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Multiplex PCR use for *Staphylococcus aureus* identification and oxacillin and mupirocin resistance evaluation

Braoios, A[1*]; Fluminhan Júnior, A.[2]; Pizzolitto, A.C.[3]

[1]Departamento de Biomedicina, Universidade Federal de Goiás – UGF, Jataí – GO;
[2]Departamento de Genética Molecular, Universidade do Oeste Paulista – UNOESTE, Presidente Prudente, SP;
[3]Departamento de Análises Clínicas, Faculdade de Ciências Farmacêuticas, UNESP, Araraquara, SP.

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ABSTRACT

Oxacillin-resistant *Staphylococcus aureus* represents a serious problem in hospitals worldwide, increasing infected patients' mortality and morbidity and raising treatment costs and internment time. In this study, the results of using the Multiplex PCR technique to amplify fragments of the genes *fem*A (specific-species), *mec*A (oxacillin resistance) and *ile*S-2 (mupirocin resistance) were compared with those of tests conventionally used to identify *S. aureus* isolates and ascertain their resistance to drugs. Fifty *S. aureus* strains were isolated from patients receiving treatment at UNOESTE University Hospital in Presidente Prudente, SP, Brazil. The 686 bp fragment corresponding to the gene *fem*A was amplified and detected in all the isolates. On the other hand, the 310 bp fragment corresponding to the *mec*A gene was amplified in 29 (58%) of the isolates. All of the isolates showed sensitivity to mupirocin in the agar diffusion test, which was corroborated by the lack of any amplicon of the 456 bp fragment corresponding to the *ile*S-2 gene, in the PCR bands. The conventional tests to identify *S. aureus* and detect resistance to oxacillin and mupirocin showed 100% agreement with the PCR Multiplex results. The use of techniques for rapid and accurate identification of bacteria and assessment of their resistance may be valuable in the control of infection by resistant strains, allowing the rapid isolation and treatment of an infected patient. However, the results demonstrate that traditional phenotypic tests are also reliable, though they take more time.

Keywords: PCR Multiplex. Oxacillin-resistant. Mupirocin. *Staphylococcus aureus*.

Autor correspondente:Prof. Dr. Alexandre Braoios- Universidade Federalde Goiás - UGF - Rod. BR 364 - km 192 – Setor Parque Industrial nº. 3800 Caixa Postal 03 - Jataí.GO - CEP.75800-000 - telefone/fax: (64) 3632 1510 e-mail:ab31@uol.com.br

INTRODUCTION

Staphylococcus aureus is one of the commonest human pathogens, especially in hospital patients. This Gram-positive bacterium is responsible for various infectious diseases, and has a great capacity to develop resistance to antimicrobial agents. Oxacillin-resistant strains represent an important health problem for infected individuals, since the therapeutic options are restricted to glycopeptides, such as vancomycin. Even vancomycin appears to be losing its effectiveness and, wh*ile* a number of new agents with broad Gram-positive activity have been licensed, none has emerged as clearly superior (Kaul et al., 2008).

The detection of *mec*A, a gene encoding a PBP (Penicillin Binding Protein) with low affinity with β-lactam antibiotics (PBP2a), by the Polymerase Chain Reaction (PCR), is considered a gold-standard technique for oxacillin resistance detection (Anand et al., 2009). According to Martineau et al. (2000), phenotypic tests for oxacillin resistance may provide unsatisfactory results, since the microorganism may carry a gene for a resistance factor, though expression of this gene may be influenced by environmental conditions and culture factors.

Some *S. aureus* strains exhibit a low level of resistance due to hyperprodution of β-lactamase, which in great quantity can also hydrolyze the penicillinase-resistant penicillins, such as methicillin and oxacillin. Such strains are named *Borderline Oxacillin-Resistant Staphylococcus aureus* (BORSA). This type of resistance is called *mec*-independent, since these strains do not have the *mec*A gene (Petinaki et al., 2001).

Mupirocin (pseudomonic acid A) is a topical antibiotic with good activity against *S. aureus*, which has been successfully used to prevent post-surgical heart infections, both in hemodialysis and burn patients (Gilbart et al., 1993; Lowy, 2003). Besides its therapeutic use, mupirocin has been used to eradicate nasal *S. aureus*. However, indiscriminate use of mupirocin can select resistant strains, and its use for nasal eradication is controversial (Ramsey et al., 1996; Lowy, 2003). According to Gilbart

et al. (1993), *S. aureus* strains resistant to mupirocin can be divided into two groups. Strains with low resistance (MIC ≥ 8 to ≤ 256 µg/mL) produce an isoleucyl-tRNA synthetase enzyme with low affinity for mupirocin, wh*ile* those with high resistance (MIC ≥ 512 µg/mL) have an additional gene (*ile*S-2) located on a conjugative plasmid. Some authors suggest that low-level and high-level resistance differ only in the location and/or number of copies of gene *ile*S-2 (Ramsey et al., 1996).

