Porphyromonas gingivalis in periodontal pockets and heart valves

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Background: There is evidence that advanced infectious chronic periodontal inflammatory disease may have an impact on general health including cardiovascular diseases. The aim of this clinical study was to evaluate the ability of Porphyromonas gingivalis to colonize heart valves and, subsequently, to assess whether there is an association between the presence of the DNA of Porphyromonas gingivalis in periodontal pockets and in degenerated heart valves.

Materials and Methods: Thirty patients were enrolled in the study and 31 valve specimens harvested during cardiac surgery operations were examined. All patients underwent a periodontal examination. To evaluate the periodontal status of the patients the following clinical parameters were recorded: the pocket depth, bleeding on probing (BOP) and approximal plaque index (API). The presence of P. gingivalis in heart valve specimens and samples from periodontal pockets was analyzed using a single–step PCR method.

Results: P. gingivalis DNA was detected in periodontal pockets of 15 patients (50%). However, the DNA of this periopathogen was found neither in the aortic nor in the mitral valve specimens.

Conclusions: This study suggests that P. gingivalis may not have an influence on the development of the degeneration of aortic and mitral valves.

Introduction

Periodontal diseases are widespread infections and may affect, in a moderate form, even up to 70–80% of the general population. The prevalence of generalized, severe periodontitis among adults during the 1980s and 1990s ranged from 5–15% globally regardless of geographic and economic considerations.1

One of the most important factors causing chronic periodontitis is the presence of periopathogens. The clinical development and expression of the disease depend on the modificable and non-modifiable factors in the susceptible host. People with diabetes mellitus and obesity are at an increased risk of periodontitis.2

Chronic periodontitis usually becomes clinically significant after the age of 30. Periodontal disease goes through periods of exacerbation and remission of inflammation.3 Transient bacteremia can not only be related to periodontal surgical procedures but can also result from daily activities such as chewing, tooth brushing, and flossing.4,5

There is evidence for a link between oral and general health, particularly regarding periodontitis and atherosclerosis, aortic intimal hyperplasia (cardiovascular lesions), diabetes, respiratory diseases, preterm and low birth weight delivery, preeclampsia, and inflammatory bowel disease.6,7 As an association between general diseases and periodontitis was found, the term “periodontal syndrome” was introduced as a “risk indicator” or a “marker”, which should be considered in prophylaxis and management of general health.8 The authors of this study underline the importance of dentist (periodontologist) and patient awareness of an increased possibility of developing or exacerbating various diseases due to periodontitis.

It has been noted in many epidemiological studies that people suffering from cardiovascular diseases have a statistically significant worse oral health than the general population.9,10 It is believed that periodontitis, which is chronic in nature but with acute phase periods, causes a small but long-lasting systemic inflammatory reaction, which in turn contributes to the development of atherosclerosis and an increased risk of cardiovascular events. Studies published in 1993 showed that an active chronic periodontal disease increases the risk of coronary artery disease by 25% compared with healthy subjects.9,10 These conclusions were confirmed in a metaanalysis carried out by Meurman et al., on the basis of a metaanalysis.11 In patients suffering from periodontitis, the risk of cardiovascular disease was increased by 20% compared with a control group of people with healthy periodontium. The same research showed that the risk of a stroke among people with periodontopathy increased 2.85-fold compared with the control group. Periodontitis cannot only be a risk factor for these diseases, but can modify other primary factors for cardiovascular events (lipid profile disorders, hypertension) as well.7

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The DNA of this periopathogen was assessed in heart valve specimens harvested during the operations. The evaluation of correlation between the presence of *P. gingivalis* in periodontal pockets and in heart valves may aid in the understanding of the role of this bacterial species in inflammatory or degenerative processes involving heart valves. *P. gingivalis* has been identified in the atherosclerotic plaque\(^5\) and it is reasonable to assume that their presence could cause inflammatory processes in heart valves too. In such a case, a new link between atherosclerosis and calcific valve degeneration could be found.

The aim of the present study was to evaluate the periodontal status and assess the presence of *P. gingivalis* DNA in periodontal pockets in patients who required surgical treatment of aortic or mitral heart valve dysfunction. Moreover, the possible co-existence of DNA of this periopathogen was assessed in heart valve specimens harvested during the operations.

