

# Prevalence of Subgingival *Staphylococcus* at Periodontally Healthy and Diseased Sites

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Staphylococci are considered members of the transient oral microbiota and are seldom isolated from the oral cavity. The aim of this study was to establish the prevalence of subgingival staphylococci in healthy and periodontal disease sites. Sterile endodontic paper points were used to isolate subgingival staphylococci in periodontally healthy and periodontally diseased sites in 30 adult subjects (n=540 sites). *Staphylococcus* spp were identified by an automated method and confirmed by conventional biochemical tests. All the samples were identified as coagulase-negative staphylococci. The results were analyzed using Mann-Whitney U, chi-square and Fisher's exact test at 5% significance level. A total of 86.7% of the subjects harbored these microorganisms in 11.7% of their periodontal sites. The most frequently isolated species was *S. auricularis*, which was isolated from 31.4% of the periodontal sites, followed by *S. epidermidis*, isolated from 21.4% of them. There was no statistically significant difference between the frequencies of these species isolated either from the healthy and the diseased sites ( $p>0.153$ ). Although staphylococci are present in the subgingival environment and contribute to the pathogenic synergism involved in periodontal diseases, the results suggest that they do not participate directly in the pathogenesis of these diseases.

Key Words: *staphylococcus*, periodontal diseases, biofilms.

## Introduction

Periodontal disease is a chronic inflammatory condition of infectious origin that affects the protecting and/or supporting periodontal tissues of the tooth. As in the case of other infections, the interactions between bacteria and the host determine the nature of the resulting disease (1).

Some bacterial species have been suggested as causing periodontal diseases, although the evidence for that remains unclear (2). Among the microorganisms implicated in these diseases are staphylococci, particularly *Staphylococcus epidermidis* and *S. aureus*. These species are frequently found in human skin and in mucous membranes (3). In addition, they are isolated in cases of prosthetic valve endocarditis and also from oral environment habitats. However, these microorganisms are not considered resident oral bacteria, but rather are generally recognized as transient organisms (2,4). Gingival sulci and, more significantly, periodontal pockets provide an environment where non-specific bacterial adhesion may occur and where microorganisms may be retained near the host bloodstream. The colonization of staphylococci species may thus be favored in these sites, leading to a possible involvement of these bacteria in periodontal diseases (5-7).

Little is known about the role of the genus *Staphylococcus* in a healthy periodontal environment, or in one with chronic gingivitis or chronic periodontitis,

despite literature reports on the isolation of *S. aureus* and coagulase-negative staphylococci (CoNS) in periodontal disease environments. For this reason, the study of these microorganisms under different periodontal conditions is important for determining their possible association with the pathogenesis of these diseases (6-8).

The aim of this study was to establish the prevalence of subgingival *Staphylococci* in periodontally healthy diseased sites.

## Material and Methods

### Patient Population

The sample was intentional and consisted of 30 patients who sought treatment at the Periodontics Service of the Department of Dentistry, Federal University of Rio Grande do Norte (UFRN), Natal, RN, Brazil.

The inclusion criteria were as follows: age between 19 and 55 years, healthy periodontal and/or chronic gingivitis and/or chronic periodontitis sites, and good general health with no sign of soft tissue pathology other than periodontal inflammation. The exclusion criteria were pregnancy, use of orthodontic appliance, history of periodontal treatment in the previous six months and use of any local or systemic antimicrobial that could affect the study outcome.

To determine the sample size and test the research instruments, a pilot study was performed with the

participation of 17 subjects, who subsequently took part in the study (data not shown). The study design was approved by the UFRN Research Ethics Committee (protocol #007/06).

### Clinical Examination

A clinical chart was completed for each subject, with all the data related to anamnesis (age, sex, glycemia and tobacco use), and the clinical parameters of the study, such as gingival bleeding index (general, GBI\_G; and by tooth, GBI\_T), probing depth, gingival recession, dental mobility, presence or absence of periodontal disease and clinical attachment level.

