

ANTIBIOTIC SUSCEPTIBILITY OF *STAPHYLOCOCCUS AUREUS* ISOLATES IN ORAL MUCOSA AND POCKETS OF PATIENTS WITH GINGIVITIS-PERIODONTITIS

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ABSTRACT

Both oral cavity and subgingival pocket are ecological niches conducive to hosting microorganisms that may act as opportunistic pathogens, such as *Staphylococcus aureus* and especially methicillin-resistant *Staphylococcus aureus* (MRSA). Early detection of MRSA is a matter of concern to Public Health. The aim of our study was to determine phenotypic and genotypic detection of methicillin resistance of *S. aureus* in oral mucosa and subgingival pocket in 102 patients with gingivitis-periodontitis. The prevalence of *S. aureus* was 10.8% ($n = 11$) in subgingival

pocket and 19.6% ($n = 20$) in oral mucosa. We obtained 31 isolates of *S. aureus* of which 13 were *mecA* positive and 18 were *mecA* negative. Detection of *mecA* gene by PCR was used as the reference method to compare the results of phenotypic methods to determine methicillin resistance. Early, accurate detection of *S. aureus* through phenotyping and genotyping methods is crucial for assessing the colonization and preventing the spread of MRSA.

Key words: *Staphylococcus aureus*, antibiotics, periodontal diseases.

SUSCEPTIBILIDAD A ANTIBIÓTICOS DE CEPAS DE *STAPHYLOCOCCUS AUREUS* AISLADAS DE MUCOSA BUCAL Y BOLSAS DE PACIENTES CON ENFERMEDAD GINGIVOPERIODONTAL

RESUMEN

La cavidad bucal y el interior de la bolsa subgingival constituyen nichos ecológicos propicios para albergar microorganismos que podrían actuar como patógenos oportunistas, como el *Staphylococcus aureus* y en particular *S. aureus* resistente a la metilina (SARM). La detección temprana de portadores reviste importancia para salud pública. El objetivo de nuestro trabajo fue determinar por métodos fenotípicos y genotípicos la metilino resistencia de cepas de *S. aureus* aisladas de mucosa bucal y bolsa subgingival de 102 pacientes con enfermedad gingivoperiodontal. Se observó una prevalencia de *S. aureus* en bolsa subgingival del

10,8% ($n = 11$) y en mucosa bucal del 19,6% ($n = 20$). Se obtuvieron 31 aislamientos de *S. aureus* de los cuales 13 fueron *mecA* positivos y 18 eran *mecA* negativos. La detección del gen *mecA* por PCR se utilizó como método de referencia para comparar los resultados de métodos fenotípicos para determinar la resistencia a metilina. La detección rápida y exacta de *S. aureus* por métodos microbiológicos fenotípicos y genotípicos es relevante para evaluar la colonización y prevenir la propagación del SARM.

Palabras clave: *Staphylococcus aureus*, antibióticos, enfermedad periodontal.

INTRODUCTION

The oral cavity and the interior of the subgingival pocket are ecological niches conducive to hosting microorganisms that may act as opportunistic pathogens.

Numerous epidemiological studies suggest that periodontal disease may be a risk factor for systemic infectious disease^{1,2}.

Over the past decade, *Staphylococcus aureus* has become increasingly methicillin-resistant^{3,4}. Methicillin-resistant *Staphylococcus aureus* (MRSA) is an

emerging pathogen that causes hospital and community-acquired infections with high rates of morbidity and mortality. In particular, there has been an increase in community-acquired methicillin-resistant *S. aureus* infections (CA-MRSA) with no apparent identified risk factors⁵. According to data from the World Health Organization Net (WHONET), the prevalence of MRSA in clinical isolates in Argentina is very high (40 to 50%)^{6,7}. Early, accurate detection of MRSA is a matter of great concern to public health, so that carriers may be identified and the spread of MRSA prevented.

