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Case Report

Shifts of subgingival bacterial population after nonsurgical and pharmacological therapy of localized aggressive periodontitis, followed for 1 year by Ion Torrent PGM platform

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ABSTRACT

The possibility of targeting the hypervariable region V3 of the 16S rRNA gene using Ion Torrent Personal Genome Machine (PGM) could provide a complete analysis of subgingival plaque samples, potentially able to identify microbiological species missed by culture-based methods. A 16-year-old female smoker patient, affected by localized aggressive periodontitis, underwent a full-mouth disinfection protocol and was inserted in a 3-month recall program. Microbiological samples were collected at baseline and at 30, 100, 365 days follow-up and analyzed by Ion Torrent PGM. *Capnocytophaga*, *Fusobacterium*, *Prevotella*, and *Treponema* were the most represented pathogens at baseline. Nonsurgical treatment and systemic antibiotics drastically lowered the anaerobic species, and their presence remained limited after 100 days, while a consistent recolonization by anaerobic bacteria was detected at 365 days. The patient showed a general improvement of periodontal conditions. Differently from polymerase chain reaction and other microarray techniques, Ion Torrent performs a quantitative analysis of the microbiota, irrespective of the searched species. An accurate definition of the shifts of the bacterial community might help periodontal researchers for a better understanding of the impact of different treatment approaches or in intercepting nonresponsive conditions.

Key words: Anaerobic bacteria, full-mouth disinfection, Ion Torrent Personal Genome Machine, localized aggressive periodontitis, nonsurgical treatment

INTRODUCTION

Localized and generalized aggressive periodontitis show different clinical features,[1,2] being also characterized by slightly diverse bacterial profiles in the subgingival microbiota.[3,4] The etiology of both diseases is widely thought to be polymicrobial,[5,6] but the role of individual species and their complex interactions with the host have not been clearly defined yet. The first microbiological analyses

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on these pathologies were conducted with conventional culture or immunohistochemical methods,[7,8] followed by a generation of molecular detection methods such as hybridization assays or polymerase chain reaction (PCR)[9,10] and even using atomic force microscopy to indagate bacterial morphology and to examine microbial interactions in subgingival biofilm.[11] The most recent advance has been cloning and sequencing of 16S rRNA genes using the fast Ion Torrent Personal Genome Machine (PGM): this approach, up-to-date reported only once in periodontology,[12] allows an open-ended exploration of bacterial populations, potentially revealing the presence of uncultivated species. The possibility of targeting the hypervariable region V3 of the 16S rRNA gene could provide a complete analysis of subgingival plaque samples, giving additional informations to evaluate the impact of different treatments or to detect the presence of resistant forms of various bacteria species. The aim of the present case report was to follow shifts in subgingival bacterial communities after nonsurgical and pharmacological therapy in a case of localized aggressive periodontitis using next generation 16S rRNA gene sequencing approach.[13]

CASE REPORT

A 16-year-old female Caucasian patient, smoker (10 cigarettes/day for 3 years), was referred to our observation showing a localized aggressive form of periodontitis.[14] After retrieving general and dental anamneses, the patient underwent oral examination to assess periodontal conditions and dental health: complete periodontal chart was performed (full-mouth plaque score [FMPS]: 38%; full-mouth bleeding score [FMBS]: 28%), together with full-mouth periapical radiographs. Then, after receiving oral hygiene instructions and motivation, the patient underwent causal therapy according to full-mouth disinfection protocol,[15] together with the following pharmacological therapy: 875 mg amoxicillin + 125 mg clavulanate twice a day and 250 mg metronidazole 3 times a day, for 10 days.[16,17] The patient was reevaluated in 30 days and then recalled every 3 months for supportive periodontal therapy, based on periodontal risk assessment.[18] New periapical radiographs and periodontal chart were collected after 1 year (FMPS: 16%; FMBS: 1%). No further pharmacological therapy was prescribed during the entire follow-up period and the patient did not quit smoking. Microbiological samples were collected at baseline and at 30, 100, 365 days follow-up from every pathological site (probing depth [PD] ≥5 mm).

