Evaluation of Peri-implant Microbiota Using the Polymerase Chain Reaction in Completely Edentulous Patients Before and After Placement of Implant-Supported Prostheses Submitted to Immediate Load

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Purpose: *To evaluate, by means of the polymerase chain reaction (PCR), the presence of* Actinobacillus actinomycetemcomitans*,* Porphyromonas gingivalis*, and* Prevotella intermedia *in the mandibular arch of completely edentulous subjects before implant placement and 4 and 6 months after the placement of mandibular implant-supported fixed prostheses.* ***Materials and Methods:*** *Fifteen patients had bacterial plaque collected with sterile paper points before implant placement (ie, when they were completely edentulous) and at 3 sites on the peri-implant sulci displaying the largest probing depths after placement of 5 implants.* ***Results:*** *For the edentulous arch,* A actinomycetemcomitans *was detected in 13.3% of subjects,* P intermedia *was detected in 46.7% of subjects, and there was no detection of* P gingivalis*. After 4 and 6 months of implant placement,* A actinomycetemcomitans *was detected in 60% and 73.3% respectively;* P intermedia *in 46.7% and 53.3% respectively; and* P gingivalis *in 46.7% and 53.3%, respectively.* ***Discussion:*** *Future diagnosis should not be restricted to distinguishing individuals at risk of peri-implant disease. Such individuals should be identified by the employment of microbiologic methods and knowledge of the multifactorial nature of the host response to the action of microorganisms.* ***Conclusions:*** *The longer the implants were in the oral cavity, the higher the occurrence of* A actinomycetemcomitans*,* P gingivalis*, and* P intermedia *in the peri-implant sulci of completely edentulous patients rehabilitated with mandibular implant-supported fixed prostheses was, without any clinical or radiographic evidence indicating peri-implant disease in the studied period.* (Before-and-After Study) Int J Oral Maxillofac Implants 2006;21:262–269

Key words: *dental implants, microorganisms, polymerase chain reaction*

The employment of osseointegrated implants in dentistry has been increasing with time, and despite the high rates of success achieved with this treatment, the reasons for failure are still a matter of concern.[1] The literature reports 3 important factors that may lead to failure: surgical trauma, occlusal overload, and bacterial infection.[2,3]

The microorganisms involved in bacterial infection include the anaerobic microorganisms of the bacterial plaque on peri-implant tissues, especially *Prevotella intermedia*, *Porphyromonas gingivalis,* and *Actinobacillus actinomycetemcomitans*. Thus, the clinical practice of implant dentistry requires the development of techniques for bacterial identification that may be routinely employed for achieving a fast and efficient diagnosis and establishing a proper treatment plan for each individual.

The polymerase chain reaction (PCR) is a relatively new method first described by Kary Mullis in the late 1980s.[4] It presents some advantages when compared to other microbiologic tests. It has become one of the most widely employed methods in molecular biology, because it is a fast and simple means to produce relatively high numbers of copies of DNA molecules obtained from minimal amounts of fragments of microorganisms' DNA.[5–10] The PCR makes possible the determination of the presence of a small number of pathogens in several sites of a same subject.[4,7] A further advantage is that microorganisms do not necessarily need to be kept viable, which sim-

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plifies the analysis and increases the sensitivity in the process of detection.[4,8–10]

The present study aimed at analyzing the putative presence of the microorganisms *A actinomycetemcomitans, P gingivalis,* and *P intermedia* by means of the PCR in completely edentulous subjects before and after placement of mandibular implant-supported fixed prostheses submitted to immediate load.

MATERIALS AND METHODS

Sample Selection

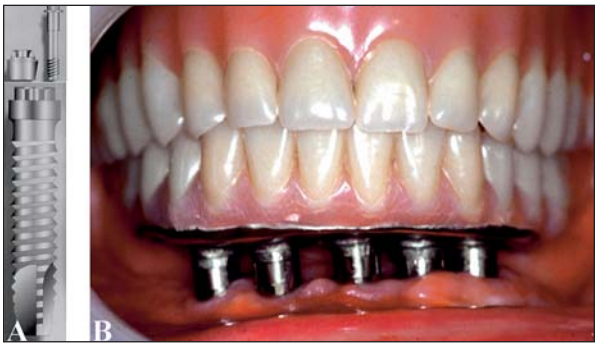
Fifteen individuals, 8 women and 7 men with a mean age of 59.9 years (range, 40 to 78 years), were selected based on the following criteria: *(1)* complete edentulism, *(2)* intent to restore their dentition with implant-supported complete dentures, and *(3)* agreement to participate in the study and signature of an informed consent document.

