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Comparison of culture and real-time PCR for detection and quantification of five putative periodontopathogenic bacteria in subgingival plaque samples

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Abstract

Objectives: Bacterial cultivation is a well-established method for analyzing plaque samples. Real-time polymerase chain reaction (PCR) is a novel rapid method for the identification and quantification of periodontopathogenic bacteria that has been recently introduced. In this study, we compared real-time PCR with conventional anaerobic cultivation.

Method: A total of 78 subgingival plaque samples were harvested from pockets $\geqslant$5 mm in 22 patients with advanced chronic periodontitis and immediately transferred into transport medium. Aliquots were evaluated with species-specific probes by real-time PCR (meridol® Perio Diagnostics, GABA) and anaerobic bacteria culture on selective media for the detection of *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Tannerella forsythensis*. The analysis was performed by two separate, blinded examiners.

Results: When real-time PCR was compared with the culture method (golden standard) for the detection of putative periodontopathogenic bacteria, the sensitivity and specificity for *A. actinomycetemcomitans* were 67% and 100%, respectively (κ: 0.79); for *F. nucleatum* 73% and 53%, respectively (κ: 0.21); for *P. gingivalis* 94% and 84%, respectively (κ: 0.77); for *P. intermedia* 33% and 94%, respectively (κ: 0.26) and for *T. forsythensis* 92% and 56%, respectively (κ: 0.51). Spearman's correlation coefficients for the quantitative results of both methods were 0.82 for *A. actinomycetemcomitans*, 0.33 for *F. nucleatum*, 0.83 for *P. gingivalis*, 0.38 for *P. intermedia* and 0.67 for *T. forsythensis*.

Conclusion: Overall, the agreement between both test methods was excellent for *A. actinomycetemcomitans* and *P. gingivalis,* fair for *T. forsythensis* and poor for *F. nucleatum* and *P. intermedia*. The discrepancies in the results may be explained by the inability of cultivation methods to distinguish between close related taxa, and the problems of keeping periopathogenic bacteria viable, which is required for standard cultivation.

Key words: bacterial culture; microbiological diagnosis; periopathogens; real-time PCR

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The World Workshop on Clinical Periodontics in 1996 described *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythensis* (former *Bacteroides forsythus*) as relevant microorganisms associated with periodontal breakdown (Zambon 1996). Since then several other bacteria such as *Prevotella intermedia*, *Treponema denti-*

cola, *Fusobacterium nucleatum*, *Peptostreptococcus micros* or *Eikenella corrodens* have also been accepted as putative periodontopathogenic microorganisms (Loomer 2004). These bacteria are mainly Gram-negative requiring anaerobic growth conditions. Therefore, for diagnostic procedures problems of sampling, transport and cultivation have to be taken into consideration. Cultivation methods are considered as the golden standard, although limitations with respect to detecting non-viable bacteria, the inability of some species to grow reliably on selective media as well as high costs narrow the use in periodontal microbiological diagnostics (Loomer 2004). Alternative differentiation methods include dark field microscopy, enzymatic and immunological assays, nucleic acid probes and polymerase chain reaction (PCR) (Loomer 2004). With nucleic acid probes a risk of cross reaction between *A. actinomycetemcomitans* and taxonomically related haemophili exists (Zambon & Haraszthy 1995). PCR methods are more specific and sensitive than cultivation, based on detection of gene-specific DNA sequences, thus a possibility to distinguish close related bacteria is given. A PCR assay for the identification of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythensis* and *T. denticola* has recently been described (Eick and Pfister 2002). The authors advocated this test as a highly sensitive and specific method for the analysis of the subgingival plaque.

Many efforts have been made to quantify target DNA molecules initially serving as template in a PCR reaction. Standard PCR lacks the ability for precise quantification because only an endpoint determination can be analyzed. Real-time PCR overcomes these problems by direct monitoring of the increasing amount of PCR products throughout the enzymatic assay. The amount of newly synthesized PCR product molecules is directly dependent on the amount of template molecules. The data for quantification are collected in exponential phases of the PCR. This allows a precise quantification of the target DNA copy number, when using internal and external standards (Bustin 2000).

