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Identification of Clinical Methicillin and Mupirocin-resistant *Staphylococcus aureus* by Multiplex-PCR

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ABSTRACT

Background: Infections resulted from multi-resistant *Staphylococcus aureus* are increasing worldwide. In the present study, a Multiplex-PCR assay for the detection of antibiotic resistance genes among *S. aureus* clinical isolates and for the concomitant identification of these isolates was described.

Methods: A total of 127 *S. aureus* isolates were collected from clinical specimens at three teaching hospitals in Tehran, Iran. Screening for methicillin and mupirocin resistance in staphylococcal isolates was performed by disk diffusion method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The presence of *femB*, *mecA*, and *iles*-2 genes was investigated by multiplex-PCR.

Results: The 62.2% and 7.8% of Staphylococcus isolates were positive for *mecA* and *ileS-2* genes, respectively. The *femB* fragment was amplified in all *S. aureus* strains tested. There is a 100% concordance between susceptibility and PCR results for isolates which harbored *ileS-2*. However, 55.1% of staphylococci were found as MRSA in the phenotypic assay.

Conclusions: The PCR assay described in this study was able detect three genes for identification of *S. aureus* and its methicillin and mupirocin resistant genotypes concomitantly in a single reaction. Hence, this method can be used on regular basis as a valuable diagnostic and surveillance tool in clinical laboratories.

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Introduction

Staphylococcus aureus is one of the most commonly found pathogenic bacteria and is responsible for many community-acquired and nosocomial infections, including septicaemia, pneumonia, wound sepsis, septic arthritis, osteomyelitis, endocarditis, and toxic shock syndrome (1-3). Treatment of these infections has become problematic due to development of multidrug resistant (MDR) *S. aureus* strains. The introduction of new antibiotics against staphylococcal infections has stimulated a considerable case of bacterial evolution in the selective pressures. The selective pressure resulted from overuse, abuse or misuse of antibiotics has led to the emergence of resistant strains and the distribution of resistance genes among pathogenic microorganisms (4-6). Thus, the use of new antimicrobial agents has continuously been followed by the appearance of new staphylococcal resistance (7, 8). Methicillin was the first semi synthetic penicillinase resistant penicillins that was introduced in 1959 to overcome the problems caused by the increased prevalence of penicillin-resistant *S. aureus* strains. Since the emergence of methicillin-resistant *S. aureus* (MRSA) strains in 1961, the prevalence of this type of *S. aureus* strains has been permanently increased worldwide (9, 10). Resistance to methicillin in *S. aureus* is mediated by the presence of *mecA* gene encoding an additional 78 kDa low affinity penicillin-binding protein PBP-2a that has a low affinity for beta-lactam antibiotics (11, 12). Several antimicrobial susceptibility assays, including cefoxitin or oxacillin disk diffusion method, oxacillin screening test and cefoxitin or oxacillin MIC test have been used for recognition of MRSA. However, there are numerous reports that these traditional antimicrobial tests are related to negative or positive false results (13-16).

Mupirocin is a carboxylic acid that has been in clinical use in hospitalized patients for prophylaxis against nasal carriage of *S. aureus* since the late 1980s. Mupirocin reversibly binds to bacterial isoleucyl-tRNA synthetase (IRS), encoded by the *ileS* gene, resulting in arresting protein synthesis (18). As a result of its extensive use, mupirocin resistance has been observed in both methicillin-susceptible and -resistant staphylococci (17). Two mupirocin-resistant phenotypes of *S. aureus* have been described: low-level resistant (LLR), with MIC ≥ 8 µg/mL to ≤ 256 µg/mL, and high-level resistant (HLR), with MIC ≥ 512 µg/mL (19). It has been suggested that LLR is due to a mutation in the native *ileS-1* gene (*mupL*), whereas HLR is usually mediated by a conjugative plasmid-associated *ileS-2* (*mupA*) gene encoding a novel isoleucyl-tRNA synthetase that is not bound by mupirocin (20, 21). In the recent years, polymerase chain reaction (PCR) technique has been developed and used for the specific and sensitive detection of microorganisms and antibiotic resistance genes in clinical microbiology laboratories (12, 13, 22). The specificity of *femB* gene detection, which encodes for an enzyme essential for formation of the pentaglycine interpeptide bridge, for the speciation of *S. aureus* has been established previously (23). In this study we set up a multiplex-PCR procedure for simultaneous identification of clinical isolates of *S. aureus* and detection of methicillin and mupirocin resistance in this microorganism.