Each subject received 5 smooth Titamax implants (Neodent Implantes Osseointegráveis, Curitiba, Brazil) with lengths of 13, 15, and 17 mm and diameters of 3.75 and 4 mm between the mental foramina (Figs 1a and 1b). Conical mini-abutments measuring 3 or 4 mm in height were placed and submitted to immediate load. No subject had employed antibiotics or oral antiseptics in the 3 months before sample collection, and all subjects submitted to radiographic follow-up during the study.

Collection of the Microbiologic Samples

Three samples of protein films were collected from 3 selected sites in the completely edentulous mandibular alveolar ridges before placement of the implants. During sample obtainment, the sites were isolated with sterile gauze that covered the surfaces of the oral mucosa on the mandibular alveolar ridges. The samples were collected by rubbing the mucosa with 3 standardized sterile paper points (no. 40 Tanari; Tanariman Industrial, Amazonas, Brazil). In each patient, 3 paper points were rubbed in 3 areas of the mandibular alveolar ridge, the anterior right and left (ie, the right and left sides of the interforaminal area, where the implants were placed) and the posterior area, for a total of 9 paper points per patient per period. These points were immediately placed in labeled plastic cryotubes that indicated the number of the subject and the area of the mandibular alveolar ridge from which the sample was collected (Figs 2a to 2d). The samples were kept at –20°C in a freezer up to processing.

Four and 6 months after placement of the mandibular implant-supported immediately loaded fixed prostheses, anamnesis was performed and the prostheses were removed to allow clinical examination and evaluation of the oral hygiene and probing depth. Oral hygiene was subjectively assessed, taking into account the presence and amount of bacterial plaque and calculus by inspection and utilization of plastic curettes, without the use of disclosing agents. The condition was scored as good (small amount of bacterial plaque and no calculus), fair (moderate amount of bacterial plaque and/or calculus), or poor (large amount of bacterial plaque and/or calculus). The osseointegrated implants were numbered from 1 to 5 from (the right to the left side of the mandible) to allow standardization of the clinical and radiographic examination (Fig 3).



Figs 1a and 1b Smooth Titamax implants of the Neodent Osseointegrated Implant System were placed in the area between the mental foramina.

The sites were isolated with sterile gauze, and the bacterial plaque and supragingival calculus at the abutments was removed with plastic curettes. The abutments were cleaned with sterile cotton wicks before collection of the subgingival plaque in order to avoid contamination.

The probing depths of the peri-implant sulci were measured on the mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual aspects of each implant by means of a plastic periodontal probe (Periowise; Premier Dental, Plymouth Meeting, PA) (Figs 4a to 4d). In each patient, the 3 sites in the sulci presenting the largest probing depths at 4 and 6 months postplacement had their peri-implant subgingival plaque collected. Sample collection was performed on different peri-implant sulci to avoid the risk of nondetection of a certain pathogen that might have been absent in some sulci yet present in others.[5]

Three paper points were individually inserted under light pressure in each site of the peri-implant sulci until resistance was felt and were kept there for 10 seconds[11] to obtain peri-implant subgingival plaque. Thereafter, these points were inserted in labeled plastic cryotubes (Figs 4e and 4f) indicating the number of the subject and the number of the implant and corresponding sites. The samples and were kept at –20°C in a freezer until processing. There were 3 times of examination—pretreatment (when

