placed in a new tube, and 1/9 volume 3 M sodium acetate and 2.5 × vol of absolute alcohol were added. After standing for 30 minutes at −20°C, the DNA suspension was centrifuged for 10 minutes at $12,000 \times g$. The supernatant was discarded and the DNA pellet was added with 1 ml of 70% alcohol. After centrifuging for 5 minutes at $12,000 \times g$, the alcohol solution was discarded and DNA pellet was dried on a vacuum drier. Finally, the dry DNA pellet was resuspended in 100 μl of TE buffer. The DNA solution was used directly for the PCR assay.

Polymerase Chain Reaction

The PCR mixture consisted of 5 μl template DNA, combination primers as 0.8 μl/20 μM (each) of K1, K2 and 0.3 μl/20 μM (each) of M1, M2, 10 μl of PCR buffer (× 10), 8 μl dNTP, 0.5 μl *Tag polymerase* (Takara Shuzo Co., Ltd. Kyoto, Japan), and 74.3 μl distilled water. The PCR mixture was overlaid with mineral oil and processed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). DNA amplification was carried out for 25 cycles in a final volume of 100 μl of reaction mixture as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. Finally, 10 μl of PCR product were analyzed by 1% agarose gel electrophoresis in tris borate buffer and stained with ethidium bromide. Following electrophoresis, the band of amplified DNA was visualized under UV light.

Hybridization

DNA from *S. aureus* resistant to tetracycline or

minocycline was transferred to nylon membranes (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by using Biodot Apparatus (Biorad Laboratories, Hercules, California). DNA-DNA hybridization was carried out with the DIG DNA Labeling and Detection Kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Digoxigenin-labeled probe which derived from PCR product of *tet*(K) and *tet*(M) was used. Hybrids were detected by using an anti-digoxigenin alkaline phosphatase conjugate with a chromogenic enzyme substrate.

Restriction Endonuclease Analysis of the Amplified DNA by *Alu* I, *Nde* I and *Hinf* I

Restriction enzyme *Alu* I, *Nde* I, and *Hinf* I were used to determine the specificity of *tet*(K) and *tet*(M) amplified DNA. The restriction enzyme was purchased from Takara Shuzo Co., Ltd. Kyoto, Japan. A mixture consisting of amplified DNA, L or H buffer 10x (Takara Shuzo Co., Ltd. Kyoto, Japan) and restriction enzyme were incubated at 37°C for 120 minutes, then incubated at 65°C for 1 minute, followed by agarose gel electrophoresis using MethaPhor Agarose 3% (FMC BioProduct, Rockland, U.S.A.).

Results

Evaluation of Primers

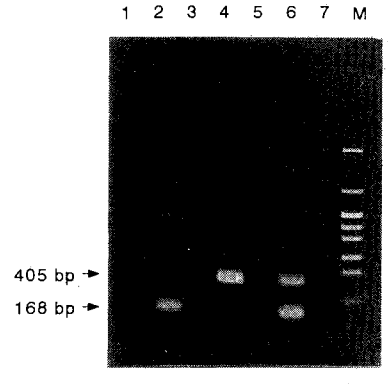
With either *tet*(K) from *S. aureus* KU1896 and *tet*(M) from *S. aureus* KU2038 as template DNA, the primers K1-K2 should generate a 168 bp PCR product, while the primer pair M1-M2 should generate a 406 bp fragment. Two DNA fragments of 168 bp and 405 bp were amplified in mixture template DNA from both control strains by combination primers K1 and K2, or M1 and M2. As seen in Fig. 1 appropriately sized fragments were generated from both genes. But a laboratory negative control strain of *S. aureus* MS353 did not generate a PCR product.

