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Carpegen® real-time polymerase chain reaction vs. anaerobic culture for periodontal pathogen identification

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Background/aims: The aim of this study was to compare two methods of microbiological diagnosis, anaerobic bacterial culture and real-time polymerase chain reaction (PCR), for the detection of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Fusobacterium nucleatum*, and *Treponema denticola*.
Methods: Seventy-two samples were collected from 18 patients who were suffering from aggressive periodontitis. The data obtained were compared for the two methods.
Results: The results obtained with real-time PCR were different from those obtained with bacterial culture. The detection differences were 3% for *A. actinomycetemcomitans*, 8.33% for *P. intermedia*, and 12.5% for *F. nucleatum*. However, the differences for *P. gingivalis* and *T. forsythia* were 51.39% and 36.11%, respectively. No comparison was possible for *T. denticola* because it cannot be identified in culture. The variations found were the result of the better detection level (10^2 pathogens) of the PCR probe. Unlike bacterial culture, PCR allows the detection of *T. denticola*, which does not forming colonies and is oxygen sensitive. For *F. nucleatum*, *T. forsythia* and *P. gingivalis*, the real-time PCR technique was more sensitive than culture.
Conclusion: Good results were obtained with the real-time PCR technique for the six periopathogens targeted. This method seems to be indicated for its simplicity, rapidity and reproducibility but it cannot analyze data for an antibiotic susceptibility test. The periodontist must therefore choose one of these two methods according to his specific clinical objective: to obtain rapid, specific detection even with weak initial concentrations (but for targeted periopathogens only) or to be non-specific and analyze the pathological activity with an antibiogram.

Key words: *Actinobacillus actinomycetemcomitans*; anaerobic bacterial culture; *Fusobacterium nucleatum*; microbiological diagnosis; periodontopathogens; *Porphyromonas gingivalis*; probe detection; real-time polymerase chain reaction; *Prevotella intermedia*; *Tannerella forsythia*; *Treponema denticola*

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Periodontitis is caused by opportunistic infectious diseases the expression of which is related to the patient's susceptibility. The reference method for determining the identity of periodontal pathogens is the cultivation method. However, certain technical limitations of anaerobic culture can be avoided by the use of molecular techniques (5, 20, 32).

Recently, real-time polymerase chain reaction (PCR) has been shown to be a sensitive, rapid method for the detection and quantification of individual microbial species (9, 21, 22, 29, 31). GABA International® Laboratories (Münschenstein, Switzerland) have developed a new concept of molecular diagnosis by real-time PCR analysis targeted on six periodontal pathogens: *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Fusobacterium nucleatum*, and *Treponema denticola*. These microorganisms are considered to be the periodontal pathogens that are implicated in or associated with periodontitis and peri-implantitis (2, 4, 17–19, 26, 27, 35, 39, 44, 46).

The objective of this study was to compare the two methods for analyzing bacterial samples: analysis using conventional anaerobic bacterial culture carried out in the Laboratory of Oral Ecosystem & Biomaterials specialized laboratory

(Toulouse, France) and analysis using the real-time PCR marketed by GABA International® (Meridol® Perio Diagnostics).

Samples for both methods were taken with two sterile paper points that were inserted into the same site (periodontal pocket) with the same protocol and at the same moment. The results were compared site-by-site, taking into account the quality and the presence or absence of pathogens.

Materials and methods

Patients and sampling sites

The population comprised 18 patients consulting for periodontitis, confirmed by clinical and radiological examination, who had received no antibiotic therapy in the previous 3 months. Age was not a selection criterion because the study was concerned simply with the quality of the non-invasive sampling methods.

Criteria for non-inclusion were antibiotic and/or periodontal treatments during the previous 3 months (which could distort the antibiogram data), and patients with less than four distinct sites in activity. Four active sites with pockets at least 5 mm deep were chosen in each patient. This choice was made after a radiographic examination where the deepest periodontal pocket in each quadrant of the arches was selected (28). The X-ray examination was performed to avoid disturbing the anaerobic subgingival flora or transferring bacteria from one pocket to another with the same periodontal probe.

The site selection criteria were the usual signs of disease activity: spontaneous bleeding, attachment loss, periodontal pocket suppuration, and gingival inflammation.

The chosen site was cleaned with sterile serum and compress to remove the supragingival bacterial biofilm, and dried with an air spray. Samples were taken with endodontic sterile paper points (Mynol® Regular Style-Fine, Sure Dent Corp., Seoul, Korea) held in sterile pliers (34): two paper points were inserted, without forcing, into the selected pocket for 20 s, until pressure resistance was felt.