Supragingival plaque was gently removed; teeth were air-dried and isolated with cotton rolls. A sterile paper point (ISO45, Roeko Dental, Langenau, Germany) was inserted for 10 s in each site, and all paper points were pooled in a sterile test tube.

Sample processing

16S rRNA gene amplification

DNA extraction was performed using the NucliSENS® easyMAG® system (Biomerieux, Gorman, North Carolina, USA). A real-time quantitative EvaGreen® dye (Fisher Molecular Biology, Waltham, Massachusetts, USA) PCR was performed with the degenerated primer 27FYM to better amplify several bacterial species, and the U534R primer, targeting the V1-V3 region of 16S gene. A nested PCR was performed with the primers B338F_P1-adaptor (B338F 5′-ACTCCTACGGGAGGCAGC-3′) and U534R_A_ barcode (U534R 5′-ATTACCGCGGCTGCTGG-3′), to prepare a 200 base template for final sequencing, with a different barcode for each sample, to amplify the bacterial V3 region.[19]

PCR sample processing

The correct size of the amplicon (260 bp) was assessed on a 2% agarose gel. Quantification of the amount of DNA was assessed with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, California, USA) and an equal quantity of PCR from each sample was used to produce the pooled library.

Ion Torrent sequencing

The pooled library was diluted at a concentration of 26 pM. Template preparation was performed using the Ion PGM Template OT2 200 kit on Ion OneTouch™ 2 System (Life Technologies, Gran Island, New York, USA), after which the enrichment percentage was carried out on Qubit® 2.0 Fluorometer. The templates were sequenced on the Ion PGM™ System machine, using the Ion PGM sequencing 200 Kit V2 (Life Technologies, New York, USA).

Sequence data analysis

QIIME 1.8.0 (Knight Lab, Boulder, Colorado, USA) was used to process the sequence data. High quality ($Q > 25$) sequences were demultiplexed and quality filtered using split_libraries_fastq.py with default parameters, except for length parameter (150 bp). Sequences were clustered into operational taxonomic units (OTU) using *de novo* OTU picking against the Greengenes

13_8 reference OTU database with a 94% and 97% similarity threshold. Alpha diversity was calculated using Shannon metrics. Statistical analysis was performed with SIMPER and ANOSIM tests.

RESULTS

At baseline, *Capnocytophaga, Fusobacterium, Prevotella*, and *Treponema* were the most represented genera in a composite microbiota (α diversity = 6). Nonsurgical treatment following full-mouth disinfection protocol together with systemic antibiotic therapy drastically lowered the anaerobic species and only a limited presence of *Capnocytophaga* could be detected at 30 days after treatment (α diversity = 2). The recolonization of the pockets by anaerobes started to be evident 100 days after treatment (α diversity = 4). Among the detected genera, *Porphyromonas, Prevotella, Dialister, Capnocytophaga*, and *Treponema* showed a greater relative abundance at 365 days posttreatment comparing to the microbiota composition at the baseline. Specifically, the presence of *Tannerella* was restored and *Aggregatibacter* was lowered; *Fusobacterium* was not present and *Veillonella* was detected. Conversely, commensal species such as *Streptococcus, Leptotrichia, Eikenella, Neisseria*, and *Haemophilus* were absent or lowered (α diversity = 5). The observed differences were not statistically significant (assessed by Kruskal–Wallis test).

