Comparison of Sampling Techniques For qPCR Quantification of Periodontal Pathogens

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The diagnosis of periodontal disease is mainly based on use of clinical and radiographic evidence. In this study we employed a quantitative PCR analysis of Aggregatibacter actinomycetemcomitans and Treponema denticola as species strongly involved in periodontal diseases, burden in periodontal pockets to detect the main sampling factors that interfere with qPCR results. From 22 patients with advanced periodontal disease, subgingival plaque was comparatively collected by paper points and periodontal Gracey curettes. Samples were collected from the same situs in presence of gingival bleeding and absence of bleeding. The concordance and agreement of results between samples were assessed. The present study demonstrates that subgingival plaque sampling with sterile absorbable paper points is often accompanied by gingival bleeding resulting in quantification biases of periodontal pathogens.

Keywords: periodontitis, qPCR, diagnosis, sampling

Oral cavity represents a highly specialized ecological niche in which more than 1000 microbial species can attach, grow and proliferate. The enormous genetic variability of oral microorganisms determine which oral microhabitat will be populated by specific species and establish normal symbiotic relationships with the host [1, 2]. The equilibrium between the host and microbial cells is very dynamic and absolutely necessary for the normal oral tissues functions. Perturbations by various environmental factors leads to establishment of a dysbiotic microbial community with elevated virulence potential and evade the local defense system [3-5]. Such mechanisms are responsible for oral diseases like caries, endodontic infections, gingivitis and periodontitis [6].

According to the polymicrobial synergy and dysbiosis model proposed by *Hajishengallis et al.* periodontal disease is initiated by a synergistic and dysbiotic microbial community rather than specific bacterial species [7]. Specific gene combinations, within the community, fulfill distinct roles that converge to shape and stabilize a disease-provoking microbiota. Inflammation of periodontal tissues is triggered by invasion and persistence of periodontal pathogens for prolonged periods of time [7-9]. The evolution of disease occur in patterns such as exacerbation periods of bone lysis are proceeded by temporal reminiscence [10, 11]. Some specific bacterial species within the subgingival communities can lead to microbial dysbiosis and are strongly correlated with periodontal disease such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* [12].

Understanding the composition and the functions of subgingival plaque is of great interest and can enable discoveries of useful clinical biomarkers for early, non-invasive, personalized diagnosis and treatment outcome monitoring of periodontitis patients [13]. In clinical practice diagnosis of periodontal disease is mainly based on clinical and radiographic evidence. Therefore, detection of disease is possible only after 1-3 mm of bone loss has already occurred, emphasizing the need for early reliable diagnostic biomarkers [14].

Detection in elevated numbers of *A. actinomycetemcomitans* and species of Socransky's *red complex* can serve as an indication for systemic antibiotherapy especially in refractory to treatment forms of disease [12, 15]. The decision of prescribing antibiotics as part of the periodontal treatment plan has to rely on solid microbiologic arguments [15[. Beside the systemic toxicity and adverse effects of antibiotics, extensive use of antibiotics is the major cause of the increasing incidence of antibiotic-resistant strains [16]. Therefore, the dental practice is a major source of uncontrollable antibiotic prescriptions [17, 18]. Antibiotics should be prescribed as an adjuvant to periodontal treatment only after proper microbiological evaluation of the periodontitis patients.

Detection of *A. actinomycetemcomitans* and *T. denticola* in periodontal pockets is associated with aggressive, rapid progressive bone loss and difficult to control disease activity. These pathogens are able to colonize extra-dental tissues from where they may translocate to pockets sites retriggering inflammatory bone loss [19, 20]. *T. denticola* also is frequently detected in

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pregnant women periodontitis and gingivitis patients which are known to bleed during paper points sampling [19-21]. Therefore systemic antibiotic administration in such cases is proven to be very beneficial in management of periodontal disease.

The main periodontal pathogens are Gram negative anaerobes, making traditional bacterial cultivation based methods, unsuitable for detection and quantification of periopathogens [12, 22]. For this purpose molecular microbiology methods such as qPCR are developed, which are incomparably more sensitive and specific than bacterial cultivation. Critical factors for microbiologic diagnosis of periodontal disease, other than detection methodologies, is the sampling process of periodontal pockets [23, 24]. A variety of clinical samples has been used for detection and quantification of periodontal pathogens, such as subgingival plaque collected by absorbable paper points and curettes, interdental space brushes, alveolar ridge mucosal swabs, tongue scrapes and saliva among others [25, 26].