**Results**

Table 1 summarizes the demographic and clinical features of the patients. There were 16 men and 14 women with an average age of 63.3 ± 9.4 y. Twenty of them suffered from hypertension, four from diabetes and eight subjects had hypercholesterolemia.

The periodontal status of the examined subjects is summarized in Table 2. Periodontitis was diagnosed in the majority of the investigated patients (27 with P category). Gingivitis was present in one of the patients and two of them had a healthy periodontium. The mean periodontal pocket depth (PPD) was 3.6 mm. Generally, the oral hygiene was poor since the mean API was 73%. Patients had on average 13.5 teeth.

The DNA of *P. gingivalis* from periodontal pockets was detected in 15 patients—all with periodontitis. However, the DNA of these bacteria was not present in any of the harvested valves Table 3.

*P. gingivalis* DNA was also absent in the additional group of patients (n = 30) who did not undergo a periodontal examination.

**Discussion**

Many studies indicate periodontitis as a possible source of a systemic release of bacterial pathogens or proinflammatory components.\(^6\)-\(^20\) Periopathogens may cause inflammatory responses due to the complex reactions they instigate.\(^5\),\(^10\),\(^21\) Literature reports support a moderate but significant association between periodontal disease and atherosclerotic vascular disease. What is more, treatment of periodontal diseases, including a control of periodontal infections, results in improved levels of markers of systemic inflammation and endothelial dysfunction.\(^22\)-\(^24\) The ability of oral pathogens to colonize coronary atheromatous plaque has already been confirmed by studies showing the calcific degeneration of heart valves, i.e., increased levels of C-reactive protein,\(^16\) interleukin-1β,\(^17\) and transforming growth factor β\(^18\) or an increased temperature in stenotic valves.\(^19\) These processes may facilitate subsequent fibrosis and calcification of the valve, which cause valve stiffness and hemodynamic dysfunction.

**Table 1. Patients’ demographics**

| Investigated group of patients (n = 30) | Gender female/male | Age (y, mean ± SD) | Harvested valves: aortic/mitral | Hypertension | Diabetes | Hypercholesterolemia |
|----------------------------------------|--------------------|-------------------|--------------------------------|--------------|----------|---------------------|
|                                        | 14/16              | 63.3 ± 9.41       | 20/11                          | 20 (67%)     | 4 (13%)  | 8 (27%)             |

**Table 2. Characteristics of periodontal status of the patients**

| Periodontal status of the patients (n = 30) | Number of teeth (mean ± SD) | API index (mean ± SD) | PPD (mean ± SD) | BGI-H: | BGI-G: | P1 | P2 | P3 |
|--------------------------------------------|-----------------------------|----------------------|-----------------|--------|--------|----|----|----|
|                                            | 13.5 ± 8.2                  | 73.3% ± 37.5         | 3.6 ± 1.6 mm    | 2 (7%) | 1 (3%) | 13 (43%) | 5 (17%) | 9 (30%) |

BGI-H, biofilm-gingival interface-healthy (PPD ≤3 mm, BOP extent score <10%); BGI-G, gingivitis (PPD ≤3 mm, BOP extent score >10%); P1, BGI deep lesion/low bleeding (PPD ≥4 mm, BOP extent scores <10%); P2, BGI deep lesion/moderate bleeding (PPD ≥4 mm, BOP extent scores from 10% to 50%); P3, BGI deep lesion/moderate bleeding (PPD ≥4 mm, BOP extent score >50%); API, approximal plaque index; PPD, periodontal pocket depth; BOP, bleeding on probing.

**Table 3. Presence of *P. gingivalis* DNA in investigated samples**

| Number of patients with periodontal examination | *P. gingivalis* DNA in periodontal pockets | *P. gingivalis* DNA in heart valves |
|------------------------------------------------|-------------------------------------------|-------------------------------------|
| 30 patients with periodontal examination       | 15                                        | 0                                   |
| 30 patients without periodontal examination    | Not examined                              | 0                                   |

*P. gingivalis* is a gram-negative, anaerobic pathogenic oral bacterium and a major etiological agent in the initiation and progression of the severe form of chronic periodontitis. This periopathogen has a number of potential virulence factors such as fimbriae, ceramides, cysteine proteinases, outer membrane proteins, and lipopolysaccharides (LPS). Through these factors it not only becomes destructive for periodontal tissues, but can also induce and enhance general inflammation.\(^12\)

