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Comparing culture, real-time PCR and fluorescence resonance energy transfer technology for detection of *Porphyromonas gingivalis* in patients with or without peri-implant infections

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Background and Objective: The aim of the study was to compare the detection of *Porphyromonas gingivalis* using a fluorescence resonance energy transfer (FRET) technology with commonly used diagnostic methods in salivary and subgingival plaque samples from subjects with dental implants. *P. gingivalis* was considered as a marker for a pathogenic microbiota.

Material and Methods: Ninety-seven adult subjects were recruited, including periodontally healthy controls with no dental implants, implant controls with no peri-implant disease and patients with peri-implant disease. Saliva and subgingival/submucosal plaque samples were collected from all subjects and were analyzed using culture, real-time PCR and FRET technology employing *P. gingivalis*-specific substrates.

Results: It was found that the *P. gingivalis*-specific substrates were highly suitable for detecting the presence of *P. gingivalis* in saliva and in subgingival plaque samples, showing comparable specificity to culture and real-time PCR.

Conclusion: We applied the FRET technology to detect *P. gingivalis* in implant patients with or without an implant condition and in controls without implants. The technique seems suitable for detection of *P. gingivalis* in both plaque and saliva samples. However, with all three techniques, *P. gingivalis* was not very specific for peri-implantitis cases. Future work includes fine-tuning the FRET technology and also includes the development of a chair-side application.

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Modern comprehensive dental care increasingly includes the consideration of dental implants for patients who need tooth-replacement therapy. For some decades dental implants have served as successful long-term predictable anchors for fixed and removable protheses in fully and partially edentulous patients (1–4). However, the procedure to replace lost natural teeth with implants offers an unwanted opportunity for bacterial colonization. The early development of biofilms on implant surfaces has been shown to be similar to that on natural teeth and on other restorative materials placed in the oral cavity (5,6). Over time, from months to years, the microbial population on the implants maturates toward a more complex microbiota (7). Thus, peri-implant mucosa may also be colonized and infected with increased numbers and proportions of oral bacterial species, analogous to the increase of microorganisms in deep periodontal pockets adjacent to natural teeth.

The inflammatory lesions that develop in the tissue around dental implants are collectively recognized as peri-implant diseases. Manifestation of peri-implant diseases represents a widespread problem. Cross-sectional studies have reported that peri-implant mucositis (i.e. bleeding on probing without concomitant alveolar bone loss) occurs in about 79% of subjects and in 50% of implants (8). The prevalence of subjects with peri-implantitis varies between 25% and 45% in several publications, in accordance with the selected population (8–12).

Studies dealing with the microbiota of failing or failed implants clearly indicate the presence of multiple pathogens that are also associated with periodontitis. Thus, the development of peri-implant diseases appears to be accompanied largely by an increase in specific bacterial species, seemingly similar to those in periodontal diseases. One of the most commonly studied periodontal pathogens (13), and hence a target bacterium in peri-implant diseases, is *Porphyromonas gingivalis* (14). The presence or absence of *P. gingivalis* in peri-implant sites may be indicative of a pathogenic microbiota, possibly one that is causing peri-implantitis (i.e. infection of tissue and bone around the dental implant).

Methods developed to detect *P. gingivalis* include enzyme assays (15), DNA probe assays (15,16), immunofluorescence (17), anaerobic culture (18) and real-time PCR assays (19,20). Bacterial culture has long been considered the ''gold standard'' diagnostic method to detect and quantify the microbiota colonizing the oral cavities and periodontal lesions. Moreover, bacterial culture is needed to create an antibiogram. However, not all bacteria can be readily cultured, and the proportional recovery of culturable species is unlikely to match their proportions in the patient. In addition, preparing cultures is time-consuming and labor-intensive because oral pathogens are often anaerobic and tend to grow slowly. Also, the use of selective media can restrict the growth of many species in periodontal and peri-implant samples. Real-time PCR is a convenient quantitative method that enables the detection of low numbers of cells. However, despite its high sensitivity and specificity, this method does not provide evidence of pathogen viability and bacterial activity.

Bacterial enzymes, such as proteases, are in theory ideally suited as biomarkers for quick and sensitive identification of microorganisms in clinical samples (15). Many of these enzymes are released into the surrounding microenviroment where they can be detected using sensitive fluorogenic and/or luminogenic substrates (21). Notably, *P. gingivalis* secretes a variety of proteases that act as virulence factors, thus allowing these bacteria to invade the host tissues. By liberating amino acids from host proteins, secreted proteases are involved in the (anaerobic) metabolism of these microorganisms (22). Recently, our group has developed fluorogenic substrates (i.e. BikKam substrates), which, because of the presence of D-amino acids in these substrates, appeared highly specific for the detection of bacteria-derived proteases (23,24). No cleavage of BikKam substrates has been found by human proteases in various body fluids (23,24). Using substrates specifically cleaved by *P. gingivalis* proteases (identified as gingipains), we have been able to detect, quickly (within minutes) and with high specificity, the presence of *P. gingivalis* in subgingival samples and saliva, without the need for enzyme isolation or sample pretreatment (24). This offers a potential diagnostic tool for the diagnosis of oral diseases, such as peri-implant infections, where we could use the presence of *P. gingivalis* as a marker for a potentially pathogenic microbiota.

