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Short communication

Sterile paper points as a bacterial DNA-contamination source in microbiome profiles of clinical samples

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ABSTRACT

Objectives: High throughput sequencing of bacterial DNA from clinical samples provides untargeted, open-ended information on the entire microbial community. The downside of this approach is the vulnerability to DNA contamination from other sources than the clinical sample. Here we describe contamination from sterile paper points (PPs) used in microbial sample collection.

Methods: Peri-implant samples from 48 individuals were collected using sterile PPs. Control samples contained only PPs or DNA extraction blank controls. 16S rRNA gene libraries were sequenced using 454 pyrosequencing. 16S rRNA gene copy numbers were measured by quantitative PCR.

Results: Nearly half of the sequencing reads belonged to two OTUs classified as *Enterococcus* (25% of reads) or *Exiguobacterium* (21%), which are not typical oral microorganisms. Of 87 peri-implant samples, only 10 samples (11%) contained neither of the two OTUs. The relative abundance of both unusual OTUs correlated with each other ($p < 0.001$; $r = 0.828$, Spearman correlation). The control samples showed that 2 of 4 (50%) of the sterile unused PPs contained bacterial DNA equivalent to 1.2×10^3 and 1.1×10^4 cells respectively, which was within the range of DNA in the clinical samples (average 1.8×10^7, SD 4.8×10^7, min 4.4×10^2, max 2.8×10^8). The microbial profile from these PPs was dominated (>83% of reads) by the two unusual OTUs.

Conclusions: Sterile PPs can contain contaminating bacterial DNA. The use of PPs as a sampling tool for microbial profiling of clinical samples by open-ended techniques such as sequencing or DGGE should be avoided.

Clinical significance: Clinicians working with PPs as sampling tools for bacterial DNA should consider using an alternative sampling tool, because sterile unused PPs can be a considerable source of foreign bacterial DNA. We recommend sterile curettes for collecting clinical samples for open-ended techniques, such as sequencing or DGGE.

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1. Introduction

High throughput sequencing such as pyrosequencing of bacterial DNA fragments from clinical samples provides open-ended information on the entire microbial community. With this molecular technology no choice of targets, as in the case of specific DNA probes, is necessary. Herewith a wealth of information on uncultivable and previously not with oral cavity associated microbiota has become available.[1] The downside of DNA-based open-ended techniques is the vulnerability to potential contamination with DNA from other sources than the clinical sample itself. Here we describe a case of severe contamination from sterile, unused paper points (PPs). PP sampling is a frequently used technique in dentistry, *e.g.*, for microbial analysis of periodontal, endodontic and peri-implant sites and lesions.

2. Materials and methods

Forty-eight patients having dental implants and regular maintenance of their dental implants were recruited for a cross-sectional study. All volunteers signed an informed consent prior to participation. The study protocol was approved by the ethical committee of the VU Medical Centre, Amsterdam (#2011/370).

Clinical measurements and sampling were performed by a trained examiner. Submucosal plaque was collected by sampling the deepest pocket or sulcus using one sterile paper point per implant (PP; Absorbent Points # 504; Henry Schein Inc, Melville, NY, USA) and sterile forceps. At least two implants per patient were sampled unless only a single implant was present. The paper points were introduced to the bottom of each peri-implant pocket/sulcus and removed after 10 s, then placed in an empty sterile microtube and stored at −80 °C. This yielded a total of 87 samples. Samples were processed for amplicon sequencing and sequenced as described previously.[2]

The preliminary sequencing data analyses revealed that a large part from the 87 sample profiles was dominated by sequences from non-oral microorganisms belonging to genus *Exiguobacterium* and genus *Enterococcus*. Approximately two years later the clinical examination and microbial sampling of the same sites was repeated for twelve of the patients (additional 23 samples) who showed a high proportion of the unusual microorganisms in their previous peri-implant samples. At this time paper points from the same manufacturer but from a different batch were used. Next to the clinical samples, sterile, unused paper points were placed in empty sterile microtubes for control purposes. Duplicate PP per each of the two sampling visits were collected and stored at −80 °C until further analysis.