Recently, real-time PCR has been used for the identification of *P. gingivalis* (Lyons et al. 2000, Sakamoto et al. 2001, Boutaga et al. 2003, Morillo et al. 2003), *A. actinomycetemcomitans* (Sakamoto et al. 2001, Morillo et al. 2003) *T. denticola* (Sakamoto et al. 2001, Asai et al. 2002, Yoshida et al. 2004) and *T. forsythensis* (Sakamoto et al. 2001).

The aim of this study was to compare real-time PCR with conventional anaerobic bacterial cultivation for the detection and quantification of *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *P. intermedia* and *T. forsythensis*.

Material and Methods

Patients and sampling sites

A total of 78 subgingival plaque samples were harvested from 22 patients with advanced chronic periodontitis. Patients had $\geqslant 20$ teeth, at least two pockets $\geqslant 5$ mm with bleeding on probing per quadrant and had not received any periodontal or antibiotic treatment during the last 6 months. All patients were in good systemic health. Subgingival plaque samples were obtained with two parallel inserted sterile paper points for 20 s and immediately transferred from the periodontal pocket into an anaerobic transport medium (Port-A-Cul, BD, Heidelberg, Germany). The plaque samples were sent to a specialized laboratory (Center for Oral Diagnostics, Kiel, Germany) where the paperpoints were placed in a reduced Wilkins–Chalgren-suspension containing vitamin K1 and hemin (Oxoid, Wesel, Germany). After vortexing for 30 s a homogenous suspension was prepared and divided into two 1.5 ml aliquots. One was used for anaerobic cultivation on selective media, the other 1.5 ml was frozen and sent on ice to another specialized laboratory (Carpegen GmbH, Münster, Germany) for evaluation by real-time PCR.

Cultivation

Cultivation, quantitative and qualitative analysis of *A. actinomycetemcomitans*, *F.* species (*F. nucleatum* and *F. periodonticum*), *P. gingivalis*, *P. intermedia/nigrescens* and *T. forsythensis* was performed as follows:

The sample for cultivation was immediately divided into equal portions of 100 µl and plated on different selective and non-selective culture media.

For *A. actinomycetemcomitans* tryptic soy–serum–bacitracin–vancomycin agar (TSBV) (5 mg/l vancomycin, 75 mg/l bacitracin) and chocolate-agar (supplemented with VITOX, Oxoid) plates were incubated in a 5% CO_2 atmosphere, 37°C. After 72 h analysis was performed with spot test, colony morphology and a biochemical identification-system (REMEL, Virotech, Rüsselsheim).

Fusobacteria species, *P. gingivalis* and *P. intermedia* were cultured on selective media (5% sheep blood with 5 mg/ml hemin and 1.0 mg/l vitamin K1) with antibiotics (0.001% w/v nalidixic acid, 2.5 mg/l vancomycin containing Brain–Heart-Infusion (BHI) Wilkens–Chalgren-Agar) and incubated for 96 h at 37°C in an anaerobic atmosphere (Gaspak-Plus-System®, BD). An identification of the microorganisms followed on the basis of their typical colony and bacterial morphology, as well as spot test, a biochemical identification system (REMEL) and their oxygen tolerance.

T. forsythensis was cultivated under anaerobic conditions on an NAM (*N*-acetyl-muraminic acid, 10 mg/l) agar. Identification was performed by means of colony and bacterial morphology. Additional tests for identification included detection of a trypsin-like activity based on the degradation of benzoyl-DL-arginine-2-naphtylamide.

The total number of the colony-forming units (CFU) was determined on the basis of serial dilution from 10^{-1} to 10^{-3} on selective media. The detection limit with the anaerobic culture used in this study was given at 10^3 CFU.

Real-time PCR

The frozen culture (1.5 ml) was sent to Carpegen GmbH, and 0.5 ml of the defrosted dilution was used for real-time PCR analysis. The cells were harvested by centrifugation (15,000 *g* at 4°C) for 10 min and immediately subjected to the automated process of the meridol® Perio Diagnostics (GABA International, Münchenstein, Switzerland) analysis. This real-time PCR-based analysis was developed and validated by Carpegen GmbH. Specificity of meridol® Perio Diagnostics was verified with purified genomic DNA from several bacterial and fungal species as well as with human DNA. Even closely related species, such as *P. intermedia* and *P. nigrescens*, did not show any cross reactivity.