According to international literature, only phenotypic analytical techniques are used to detect *Staphylococcus aureus* in samples from oral mucosa and periodontal pocket, using a single solid selective culture medium such as mannitol salt agar or Baird-Parker agar to isolate it, and traditional biochemical tests such as catalase and coagulase to identify it^{8,9}. The presence of the *nuc* gene allows *S. aureus* to be distinguished from other *Staphylococcus* species. The main mechanism in methicillin resistance is the expression of a penicillin-binding protein (PBP) called PBP2a, encoded by the *mecA* gene and characterized by its very low affinity for β -lactam antibiotics. Detection of the *mecA* gene or its product, penicillin binding protein (PBP2a), is considered the gold standard^{10,11} for MRSA confirmation. Recent investigations suggest that disk diffusion using cefoxitin is superior to most previously recommended phenotypic methods, including oxacillin disk diffusion and oxacillin screen agar testing¹². Borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) isolates are characterized by MICs to oxacillin close to or just above resistance breakpoints.

The aims of this study were to: a) determine the presence of *Staphylococcus aureus* strains isolated from oral mucosa and pocket samples from patients with gingivitis-periodontitis, and their sensitivity to antimicrobial agents b) evaluate the results obtained by molecular detection methods and the methicillin-resistance of those isolates, using the respective phenotypic methods for identification and determination of antibiotic susceptibility.

MATERIALS AND METHODS

Source of isolates

The *S. aureus* isolates evaluated in this study were from 102 immunocompetent adult patients with gingivitis-periodontitis, who visited the outpatient offices at the School of Dentistry of the University of Buenos Aires. Subjects were aged 18 to 70 years, and average age was 42.6 ± 15.1 (54.1% female and 45.9% male). The gingival and periodontal evaluation included clinical and radiographic examinations and measurement of clinical indicators with a pressure-controlled periodontal probe: attachment loss (AL), probing depth (PD), plaque index¹³ and gingival index¹⁴. The measurements were taken on all teeth except the third molar, at 4 sites per tooth (mesial, vestibular, distal and palatal/lingual).

The following exclusion criteria were applied: patients with systemic diseases altering the gingival-periodontal state; patients who had taken antibiotics, anti-inflammatory or anti-fungal drugs within six months prior to the study, and patients who had received periodontal treatment within six months prior to the study. Samples were taken before basic periodontal therapy was performed¹⁵.

The reference strains used were *S. aureus* ATCC 43300 (methicillin-resistant) and *S. aureus* ATCC 25923 (methicillin-sensitive).

Sampling and microbiological method

Oral cavity and subgingival samples were taken after the patient had rinsed his/her mouth with distilled water. Samples from the subgingival pocket were taken with a Gracey 7/8 curette, after relatively isolating the zone with cotton rolls and high-power suction and removing supragingival biofilm. A sample of oral mucosa (mucosa, tongue and cheek) was taken from each patient with a sterile cotton swab. All samples were placed in sterile PBS (phosphate buffer solution, pH 7.4) transport medium, and kept at 4° C until processing. The material was immediately sent to the microbiology laboratory. The samples were cultured in selective hyper-salted broth (containing 5 g proteose-peptone (Merck, Darmstadt, Germany), 10 g meat extract (Merck, Darmstadt, Germany), 65 g NaCl (Merck, Darmstadt, Germany) and distilled water to 1 liter, pH 7.5) and selective, differential solid media such as mannitol salt agar (Biokar Diagnostics, Beauvais, France), CHROMagar MRSA (CHROMagar Company, Paris, France) and CHROMagar Staph aureus (CHROMagar Company, Paris, France). Microscope studies with Gram coloring and biochemical tests were used to identify genus and species. A coagulase test (bioMérieux, Marcy-L'Etoile, France) and a Microgen™ STAPH-ID System kit (Microgen Bioproducts, Cambale, UK) were used.

Sensitivity to antimicrobial agents tests

To evaluate the susceptibility of *Staphylococcus aureus* to antimicrobial agents, diffusion tests and MIC were used according to the Clinical Laboratory Standard Institute (CLSI) standards.¹⁶ The diffusion method in Mueller-Hinton agar (Merck, Darmstadt, Germany) supplemented with 2% sodium chloride (NaCl) was performed using disks (Laboratorios Britania, Buenos Aires, Argentina) with 1 μ g oxacillin (OXA) and 30 μ g cefoxitin (FOX)^{15,16}.

