Epidemiologic Study of methicillin-resistant Staphylococcus aureus by Coagulase Gene Polymorphism

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Received 16 Mar 2005
Accepted 16 Nov 2005

ABSTRACT: An epidemiologic study of methicillin-resistant Staphylococcus aureus (MRSA) was conducted by antibiotype, coagulase gene typing and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. A total of 129 MRSA strains were isolated from 17 hospitals in the regions of the central, northern, northeastern and eastern Thailand during November 2003 – March 2004. Antimicrobial susceptibility testing with a panel of 10 antimicrobial agents showed 9 different antibiotypes. The antibiotypes 1 and 2 were the most common phenotypes with 44.2% and 35.6% of the isolates, respectively. Coagulase gene typing of MRSA strains generated 4 different genotypes: I, II, III, IV, the PCR products of which were 492±20, 654±20, 735±20 and 816±20 bp with the percentages of 1.5 (2/129), 2.3 (3/129), 82.2 (106/129) and 14 (18/129), respectively. Coagulase gene PCR-RFLPs exhibited 4 patterns: A, B, C and D, with AluI digested PCR product fragments at 220±20 and 220±20 bp (pattern A); 400±20 and 220±20 bp (pattern B); 420±20 and 220±20 bp (pattern C); and 510±20 and 220±20 bp (pattern D). The percentage values for each pattern were compatible with those from the coagulase gene typing method. The results indicated that antibiotypes 1, 2, coagulase gene type III and PCR-RFLP pattern C were the epidemic strains while the rest were sporadic strains.

KEYWORDS: epidemiologic study, methicillin-resistant Staphylococcus aureus, antibiotype, coagulase gene typing, polymerase chain reaction-restriction fragment length polymorphism.

INTRODUCTION

Methicillin-resistant Staphylococcus aureus (MRSA) which causes nosocomial infections, is among the most important multi-resistant pathogens worldwide1-2. A variety of typing techniques have been developed to discriminate the related strains from unrelated strains. Accurate and rapid typing are crucial for the control of MRSA outbreaks3. Both phenotypic and genotypic characterizations can be used to identify epidemic MRSA. A bacteriophage typing has been internationally accepted since 19514-5. However, some MRSA strains may not be typable with phages6. The antibiotype has been the main typing tool in many hospital outbreaks because the technique is widely available and standardized. A large number of molecular methods have been developed for epidemiologic typing of MRSA strains, including pulsed-field gel electrophoresis (PFGE), polymerase chain reaction (PCR) of the coagulase (coa) gene, the protein A (spa) gene and hypervariable (HVR) regions adjacent to the mecA gene, DNA sequencing and polymerase chain reaction-restriction fragment length polymorphism of the coa and spa genes7-12,12,12. PCR products of the coa and spa genes can be further discriminated by AluI/CfoI/HaeIII and Rsal digestion, respectively10,11,12. Macrorestriction followed by PFGE is considered to be one of the most reliable, discriminatory and reproducible typing procedures, which allows the detection of a high degree DNA polymorphism. However, PFGE is costly, technically complex, time-consuming and lacks an agreed criterion for the interpretation of banding patterns8,9,12,13,14,15. Variations in the sequence of the coa and spa genes and the HVR adjacent to the mecA gene, have been the basis for the most widely used forms of PCR typing for MRSA13, which show a good correlation with PFGE typing12,13,16,17. The heterogeneous coa gene region contains 81-base pairs tandem repeats at the 3′ ends. The X region in the spa gene includes a variable number tandem repeat (VNTR) of 24-basepairs. PCR amplifications of these particular regions produce DNA fragments of different sizes and are highly polymorphic with regard to the number and sequence of the repeats6,12,17. Therefore, the aim of this study was to investigate epidemiologic typing of MRSA.
isolates in some regions of Thailand by using antibiotic, coagulate gene typing and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the coagulate gene.

**MATERIAL AND METHODS**

**Bacterial Isolates**
A total of 129 MRSA isolates were obtained from clinical specimens, such as pus, sputum, urine and haemoculture, from 17 hospitals in four regions of Thailand (Central, North, Northeast and East). All isolates were collected between November, 2003 and March, 2004. Two coagulate-negative staphylococci isolates were collected between November, 2003 and March, 2004. Two coagulate-negative staphylococci isolates were included as negative controls for PCR testing of the coagulate gene.

