

## Multiplex PCR Assays for the Detection of Clinically Relevant Antibiotic Resistance Genes in *Staphylococcus aureus* Isolated from Malaysian Hospitals

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**Abstract:** Multiple drug resistant *Staphylococcus aureus* is one the most common nosocomial pathogen worldwide. The timely identification of this hospital acquired pathogen and detection of the various antibiotic resistant genes harbored is one of the most important function of the microbiology laboratory. In this study, we report the development of a multiplex PCR system for the diagnosis of *S. aureus* and the detection of clinically relevant antibiotic resistance genes harbored by some isolates. This system was designed to identify *S. aureus* at species level and to detect methicillin, gentamycin, erythromycin, vancomycin and mupirocin resistant genes, respectively from a single colony in a single tube reaction. All isolates amplified a 108 bp fragment (conserved in *S. aureus*) confirming the identity of *S. aureus*, 23 isolates produced a band at the position of 533 bp, 28 isolates at 139 bp and 30 isolates at 174 bp evidencing the presence of *mecA* (methicillin or oxacillin resistance), *ermA* (erythromycin resistance), *aac* (6')-aph (2'') (gentamycin resistance) genes. None of the isolates amplified *van A* (vancomycin resistance) and *ileS-2* (mupirocin resistance) genes showing the absence of their resistance in the isolates studied. These genotypic results when compared with classical antibiotic susceptibility tests showed less correlation. Overall, we found a correlation between phenotypic and genotypic methods of 60% for methicillin, 36.7% for gentamycin, 43.3% for erythromycin, 100% for vancomycin and mupirocin. This suggests that classical antibiotic sensitivity test is not accurate, but need to be supplemented with other methods to be applied in a clinical laboratory. The system developed in this study offers a rapid, simple specific and accurate detection of multiple antibiotic resistant genes in clinical *S. aureus* isolates and thus could be systematically applied as a diagnostic test in clinical microbiology laboratories, facilitating the design and use of antibiotic therapy.

**Key words:** *Staphylococcus aureus*, antibiotic resistance, multiplex PCR, primer, nosocomial, University Putra Malaysia

### INTRODUCTION

The extensive use of antibiotics over the last 50 years has led to the emergence of bacterial resistance and to the dissemination of resistance genes among pathogenic microorganisms (Amabile, 1993; Murray *et al.*, 1999). The progressive emergence and rapid dissemination of antibiotic resistance in *S. aureus* and its association with the use and consumption of antibiotics constitute a major health concern and have been considered a global crisis

(Chambers, 1997; Hashimoto *et al.*, 1994; Martinez and Baquero, 2000). *S. aureus* is the casual agent of most staphylococcal infections and is associated with serious community-acquired and nosocomial diseases. It causes superficial, deep-skin, soft-tissue infections, endocarditis and bacteremia with metastatic abscess formation and a variety of toxin-mediated diseases including gastroenteritis, staphylococcal scalded-skin syndrome and toxic shock syndrome (Fidalgo *et al.*, 1990; Roberts *et al.*, 1991). Serious complications occur because of multiple-

antibiotic-resistant *S. aureus*. As *S. aureus* is representative of many drug resistant bacteria, it is necessary to study the ways to control this bacterium and their ability to counteract antibiotic effects. The first antibiotic used in the control of *S. aureus* Penicillin G developed resistance within 2 years of its introduction and has increased gradually over the past 50 years. Then came the antibiotic methicillin designed to target penicillin resistant strains of *S. aureus*. Initially, it proved effective, but soon after the widespread use of this antibiotic, methicillin resistant strains were discovered and were found to spread rapidly throughout the world in 1980's (Chambers, 1997). One of the few antibiotics that are still active against MRSA is mupirocin (pseudomonic acid A), a natural antibiotic derived from *Pseudomonas fluorescens*. Mupirocin has been used successfully as a topical agent to eradicate colonization of MRSA in patients as well as in hospital workers (Hill *et al.*, 1988; Bastos *et al.*, 1999). Until recently, the antibiotic vancomycin has been the last weapon against strains of *S. aureus* that shows resistant to all other antibiotics (especially MRSA). After extensive research, it was found that *S. aureus* acquired resistance to vancomycin also. In 1997, a vancomycin-resistant *S. aureus* was isolated in Japan (Hiramatsu *et al.*, 1997). With the discovery of vancomycin resistant, the possibility of a strain of *S. aureus* resistant to all antibiotics has become extremely feasible. Worldwide, many strains of *S. aureus* are already resistant to all antibiotics and thus the organism has progressed one step closer to becoming an unstoppable killer. Multiple antibiotic resistant *S. aureus* strains constitute a major health care problem; therefore, the availability of sensitive and specific methods for the accurate detection of antibiotic resistance in these bacteria has become an important tool in clinical diagnosis. The use of PCR for the sensitive and specific detection of microorganisms and antibiotic resistance genes is increasing in clinical microbiology laboratories. Therefore, the goal of our study was to develop a simple and rapid multiplex PCR system to allow the detection of methicillin, gentamycin, mupirocin, vancomycin and erythromycin resistant genes in *S. aureus*. This simple and rapid MPCR system for the accurate identification of multiple antibiotic resistant *Staphylococcus aureus* could be systematically applied as a diagnostic test in clinical microbiology laboratories, facilitating the design and use of antibiotic therapy.