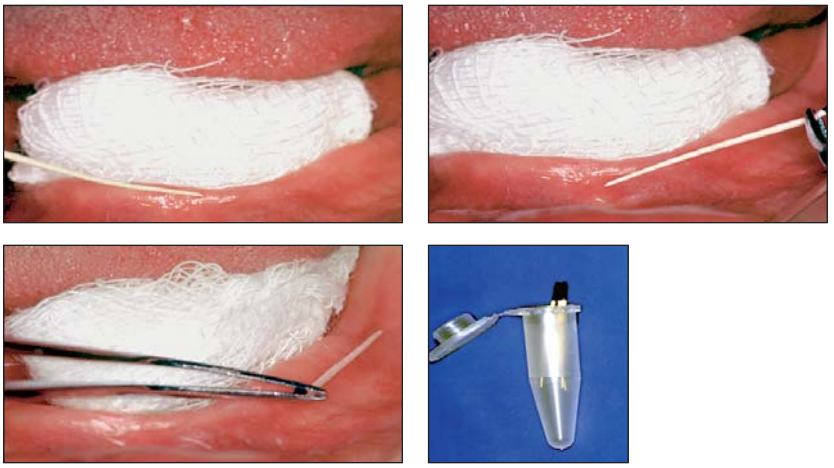


Fig 2 *(a to c)* Collection of protein films of 3 samples of 3 areas (anterior right, anterior left, and posterior) on the completely edentulous mandibular alveolar ridge. *(d)* Samples were placed in labeled plastic cryotubes.

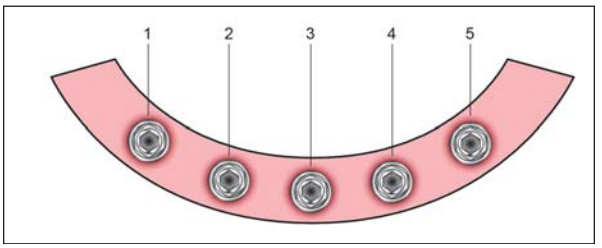


Fig 3 Numbering of the osseointegrated implants on the mandibular alveolar ridge.

the patients were completely edentulous), 4 months postplacement, and 6 months postplacement. Ninety samples obtained from the oral cavities of 15 individuals were analyzed from a total of 135 samples; the remaining 45 samples (15 from each period) were stored for utilization if some samples were lost.

Bacterial plaque and/or calculus were cleaned off with plastic curettes and tap water before reinsertion of the prostheses. All appointments included oral hygiene instructions. Patients were asked not to use oral antiseptics to avoid changes in the oral microbiota during the study.

DNA Extraction and Analysis

Each plastic cryotube containing a sample received

- 100 μL of TAS (50 mmol/L Tris hydrochloride [HCl], pH 8.0; 50 mmol/L EDTA; and 150 mmol/L sodium chloride [NaCl])
- 10μL of 10% sodium dodecyl sulfate (SDS)
- 2μL of proteinase K

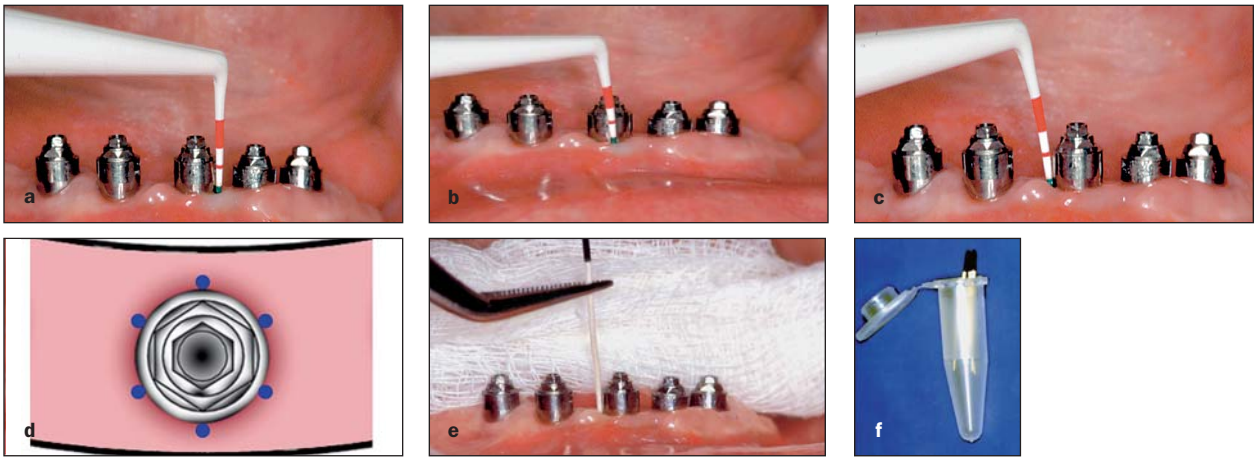
The cryotube was kept in water-bath at 60°C for 1 hour. Thereafter 50 μL of phenol and 50 μL of chloroform were added. This was followed by centrifugation (Microcentrifuge model 213; Fanem, São Paulo, Brazil) for 3 minutes at 10,000 rpm. Fifty microliters of chloroform was added to the supernatant followed by centrifugation for 3 minutes at 10,000 rpm. This procedure was repeated once. Potassium acetate and 100% ethanol were then added to the supernatant, which was then maintained at 4°C in a refrigerator overnight or in the freezer for 2 hours. After refrigeration, the supernatant was centrifuged for a further 10 minutes at 10,000 rpm, and the liquid was discarded. This procedure was followed by addition of 70% ethanol, centrifugation at 10,000 rpm for 3 minutes, and elimination of the supernatant. Afterward, the cryotube was placed in a culture oven (model 002 CB; Fanem) at 37°C for 5 minutes and TE (10 mmol/L Tris-Cl, pH 8.0; and 1mmol/L EDTA, pH 8.0) was added to the dried material, which was then kept in a refrigerator for future analysis.