In addition, the specificity of amplified product *tet*(K) was evaluated after digestion with the restriction enzyme *Alu* I, generating two DNA fragments (80 bp, 89 bp). And the specificity of amplified product *tet*(M) was evaluated after digestion with the restriction enzyme *Nde* I and *Hinf* I, generating two DNA fragments (129 bp, 277 bp) and (257 bp, 149 bp), respectively (data not shown). To evaluate the specificity of *tet*(K) and *tet*(M) genes, we also hybridized the DNA bacteria with *tet*(K) and *tet*(M) PCR product digoxiginin-labeled probes. The *tet*(K) DNA template hybridized only with the *tet*(K) PCR product digoxiginin-labeled probes, while the *tet*(M) DNA template hybridized only with the *tet*(M) PCR product digoxiginin-labeled probes. One tetracycline susceptible control strain which failed to give PCR product, did not hybridize with the digoxiginin-labeled probes (Fig. 2).

Correlation between Presence of Resistance Genes and MICs of Tetracycline and Minocycline in *S. aureus*

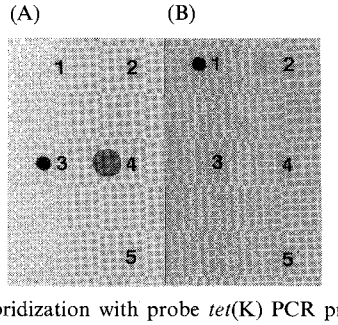
The correlation between tetracycline or minocycline resistance of *S. aureus* clinical isolates and the presence of *tet*(K) or *tet*(M) gene is shown in Fig. 3. The MICs of tetracycline and minocycline against *S. aureus* strains harboring the different *tet* genes, alone or in combinations, were determined. None of thirty-five strains of *S. aureus* with MICs of tetracycline 0.39 μg/ml and minocycline 0.05 μg/ml were amplified for *tet*(K) or

Fig. 1. Electrophoretic analysis of PCR products obtained by amplification of control laboratory strains by combination of specific primers K and M.



DNA template for lanes 1 and 2, *tet*(K) from *S. aureus* KU1896; lanes 3 and 4, *tet*(M) from *S. aureus* KU2038; lane 5, MS353 that is negative control of *tet*(K) and *tet*(M); lane 6, mixture *tet*(K) and *tet*(M); lane 7, control primers, and lane 8, Marker pHY; lanes 1 and 4 used primer M; lanes 2 and 3 used primer K.

Fig. 2. Analysis of DNA from *S. aureus* after transfer to nitrocellulose paper and hybridization with PCR product digoxiginin-labeled probe.



A: Hybridization with probe *tet*(K) PCR product from *S. aureus* KU1896. Lane 1, *tet*(M) PCR product KU2038; lane 2, DNA *tet*(M) KU2038; lane 3, *tet*(K) PCR product from KU1896; lane 4, DNA *tet*(K) KU1896; lane 5, DNA from MS353 that is negative control of *tet*(K) and *tet*(M).

B: Hybridization with probe *tet*(M) PCR product from *S. aureus* KU2038 with the same fragments as in A, respectively.

Fig. 3. Scattergram of agar dilution MICs of tetracycline and minocycline.

○, *tet*(K) and *tet*(M) negative; ●, *tet*(K) negative; □, *tet*(M) positive; ■, *tet*(K) and *tet*(M) positive.