In some patients with heart valve dysfunction (stenosis or regurgitation), a surgical replacement of the valve is needed. Although some of the valve problems are due to an acute process, i.e., acute endocarditis, myocardial infarction, or trauma, chronic processes are a major cause of valve dysfunction. The heart valve is subjected to mechanical stress, which may lead to the damage of the endothelium covering the valve.\(^13\) It may facilitate the penetration and accumulation of lipids in the valve,\(^14\),\(^15\) which, in turn, may cause or exacerbate the inflammatory processes in valve leaflets. Some studies confirm the inflammatory origin of the calcific degeneration of heart valves, i.e., increased levels of C-reactive protein,\(^16\) interleukin-1β,\(^17\) and transforming growth factor β\(^18\) or an increased temperature in stenotic valves.\(^19\) These processes may facilitate subsequent fibrosis and calcification of the valve, which cause valve stiffness and hemodynamic dysfunction.
presence of *P. gingivalis* RNA/DNA in atheromatous plaque or in samples taken from coronary vessels.\textsuperscript{22-25} Ishihara found *P. gingivalis* 16S rRNA in 47.1\% of subgingival samples and in 5.8\% of coronary artery samples from patients with less than four periodontal sites, and in 58.8\% of subgingival and in 29.4\% of coronary artery samples from patients with four or more periodontal sites.\textsuperscript{26} Mahendra found *P. gingivalis* DNA in 45.1\% of atherosclerotic plaque samples.\textsuperscript{27}

It has been reported that *P. gingivalis* accelerated atheroma formation.\textsuperscript{28-30} caused an increase in systemic inflammatory marker levels,\textsuperscript{29,21} invaded endothelium and vascular smooth muscle cells,\textsuperscript{31,32} and appeared to alter endothelial function.\textsuperscript{33,34} *P. gingivalis* activates endothelial cells and upregulates various adhesion molecules, and thus, increases the likelihood of macrophage diapedesis and subsequent conversion to foam cells. All of these processes lead to the progression of atheroma formation.\textsuperscript{30} *P. gingivalis* influences the development of intimal hyperplasia in the aorta through the upregulation of S100 calcium-binding protein A9.\textsuperscript{4} It was also demonstrated that a *P. gingivalis* infection causes an increased vasoconstrictor response to phenylephrine both in mice with spontaneous atherosclerosis and in healthy mice.\textsuperscript{35} However, the virulence mechanisms are different for various *P. gingivalis* strains and a detailed examination of these processes needs to be performed.\textsuperscript{32}

In this study, the assessment of the presence of *P. gingivalis* DNA in cardiac valves in relation to its presence in periodontal pockets was performed. Overall, in our research, severe periodontitis was diagnosed only in six patients. Only two subjects had a pocket depth of six or more millimeters. Out of the 30 investigated patients, 15 had positive samples for *P. gingivalis* DNA from the periodontal pockets. However, *P. gingivalis* DNA was not detected in the aortic/mitral valve specimens harvested from these patients. Moreover, in the additional 30 valve specimens (13 mitral and 17 aortic) which were examined in patients who did not undergo periodontal examination, no *P. gingivalis* DNA was found. In a study published by Raffaelli, the presence of DNA of 8 selected periodontal pathogens (including *P. gingivalis*) in patients with chronic periodontitis was examined in both blood and aortic valves. The periodontal pathogen was present neither in valve specimens nor in the whole blood samples.\textsuperscript{36} In another study, Nakano investigated a group of 35 patients for the presence of periodontitis-related bacteria in specimens extracted from heart valves as well as those obtained from the supragingival plaque.\textsuperscript{37} *P. gingivalis* was detected in four heart valves, *Treponema denticola* in 14, and *Prevotella intermedia* in two of the investigated valve samples. Conversely to other studies, the authors did not find any periopathogens in the atheromatous plaque taken from a separate group of 27 patients.\textsuperscript{35,36}

We did not find the presence of *P. gingivalis* DNA in either aortic or mitral valves in a group of 60 patients (30 patients without concomitant periodontal examination), even though this DNA was detected in periodontal pockets of 15 patients. It is known that *P. gingivalis* possesses various properties, which can promote atherosclerosis, invade endothelial cells, and enhance atheroma formation. The lack of DNA of these bacteria in heart valves with calcific degenerations may indicate no relationship between the presence of *P. gingivalis* and valve dysfunction. This pathogen seems not to play any role in the damage or inflammation of heart valves.