PCR Detection

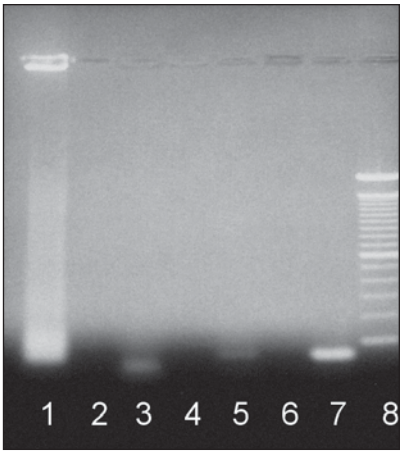
The following was placed in a sterile cryotube:

- Two μL of genomic DNA
- Nucleotides
  - Deoxycytidine triphosphate (dCTP)
  - Deoxyadenosine triphosphate (dATP)
  - Deoxyguanine triphosphate (dGTP) and
  - Feoxythymidine Triphosphate (dTTP)
- The polymerase enzyme (Gibco-Life Technologies, Gaithersburg, MD)
- The oligonucleotides (Gibco-Life Technologies) (Table 1)
- The buffer solution (10× PCR buffer—500 mmol/L KCl, 15 mmol/L $MgCl_2$, 100 mmol/L tris-HCl; pH 9.0)

Amplification was performed in a DNA Thermal Cycler (Gene Amp.-PCR System 2400; Perkin Elmer, Wellesley, MA) programmed for 94°C (5 minutes), and the temperature was lowered to 65°C, which is the annealing temperature adequate for each primer pair, for 30 seconds. Finally, the temperature was raised to 72°C (2 to 5 minutes) to allow completion of DNA extension. Repetition of these procedures of



Figs 4a to 4d The sulci were probed at the mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual aspects of each implant in the mandibular alveolar ridge by means of insertion of a plastic periodontal probe (Periowise).
Figs 4e and 4f Paper points were individually inserted and placed in labeled plastic cryotubes.
Fig 5 *(Right)* Detection of the presence or absence of *A actinomycetemcomitans*, *P gingivalis*, and *P intermedia* and achievement of the outcomes of each subject.



denaturation, annealing, and synthesis for 30 cycles led to the achievement of millions of copies of a certain double-stranded DNA sequence. After completion of the cycles, the material was kept in a freezer at –20°C until reading in the electrophoresis gel.

Electrophoresis

The DNA samples were placed on an agarose gel (Sigma-Aldrich, St Louis, MO) prepared at a concentration of 0.8 g after solidification. The electric current for migration was employed at 100 Kw for 1 hour. The DNA ladder (100 bp) was simultaneously placed on the same gel.

Interpretation of the Electrophoresis

After electrophoresis, the fluorescence of the gel was analyzed on an ultraviolet (UV) light transilluminator (Pharmacia LKB Macro). Evidence of fluorescence on the gel was regarded as positive, and the absence of fluorescence was regarded as negative. This procedure was performed for all samples of subjects for the reference bacteria. The gel was photographed with a Polaroid camera (model MP4, Polaroid, Waltham, MA) for detection of the presence or absence of the respective bands (Fig 5):

- Groove 1: *A actinomycetemcomitans* (reference bacteria
- Groove 2: patient sample without *P intermedia*
- Groove 3: *P intermedia* (reference bacteria)
- Groove 4: patient sample without *P gingivalis*
- Groove 5: *P gingivalis* (reference bacteria)
- Groove 6: blank control
- Groove 7: patient sample positive for *A actinomycetemcomitans*
- Groove 8: DNA ladder

Statistical Analysis

Statistical analysis of the comparison between the mean peri-implant probing depths was conducted with paired *t* tests at a significance level of 5%. Evaluation of the colonization of *A actinomycetemcomitans*, *P gingivalis,* and *P intermedia* for the 3 study periods was carried out with the McNemar test at a significance level of 5%.