To determine the MIC for OXA in solid medium against *S. aureus* isolates, we used Mueller-Hinton agar (Merck, Darmstadt, Germany) supplemented with 2% de NaCl, to which increasing concentrations of OXA were added. The presence of colonies was considered positive, and lack of growth, negative. The *S. aureus* isolates with oxacillin MIC 2 to 8 µg / ml and without the *mec A* gene were considered as oxacillin borderline (BORSA)¹¹.

Molecular methods

A multiple-PCR was performed to evaluate the presence of the *nuc* gene, in order to distinguish *Staphylococcus aureus* from other *Staphylococcus* species, and the methicillin-resistant gene *mecA* was amplified^{20,21}.

DNA was extracted from a strain cultured in brain heart broth (Merck, Darmstadt, Germany) incubated for 18 hours at 37°C²². *S. aureus* ATCC 25923 and *S. aureus* ATCC 43300 were used as negative and positive controls, respectively, for the *mecA* gene. For the Multiple-PCR we used primers *MecA* 1 (AGT TCT GCA GTA CCG GAT TTG C), *MecA* 2 (AAA ATC GAT GGT AAA GGT TGG C)¹⁴, *nuc*-1 (GCG ATT GAT GGT GAT ACG GTT) and *nuc*-2 (AGC CAA GCC TTG ACG AAC TAA AGC)²³ following the protocol described by Geha G et al.²⁴ The PCR products were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide, for viewing under UV light.

Statistical analysis

Sensitivity, Specificity and Essential Correlation were calculated for the different phenotypic methods used to determine methicillin resistance.

Sensitivity: number of resistant strains detected by each method divided by total strains showing presence of the *mecA* gene by PCR, as a percentage. Specificity: number of sensitive strains shown by each method divided by total strains that did not show presence of the *mecA* gene by PCR. Essential Correlation (C): determined using the presence of the *mecA* gene as a reference, by means of the following formula:

$$C = \frac{(N-Fs -Fr) \times 100}{N}$$

where N is the number of strains studied, Fs are the false sensitive results and Fr are the false resistant results.

RESULTS

Table 1 shows the means, standard deviations and 95% confidence intervals for average probing depth, attachment loss, gingival index and plaque index for the 102 subjects at the time of sampling.

Average clinical indices were compared by means of the ANOVA test.

All patients with chronic gingivitis and periodontitis were positive for bleeding on probing.

The correlation between identification of *S. aureus* with traditional phenotypic tests and detection of the *nuc* gene was 100%.

Among the 102 patients with gingivitis-periodontitis, *S. aureus* had a prevalence of 10.8% (n = 11, CI 95%: 5.8-18.9) in pocket and 19.6% (n = 20, CI 95%: 12,7-28,9) in oral mucosa; methicillin-resistant *S. aureus* (MRSA) was isolated from the pocket in 3.9% (n = 4, CI 95%: 1.1-9.7) and from oral mucosa in 8.8% (n = 9, CI 95%: 4.1-16.1).

Table 2 shows total patients with gingivitis-periodontitis who were positive for culture of the genus *Staphylococcus*. Among the 20 patients with gingivitis, prevalence was 45.0% (n = 9) in pocket and 65.0% (n = 13) in oral mucosa; while for the 82 patients with chronic periodontitis, prevalence was 32.9% (n = 27) in the pocket and 47.6% (n = 39) in oral mucosa. For both sites there was predominance of coagulase-negative *Staphylococcus* (CNS) over *S. aureus*; CNS prevalence was 25.5% (n = 26, CI 95%: 17.6-35.3) in the pocket and 34.3% (n = 35, CI 95%: 25.4-44.4) in oral mucosa. Association between *S. aureus* and CNS was found at both sites, and its prevalence was 1.0% (n = 1, CI 95%: 0.0-5.3) in the pocket and 3.0% (n = 3, CI 95%: 0.6-8.4) in oral mucosa.

