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Detection of antimicrobial resistance by means of phenotypic and genotypic tests in Staphylococcus aureus recovered from central vascular catheters

Detecção de resistência a antimicrobianos através de testes fenotípicos e genotípicos em Staphylococcus aureus

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RESUMO
Staphylococcus aureus resistentes a múltiplos antibióticos representam um grande problema no controle de infecção hospitalar. O perfil de resistência aos antimicrobianos de S. aureus, presente em cateteres vasculares centrais de pacientes internados em uma Unidade de Terapia Intensiva de um Hospital Universitário do Triângulo Mineiro, foi avaliada por testes antimicrobianos, através do qual foi possível detectar uma elevada resistência a penicilina (94,7%) e à ampicilina (86,8%), além de uma cepa resistente à vancomicina. A avaliação da resistência a oxacilina foi confirmada através de PCR pela presença do gene mecA. A associação dos resultados obtidos no ensaio fenotípico com a presença do gene mecA, foi confirmada através da tabela de contingência e o do teste ×2 com correcção de Yates. Em 49 amostras avaliadas, 23 apresentaram resistência a oxacilina, e foi possível detectar a presença do gene de resistência mecA em 21 amostras. O teste por RAPD permitiu a separação dos grupos fenotípicas em dois padrões de grupos diferentes, as que apresentam resistência e as sensíveis à substâncias antimicrobianas, com uma divergência de 73,3%. Os marcadores moleculares para a detecção de resistência a oxacilina, como o gene mecA, foram encontrados por ser mais sensível do que os marcadores fenotípicos.
Palavras-chave: Staphylococcus aureus. Farmacorresistência Bacteriana.

ABSTRACT
Staphylococcus aureus resistant to multiple antibiotics represent an important problem in nosocomial infection control. The profile of antimicrobials resistance of *S. aureus* isolated from central vascular catheters from Intensive Care Unit (ICU) patients (Triangulo Mineiro University College Hospital). The *S. aureus* were evaluated by antimicrobial tests, detection of the *mec*A gene and RAPD (Random Amplified Polymorphic DNA), of the resistance to penicillin (94,7%) and ampicillin (86,8%) was high. One isolate presented resistance to vancomyci. The association of results obtained by phenotypic test with the presence of the *mecA* gene was evaluated. Out of 49 *S. aureus* evaluates, 23 (47%) presented resistance to oxacilin, and it is possible to detect the presence of the resistance *mec*A gene in 21 (43%). The by RAPD patterns allowed established into two different phenotypic groups, the ones that presented resistance and the ones susceptible to antimicrobial substances, with a dissimilarity of 73,3%. Molecular markers for detection of resistance to oxacilin, as the *mecA* gene, were found to be more sensitive than the phenotypic markers.
Key words: Staphylococcus aureus. Drug Resistance, Bacterial.

INTRODUCTION

Staphylococcus aureus is considered one of the main pathogens that cause nosocomial infections. The clinical manifestations range from traumatic infections to septicemia. With the introduction of methicillin in the therapeutic of staphylococcal infections, in the beginning of the 1960's, a constant increase of isolated Methicillin-Resistant *Staphylococcus aureus* – (MRSA). These infections by MRSA represent an important problem for the health institutions, large or small, since the predisposing factors for these infections, such as previous antimicrobial treatment, long stay in hospital, mechanical ventilation, ICU attendance, intravascular catheterization and general surgical procedures are common in the medical activity [1]. Approximately 20% to 40% of patients with central vascular catheter develop local infection, and 3% to 10% develop bacteraemia [2]. It is estimated that 30% of all endemic nosocomial bacteraemias and most candidiasis are related to the infusion therapy through vascular

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catheters. Since it is known that the length of vascular catheterization is, probably, the greatest determining factor of that kind of infection and its incidence, it can be certainly diminished with the employment of a careful methodology [3].

The intrinsic resistance of the staphylococcus to meticilin/oxacilin results from PBPs (Protein Binding Penicillins) present in the cellular wall, which express themselves starting from an acquired chromosomal gene, *mecA*, which codifies the PBP2' or 2a, whose affinity with the beta-lactamic antibiotics is very low. The resistance of staphylococcus to beta-lactamic antibiotics can be due to some environmental factors such as pH, temperature and osmolarity [4].

There is considerable genetic heterogeneity in natural populations of *S. aureus* [5, 6] which can be explored to investigate the dissemination of strains of *S. aureus* of human and animal origin. The evaluation of these heterogeneous traits can be achieved by using the molecular marker RAPD (*Random Amplified Polymorphic DNA*), utilized to differentiate the isolated ones at intra-specific level [7].