RESULTS

The reasons for tooth loss are depicted in Fig 6. It should be noted that 9 subjects (60%) became com-

Table 1 Nomenclature and Composition of the Species-Specific Primer Pairs for PCR

Primers	Oligonucleotide sequence 5' → 3'
A actinomycetemcomitans	(5' GCT AAT ACC GCG TAG AGT CGG 3')
	(5' ATT TAC CAC CTA ACT TAA AGG 3')
P gingivalis	(5' AGG CAG CTT GCC ATA CTG CG 3')
	(5' ACT GTT AGC AAC TAC CGA TGT 3')
P intermedia	(5' AAC GGC ATT ATG TGC TTG CAC 3')
	(5' CTC AAG TCC GCC AGT TCG CG 3')

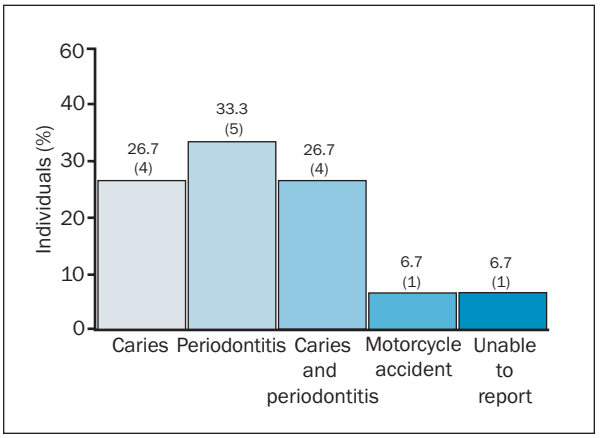


Fig 6 Reasons for the tooth loss reported by the 15 subjects (in percentages and numbers of individuals).

pletely edentulous because of periodontitis (including 5 individuals who lost their teeth because of periodontitis alone and 4 who reported having lost their teeth because of both caries and periodontitis). Among the 5 subjects that lost their teeth because of periodontitis alone, *P gingivalis* was the most frequently detected microorganism in the peri-implant sulci at 4 and 6 months after placement of the implants; it was detected in 4 of the 5 individuals.) *A actinomycetemcomitans* was detected in 3 of the 5 subjects 6 months after placement of the implants. The values for probing depths in the 450 peri-implant sulci of 15 subjects at 4 and 6 months after placement of the mandibular implant-supported fixed prostheses submitted to immediate load can be seen in Fig 7. Some areas presented bleeding upon peri-implant probing; however, this study did not aim to correlate inflammation/bleeding with the presence of pathogens. Probing depths ranged from 1 to 5 mm, with a prevalence of sites with peri-implant probing depths from 2 to 3 mm and few sites with 1, 4, or 5 mm. Comparison of the mean peri-implant probing depths of 2.63 mm at 4 months and 2.50 mm at 6 months after placement of the implants was accomplished by means of the paired *t* test; the difference was not found to be statistically significant ($t = 1.628$; $P = .127$).

Figure 8 demonstrates the percentages of individuals who presented *A actinomycetemcomitans*, *P gingivalis,* and *P intermedia* for the 3 study periods. *A actinomycetemcomitans* was the predominant microorganism in the peri-implant sulci at 4 and 6 months. Statistically significant differences were found for presence of *A actinomycetemcomitans* before implant placement and 4 months postplacement ($P = .023$). The difference in presence of *A actinomycetemcomitans* before implant placement and after 6 months was also significant ($P = .007$).

The same was observed for *P gingivalis*—presence of this bacterium was significantly increased at 4 and 6 months postplacement compared with the completely edentulous state ($P = .023$ and $P = .008$, respectively). However, there was no statistically significant difference between presence of either *A actinomycetemcomitans* or *P gingivalis* at 4 months versus presence at 6 months ($P = .267$ and $P = .789$, respectively).