Material and method

Clinical samples and laboratory conventional tests

A total of 127 nonduplicate *S. aureus* strains, isolated from May to November 2013 from patients with various infections in three university hospital microbiology laboratories in Tehran, were included in this cross-sectional study. These isolates were from various clinical specimens,

including blood (22.4%), skin lesions (13.7%), broncho-alveolar lavage (5.6%), urine (21.1%), sputum (4.1%), cerebrospinal fluid (1.9%), cynovial fluid (4.8%), and pus (26.3%) from the hospitalized patients. All staphylococci were presumptively identified as *S. aureus* by standard biochemical tests for colony morphology, Gram staining, catalase, coagulase, DNase, and novobiocin sensitivity tests. All isolates were also evaluated for the presence of the *femB* gene by PCR. Screening for methicillin and mupirocin resistance in staphylococcal isolates was performed by disk diffusion method on Mueller-Hinton agar plates (Merck, Germany), according to CLSI recommendations (24). MRSA strains were identified by a 30-µg cefoxitin disk (Mast, UK) and also, 200-µg disk of mupirocin (Mast, UK) was used in order to detect high level resistance.

Multiplex-PCR for detection of genes

The presence of the intrinsic *femB* gene and two antibiotic resistance determinants (*mecA* and *ileS-2*) were investigated by multiplex-PCR in a Mastercycler gradient instrument (Eppendorf, Germany). Genomic DNA was extracted from Staphylococcus colonies grown overnight on the brain heart infusion (Merck Co., Germany) agar plates, using the Genomic DNA Purification Kit (Fermentase Co., Lithuania). The three pairs of primers used for the reactions are listed in Table 1.25 Each reaction contained mixture contained 12.5 µL of 2× MasterMix (SinaClon, Iran), including 1× reaction buffer, 3 mmol/L $MgCl_2$, a 0.2 mmol/L concentration of each of the four dNTPs, and 1.25 IU *Taq* DNA polymerase, 0.8 µM of each three pairs primer, 1 µL of template DNA (0.5 µg), and 9.5 µL of sterile distilled water up to 25 µL. DNA amplification was performed with the PCR cycling conditions as follows: initial denaturation at 94°C for 5 min, followed by 32 cycles of amplification (denaturation at 94°C for 45 s, annealing at 52°C for 40 s, and extension at 72°C for 1 min), with a final extension step at 72°C for 5 min.

electrophoresis in 0.5× TBE for 100 min at 90 V and 30 mA. The standard strains used for quality control were as follows: *S. aureus* ATCC 43300 as positive control for *mecA* gene, *S. aureus* ATCC BAA 1708 as positive control for *ileS-2* gene, and *S. epidermidis* ATCC 12228 as negative control for all *mecA*, *ileS-2*, and *femB* genes tested.

Result

Detection of selected S. aureus genes

During the 6-month study period, 127 non-consecutive isolates of *S. aureus* were obtained. The *femB* gene was detected in all 127 isolates tested (100%), confirming them as *S. aureus*. In this study we found that the incidence of the two resistance determinants tested was 79 (62.2%) strains of *S. aureus* contained *mecA* gene and 10 (7.8%) were positive for *ileS-2* in the total of 127 isolated strains. Amplification of *femB*, *mecA*, and *ileS-2* genes produced separate bands corresponding to their respective molecular sizes that were easily recognizable (Fig 2). The 7.8% of included *S. aureus* with amplified *ileS-2* gene in PCR emphasis the fact that they belong to high mupirocin-resistant phenoyype. The *femB* fragment was always amplified in the case of *S. aureus* strains and not at all in the case of *S. epidermidis* ATCC 12228 control strain.

Relationship between antimicrobial screening test and PCR assay

We compared cefoxitin and mupirocin susceptibility results found by the disk diffusion method for all Staphylococcus isolates with the results achieved by the multiplex-PCR assay for the identification of antibiotic resistance genes (Table 1). All 10 isolates in which resistance to mupirocin was detected by the susceptibility test, were later confirmed by our PCR assay. However, 70 (55.1%) isolates were found as MRSA in the phenotypic assay.

Table 1. The nucleotide sequences of the primers used for PCR reaction

Gene	Primer sequence (5'→3')	Ampliqon size (bp)
mecA	F, GTA GAA ATG ACT GAA CGT CCG ATA A R, CCA ATT CCA CAT TGT TTC GGT CTA A	310
femB	F, TTA CAG AGT TAA CTG TTA CC R, ATA CAA ATC CAG CAC GCT CT	651
ileS-2	F, TAT ATT ATG CGA TGG AAG GTT GG R, AAT AAA ATC AGC TGG AAA GTG TTG	456

Table 2. Correlation between phenotypic groups and PCR results

Species	Resistance phenotype		No. of isolates	Presence of fragment		
	Methicillin	Mupirocin		*mecA*	*ileS-2*	*femB*
S. aureus	**R**	**S**	**70**	+	-	+
	S	**S**	**48**	-	-	+
	S	**R**	**9**	+	+	+