In the present study we compared the practical feasibility of the *P. gingivalis*-specific fluorogenic substrates with methods currently used (culture and real-time PCR) to detect *P. gingivalis* in saliva and plaque samples from the peri-implant sites of both diseased patients and healthy individuals. In addition, we tested the hypothesis that the presence of *P. gingivalis* in saliva would be associated with infections at the peri-implant site, thus testing the feasibility of using saliva as a diagnostic fluid for peri-implant health status.

Material and methods

Subject sample

Ninety-seven adult subjects (age-range 29–80 years) were recruited for the present study. Data on demographics and smoking habits were collected using a self-reported questionnaire. The study population consisted of periodontally healthy controls with no dental implants, nondiseased implant controls and patients with peri-implant disease referred for diagnosis and treatment of a peri-implant condition to the Departments of Periodontology or Oral Function and Prosthetic Dentistry, Academic Centre for Dentistry Amsterdam (ACTA), or to the Centre for Implantology and Periodontology, Amstelveen, The Netherlands.

The definition and diagnosis of peri-implant diseases was based on the clinical and radiographic criteria described previously (25,26): (i) peri-implant mucositis (the presence of inflammation in the mucosa at an implant with no signs of loss of supporting bone); and (ii) peri-implantitis (in addition to inflammation in the

mucosa, peri-implantitis is characterized by loss of > 2 mm of supporting bone, as evidenced on new diagnostic radiographs compared with the time point that the implant–suprastructure interface was established).

Controls included subjects who showed no radiographic signs of alveolar bone loss and/or displayed fewer than five pockets of ≥ 5 mm, concomitant with a clinical attachment level of ≥ 2 mm, following periodontal measurements accessed within 3 mo before sample collections. These subjects could include successfully treated periodontal patients (see below). The presence of gingivitis was not an exclusion criterion for a control.

The clinical parameters assessed included the number of teeth and the number of implants present, pocket probing depth, gingival recession and bleeding on probing at six different sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual) of each implant/tooth present, excluding third molars. Bleeding of the peri-implant mucosa was scored dichotomously as present or absent upon completion of probing (27).

On the basis of periodontal measurements and dental radiographs (intra-oral bitewing, peri-apical X-rays and/or orthopantomographs), controls were divided into the following three groups: subjects with no implants but potential candidates for implant therapy [control, no implant (C-NI)]; subjects with an implant placed and loaded for ≤ 6 mo [control with recent implant (C-RI)]; and subjects with a steadily healthy implant with a suprastructure and a functional period of at least 6 mo [control with stable implant (C-SI)].

Exclusion criteria for patients with peri-implant disease and healthy controls were: (i) a recent history of, or the presence of, any acute infection; (ii) tooth extraction and trauma < 2 wk preceding the sample; (iii) systemic antibiotic treatment during the preceding 3 mo; (iv) pregnancy; and (v) systemic diseases that might influence the condition of the periodontal tissues and the subgingival microflora. Smoking habits were defined as follows: current smokers were participants who smoked at least one cigarette per day, while nonsmokers were defined as those who had never smoked or as those who had stopped smoking at least 3 mo before the study. A previous history of periodontal disease did not represent an impediment for entry to this study, but it was recorded.

The current study population was made up of five groups, as follows: (i) peri-implant mucositis group (mucositis) (n = 20); (ii) peri-implantitis group (implantitis) (n = 20); (iii) periodontal healthy group with no implant (C-NI) (n = 19); (iv) nondiseased implant with recent implant placement group (C-RI) (n = 19); and (v) nondiseased implant with a steadily healthy implant for at least 6 mo (C-SI) (n = 19).

The research project was approved by the Medical Ethics Committee of the VU University Medical Center, Amsterdam (3rd February 2011, #2011/022). All volunteers signed an informed consent to participate.

Bacterial sampling

Sampling techniques, as well as sample storage, dispersion and dilutions, were performed through routine procedures and established techniques (18,27–30). In brief, stimulated whole saliva was collected from all volunteers; this was achieved by asking the subject to chew on a piece of inert paraffin (PARAFILM® M barrier film; VWR International, Amsterdam, the Netherlands) for 5 min after rinsing his/her mouth with saline to remove food residue before sample collection. The peri-implant site with the deepest inflamed pocket was selected for bacterial sampling. For controls, the disto-lingual or disto-palatal peri-implant site was selected in the C-RI and C-SI groups, while in the C-NI group, a caries-free gingival sulcus adjacent to the edentulous candidate implant area was selected. After removing supragingival plaque with sterile curettes using coronal strokes starting from the gingival margin, two sterile paper points (Absorbent Points Cell Pk #504 Fine; Henry Schein UK Holdings Ltd., Southall, Middlesex, UK) were introduced to the bottom of each peri-implant or periodontal pocket and removed after 10 s. The paper points were transferred to sterile Eppendorf tubes containing 1.5 mL of reduced transport fluid.