For *P intermedia,* nonsignificant differences were seen between bacterial presence before implant placement and 4 months ($P > .99$) or 6 months after placement ($P = .789$). There was also no difference between bacterial presence at 4 months and presence at 6 months ($P = .789$). With lack of a control for even semi-quantitative analysis, comparisons from 1 period to the next must be made with caution.

Subjective evaluation of the oral hygiene in the 15 subjects at 4 and 6 months after placement of mandibular implant-supported fixed prostheses under immediate load is demonstrated in Fig 9. Only 2 subjects appeared to present improvement in oral hygiene status, which was poor on the initial evaluation and improved to good.

DISCUSSION

The PCR has the potential to be used for microbiologic diagnosis/identification of pathogens related to the development and perpetuation of peri-implant lesions.

In the groups of the present study, investigation of the reasons that led to tooth loss (Fig 6) was important, since it is known that, after extraction of all teeth, many microorganisms disappear from the oral cavity, especially anaerobic pathogens, because of the absence of sites available.[12]

This study employed the PCR in samples from completely edentulous individuals with complete dentures without dental implants, which revealed that *A actinomycetemcomitans* was present in the

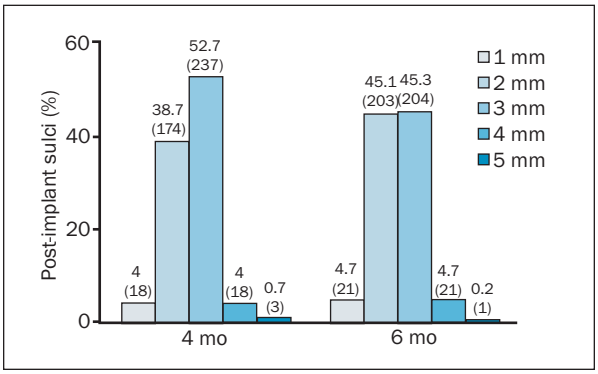


Fig 7 Probing depths (in mm) of 450 sites on the peri-implant sulci of the 15 individuals at 4 and 6 months after the placement of mandibular implant-supported fixed prostheses submitted to immediate load. The mean probing depths were 2.63 mm at 4 months and 2.50 mm at 6 months (paired *t* test; $t = 1.628$; $P = .127$).

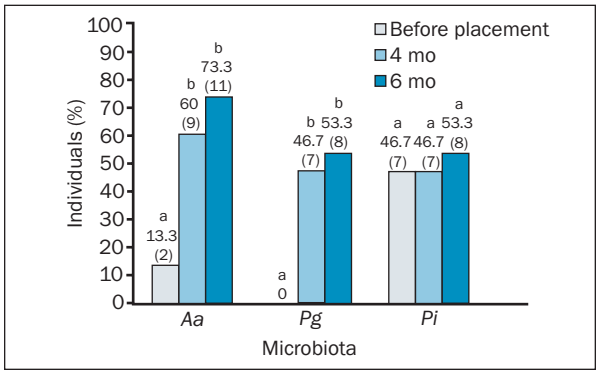


Fig 8 Distribution of the presence of *A actinomycetemcomitans*, *P gingivalis*, and *P intermedia* for the 3 study periods. For each bacterium, stages with a same letter are not statistically significantly different from each other (McNemar test).