To obtain the data, the patients were subjected to a clinical examination in which the probing depth was measured using a Williams periodontal probe (Trinity, São Paulo, SP, Brazil) at each of the following periodontal sites: mesial-buccal (MB), buccal (B), distal-buccal (DB), mesial-lingual (ML), lingual (L) and distal-lingual (DL). The modified O'Leary gingival (9) index and the clinical attachment level of each tooth were then measured.

The subjects underwent blood glucose monitoring with a glucometer (ACCU-CHEK Softclix, Roche®, Rio de Janeiro, RJ, Brazil), which provided a quick measurement of the blood glucose concentration in each patient.

### Subgingival Biofilm Collection

Three teeth were randomly selected (raffle) according to the health and disease conditions of the oral environment. All teeth were included, except for maxillary and mandibular third molars, poorly positioned teeth, teeth with extensive caries and root caries. This collection was performed at 6 sites per tooth, totaling 18 periodontal sites per subject. A single operator, trained in the study criteria, performed the collection. The sites chosen for sampling were isolated with sterile gauze and gently air-dried to remove any saliva. After carefully removing supragingival biofilm from the dental surface with gauze and sterile curettes (Trinity), so that the instruments did not touch the gingival margin to prevent bleeding, the samples of subgingival biofilm were obtained by inserting sterile endodontic paper points (Johnson, East Windsor, NJ, USA) into the gingival sulcus or periodontal pocket for 30 s (7). The samples were individually placed in Eppendorf vials (KASVI, Curitiba, PR, Brazil) containing sterile saline solution (0.5 mL) and preserved in ice for subsequent bacteriological analysis.

Care was taken to ensure that the absorbent paper points were not contaminated by the subject's own microbiota prior to or after insertion into the crevice/pocket by isolating the gingival margin with cotton rolls and discarding samples that touched the patient's skin or lips. The subject's nostrils were isolated to prevent contamination of the absorbent paper points during

insertion and removal. Further, the saturated absorbent paper points were discarded, as this was a potential indication of saliva contamination. When a sample was discarded, an alternative site was selected and sampled.

To minimize contamination from the clinicians involved in the sample collection, each wore a tight-fitting surgical mask and gloves. In addition, all instruments and sampling materials were sterilized and opened only at the time of sampling. All transport and isolation media were sterilized and strictly controlled for quality.

### Culture and Identification

The samples were processed no later than 30 min after subgingival biofilm collection. Each sample was vortex-mixed for 30 s to disperse the bacteria from the paper point. A 0.1-mL aliquot of this suspension was plated in duplicate onto manitol salt agar (Prodimol Biotecnologia, Belo Horizonte, MG, Brazil). All plates were incubated at 37 °C in air for 24–48 h. After this period of incubation, a macroscopic counting of the colony-forming units (CFU) was performed on the different morphological colony types obtained per periodontal site. Staphylococci were identified by colony morphology, Gram stain, catalase reaction, bacitracin susceptibility and coagulase tube test. The *S. aureus* ATCC 25923 strain was used as positive control in all tests.

Species identification was performed using the Staph System (VITEK; Bio-Mérieux, Marcy-l'Étoile, France) and the cultures were confirmed by the conventional method (8), which consists in a set of biochemical tests that determine the production of ornithine decarboxylase, urease, pyrrolidonylarylamidase-PYR, as well as anaerobic growth in thioglycolate (Merck, Darmstadt, Germany), susceptibility to novobiocin (5 µg, DME, Araçatuba, SP, Brazil), and acid production from carbohydrates (trehalose, manitol, mannose, maltose, xylose; Reagen, Rio de Janeiro, RJ, Brazil). Test readings were obtained after 24, 48, and 72 h of incubation at 37 °C.

### Statistical Analysis

The data were analyzed using STATA 10.0 software. The results were analyzed by the Mann-Whitney U non-parametric, chi-square or Fisher's Exact test. The significance level was set at 5% for all the analyses.