After collection, an aliquot from each of the 97 salivary and 97 subgingival samples was cultured for detection of *P. gingivalis* within 24 h after sampling; the remainder of all samples were frozen at −20°C until further laboratory analysis.

Culture

Culture of all samples and identification of *P. gingivalis* colonies was performed through routine procedures and established techniques (18,28–30). In brief, a total of 100 μL of the subgingival plaque and saliva samples was used for culture after 10-fold serial dilution in sterile phosphate-buffered saline. Samples were grown anaerobically (80% N_2, 10% H_2, 10% CO_2) at 37°C on 5% horse blood agar plates (Oxoid No. 2; Oxoid, Basingstoke, UK) enriched with hemin (5 mg/L) and menadione (1 mg/L) for detection of *P. gingivalis*. *P. gingivalis* was identified on the basis of gram staining, anaerobic growth, the inability to ferment glucose, the production of indole and a positive hemagglutination test with 3% sheep erythrocytes. The total number of colony-forming units (CFU) was counted on blood agar plates and the number of *P. gingivalis* colonies was expressed as a percentage of this total number.

Real time-PCR assay

Real-time PCR was performed in our laboratories using established techniques, which are also applied to routine diagnostic procedures for periodontal patient care (19,31,32). In short, DNA was extracted from a 100-μL sample of subgingival plaque or from a 100-μL sample of saliva using a commercial kit (MagNA Pure LC DNA isolation kit III; Roche Diagnostics, Indianapolis, IN, USA) in MagNA Pure LC (Roche Diagnostics) according to the instructions provided by the manufacturer. Real-time PCR amplification reactions were carried out in a reaction mixture of 20 μL consisting of 4 μL of sample

lysate and 16 μL of reaction mixture containing LightCycler PCR mix, PCR water and primers (forward, 5′-GCG-CTCAACGTTCAGCC-3′; and reverse, 5′-CACGAATTCCGCCTGC-3′) and the probe (LC610-CACTGAACTCA-AGCCCGGCAGTTTCAA-BBQ) for *P. gingivalis*. The conditions for real-time PCR amplification in a Light Cycler 480 (Roche Diagnostics) were as follows: initial denaturation at 95°C for 5 min, 45 amplification cycles (of denaturation at 95°C for 10 s, and annealing and extension at 60°C for 20 s), followed by one cycle of cooling at 40°C for 15 s.

FRET technology

Recently we established the detection of activity of *P. gingivalis* enzymes in both artificial (spiked) oral samples as well as in subgingival plaque samples from periodontal patients using the FRET technology (24). Therefore, by applying the fluorogenic BikKam substrates specifically tailored for *P. gingivalis* (Table 1) (24), we further explored here the applicability of FRET technology. Reactions were performed in nonaffinity, black, 96-well plates (Greiner, Recklinghausen, Germany). Enzyme activity in the samples was determined by incubating 16 μM substrate with 45 μL of saliva and 45 μL reduced transport fluid from paper point samples, supplemented with 5 mM cysteine at 37°C per each well. *P. gingivalis* culture supernatant was used as a positive control. Plates were read on a fluorescence microplate reader (Fluostar Galaxy, BMG Laboratories, Offenburg, Germany), with fluorescence readings taken every 3 min, at 485-nm excitation and 530-nm emission wavelengths, over a 90-min time period. The experiments were run in duplicate and the average result was calculated for use in further statistical analyses. The slope of the relative fluorescence (RF) divided by time (RF/min) was used to define the enzyme activity. Samples with an RF/min value of ≥ 5 were considered positive for *P. gingivalis*, as described previously (23,24).

Table 1. Porphyromonas gingivalis-specific D-amino acids containing fluorescence resonance energy transfer (FRET) substrates

FRET substrate	Sequence
BikKam 9	FITC-Arg-DAsp-KDbc
BikKam 10	FITC-Arg-DGlu-KDbc
BikKam 11	FITC-Arg-DHis-KDbc
BikKam 12	FITC-Arg-DLys-KDbc
BikKam 13	FITC-Arg-DArg-KDbc

Arg, arginine; DAsp, D-aspartic acid; DGlu, D-glutamic acid; DHis, D-histidine; DLys, D-lysine; FITC, fluorescein isothiocyanate; KDbc, Lysine-Dabcyl.

The supernatants of the following *P. gingivalis* strains were used as a positive control in this study: W83, X-2, A7A1-28, ATCC 49417, HG1690, HG1691 and 34-4 (24,33). These *P. gingivalis* strains were cultured on blood agar plates under anaerobic conditions in 5% CO_2 for 48 h at 37°C. Then, single colonies were inoculated into Brain Heart Infusion broth (Difco Laboratories, Detroit, MI, USA) supplemented with hemin-menadione, and cultured at 37°C under anaerobic conditions. After 24–72 h, the cells were pelleted by centrifugation for 10 min at 10,000 *g*. Supernatants, containing secreted enzymes, were sterilized by filtration through a 0.22-μM filter (MilliPore UK Ltd., Watford, UK). These *in vitro* samples were used directly or frozen at −20°C for later use.