One of the two paper points was then placed in a 2-ml bottle of reduced transport medium VGMA-III of Möller (25), the other paper point was placed in a specific tube for analysis with real-time PCR in a specialized molecular laboratory (Carpegen® GmbH, Münster, Germany).

The total bacterial load and the qualities of the bacteria revealed by each method were examined.

Anaerobic culture procedures

Culturing procedures were carried out in the Laboratory of Oral Ecosystem & Biomaterials (Faculty of Dentistry, Toulouse, France). The reduced transport medium used was Möller's VGMA-III modified by Slots (25, 33, 37).

To liquefy the VGMA III transport medium, the 2-ml bottles were reheated at 37°C for 15 min. After mixing for 30 s at maximal speed on a Vortex mixer, the 2-ml bottles containing glass beads were opened in an anaerobic chamber (Bactron® IV Anaerobic Environmental Chamber, Sheldon Mfg, Cornelius, OR) and samples were serially diluted tenfold in Wilkins–Chalgren® broth (WC®, Oxoid, Basingstoke, Hampshire, UK).

Bacteria were cultivated on different selective and non-selective culture media. Following Slots' rapid identification method (37, 38), we used appropriate dilutions (10^{-2} and 10^{-3}) to allow the specific growth and isolation of *A. actinomycetemcomitans* (TSBV medium) (36) and *F. nucleatum* (CVE medium) (45). 100 µl of 10^{-3} and 10^{-4} dilutions were sown on a non-specific blood agar plate (10) supplemented with 0.0002% menadione sodium bisulfite, 0.4% hemin chloride and 10 mg/l *N*-acetyl-muraminic acid. This allowed a total bacterial count and the culture and identification of black-pigmented '*Bacteroides*' (i.e. *P. gingivalis*, *P. intermedia*) and non-pigmented '*Bacteroides*' (i.e. *T. forsythia*).

The composition of each selective medium was as follows.

TSBV medium (for the isolation of *A. actinomycetemcomitans*) comprised sterile water, 40 g/l tryptic soy agar (Soybean-Casein) (Difco™, Sparks, MD), and 1 g/l yeast extract (Difco); the solution was adjusted to pH 7.2 and autoclaved at 120°C for 20 min. Once cooled to 56°C 10% sterile horse serum (bioMérieux, Marcy l'Etoile, France), 75 µg/ml bacitracin (Sigma-Aldrich, Steinheim, Germany) and 5 µg/ml vancomycin (Sigma) were added.

CVE medium (for the isolation of *F. nucleatum*) comprised 1% Trypcase Soja (bioMérieux), 0.02% tryptophan (Sigma), 1.5% granulated agar (Difco), 0.5% yeast extract (Difco), 0.5% sodium chloride (Sigma) and 0.2% glucose (Sigma). The solution was autoclaved at 120°C for 20 min. When the temperature had fallen to 56°C, 5% sterile defibrinated sheep blood (Diagnostics Pasteur, Marnes La Coquette, France), 4 µg/ml erythromycin (Sigma) and 5 µg/ml crystal violet (Sigma) were added.

Agar plates were placed in an anaerobic chamber for 5–6 days at 37°C (N_2 80%, CO_2 10%, H_2 10%). Identification of putative anaerobic bacteria was carried out according to Bergey's manual criteria (11) as follows: colonial morphology and consistency, colonial color, colonial long-wave ultraviolet fluorescence (λ=365 nm), cell mobility and morphology, Gram-staining, catalase, and oxidase slide tests. This identification was first performed with the naked eye, then with a binocular magnifying glass and, finally, under the microscope (oil immersion) to clarify any doubts on species identification. If no definitive identification was made, isolates were characterized by means of the API 32-A system® (bioMérieux) and aerotolerance.

When the culture of a sample showed more than 10^5 bacteria/ml, the sample was considered significant. The well-identified periopathogenic bacteria were placed in sub-cultures. Then, an antibiotic susceptibility test (antibiogram) was performed.

Real-time quantitative PCR

This test method detected the bacterial DNA of the following six periopathogens, alive or dead: *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia*, *F. nucleatum*, and *T. denticola*. In addition to this determination, the total bacterial load in the samples was also assessed.

As previously explained, we used exactly the same sampling technique as for the culture method, inserting Roeko® cones (Roeko, Langenau, Germany) at the same time and in the same periodontal site. We directly deposited the sampling cones in Sarstedt AG & Co. (Numbrecht, Germany) transport tubes. The plaque samples were sent to the specialized molecular laboratory (Carpegen GmbH, Münster, Germany). The cells were subjected to the automated process of Meridol® Perio Diagnostics analysis (GABA International, Münchenstein, Switzerland).