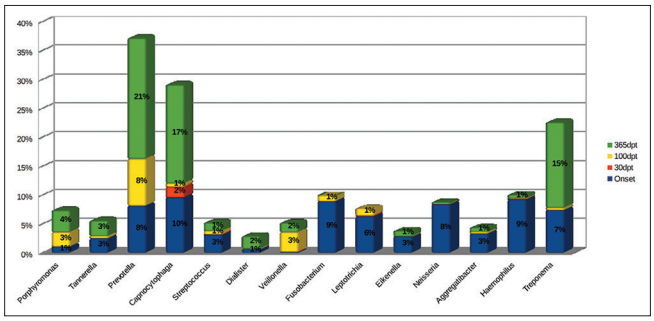

Figure 1: The genus-level composition of the subgingival bacterial population (%) at the different time points (dpt = days posttreatment)

DISCUSSION

In the present case [Figure 1], it appears clearly how the subgingival microbiota changed in quantity and quality from pretreatment to posttreatment. As previously reported,[20,21] recontamination of the affected sites was the main issue pushing researchers for developing protocols such as "full-mouth disinfection," in all its updated versions.[15,22,23] Nevertheless, some authors consider the real advantages of this procedure as a questionable and controversial matter.[24,25] In this patient, shifts of subgingival microbiota determined both clinical and radiographical improvement of the periodontal conditions [Figure 2]. FMPS and FMBS decreased along time as well as the probing depth of the pathological pockets. However, the increasing of anaerobic pathogens appeared remarkable at 365 days after treatment, suggesting that the 3-month recall program alone could be not sufficient for maintaining stable periodontal conditions over time. It is interesting to note that the bacterial shift toward a higher prevalence of pathogen species was not accompanied by clinical signs of inflammation: microbiological analysis seems to predict the necessity of further therapies before the clinical scenario becomes evident.

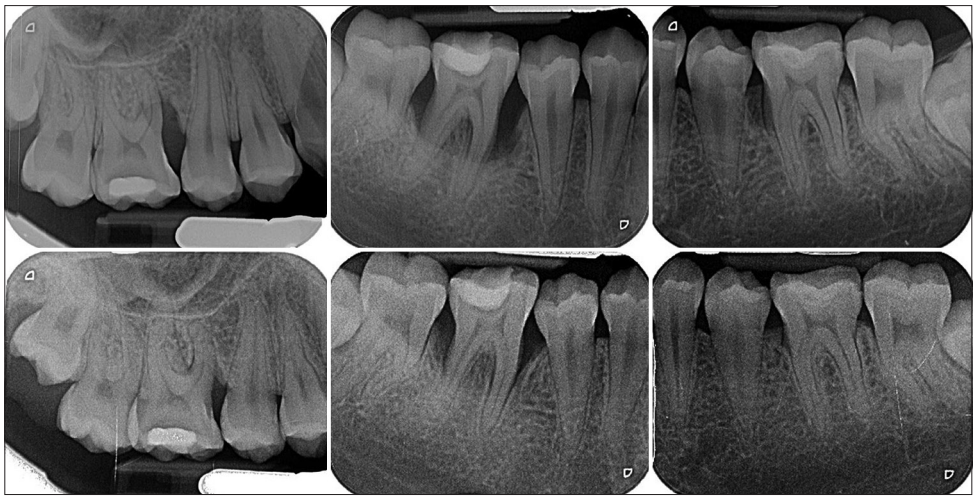

Figure 2: Periapical radiographs comparing baseline condition (upper line) with the situation 1 year after nonsurgical and pharmacological therapy (lower line)

The main advantage of the Ion Torrent PGM platform is basically due to the high throughput of the technique able to identify microbiological species missed by culture-based method. Differently from PCR and other microarray techniques, Ion Torrent performs a quantitative analysis of the microbiota, irrespective of the searched species. The metagenomic analysis applied in this case report could open possible new perspectives in exploring differences of the subgingival microbiota in periodontal patients: changes in community profiles and metrics could be diagnostically more predictive than the detection of selected periodontal pathogens, such as in PCR or in culture-based methods. An accurate definition of the shifts in the bacterial community could help periodontal researcher for a better understanding of the impact of different treatment approaches or in intercepting nonresponsive conditions.

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Conflicts of interest
There are no conflicts of interest.

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