Statistical analysis

Statistical analysis of the data was performed with PASW STATISTICS 18.0 (SPSS Inc., Chicago, IL, USA). Means, standard deviations and frequency distributions were calculated. The sensitivity and specificity of the three techniques applied to subgingival plaque and saliva samples were determined using 2 × 2 contingency tables.

Results

Study population

The demographic and clinical characteristics of the subjects included in the study are presented in Table 2. Forty patients (22 women and 18 men; 37–74 years of age) suffering from either peri-mucositis or peri-implantitis and 57 healthy controls (27 women and 30 men; 29–80 years of age) participated in the current study. The patients in the mucositis, implantitis and C-SI groups were older than those in the C-NI and C-RI groups. Forty-nine women and 48 men participated in the current study. The majority of subjects (85%) were Caucasian. Of the 97 participants, 18% were current smokers, and the highest number of smokers was found in the C-NI group. Almost 30% of the total study population had a previous history of periodontal disease.

All the study subjects had an average of at least 15 teeth present, with the averages ranging from a minimum of 15.5 for the implantitis group to a maximum of 24.9 for the C-NI group. For this study population the average number of implants was 2.7. Notably, the C-NI group had no dental implants by definition and served as a reference group with an edentulous area that was potentially suitable for implant therapy.

A mean of 2.2 sites with pocket probing depths ≥ 5 mm was detected for the whole dentition, while 41 (42.3%) sites sampled had pocket probing depth values of ≥ 5 mm. In general, the patients with peri-implant disease showed the highest mean value for the percentage of bleeding on probing (20.1% and 15.6% for mucositis and implantitis groups, respectively), thus reflecting the inflammatory state of this group of patients.

Prevalence of *P. gingivalis*

Table 3 depicts the prevalence of *P. gingivalis* as detected by anaerobic culture, real-time PCR and FRET for the 97 subgingival plaque samples. When culture and FRET were used as the detection methods, *P. gingivalis*-positive samples were detected more often in patients with peri-implant disease than in the healthy control groups. In fact, the subgingival plaque samples from six out of 20 implantitis patients were culture positive for the presence of *P. gingivalis*. The peri-implant mucositis group also contributed with one *P. gingivalis*-positive sample. Only four (7%) of 57 samples were positive for *P. gingivalis* when C-NI, C-RI and C-SI were grouped together.

Table 2. Demographic and clinical data for the study population

	Implant patients with peri-implant disease (n = 40)			Implant patients with no peri-implant disease (n = 38)	
	Mucositis (n = 20)	Implantitis (n = 20)	C-NI (n = 19)	C-RI (n = 19)	C-SI (n = 19)
Age (years)	59 ± 8.3	57.4 ± 9.1	47.2 ± 12.6	53.1 ±10.5	58.7 ± 11.9
Gender					
Female	13 (65)	9 (45)	7 (36.8)	14 (73.7)	6 (31.6)
Male	7 (35)	11 (55)	12 (36.8)	5 (26.3)	13 (68.4)
Ethnicity					
Caucasian	19 (95)	16 (70)	16 (84.2)	16 (84.2)	18 (94.7)
Other	1 (5)	6 (30)	3 (15.8)	3 (15.8)	1 (5.3)
Smoking status					
Nonsmoker	18 (90)	16 (80)	13 (68.4)	15 (78.9)	17 (89.5)
Smoker	2 (10)	4 (20)	6 (31.6)	4 (21.1)	2 (10.5)
History of periodontitis	4 (20)	3 (15)	11 (57.9)	7 (36.8)	4 (21.1)
Teeth	18.1 ± 8.7	15.5 ± 8.6	24.9 ± 2.2	22.4 ± 4.3	16.7 ± 10
Implants	3.2 ± 2.3	4.7 ± 3.0	0.0	2.1 ± 1.3	3.2 ± 1.8
Sites with pocket probing depth ≥ 5 mm					
All teeth	3.2 ± 2.3	2.2± 6.3	2.7 ± 2.3	1.7 ± 1.5	0.8 ± 1.1
Sampled sites	11 (55)	20 (100)	5 (26.3)	1 (5.3)	1 (5.3)
Bleeding on probing (%)					
All teeth	20.1 ± 18.5	15.6 ± 13.1	11.9 ± 5.5	13.6 ± 6.5	7.1 ± 5.2
Sampled sites	20 (100)	20 (100)	6 (31.6)	0 (0)	0 (0)

Values are given as mean ± standard deviation or n (%).
C-NI, periodontal healthy with no implant; C-RI, periodontal healthy with recent implant placement; C-SI, steadily healthy implant for at least 6 mo; mucositis, peri-implant mucositis group; implantitis, peri-implantitis group.

