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Bacterial Culture and DNA Checkerboard for the Detection of Internal Contamination in Dental Implants

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Abstract

Purpose: The aim of this in vitro study was to evaluate the bacterial leakage along the implant–abutment interface by the conventional bacterial culture and DNA Checkerboard hybridization method.
Materials and Methods: Twenty Branemark-compatible implants with a 3.75-mm diameter and external hexagonal platform were randomly placed in two groups of ten implant–abutment assemblies each. One group was used to analyze bacterial counts by DNA Checkerboard hybridization and the other by a conventional bacterial culture. Suspensions of *Fusobacterium nucleatum* (3 μl) were injected into the grooved internal cylinders of each implant assembly, and the abutment was connected by a 32 Ncm torque. The combined implant–abutments were individually placed in tubes containing the CaSaB culture medium and incubated in a bacteriological constant temperature oven for 14 days. The samples were observed daily as to the presence of turbidity, and after the designated time the microorganisms were collected from the implant interiors and analyzed by the two methods.
Results: After 14 days, six implant–abutment assemblies showed turbidity. Both methods indicated reduced microorganism counts in samples from the interior of the implant–abutment assemblies after incubation in the culture medium; however, the number of counts of *F. nucleatum* was higher by the DNA Checkerboard method when compared to the group analyzed by conventional bacterial cultures ($p < 0.05$).
Conclusion: The DNA Checkerboard method was shown to be more sensitive than conventional cultures in the detection of microorganisms.

Bone-integrated implants, surgically located in the alveolar bone, are an efficient support both for fixed and removable prostheses.[1,2] Bacterial infection can interfere with osseointegration during the healing phase of the surgical intervention and can cause peri-implantitis of the osseointegrated implant.[3,4]

The time period for contamination and bacterial stability after implant exposure to the oral cavity is still controversial, and although some investigators report the presence of microorganisms after 2 or 3 months,[5] others claim this takes place only 14 days after exposure to the oral media.[6] Infectious processes close to the bone apex may develop even during the surgical implant installation and proceed during the full functional period.

The positioning of the implant–abutment interface in the bone supracrestal area is etiologically important,[3,7,8] since a risk factor in the development of peri-implantitis is screw loosening, which would allow penetration of periodontal pathogens into the assembly interior.[8] It is accepted that the connection between implants and abutments exhibits areas where bacterial niches can develop and cause peri-implant tissue inflammation.[9]

No direct and simple method allows the clinician to quantify microbial colonization in the peri-implant tissues and implant–abutment interface.[10] The method most often used for this purpose is conventional bacterial culture, which is selective or even restricted to specific and well-known microbial species.[11]

Bacterial DNA analysis has recently allowed a fast and reliable identification and quantification of microorganisms in the oral cavity, greatly facilitating periodontal and peri-implant disease diagnosis.[12-14] DNA Checkerboard hybridization has

been frequently employed to simultaneously and rapidly identify a large number of bacterial species without losing accuracy.[15]

The aim of this in vitro study was to evaluate the bacterial leakage along the implant–abutment interface by two methods of detecting bacterial contamination, conventional microbiological cultures and DNA Checkerboard hybridization.

Materials and methods

Sample preparation

To evaluate bacterial contamination along the implant–abutment interface, 20 Branemark-compatible implants with a 3.75-mm diameter external hexagonal platform (SIN®, São Paulo, Brazil) were selected for connection to 20 pre-machined cobalt–chromium alloy (Co–Cr) abutments with plastic sleeves cast with nickel–chromium alloy (Ni–Cr; VeraBond II, AalbaDent, Fairfield, CA). The prosthetic abutments were wax-embedded, simulating a base construction for ceramic application, with a standardized volume and cast in a Ni–Cr alloy. After cast, the abutments were sealed in surgical-type envelopes and autoclave-sterilized at 121°C for 30 minutes. Ten of the combined implant abutments were tested by conventional microbiological methods and ten by DNA Checkerboard hybridization.

