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Short communication

Assessment of viable periodontal pathogens by reverse transcription quantitative polymerase chain reaction

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Background and Objective: Molecular biological methods for the detection of periodontitis-associated bacteria based on DNA amplification have many advantages over classical culture techniques. However, when it comes to assessing immediate therapeutic success, e.g. reduction of viable bacteria, DNA-based polymerase chain reaction is unsuitable because it does not distinguish between live and dead bacteria. Our objective was to establish a simple RNA-based method that is easily set up and allows reliable assessment of the live bacterial load.

Material and Methods: We compared conventional quantitative real-time PCR (qPCR), propidium monoazide-qPCR and reverse transcription qPCR (RT-qPCR) for the detection of periodontal pathogens after antibiotic treatment *in vitro*. Applicability was tested using clinical samples of subgingival plaque obtained from patients at different treatment stages.

Results: The bacterial load was remarkably stable over prolonged periods when assessed by conventional qPCR, while both propidium monoazide intercalation as well as cDNA quantitation showed a decline according to decreasing numbers of viable bacteria after antibiotic treatment. Clinical samples of subgingival plaque were directly subjected to DNase I treatment and RT without previous extraction or purification steps. While the results of the DNA- and RNA-based methods are comparable in untreated patients, the classical qPCR frequently detected substantial bacterial load in treated patients where RT-qPCR no longer indicates the presence of those pathogens. The disagreement rates ranged between 4 and 20% in first visit patients and 8–50% in the group of currently treated patients.

Conclusion: We propose to use RNA-based detection methods to verify the successful eradication of periodontal pathogens.

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The oral bacterial biofilm contributes significantly to the development and progression of periodontitis (1,2). Molecular biological methods by which specific bacteria can be detected are becoming increasingly accepted as supporting tools to aid the choice of treatment (3–5). These DNA-based methods, mostly employing conventional or real-time polymerase chain reaction (PCR) have the advantage of high sensitivity, high specificity and offer the possibility of quantitative analysis. However, PCR methods using chromosomal DNA as the target molecule have the disadvantage that they cannot discriminate between viable and dead bacteria, thus making this technique unsuitable to monitor the therapeutic progress related to bacterial load. The integrity of the genomic DNA is essential for the survival and reproductive success of an organism, which is why DNA is highly protected by a number of mechanisms (6). RNA, on the other hand, has mostly temporary function, e.g. as messenger RNA, and its degradation is closely regulated and occurs more or less quickly, depending on the RNA species. RNA-degrading enzymes are omnipresent. Therefore, certain precaution measures have to be in place, which makes working with RNA somewhat more difficult. As ribosomal (rRNA) RNAs are both structural and major functional parts of the ribosomes, the protein synthesis machinery, they are among the most stable RNA species, making them a popular target for molecular biological methods (7,8).

To overcome the drawback of high DNA stability even in non-viable cells it was proposed to use propidium monoazide (PMA) that specifically penetrates non-viable cells, intercalates with dsDNA and by that efficiently inhibits PCR amplification. In other words within a pool of viable and dead bacteria, only the viable cells will be detected by PCR. Loozen and colleagues recently showed that this method works well with live and heat-killed suspensions of oral pathogenic bacteria (9). Nevertheless, the question of practicability remains. The dentist will usually take a plaque sample using paper points and dissolve them in a suitable buffer before further analysis. If viability and membrane integrity were to be preserved, at least for a limited period this would likely require a more sophisticated protocol. Moreover, as we are dealing with a pool of many different bacterial species, a single preservation method might not be suitable and/or could be selective for certain species. Other possible solutions such as combined immunofluorescence and fluorescence *in situ* hybridization (10) or RNA-oligonucleotide quantification (11) appear valuable, but technically demanding and therefore more suitable for research purposes than clinical application. Here we propose a simplified RNA method to provide basic insight into the viable microbial load within a patient's plaque sample.

Material and methods

Antibiotic treatment of bacterial cultures

Actively growing cultures of *Porphyromonas gingivalis* [DSM20709, approximate concentration 1×10^6 CFU/mL in chopped meat medium with carbohydrates (DSMZ medium no. 110)] and *Aggregatibacter actinomycetemcomitans* [DSM8324, approximate concentration $5x10^5$ CFU/ml in Colombia blood medium (DSMZ medium no. 693)] were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Control samples were taken immediately upon arrival to ensure their viability. Amoxicillin and metronidazole (both Sigma-Aldrich, Vienna, Austria) were added to the remaining cultures at final concentrations of 30 μg/mL to *A. actinomycetemcomitans* and *P. gingivalis*, respectively. Additional doses of antibiotics (same concentration) were added every other day. Bacteria were maintained in an aerobic (5% CO_2) incubator at 37°C for the duration of the experiment, i.e. 3 days and 7 days for *P. gingivalis and A. actinomycetemcomitans*, respectively. Sampling took place before (0 h), and at several time points after the addition of antibiotics. The non-antibiotic treated sample was used as control (expressed as 100%). The experiment was repeated twice.