**Identification of MRSA**
MRSA isolates were identified by standard microbiological methods including Gram stain, catalase, coagulate, DNase and growth on mannitol salt agar. Resistance to oxacillin was determined by the disc diffusion method according to the National Committee for Clinical Laboratory Standard (NCCLS) guidelines.

**Antimicrobial Susceptibility Testing**
Antimicrobial susceptibility testing was performed by the disc diffusion method for erythromycin (15 μg), gentamicin (10 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), amikacin (30 μg), chloramphenicol (30 μg), rifampicin (5 μg), ciprofloxacin (5 μg), vancomycin (30 μg) and teicoplanin (30 μg) according to NCCLS guidelines.

**DNA extraction**
Genomic DNA was isolated by using DNAzol reagent (Invitrogen). One loopful of MRSA cells was harvested from a blood agar plate, suspended in 100 μl UltraPure water (Gibco BRL) and gently mixed with 900 μl of DNAzol reagent. The sample was centrifuged at 10,000 rpm for 10 min at 4°C. The DNA was precipitated from the supernatant by adding 500 μl of absolute ethanol and mixing by inversion at room temperature for 1-3 min. The DNA pellet was washed with 1 ml of 75% ethanol, air-dried and dissolved in 100 μl 8 mM NaOH. The DNA was kept at -20°C until use.

**Coagulate Gene Typing by Polymerase Chain Reaction**
The entire 3'-tandem repeat end region of the coagulate gene was amplified with two primers, as described by Hooky J et al. The forward primer 5'-ATA GAG ATG CTG GTA CAG G 3' (1513 to 1531; nucleotide numbering according to the work of Kaida et al. MRSA 213, accession no. X16457) and the reverse primer 5'-GCT TCC GAT TGT TCG ATG C 3' (2188 to 2168) were chosen. Five microliters of DNA solution was added to a 45 μl PCR mixture containing 35 μl of UltraPure water, 5 μl of 10X PCR buffer, 1.5 μl of 50 mM MgCl₂, 1 μl of 10 mM deoxyribonucleotide triphosphate (Invitrogen), 1 μl of 10 mM solutions of each primer and 0.5 μl of 5 unit/μl TaqDNA polymerase (Invitrogen). DNA amplifications were performed in a P.E 2400 Thermal cycler (Perkin-Elmer 2400) as the following cycling parameters: an initial step at 94°C for 3 min; 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; and a final step at 72°C for 5 min. PCR products (10 μl) were subjected to electrophoresis on 2% (W/V) agarose gel (Seakem) and the sizes of the PCR products were determined by comparison to the 100-1,500 base pairs ladder DNA marker (Roche Applied Science). The gel was stained with ethidium bromide and

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>ERY</th>
<th>GEN</th>
<th>TET</th>
<th>SXT</th>
<th>AMK</th>
<th>CHL</th>
<th>RIF</th>
<th>CIP</th>
<th>VAN</th>
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<tr>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
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<tr>
<td>Ant 3</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
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<td>Ant 4</td>
<td>R</td>
<td>R</td>
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<td>R</td>
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<td>S</td>
<td>R</td>
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<td>R</td>
<td>S</td>
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<tr>
<td>Ant 6</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
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<td>R</td>
<td>R</td>
<td>S</td>
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<td>Ant 8</td>
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<td>R</td>
<td>R</td>
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<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
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</table>

*Abbreviations for susceptibility: R, resistant; S, susceptible.
Abbreviations for antimicrobial agents: ERY, Erythromycin; GEN, Gentamicin; TET, Tetracycline; SXT, Trimethoprim-sulfamethoxazole; AMK, Amikacin; CHL, Chloramphenicol; RIF, Rifampicin; CIP, Ciprofloxacin; VAN, Vancomycin; TEC, Teicoplanin.*

Table 1. Antimicrobial susceptibilities of MRSA isolates in this study.
photographed on a UV illuminator.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the Coagulase Gene

Ten microliters of PCR product was digested with 2 units of restriction endonuclease Alu I (Invitrogen) at 37°C for 1 hr 30 min. The restriction digested fragments were electrophoresed on 2% agarose gels, which were then stained with ethidium bromide and visualized under UV light.