## MATERIALS AND METHODS

**Bacterial isolates:** A total of thirty isolates of *Staphylococcus aureus* were used in this study. Included

were 2 reference strains of multiple drug resistant *S. aureus* obtained from University Hospital Petaling Jaya. All of them were obtained from different patients visiting Hospital Seremban, Hospital Miri, Sarawak and from the Laboratory Gribbel's Petaling Jaya.

**Antimicrobial susceptibility testing:** Disc diffusion tests were performed for each of the 30 isolates identified as *S. aureus*. Discs containing 10 µg of gentamycin, 15 µg of erythromycin, 5 µg of methicillin, 5 µg of mupirocin and 30 µg of vancomycin (all from OXOID, England) were placed on the Muller Hinton agar streaked with swab dipped in the *S. aureus* culture. The agar plates were incubated for 18-24 h at 37°C to study the antimicrobial susceptibility pattern of *S. aureus* to each of the antibiotic tested.

**DNA preparation:** After overnight culture on Blood agar plates, one colony of each sample was resuspended in 25 µL of sterile ultra pure water and the suspension was then placed in a 100°C heat block for 12 min. From this suspension, a 1 µL volume was directly used as a template for PCR amplification.

**Primers:** The 6 primer sets used in this study shown in Table 1 have been described (Martineau *et al.*, 1998, 2000; Louie *et al.*, 2000; Perez-Roth *et al.*, 2001) and were synthesized from a commercial source ( $\alpha$  DNA).

**Multiplex polymerase chain reaction:** The multiplex PCR was performed according to Perez-Roth *et al.* (2001) in a total volume of 25 µL containing 80 mM MgCl<sub>2</sub>, PCR buffer, 3.5 mM DNTP mix (Fermentas), 10 picomole µL<sup>-1</sup> of each of the primers listed in the Table 1 (OPERON Technologies) and 1 unit of Taq polymerase (BioSyntech Technologies) with 1 µL of bacterial suspension obtained from the rapid DNA extraction method described above. Amplifications were carried out by using a thermal cycler (Biometra-Trio Thermoblock) with the following thermal cycling profile: an initial denaturation step at 94°C for 5 min was followed by 10 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec and extension at 72°C for 45 sec) and 25 cycles of amplification (denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec and extension at 72°C for 1 min) ending with a final extension step at 72°C for 10 min. After amplification, 10 µL of the reaction mixture was loaded onto a 2% agarose gel and electrophoresed to estimate the sizes of the amplification products with a 100-bp molecular size standard ladder (MBI Fermentas). The gel was then stained with ethidium bromide and photographed under UV illumination.

Table 1: Primers used for multiplex PCR for the identification of *S. aureus* and detection of its antibiotic resistant genes

Target	Primer sequences (5'-3')	Amplicon size (bp)
<i>S. aureus</i> (Sa442 fragment)	AAT CTT TGT CCG TAC ACG ATA TTC TTC ACG CGT AAT GAG ATT TCA GTA GAT AATACA ACA	108
aac (6')-aph (2'')	TTG GGA AGA TGA AGT TTT TAG A CCT TTA CTCCAA TAA TTT GGC T	174
ermA	TAT CTT ATC GTT GAG AAG GGA TT CTA CAC TTG GCT TAG GAT GAA A	139
mecA	AAA ATC GAT GGT AAA GGT TGG C AGT TCT GCA GTA CCG GAT TTG C	533
ileS-2	TAT ATTATG CGA TGG AAG GTT GG AAT AAA ATC AGC TGG AAA GTG TTG	456
VanA	CAT GAA TAG AAT AAA AGT TGC AAT A CCC CTT TAA CGC TAA TAC GAT CAA	1030