DNA was extracted from both, clinical samples and control PPs, and quantified by 16S rRNA gene qPCR as described previously.[2] Additionally, in the second sequencing run the duplicate DNA-extraction blank controls were included in all forthcoming steps. These control samples, further called Blank 1 and Blank 2, were exposed to all the reagents used for DNA extraction, purification and quantification, as well as for amplicon PCR and sequencing.

Barcoded amplicon libraries of the small subunit ribosomal RNA gene hypervariable region V5-V7 were generated for each individual clinical sample and each of the controls, pooled and sequenced as described previously.[2] Sequencing data were quality filtered and analyzed using QIIME version 1.5.0[3] as described in Kraneveld et al. (2012).

Descriptive statistics and statistical analyses were performed using statistical software (SPSS PASW Statistics version 20.0, Chicago, IL, USA).

3. Results

From the original sequencing dataset of 87 peri-implant samples, 46% of nearly 600000 sequencing reads were classified as either genus *Enterococcus* (25% of reads, OTU #114; BLAST search result: 99% identity with *Enterococcus casseliflavus/E. canintestini/E. dispar/E. gallinarum*) or *Exiguobacterium* (21% of reads, OTU #686; BLAST search result: 99% identity with *Exiguobacterium aurantiacum/E. mexicanum*) (Fig. 1A). Only 10 samples (11%) contained neither of the two OTUs, further named EE. *Exiguobacterium* was found in 68% of the samples, and *Enterococcus* in 89% of the samples. Both OTUs showed a strong positive correlation with each other ($p < 0.001$; $r = 0.828$; Spearman's correlation). In four samples (4.6%) a high proportion of *Enterococcus* was found without any reads classified as *Exiguobacterum* (Fig. 1B).

By analyzing the clinical data (bleeding on probing, pocket depth, amount of plaque, radiographic bone destruction–data not shown), we found that in the samples taken from 2–3 mm sulci a significantly higher proportion of EE was observed than from the deepest pockets (≥5 mm) ($p = 0.004$; Mann–Whitney test) (Fig. 2A). There was a trend for more bacterial DNA in the samples from the deep pockets (≥5 mm) than from the 2–3 mm sulci ($p = 0.06$) (Fig. 2B). There was no significant correlation between the proportion of EE and the amount of bacterial DNA in the samples ($p = 0.1342$, $r = 0.1638$, Spearman correlation) (Fig. 2C).

In twelve of the patients with high EE proportions the same peri-implant pockets were resampled two years later. Of all reads, 12% were classified as EE, and only 4 of 23 samples (17%) contained neither of the two OTUs. Eleven samples (48%) contained reads classified as *Exiguobacterium*.

Next, we analyzed the data from the control samples – two DNA-extraction and PCR blank control samples (Blank 1 and Blank 2) and four sterile, unused PPs. Among the control PPs, two PPs (PP1 and PP2) contained bacterial DNA equivalent to 1×10^3 and 1.1×10^4 cells respectively, which was within the range of DNA in the clinical samples (average 1.8×10^7, SD 4.8×10^7, min 4.4×10^2, max 2.8×10^8). In the remaining two PPs and Blank 1 and 2 the DNA amount out of the linear range of the standard curve of 16S rRNA gene qPCR (less than 1000 cells) was measured. Sequencing output – the number of reads per sample–corresponded to the DNA results (Fig. 3). The PP1 and PP2 contained 2643 and 5977 reads, of which 90% and 83% were classified as EE, respectively (Fig. 3). Blank 1 and Blank 2 contained a DNA amount equivalent to 2.1×10^3 cells and less than 1000 cells and resulted in 265 and 116 reads, respectively. None of these reads were classified as EE. The OTU-profile was relatively diverse (11–16 OTUs) and was traceable in all control samples (Fig. 3).