The *fem*A gene encodes a protein (*Fem*), essential for the expression of resistance to methicillin. In spite of this involvement in resistance, the gene *fem*A is also present in methicillin-sensitive strains. A homologous *fem*A gene has also been described in other *Staphylococcus* species. However, there are inter-species variants of this gene that may be used as species-specific primers (Vannuffel et al., 1999).

Variants of the PCR technique have been proposed, in order to amplify and detect more than one gene in the same reaction. Using this approach, named PCR Multiplex, it is possible to identify and assess microorganism resistance and virulence in the same reaction (Pereira et al., 2008; Markoulatos et al., 2002).

In the present study, the PCR Multiplex technique was employed to detect the genes *fem*A (species-specific), *mec*A (methicillin/oxacillin resistant) and *ile*S-2 (mupirocin resistant), with the aim of developing a more efficient validated technique for the diagnosis of *Staphylococcus aureus* infections.

MATERIAL AND METHODS

Samples: Fifty *S. aureus* isolates from patients attended at the University Hospital "Dr. Domingos Leonardo Cerávolo" (UNOESTE) in Presidente Prudente, SP, Brazil, from July to December 2002, were analyzed. The Multiplex PCR technique was validated against the microorganisms ATCC 29213 (*S. aureus* sensitive to mupirocin and methicillin), ATCC 43300 (*S. aureus* resistant to methicillin and sensitive to mupirocin), and the transconjugated strain RN8411, containing the plasmid pMG1, named strain JJ1 (*S. aureus* oxacillin sensitive and mupirocin resistant).

Bacterial identification: Conventional identification was performed by microscopic morphology, slide catalase test, tube coagulase test, heat-stable nuclease and mannitol fermentation test (Winn et al., 2006).

Oxacillin and mupirocin resistance testing: This was carried out by the diffusion method in Mueller Hinton (MH) agar (Oxoid), with antibiotic discs (oxacillin 1µg/mL and mupirocin 5 µg/mL – Oxoid). Additionally, the isolates were spread on MH agar, supplemented with 6µg/mL oxacillin (Sigma) and 4% NaCl (*screening agar*), for oxacillin resistance confirmation (NCCLS, 2000).

Minimal inhibitory concentration determination (MIC) for oxacillin: The oxacillin MIC for each isolate was determined by the broth macrodilution method, using MH broth (Oxoid), supplemented with 2% NaCl and oxacillin, in serial dilutions from 0.12 µg/mL to 256 µg/mL (NCCLS, 2000).

Bacterial DNA extraction and Purification: For DNA extraction, the microorganism was grown in trypticase soy broth (TSB - Difco) at 35°C for 18 hours; then 1 mL bacterial suspension was centrifuged at 14,000 rpm for 1 min. The supernatant was drawn off and pretreated with 10 mg/mL lysozyme (Calbiochem), after which the extraction was carried out with a GFX Genomic Blood (Amersham Biosciences) kit. The DNA was kept at – 20°C until the moment of use. The bacterial DNA extraction procedure followed the manufacturer's instructions and has been used by other researchers (Fabiano et al., 2008).

Amplification Reaction: The PCR mix consisted of buffer (50 mM KCl, 20 mM Tris-HCl [pH 8.4]), each of the four deoxyribonucleotide triphosphates (dNTPs) at 0.2 mM, 3 mM $MgCl_2$, 25 pmol of primers (Invitrogen) MecA1 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A-3') and MecA2 (5'-CCA ATT CCA CAT TGT TTC GGT CTA A-3') for amplification of a 310 bp fragment of the gene *mec*A (resistance to oxacillin); MupA (5'-TAT ATT ATG CGA TGG AAG GTT GG-3') and MupB (5'-AAT AAA ATC AGC TGG AAA GTG TTG-3') for amplification of a 456 bp fragment of the gene *ile*S-2 (resistance to mupirocin); 50 pmol of primers F1 (5'-CTT ACT TAC TGG CTG TAC CTG-3') and F2 (5'-ATG TCG CTT GTT ATG TGC-3') for amplification of a 686 bp fragment of the gene *fem*A (species-specific) and, finally, 1.25 U of *Taq* polymerase (Gibco). The final reaction volume was 25 µL. The amplification reaction involved an initial denaturation phase at 94°C for 5 min, followed by 10 amplification cycles (denaturation at 94°C for 30 s, annealing at 64°C for 30 s and elongation at 72°C for 45 s), then 25 further amplification cycles (94°C for 45 s, 50°C for 45 s and 72°C for 1 min) and a final elongation phase at 72°C for 10 min. After the reaction, 5 µL of the final product was resolved into amplified fragments by electrophoresis in 2% agarose gel at 100 V (45 mA) for 1 hour. To estimate the molecular weights of fragments, a 100-bp molecular weight ladder was run on each gel. The gel was stained with ethidium bromide, and the amplicons observed under UV radiation (Pérez-Roth et al., 2002; Vannuffel et al., 1999).