Considering the principle that nosocomial infections by *S. aureus* can present phenotypic and genotypic diversity, we can expect the existence of a correlation of these differences with the infection control. We aimed to verify the phenotypic and genotypic variability of *Staphylococcus aureus* isolated from central vascular catheters (CVC) from Intensive Care Unit patients of the Triangulo Mineiro Federal University (UFTM) College Hospital.

PATIENTS, MATERIALS AND METHODS

Forty-nine strains of *Staphylococcus aureus*, previously isolated and identified by the pathology Service of the UFTM, from samples obtained in the CVC of ICU patients in the period from January 2005 to February 2006, were analyzed. This study was approved by Ethical Research Committee from Triangulo Mineiro Federal University.

The collected CVC were immersed in *Brain Heart Infusion* (BHI) for 30 minutes, and cultivate in blood agar and mannitol agar and incubated at 37º for 24 hours.

Colonies with characteristics suggestive of staphylococcus were re-isolated in BHI agar and, after 24 hours at 37°C, submitted to Gram stain, catalase and coagulase tests.

The antimicrobials tests were done by disc diffusion method. The *S. aureus* were cultivated in Brain Heart Infusion (BHI) culture medium at 37º until they reached the 10^8 UFC/ml concentrations, utilizing as reference to the turbidity scale 0.5 of the McFarland scale. The suspension was inoculated in Mueller-Hinton Agar (MHA), and the antimicrobials discs (ampicillin, penicillin, oxacillin, vancomycin and ampicillin/sulbactam) were added, and the plates kept at 37ºC for 24 hours. The results interpretation were classified as susceptible or resistant, according to NCCLS [8].

The PCR technique was employed for the confirmation of resistance to oxacillin by the of *mecA* gene presence. The genomic DNA of the resistant isolates was obtained according to Ausubel et al. [9] with some modifications. Colonies cultivated in Muller-Hinton agar for 24 h at 37ºC were suspended in 100 ul of buffer (10mM Tris-HCl and 1mM EDTA, pH8.0) in order to establish a final concentration of $3x10^8$ UFC/ml using 0.5 of the McFarland scale. For the cellular lyse 50 ul of lysozyme (20mg/ml) were added and incubated for 45 minutes at 37°C, followed by the addition of 20 ul of SDS (Sodium Dodecyl Sulphate) 20 % and 5 ul of proteinase K (20mg/ml). After incubation at 37°C for one hour, 200 ul of NaCl 5M were added and shaken manually for 15 seconds. The suspension was centrifuged at 10.000g for 15 minutes at 4ºC and the supernate transferred to another micro-tube. One hundred microliters of phenol/chloroform (1:1) followed by 100ul of chloroform:isoamilic alcohol (24:1) were added to release and separation of proteins, followed by centrifugation at 10.000g for15 minutes. The supernatant was transferred to another micro-tube.

The DNA was precipitated with 800 ul absolute ethylic-alcohol, followed by centrifugation at 10.000g for 15 minutes at 4°C. The pellet was incubated at 42º for 40 minutes with 20 ul of RNAse (10mg/ml) washed twice with ethylic alcohol 70%, and dried at room temperature. The pellet was re-suspended in 30 ul of milliQ water. The extracted nucleic acid was maintained at–20°C until the moment of the analysis by PCR and by RAPD.

The amplification of the *mecA* gene was achieved according to Murakami et al. [10], 10pmol of the *primers* 5' were used AAAATCGATGGTAAAGGTTGGC 3' and 5' AGTTCTGCAGTACCGGATTTGCC 3' (Invitrogen), 50ng of the extracted DNA, 1U of Taq polymerase, 10mM of dNTP, 2.5mM $MgCl_2$, Buffer10X of the Taq, completing the volume to 20 ul with extra pure water. The PCR reaction was processed in 40 cycles constituted of denaturation cycles at 94°C for 30s; annealing at 55°C for 30s; extension at 72°C for 1 minute; a final extension cycle at 72°C for 5 minutes and 4°C for indefinite time. A volume of 10 ul of the PCR product was applied in agarose gel at 1% with Ethide Bromide for the fragment visualization under UV, and the gel was photographed on VDS Pharmacia. The 100pb (Ludwig) DNA Jadder was used for comparison. As negative control *S. aureus* ATCC 25923 was utilized.