**Conclusions**

It can be concluded that there may be no association between the presence of *P. gingivalis* and calcific degeneration of heart valves. Since some studies confirmed not only the presence of *P. gingivalis*. DNA in atherosclerotic plaque, but also the contribution of this periopathogen to the formation of atherosclerosis by direct and indirect mechanisms, further studies that analyze the colonization mechanisms of *P. gingivalis* should be performed. This could help to find new differences in the pathogenesis of atherosclerosis and calcific degeneration of heart valves, and the correlation between periodontitis and valve pathologies. Subsequently, it may lead to a better understanding of these diseases and the development of more selective treatment methods.

**Materials and Methods**

Thirty dentate patients from the Department of Cardiac Surgery were prospectively included in the study, all of which were scheduled for aortic and/or mitral valve surgery replacement due to valve dysfunction (stenosis, regurgitation, or both). Twenty aortic valves (16 stenotic valves with or without concomitant regurgitation and 4 with pure regurgitation) and 11 mitral valves (7 stenotic with or without coexisting regurgitation and 4 with pure regurgitation) removed during cardiac surgery procedures were examined for the presence of *P. gingivalis* DNA (2 valves: aortic and mitral were taken from 1 patient). The collected medical data included clinical variables such as: the type of harvested valve, past medical history and the details of past dental care. A complete dental/periodontal examination was performed by a single dentist. The periodontal status was assessed according to a simplified periodontal disease classification of Offenbacher et al., 2008.\textsuperscript{3} Pocket depth was determined at four sites per tooth. Oral hygiene was examined according to the API (Lange et al., 1978)\textsuperscript{38} and the total number of teeth was evaluated. Subgingival samples were collected from two gingival sulcus sites or the two deepest gingival/periodontal pockets from each patient. The place around the chosen pocket was dried and isolated from saliva. Sterile paper points were inserted into the sulcus/pocket for 10 s, then transferred to a sterile Eppendorf tube and frozen at −20 °C. Aortic and mitral valves harvested during the surgical treatment were placed in a sterile tube and frozen at −20 °C. Thirty valve specimens from patients who did not undergo a periodontal examination were additionally analyzed for the presence of the *P. gingivalis* DNA.

**DNA extraction protocol**

The laboratory tests were performed in the Department of Forensic Medicine, Molecular Techniques Unit at Wroclaw Medical University. Ten to thirty milligrams of heart valves extracted during cardiac surgery procedures were cut in to the
small pieces. Next, in order to extract the DNA from heart valves, the E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek) protocol was conducted according to the manual.\(^\text{39}\) No washing of heart valve samples was performed. The DNA from the oral cavity was obtained using paper points. Then, an isolation of whole genomic DNA from oral samples was done with the use of a modified CTAB method (called: cetyltrimethylammonium bromide, hexadecyltrimethylammonium bromide).\(^\text{30}\)

Each paper point was placed in a 1.5 mL Eppendorf tube filled with 400 μL of sterile water (Sigma Aldrich Reagent Water Molecular Biology), and the mixture was stirred in a vortex apparatus. Afterwards, paper points were removed from the tubes and 70 μL of 10% SDS (sodium dodecyl sulfate), and 50 μL of proteinase K at a concentration of 1 mg/mL (Sigma Aldrich) were added. Subsequently, the mixture was vortexed vigorously and incubated in the thermo block for 10 min at 65 °C. Next, 100 μL of 5M NaCl and 100 mL of CTAB/NaCl (0.274 M CTAB [Hexadecyl trimethylammonium bromide], 0.877 M NaCl, Sigma Aldrich) was added to each tube, and the mixture was shaken and incubated for 10 min in 65 °C. Then, 750 μL of chloroform isooamyl alcohol was added (chloroform: isooamyl alcohol mixture 24:1, Fluka Analytical) to each tube and vigorously shaken. After centrifugation for 5 min of incubation at −20 °C the material was centrifuged, the supernatant was removed and the DNA pellet was dried at room temperature for 30 min at 65 °C. Aldrich) to precipitate the DNA. After 30 min of incubation in 20 μL of 5M NaCl and 100 μL of 5M NaCl and 100 μL of 5M NaCl and 100 μL of CTAB/NaCl (0.274 M CTAB [Hexadecyl trimethylammonium bromide], 0.877 M NaCl, Sigma Aldrich) was added to each tube, and the mixture was shaken and incubated for 10 min in 65 °C. Then, 750 μL of chloroform isooamyl alcohol was added (chloroform: isooamyl alcohol mixture 24:1, Fluka Analytical) to each tube and vigorously shaken. After centrifugation for 5 min of incubation at −20 °C the material was centrifuged, the supernatant was removed and the DNA pellet was dried at room temperature under sterile conditions. The precipitated DNA was dissolved in 20 μL of distilled water of a quality adapted to molecular biology (Gibco, Invitrogen Paisley). The isolated DNA was subjected to amplification within several hours of isolation in order to avoid degradation of the genetic material. The presence of the DNA was controlled by spectrophotometric measurement at a wavelength of 260 nm. The concentration of the obtained DNA was between 15 and 20 ng/μL. The DNA was stored at −20 °C until the PCR reaction was performed.

