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Analysis of the presence of pathogens which predict the risk of disease at peri-implant sites through polymerase chain reaction (PCR)

Análise por reação em cadeia da polimerase (PCR) da presença de patógenos preditores de risco em sítios periimplantares

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ABSTRACT: The presence of DNA of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia* in the peri-implant sulcus samples of 19 partially edentulous patients was analyzed by polymerase chain reaction (PCR) and related to the depth of the peri-implant sulcus, bleeding on probing, and probable risk of disease. Ten of those patients presented a history of periodontal disease and nine of those did not. The DNA amplification of these pathogens was observed in seven samples, of which four were from patients without history of periodontal disease. The results suggest that even when significant inflammatory signs are absent the qualitative detection may indicate risk of peri-implantitis, requiring more strict postoperative control.

DESCRIPTORS: *Actinobacillus actinomycetemcomitans*; *Porphyromonas gingivalis*; *Prevotella intermedia*; PCR.

RESUMO: A presença dos ADN de *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* e *Prevotella intermedia* em amostras coletadas de sulco periimplantar de 19 pacientes parcialmente desdentados foi analisada pela reação em cadeia da polimerase (PCR). Dentre esses 19 pacientes, dez apresentavam histórico de doença periodontal e nove não apresentavam antecedentes. Os resultados obtidos nesta análise foram relacionados com a profundidade do sulco periimplantar, o sangramento à sondagem e o provável risco de doença. Constatou-se que houve a amplificação do ADN das bactérias-alvo em sete amostras, sendo quatro de pacientes sem histórico de periodontopatia. Este resultado sugere que mesmo na ausência de sinais inflamatórios significantes, essa detecção qualitativa pode indicar risco de periimplantite, requerendo manutenção pós-operatória mais rigorosa.

DESCRITORES: *Actinobacillus actinomycetemcomitans*; *Porphyromonas gingivalis*; *Prevotella intermedia*; PCR.

INTRODUCTION

Peri-implantitis occurs in a few dental implants caused by infection and/or by the action of excessive load, jeopardizing osseointegration. Microbiologic exams can greatly help in the treatment of infectious lesions, because they allow the recognition of the pathogens and thus of the appropriate antimicrobial medication that can be chosen to fight the disease.

Only 20 years after the pioneer research of Rams, Link[25] (1983), there are several scientific papers in the literature that demonstrate that: a) the microbiota of healthy peri-implant sites is similar to that of healthy periodontal sites (large number of Gram-positive facultative saccharolytic cocci and rods); b) the microbiota of peri-implantitis sites is similar to that of periodontitis sites, presenting expressive prevalence of Gram-negative anaerobic proteolytic rods and spirochetes (bacterial shift); c) the periodontal pocket can be a reservoir of pathogens capable of infecting the peri-implant area, putting partially edentulous patients at greater risk than totally edentulous patients.

However, despite these researches, according to Quirynen *et al.*[24] (2002), the effect of periodontal disease in implant users is still unknown. This opinion justifies the propositions of our study, which are:

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1. To identify, by using the polymerase chain reaction (PCR), the presence of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia*, signs of the risk of developing the disease, in clinically healthy peri-implant sulci of patients with and without a history of periodontal disease, in order to determine if this condition has a decisive influence on the colonization of these bacteria;
2. To confirm the importance of postoperative control in relation to the risk of developing the disease, especially in patients with a history of periodontitis.

MATERIAL AND METHODS

Patients and peri-implant clinical exam

After the approval of the Research Ethics Committee of Experimentation on Humans, University of Santo Amaro (São Paulo, Brazil), 19 partially edentulous patients were selected; these patients had been dental implant users for at least one year and had not received treatment with antibiotics or immunosuppressants during the last three months or periodontal treatment during the last two months previous to the study. Ten patients had a history of periodontitis. Seven out of the 19 patients were males and the ages of the 19 patients ranged from 30 to 64. All selected patients signed a specific consent form of agreement and after the formal procedures they were examined with a millimeter teflon probe (Hu-Friedy, Chicago, USA) to determine the depth of the peri-implant sulcus and the presence of bleeding on probing.