Propidium monazide treatment

Propidium monoazide was purchased from Biotium (VWR Int., Vienna, Austria) and added to bacterial solutions at a final concentration of 100 μM. The PMA–bacteria mixture was incubated in the dark for 30 min followed by a 3 min exposure to a 500 W halogen light source at a distance of 10 cm. The tubes were placed on ice to avoid heating. After centrifugation at $15{,}000 \times g$ for 3 min the pellet was resuspended in 100 μL of our proprietary bacterial lysis solution (Lambda GmbH, Rainbach, Austria).

DNA extraction

One-hundred microliters of the diluted, PMA-treated bacteria or 5 μL of pure bacteria lysates (either from bacterial cultures or subgingival plaque samples) were used for DNA extraction using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich) according to the manufacturer's instructions.

RNA extraction

We used Ambion® PureLink® RNA Mini Kit (Life Technologies, Vienna, Austria) for RNA purification following the manufacturer's instructions.

Subgingival plaque samples

Clinical samples were surplus samples from ongoing clinical studies on periodontitis markers and performance of a point of care test for which ethics commission and written consent of the patients has been granted. Personal data were rendered anonymous, only the overall treatment status (non-treated, in treatment, recall) was made available for research purposes. Subgingival plaque samples were taken using sterile paper points (ISO 35, Diantent, Almere, the Netherlands). Two paper points from different sites were pooled into one tube and immediately submerged into

160 μL of our proprietary lysis solution (Lambda GmbH, Rainbach, Austria). Samples were stored in the lysis buffer at −20°C for a maximum of 2 wk until analysis.

DNase I treatment and reverse transcription

Eight microliters of the purified bacterial RNA or of lysed subgingival plaque sample were treated with 1 μL (1 U/μL) DNase I (Fermentas, Sankt Leon-Rot, Germany) for 30 min at 37°C in a total volume of 20 μL. The reaction was stopped by addition of 1 μL EDTA (50 μM) and incubation at 65°C for 10 min.

Five microliters of total RNA (DNase I treated) were subjected to reverse transcription (RT) using *SuperScript® VILO™ cDNA Synthesis Kit* (Life Technologies) following the manufacturer's instructions.

To simplify the method and make it suitable for patient samples we adapted the above protocol to perform direct DNase I treatment of subgingival plaque samples. The samples were taken using commercial paper points. Bacterial dissolution and lysis was achieved by immersing the paper points in our proprietary lysis solution and heating the samples to 95°C for 6 min. The lysis solution stabilizes both DNA and RNA, which results in inhibiting effects on some downstream applications. We therefore performed a control experiment to evaluate whether it was possible to directly perform DNase I treatment and subsequent cDNA synthesis out of the specimen lysates by comparing the RT-quantitative PCRs (RT-qPCRs) using purified as well as crude RNA of the same sample. As we did not detect any significant differences in the final PCR results (data not shown), nor a problem with DNA contamination, we continued using the crude RNA lysates.

Real-time polymerase chain reaction

Real-time PCR was performed using bacterial DNA (derived from bacterial cultures or subgingival plaque samples), bacterial DNA after PMA treatment (PMA-DNA) or cDNA (derived from bacterial cultures or subgingival plaque samples) using SYBR Green Supermix (Quanta Bioscience via VWR Int., Vienna, Austria). The PCR reaction mixture was prepared in a total volume of 25 μL, using 0.5 μL forward primer (2 μM), 0.5 μL reverse primer (2 μM) and 1 μL template. The reactions were set up in 96-well optical plates (Bio-Rad, Vienna, Austria) and amplified using a C1000 Thermal Cycler with CFX96 Real Time System (Bio-Rad). We used proprietary primers (see sequences in Table 1) targeting the 16S rRNA region to perform species-specific amplification of *Treponema denticola*, *Tannerella forsythia*, *P. gingivalis*, *Prevotella intermedia*, and *A. actinomycetemcomitans*. Cycling conditions were 10 min at 94°C; 40 cycles of 30 s at 94 °C, 40 s at 60°C or 63°C or 67°C for *T. denticola*, *T. forsythia*, *P. gingivalis* and *P. intermedia* or *A. actinomycetemcomitans*, respectively, 40 s at 72°C and a final extension at 72°C for 3 min. A standardized positive control, derived from bacterial cultures of known CFU, a negative control as well as RT-controls (non-RT RNA preparations, after DNase I treatment) for the RNA-based PCRs were included in the runs. All samples were run in duplicate.