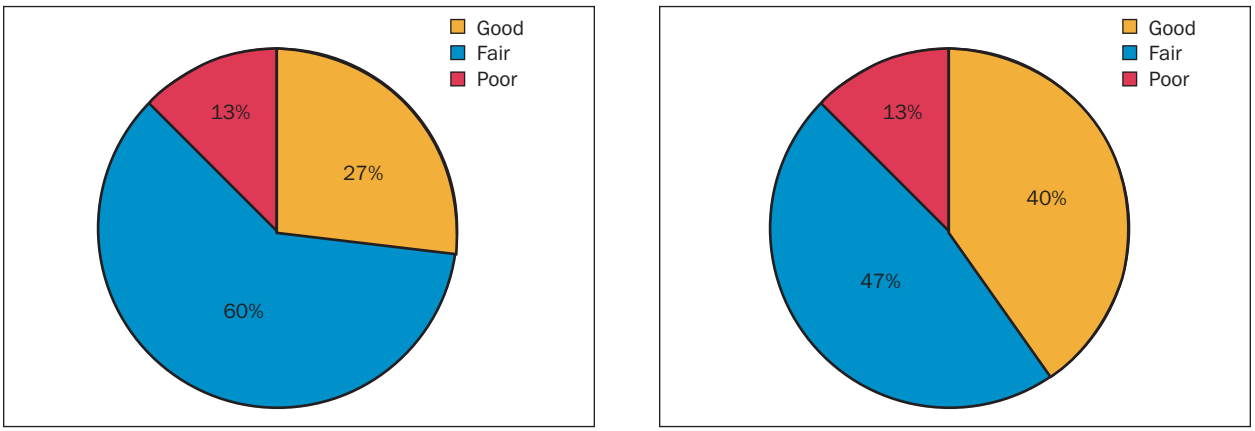


Fig 9 Oral hygiene scores of the 15 individuals, at *(a)* 4 and *(b)* 6 months after placement.

mucosa of the mandibular alveolar ridges in 2 of the 15 subjects (13.3%) (Fig 8). Several studies in the literature have reported nondetection of *A actinomycetemcomitans* by means of dark field microscopy or culture on the oral mucosa of completely edentulous individuals without implants.[12–15] Danser and associates[12] detected *A actinomycetemcomitans* on the mucosa and in saliva and supragingival plaque before tooth extraction in 2 subjects (25%), but the bacteria were not detected 1 or 3 months after tooth extraction in any individual. However, in another study of Danser and associates[3] this microorganism was detected by means of culture in 1 of 20 completely edentulous subjects without implants (5%) a short period after the extraction of teeth with a previous history of periodontitis. Their data revealed that this subject presented *A actinomycetemcomitans* while still dentate. This microorganism was not found 1 year after implant placement.

P gingivalis was not found in this study by means of the PCR on the mucosa of completely edentulous individuals wearing complete dentures without dental implants (Fig 8). This finding is in agreement with the findings revealed in Könönen and colleagues'[14] culture study of 50 subjects and in Danser and associates'[12] study of 8 subjects (100%) at 1 and 3 months after complete tooth extraction. In the latter study, the microorganism had been observed in 6 of 8 dentate individuals. Moreover, Danser and associates[3] did not detect *P gingivalis* in 2 dentate subjects either before or after these subjects became completely edentulous, without implants.

The low detection of *A actinomycetemcomitans* and the absence of *P gingivalis* in completely edentulous subjects in this study may be explained by the fact that colonization with these microorganisms probably did not occur before tooth extraction. If it did occur, these bacteria might be present in oral mucosa in amounts below the detection level or even in nonsampled sites. However, Danser and coworkers[15] evaluated the oral microbiota of 26 completely edentulous individuals without implants by means of culture and

detected *P gingivalis* in only 2 individuals, on the tonsils in 1 and in the saliva of the other. According to these authors, the disappearance of putative periodontopathogens immediately after complete tooth extraction might be a temporary event; they might be found once again in a future evaluation.

P intermedia was detected by PCR on the oral mucosa of the mandibular alveolar ridge in 46.7% of the subjects (7 individuals) prior to implant placement (Fig 8). This is in agreement with the findings of Danser and colleagues,[12] who conducted a culture study on completely edentulous individuals without implants and with a previous history of periodontitis. The presence of *P intermedia* was detected on the oral mucosa and in the saliva of 4 individuals (50%) after tooth extraction.

In another study by Danser and coworkers[15] 26 completely edentulous subjects, wearers of complete dentures without implants, were evaluated, and *P intermedia* was detected in the culture medium for 7 individuals (27%). On the other hand, a later study by Danser and colleagues[3] did not detect this microorganism by means of culture a short period after total tooth extraction, even though the patients presented *P intermedia* while still dentate. The outcomes of the present study demonstrated a greater presence of *P intermedia* on the oral mucosa of the mandibular alveolar ridges in completely edentulous patients (Fig 8) when compared to other studies.

Further studies are required to evaluate whether the presence of such microorganisms in completely edentulous individuals constitutes a risk factor for long-term survival of future dental implants,[15] since the oral mucosa is regarded as a reservoir for bacteria in the peri-implant sulci.[3,16,17] Microbiologic evaluation at 4 and 6 months after placement of the mandibular implant-supported fixed prostheses revealed the presence of *A actinomycetemcomitans* in 9 (60%) and 11 individuals (73.3%), respectively; this may be explained by the higher sensitivity of the PCR. The present results disagree with the findings of Ong and associates,[18] who detected *A actinomycetemcomitans* in the culture medium for only 1 implant (5.2%) in 19 subjects evaluated in a period of 30 months after implant placement. The present results also disagree with the results of George and associates[19] who found positive values in 14 of 114 sites (12.3%) examined by latex agglutination in completely edentulous individuals with stable implants.