The main validated test parameters of meridol® Perio Diagnostics are:

- the detection limit for each of the five pathogens is 100 bacteria within a patient's sample;
- the linear range for quantification comprehend seven orders of magnitude for each pathogen;
- the coefficient of variation is 15%.

The test method detects and quantifies six periodontal pathogens (*A. actinomycetemcomitans, F. nucleatum* ssp., *P. gingivalis, P. intermedia, T. forsythensis* and *T. denticola*) and the total bacterial load. Results for *T. denticola* and total bacterial load were not used for the comparative study because the corresponding data from cultivation were not available.

The bacterial genomic DNA was isolated and purified with the AGOWA® mag DNA Isolation Kit Sputum (AGOWA GmbH, Berlin, Germany). The protocol followed the manufacturer's instructions with minor changes to adjust the procedure to the automated isolation with a pipetting robot (Tecan, Genesis Workstation; Tecan Schweiz AG, Switzerland). Primers and probes for meridol® Perio Diagnostics were designed to match highly specifically to ribosomal DNA (rDNA) of the five bacterial pathogens. The exact primer and probe sequences were selected with the Primer Express software (Applied Biosystems, Foster City, CA, USA), which checks the primer and probe sets for matching the guidelines that are recommended for real-time PCR with TaqMan® probes. The primers and probes were purchased from Applied Biosystems. Real-time PCR was carried out with 2 µl of the isolated DNA as template in a reaction mixture containing the appropriate primer probe sets and the TaqMan® Universal PCR Mastermix. The PCR was carried out in a ABI 7900 HT (Applied Biosystems) real-time PCR cycler in 384 well plates.

Statistical analysis

The analysis of the plaque samples with the two identification methods was performed by two separate, blinded examiners. To be able to compare the two methods, the results were presented as a score system in the following ranges: 0 indicating no detection, 1 indicating a range $\geqslant 10$ but $<10^2$ bacteria/plaque sample, 2 indicating a range $\geqslant 10^2$ but $<10^3$ bacteria/plaque sample, 3 indicating a range $\geqslant 10^3$ but $<10^4$ bacteria/plaque sample, 4 indicating a range $\geqslant 10^4$ but $<10^5$ bacteria/plaque sample, 5 indicating a range $\geqslant 10^5$ but $<10^6$ bacteria/plaque sample and 6 indicating a range $\geqslant 10^6$ bacteria/plaque sample.

In the present study the level of detection for anaerobic culture was 10^3 CFU and 10^2 bacteria/plaque sample for the real-time PCR-based test. For this reason the results of the anaerobic culture were compared both with the original results (detection level 10^2 bacteria/plaque sample) and with the adjusted data (threshold level 10^3 bacteria/plaque sample) of the real-time PCR.

The sensitivity was determined as the number of samples found positive both by anaerobic cultivation and by real-time PCR divided by the numbers of positive results by the cultivation method. The specificity was calculated as the number of negative results found concomitant by both tests divided by the numbers of negative results by the cultivation method.

For measuring the agreement between the anaerobic cultivation and real-time PCR kappa-statistics (κ) was used. For the description of the agreement of the quantitative results of both methods the Spearman correlation coefficient (SPSS software package, version 11.0) was calculated.

Results

A. actinomycetemcomitans

Real-time PCR detected many more *A. actinomycetemcomitans* positive samples than cultivation. Only six of 38 samples positive for *A. actinomycetemcomitans* by the novel PCR technology were also positive for this bacterial species by culture, resulting in a sensitivity of 100% and a specificity of only 55.6% for real-time PCR. Adjusting the threshold of detection, exact agreement between both methods was found in 97.4% with a corresponding sensitivity of 66.7% and a specificity of 100% (Table 1). The respective κ values were improved from 0.16, representing a poor agreement, to 0.79 representing an excellent agreement.

F. nucleatum

Comparison of the results for *F. nucleatum* showed a sensitivity of 98.4% and a specificity of 13.3% for the PCR method when compared with cultivation results. Increasing the threshold of detection for real-time PCR resulted in a sensitivity of 73.0% and a specificity of 53.3%. The corresponding κ value remained low (0.17 *versus* 0.21), describing a poor agreement of the methods (Table 2).