The most widely used sampling process is collection of subgingival plaque by insertion in the deepest periodontal pockets of sterile absorbable paper points after isolation from saliva of the sampling site with cotton rolls [27]. During collection of subgingival periodontal pathogens by paper points, a critical step is the introduction of the paper point in the pocket in order to avoid contamination with supragingival plaque and to avoid gingival bleeding. The paper points possess a narrow absorption limit emphasizing the importance of avoiding bleeding during sampling. In case of gingival bleeding the paper points absorb blood instead of gingival crevicular exudate compromising the outcome of microbiological investigations [27-30]. Very often, clinicians deal with gingival bleeding during sample collection because of inflammation, which in many cases avoiding this situation is impossible, especially in pregnant women or patients with hepatitis among others [31, 32]. Taking the above into consideration, the aim of the present study is to investigate the influence of gingival bleeding during periodontal pockets sampling on the outcome of qPCR detection and quantification of main periodontal pathogens.

Experimental part

A total of 22 adult patients, 27-65 years of age, with generalized chronic periodontitis were recruited for the present study. This study was approved by the ethic commission of Grigore T. Popa University of Medicine and Pharmacy, Iasi and each patient gave the written consent for enrolment in the present study.

The clinical investigation and assessment of clinical periodontal status was done by a single precalibrated specialized periodontist to avoid variations in periodontal parameters measurement. Alveolar bone loss was assessed by digital orthopantomography. In the study were not included patients with systemic health disorders or administration of systemic antibiotics 6 months before the samples collection.

Samples collection

Each patient provided 3 samples, the first sample was collected by absorbable paper points in gingival bleeding conditions, the second in absence of gingival bleeding by paper points and the third sample was collected by a sterile periodontal Gracey curette. For sampling technique four periodontal pockets were sampled and pooled into one Eppendorf 2 mL tube. Before sampling each tooth was air dried and isolated from saliva by cotton rolls. Sterile absorbable paper points ISO #40 (Roeko, Germany) were inserted into the pocket, maintained for 30 s and introduced in the 2 mL Eppendorf tube. In the case of curette sampling, the periodontal curette was inserted into the periodontal pocket with the active part as deep as possible and the plaque was collected as a single stroke on the dental root surface. The working end of the curette was wiped off with one paper point into a 2 mL Eppendorf tube.

DNA extraction

DNA extractions were performed with the QIAamp DNA Mini Kit (Qiagen, Germany) following manufacturer instructions for Gram positive bacteria in order to be able to quantify the total bacterial load in subgingival plaque samples. After extraction, the yield and the purity of DNA was assessed by NanoPhotometer® (Implen Gmbh, Germany) readings of optical densities (OD) at 260 nm and 280 nm. The ratio of OD_{260}/OD_{280} was ussed as an indicator of the DNA purification efficiency, and values of 1.7-2.0 represents acceptable DNA purity for PCR amplification.

Quantitative PCR assays

In order to quantify the total bacterial load a *real-time* PCR assay using a double stranded DNA dye. The reaction composition was: 5μL of GoTaq® qPCR Master Mix solution, 30 nM final concentration ROX reference dye, 200 nM primers final concentration, 1 μL of target DNA and up to 10 μL PCR-grade water. The following primers were used, 5'-TCCTACGGGAGGCAGCAGT-3' and 5'-GGACTACCAGGGTATCTAATCCTGTT-3'. The quantification was performed accoring to a constructed standart curve plasmid-based, amplicon sequence inserted (Primerdesign, UK). Thermal cycling conditions were as follow: initial denaturation at 95°C-10 min, 40 cycles at 95°C-10s, 60°C-15s and 72°C- 40s. For quantification of *A. actinomycetemcomitans* and *T. denticola* a TaqMan probe assay was employed using a GoTaq® Probe qPCR Master Mix solution. We employed the same sequence of primers and probes, as well as the the same reaction conditions as indicated by *Hyvarinen et al* [33].

Results and discussions

The yield and the purity of DNA obtained from subgingival plaque samples are listed in table 1. All the samples provided amplifiable DNA in sufficient quantity for a very large number of PCR reactions. In case of the samples collected by paper points in bleeding conditions the mean quantity of recovered DNA was 3.67μg comparatively to 5.47 and 8.93 μg in absence of bleeding and in samples collected with curettes respectively. The $OD_{260/280}$ values are homogenous in all the sample types.

The number of total bacteria varies greatly according to sample type, and as expected the lowest number of bacteria, are found in samples with gingival bleeding. Curette samples yielded on average $2.75x10^8$ bacteria and paper points $6.76x10^7$ bacteria in absence of gingival bleeding and in presence of gingival bleeding only $1.74x10^4$ bacteria. The maximum number of bacteria recovered by paper points in presence of gingival bleeding was $2.88x10^5$, which is much smaller compared by the other types. The mean counts and the detection range of *A. actinomycetemcomitans* and *T. denticola* are listed in table 2.

