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Molecular Analysis of Oral Bacteria in Heart Valve of Patients With Cardiovascular Disease by Real-Time Polymerase Chain Reaction

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Abstract: Structural deficiencies and functional abnormalities of heart valves represent an important cause of cardiovascular morbidity and mortality, and a number of diseases, such as aortic stenosis, have been recently associated with infectious agents. This study aimed to analyze oral bacteria in dental plaque, saliva, and cardiac valves of patients with cardiovascular disease. Samples of supragingival plaque, subgingival plaque, saliva, and cardiac valve tissue were collected from 42 patients with heart valve disease. Molecular analysis of *Streptococcus mutans*, *Prevotella intermedia*, *Porphyromonas gingivalis,* and *Treponema denticola* was performed through real-time PCR. The micro-organism most frequently detected in heart valve samples was the *S. mutans* (89.3%), followed by *P. intermedia* (19.1%), *P. gingivalis* (4.2%), and *T. denticola* (2.1%). The mean decayed, missing, filled teeth (DMFT) was 26.4 ± 6.9 (mean $\pm$ SD), and according to the highest score of periodontal disease observed for each patient, periodontal pockets > 4 mm and dental calculus were detected in 43.4% and 34.7% of patients, respectively. In conclusion, oral bacteria, especially *S. mutans*, were found in the cardiac valve samples of patients with a high rate of caries and gingivitis/periodontitis.

(*Medicine* 94(47):e2067)

INTRODUCTION

Structural deficiencies and functional abnormalities of heart valves are conditions that need cardiovascular surgery. These abnormalities may be caused by congenital diseases or by a variety of acquired diseases that result in valvular stenosis, valvular insufficiency, or both.[1]

The rheumatic heart disease (RHD), the most common heart valve disease in underdeveloped countries, is a condition that causes damage to the valve function, due to an abnormal immune response to group A streptococcal infection, especially during infancy.[2] Patients with RHD have valvular lesion caused by the rheumatic valve involvement (stenosis and/or regurgitation) or secondary to ventricular dilatation, leading to mitral or tricuspid insufficiency.[3]

The aortic stenosis, the most common valve disease in industrialized countries, had been considered, for many years, a degenerative disease that would appear with aging, and that was caused by the passive accumulation of calcium on the surface of the valve leaflet. Recent studies, however, have demonstrated that this disease represents an active process that may be divided into 2 distinct phases: an early initiation phase, similar to atherosclerosis, and a later progression phase that involves pro-calcifying and pro-osteogenic factors.[4–6]

In the last few decades, these conditions have been the object of great attention in the cardiology field, mainly due to changes in their presentation profile and treatment. These changes came as a result of a significant reduction in the incidence and sequelae of rheumatic fever, increase in life expectancy, technological advancement, and the discovery of new causes for valve diseases, including the alleged role of infectious agents in their pathogenesis.[7]

Infectious agents are well known to cause infective endocarditis, another major cause of valve replacement, but there are recent evidence also connecting these pathogens to valvular stenosis and/or regurgitation. In this context, an association between *Chlamydophila pneumonia* infection and aortic stenosis

has been suggested by Nyström-Rosander et al[8] and Turgeman et al.[9] Conversely, Kaden et al[10] did not find this association.

Oral micro-organisms have already been indirectly associated with other cardiovascular diseases (CVD), such as atherosclerosis. Oral bacteria involved in the etiopathogenesis of chronic periodontitis are known to cause an immune and moderate systemic inflammatory response, elevating the serum concentration of multiple cytokines and inflammatory markers that are abundantly produced in pathological periodontal tissues and strongly associated with the pathogenesis of some CVDs.[11]

The presence of oral bacteria in valvular tissue with or without clinical endocarditis have been investigated through sensitive molecular exams, such as the polymerase chain reaction (PCR), in order to investigate the mechanisms that can link oral infections to CVD.[12–14] In addition to identifying oral bacteria DNA in valvular tissue, Nomura et al[15] have reported the mechanisms that led cariogenic bacteria to colonize heart valves.