Statistical analysis

We used Prism 4 (GraphPad) to analyze the time course experiments by two-way ANOVA followed by Bonferroni post-tests. $p \leq 0.05$ was considered significant.

Results and Discussion

To test our hypothesis that using rRNA as a target molecule in real-time PCR instead of DNA will be suitable to monitor the decline of viable bacteria, we first used *Escherichia coli* treated with ampicillin as a model to set up the system. We found that there was no significant difference between the results of PMA-qPCR, previously reported as a tool to detect viable bacteria only (9), and our RT-qPCR. Both PMA-qPCR as well as RT-qPCR showed a very similar course of declining viable bacteria counts, which went almost down to the detection limit (approximately 10^2 CFU/reaction) at day 5. However, according to the classical DNA-based qPCR, there was no apparent effect of the antibiotic treatment within the 5 d evaluation period (data not shown).

Next, we applied the above established methods to known periodontopathic bacteria. We chose to use cultures of *P. gingivalis* and *A. actinomycetemcomitans*, performed antibiotic treatment, and documented the resulting dying-off by qPCR, PMA-qPCR and RT-qPCR. Non-treated culture aliquots were used as controls for each time point. Data are expressed as percentage of control. A significant decrease of viable *A. actinomycetemcomitans* was first observed after 72 h by either using PMA-qPCR or RT-qPCR methods (Fig. 1A) with continuous decline down to 8% of the untreated initially cultured bacteria, by RT-qPCR and about 50% viable bacteria by PMA-qPCR, respectively. However, conventional qPCR showed remarkably stable DNA content with only minor reduction of about 2% from baseline (Fig. 1A). Within the culture of *P. gingivalis* we observed a much faster reduction of viable cells. All three methods detected a decline

Table 1. Primer sequences

Species/primer name	Sequence 5′–3′
T. denticola forward	GTACCCAATGCAGTTTACGA
T. denticola reverse	CAAGGCAACGATGGGTAT
T. forsythia forward	TGCTTCAGTGTCAGTTATACCT
T. forsythia reverse	ATTTTATTGCATGTACCTTGT
P. gingivalis forward	GGGCGATACGAGTATTGC
P. gingivalis reverse	TCAGTGTCAGTCGCAGTATG
P. intermedia forward	TCTGATTAGCTTGTTGGTGC
P. intermedia reverse	AGCCGGTCCTTATTCGAAG
A. actinomycetemcomitans forward	CTACCAAGCCGACGATCGC
A. actinomycetemcomitans reverse	GGCATGCTATTAACACACCAACC

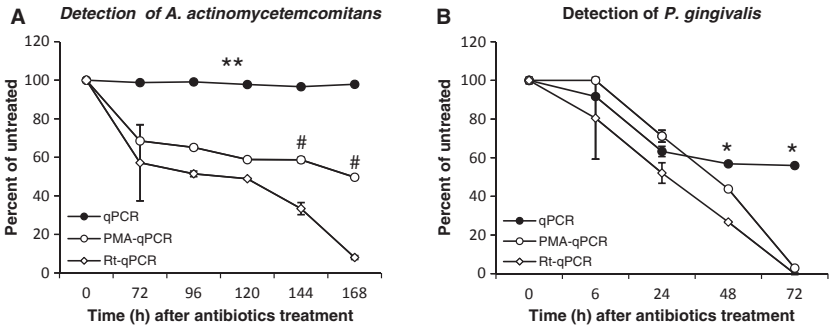

Fig. 1. Species-specific detection of *A. actinomycetemcomitans* (A) and *P. gingivalis* (B) at different time points (h) after antibiotic treatment as assessed by qPCR (filled circles), PMA-qPCR (open circles) and RT-qPCR. Untreated sample aliquots were taken immediately upon arrival and served as 0 h control. Data are presented as treated/untreated (%) (percentage of baseline) ± SEM, (A) $^{**}p < 0.001$ for time points 72–168 comparing qPCR to PMA-qPCR and RT-qPCR, respectively; $^{\#}p < 0.05$ comparing PMA-qPCR and RT-qPCR. (B) $^{*}p < 0.001$ comparing qPCR to PMA-qPCR and RT-qPCR, respectively. PCR, polymerase chain reaction; qPCR, quantitative PCR; PMA, propidium monoazide; RT, reverse transcription.

between 30% and 50% from baseline already 24 h after addition of antibiotics. While in the PMA-qPCR and RT-qPCR the decline continued to, basically, 0% survivors after 72 h, the DNA-based PCR remained at a level of 60% (Fig. 2B).