Similar findings were observed for the FRET assay; a total of five *P. gingivalis*-positive samples were obtained from the patients with peri-implant disease (with four out of five positive samples originating from the implantitis group), while only one sample from the healthy controls was proved to be *P. gingivalis* positive.

A higher prevalence of *P. gingivalis* was found by real-time PCR in samples from the C-NI group than in samples from the other groups. The highest enzyme activity, found by FRET assays, among all subgingival samples was recorded in the implantitis group (Table 3). Interestingly, a *P. gingivalis*-positive result was obtained with each of the three different techniques for the same subgingival plaque samples of only four individuals of the total study population.

Table 4 shows the prevalence of *P. gingivalis* in saliva samples, as determined by anaerobic culture, real-time PCR and FRET assays. Culture showed the lowest prevalence of *P. gingivalis*-infected samples among all the study groups. For instance, *P. gingivalis* was not found by bacterial culture in any saliva sample from the 19 C-SI group subjects. In contrast, real-time PCR and the FRET technique seemed more capable of detecting *P. gingivalis* in saliva. In fact, six and four *P. gingivalis*-positive samples were detected by real-time PCR in the mucositis and implantitis groups, respectively, while FRET detected larger numbers of *P. gingivalis*-positive samples, respectively eight (40%) and six (30%) in the mucositis and implantitis groups.

A *P. gingivalis*-positive result was achieved for each of the three different techniques in the same salivary sample in only two patients. Positive results for the same sample were obtained more often with real-time PCR and FRET than with other combinations of the three techniques: for example, six out of eight samples were in agreement for the C-NI group.

Comparison of anaerobic culture, real-time PCR and FRET assays

There were discrepancies between the results obtained with the three techniques for the detection of *P. gingivalis* from subgingival plaque samples (Table 3). In Table 5 we present the results of the 97 subgingival plaque samples: the calculated sensitivity and specificity were 77% and 92%, respectively, for real-time PCR compared with anaerobic culture (Table 5A). Comparison of bacterial culture and the FRET assay is shown in Table 5B: a specificity of 100% was obtained for analysis of the subgingival plaque samples, but a sensitivity of 67% was obtained with culture serving as gold standard. In Table 5C the results of real-time PCR and FRET methods were compared. Similarly to the results in Table 5B the specificity was close to 100%, but the sensitivity of the FRET assay was 58% for the detection of *P. gingivalis* from subgingival plaque samples around implants or natural teeth, with real-time PCR serving as the gold standard.

Comparison of the three methods for detecting *P. gingivalis* in salivary samples is summarized in Table 6. Among the three detection methods, the comparison between culture and real-time PCR gave the highest score for sensitivity and specificity in saliva (83% and 72%, respectively, for real-time PCR compared with anaerobic culture). Comparison between bacterial culture and the FRET assay, shown in Table 6B, yielded a sensitivity of 63% and a specificity of 75%. The number of positive results determined by real-time PCR and the FRET assay, as summarized in Table 6C, yielded sensitivity and specificity values for the FRET analysis, compared with real-time PCR, of 67% and 78%, respectively.

Comparison of detection of *P. gingivalis* in saliva and subgingival plaque samples

The correlation of all positive and negative results for the subgingival plaque and salivary samples was further analyzed using the three techniques (Table 7). When real-time PCR was used, 14 out of 15 subjects who harbored *P. gingivalis* in their subgingival plaque samples also had *P. gingivalis* in their saliva (Table 7B). The detection of *P. gingivalis* in saliva reflected its presence also in the subgingival plaque

Table 3. Prevalence of *Porphyromonas gingivalis* in subgingival plaque samples from selected sites of the various study groups

	Implant patients with peri-implant disease (n = 40)			Implant patients with no peri-implant disease (n = 38)	
	Mucositis (n = 20)	Implantitis (n = 20)	C-NI (n = 19)	C-RI (n = 19)	C-SI (n = 19)
Culture					
P.g. prevalence	1 (5)	6 (30)	1 (5.3)	1 (5.3)	2 (10.5)
P.g. count (CFU/mL × 10^6)	0.76	0.24 ± 0.45	0.10	64	0.24 ± 0.27
Real-time PCR					
P.g. prevalence	1 (5)	4 (20)	6 (31.6)	2 (10.5)	2 (10.5)
P.g. counts/mL × 10^6	26	8.13 ± 9.41	0.49 ± 0.68	8 ± 11.3	7.04 ± 9.84
FRET					
P.g. prevalence	1 (5)	4 (20)	1 (5.3)	0 (0)	0 (0)
Enzyme activity (RF/min)					
BikKam 9	1.24	10.69 ± 15.85	1.29	0	0
BikKam 10	1.67	24.68 ± 38.06	3.76	0	0
BikKam 11	5.65	58.52 ± 38.05	7.70	0	0
BikKam 12	8.24	59.70 ± 74.34	10.82	0	0
BikKam 13	4.85	53.82 ± 77.86	7.57	0	0
P.g. positive in culture, in real-time PCR and in FRET	1	2	1	0	0
P.g. positive in culture and in real-time PCR	1	3	1	1	1
P.g. positive in culture and in FRET	1	3	1	0	0
P.g. positive in real-time PCR and in FRET	1	2	1	0	0

Values are given as n (%) of subjects who are *P. gingivalis* positive, mean total CFU/mL × 10^6 ± standard deviation for culture, counts/mL × 10^6 ± standard deviation for real-time PCR, or relative fluorescence/min ± standard deviation for FRET.