Results

Antibiotypes

Nine different antibiotypes (Ant1 - 9) were identified using a panel of 10 antimicrobial agents (Table 1). The most common antibiotypes, namely antibiotypes 1 and 2, were found in 44.2% (57/129) and 35.6% (46/129) of the isolates, respectively. Antibiotype 1 was sensitive to chloramphenicol, vancomycin and teicoplanin, and resistant to erythromycin, gentamicin, tetracycline, rifampicin, trimethoprim-sulfamethoxazole and amikacin. Antibiotype 2 was similar to antibiotype 1 except that it was sensitive to rifampicin. Antibiotypes 1 and 2 were involved in epidemic outbreak, while the others (antibiotypes 3 - 9) were found in sporadic cases.

Coagulase Gene Typing and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of Coagulase Gene

PCR amplification of the coagulase gene from 129 isolates generated 4 different types of products: I, II, III and IV, which had sizes of 492±20, 654±20, 735±20 and 816±20 base pairs (Figure 1A) and were found at 1.5% (2/129), 2.3% (3/129), 82.2% (106/129) and 14% (18/129), respectively (Table 2). Two coagulase-negative staphylococci, E. coli, Ps. aeruginosa produced no DNA products upon PCR amplification. PCR-RFLP analysis of the coagulase gene yielded 4 patterns of Alu I digested PCR products: A, B, C and D, which had DNA products at 220±20 and 220±20 base pairs (pattern A); 400±20 and 220±20 base pairs (pattern B); 420±20 and 220±20 base pairs (pattern C); and 510±20 and 220±20 base pairs (pattern D), respectively (Figure 1B). The percentages of these patterns were compatible with those of the coagulase gene typing method. The band of 220±20 base pairs was a common one found in all patterns, while the others varied in size. Coagulase genotype III / PCR-RFLP pattern C was the most common (n =106; 82.2%) and accounted for all epidemic strains, while the rest of coagulase gene types / PCR-RFLP patterns were found in sporadic strains. Figures 2 and 3 show the coagulase gene type / PCR-RFLP pattern for

Table 2. Coagulase gene typing and PCR-RFLP pattern of MRSA.

<table>
<thead>
<tr>
<th>Coagulase gene typing / PCR-RFLP pattern</th>
<th>Size of coagulase gene PCR product</th>
<th>Size of Alu I fragments</th>
<th>No. of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I / A</td>
<td>492 ± 20</td>
<td>220 ± 20, 220 ± 20</td>
<td>2 (1.5)</td>
</tr>
<tr>
<td>II / B</td>
<td>654 ± 20</td>
<td>400 ± 20, 220 ± 20</td>
<td>3 (2.3)</td>
</tr>
<tr>
<td>III / C</td>
<td>735 ± 20</td>
<td>420 ± 20, 220 ± 20</td>
<td>106 (82.2)</td>
</tr>
<tr>
<td>IV / D</td>
<td>816 ± 20</td>
<td>510 ± 20, 220 ± 20</td>
<td>18 (14)</td>
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</table>
epidemic and sporadic strains, respectively. Almost all of the antibiotype 1 (n=51) and antibiotype 2 (n=38) isolates had coagulase gene type III and PCR-RFLP pattern C (Table 3).

**DISCUSSION**

Rapid and accurate differentiation of MRSA associated with nosocomial infection is crucial to epidemiologic surveillance and hospital infection control. This may clarify whether strains from the environment or from staff members are related to those that cause infection, and whether isolates from one patient belong to one genotype. In this study, we have evaluated the use of antibiotype and PCR based typing methods (coa gene typing and PCR-RFLP of the coa

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**Table 3.** Correlations between antibiotypes and PCR based method.