Sample collection

After the partial isolation of the area with cotton rolls and the removal of the supra-gingival biofilm with teflon curettes (Hu-Friedy, Chicago, USA) we proceeded to collect the material. Two cones of sterilized absorbent paper #40 (Dentsply®, Petrópolis, RJ, Brazil) were introduced in the peri-implant sulcus as deep as possible for 60 seconds[7] and transferred to an Eppendorf tube (Fisher Scientific, Pittsburgh, PA, USA) containing 400 µl of Milli-Q ultra-pure sterilized water (Millipore Ltda., São Paulo, Brazil), maintained at 4°C or transported immediately to the Laboratory of Anaerobes, Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo.

Polymerase chain reaction (PCR)

PCR was used to detect the DNA of the target bacteria with species-specific initiators (Invitrogen do Brasil Ltda., São Paulo, SP, Brazil) derived from the sequence of the gene 16S rDNA. The DNA of the bacteria present in the samples was extracted by boiling for 15 minutes[5]. Then the samples were centrifuged at 14,000 g for 10 minutes and the supernatant containing DNA was used immediately or stocked at –20°C. The amplification of the DNA was accomplished in final volumes of 25 µl containing 10 µl of the DNA extracted from each material, and the following substances: 2.5 µl of PCR buffer (10 X) (Boehringer Mannheim, Indianapolis, IN, USA), 1.25 µl of $MgCl_2$ (50 mM) (Invitrogen do Brasil Ltda., São Paulo, SP, Brazil), 1.0 µl of the dNTP mixture (0.2 mM) (Invitrogen do Brasil Ltda., São Paulo, SP, Brazil), 1.0 µl of each specific initiator (0.4 µM), 0.25 µl of Taq DNA polymerase (0.5 IU) (Invitrogen do Brasil Ltda., São Paulo, SP, Brazil), and 8.0 µl of Milli-Q sterilized H_2O. The pairs of initiators were synthesized according to Ashimoto *et al.*[3] (1996) and Avila-Campos *et al.*[4] (1999) (Table 1).

The reaction was accomplished in a thermocycler (Perkins Elmer, Gene Ampl PCR System 9700, São Paulo, Brazil) programmed for a cycle of 94°C for 5 minutes; 30 cycles of 94°C for 30

TABLE 1 - Specific Initiators, hybridization temperature and amplified products used in the PCR test for the target bacteria selected.

	Initiators' sequence 5' → 3'	Hybridization temperature	Amplified products
Actinobacillus actinomycetemcomitans	GCA GGA TCC ATA TTA AAT CTC CTT GT GCG GTC GAC AAC CTG ATA ACA GTA TT	55°C	0.5 kb
Porphyromonas gingivalis	GGC TTG AGT TCA GCG GCG GCA G CCC CGA AGG AAG ACG GTT TTC ACC ATC AG	60°C	0.6 kb
Prevotella intermedia	AAC GGC ATT ATG TGC TTG CAC CTC AAG TCC GCC AGT TCG CG	50°C	0.4 kb

seconds; 50°C, 55°C or 60°C (according to each pair of specific initiators) for 30 seconds; and a cycle of 72°C for 5 minutes. The products of the amplification were submitted to electrophoresis (Biorad®, São Paulo, Brazil) in agarose gel (Invitrogen do Brasil Ltda., São Paulo, SP, Brazil) at 1%, in current source, at 70 V for 150 minutes. After this procedure, the gel was colored with bromide ethidium (0.5 μg/ml) (Invitrogen do Brasil Ltda., São Paulo, SP, Brazil), submitted to a trans-illuminator with ultraviolet light (Invitrogen do Brasil Ltda., São Paulo, SP, Brazil) and photographed (Digital Kodak Science System-DC 120, Rochester, NY, USA). For the negative control of the amplification, the DNA was substituted by water and, for the positive control, the DNA of recognized samples of each researched species was used. As a marker of molecular weight, 1 kb DNA ladder (Gibco-BRL®, São Paulo, Brazil) was used.