These results confirm previous findings that DNA is only slowly degraded after loss of viability (12,13) and might under certain circumstances even be detectable years after cell death (12). The *in vitro* data suggest that the PMA- and RT-qPCRs perform in a very similar way, with no significant difference up to 5 d following antibiotic treatment, indicating that both methods selectively detect viable cells only. Nonetheless, we did find a more rapid decline of RNA by RT-qPCR at later time points, i.e. 144 h and 168 h compared to PMA-qPCR in *A. actinomycetemcomitans* cultures, probably related to RNA stability.

To this stage, we used DNA and RNA purified from bacterial cultures for the downstream processes. Next, we compared our standardized qPCR (DNA-based, using purified DNA) to the simplified RNA-based RT-qPCR in a set of 49 subgingival plaque samples. To get an idea of the comparability of the two methods in patients at different treatment stages, we divided the patients in to an untreated and a treatment group. Additionally, we extended the species panel to a total of five pathogens, i.e. *T. denticola*, *T. forsythia*, *P. ginigvalis*, *P. intermedia* and *A. actinomycetemcomitans*. The prevalence of the individual pathogens in the study population was 75.5% (37 of 49), 97.9% (48 of 49), 79.5% (39 of 49), 32.6% (16 of 49) and 10.2% (5 of 49) for *T. denticola*, *T. forsythia*, *P. ginigvalis*, *P. intermedia* and *A. actinomycetemcomitans*, respectively using DNA-based qPCR. Overall, pathogen-specific DNA was amplified at remarkable uniform levels in all specimens, while the data obtained by the RNA-based method was much more diversely distributed (Fig. 2, all panels). However, the purpose here was not to obtain quantitative data but rather evaluate the comparability of the methods. Therefore, we defined an area between threshold cycles (C_q) 15 and 35 as the range of positive bacterial detection (Fig. 2A and B, light gray area), C_q values above 35 were considered negative (Fig. 2A and B, dark gray area). The detection limit of our qPCR is 10^2 CFU/assay. We commonly detect bacteria in subgingival plaque samples in the range of 10^4–10^7 CFU corresponding to C_q values between 17 and $\leq$ 35.

Looking at the two patient groups in more detail, we found that the DNA- and RNA-based detection methods were best compared in specimens derived from untreated (first visit) patients (Fig. 2A). In this subset, the majority of cases showed accordant positive (light gray area) or negative (dark gray area) signals for the presence of the individual pathogens using DNA-based qPCR (black circles) and RT-qPCR (open diamonds). The levels of disagreement ranged from 4% in *P. intermedia* to 20% in *P. gingivalis* and *T. denticola*. The best agreement of the two methods was 88% observed for *T. forsythia* in untreated patients. However, for the same species we also found the highest disagreement of 50% in currently treated patients. The agreement for *A. actinomycetemcomitans* was 80% in total. There was only one first-visit patient where we detected *A. actinomycetemcomitans* with both methods (data not shown). The level of agreement depends on the prevalence of individual bacterial species in the study population, but may also be species-specific. While in all other species the level of disagreement increased in the treatment population this was not the case for *P. gingivalis*.

Overall, the analysis of a set of clinical samples showed a peculiar difference between the distribution levels of pathogen DNA versus RNA. As we used surplus samples of an unrelated periodontitis study, the study design does not allow for thorough statistical analyses. We had access to healthy control samples, which were recruited for the clinical study, but chose not to include these subjects in our investigation. They were all DNA-negative for periodontal pathogens and had total germ loads between 1 and 2×10^4 CFU/sample.

Although we compared individual C_q values that may naturally lead to some differences, the overall picture showing positive or negative detection of pathogen nucleic acids remains valid and is well represented in Fig. 2. Our results also show that the simplified RT-qPCR can successfully be applied to patient samples. In cases where viable bacteria were detected and no therapeutic intervention had taken place the results of the conventional qPCR and RT-qPCR were identical in about 80% of the cases,