## Results

### Staphylococci Isolation

A total of 86.7% of the subjects harbored staphylococci in the subgingival dental biofilm on at least one periodontal site. Sample identification showed that all staphylococci were coagulase-negative and none was identified as *S. aureus*. Individual analysis of the 540 periodontal sites

showed an occurrence of staphylococci in as little as 11.7% of all sites examined. The mean number of periodontal sites *per individual* that harbored staphylococci was 2.16.

There was a tendency towards a lower staphylococci isolation rate in the most serious forms of periodontal disease, but with no significant association ( $p=0.672$ ) between the condition of the site and the presence of staphylococci. A low occurrence of staphylococci was also found in all types of periodontal sites, but without a statistically significant association ( $p=0.672$ ).

Furthermore, no statistically significant relation was observed between the presence of these microorganisms and gender ( $p=0.42$ ), periodontal disease ( $p=0.78$ ), tobacco use ( $p=0.70$ ), periodontal disease severity ( $p=0.62$ ), dental mobility ( $p=0.45$ ) and gingival bleeding at the site ( $p=0.87$ ). In the present study, no significant correlation was detected between the presence of staphylococci in the periodontal sites examined and age ( $p=0.54$ ), amount of blood sugar (mg/dL) ( $p=0.33$ ), GBI\_G ( $p=0.75$ ), GBI\_T ( $p=0.87$ ), probing depth ( $p=0.10$ ), gingival recession ( $p=0.59$ ) and attachment level ( $p=0.21$ ).

The most frequently isolated species was *S. auricularis*, which was isolated from 31.4% of the periodontal sites, followed by *S. epidermidis* (21.4%), *S. hominis* (12.9%), *S. capitis* (10%), *S. simulans* (7.1%), *S. cohnii* (5.7%), *S. warneri* (5.7%), *S. haemolyticus* (2.9%) and *S. saprophyticus* (2.9%) were also present in the periodontal sites, albeit in much smaller numbers. There was no significant difference in the

frequency of isolation of these species between the diseased sites and both sets of healthy sites ( $p>0.153$ ), except for *S. simulans*, which was more frequent in patients with chronic gingivitis ( $p=0.01$ ). *S. capitis* was more commonly isolated from sites with chronic periodontitis ( $p=0.07$ ), although this difference was not statistically significant (Fig. 1).

### Staphylococci Colonization Levels

To analyze this variable were used the data related to the number of colony-forming units (CFU) per site. It was found that 50% of the samples had  $4 \times 10^1$  or fewer CFUs, varying from 0 to uncountable in all the periodontal sites examined. It was also observed that 5.5% ( $n=30$ ) of the sites had more than  $1 \times 10^2$  CFUs of staphylococci. Out of these, 17 (56.6%) were from healthy sites, 8 (26.6%) from gingivitis sites, 4 (13.3%) from sites with slight chronic periodontitis, and 1 (3.3%) from a site with moderate chronic periodontitis (Fig. 2).

No statistically significant relation was observed between the colonization levels of these microorganisms and sex ( $p=0.28$ ), periodontal disease ( $p=0.68$ ), tobacco use ( $p=0.53$ ), periodontal disease severity ( $p=0.82$ ), dental mobility ( $p=0.52$ ), gingival bleeding at the periodontal site ( $p=0.91$ ), condition of the periodontal site ( $p=0.92$ ) and examined periodontal site ( $p=0.60$ ). Similarly, no statistically significant relation was detected between quantitative CFUs and age ( $p=0.51$ ), amount of blood sugar (mg/dL) ( $p=0.44$ ), GBI\_G ( $p=0.66$ ), GBI\_T ( $p=0.97$ ),