## RESULTS

Multiplex PCR for the detection of antibiotic resistant genes in *Staphylococcus aureus*: Amplifications of *mecA* (533 bp), *ileS-2* (456 bp), *aac (6')-aph (2'')* (174 bp), *ermA* (139 bp), *van A* (1030 bp) genes and *S. aureus* specific fragment produced distinct bands expect for *ileS-2* and *van A* corresponding to their respective molecular sizes that are easily recognizable (Fig. 1). The *S. aureus* specific primers amplified a 108 bp fragment in all the isolates confirming the identity of *S. aureus*. This protocol including the rapid extraction method from a single colony and electrophoretic analysis of the amplified products on an agarose gel, was performed in about 4 1/2 h.

**Correlation between Multiplex PCR assay (MPCR) and susceptibility testing:** The 2 methods, which are MPCR and the disc diffusion test were compared for antibiotic susceptibility. We compared methicillin, gentamycin, erythromycin, vancomycin and mupirocin results determined by the disk diffusion method with the results obtained by the MPCR system for the detection of antibiotic resistance genes. Results from the 6 MPCR does not correlate very well with the disc diffusion test. Out of the 30 isolates studied 23 were resistant to methicillin, 30 to gentamycin and 28 to erythromycin by PCR assay, where as susceptibility testing showed 11 isolates resistant to methicillin, 10 to gentamycin and 12 to erythromycin. Moreover, 14, 18, 19 isolates that showed sensitive pattern by disc diffusion method showed the presence of resistant genes, respectively for methicillin erythromycin and gentamycin. It is shown that the disc diffusion method gave different results for these isolates. None of the isolates showed resistance to mupirocin and vancomycin by either method. Overall, we found a correlation between phenotypic and genotypic methods of 60% for methicillin, 36.7% for gentamycin, 43.3% for erythromycin, 100% for vancomycin and mupirocin.

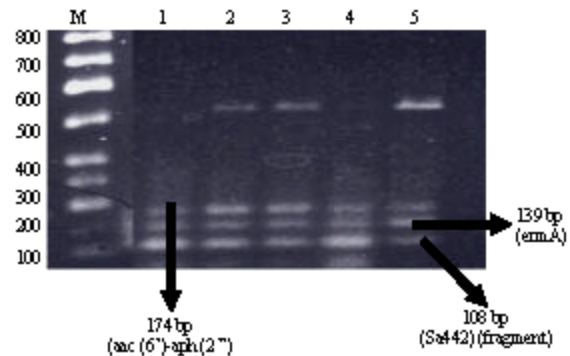


Fig. 1: Lane M represents the 100 bp marker. Lanes 1-5 shows the presence of methicillin, gentamycin and erythromycin resistance genes in *S. aureus*. A band of 108 bp confirm the identity of *S. aureus*

## DISCUSSION

Nosocomial infections caused by multi resistant staphylococci are a growing problem for many health care institutions (Kloos and Bannerman, 1999). Of all the species of staphylococci, *S. aureus* and *S. epidermidis* have the greatest pathogenic potential. According to a recent report by the World Health Organization, Drug-resistant infections in rich and developing nations alike are threatening to make once treatable diseases incurable. This chilling announcement fits most accurately *Staphylococcus aureus*, the number one cause of potentially life-threatening hospital-borne infections in the United States and all over the world. Therefore, it is highly essential to raise a technology for the accurate and rapid detection of resistant isolates constituting a critical goal of clinical microbiology. During the last decade, several studies have demonstrated the extremely high capacity of PCR for specifically detecting bacteria and genes of interest (Salisbury *et al.*, 1996). That ability has revealed PCR as a powerful tool in clinical microbiology studies (Cockerill, 1999). Genotyping/identification of *S. aureus* and its antibiotic resistant genes has been used based on the detection of different specific target sequences like Sa442 fragment (Martineau *et al.*, 1998),

nuc gene (Brakstad *et al.*, 1992) femA or femB (Berger-Baichi *et al.*, 1992; Kizaki *et al.*, 1994) mecA (methicillin or oxacillin resistance), ermA (erythromycin resistance), aac (6')-aph (2'') (gentamycin resistance), van A (vancomycin resistance), ileS-2 (mupirocin resistance) genes (Martineau *et al.*, 2000; Perz-Roth *et al.*, 2001). The goal of our study was to develop a rapid and accurate system for the simultaneous identification of *S. aureus* strains and the detection of genes conferring methicillin, erythromycin, gentamycin, vancomycin and mupirocin resistance.