The RAPD method for this study was done by PCR utilizing random short primers of the Operon Technologic (OPA 01, OPA 04, OPA 07, OPA 08, OPA 09, OPA 10, OPA11 and OPA 20). For each reaction 5ng of extracted DNA, 1U of Taq polymerase, 10mM of dNTP, 2.5mM $MgCl_2$, buffer 10X of Taq were utilized and the volume completed with ultra pure water to 20 ul. The

reaction was processed in MJ Research PTC – 100 thermocyclator, programmed with the following amplification conditions: 3 initial cycles of 94ºC (1 minute) denaturation, 35ºC (1 minute) anealling of the *primer*, 72ºC (2 minutes) extension by Taq polymerase followed by 34 cycles of 94ºC (1 minute), 35ºC (1 minute), 72ºC (2 minutes); 1 cycle of 72ºC (2 minutes) for final extension and 4ºC for unlimited time. The amplified products were submitted to electrophoresis in agarose gel 1.5% stained with of Ethide Bromide.

For the analysis of the data, the Systat program, version 10.1 (2004) was used for the analysis of the polymorphic edges and cluster formation as well as the Contingency Table and the x^2 test with Yates correction for the association of the obtained data [11].

RESULTS

Among the 49 *S. aureus* submitted to the susceptibility test, for the 5 antimicrobials, 11 (22.5%) revealed to be sensitive to all the of them. The 38 remaining strains were gathered in 10 distinct resistance groups, and the group 1 (OXA / PEN / AMP / APS) was predominant. One *S. aureus* was resistant to all the antimicrobials, including vancomycin (Table 1).

The resistance distribution of the samples showed an elevated index of resistance to penicillin (94.7%) and ampicillin (86.8%) (Table 2).

Among 49 strains included in the study, 33 were *mecA*-positive (67.3%) and 16 *mecA*-negative (32.7%). Considering just the isolates that have resistance to some antimicrobial, 86.8% presented the *mecA* gene (Table 3).

The data were disposed in a Contingency Table according to Callegar-Jacques [11], with the variable values that stand for presence of phenotypic resistance to the antimicrobial oxacilin and presence of the *mecA* gene. The data indicate that the frequency of strains of *S. aureus* resistant to oxacilin is quite high (46.9% or 23 in 49). It is also possible to verify that the presence of the *mecA* gene is related to the presence of resistance to oxacilin (91.3% or 21 in 23). In order to validate these results, the statistical test x^2 was applied with Yates correction [11]. The $x^2_{Yates} = 9.35$ exceeds the critical value of $gl = 1$ and $á = 0.01$ (6.63) thus rejecting the independence between the presence of the *mecA* gene and the resistance to oxacilin.

The presence of the *mecA* gene was detected in 12 strains (46.1%) of all the samples that had been

Table 1 - Antimicrobial Resistant Groups in the S*. Aureus* strains isolated from the CVC of ICU patients of the UFTM College Hospital, Uberaba/MG, 2007.

Groups	Phenotypic of resistance to antimicrobials*	Strains isolated with the resistance characteristic	
		№	%
1	OXA / PEN / AMP / APS	13	26,6
2	OXA / PEN / AMP	05	10,2
3	OXA / PEN	02	4,1
4	OXA / APS	01	2,0
5	PEN / AMP / APS	02	4,1
6	PEN / AMP	11	22,5
7	PEN / APS	01	2,0
8	OXA / PEN / AMP / APS / VAN	01	2,0
9	OXA / AMP	01	2,0
10	PEN	01	2,0
11	**	11	22,5
Total of samples		**49**	**100,0**

* Oxacilin (OXA), Penicillin (PEN), Ampicillin (AMP), Ampicillin/Sulbactam (APS), Vancomycin (VAN); ** Susceptible to all the tested antimicrobials.

TABLE 2 - Percentage and Profile of Resistance of the *S. Aureus* Strains.

Antimicrobials	% of resistance *	*mecA*-positive gene	*mecA*-negative gene
Oxacilin	60,5	91,3%	8,7%
Penicillin	94,7	66,7%	33,3%
Ampicillin	86,8	66,7%	33,3%
Vancomycin	2,6	100%	0%
Ampicillin/Sulbactam	47,4	77,7%	22,3%

* Only the samples that showed resistance were considered.

Table 3 - Rresistance Contigency of Oxacilin of the Meca Gene in Strains of the *S. aureus*.

Staphylococcus aureus (strains)	Presence of the *mecA* gene		Total
	Yes	No	
Resistant to oxacilin	21	2	23
Susceptible to oxacilin	12	14	26
Total	33	16	**49**

classified as negative for phenotypic resistance to oxacilin.