A pure culture of *Fusobacterium nucleatum* (ATCC 25586, Manassas, VA), incubated in anaerobic conditions, was used for microbiological evaluation. Bacterial suspensions were prepared by cultivating the microorganism in TSBy (Tryptic Soy Broth yeast, Difco Laboratories, Detroit, MI) for 48 hours at 35°C and further dilution in nutrient broth to a McFarland Standard density of 0.5 (1×10^8 CFU/ml). This species was chosen because it is involved in developing of peri-implantitis.[16]

For the microbiological tests, suspensions of *F. nucleatum* (3 μl) were inoculated under sterile conditions into the implant inner compartment using a micropipette (Gilson, Inc. Middleton, WI). The abutments were then carefully connected to the implants with titanium alloy screws and tightened to 32 N/cm, according to the manufacturer's instructions. During microorganism inoculations and for the abutment connection, the implants were held with sterile pliers to allow a firm torque action. The upper part of the assemblies was then sealed with a layer of gutta percha (Meta Biomed Co., Ltd, Cheongju, South Korea) and cyanoacrylate adhesives (Super Bonder, Henkel do Brazil Ltd, Itapevi, Brazil). Assembled components were immersed for 30 seconds in 5 ml sterile TSBy for evaluation of external contamination. Tubes producing cloudy broth (indicative of colonization) were excluded from further observation after bacterial growth was evaluated in culture plates.

The remaining assemblies were then completely immersed in 5 ml of the nutrient and incubated at 35°C in anaerobic conditions for 14 days to evaluate bacterial leakage through the implant–abutment interfaces. Samples were examined every 24 hours to check turbidity of the medium surrounding the assemblies. In positive instances, 0.1 ml of the nutrient was plated on the SB20 medium (Bacitracin Sacarose Agar, Difco Laboratories, Detroit, MI) and a resulting growth of *F. nucleatum* was considered as a proof of leakage from the interface to the surrounding liquid.

After 14 days of incubation, the implant–abutment assemblies were aseptically removed from the nutrient-containing tubes, placed on absorbing paper, and externally dried with air jets. To carefully untie the screws and remove the abutments, the assemblies were held by special needle-holding tweezers. Microbrushes (KG Brush, KG Sorensen, São Paulo, Brazil) were used to collect samples from implant internal grooves and from the screw threads. The material-containing brushes were transferred for group 1 experiments to 1 ml of Sorensen Phosphate buffer and for group 2 to 150 μl of TE (Tris–HCl, 1 mM EDTA, pH 7.6), followed by the addition of 150 μl 0.5 M NaOH and kept at −20°C until further processing by DNA Checkerboard hybridization according to do Nascimento et al.[17] To evaluate the efficiency of the collection method by the microbrushes, a similar experiment was conducted with the collection of samples being carried out immediately after inoculation of *F. nucleatum.*

Culture evaluation

From group 1, tubes containing microbrush-removed material were shaken for 2 minutes at high speed (Mistron, Tectronix, Richardson, TX) to disaggregate the contents, which were further analyzed according to the criteria described by Westergren and Krasse.[18]

The culture medium selected to evaluate implant contamination by *F. nucleatum* was SB20 (Difco Laboratories). Decimal serial dilutions were made aseptically in a laminar flow chamber from 500 μl of the initial inoculum (Sorensen phosphate buffer) into 4.5 ml TBSy up to 10^{-4}; 50 μl of each dilution were plated on Petri dishes (20×100 mm^2) containing culture medium SB20 with the help of a centesimal pipette and incubated under anaerobic condition.

DNA Checkerboard evaluation

Preparation of samples and membranes

After thawing, the samples were vortexed for 2 minutes at room temperature, the microbrushes removed, and the samples boiled for 5 minutes. After cooling, 800 μl of 5 M ammonium acetate was added to each tube, and the full contents added to the extended slots of a MiniSlot apparatus (Immunetics, Boston, MA), and then concentrated onto a 15×15 cm^2 nylon membrane (Hybond N + Amersham Biosciences, UK) followed by baking for 2 hours at 80°C. As control samples, mixtures of genomic DNA corresponding to either 10^5 or 10^6 bacterial cells of each analyzed species were assembled, denatured, precipitated, and applied into two control slots.