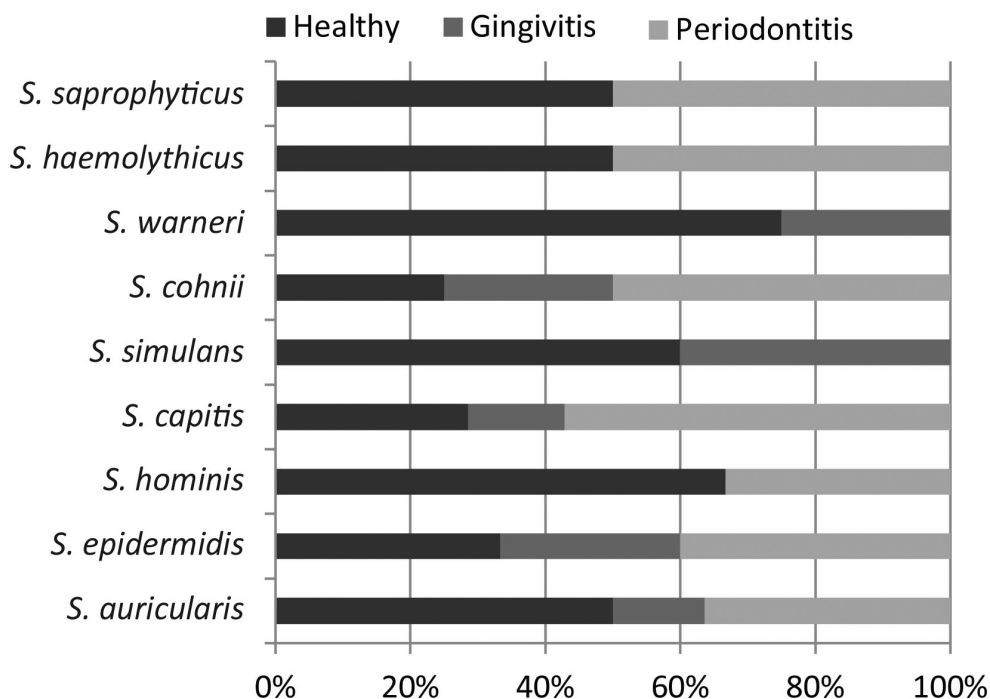


Figure 1. Distribution of the staphylococci species according to periodontal condition.

probing depth ( $p=0.19$ ), gingival recession ( $p=0.85$ ), and attachment level ( $p=0.29$ ).

## Discussion

The oral cavity may represent a poorly recognized environment for staphylococci which, under suitable conditions, may cause local or systemic infections (10). Periodontal pockets provide a site where non-specific bacterial adhesion may occur, and where these bacteria may be retained in the oral cavity close to the bloodstream. In patients with periodontitis, the mean dentogingival surface area is 8-20 cm<sup>2</sup> compared with 5 cm<sup>2</sup> in healthy individuals. Furthermore, microulceration of the sulcular epithelium and of the pocket lining facilitates bacteremia and systemic diffusion of bacterial and immunocomplex products (3).

*Staphylococcus* spp bacteria have been isolated from subgingival sites of patients with periodontitis, although it remains unclear whether there is a causal relation between the presence of these bacteria and chronic periodontal disease, (2). However, because few subgingival biofilm samples containing *staphylococci* have been collected from healthy sites, it has been impossible to determine if staphylococci isolation is related to disease or if they are putative microorganisms of subgingival sites (6,7,11).

Based on evidence that the periodontal pocket may be an ecological niche suitable for harboring opportunistic microorganisms, this study determined the prevalence of *Staphylococcus* spp from subgingival biofilm. Several subjects (86.7%) harbored microorganisms of this genus in the examined periodontal sites. Although there are discrepancies in the literature regarding the prevalence of subgingival *staphylococci*, the results of this study concur with those of Murdoch et al. (3), who isolated staphylococci from at least one diseased site in 54% of periodontal patients, and Loberto et al. (8), who isolated

staphylococci from the subgingival biofilm in 37.5% of subjects with chronic periodontitis. Rams et al. (6) isolated staphylococci from 50.4% of patients with advanced adult periodontitis, and Dahlén and Wikström (12) isolated *S. epidermidis* from 54.4% of patients. In both these studies, the patient cohort was >500 patients. In other studies, the recorded prevalence of staphylococci was lower, 5.6% (13) and 28% (7).