Table 1: Patients' periodontal clinical parameters (Mean ± SD and CI 95%) at the time of sampling according to periodontal health condition*.

Clinical Parameters	Gingivitis	Chronic Periodontitis
Probing Depth (mm)	3.9 ± 0.79 (3.5-4.2)	5.6 ± 1.7** (5.2-6.0)
Attachment Loss (mm)	0	5.9 ± 2.5 (5.3-6.5)
Plaque Index ^a	0.90 ± 0.80	1.70 ± 0.11
Gingival Index ^b	0.95 ± 0.30	2.10 ± 0.16

* with Gingivitis N= 20; with Chronic Periodontitis N=82.

** ANOVA Test p<0.001. a: Silness and Løe 1964; b: Løe and Silness 1963.

Table 2: Distribution of absolute (n) and relative (%) frequencies of the genus *Staphylococcus* from isolates from oral mucosa and subgingival pocket in patients with gingivitis-periodontal disease (n =102).

<i>Staphylococcus</i>	Isolates							
	Gingivitis				Chronic Periodontitis			
	Oral Mucosa		Gingival Pocket		Oral Mucosa		Periodontal Pocket	
	n	%	n	%	n	%	n	%
MSSA ¹	1	5.00	0	0.00	7	8.54	6	7.32
MRSA ²	1	5.00	1	5.00	7	8.54	3	3.66
BORSA ³	0	0.00	0	0.00	1	1.22	0	0.00
MSSA/SCN	1	5.00	0	0.00	1	1.22	1	1.22
MRSA/SCN	0	0.00	0	0.00	1	1.22	0	0.00
CNS ⁴	10	50.00	8	40.00	22	26.83	17	20.73
Total patients with positive culture	13	65.00	9	45.00	39	47.56	27	32.93

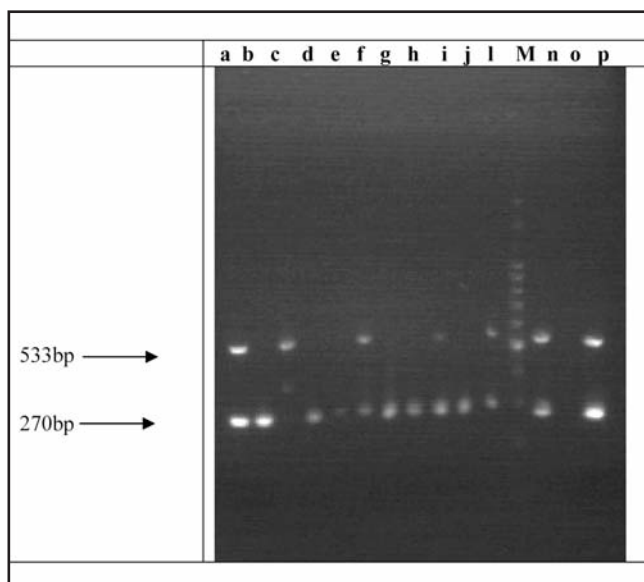
¹ MSSA: Methicillin-sensitive *Staphylococcus aureus*; ² MRSA: Methicillin-resistant *Staphylococcus aureus*

³ BORSA: Borderline oxacillin-sensitive *Staphylococcus aureus*; ⁴ CNS: Coagulase-negative *Staphylococcus*

Table 3: Sensitivity to antimicrobial agents of *Staphylococcus aureus* strains (nuc gene positive). Results of the evaluation by disc diffusion method, MIC in solid oxacillin medium, and detection of the Mec A gene.

Number of strains	Number of strains with positive (+), negative (-), Resistant (R) or Sensitive results (S) using the following methods							
	Mec A PCR		MIC in solid oxacillin medium		1µg OXA disk diffusion		30µg FOX disk diffusion	
	+	-	R	S	R	S	R	S
MSSA ¹⁷	0	17	0	17	1	16	0	17
MRSA ¹³	13	0	13	0	13	0	13	0
BORSA ¹	0	1	1	0	1	0	0	1

* The percentages for sensitivity, specificity and essential correlation were, respectively: 100, 89 and 94 for the OXA disk diffusion method; 100, 94 and 97 for OXA MIC in solid medium; 100, 100 and 100 for the FOX disk diffusion method.