Prehybridization and hybridization

Membranes were pre-wetted in $2 \times$ SSC (0.03 M Na_3 citrate; 0.3 M NaCl) followed by pre-hybridization at 60°C overnight in a hybridization solution [5× SSC; 0.1% SDS; 5% dextran sulfate and a 20-fold dilution of liquid block (GE Healthcare, Bucks, UK)]. After prehybridization, the membranes were placed in a Miniblotter 45 (Immunetics), with the DNA "lanes" at a 90° angle to the device channels. Defined amounts of

Table 1 Assemblies presenting positive and negative results in the bacterial leakage test, and assemblies excluded due to external contamination

Implant	First contamination test	Bacterial leakage though the implant abutment interface
1	−	−
2	−	+
3	−	+
4	+	Excluded
5	−	−
6	−	−
7	−	+
8	−	−
9	−	−
10	−	−
11	−	−
12	−	+
13	−	−
14	−	+
15	−	−
16	−	−
17	+	Excluded
18	−	+
19	−	−
20	−	−

fluorescein-labeled whole genomic probes (30 to 280 ng, depending on the probe) were diluted in 150 μl of hybridization solution applied in the individual lanes of the Miniblotter, and the whole apparatus was placed in a sealed plastic bag containing sheets of wetted towel paper. Hybridization was performed overnight at 60°C, with gentle agitation. The following day, the membranes were washed once in 1 × SSC, 0.1% SDS at 65°C for 15 minutes and once in 0.1 × SSC, 0.1% SDS at 65°C for 15 minutes.

Detection of the fluorescein-labeled probes

After washing, the hybrids were detected by chemiluminescence using the "Gene Images CDP-Star detection module" (GE Healthcare) as follows: membranes were blocked for 1 hour, at room temperature, with agitation in a blocking solution [1:10 dilution of liquid blocking agent (GE Healthcare) in buffer A (100 mM Tris–HCl; 300 mM NaCl, pH 9.5)]. The blocking solution was removed, and the membranes incubated for 1 hour more, at room temperature, with an anti-fluorescein-alkaline phosphatase conjugate antibody (GE Healthcare), diluted 1:5000 in buffer A containing 0.5% bovine serum albumin. After washing with agitation three times, 10 minutes each, in buffer A-containing Tween 20 (0.3%), the membranes, free of excess washing solution, were treated with the CDP-Star detection reagent, and after draining the excess were sealed in a plastic bag. Chemiluminescence signals were detected by exposing the membrane to ECL Hyperfilm-MP (GE Healthcare) for 10 minutes. Hybridization signals were visually evaluated by comparison with the standards for the test species. Typically, the signals would be recorded as: 0, not detected; 1, $<10^5$ cells; 2, $\sim10^5$; 3, 10^5 to 10^6; 4, $\sim10^6$; and 5, $>10^6$ cells.

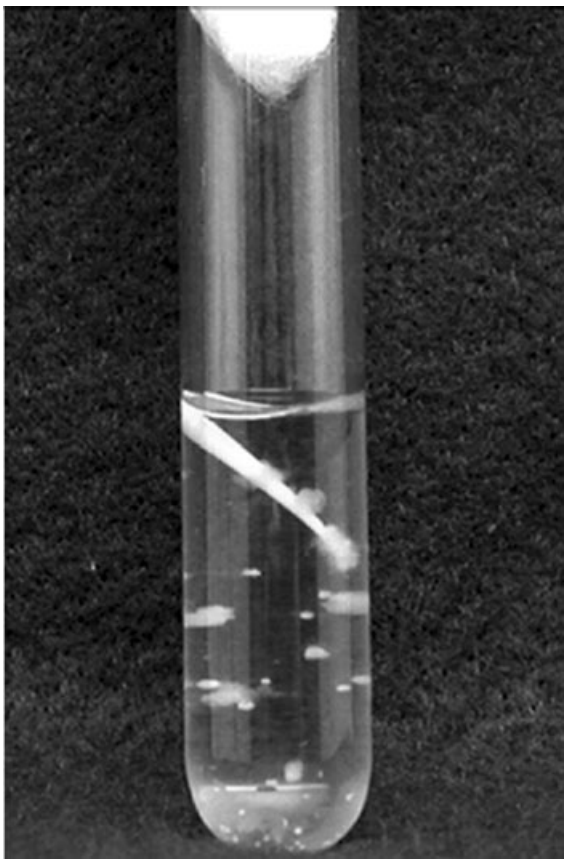

Figure 1 Growth of colonies on microbrushes used to collect samples from the internal parts of the implants.

Data analysis

Data from the microbiological tests were analyzed using the Mann–Whitney nonparametric test. To evaluate the influence of time on bacterial quantification, the Wilcoxon nonparametric test was performed using the software Number Cruncher Statistical Software (NCSS, Kaysville, UT). A value of $p < 0.05$ was considered statistically significant.