Periodontal disease represents the main reason of dental extraction and despite extensive research on the subject, efficient modalities of clinical management of disease are still lacking [34, 35]. In the last years improvements in

Sample	DNA yield (µg)	DNA purity $OD_{260/280}$	Total Bacteria
Curette	8.93 (4.23-23.15)	1.87 (1.72-1.97)	$2.75x10^8$ ($8.51x10^5$-$2.95x10^9$)
Paper points bleeding	3.67 (1.28-5.45)	1.84 (1.68-1.95)	$1.74x10^4$ ($6.92x10^2$-$2.88x10^5$)
Paper point no bleeding	5.47 (3.18-12.45)	1.93 (1.87-2.01)	$6.76x10^7$ ($8.32x10^5$-$1.05x10^9$)

Table 1
DNA YIELD AND TOTAL BACTERIA COUNTS ACCORDING TO THE SAMPLING PROCEDURE. THE VALUES ARE EXPRESSED AS MEAN VALUES AND RANGE.

Sample	*A. actinomycetemcomitans*	*T. denticola*	Positive samples (n=22)
Curette	$3.24x10^4$ ($4x10^2$-$6.23x10^5$)	$4.96x10^5$ ($1x10^2$-$1.11x10^7$)	A.a= 3 T.d= 14
Paper points bleeding	$4.68x10^2$ ($2.24x10^2$-$3.26x10^3$)	$1.78x10^3$ ($1x10^2$-$9.24x10^3$)	A.a= 2 T.d= 8
Paper point no bleeding	$4.86x10^4$ ($4.26x10^2$-$6.83x10^5$)	$3.67x10^5$ ($1x10^2$-$5.82x10^6$)	A.a= 5 T.d= 14

Table 2
PERIODONTAL PATHOGENS COUNTS ACCORDING TO THE SAMPLING PROCEDURE. THE VALUES ARE EXPRESSED AS MEAN VALUES AND RANGE

terms of sensitivity and specificity in diagnosing periodontitis were achieved due to advances in developing molecular microbiology methods [33[. Further improvement of periodontitis diagnosis in terms of predictory power, is possible by adjusting the sampling process of subgingival bacteria [36].

Early cultivation based studies investigated the differences in bacterial composition of paper points and curette subgingival samples [37, 38]. In terms of numbers of colony forming units, paper points proved superior to curette samples indicating that only the loosely adherent microorganisms in the pocket could be collected by paper points [39]. Paper points collect microorganism from the outer layer of the subgingival biofilm and fails to sample the apical part of the periodontal pocket because the tip of the paper point reaches saturation in absorption efficiency before reaching the most apical part of the periodontal pocket (data not shown). On the other hand, curette sampling can alter the biofilm composition during sampling but bacteria adherent to the tooth and apically positioned, are more successfully collected in comparison to paper points [31, 40]. Blood contamination of paper points interfering with bacterial load determination is reported by *Smola et al* [31]. Pooling paper point samples with curette samples may uncover a more real microbiological representation of the sampled periodontal pocket and further investigations are needed.

The results of qPCR assays can be biased by numerous factors including DNA extraction method, sample collection and storage [41, 42]. Sampling the periodontal pockets is of crucial importance in obtaining reliable qPCR analysis of oral fluids in scope of diagnosing periodontitis.

The counts of *T. denticola* as well as the number of positive samples are in excellent agreement between curette and standard paper point samples. The low variation in count number between standard samples are due to the endogenous factors of PCR methodology which is known that absolute reproducibility of qPCR results in terms of quantification is impossible to achieve. In case of paper point samples collected in gingival bleeding conditions in 6 otherwise positive patients, detection of *T. denticola* failed. The mean counts of *T. denticola* in those samples was much lower compared to the other sample types plus the 6 false negative results, clearly shows the interference of gingival bleeding during paper point sampling on the microbiological diagnosis of periodontal disease.

A. actinomycetemcomitans was detected in 3 curette samples, 5 standard paper point samples and 2 compromised paper point samples. The poor recovery of this particular periodontal pathogens by curette sample was also observed in other studies (43, 44). The 2 false negative results in curette and compromised paper point samples supports our hypothesis that sampling in absence of gingival bleeding is essential for reliable molecular microbiology investigation of periodontal disease with serious clinical implications in the management of periodontitis patients. Therefore, the sampling by paper points can be totally compromised by gingival bleeding and mislead bacterial quantification by paper points. Clinicians should be aware by the biases that can be introduced in analytical process by sampling periodontal pockets in gingival bleeding conditions.

Conclusions

Detection and quantification of periodontal pathogens is of great importance in the clinical management of periodontal disease. The increasing incidence of antibiotic-resistant strains impose great challenges in public healthcare system and periodontal disease represents a considerable source of uncontrollable antibiotic prescriptions. The decision of systemic antibiotics administration to periodontitis patients should be based on microbiological investigation.

Compromised by gingival bleeding paper point subgingival plaque samples fails to detect periodontal pathogens as *Aggregatibacter actinomycetemcomitans* and *Treponema denticola.* This study clearly demonstrates that gingival bleeding during paper point sampling of periodontal pockets introduce detection and quantification biases of periodontal pathogens by misleading the periodontitis diagnosis compromising in this way the periodontal treatment.

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