The bacterial genomic DNA was isolated and purified following the manufacturer's instructions. Primers and probes for Meridol® Perio Diagnostics were designed to be a highly specific match for the ribosomal DNA (rDNA) of the periodontal pathogens.

Real-time PCR was carried out using 2 µl of the extracted DNA as the template in a reaction mixture containing the appropriate primer probe sets and the TaqMan® Universal PCR Master Mix (16, 31). The sensitivity of this method is greater than that of the culture method: the bacterial detection threshold is 100 bacteria with a variation coefficient of 15%.

Table 1. Mean proportion of the periodontal pathogens according to the two methods of detection

	Culture		Real-time PCR		
	% (by site)	Proportion, mean ± SD	% (by site)	Proportion (%), mean ± SD	% ($>10^3$)
Actinobacillus actinomycetemcomitans	0	0	33.33	0.08 ± 0.02	2.77
Porphyromonas gingivalis	8.33	2.99 ± 6.69	84.72	4.91 ± 8	59.72
Tannerella forsythia	33.33	6.39 ± 6.9	75	3.92 ± 5.74	69.44
Treponema denticola	0	0	80.55	3.64 ± 5.24	70.83
Fusobacterium nucleatum	45.83	9.21 ± 15.54	77.78	0.41 ± 0.91	58.33
Prevotella intermedia	30.55	12.06 ± 21.08	36.11	1.06 ± 2.49	22.22

Table 2. Total bacterial load

	Culture	Real-time PCR
Minimum	3.5×10^5	1×10^5
Maximum	4×10^8	2.3×10^8
Mean	5.48×10^7	4×10^7
Standard deviation	5.32×10^7	4.95×10^7

Two days after sampling, we received a report of the analysis showing the number of micro-organisms detected and the total bacterial load.

Statistics

While the detection threshold was only 10^2 bacteria for real-time PCR analysis, for anaerobic culture it was 10^3. So, to compare the two methods, we have analyzed original results and adjusted the data to the level of the anaerobic culture procedure (10^3; Table 1). EXCEL software was used to classify the results and calculate the means and standard deviations. The results are given in the form of histograms and tables.

Results

The results obtained with each method were analyzed by the proportion of each species, and then compared to the total flora (Tables 1 and 2). From the 18 patients, we collected 72 analyses by bacterial culture and 72 analyses by real-time PCR.

For all the periopathogens targeted, the molecular technique gave more powerful results than the culture, except for *P. intermedia*, for which the quantity of periopathogen identified was slightly higher by culture. Relative similarities were found for *A. actinomycetemcomitans* and *F. nucleatum* (Fig. 1). However, a great difference was noted for both *P. gingivalis* (51%) and *T. denticola* (70%); the latter was naturally absent in culture because it does not form colonies. With regard to *T. forsythia*, the molecular technique identified a larger number of bacteria than the culture method (36.11% more).

Porphyromonas gingivalis

The presence of *P. gingivalis* was very variable: we found a 51.39% difference between the two methods. This discrepancy is considerable given the pathogenicity of this bacterium. On the other hand, *P. gingivalis* was present in 2.99% of the total cultivable flora, and 4.91% with the probes (5.99% in corrected data) but the proportion remained small. The standard deviation was 3, a small variation compared to the average.

One result gave identical percentages for the total flora for both techniques, two cases were very close and three showed a high percentage with the probes and a low percentage with the cultures.

Tannerella forsythia

As mentioned above, there was a 36.11% global difference between the two methods for *T. forsythia*, which is considerable. However, there were great variations in the results: 16 sites (pockets) showed high similarity (difference <0.5%) including one site with identical results; 12 sites gave various results, including 10 pockets where culture detection was higher than by the probes (24% of difference); seven sites showed detection <1% for the probes but varying from 7 to 20% for the cultures (including two pockets with 0% *T. forsythia* for the probes, and 15–24% for the cultures).

In the last cases, live bacteria were cultured in high concentrations whereas the probes had not detected them. One could wonder whether the sampling was valid but as it was significant for the other pathogenic bacteria, with a total bacterial load $\geq 10^6$/ml, we can consider it valid.