The RAPD, by means of 8 short *primers* among the 11 genotypes (11 groups) defined by the same phenotypic characteristic, found in the antibiogram, had as result 76 amplified edges, among which 18% were polymorphic. That percentage is normal, considering the differences between populations.

With the results obtained from all the random amplifications, a dendogram of disagreement percentage was built, which permitted the grouping of the strains in two different grouping patterns, the ones that had resistance and the ones susceptible to antimicrobials, with dissimilarity of 73.3%. The limiting genetic distance was found for the groups 8 and 11, resistant to all the tested antimicrobials, including the vancomycin and susceptible to all the antimicrobial, respectively.

Within the pattern of resistance, there were three grouping patterns that presented a high degree of genetic relation.

DISCUSSION

The results confirm the prevalence of *S. aureus* multiresistant strains in the UFTM College Hospital and demonstrate the wide spectrum of resistance in face of the antimicrobials usually employed in the clinical practice [12].

The elevated index of resistance to penicillin and to ampicillin corroborate the works developed by Booth et al. [13], Tahnkiwale et al. [14] and Kaszanyitzky et al. [15]. The resistance index to vancomycin was of (2.6%), similar results were found by Linden [16] in the USA, Andrade et al. [17] and Bernardes, et al. [18] in Brazil. The routine and prolonged treatment with that antimicrobial can be one of the factors that contribute to the selection of resistance [19]. The decrease of sensitivity to vancomycin is associated with an activation of the cellular wall synthesis, when there is a hyper-production of penicillin-binding proteins, PBP2 and PBP2a, resulting in the thickening of the bacterial wall of *S. aureus*, thus making difficult the incoming of the glycopeptides to the bacterial cell internal part. The other mechanism of resistance to vancomycin on the part of the *S. aureus* presently described is the one of the acquisition of a gene of resistance classically described in *Enterococcus spp*, the *vanA* gene [20].

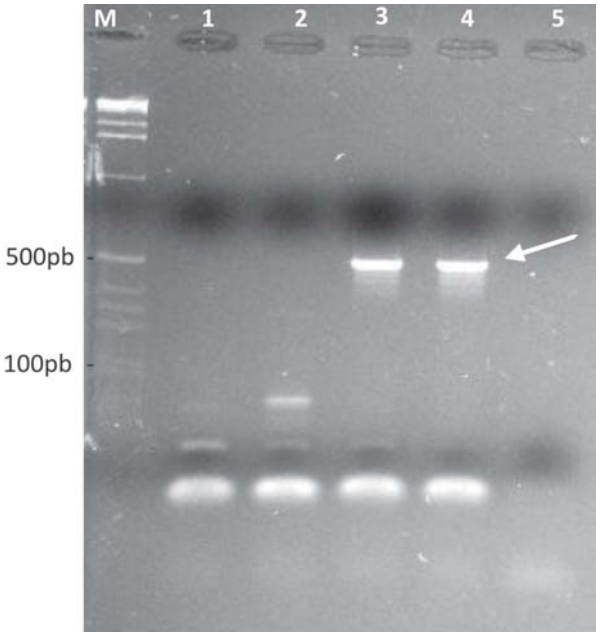

Figure 1 – Fragments of 513 pb of the *mecA* gene (seta), of *Staphylococcus aureus* strains. Agarose gel 1.5% colorized with Ethide Bromide. M: marker of molecular weight (100 pb); 1 and 2: *mecA*-negative strains; 3 and 4: *mecA*-positive strains; 5: negative control.

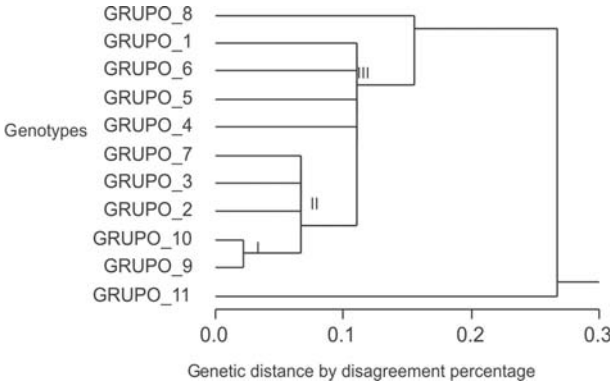

Figure 2 – Dendogram representative of the genetic distance by disagreement percentage and groupings by the UPGMA method among the 11 genotypes utilizing 8 *primers*. Resistance separates the groups: Group 8 (OXA/PEN/AMP/APS/VAN); Group 1 (OXA/PEN/AMP/APS); Group 6 (PEN/AMP); Group 5 (PEN/AMP/APS); Group 4(OXA/APS); Group 7 (PEN/APS); Group 3 (OXA/PEN); Group 2 (OXA/PEN/AMP); Group 10 (PEN); Group 9 (OXA/AMP) e o Group 11 – susceptible (OXA/PEN/AMP/APS/VAN). I, II and III groups of most genetic similarity

The incorrect use of antibiotics associated to the natural selection of the microorganisms probably resulted in the multi-resistance phenomenon. According to Meng et al. [21] the resistance to drugs is related mainly with the excessive use of antibiotics and with

the sub-therapeutical application of antimicrobials for disease prevention [22, 23].