The mean total counts, mean counts of *P. gingivalis* species and mean enzyme activity for each substrate were calculated only including *P. gingivalis*-positive individuals. Samples with an enzyme activity [relative fluorescence (RF) divided by time (RF/min)] value of ≥ 5 were considered positive for *P. gingivalis* (23,24).

CFU, colony-forming unit; C-NI, periodontal healthy, no implant group; C-RI, periodontal healthy with recent implant placement; C-SI, steadily healthy implant for at least 6 mo; FRET, fluorescence resonance energy transfer; implantitis, peri-implantitis group; mucositis, peri-implant mucositis group; *P.g.*, *Porphyromonas gingivalis*.

samples for two out of 10 and two out of five patients when culture and FRET technology, respectively, were used as the selected method (Table 7A,C). From Table 6 it is clear that real-time PCR gives the highest number (n = 15) of *P. gingivalis*-positive subgingival biofilm samples, while FRET assays give the highest number (n = 34) of *P. gingivalis*-positive saliva samples compared with anaerobic culture and PCR (n = 5 and n = 30 positive saliva samples, respectively).

Discussion

The purpose of the study was to investigate the feasibility of detecting *P. gingivalis* from peri-implant sites and saliva of implant patients using specific FRET substrates in comparison with culture and real-time PCR. In addition, we studied whether the presence of *P. gingivalis* in saliva could be linked to *P. gingivalis* infections at peri-implant site/pocket samples, thus, in fact, testing the feasibility of saliva as a diagnostic fluid of peri-implant health status; in this case *P. gingivalis* would serve as marker bacterium representing a pathogenic microbiota that affects the success rate of implants. The ultimate goal of this study was to form a base for the development of a diagnostic technology that would enable the dental practitioner to perform a chair-side diagnostic salivary test to identify the presence of *P. gingivalis*, serving as a microbial marker for a peri-implant infection.

Bacterial culture is considered as the ''gold standard'' diagnostic method of detecting and quantifying the microbiota colonizing the oral cavities and to create an antibiogram. Anaerobic culture is specific in its ability to distinguish species. However, it has limitations compared with real-time PCR; culture is time-consuming and laborious, and it has a relatively low level of sensitivity. Real-time PCR is reliable and able to detect low numbers of bacterial cells, but bacterial DNA needs to be extracted and isolated from the sample, and these processes can be costly and laborious. Furthermore, although real-time PCR has been described as a very specific technique (19,34), it is conceivable that cross-reactivity with other (unknown) species may occur as the oral and subgingival microbiota is extensive and diverse (35). In contrast, the approach using FRET analysis, as presented in this study, is easy to perform and requires fewer experimental steps and less time. Owing to the specific character of the BikKam substrates there is no need for time-consuming enzyme pre-enrichment and purification, thus offering the potential for development into a chair-side test. Moreover, the bacteria as a source of the enzymes need not necessarily be viable, as long as there is bacteria-derived enzyme activity.

We found no straightforward correlation among the results obtained using the three techniques: in the present study, five of 97 subgingival plaque samples and 21 of 97 salivary samples were *P. gingivalis* positive (> 10^4 CFU/mL) by real-time PCR but negative by culture. Isolation of *P. gingivalis* was performed on a non-selective medium, which would hamper the detection of small numbers of the microorganism in the presence of a large background of bacterial cells. Besides, an anaerobic environment is difficult to maintain during sample collection. In addition, in contrast to bacterial culture, real-time PCR also detects nonviable bacterial cells present in the sample (i.e. it does not

Table 4. Prevalence of *Porphyromonas gingivalis* in salivary samples from patients in the various study groups[a]