RESULTS

The results of the analysis of the clinical parameters and of the microbiologic procedure are described in Table 2.

Seven peri-implant samples were positive for the target microorganisms.

Associations of these pathogens were observed in only three of the 19 samples; the DNAs of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* were detected only in sample 4 (history of periodontal disease, sulcus depth of 5 mm and bleeding on probing). In sample 5 (without history of periodontal disease, depth of 2 mm and bleeding on probing), the DNAs of both *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* were found. Sample 18 (without history of periodontal disease, depth of 3 mm and bleeding on probing) contained the DNAs of *Porphyromonas gingivalis* and *Prevotella intermedia*.

TABLE 2 - Anamnesis, clinical and microbiologic data (PCR) of the 19 patients analyzed.

Patients	HPD	DPIS	BP	Smoke	Age	Sex	TS	Results
Sample 1	Yes	2 mm	Yes	No	63	M	24 months	-
Sample 2	Yes	3 mm	Yes	No	53	F	18 months	-
Sample 3	Yes	3 mm	Yes	No	46	F	36 months	-
Sample 4	Yes	5 mm	Yes	No	53	M	30 months	*Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans*
Sample 5	No	2 mm	Yes	No	30	F	36 months	*Prevotella intermedia* and *Actinobacillus actinomycetemcomitans*
Sample 6	Yes	1 mm	No	No	63	M	12 months	-
Sample 7	Yes	3 mm	Yes	No	64	F	24 months	-
Sample 8	No	1 mm	No	No	58	F	18 months	*Porphyromonas gingivalis*
Sample 9	Yes	2 mm	No	No	53	F	48 months	*Porphyromonas gingivalis*
Sample 10	No	2 mm	No	No	49	M	22 months	-
Sample 11	Yes	1 mm	Yes	Yes	50	F	12 months	*Porphyromonas gingivalis*
Sample 12	No	4 mm	No	Yes	39	M	60 months	-
Sample 13	Yes	2 mm	No	No	52	M	36 months	-
Sample 14	No	3 mm	No	No	63	F	18 months	-
Sample 15	Yes	3 mm	No	Yes	61	F	48 months	-
Sample 16	No	2 mm	No	No	37	F	36 months	-
Sample 17	No	1 mm	No	No	53	F	36 months	*Porphyromonas gingivalis*
Sample 18	No	3 mm	Yes	No	44	F	48 months	*Porphyromonas gingivalis* and *Prevotella intermedia*
Sample 19	No	1 mm	No	No	43	M	120 months	-

HPD = History of periodontal disease; DPIS = Depth of the peri-implant sulcus; BP = Bleeding on probing; TS = Time after the implant surgery.

Porphyromonas gingivalis was the only target pathogen detected in sample 8 (without history of periodontal disease, depth of 1 mm and no bleeding on probing), sample 9 (history of periodontal disease, depth of 2 mm and no bleeding on probing), sample 11 (history of periodontal disease, depth of 1 mm and bleeding on probing), and sample 17 (without history of periodontal disease, depth of 1 mm and no bleeding on probing).

In summary, the DNA of at least one of the target pathogens was detected in only three of the 10 peri-implant samples of patients with history of periodontitis. In the other seven samples, those species were not detected, but they were found in four of the nine patients without history of periodontal disease.

DISCUSSION

Biofilms associated with periodontitis and peri-implantitis frequently harbor high levels of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans*[7,10,11,24,29]. These pathogens are also related to the risk of periodontitis[11,26,29]. These observations led us to research the presence of these mentioned bacteria in seemingly healthy peri-implant sites and to try to establish a relationship between them and the risk of disease.

With this purpose, we chose the PCR, a highly sensitive molecular method that detects minimum amounts of the target DNA. PCR is also reproducible and relatively easy to execute as well as particularly valuable in the detection of non-cultivable organisms or those that are difficult to be distinguished in the cultivation[4,28].