RESULTS

In this study, 50 *S. aureus* strains were isolated from catheter (34%), blood (28%), surgical incision (14%), lung (7%) and other clinical specimens (17%). They were identified as *S. aureus* by conventional phenotypic tests. There was 100% agreement between the conventional identification results and the amplification of the 686 bp fragment of the species-specific gene *fem*A (Fig. 1).

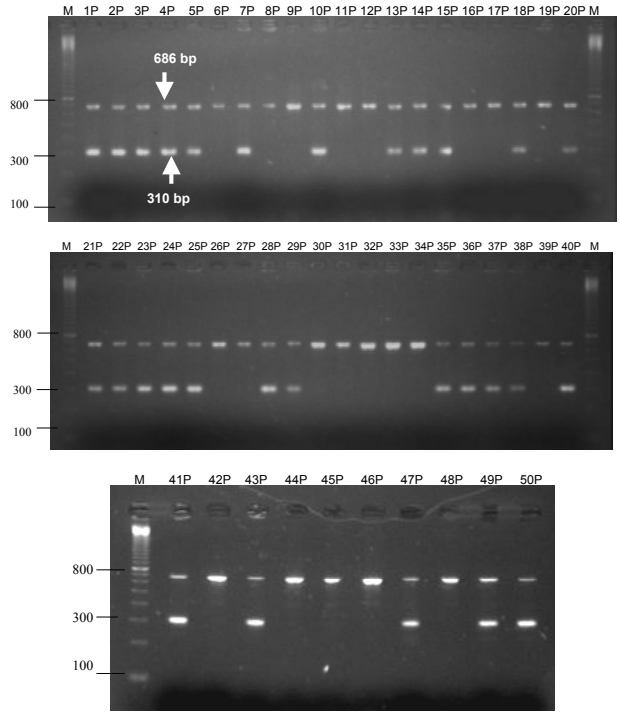
The agar diffusion test for oxacillin and mupirocin resistance was carried out and, besides that, all of the isolates were seeded on screening agar to confirm oxacillin resistance. From the total of 50 *S. aureus* strains isolated from patients with infectious disease, 29 (58%) showed resistance to oxacillin by the agar diffusion method, and also grew on oxacillin-resistance screening agar.

All of the oxacillin-resistant isolates detected by the agar diffusion method grew on agar with oxacillin (*screening agar*). Furthermore, a 310 bp fragment of the gene *mec*A, which codes for resistance to oxacillin, was amplified from

all of the 29 resistant isolates (Fig 1). Conversely, none of the 20 isolates (40%) that showed sensitivity to oxacillin in the phenotypic tests exhibited any band for the gene *mec*A in the Multiplex PCR test. Thus, both oxacillin-resistant and susceptible strains showed 100% agreement between phenotypic and PCR results.

Only one isolate (2%) was considered BORSA (*Borderline Oxacillin Resistant Staphylococcus aureus*) for having shown an inhibition halo for oxacillin of 11 mm and MIC of 2µg/mL, and this strain also produced no PCR band for the gene *mec*A. All 50 isolates were mupirocin-sensitive in the agar diffusion test and this was corroborated by the lack of amplification of the 456 bp fragment of the gene *ile*S-2, which codes for resistance to mupirocin.

The minimal inhibitory concentration for oxacillin varied from 0.12 to 1 µg/mL for oxacillin-sensitive isolates (MSSA) and 32 to 128 µg/mL for resistant isolates (MRSA). The data concerning oxacillin MICs can be seen in Table 1.



M: 100 bp molecular weight ladder

Figure 1: Multiplex PCR for *fem*A (686 bp), *mec*A (310 bp) and *ile*S-2 (456 bp) genes of *S. aureus* strains isolated from patients.

Table 1: Minimal Inhibitory Concentration (MIC) of oxacillin for 50 *S. aureus* isolates from patients at UNOESTE University Hospital (Presidente Prudente, SP), in July-December 2002.

	MIC of oxacillin (µg/mL								
S. aureus	0.12	0.25	0.5	1	2	32	64	128	Total
MSSA	5	2	8	5	-	-	-	-	20 (40%)
BORSA	-	-	-	-	1	-	-	-	1 (2%)
MRSA	-	-	-	-	-	2	20	7	29 (58%)

DISCUSSION

Teixeira et al. (1995), in a study carried out in various Brazilian hospitals, reported that on average, 56% of the *Staphylococcus aureus* isolates were resistant to oxacillin. Thus, the oxacillin-resistant *S. aureus* frequency at the UNOESTE University Hospital (58%) is similar to that found in other hospitals in the country.