	Implant patients with peri-implant disease (n = 40)			Implant patients with no peri-implant disease (n = 38)	
	Mucositis (n = 20)	Implantitis (n = 20)	C-NI (n = 19)	C-RI (n = 19)	C-SI (n = 19)
Culture					
P.g. prevalence	1 (5)	1 (5)	1 (5.3)	2 (10.5)	0 (0)
P.g. count (CFU/ mL $\times$ 10^6)	5.90	0.48	0.28	45.20 ± 62	0
Real-time PCR					
P.g. prevalence	6 (30)	4 (20)	8 (42.1)	8 (42.1)	4 (20.1)
P.g. counts/mL $\times$ 10^6	0.72 ± 0.74	1.76 ± 2.55	0.96 ± 0.96	1.15 ± 0.92	14.52 ± 21.24
FRET					
P.g. prevalence	8 (40)	6 (30)	8 (42.1)	5 (26.3)	7 (36.8)
Enzyme activity (RF/min)					
BikKam 9	5.79 ± 4.16	3.97 ± 2.68	8.12 ± 4.68	4.76 ± 19.10	4.18 ± 2.74
BikKam 10	0.54 ± 1.46	-0.44 ± 1.43	4.08 ± 5.51	-4.75 ± 10.32	1.34 ± 3.10
BikKam 11	5.01 ± 3.87	4.80 ± 5.90	8.65 ± 8.52	-0.81 ± 10.70	4.03 ± 3.07
BikKam 12	3.30 ± 1.41	3.31 ± 3.30	9.19 ± 8.83	15.80 ± 27.00	2.55 ± 1.54
BikKam 13	5.27 ± 2.73	8.55 ± 5.44	1.03 ± 8.80	10.20 ± 11.50	6.34 ± 4.20
P.g. positive in culture, in real-time PCR and in FRET	0	1	1	0	0
P.g. positive in culture and in real-time PCR	0	1	1	2	0
P.g. positive in culture and in FRET	0	1	1	0	0
P.g. positive in real-time PCR and in FRET	2	3	6	2	2

Values are given as n (%) of subjects who are *P. gingivalis* positive, as mean total CFU/mL $\times$ 10^6 ± standard deviation for culture, as counts/ mL $\times$ 10^6 ± standard deviation for real-time PCR, or as relative fluorescence/min ± standard deviation for FRET.

The mean total counts, mean counts of *P. gingivalis* species and mean enzyme activity for each substrate were calculated only including *P. gingivalis*-positive individuals.

Samples with an enzyme activity [relative fluorescence (RF) divided by time (RF/min)] value of ≥ 5 were considered positive for *P.* gingivalis (23,24).

CFU, colony-forming unit; C-NI, periodontal healthy, no implant group; C-RI, periodontal healthy with recent implant placement; C-SI, steadily healthy implant for at least 6 mo; FRET, fluorescence resonance energy transfer; implantitis, peri-implantitis group; mucositis, peri-implant mucositis group; *P.g. Porphyromonas gingivalis*.

differentiate between intact DNA from viable and nonviable cells). Furthermore, we observed that real-time PCR gave a higher rate of *P. gingivalis* in subgingival plaque samples in samples for the C-NI group than for the other groups. We speculate that this occurred because of the high number of patients with a previous history of periodontal disease in this group. This finding supports the notion that the residual pockets, although receiving treatment, may act as reservoirs of *P. gingivalis*. The highest enzyme activity recorded in subgingival plaque samples by the FRET assays was obtained for the implantitis group. This suggests that a higher level of *P. gingivalis* is present in peri-implantitis patients. However, the results obtained using the FRET method, which also has the potential to detect viable bacterial cells, did not always match the results obtained by real-time PCR in subgingival plaque samples. In fact, FRET-positive/real-time PCR-negative and FRET-negative/real-time PCR-positive discrepancies negatively influenced the sensitivity and specificity of the FRET-based technology for the tested oral bacteria, assuming that in these comparisons real-time PCR is truly a gold standard. However, we suggest that for saliva samples, FRET assays may prove to be superior over real-time PCR, given the possibility that bacterial DNA has been degraded in saliva. The inclusion of more and other specific substrates in future research may shed light on this issue.

When culture was used as a gold standard, the FRET method demonstrated in subgingival plaque samples a low sensitivity (67%) as a result of the FRET-positive/culture-negative ratio. The number of false positives generated by FRET could represent cross-reactivity with other bacterial species of which we are not aware and that could produce active proteases specific for the *P. gingivalis* peptides (Table 1) (24). However, the high specificity (100%) excluded the chance to falsely detect *P. gingivalis* using the FRET technology in subgingival plaque samples. Comparable results were obtained when real-time PCR served as a gold

Table 5. Comparison of the prevalence of *Porphyromonas gingivalis* in subgingival plaque samples using: (A) anaerobic culture vs. real-time PCR, (B) anaerobic culture vs. fluorescence resonance energy transfer (FRET) assays and (C) real-time PCR vs. FRET assays

	Positive	Negative	Total
(A) Culture vs PCR			
Positive	7	8	15
Negative	3	79	82
Total	10	87	97
(B) Culture vs FRET			
Positive	5	0	5
Negative	5	87	92
Total	10	87	97
(C) PCR vs FRET			
Positive	4	1	5
Negative	11	81	92
Total	15	82	97

(A): sensitivity = 77% and specificity = 92% (culture was the gold standard).
(B): sensitivity = 67% and specificity = 100% (culture was the gold standard).
(C): sensitivity = 58% and specificity = 99% (real-time PCR was the gold standard).