Heller et al. (14) analyzed the prevalence and infection levels of 51 microbial species (including *S. aureus*) in the subgingival biofilm of 260 patients with chronic or aggressive periodontitis with the aim of comparing both diseases. They observed that *S. aureus* was more prevalent in the subgingival biofilm of patients with chronic periodontitis compared to those with aggressive periodontitis. In contrast, other authors (15) found a strong association between *S. aureus* and aggressive periodontitis in non-smokers. In the present study, no significant difference was observed between the diseased and the healthy subgingival sites of periodontitis patients as regards the frequency of isolation of staphylococci or CFU counts. The present results are in accordance with those reported elsewhere (3,8). This suggests that staphylococci may be isolated from the subgingival environment irrespective of disease status.

In the present study no relation between gender and the isolation of subgingival staphylococci was observed, even though there was a predominance of isolation in men. This lack of correlation has also been reported in previous studies (3,8,12).

All the samples were identified as coagulase-negative staphylococci. In some studies a higher percentage of *S. epidermidis*, followed by that of *S. aureus*, was observed (3,6,8,11,16), whereas in others, *S. aureus* was either not found or isolated in tiny amounts from subgingival microbiota (17-20). In the present study, *S. auricularis*

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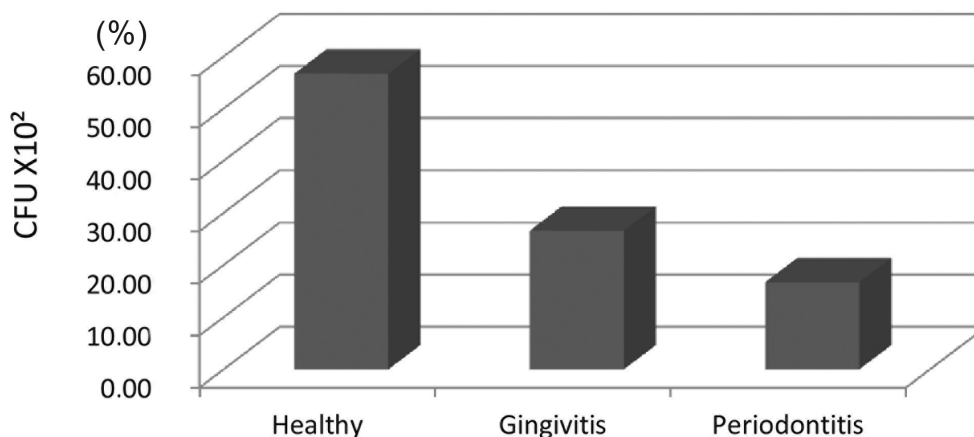


Figure 2. Prevalence of staphylococci in subgingival biofilm according to the number of colony-forming units in the periodontal sites.

was identified in a higher percentage, followed by *S. epidermidis*. There was no significant difference in the frequency of isolation of these species between diseased and healthy sites, and each species was isolated in very small amounts. *S. auricularis* has been rarely isolated in oral cavity infectious diseases (21-23), and if it has been isolated, its numbers are low.

Koksai et al. (24) isolated *S. auricularis* from cultures of blood from septic patients in Turkey. Although this species is not usually isolated from the oral cavity, it was observed that it inhabits the external auditory canal, involved in some infectious processes which may facilitate its presence in the oral cavity by transient colonization.

Although the number of other species of staphylococci isolated in this study was small, the numbers of isolated *S. simulans* were significantly higher in patients with chronic gingivitis. *S. capitis* was also more frequently isolated from sites with chronic periodontitis ( $p=0.07$ ), although this difference was not statistically significant. Other studies also found small amounts of this species in the gingival sulcus and periodontal pocket (3,6,8,17).