P. gingivalis

P. gingivalis was detected almost twice as often by real-time PCR when compared with cultivation. This was reflected by a high sensitivity of 97.1% and a low specificity of 20.9% of the novel test as compared with the culture results. However, adjustment of the detection threshold maintained a high sensitivity (94.3%) and increased the specificity to 83.7%. Concomitantly, the κ value was improved from 0.17 to 0.77, representing excellent agreement between both methods (Table 3).

P. intermedia

Sixty-one of 78 samples scored positive for *P. intermedia* by real-time PCR, as opposed to 42 following cultivation. Therefore, sensitivity was found to be 81.0% and specificity 25%. With an adjusted threshold of detection a sensitivity of 33.3% and a specificity of

Table 1. Comparison between anaerobic culture and real-time PCR for the detection of *Actinobacillus actinomycetemcomitans* in subgingival plaque samples

Anaerobic culture result	No. (%) of samples with the real-time PCR result at a detection level of 10^2 bacteria/plaque sample*		Total	No. (%) of samples with the real-time PCR result at a detection level of 10^3 bacteria/plaque sample†		Total
	Positive	Negative		Positive	Negative	
Positive	6 (7.7)	0 (0)	6	4 (5.1)	2 (2.6)	6
Negative	32 (48.7)	40 (51.3)	72	0 (0)	72 (92.3)	72
Total	38	40	78	4	74	78

*Sensitivity 100%; Specificity 55.56%; κ 0.16.
†Sensitivity 66.67%; Specificity 100%; κ 0.79.

Table 2. Comparison between anaerobic culture and real-time PCR for the detection of *Fusobacterium nucleatum* in subgingival plaque samples

Anaerobic culture result	No. (%) of samples with the real-time PCR result at a detection level of 10^2 bacteria/plaque sample*		Total	No. (%) of samples with the real-time PCR result at a detection level of 10^3 bacteria/plaque sample†		Total
	Positive	Negative		Positive	Negative	
Positive	62 (79.5)	1 (1.3)	63	46 (59)	17 (21.8)	63
Negative	13 (16.7)	2 (2.6)	15	7 (9)	8 (10.3)	15
Total	75	3	78	53	25	78

*Sensitivity 98.41%; Specificity 13.33%; κ 0.17.
†Sensitivity 73.02%; Specificity 53.33%; κ 0.21.

Table 3. Comparison between anaerobic culture and real-time PCR for the detection of *Porphyromonas gingivalis* in subgingival plaque samples

Anaerobic culture result	No. (%) of samples with the real-time PCR result at a detection level of 10^2 bacteria/plaque sample*		Total	No. (%) of samples with the real-time PCR result at a detection level of 10^3 bacteria/plaque sample†		Total
	Positive	Negative		Positive	Negative	
Positive	34 (43.6)	1 (1.3)	35	33 (42.3)	2 (2.6)	35
Negative	34 (43.6)	9 (11.5)	43	7 (9)	36 (46.2)	43
Total	68	10	78	40	38	78

*Sensitivity 97.14%; Specificity 20.93%; κ 0.17.
†Sensitivity 94.29%; Specificity 83.72%; κ 0.77.

Table 4. Comparison between anaerobic culture and real-time PCR for the detection of *Prevotella intermedia* in subgingival plaque samples

Anaerobic culture result	No. (%) of samples with the real-time PCR result at a detection level of 10^2 bacteria/plaque sample*		Total	No. (%) of samples with the real-time PCR result at a detection level of 10^3 bacteria/plaque sample†		Total
	Positive	Negative		Positive	Negative	
Positive	34 (43.6)	8 (10.3)	42	14 (18)	28 (35.9)	42
Negative	27 (34.6)	9 (11.5)	36	2 (2.6)	34 (43.6)	36
Total	61	17	78	16	62	78

*Sensitivity 80.95%; Specificity 25%; κ 0.06.
†Sensitivity 33.33%; Specificity 94.44%; κ 0.26.

94.4% was observed. κ-values were rather low for both analyses (0.06 *versus* 0.26) (Table 4).