Table 6. Comparison of the prevalence of *Porphyromonas gingivalis* in salivary samples using: (A) anaerobic culture vs. real-time PCR, (B) anaerobic culture vs. fluorescence resonance energy transfer (FRET) assays and (C) real-time PCR vs. FRET assays

	Positive	Negative	Total
(A) Culture vs PCR			
Positive	4	26	30
Negative	1	66	67
Total	5	92	97
(B) Culture vs FRET			
Positive	2	31	33
Negative	3	61	64
Total	5	92	97
(C) PCR vs FRET			
Positive	15	19	34
Negative	15	48	63
Total	30	67	97

(A): sensitivity = 83% and specificity = 72% (culture was the gold standard).
(B): sensitivity = 63% and specificity = 75% (culture was the gold standard).
(C): sensitivity = 67% and specificity = 78% (real-time PCR was the gold standard).

Table 7. Comparison of the prevalence of *Porphyromonas gingivalis* in subgingival plaque samples with the prevalence in salivary samples using (A) anaerobic culture, (B) real-time PCR and (C) fluorescence resonance energy transfer (FRET) assays

	Subgingival plaque		
Saliva	Positive	Negative	Total
(A) Culture			
Positive	2	3	5
Negative	8	84	92
Total	10	87	97
(B) Real-time PCR			
Positive	14	16	30
Negative	1	66	67
Total	15	82	97
(C) FRET			
Positive	2	32	34
Negative	3	60	63
Total	5	92	97

(A): sensitivity = 56% and specificity = 97% (subgingival plaque samples was the gold standard).
(B): sensitivity = 94% and specificity = 84% (subgingival plaque samples was the gold standard).
(C): sensitivity = 63% and specificity = 74% (subgingival plaque samples was the gold standard).

standard for detection of *P. gingivalis* in subgingival plaque samples in comparison with FRET assays (sensitivity = 58%, specificity = 99%). In contrast, the specificity of FRET assays was lower in saliva (specificity = 75% in anaerobic culture and specificity = 78% with real-time PCR as the gold standard). Therefore, once again we need to appreciate that it may be not justified to use culture and real-time PCR as gold standards when analyzing the microbiota of saliva samples; each has its own limitations, as outlined above, and the results are also dependent on the site or the organ sampled. Any new technique for the analysis of the oral microbiota faces the problem of being compared with a "gold" standard that is not a perfect system to start with. Only complete sequencing of the oral microbiome may overcome this to a great extent. However, for this purpose intact bacterial DNA is still needed. Therefore, we feel confident to suggest that, in addition to culture and real-time PCR, *P. gingivalis* can also reliably be detected in saliva by employing the FRET technology.

Notwithstanding that the literature seems to support an association between *P. gingivalis* and peri-implant infections, also in studies where paper points were used as a detection method (36), we found that only a limited number of peri-implant pockets in patients suffering from peri-implantitis were positive for the presence of *P. gingivalis*, regardless of the detection method (six out of 20 by culture, and four out of 20 by real-time PCR or FRET). We postulate two possible explanations. First, the use of paper points might not offer the best method to obtain representative bacterial samples in peri-implant sites or pockets. In many instances the implant superstructure might prevent proper access to the peri-implant sulci, thus hampering the sampling procedure and leading to erratic results. In addition, the bacterial infection is "hiding" within the screw threads of the implant. Second, *P. gingivalis* is an inadequate bacterial marker for peri-implantitis, based on subgingival plaque sampling. We have to recognize that peri-implantitis is a polymicrobial infection and multiple species could – individually or in combination – be associated with this complication of implant dentistry. Therefore, at this point more marker bacterial species should be included in the study of the complex peri-implant microbiota, and this knowledge will serve for future work expanding FRET technology in saliva.

It could be considered a weakness of this study that only one pocket was sampled for each individual and we compared the outcomes with salivary sampling. In fact, when considering the number of subgingival samples needed to detect the presence of *P. gingivalis* in periodontitis patients, selection of the deepest pocket in each quadrant is the most efficient method of sampling (37). However, in studies on peri-implantitis this is not possible because peri-implantitis is present most often at only one implant.

In the current study we used D-amino acids containing substrates that can be used for enzyme-based diagnostic purposes; these substrates appear to be

specific for bacterial proteases (23,24). Even with the current limited set of substrates, promising results have been obtained. More specific D-amino acids containing FRET substrates can be designed for the refinement of *P. gingivalis* sensitivity and the identification of other bacterial species. We suggest that the FRET technique may have special value in salivary bacterial diagnostics and peri-implantitis or peri-mucositis. Also, the risk for this condition might be an important target.

In conclusion, we applied the FRET technique to detect *P. gingivalis* in implant patients with or without an implant condition, and in controls without implants. The FRET technique might also be suitable for detecting *P. gingivalis* in saliva samples: overall, FRET assays showed a higher rate of *P. gingivalis*-positive saliva samples. However, *P. gingivalis* is not very specific for peri-implant cases, as subjects with peri-mucositis and controls can also harbor this species in their saliva. Future work includes fine-tuning the FRET technology, and development of this technology into a chair-side application and multispecies testing. The current pilot study indicates that further investigations into additional D-amino acid substrates and other bacterial markers are warranted to increase the diagnostic strength and applicability.

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