Results

Two of the 20 assemblies used in this study, one from each group, were excluded because of external contamination during microorganism inoculation. Six of the remaining 18 assemblies evaluated, three from each group, showed signs of bacterial leakage though the implant–abutment interface after 14 days of incubation. Table 1 shows the total number of assemblies and the number of excluded ones, both by contamination and bacterial leakage from the inner parts of the implants.

Growth of colonies originating from the material inoculated into the implants and collected with microbrushes in group 1 may be seen in Figure 1. Figure 2 shows the hybridization reaction of groups 2 and 3.

For statistical comparison, numbers of CFUs corresponding to the counts in the TSBy medium were converted into scores.

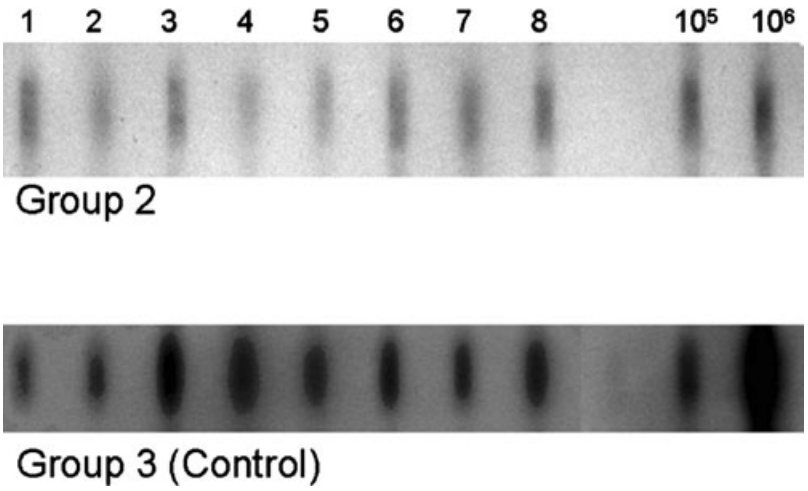

Figure 2 Chemiluminescent signals from the DNA Checkerboard hybridization reaction from groups 2 (top) and 3 (bottom).

Summarized data originated from the application of the two methods; for samples collected immediately or 14 days after implant inoculation see Table 2. Low variation in the score values was observed for samples collected immediately after contamination.

In general, the values obtained by DNA Checkerboard hybridization were significantly higher than the ones by the conventional method ($p < 0.001$). In both methods, however, microorganism detection was smaller in samples collected 14 days after implant internal contamination (groups 1 and 2) when compared to immediate collection ($p < 0.05$).

Discussion

Adequate adaptation between abutment and implant is one of the primary objectives during prosthesis installation on implants.[19] Empty spaces between components favor movement of microorganisms both from the external medium to the interior of the implant and in the opposite direction.[3,20] The size of these empty spaces on the implant–abutment interface has been investigated by other authors who tried to correlate its increase with the frequency and level of bacterial infiltration.[3,21,22] The aim of this in vitro study was to evaluate the bacterial leakage along the implant–abutment interface by conventional bacterial culture and DNA Checkerboard hybridization method.

Of the 18 assemblies evaluated during a 14-day period, three in each group showed evidence of leakage of *F. nucleatum* through the implant–abutment interface. The detection of implant external contamination is evidence that the presence of micro-crevices may be a physical way for bacterial passage to the external medium. This had been already demonstrated, in vitro, in earlier work but with higher contamination

Table 2 Estimated numbers of bacterial cells and respective scores resulting from the DNA Checkerboard hybridization and culture methods, from samples collected immediately and 14 days after implant inoculation

	Checkerboard[a]				Culture[a]			
	Immediately[b]		After 14 days[b]		Immediately[b]		After 14 days[b]	
Sample	Score	Abs. Num.	Score	Abs. Num.	Score	Abs. Num.	Score	Abs. Num.
1	3	5.50	0	0.00	1	0.34	0	0.00
2	3	5.50	3	5.50	1	0.40	1	0.13
3	3	5.50	3	5.50	1	0.54	0	0.00
4	3	5.50	0	0.00	1	0.21	1	0.23
5	3	5.50	2	1.00	1	0.40	0	0.00
6	3	5.50	4	10.00	1	0.42	0	0.00
7	3	5.50	2	1.00	1	0.30	1	0.13
8	3	5.50	3	5.50	1	0.62	0	0.00
9	3	5.50	0	0.00	1	0.54	0	0.00
10	3	5.50	–	–	1	0.42	–	–
Median	3	5.50	2	1.00	1	0.41	0	0.00