Discussion

Multi-resistant *S. aureus* strains now account for important causes of hospital- and community-acquired infections (12, 26) In addition, the emergence and spread of MRSA strains in the last decade, most likely due to the continued over-use of broad-spectrum β-lactam antibiotics, has increased worldwide as well as in Iran (12, 27) On the other hand, there are several reports on the emergence or even increased incidence of *S. aureus* strains resistant to newer antimicrobials, like mupirocin in hospitalized patients throughout the world (28-30). Mupirocin has been used for the treatment of different types of staphylococcal skin infections and has served as an main antimicrobial in the control of MRSA outbreaks because of its useful effect in the eradication of MRSA nasal carriage in patients and health care workers (20). For these reasons, precise and rapid detection of such problematic resistant isolates is a critical goal of clinical microbiology, and is necessary for prompting effective therapy, reducing the risk of patient's mortality, as well as performing continuous surveillance programs (13, 22). During the last decade, many studies have revealed the extremely high ability of PCR for specially detecting bacteria and genes of interest (31). There are several studies showing the capability of the PCR technique for the identification of *S. aureus* strains and for the detection of antibiotic resistance genes (13, 24, 32).

In the current study, we used an multiplex-PCR to identify simultaneously *S. aureus* strains and detect the genes rendering methicillin and mupirocin resistance, namely, *mecA* and *ileS-2*, respectively. We employed primers targeted to the *femB*, a gene which previous studies had showed the feasibility of that for the definitive identification of *S. aureus* species (22, 24). In contrast to other authors (33, 34), we have found a 100% concordance between mupirocin susceptibility testing and PCR results for our included isolates. In other cases (34), inconsistencies between the PCR results and the mupirocin MICs appeared to be due to the selection of bacterial colonies with mixed mupirocin susceptibilities derived from lack of expression of the *ileS-2* gene in a proportion of the cells. Because these, it is thought that only a combination of both approaches should be used for a reliable identification of mupirocin-resistant *S. aureus* isolates. Based on our result, the rate of MRSA identified by cefoxitin disk diffusion method was 55.1%; whereas, 62.2% of Staphylococcus isolates detected by PCR protocol. This false negative result has been reported previously by other authors, like Pillai *et al* (16), Najar-Peerayeh *et al* (35) and Mohanasoundaram *et al* (14). At the moment, due to the emergence of mupirocin-resitant *S. aureus* strains in different geographic regions or patient groups (36, 37), and the increased incidence and distribution of MRSA, it is absolutely necessary that fast and sensitive laboratory methods be accessible for the immediate detection of multi-resistant MRSA. For that purpose, the PCR assay described in this study is enough best method with respect to accuracy, highly sensitivity and specificity, rapidity, and viability. Therefore, considering that it represents an easy and cost effective method, PCR could be systematically considered as a valuable diagnostic tool, especially in hospitals in areas where multi-resistant MRSA is endemic, helping timely and appropriate treatment and infection control.

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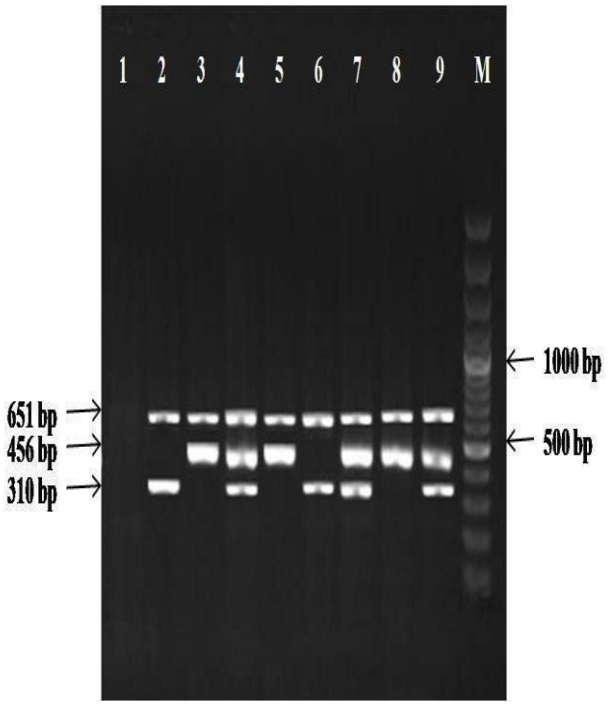

Figure 1. Gel electrophoresis profiles showing multiplex-PCR products of some *Staphylococcus* strains. Lane 1: negative control (*S. epidermidis* ATCC 12228); Lanes 2, 3: positive controls (*S. aureus* ATCC 43300 and *S. aureus* ATCC BAA 1708, respectively); Lanes 4, 7, 9: methicillin-resistant and mupirocin-resistant *S. aureus* isolates; Lanes 5 , 8: mupirocin-resistant *S. aureus* isolates; 6: methicillin-resistant *S. aureus* isolate; M: 1 kbp DNA size marker.

Conflict of interest

None declared conflicts of interest.

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