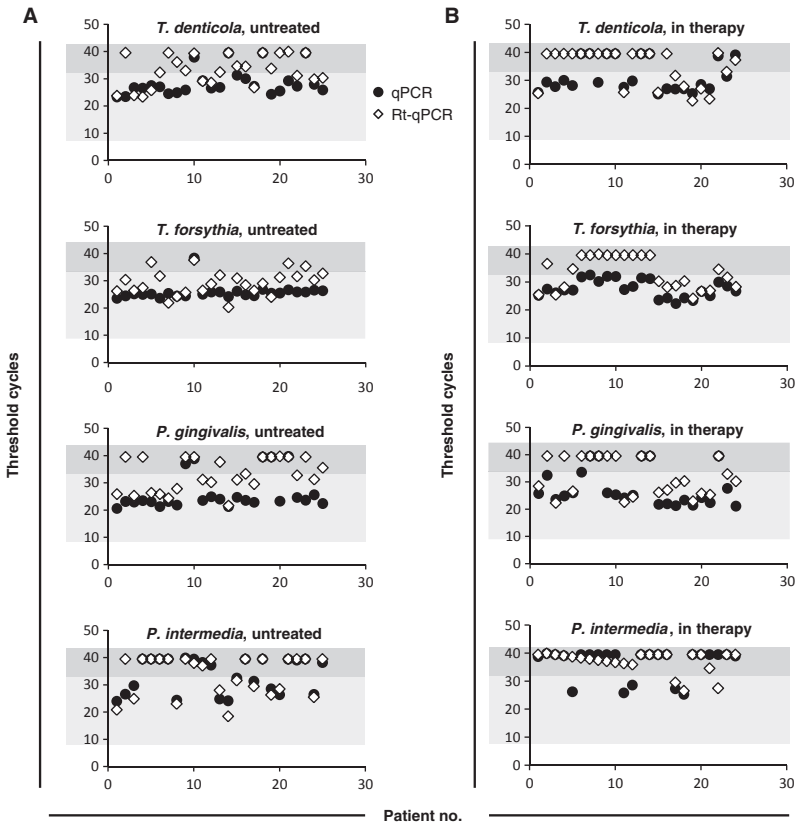

Fig. 2. Subgingival plaque samples of 49 patients, 25 untreated (first visit) patients (A) and 24 patients currently treated for periodontitis (B) were analyzed for the presence of *T. denticola*, *T. forsythia*, *P. ginigvalis*, *P. intermedia* by qPCR (black circles) and RT-qPCR (open diamonds). Threshold cycle (C_q) values by qPCR were directly compared to C_q values by RT-qPCR. A cut-off for positivity was set at a C_q of 35 (light gray background), if more than 35 cycles were needed to detect nucleic acid amplification the samples were considered negative (dark gray background). PCR, polymerase chain reaction; qPCR, quantitative PCR; RT, reverse transcription.

showing that in principle both methods perform equally. However, in patients already treated for periodontitis the RNA-based method presumably reflects more accurately the status of (declining) bacterial load, while the DNA-based method might convey up to 50% false positive results.

Polymerase chain reaction is a fast, sensitive and specific way to detect periodontal pathogens at the species level and has been shown by several authors to be superior to bacterial culture methods (5,14,15), even though it cannot (at the moment) determine antibiotic resistance and can only detect a limited and predefined set of species. Using RNA as target molecule has the reputation of being technically demanding and more difficult to relate to the actual abundance of bacteria. However, for the purpose of assessing the progress and outcome of periodontal therapy it might not be necessary to actually quantify the bacterial load on a CFU basis, but rather set a threshold of positive and negative detection. In fact it is common practice to give diagnostic test results in such a qualitative format.

When it comes to choice of the most suitable and efficient periodontitis therapy many dentists prefer to know the status of bacterial load and their pathogen affiliation (16). In patients suffering from aggressive periodontitis the bacterial status especially the presence of *P. gingivalis* and *A. actinomycetemcomitans* (as virulent bacteria) should be confirmed by microbial diagnostics. These patients may benefit from a targeted and early antibiotic therapy (as initial therapy) compared to patients who receive antibiotics only after 6 mo and unsuccessful mechanical therapy (17). Even from the patients point of view it appears plausible and will most likely improve compliance to the therapy, if it is based on the known presence of certain pathogens. The use of PCR-based assays for the detection of specific periodontopathic bacteria is becoming state of the art but might lead to an overestimation of the actual bacterial load. Previously proposed solutions using ethidium monoazide or PMA intercalation, combined immunofluorescence and fluorescence *in situ* hybridization or RNA-oligonucleotide quantification (9–11,18) have proven their validity. Yet, most of those methods require samples to be immediately processed, special expertise and equipment. We found that our simplified RT-qPCR protocol was easily applied to patient samples, adding only one more, simple step to the classical qPCR but offering more accurate information on the viable bacterial load. Nevertheless, the results presented in this work do raise questions as to the mechanisms involved in the degradation of both DNA and RNA in the context of the oral biofilm and the use of either mechanical treatment or antibiotics, which warrants further studies using subgingival plaque samples.

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