[a]Statistical difference among the scores detected by the Mann-Whitney test ($p < 0.05$).
[b]Statistical difference among the scores detected by the Wilcoxon Signed-Rank Test ($p < 0.05$).

indexes.[3,8,23,24] In a recent in vitro study, do Nascimento et al[25] showed similar infiltration indexes through the interface of implants and cast or machine-milled abutments. Passage of microorganisms through the implant–abutment interface has also been shown in other in vivo studies.[26,27]

The low rate of movement of microorganisms from the implant internal region to the exterior in the present study may have been due to the coronal obliteration of the prosthetic components,[28] constructed to prevent leakage through any interface other than the implant–abutment one. Quirynen et al[23] detected higher bacterial contamination in the interior of implants, which were totally submersed in the culture medium without occlusal closing, than in the ones immersed up to the interface. These authors thus showed that bacterial leakage through the prosthetic screw may also interfere in the percolation of cells into the interior of implants.

The 14-day period to observe implant external contamination confirms the study by Koka et al[6] who verified that subgingival bacterial colonization proceeds in the same time interval. Nakazato et al,[29] however, showed that it takes only 4 hours for bacterial colonies to be seen on abutment surfaces.

The earlier studies described evaluated microorganism leakage through the implant–abutment interface using conventional bacterial cultures. In this study, the traditional microbiological analysis in culture media was compared to the DNA Checkerboard hybridization. This last technique, initially described by Socransky et al,[12] has frequently been used in microbiological studies to characterize periodontal and peri-implant microbiota.[14,15,30,31]

The traditional culture method allows the detection of unexpected bacterial strains growing in different culture media, which could be an advantage. The DNA Checkerboard hybridization, however, is easily performed and permits analysis of several clinical samples, identifying up to 40 bacterial species in one membrane. The major advantages of this method are its quickness and its detection of fastidious and hard-growing species.[31]

To compare data from different methods of analysis, the results were standardized by being expressed as scores using the same intervals. Papapanou et al[32] used the same procedure to compare different analytic methodologies.

In the present study, CFU numbers obtained with the DNA Checkerboard technique were higher than the ones with conventional culture. With both techniques, the CFU numbers were lower 14 days after inoculation in comparison with baseline values. A similar result was obtained by van Steenberghe et al[33] in a clinical study showing lower detection in the subgingival microbiota by the traditional culture method when compared to DNA hybridization. A factor that may have influenced bacterial growth in culture media in the present study was the microaerophilic character of the microorganism used. Different levels of detection were also reported by Heijdenrijk et al,[30] who concluded that serial dilution in microbiological procedures, especially with anaerobes, leads to smaller cell number counts in conventional cultures.

While conventional cultures detect only viable bacterial cells, DNA hybridization compiles both viable and nonviable cells, and this may be a possible explanation for the differences detected by the two methods.

Mombelli et al[5] described limitations in bacterial cell quantification in the conventional method observing a variation of up to 24% in the CFU counting values in one single sample, as evidence of the limited precision of the method, especially when dealing with species with high aggregation tendencies.

It is known that subproducts of bacterial metabolism can destroy or damage cell genetic material, hindering effective bounding to the DNA probes in the DNA Checkerboard hybridization, an interference that increases the longer the cell stays in contact with the residues.[34] It should be emphasized that this cellular damage also influences counts of viable organisms detected in the Petri dishes.

All the factors described may have a bearing on the low number of microorganisms encountered in the implant interior, but new in vivo longitudinal studies are necessary to establish their influence in long-term clinical situations.

Conclusion

The DNA Checkerboard hybridization was shown to be more sensitive than the conventional bacterial culture method to detect in vitro contamination of dental implants by *F. nucleatum*. The detection of bacteria in the internal parts of the implants by either method diminishes with time, suggesting reduction of bacterial viability and damage of genetic material. The DNA Checkerboard hybridization technique may present advantages over the culture methods in the identification and quantification of bacteria associated with implant components and peri-implant tissues.

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