T. forsythensis

Real-time PCR for *T. forsythensis* detected all 51 culture positive samples, resulting in a sensitivity of 100%. However, only five of 27 culture negative samples were confirmed as negative by real-time PCR, leading to a specificity of only 18.5%. Following the adjustment of the detection threshold for the real-time PCR, the corresponding sensitivity was 92% and the specificity 55.6%. The κ value was only 0.23 when using a threshold of detection of 10^2 bacteria/plaque sample representing a poor agreement between the methods. With an adjustment of the threshold level, κ improved to 0.51 representing a fair agreement between the methods (Table 5).

Table 6 shows the relationship between quantitative results determined by anaerobic culture and real-time PCR for the five bacterial species. The corresponding Spearman's correlation coefficients ranged from 0.83 for *P. gingivalis*, 0.82 for *A. actinomycetemcomitans* to 0.33 for *F. nucleatum* (Table 6).

Discussion

In the present study, the agreement between anaerobic cultivation and real-time PCR was analyzed with two different threshold levels. The detection level for cultivation was 10^3 CFU, for real-time PCR two different threshold levels (10^2 or 10^3 bacteria/plaque sample) were used in the comparison of the two methods.

The reason for adjusting the threshold level to 10^3 is determined by the standard anaerobic cultivation, which does not allow a higher sensitivity. Even though real-time PCR used in this study had a lower detection limit of 10^2 bacteria/plaque sample, a threshold level of 10^3 was employed to perform an unbiased comparison between cultivation and real-time PCR. Furthermore, most of the periodontopathogenic bacteria can be found in periodontal healthy subjects, although in low amounts (Ximénez-Fyvie et al. 2000). It has also been demonstrated, that the mean numbers of target bacteria were higher in patients than in controls (van Winkelhoff et al. 2002). As stated by Socransky and Haffajee (1992) is it essential for the development of periodontitis that a pathogenic species is present, but still disease will not necessarily take place as long as the pathogen does not exceed the threshold for the host.

A. actinomycetemcomitans and *P. gingivalis*

At present, there are only few reports in the literature on the use of real-time PCR as an identification method for periodontal pathogens. Morillo et al. (2003) developed a real-time PCR based on SYBR® Green for the identification of *A. actinomycetemcomitans* and *P. gingivalis* strains from the American Type Culture Collection (ATCC). They found a good sensitivity and specificity for both species with this assay. A good agreement (κ values for *A. actinomycetemcomitans* and *P. gingivalis*: 0.615 and 0.600) between cultivation and real-time PCR for detection of these two bacteria was found by Sakamoto et al. (2001). They investigated a total of 40 samples, taken at the two deepest pockets in each quadrant in five patients. These results are compliant with the

Table 5. Comparison between anaerobic culture and real-time PCR for the detection of *Tannerella forsythensis* in subgingival plaque samples

Anaerobic culture result	No. (%) of samples with the real-time PCR result at a detection level of 10^2 bacteria/plaque sample*		Total	No. (%) of samples with the real-time PCR result at a detection level of 10^3 bacteria/plaque sample†		Total
	Positive	Negative		Positive	Negative	
Positive	51 (65.4)	0 (0)	51	47 (60.3)	4 (5.1)	51
Negative	22 (28.2)	5 (6.4)	27	12 (15.4)	15 (19.2)	27
Total	73	5	78	59	19	78

*Sensitivity 100%; Specificity 18.52%; κ 0.23.
†Sensitivity 92.16%; Specificity 55.56%; κ 0.51.

Table 6. Comparison of anaerobic culture and real-time PCR for the quantification of *Actinobacillus actinomycetemcomitans* (A.a.), *Fusobacterium nucleatum* (F.n.), *Porphyromonas gingivalis* (P.g.), *Prevotella intermedia* (P.i.) and *Tannerella forsythensis* (T.f.)