The peri-implant sites of patients with periodontitis can present high concentrations of pathogens in the periodontal pockets[2,10,19,30]. Therefore, periodontal lesions considered irreversible can determine the indication of extraction of unhealthy teeth to avoid infection of future peri-implant sites[8]. The presence of severe periodontitis should not hinder the insertion of implants; however, this is a high risk situation that should be minimized by previous periodontal treatment as well as appropriate post-surgical control, which, ideally, should include the analysis of the microbiota[6,10,12,15]. The need of that analysis was emphasized by Alcoforado *et al.*[1] (1991), who, besides isolating periodontopathogens from sites with peri-implantitis, showed the presence of high proportions of *Candida albicans* and exogenous bacteria (Gram-negative enteric rods, *Staphylococcus* spp. and *Pseudomonas aeruginosa*), probably due to the inadequate administration of antibiotics that affect the resident microbiota. The microbiologic exam is often indicated when clinical and radiographic signs of disease exist[23], but our results suggest that it should also be indicated to identify the risk of the disease.

We should consider that pathogens are usually found at a proportion lower than 1.0% (supplemental microbiota) in healthy sites[9] where they are controlled by the host's defense[2,9]. Likewise, the observation of pathogens at peri-implant sites does not necessarily mean failure of the implant[18]. That statement partially explains some unexpected results found in this research, such as the detection of the target pathogens in clinically healthy sites and in patients without history of periodontal disease.

Another aspect worthy of emphasis is that hosts with similar levels of pathogens can present different clinical manifestations due to individual variations in their inflammatory response. People with polymorphism in genes that codify the production of interleukins and the tumor necrosis factor-alpha (response mediators) are more susceptible to periodontitis[17]. This fact can probably be extrapolated to include peri-implantitis, which is equally dependent on the interaction between the levels of microbial aggression and the response of the host. Thus, even if pathogens are present in higher proportions, clinical signs are not always present or do not arise with the same intensity. However, inadequate biofilm control allows the gradual installation of larger numbers of pathogens that produce metabolites, which, in addition to destroying the tissues, incite defense cells to secrete mediators that contribute to an increase in tissue destruction.

The sulcus depth and bleeding on probing are related to the quality of the peri-implant microbiota; when the depth is deeper than 3 mm a numeric increase of anaerobes like spirochetes and several Gram-negative bacteria, mainly rods, clinically expressed by bleeding on probing and, sometimes, by suppuration, is observed[18,21]. Therefore, the clinical state of peri-implant sites, mainly in patients with periodontitis previous to the surgery, needs to be constantly evaluated. Infections in the peri-implant tissues caused by the biofilm do not react adequately to antibiotic treatment, sometimes forcing their removal to control the process. However, the modern systems of dental implants make it possible to control bacterial colonization. With this purpose, Gromatzky, Sendyk[15] (2002)

established a modern protocol seeking to preserve osseointegration.

The absence of relationship amongst the presence of pathogens, clinical signs of severe inflammation and history of periodontitis, observed in this research, contradicts reports of several authors[2,8,13,14,16,20,22,27] and the following considerations try to explain that discrepancy:

1. undetection does not mean that microorganisms are not present in the examined material, due to the possibility of technical failure in collection, transport and laboratorial processing of the material, sometimes making it necessary to repeat the exam;
2. PCR detects low amounts of DNA of target microorganisms, including even the non-viable ones; therefore, the result also shows the past presence of this microorganism in the examined material;
3. the possibility of random collection of some clones with defective or absent genes that codify the production of virulence factors should be considered. The pathogenic species show variations in the degree of production of those factors and supplementary genetic exams are necessary to distinguish the most virulent clones.

CONCLUSIONS

1. PCR proved efficient to identify the DNA of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* even in peri-implant sulci with reduced depths.
2. The detection of DNA of the bacterial species studied in apparently healthy peri-implant sites can indicate peri-implantitis risk, making the establishment of a more strict preventive control in those patients compulsory, in order to guarantee the success of the treatment.

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