The *Staphylococcus* strains isolated were identified and their susceptibility to antibiotics evaluated by Multiplex-PCR (Fig. 1).

Table 3 shows the results of susceptibility to antimicrobial agents using phenotypic and genotypic methods for the 31 *S. aureus* isolates.

Fig. 1: Electrophoresis on agarose gel containing 533-bp amplicons corresponding to the *mecA* gene and 270-bp amplicons corresponding to the *nuc* gene. Lanes: a, strain MRSA-42 oral cavity; b, strain MSSA-13 oral cavity; c, CNS *mecA* positive oral cavity; d, strain MSSA-3 oral cavity; e, strain MSSA-3 periodontal pocket; f, strain MRSA-89 periodontal pocket; g, strain MSSA-71 periodontal pocket; h, strain MSSA-71 oral cavity; i, strain MRSA-20 periodontal pocket; j, strain MSSA-20 oral cavity; k, strain MRSA-73 oral cavity; M, molecular weight marker (100bp Marker); n, strain MRSA-87 periodontal pocket; o, negative reagent control; p, *S. aureus* ATCC 43300.

DISCUSSION

The oral cavity and subgingival pockets of patients with gingivitis-periodontitis are a possible reservoir of opportunistic pathogens such as *S. aureus*, the genus *Candida* and *Pseudomonas aeruginosa*, among others^{25,26}. *S. aureus* and *Staphylococcus epidermidis* are frequently reported as pathogens responsible for infections. However, they are seldom or not at all studied from the oral cavity and periodontal pockets. Subgingival *Staphylococcus* does not necessarily represent an infection, but it does form part of the indigenous microbiota. Slots et al.²⁷ found *Staphylococcus* spp. in refractory periodontitis 19 years ago in 28.3% of individuals aged 25 to 60 years. In this study, which analyzed a large number of samples, *S. aureus* was the second most frequent sub-gingival *Staphylococcus* species following *S. epidermidis* belonging to the CNS group. Our methodology enabled a higher percentage of *S. aureus* to be recovered from the periodontal pocket than the methodology used by Loberto J et al.²⁸ This might be explained by the different prevalence values according to the geographic area of the population studied, but may also be due to the fact that the method we used for recovering *S. aureus* employs more than one culture medium for its isolation. We found similar percentages to those reported in the literature for CNS and *S. aureus* in the oral cavity. In a study on patients with treated periodontitis, Rams et al.^{21,30} report about

50% of subgingival *Staphylococcus* isolates and 22.3% *S. aureus*. In our study, all the results of the biochemical tests for identifying *S. aureus* match the results of the evaluation of the *nuc* gene that encodes thermonuclease.

According to the literature, and in agreement with our results, cefoxitin disk diffusion can accurately predict the presence of the *S. aureus mecA* gene^{7,29}. Detection of the *mecA* gene is considered to be the reference method for evaluating *S. aureus* methicillin resistance^{30,31}. It allows MRSA to be identified and distinguished from borderline oxacillin resistant strains (BORSA). Clinical evidence for failure with lactam therapy has been lacking for patients with borderline oxacillin resistant *S. aureus* (BORSA) infections. Skinner et al. describe a failure of cloxacillin for a patient with endocarditis due to BORSA³². This is the first report of local prevalence of *S. aureus* and MRSA in samples from oral mucosa and subgingival pocket in patients with periodontal disease. Moreover, a significative amount of MRSA was found. Today there are microbiological tools that allow the relative frequency of these pathogens in different niches of the oral mucosa to be established, in particular, in periodontal pocket in patients with periodontal disease. Prompt and accurate identification of MRSA is essential in order to manage infections they cause effectively and important to prevent them from spreading.¹²

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