Considering the recent findings, the aim of this study is to identify cariogenic and periodontopathogenic micro-organisms in the dental plaque, saliva, and cardiac valves of patients undergoing valve replacement surgery, regardless of the disease, in order to contribute with epidemiologic data regarding the presence of oral bacteria in cardiac valves. Detailed evaluation of oral health status of all patients, including presentation of caries and periodontal disease history, by dental examination, will also be reported. This data could provide evidence of a connection between oral diseases and the frequency of oral micro-organisms in valve samples.

MATERIAL AND METHODS

Calibration, Oral Examination, and Collection of Samples

Patients admitted to Hospital Universitário Walter Cantídio and Hospital de Messejana Dr. Carlos Alberto Studart Gomes (Fortaleza, Ceará, Brazil) who were consecutively scheduled for cardiovascular surgery of valve replacement between March 2012 and September 2012 were enrolled in this study, which accounted for a convenience sample of 42 patients. All participants gave their informed consent and this study was approved by the Ethics Committee of the Hospitals where the research was performed.

Two examiners (F.A.F.O and C.P.F) were calibrated to evaluate teeth condition and current periodontal status of the patients, using the indexes DMFT (decayed, missing, and filled teeth) and PSR (periodontal screening and recording), respectively. For inter-examiner calibration, both examiners evaluated the same 10 volunteers that were not enrolled in the study. After 1 week, the same patients were re-evaluated to assess intra-examiner calibration. The Kappa values ranged from 0.80 to 0.97.[16]

Before the cardiovascular surgery, the previously calibrated examiners performed bedside oral examination. Information regarding smoking history, hypertension, diabetes mellitus, dyslipidemia, and other comorbidities were obtained through medical charts and anamnesis. Only 1 patient could not undergo the periodontal examination due to his poor physical condition.

For dentate patients, during oral examination, supragingival and subgingival dental plaque were collected.[17] Only for edentulous patients, saliva samples were collected by rubbing sterilized absorbent paper points in the oral mucosa and on the alveolar ridge, palate, and tongue. The oral samples were stored in a sterile vial containing phosphate-buffered saline (PBS) at −20°C for subsequent molecular analysis.

A total of 47 cardiac valves were collected, aseptically, during valve replacement surgeries. A fragment of each sample was stored in a sterile container containing PBS at −20°C for further analysis using real-time PCR.

DNA Extraction and Real-Time PCR

DNA extraction and molecular analysis through real-time PCR were carried out, respectively, at the Laboratory of Human Cytogenetics and Laboratory of Human and Medical Genetics of the Federal University of Pará (Belém, Brazil).

A total of 114 samples of supragingival and subgingival dental plaque, saliva, and cardiac valves (∼30 mg) were transferred to sterile plastic tubes of 2 mL containing a buffer, maintaining aseptic handling, and posteriorly homogenized. DNA extraction proceeded according to a standard protocol that used proteinase K and cetyltrimethylammonium bromide (CTAB) to remove complex polysaccharide.[18]

The samples of extracted DNA were subjected to real-time PCR for the detection of DNA from 4 different bacterial species: *S. mutans, P. gingivalis, P. intermedia,* and *T. denticola.* TaqMan probes to 16S bacterial ribosomal DNA (Table 1) were specifically designed for this study (Life Technologies®). For real-time PCR, 1 μL of genomic DNA (5 ng) was added to 8 μL of TaqMan® *Universal PCR Master Mix* (Applied Biosystems®), 0.35 μL of probe, and 3.15 μL of water. The final volume of 14 μL was obtained by adding IPC (1 μL) and IPC DNA (0.5 μL), which was used as an internal control for the reaction. Amplification was carried out in a Thermal Cycler 7500 real-time PCR System (Applied Biosystems®). Bacterial DNA was replaced by water as a negative control for the reaction. The real-time PCR protocol consisted of an initial step of denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min.