The present study detected *P gingivalis* in 46.7% and 53.3% of the sample (7 and 8 individuals) at 4 and 6 months after placement of mandibular implant-supported fixed prostheses, respectively. This demonstrates the probable superiority of the PCR in the ability to detect bacteria when compared to dark field microscopy, culture, and immunochemistry. This bacterium was not detected after placement of clinically healthy implants in totally edentulous subjects in studies[3,16–18] in which these other methods were used.

In the present study, *P intermedia* was detected with analysis of 60 samples with the PCR in 46.7% and 53.3% of the sample (7 and 8 individuals) at 4 and 6 months after placement of mandibular implant-supported fixed prostheses, respectively. In a study by Mombelli and coworkers,[16] *P intermedia* was found in low percentages by means of dark field microscopy and culture after the placement of implants in the mandibles of completely edentulous individuals. In a study of cultures, Ong and colleagues[18] detected this microorganism in 7 healthy sites in 5 of 19 individuals (26%). Danser and associates[3] found *P intermedia* by means of culture in 5 of 11 individuals (45%) with peri-implant pockets. In a study of cultures by Mombelli and Mericske-Stern,[17] *P intermedia* was detected in 8.8% of 36 samples of plaque from the peri-implant sulci of 19 subjects.

At the last time point studied (6 months after placement of mandibular implant-supported fixed prostheses), it was observed that more than 50% of the individuals in the study had all 3 microorganisms (Fig 8). This high level of detection could be the result of the sensitivity of the method of microbiologic analysis used (PCR), physical factors (humidity, temperature, and pH), nutritional aspects, and the carbon dioxide and oxygen concentrations on the peri-implant sulci of these subjects, which are adequate for the survival, growth, and maintenance of these microorganisms.

In the present study, among the 3 microorganisms evaluated, *P gingivalis* was the most detected at 4 and 6 months in the peri-implant sulci of the individuals that lost their teeth because of periodontitis (4 of 5), followed by *A actinomycetemcomitans* in 3 subjects at 6 months after prostheses placement.

In completely edentulous subjects with implants, the mean probing depths were smaller than 3 mm in studies by Adell and coworkers,[20] Bower and associates,[21] and Mombelli and Mericske-Stern.[17] These results are similar to those observed in the present study, in which most peri-implant sulci revealed probing depths of around 3 mm, with means of 2.63 and 2.50 mm 4 and 6 months prosthesis placement. Comparison of these means did not reveal any statistically significant differences, which may be the result of the short period between the probing depth measurements and the relatively short period the implants were in the oral cavity, which permitted the peri-implant tissues to remain clinically healthy.

The previous colonization of completely edentulous sites may likely influence the microbiota found adjacent to implants, as demonstrated in the present

study. There is a need for long-term follow-up of the subjects for a better explanation of this aspect.

If tests to differentiate between virulent and nonvirulent species, exogenous and endogenous microorganisms, and susceptible and resistant individuals could be developed, as well as tests to determine the number of pathogens required to induce the disease, the ability of the clinician to interfere with the infection chain and reduce disease onset would be remarkably improved.[22]

CONCLUSIONS

- In the completely edentulous stage before implant placement, 13.3% of the sample (2 individuals) presented with *A actinomycetemcomitans*, and 46.7% (7 individuals) with *P intermedia*. *P gingivalis* was not found.
- After placement of mandibular implant-supported prostheses submitted to immediate load, *A actinomycetemcomitans* was detected in 60% of the sample (9 individuals) after 4 months and 73.3% of the sample (11 individuals) after 6 months; *P gingivalis* was detected in 46.7% (7 individuals) and 53.3% (8 individuals); and *P intermedia* in 46.7% (7 individuals) and 53.3% (8 individuals), respectively.
- The longer the implants were in the oral cavity, the higher the occurrence of *A actinomycetemcomitans*, *P gingivalis,* and *P intermedia* in the peri-implant sulci of completely edentulous patients rehabilitated with mandibular implant-supported fixed prostheses, without any clinical or radiographic evidence indicating peri-implant disease in the studied period.

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