PCR reaction

DNA samples isolated from biological material taken from both the oral cavity and heart valves were tested for human β-globin in order to confirm correct isolation of the DNA. The pair of primers taken from the publication of Tristao W. et al. were used, with a sequence as follow: GH20 (GAAGAGCCAA GGACAGGTAC) and PC04 (CAACTTCTAC CAGCTTCACC).\(^\text{41}\) The concentration of primers was 10 pmol/μL (Syngen Biotech Poland.)

Specific 16S rRNA-based polymerase chain reaction primers were used to detect \textit{P. gingivalis}, as described by Slots et al. with the forward primer 5′AGGCAGCTTG CCATACTGCG, and the reverse primer 5′ACTGT TTAGCA ACTACCGATG.\(^\text{42}\) The amplification reactions were performed in 25 μL reaction volumes with 0.2 μM of forward and reverse primers, 0.2 mM of each dNTPs, 2 U of DFS-Taq DNA polymerase (BIORON) and ~40 ng of template DNA, with the use of 50 μL of Dream Taq Green PCR Master Mix (2×) (Fermentas) and 10 μL of ca. 2.5–10 μM patient’s DNA.

The thermal profile consisted of initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 10 s, an annealing at 59 °C for 20 s, and an extension at 72 °C for 1 min. PCR conditions were adjusted on the reference strain of \textit{Porphyromonas gingivalis} W83. During the PCR reaction the DNA extracted from oral samples and heart valves was compared with a negative control (mix of primers from Dream Taq Green PCR Master Mix [2×] [Fermentas]) and to a positive control (DNA of \textit{P. gingivalis} strain W83).

Electrophoresis

The agarose gel electrophoresis was performed in order to visualize the reaction product. The agar was dissolved in the TAE buffer to receive a 1% solution. After preparing the agarose gel, 45 mL of the liquid agarose in the TAE buffer was collected and 2 μL of Midori Green DNA Stain (Nippon Genetics Europe GmbH) was added. A molecular standard size Gene Ruler 100-bp DNA Ladder (Fermentas) was used.

Proper DNA extraction was confirmed by the presence of β-globin (268 bp).\(^\text{41}\) Positive results were more clearly seen in samples obtained from heart valves than those from the oral cavity (Fig. 1). This may be attributed to the fact that the latter samples contained less biological material.

The samples that had 405 bp under the UV lamp (Canon) and on UV Sample Tray in Gel Doc Ez (BioRad) were considered positive for \textit{P. gingivalis} DNA (Fig. 2).

Statistical analysis

The results were presented as mean values ± standard deviations (SD).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 1. Gene Ruler 100 bp; 2–14, results for β-globin reaction obtained from heart valves (hv); 15–27 results for β-globin reaction obtained from DNA extracted from oral samples (os). Positive results observed on 268 bp; 28, negative control (H2O PCR water, nuclease free instead of DNA).

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Figure 2. 1, 18-Gene Ruler 100 bp; 2–17, patients results; 2, 3, 8, 9, 12, 14, positive results obtained from the DNA extracted from oral samples (405 bp); 19, positive control (reaction made with the use of DNA of W83 P. gingivalis); 20, negative control (H2O PCR water, nuclease free).