Site-by-site analysis

In general, site-by-site-analysis results of bacterial presence were different for both the cultures and the probes. However, the proportion of the total flora (total load) was relatively similar in the two techniques (Table 2). Although very variable (0–12%), the percentage of periopathogens was small when compared to total flora in

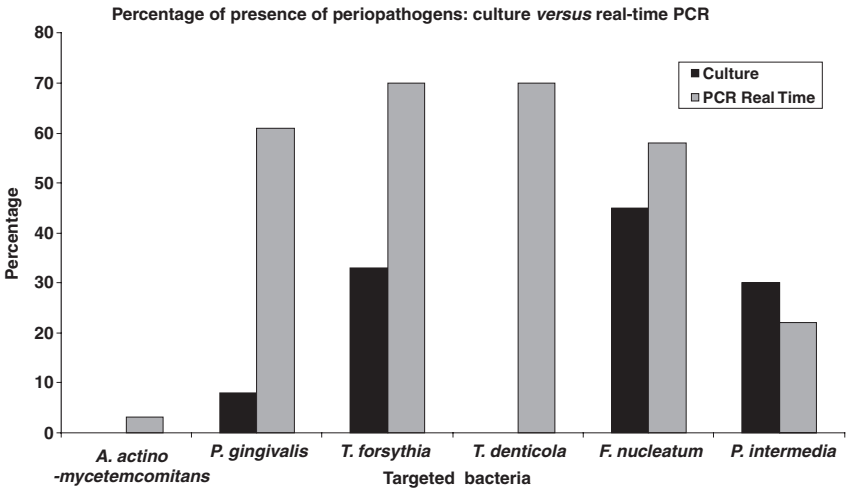

Fig. 1. Comparative table of the two methods according to the periodontal pathogens. Percentage of the number of sites in which the target bacterium was detected.

the two techniques. This variation within the groups may be very high: up to 21% for *P. intermedia* and 15% for *F. nucleatum* (Table 1).

Discussion

All the samples were successfully analyzed and presented a large total flora, which shows that each sample was statistically significant. The periopathogens identified seem to correspond to the microflora commonly described in the literature.

There was a difference in periopathogen distribution from one patient to another. No direct link was observed between disease gravity and presence of pathogenic bacteria: some patients suffered from aggressive periodontitis with few pathogens while others reacted only to a great quantity of pathogens. The pathogenic species has to be present for periodontitis to develop but disease will not necessarily occur as long as the pathogen does not exceed a well-determined and specific threshold for each host (1, 41, 42).

Porphyromonas gingivalis

The results seem to be very different for *P. gingivalis* but when the proportions of the total flora are considered, a good correlation appears between the two techniques for this pathogen. This anaerobic, black-pigmented species is extremely oxygen sensitive and it is possible that the cultures lost live cells during sampling, transport or sowing (subculture).

Actinobacillus actinomycetemcomitans

Comparable results were obtained for *A. actinomycetemcomitans* with a difference of only 2.77% between the two methods.

The quantity of *A. actinomycetemcomitans* is very small compared with the total flora: 0.08% for the probes and 0% for the cultures. These results are very different from the findings of Jervøe-Storm et al. (12) but similar to those of Lau et al. (16). The quantity of this capnophilic periopathogen did not generally reach the threshold of culture detection. Besides, in the population studied here there was a very large majority of Caucasian patients. Haubek et al. (6–8) and Poulsen et al. (30) have shown that *A. actinomycetemcomitans* is a very rare periopathogen in northern Europe. The geographic and ethnic distribution of this bacterium could therefore explain the very weak prevalence of *A. actinomycetemcomitans* in our study.

Fusobacterium nucleatum and *Prevotella intermedia*

There were different results for *F. nucleatum* and *P. intermedia*. The probes did not detect these pathogens whereas the cultures highlighted a strong titration in 10% of cases. For these two species, the question arises as to the reliability of detection, either by probes or by conventional culture.

The most probable theory is related to the fact that the culture medium is not specific for *P. intermedia* or *Prevotella nigrescens*. These two pathogens are very similar: they have the same metabolism, and show no difference of pathogenicity. Whereas the probes are highly specific for *P. intermedia* and differentiate the two periopathogens, the cultures do not differentiate them at all. There could thus be a high titration result in culture but an absence in the probes.

In the same way, for *F. nucleatum* and *Fusobacterium periodonticum*, the culture medium is not specific (although it remains possible to improve the human recognition by observing colonies with ultraviolet light). The analysis can thus induce an overestimate by confusion of colonies, which is not the case for the probes.

Tannerella forsythia

The difference of 36.11% in the detection of *T. forsythia* between culture and real-time PCR technique is certainly the result of the fact that this bacterium is fastidious to cultivate (3). Besides, there was a very small proportion of this periopathogen in our samples. According to some bacteriologists (L. Dubreuil, Lille, France, unpublished data), it seems nowadays that menadione and hemin are inhibitory for some isolates of *T. forsythia*: if this is the case, it could explain the weak percentage of *T. forsythia* isolates in our study.