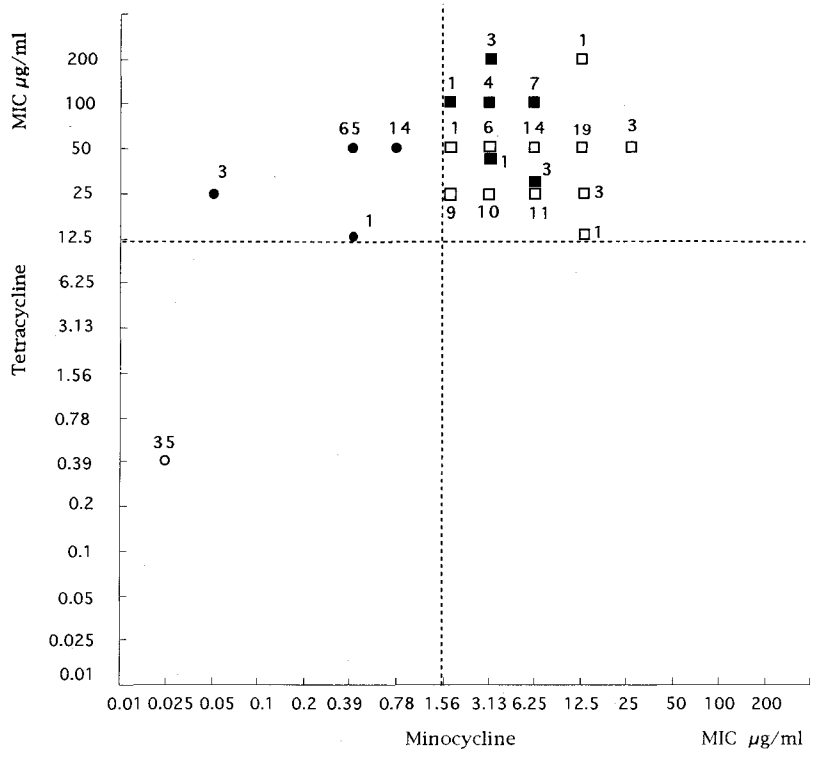
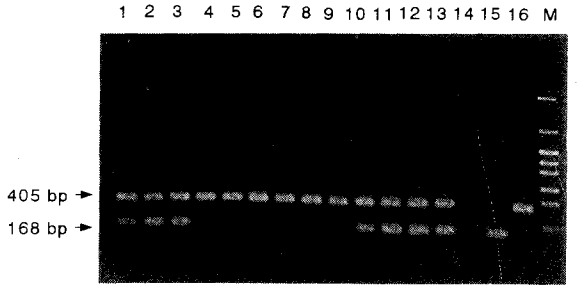


Table 2. Detection of *tet*(K) and *tet*(M) genes in 97 tetracycline and minocycline resistant *S. aureus* from Asian countries.

Country of origin	Number of gene detected by PCR		
	tet(K)	tet(M)	*tet*(K) + *tet*(M)
Japan (n=55)	0	54	1
Indonesia (n=23)	0	14	9
China (n=5)	0	0	5
Korea (n=6)	0	6	0
Thailand (n=8)	0	4	4

n: Number of strains tested.

Fig. 4. Electrophoretic analysis of PCR amplified products obtained by amplification of clinical isolates of *S. aureus* strains from Asian countries by combination of specific primers K and M.



DNA template for lanes 1~3, Indonesian strains; lanes 4~7, Japanese strains; lanes 8~9, Korean strains; lanes 10~11, Chinese strains; lanes 12~13, Thai strains; lane 14, MS353 (negative control); lane 15, KU1896 (control *tet*(K)); lane 16, KU 2038 (control *tet*(M)); and lane 17, Marker pH Y.

tet(M) gene with the primers. The MICs of tetracycline and minocycline for eighty-two *S. aureus* strains harboring only *tet*(K) were $\geqq 25\,\mu g/ml$ and $\leqq 1.56\,\mu g/ml$, respectively. However one strain harboring *tet*(K) has MIC for tetracycline $12.5\,\mu g/ml$. The MICs of tetracycline and minocycline for seventy-seven *S. aureus* strains harboring only *tet*(M) were $\geqq 25\,\mu g/ml$ and $\geqq 1.56\,\mu g/ml$, respectively. But one strain is harboring *tet*(M) has MIC for tetracycline $12.5\,\mu g/ml$. And of ninety-seven strains harboring *tet*(M), 19 strains of tetracycline and minocycline resistant *S. aureus* had two genes *tet*(M) and *tet*(K). There was a two to fourfold difference in MICs for tetracycline against *S. aureus* strains harboring both tetracycline resistant genes *tet*(M) and *tet*(K) (100, $200\,\mu g/ml$, respectively).