In this study, only one BORSA strain was isolated. The differentiation of MRSA from BORSA strains is crucial for therapeutic advice. MRSA strains produce PBPs with low affinity to beta-lactam antibiotics, and this production is induced by these antimicrobials, except for methicillin and oxacillin (Lowy, 2003). The choice of therapy for the treatment of infections caused by MRSA strains is restricted to the glycopeptides in general (Kaul et al., 2008). On the other hand, BORSA strains are beta-lactamase hyperproducers and do not produce a modified PBP (PBP2a); that is, they have *mec*-independent resistance (Nicola et al., 2000). Hence, the combination of a β-lactam and a β-lactamase inhibitor could be useful in treating infections caused by these strains (Fluit et al., 2001), implying the less frequent use of glycopeptides for the treatment.

All isolates were susceptible to mupirocin, demonstrating that this antimicrobial can be used as an alternative treatment. However, it is exclusively for topical treatment and thus of limited usefulness (Krishman et al., 2002).

In this study there was perfect correlation between the phenotypic (conventional) and genotypic (Multiplex PCR) results for identification of *S. aureus* and detection of oxacillin and mupirocin resistance. Pérez-Roth et al. (2002) also reported complete consistency between the results of phenotypic tests and Multiplex PCR used to identify *S. aureus* and assess its resistance to oxacillin and mupirocin. PCR does not differentiate the BORSA strains, since they do not have the *mec*A gene. Distinguishing BORSA from MRSA strains is crucial for therapeutic orientation, to minimize the use of vancomycin.

Wh*ile* the Multiplex PCR technique was being standardized, the amplified fragments showed great differences in band intensity. This problem was solved by using different concentrations of each primer pair. Bej et al. (1990) reported that when various fragments of different sizes are to be amplified in the same PCR reaction, greater concentrations of the primers used to amplify the bigger fragments should be added, since the smaller ones will be amplified more readily and in greater quantity, resulting in significant differences in the intensity of the generated fragments.

Multiplex PCR can be of great use for the diagnosis of multi-resistant bacterial infections, since it detects the resistance genes, whilst phenotypic techniques detect the results of gene expression. The expression of a gene can be influenced by both environmental factors and bacterial culture conditions. Thus, phenotypic techniques may give doubtful results and be hard to interpret.

The use of molecular techniques has revolutionized several areas of science and brought greater security and reliability in microbial identification and the detection of antimicrobial resistance. However, these techniques are expensive for most small laboratories. Our results show that traditional techniques can give comparable results to the more advanced techniques, enabling their safe use and reliability. Another phenotypic technique that can be used to detect oxacillin resistance is the agar diffusion test

with disks of cefoxitin, reported recently as an excellent technique for this purpose (Anand et al., 2009).

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RESUMO

Utilização de PCR-multiplex para identificação de Staphylococcus aureus e avaliação da resistência à oxacilina e mupirocina

***Staphylococcus aureus* resistente à oxacilina representa um problema grave em hospitais de todo o mundo, aumentando a morbidade e mortalidade de pacientes infectados e, elevando os custos do tratamento e tempo de internação. Neste trabalho, foram comparados os resultados da técnica de PCR Multiplex para a amplificação dos fragmentos dos genes *fem*A (espécie-específico), *mec*A (resistência à oxacilina) e *ile*S-2 (resistência à mupirocina) com os resultados da identificação e testes convencionais para avaliação da resistência. Cinqüenta *S. aureus* foram isolados de pacientes atendidos no Hospital Universitário "Dr. Domingos Leonardo Cerávolo" da Unoeste, em Presidente Prudente, SP, Brasil. Houve amplificação do fragmento 686 pb, correspondente ao gene *fem*A para todos os isolados. Por outro lado, houve amplificação do fragmento 310 pb correspondente ao gene *mec*A em 29 (58%) dos isolados. Todos os isolados mostraram sensibilidade à mupirocina observados no teste da difusão em ágar, e também pela ausência de amplificação do fragmento 456 pb, correspondente ao gene *ile*S-2. Os resultados dos testes convencionais para identificação de *S. aureus* e avaliação de resistência à oxacilina e mupirocina mostrou 100% de concordância com os resultados da PCR Multiplex. A utilização de técnicas mais rápidas e precisas para identificação e avaliação de resistência pode ser valiosa para o controle de infecção por cepas resistentes, permitindo rápido isolamento e tratamento do paciente. Entretanto os resultados demonstram que testes fenotípicos tradicionais também são confiáveis, apesar do maior tempo de execução.**

Palavras-chave: PCR Multiplex. Resistência à oxacilina. *Staphylococcus aureus*. Mupirocina.

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