Anaerobic culture vs. real-time PCR

Bacterial anaerobic culture is based on living cells. This technique enables us to search for all the microorganisms present in a non-specific way. Since it is not directed towards precise pathogenic targets, it remains the most objective technique (gold standard). Thus, many unexpected pathogen colonies appear during culture, whereas routine probes cannot detect them. This fact can be crucial, in particular in the presence of *Candida*, *Enterobacter cloacae* or *Pseudomonas* periodontal infections (40) when large amounts of antibiotics have been prescribed in the past (which will worsen the situation, making antifungal drugs or hydroquinolones the only appropriate prescription).

On the other hand, compared to the probes, there was a loss of information for weak concentrations in cultures. This can be explained by a threshold of detection of 10^3 for the culture and 10^2 for the molecular technique. The loss of information can also be the result of the death of bacteria during sampling or transport. However, that surely represents an infinitesimal quantity because the cultivated total bacterial load found by the two techniques is comparable. This indicates that there was very little mortality in the samples.

In the real-time PCR, the probes and/or primers detect the DNA of both living and dead bacteria. This major difference could explain why the cultures were still sometimes negative while probe detections were positive. If this assumption is correct, the probes enable us to highlight the bacterial history of the pocket for the target periopathogens.

Criteria of choice

Which technique is the best reflection of the clinical situation? Should we search for live bacteria or evaluate the DNA traces persisting in the periodontal pockets whatever the state of the bacteria?

Anaerobic culture seems to give a clearer idea of the evolutionary potential of the pocket while real-time PCR could be the reflection of the periodontitis history. On the other hand, we have no indication of how long the dead bacteria may have been in the pocket, and thus of the importance and chronology of the last disease activity. The probes enable us to highlight the most pathogenic bacteria that exist or have existed in the sample, even if the concentration is weak. Therefore, for the target periopathogens, and only the target ones, we have a better sensitivity with this technique.

The duration of the analysis depends on the detection technique used. Real-time PCR can provide results in 2 h, whereas anaerobic culture requires 7–8 days to confirm the presence of putative periopathogens (and nearly an extra week for the antibiogram data).

The cultures have the important advantage of allowing an antibiotic sensitivity

test to be carried out. In practice, we can sow identified bacteria in the presence of specific antibiotic dilutions. Although the interest of this *in vitro* technique remains relative, because of the presence of biofilm and the complexity of the oral flora (43, 44), we still have no other tested scientific method by which to analyze the sensitivities of pathogens to antibiotics.

The use of probes requires reference to the scientific literature to prescribe the most suitable antibiotic (broad consensus). These references are generally based on statistical evaluations of culture results from several geographical and ethnic origins but we will never know the real sensitivity of each detected pathogen to antibiotics. Besides, Mellado et al. (23, 24), Lakhssassi et al. (13), and Lakhssassi & Sixou (14, 15), have brought to light the high inter-individual and, above all, intra-individual variations in the susceptibility of some periopathogens to antibiotics (in particular for *P. intermedia*). This fact emphasizes the fundamental importance of conventional bacterial culture and antibiogram for each patient suffering from aggressive periodontitis.

Conclusion

We found a similarity between the two methods of analysis to objectify the presence of the six target periodontal pathogens (after concentration correction).

The intrinsic technical limitations of anaerobic bacterial cultures cause a loss of quantitative information because at least 10^3 pathogens are required for detection. However, this reference technique has two important advantages: bacterial detection is not targeted, and we can carry out an antibiotic susceptibility test. Thus, anaerobic culture brings more information and allows the right antibiotherapy to be implemented.

The real-time PCR technique gave relatively similar results, the variations found being related to its lower detection threshold (10^2). This technique can make it possible to detect the presence of *T. denticola* whereas culture cannot. For *F. nucleatum*, *T. forsythia*, and *P. intermedia*, real-time PCR seems more sensitive and more discriminating than anaerobic culture. Taking into account these results, the reliability of molecular detection of the six targeted periopathogens authorizes its widespread but not systematic use thanks to its simplicity, rapidity, and reproducibility. We hope that the number and quality of probes and primers will increase to cover all the periodontal pathogens.

With regard to the results of this study, the conventional and molecular techniques seem complementary. They allow a precise bacteriological diagnosis to be established, on condition that they are used according to the therapeutic objectives: to allow the detection of very small quantities of target pathogens, or to be non-specific according to the diversity of the periodontitis flora. In our opinion, real-time PCR is the most suitable technique for the detection and quantification of persisting periopathogens during the control and maintenance phases while reassessing periodontal treatment (32).

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