The genotypic detection for PCR presented succeptibility and specificity of 91.3%. Similar results were found by Grisold et al. [24]. The strains classified as resistant to oxacilin and negative for the *mecA* gene (8.7%), could have been selected for resistance through another mechanism, as the beta-lactamase hyper-production or the alteration in another PBP apart from PBP2 [25, 26].

The presence of the false-negative for the *mecA* gene was detected in 46.1%, superior value to the found one for other authors, as Marshall [27] and Pfaller [28], who showed values of 12% and 18%, respectively. The high index found could be explained, because a phenotype of resistance to oxacilin is very variable and depends on the expression of the *mecA* gene. That variability is known as heteroresistance phenotype, where in all the heterogenically resistant bacterial population, all the cells carry the *mecA* gene, which is a genotypic marker of the resistance; however, not all strains express the resistance phenotypically in a similar way [29]. The results prove the wide distribution of the resistance gene in the beds of the UFTM College Hospital ICU, and that is a tendency in nosocomial environment [24].

Each *S. aureus* resistant to oxacilin (MRSA) has a characteristic profile of the proportion of cells that grow in the presence of specific concentrations of oxacilin and of different environmental conditions [29, 30]. Genes homologous to the blaZ gene regulators regulate the expression of resistance to oxacilin in S. aureus. That gene codifies the â-lactamases production. The *mecI* and *mecR1* genes regulate the response of the *mecA* to the beta-lactamic in a way similar to the regulation of the *blaZ* by the *blaR1* and *blaI* genes, in face of the exposure to penicillin [30, 31]. Several methods have been utilized for the detection of the resistance to oxalicin in *Staphylococcus aureus*, but according to Chambers [31], the molecular method through PCR is the only one considered as *gold standard* for detection of the resistance [32].

The grouping I was the one with most genetic similarity among the studied genotypes, having a genetic distance of only 0.022 (2.2%). In this grouping, two phenotypic groups of resistance were gathered. Group 10 resistant to penicillin and group 9 resistant to oxacilin and ampicillin, demonstrating the similarity of groups that did not possess multiresistance. Besides, the resistance mechanism of those groups (â-lactamic) is analog, that is to say, both inhibit the "PBP"s ("P*rotein Binding Penicillins*") that actuate in the formation of the cellular wall and, at inhibiting the "PBP"s, they impede the formation of the peptidoglycan layer on the cellular wall, initiating the bacterial death [33]. On the other hand, in the grouping II, where all the groups present resistance to penicillin, genetic similarity of 93.3 % was obtained. The grouping III, with genetic similarity of 88.9 %, presents groups with resistance to at least two antimicrobials, one of them is ampicillin. Another common characteristic between the groups 1, 4, 5 and 8 is the resistance to the antimicrobial sulbactam, a beta-lactams inhibitor [34]. The data show a small genetic disagreement distance between the analyzed samples, characterized for being samples of the same species and having all been isolated from the same kind of infected material. There is greater genetic similarity between groups that present the same kind of resistance, thus confirming the phenotypical analyses.

The molecular characterization of *S. aureus* genotypes has become a very important tool for the understanding of the mechanisms of resistance of that species and, consequently, to provide subsidies for the adequate and efficient use of the antimicrobials [35].

In conclusion, the present results revealed that, the *S. aureus* with mecA gene presented more resistant to oxacilin (21/33 or 63.6%) than susceptible to it (12/33 or 36.4%). The value-*P* associated to this conclusion is $0.001 < P < 0.01$; molecular markers for the detection of resistance to oxacilin, as PCR for the *mecA* gene, are more sensitive than the phenotypic markers and the RAPD-PCR, for being a quick technique of easy execution, can be used for the typing of great part of the bacteria of interest in the medical practice. The appearance of VRSA corroborates the need for strategies in order to avoid/prevent the propagation of microorganisms resistant to antibiotics and control the use of antimicrobial drugs in health assistance environments.

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