<table>
<thead>
<tr>
<th>Antibiotypes (n)</th>
<th>Coagulase gene type (n)</th>
<th>Coagulase PCR-RFLP pattern (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (57)</td>
<td>coa3 (51), coa2 (3), coa4 (3)</td>
<td>RFLP-C (51), RFLP-B (3), RFLP-D (3)</td>
</tr>
<tr>
<td>2 (46)</td>
<td>coa3 (38), coa4 (6), coa1 (2)</td>
<td>RFLP-C (38), RFLP-D (6), RFLP-A (2)</td>
</tr>
<tr>
<td>3 (12)</td>
<td>coa3 (10), coa4 (2)</td>
<td>RFLP-C (10), RFLP-D (2)</td>
</tr>
<tr>
<td>4 (5)</td>
<td>coa3 (5)</td>
<td>RFLP-C (5)</td>
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<td>5 (2)</td>
<td>coa4 (2)</td>
<td>RFLP-D (2)</td>
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<td>6 (2)</td>
<td>coa4 (2)</td>
<td>RFLP-D (2)</td>
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<td>7 (1)</td>
<td>coa4 (1)</td>
<td>RFLP-D (1)</td>
</tr>
<tr>
<td>8 (1)</td>
<td>coa4 (1)</td>
<td>RFLP-D (1)</td>
</tr>
<tr>
<td>9 (1)</td>
<td>coa4 (1)</td>
<td>RFLP-D (1)</td>
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</tbody>
</table>
gene) in an epidemiological study of MRSA isolated from four regions of Thailand. Antibiotyping was a good epidemiological marker, showing a highly significant correlation with coa gene typing and PCR-RFLP of the coa gene. We found that antibiotype 1 and 2 were correlated to coa gene type III and PCR-RFLP pattern C. Antimicrobial susceptibility testing is the simplest epidemiologic typing method, the results are easy to interpret and minimal laboratory skills and equipment are required. Nevertheless, its main disadvantage consists of the variability in resistance expression, which is susceptible to instability due to loss of extrachromosomal genetic elements.\(^9,17\)

Coagulase enzyme activity is the principle criteria for diagnosis of S. aureus from other staphylococci. This enzyme can convert fibrinogen to fibrin. The coagulase gene consists of three distinct regions: (i) the N-terminus containing the prothrombin-binding site, (ii) a central region which is highly conserved, and (iii) a C-terminal region composed of 81-bp tandem repeated units, which each encode 27-amino acid residues. The C-terminal repeated units comprised four, five, six, seven and eight units of the 81-tandem repeat.\(^8\) Therefore, the size of the region of the coaglase gene is variable in MRSA strains. The heterogeneity in the number of tandem repeat units of the coaglase gene would be a potential target for typing of MRSA.\(^13,16,17\) We found that the epidemic strains had coa genotypes II, in which the PCR product was 735±20 base pairs. The other, sporadic cases had coa gene types I, II and IV.

We used AluI restriction endonuclease, which recognizes AT/GC sequences, for RFLP typing, which can be equally well assessed by CfoI or HaeIII digestion of PCR product.\(^6,10,19\) Four distinct PCR-RFLP patterns were observed among the 129 strains. The number of fragments produced upon AluI digestion were one to two bands. These results differ from previous studies, in which AluI PCR-RFLP fragments varied from one to four bands using the same primer sequences.\(^6,10,19\) However, the results of epidemic typing of the PCR-based methods are correlated. Both methods have been reported as attractive typing methods for clinical laboratories.\(^11,22\) They require only small quantities of crude DNA and isolates can be compared easily by the number and size of PCR products and their AluI restriction fragments. The low discriminatory power of the PCR-RFLP method makes it less suitable as a single typing method, but this could be overcome by the use of other enzymes to expand the number of bands.\(^23\)

In conclusion, antibiotyping is valuable, especially in routine work, as a first-line screening method to determine strain relatedness. It may allow rapid and early recognition of a previously defined epidemic strain in a particular hospital setting. Also, PCR-based method (coa genotyping and PCR-RFLP of the coa gene) provide easy, inexpensive and reasonable results for epidemiological studies during nosocomial outbreaks and are able to distinguish between the epidemic and sporadic strains.

ACKNOWLEDGMENTS

We thank the staff members of Microbiology Laboratory, Department of Clinical Pathology of Pathum Thani Hospital, Sawanpracharuk Hospital, Nakhon Nayok Hospital, AngThong Hospital, Sing Buri Hospital, Nakonpink Hospital, Petchabun Hospital, Chiang Rai Hospital, Khon Kaen Hospital, Yasothon Hospital, Sakon Nakhon Hospital, Udon Thani hospital, Saphasitthiprasong Hospital, Chon Buri and Chao Praya Apaiyaphubeth Hospitals for providing MRSA isolates. This work was funded by Huachiew Chalermprakiet University.

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