Our study describes multiplex PCR system that can be used to detect clinically relevant resistance genes frequently encountered in *Staphylococcus aureus*. This system was applied to *S. aureus* isolates isolated from different patients and from different hospitals in Malaysia. For the identification of *S. aureus*, we employed PCR primers targeted to the Sa442 fragment, since it is highly conserved in *S. aureus* and also previous studies had demonstrated the feasibility of these primers for the species-specific and ubiquitous identification of this bacterial species (Martineau *et al.*, 1998).

Although, previous studies have reported the application of PCR for the accurate detection of the various antibiotic resistant genes and the possibility of simultaneous identification of *S. aureus*, no reports were evidenced for the identification of *S. aureus* and the detection of methicillin, erythromycin, gentamycin, vancomycin and mupirocin resistance in a single tube using the DNA obtained from the rapid DNA extraction method described above. It is highly important and necessary to identify the clinical pathogens in clinical microbiology laboratories at the earliest; therefore, it is very crucial to have a simple and rapid method for DNA extraction. Although, there were several reports describing this method of extracting DNA from overnight cultures (Nunes *et al.*, 1999; Tokue *et al.*, 1992; Vannuffel *et al.*, 1995), in our study, we report a rapid method for bacterial DNA extraction method directly from a single colony in as little as 12 min. This method yielded excellent quality target DNA for PCR amplifications. The high specialty about our method is that good qualities of expected PCR fragments were obtained when amplifications were performed with as low as 1  $\mu$ L of template with 6 sets of primers. Furthermore, we compared the performance of these PCR assays with the classical method of susceptibility testing. Overall, we found a correlation between phenotypic and genotypic methods of 60% for methicillin, 43.3% for erythromycin, 36.7% gentamycin, 100% for mupirocin and vancomycin resistance. From this study it is clear that disc diffusion method gave different results for erythromycin, methicillin and gentamycin resistance, which gives an indication that the phenotypic methods are not 100% reliable.

Moreover, the classical susceptibility testing requires 2-3 days to get the results, it has additional shortcomings: firstly since different bacterial species differ in their susceptibility to a given antibiotic, breakpoints of different values must be tested, secondly susceptibility tests may be highly dependent on experimental conditions and thirdly these results are not stable, when each isolate was tested with the same antibiotic in triplicates under the same condition, each of it gives different readings. From the results obtained in this study, we have come to the conclusion that it is absolutely necessary that fast and sensitive laboratory methods be available for the accurate and the immediate detection of multiple antibiotic resistant *S. aureus*. Overall our system described is highly sensitive, accurate, rapid, feasible and cost-effective; therefore, it could be systematically applied in clinical microbiology laboratories for the identification of multiple drug resistant. This will contribute the reduction of treatment with broad-spectrum antibiotics, thereby reducing the emergence of resistance and also initiating the appropriate treatment without delay.

## CONCLUSION

Multidrug-resistant *Staphylococcus aureus* has become the most prevalent nosocomial pathogen worldwide that can be determined to be public. It is important to identify *S. aureus* and detect the presence of antibiotic resistant genes it harbors immediately to facilitate the therapy with the correct antibiotic. This study reports the development of a multiplex PCR system for identification of *S. aureus* and detection of various antibiotic resistant genes. The results when compared with the results obtained from the disc diffusion method gave low correlation, suggesting that classical method need to be supplemented with the molecular technique for confirmation of clinical laboratory tests. Moreover, classical methods takes 2-3 days to get the results, which delays the treatment, whereas PCR method gives the result within 4-5 h paving way for the earliest treatment with the correct antibiotic. Therefore, this study demonstrates the practical value, sensitivity, rapidity, specificity and accuracy of the PCR method for the diagnosis and treatment of *S. aureus* infections.

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