Detection of *tet*(K) and *tet*(M) in Clinical Isolates of *S. aureus*

The distribution of *tet*(K) or and *tet*((M) genes in tetracycline and minocycline resistant of *S. aureus* strains

from Asian countries are shown in Table 2. We examined a total of ninety-seven different clinical isolates of *S. aureus* resistant to tetracycline and minocycline from Asian countries. Interestingly, we found that 39.1% of *S. aureus* resistant to tetracycline and minocycline isolated from Indonesia carried both *tet*(K) and *tet*(M) genes. All the strains from China and 50% of strains from Thailand clinical isolates of *S. aureus* resistant to tetracycline and minocycline were also carried both *tet*(K) and *tet*(M) genes. While in contrast, 99.2% clinical isolates of *S. aureus* strains resistant to tetracycline and minocycline from Japan and all strains from Korea harbored only *tet*(M) gene (Fig. 4).

Discussion

The PCR assay has recently been used to detect tetracycline-resistant genes, such as, *tet*(M) by BLANCHARD, A. *et al.* in 1992[9], *tet*(M) and *tet*(O) by ROBERTS, M. C. *et al.* in 1993[10], and *tet*(K) and *tet*(L) by PANG, Y. *et al.* in 1994[11]. In this study, a combination specific primers were designed for detection of *tet*(K) and *tet*(M) determinants from *S. aureus* in a single reaction. The specific pair of primers (K1-K2) worked only for *tet*(K) determinant and the specific pair of primers (M1-M2) worked only for *tet*(M) determinant. These combination primers gave an appropriate PCR product with *tet*(M) determinant, as well as, 215 different clinical isolates of *S. aureus* examined. We found these primers gave a high specificity to detect *tet*(K) and *tet*(M) genes in *S. aureus*. In addition, the specificity of these PCR amplified product has been determined by using restriction endonuclease analysis and DNA-DNA hybridization with digoxigenin-labeled probes.

There was an excellent correlation between the resistant phenotypes of the strains studied, as determined by MICs, and the genotypes inferred from PCR experiments using this combination of specific primers. Strains harboring *tet*(K) were resistant to tetracycline and susceptible to minocycline and *S. aureus* strains harboring *tet*(M) were resistant to both antibiotics. There was a high MIC of tetracycline against *S. aureus* harboring both *tet*(M) and *tet*(K). They were increased two to fourfold in strains harboring both resistant determinant. This observation was similar to the result of a previous investigation, which indicated that the two genes contribute in an additive fashion, to the degree of resistance to tetracycline[14]. The reasons for this differential gene expression depending upon the bacterial host still remains unknown. The use of specific primers allowed the differentiation of two genotypes *tet*(M) or *tet*(K) and *tet*(M), among *S. aureus* strains. Study of the prevalence of tetracycline resistant determinants in *S. aureus* indicated that *tet*(K) and *tet*(M) were wide spread and that *tet*(L) and *tet*(O) were rare[10]. Further study could be done on these primers for detecting other tetracycline-resistant determinants in different species bacteria.

In studying clinical isolates of *S. aureus* resistant to minocycline and tetracycline from various countries in Asia, we found that strains from Indonesia, China and Thailand carried both *tet*(K) and *tet*(M) predominated, while strains from Japan and Korean carried only *tet*(M) gene. The reasons of this phenomenon is unknown, but a different coagulase-typing between these two groups could be studied much further. From the epidemilogy study of coagulase typing of *S. aureus*, we found that predominant clinical isolates of *S. aureus* from Japan were coagulase-type II and VII, and clinical isolates of *S. aureus* from Korea were coagulase-type III and VII. But in contrast, clinical isolates of *S. aureus* from Indonesia[2], China and Thailand were coagulase-type IV (unpublished data). The approach using combination primers *tet*(K) and *tet*(M) in PCR assay provides a useful for epidemiology study to trace strains harboring tetracycline-resistant determinant in the future.

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