Statistical Analysis

The distribution pattern of the quantitative data of the sample was evaluated through the Shapiro–Wilk test. Posteriorly the quantitative data were submitted to the Student *t* test

TABLE 1. List of Primers and Probes Designed for the Identification of Different Cariogenic and Periodontopathogenic Micro-organisms

Micro-organisms	Primer/Probe TaqMan (sequence 5′-3′)
S. mutans	
Forward	GGCTCTCTGGTCTGTCACTGA
Reverse	TCGTTTACGGCGTGGACTAC
Probe	FAM-CACGCTTTCGAGCCTC-NFQ
P. intermedia	
Forward	AGATATCATGACGAACTCCGATTGC
Reverse	CGAGCTTAAGCGTCAGTAACACT
Probe	FAM-CCGTACGCTGCCTTC-NFQ
P. gingivalis	
Forward	AACACGTCCGCCAACAGA
Reverse	CTATCCTTGTCCTTGACGGTGTAG
Probe	FAM-CCCCTCCTGCAGATCT-NFQ
T. denticola	
Forward	TCCTTCTGTTCTTTCCTGAAACTCTTTT
Reverse	GGGATTCGAACCCCTGACCTA
Probe	FAM-CAAAGCCGCCGCTCTA-NFQ

and expressed as mean ± SEM. The quantitative demographic variables were expressed as the mean ± SD.

Qualitative nominal variables were expressed as absolute frequency (relative frequency) and analyzed by the Fisher exact test or the chi-square test with Bonferroni correction. When possible, the prevalence ratio expressed as the prevalence ratio (confidence interval minimum − maximum) was calculated.

The statistical software EpiInfo 3.5.1 for Windows (CDC Atlanta) was used and a significance level of $P < 0.05$ was established for all analyses.

RESULTS

For the present study, a total of 114 oral samples (supragingival plaque, subgingival plaque, and saliva) and cardiovascular samples (heart valves) were collected from 42 patients with a mean age of 55.6 ± 13.8 years. Regarding the medical conditions that led to valve replacement surgery, mitral regurgitation (23.4%) and aortic stenosis (21.2%) were the most common (Table 2).

Detailed oral examination for the investigation of dental caries and periodontitis was carried out in all 42 individuals. The mean number of teeth missing due to caries was 23.52 ± 9.41 per patient, and all patients have already had a previous experience of caries resulting in tooth loss. According to the highest degree of periodontal disease observed in the individual, excluding edentulous patients (44.0%), periodontal pockets >4 mm (43.4%), and dental calculus (34.7%) were present in a greater number of patients. Other demographic,

medical, and dental characteristics of the patients are summarized in Table 2.

Molecular analysis of oral samples revealed high frequency of *S. mutans* and *P. intermedia* in supragingival plaque, saliva, and subgingival plaque of dentate and edentulous patients (ranging from 60.0% to 100.0%), whereas *P. gingivalis* and *T. denticola* were present in fewer oral samples (ranging from 17.6% to 64.0%) (Fig. 1). Distribution profile of oral bacteria between dentate and edentulous patients revealed significant differences for *P. gingivalis* ($P = 0.024$) and *T. denticola* ($P = 0.037$). According to the probing depth, *P. gingivalis* ($P = 0.002$) and *T. denticola* ($P = 0.044$) were found with a higher frequency in patients with periodontal pockets >4 mm, with a rate >75.0%.

Regarding the presence of oral bacteria in the heart valves, all 4 micro-organisms were found in at least 1 sample, and only 5 valves (10.6%) were not infected with cariogenic or periodontopathic bacteria. The micro-organism most frequently found in the valve samples was the *S. mutans* (89.3%), followed by *P. intermedia* (19.1%), *P. gingivalis* (4.2%), and *T. denticola* (2.1%) (Fig. 1). Significant difference was observed between the frequency of *S. mutans* and the studied periodontopathic bacteria in the valve tissue ($P < 0.001$) (Fig. 1). Likewise, *P. intermedia* was significantly more present in the valve tissue compared to *P. gingivalis* ($P = 0.025$) and *T. denticola* ($P = 0.007$). Differing from other bacteria (*P. intermedia*, $P < 0.001$; *P. gingivalis*, $P < 0.001$; *T. denticola*, $P < 0.001$), there was no significant difference between the presence of *S. mutans* in heart valve and dental plaque or saliva ($P = 0.060$) (Fig. 1). There was no significant difference between the frequency of oral bacteria in the heart valves regarding dental condition (dentate, edentulous) ($P = 0.504$), anatomical location (aortic or mitral) ($P = 0.596$), and clinical diagnosis (stenosis, insufficiency, or both) ($P = 0.256$).