	Spearman's correlation coefficient at a detection level of 10^2 bacteria/plaque sample	Spearman's correlation coefficient at a detection level of 10^3 bacteria/plaque sample
A.a.	0.45	0.82
F.n.	0.34	0.33
P.g.	0.78	0.83
P.i.	0.22	0.38
T.f.	0.68	0.67

results in the present study, showing excellent agreement and high correlation coefficients for the comparison of both methods for *A. actinomycetemcomitans* and *P. gingivalis*. Furthermore, the present results are in concert with the findings of Boutaga et al. (2003) who investigated 259 pooled plaque samples, taken from the deepest pocket in each quadrant harvested from 259 patients, for the presence of *P. gingivalis*. They also used cultivation as the golden standard and found a sensitivity of 100% and a specificity of 94% comparing cultivation and real-time PCR based on TaqMan-probes. With the adjusted detection threshold in the present study, also high values for sensitivity (94.3%) and specificity (83.7%) could be obtained. Interestingly, Boutaga et al. (2003) employed a detection limit of 10^4 CFU/ml for the cultivation, whereas in our study we found an excellent agreement between both analytic methods, but at a lower detection limit of 10^3 CFU.

F. nucleatum

For *F. nucleatum* the agreement between both microbiological identification methods was poor. This can be explained by the fact that the standard anaerobic cultivation used in this study was not able to distinguish between different species of *Fusobacterium*. The bacteria in the taxonomic group of *Fusobacteria* are very closely related with each other (Tanner et al. 1994, Conrads et al. 2002). Therefore, it was not possible for cultivation to differentiate between e.g. *F. nucleatum* and *F. periodonticum*. A molecular biological method such as PCR is required for a differentiation between such bacteria (Bolstad & Jensen 1993). This explains the high degree of detection by the anaerobic cultivation in the present study (63 positive of 78 samples) in contrast to the results with the real-time PCR (53 positive of 78 samples). It is appropriate to assume, that the differences in these results, were because of a detection of other *Fusobacteria* than *F. nucleatum* with the cultivation method.

P. intermedia

Similar problems occurred when analyzing *P. intermedia*. Only a poor agreement between both test methods could be demonstrated. Whereas the culture method could not distinguish between *P. intermedia* and *P. nigrescens*, this distinction could be made by real-time PCR. As described by Tanner et al. (1994) and Kuboniwa et al. (2004) differentiation between *P. intermedia* and *P. nigrescens* is very difficult using routine phenotypic tests, however they can be identified by their unambiguously DNA profiles. Thus, for both *F. nucleatum* and *P. intermedia* a relatively high number of false positive findings was obtained by standard anaerobic cultivation as compared with real-time PCR, explaining the lack of agreement between the results of both methods.

T. forsythensis

Comparing the results of culture and real-time PCR for the detection of *T. forsythensis* the agreement was only fair. It is likely that false negative results for *T. forsythensis* were obtained by anaerobic cultivation, because of the fact that *T. forsythensis* is a very difficult bacteria to cultivate (Ezzo & Cutler 2003). In accordance with the present study only a fair agreement between cultivation and real-time PCR for the detection of *T. forsythensis* (κ: 0.545) was reported by Sakamoto et al. (2001) who analyzed 40 samples harvested in five patients.

The results of the present study indicate, that the use of cultivation for the analysis of specific bacteria in subgingival plaque samples may have some limitations. These are mainly caused by the anaerobic nature of periodontopathogenic bacteria, as already described. Novel molecular biological diagnostic tools may be able to overcome the problems. A comparison between cultivation and a PCR-based method was performed by Eick & Pfister (2002). They showed advantages with the DNA probe, and suggested that nucleic acid techniques should replace cultivation methods as gold standard in microbial diagnosis of progressive periodontitis. The results of the present study seem to support their suggestion. In addition, real-time PCR does not only provide rapid detection but also quantification.

On the other hand, molecular biological identification techniques can only detect bacteria for which the test is designed. No additional information can be gained, such as coincidental detection of unexpected bacteria or the testing of antibiotic susceptibility.

In summary, in the present study a novel test based on real-time PCR was found to be a sensitive and specific identification method, with the addi-

tional possibility to perform a quantification of specific bacteria in the subgingival plaque samples. Depending on the bacteria studied, we found an excellent to poor agreement between both test methods. The discrepancies in the results may be explained by the inability of cultivation methods to distinguish between close related bacteria, the different threshold levels of both methods and the problems of keeping periopathogenic bacteria viable, which is required for standard cultivation.

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