Of all the 540 periodontal sites examined separately, only a small percentage of them presented staphylococci (11.7%), and there was a tendency toward an even lower isolation rate of staphylococci in the most severe forms of periodontal disease. This might be a result of the characteristics of the studied environments. However, the microorganisms that colonize sites with chronic periodontitis do not depend on oxygen for survival, i.e., they are generally anaerobic. This fact reinforces the idea that the smaller amounts of staphylococci isolated in sites with more advanced periodontal disease are due to the characteristic of the periodontal microenvironment, which hampers the colonization by these bacteria.

Different authors (13,25) observed a low prevalence of *S. aureus* (<5%) in individuals with different probing depths. Loberto et al. (8) isolated staphylococci in 33% of individuals with probing depth  $\geq 5$ mm, but did not find a correlation between the presence of the microorganisms and probing depth. In the present study, there was no association between the depth of the pocket or of the gingival sulcus and the amounts of isolated staphylococci.

Few studies have reported on the staphylococcal colonization levels observed in periodontally compromised and healthy subjects. Confirming the results of this study, no statistically significant relation was detected between CFU counts and the presence of these microorganisms. Ohara-Nemoto et al. (26) found numbers of staphylococci ranging from  $10^2$  to  $10^5$  CFU  $g^{-1}$ , albeit from supragingival plaque.

In conclusion, the prevalence of the staphylococci in the individuals was considered high overall, but low in the periodontal sites, irrespective of the existing periodontal

condition (healthy or diseased). A tendency towards isolating these microorganisms in healthy sites and in those with less advanced forms of the disease was observed. This is believed to be a result of environmental characteristics. Only coagulase-negative staphylococci at low concentrations was detected in the examined periodontal sites. Most of the examined sites had low staphylococci colonization levels, irrespective of the existing periodontal condition. Although staphylococci are present in the subgingival environment and contribute to the pathogenic synergism of periodontal diseases, the results of this study suggest that they do not participate directly in the pathogenesis of these diseases.

The obtained data show that there is no specificity for the isolation of one species over another, regardless of the periodontal status of the site. Differences between studies may reflect distinctive microbial profiles between patients with different types of periodontal diseases, but geographical and genetic aspects should also be considered. Outcome differences may also result from different methodological approaches to clinical diagnostic criteria, sample size, material collection and microbiological technique.

## Resumo

Os estafilococos são considerados membros da microbiota oral transitente e são pouco isolados da cavidade oral. O objetivo deste estudo foi estabelecer a prevalência de estafilococos subgingivais em sítios saudáveis e com doença periodontal. Pontas endodônticas de papel absorvente estéreis foram usadas para isolar estafilococos subgingivais de sítios periodontais doentes e saudáveis, em 30 indivíduos adultos ( $n=540$  sítios). Os *Staphylococcus* spp foram identificados por método automatizado e confirmados por provas bioquímicas convencionais. Todas as amostras foram identificadas como estafilococos coagulase-negativa. Os resultados foram analisados através dos testes de Mann-Whitney U, Qui-quadrado ou Exato de Fisher para um nível de significância de 5%. Um total de 86,7% dos indivíduos albergavam estes microrganismos em 11,7% de seus sítios periodontais. A espécie mais frequentemente isolada foi *S. auricularis*, a qual foi isolada em 31,4% dos sítios periodontais, seguida pela espécie *S. epidermidis*, isolada de 21,4% dos sítios. Não houve associação significativa na frequência de isolamento das espécies, seja nos sítios periodontais saudáveis ou doentes ( $p>0.153$ ). Embora os estafilococos estejam presentes no ambiente subgingival e contribuam para o sinergismo patogênico envolvido em doenças periodontais, sugere-se que eles não participem diretamente na patogênese destas doenças.

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