DISCUSSION

For many years, studies have been developed with an aim to investigate the possible connection between periodontal disease and cardiovascular disease, through the evaluation of inflammatory markers that are common to both pathologies. However, recent studies, such as the study of Nakano et al,[12] attempted to elucidate the direct mechanisms that link oral diseases to CVDs, and, according to this author, the presence of oral bacteria in the bloodstream (bacteremia) is probably one of the initiators of biological events that justify this association.

TABLE 2. Demographic, Clinic, and Dental Characteristics of Patients With Heart Valve Diseases

Variables	Samples
Demographic	42 patients
Age	55.6 ± 13.8 (27−81)
Sex	
Male	23 (54.8)
Female	19 (45.2)
Clinical diagnosis	47 heart valves
Aortic stenosis	10 (21.2)
Aortic insufficiency	8 (17.0)
Double aortic lesion	6 (12.7)
Mitral stenosis	8 (17.0)
Mitral insufficiency	11 (23.4)
Double mitral lesion	4 (8.5)
Dental profile	42 patients/252 sextants
DMFT	26.4 ± 6.9 (6−32)
PSR	
Excluded sextants ($\leq$ 1 tooth)	169 (67.1)
Sextants	83 (32.9)
Health sextants	6 (7.3)
Bleeding sextants	26 (31.3)
Sextants with dental calculus	20 (24.1)
Sextants with periodontal pockets 3.5−5.5 mm for 4−5 mm	17 (20.5)
Sextants with periodontal pockets>5.5 mm for $\geq$6 mm	8 (9.5)
Lost information	6 (7.3)

Qualitative dada expressed by ''n(%)''.

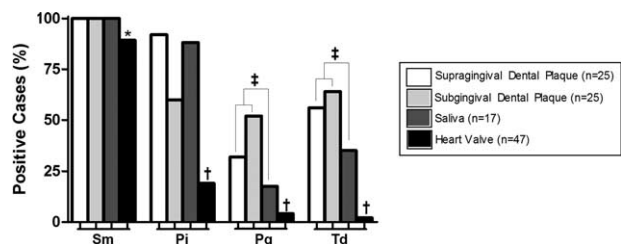**FIGURE 1.** Percentage distribution of cariogenic and periodontopathic bacteria in dental plaque, saliva, and heart valve samples. *$P < 0.05$ versus valve sample of Pi, Pg, and Td. †$P < 0.05$ versus supraginginval dental plaque, subgingival dental plaque, and saliva sample for each oral bacteria. ‡$P < 0.05$ oral sample of dentate patients versus the oral sample of edentulous patients (saliva) for each oral bacteria.

The involvement of cariogenic bacteria in the pathogenesis of some CVDs has been studied, and the reported detection rate of *S. mutans* in cardiovascular samples has been superior to the detection rate of periodontopathic bacteria.[12] In the present study, a high detection rate of *S. mutans* in the heart valve samples, as well as dental plaque and saliva samples was observed. The higher frequency of *S. mutans* in the heart valves compared to other studies may be related to the great previous experience of dental caries among the participants of the present study. It was also observed a high percentage of tooth loss due to caries, and epidemiological studies have demonstrated that the absence of teeth may be a risk factor associated with many cardiovascular diseases, including aortic stenosis.[19] In our research, the high detection rate of *S. mutans* in dental plaque samples and saliva samples may suggest that the *S. mutans* found in the valve samples was originated from the oral cavity.

In the present study, edentulous patients also presented a high frequency of the cariogenic bacterium in saliva and cardiac valve samples. These bacteria can be found in the oral cavity even after complete tooth loss, adhered to soft tissue or dentures.[20] The colonization of *S. mutans* in the cardiac tissue of these patients may have happened previously to complete tooth loss. Another theory is that these bacteria may have entered the bloodstream after complete tooth loss through soft tissue trauma. The possible occurrence of bacteremia in edentulous patients needs further investigation.

Statistically significant difference was found between the frequency of *S. mutans* and the other investigated micro-organisms in the heart valves. In the past few years, *in vitro* and *in vivo* experimental studies have reported that *S. mutans* has the ability to modify the expression of certain genes influenced by plasma components, in order to obtain advantages while inside the bloodstream.[21] These findings may justify this bacterium's high ability to settle in the heart valve tissue.

Jung et al[22] reported the role of AtlA, a recent discovered fibronectin binding protein, in the *S. mutans* resistance to fagocitosis and in its ability to survive in the bloodstream. Its maturation is enhanced by physiological serum calcium concentration. Fibronectin, elastin, laminin, and collagen are considered the most common extracellular matrix components and may serve as receptors for *S. mutans*.[23]

The discovery of bacterial surface structures such as the proteins P1, WapA, GtfB, GtfC, GtfD, Cnm, and Cbm, has been important to understand the mechanisms of attachment of these pathogens to the cardiovascular tissue, with subsequent invasion of endothelial cells, induction of inflammation with the production of cytokines, platelet aggregation, and foam cell formation.[24–27]

Jung et al[28,29] and Matsumoto-Nakano et al[24] have already reported the important interaction between this bacterium and the platelets in heart valve tissue through experimental studies, where they show that platelet aggregation is stimulated by this micro-organism.

Pariodontophatic bacteria were found less frequently in heart valve tissue, when compared to the gram-positive bacterium *S. mutans*, corroborating the findings of Nakano et al.[14] Great variability is reported in the literature regarding the prevalence of these bacteria in heart valve tissues and atherosclerotic plaques, and the reported frequency is either superior or inferior to that observed in the present study.[12,14,30–32] Many authors that found a higher frequency of these periodontal bacteria in cardiovascular tissues, compared to the present study, have included in their studies only dentate patients with moderate to severe periodontitis.

In the present study, the majority of the dentate patients presented periodontal disease, exhibiting dental calculus and periodontal pockets >3.5 mm for >4 mm. This fact was determinant for the periodontal bacteria of the orange complex (*P. intermedia*) and red complex (*P. gingivalis*, *T. denticola*) to be detected at a high frequency in the dental plaque, specially subgingival plaque removed from periodontal pockets >6 mm, corroborating previously reported findings.[20,33]

Among the gram-negative micro-organisms, *P. intermedia* was significantly more detected in the heart valve tissue ($P < 0.05$). The high detection rate of this micro-organism in the oral samples of dentate (supragingival and subgingival dental plaque) and edentulous patients (saliva) probably explains its higher frequency in the heart valve samples, compared to the other periodontopathic bacteria. The hypothesis that periodontopathic bacteria can infect cardiovascular samples prior to complete tooth loss of a patient that had periodontal disease may be considered, as suggested by some authors, such as Zaremba et al.[34]

The frequency of periodontopathic bacteria in the heart valve samples was considered low, when compared to their frequency in the oral samples, which may suggest that these micro-organisms have greater difficulty in surviving inside the bloodstream and adhering to heart valve tissue, compared to *S. mutans*.

P. gingivalis has received special attention among the periodontopathic bacteria regarding its association with CVDs, although it was found at a low frequency in the present study. This bacterium is detected at a high frequency in studies that investigate its presence in atherosclerotic plaques, and *in vitro* and *in vivo* experimental studies have demonstrated that *P. gingivalis* accelerates the process of atherosclerotic plaque formation through different mechanisms.[32,35,36]

In the present study, real-time PCR was used because it has previously demonstrated high sensitivity and efficacy in detecting bacterial DNA in valve tissue and oral samples, even at low levels.[12,37] Nevertheless, the polymerase chain reaction may also detect DNA of dead bacteria, which poses a doubt regarding the role of the detected bacteria in the pathogenesis of the disease. It is unlikely that the bacteria present in the heart valve tissue are innocuous, as recent laboratorial studies have given support to the idea that these bacteria may have a direct influence in the initiation and progression of the disease. However, new studies are essential to confirm this association. The evaluation of the expression of different biomarkers (inflammatory, thrombotic, and osteogenic) in human heart valves affected with chronic diseases and its correlation with the presence and frequency of oral bacteria in those samples may provide a positive association between the infection and the intensity of the local inflammatory process. This would be an important finding to confirm an active role of the oral bacteria in the progression of these diseases.

In summary, cariogenic and periodontopathic bacteria were found in dental plaque, saliva, and cardiac valve samples of patients with cardiovascular disease. The detection rate of *S. mutans* in cardiovascular samples was superior to the detection rate of periodontopathic bacteria. This finding may be related to the great previous experience of dental caries among the participants of the present study and *S. mutans* possible ability to survive in the bloodstream and attach to extracellular matrix components.

ACKNOWLEDGMENTS

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