Clinical and microbiological parameters of naturally occurring periodontitis in the non-human primate *Macaca mulatta*

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**ABSTRACT**

**Background:** Non-human primates appear to represent the most faithful model of human disease, but to date the oral microbiome in macaques has not been fully characterized using next-generation sequencing.

**Objective:** In the present study, we characterized the clinical and microbiological features of naturally occurring periodontitis in non-human primates (*Macaca mulatta*).

**Design:** Clinical parameters of periodontitis including probing pocket depth (PD) and bleeding on probing (BOP) were measured in 40 adult macaques (7–22 yrs), at six sites per tooth. Subgingival plaque was collected from diseased and healthy sites, and subjected to 16S rDNA sequencing and identification at the species or higher taxon level.

**Results:** All macaques had mild periodontitis at minimum, with numerous sites of PD ≥ 4 mm and BOP. A subset (14/40) had moderate-severe disease, with ≥2 sites with PD ≥ 5mm, deeper mean PD, and more BOP. Animals with mild vs moderate-severe disease were identical in age, suggesting genetic heterogeneity. 16S rDNA sequencing revealed that all macaques had species that were identical to those in humans or closely related to human counterparts, including *Porphyromonas gingivalis* which was present in all animals. Diseased and healthy sites harboured distinct microbiomes; however there were no significant differences in the microbiomes in moderate-severe vs. mild periodontitis.

**Conclusions:** Naturally occurring periodontitis in older macaques closely resembles human adult periodontitis, thus validating a useful model to evaluate novel anti-microbial therapies.

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Introduction

Periodontitis is a microbiome-driven disease that mainly affects older individuals and results in chronic immune activation, inflammation, soft tissue and bone destruction, and ultimately tooth loss. Accumulating evidence indicates that periodontitis contributes to the risk and severity of systemic conditions in which inflammation plays a key role, including type 2 diabetes, atherosclerosis and cardiovascular disease, pre-term birth, arthritis, and certain cancers [1–3]. The current preventive regimens and treatments for periodontitis are focused on removal of bacterial biofilms through oral hygiene measures and surgical procedures. Such approaches are sometimes ineffective, and are labour intensive, costly, and difficult to implement from a public health perspective.

Given the microbial aetiology of periodontitis, new anti-microbial agents and vaccines represent a logical strategy for disease prevention and to augment current treatment regimens. Appropriate pre-clinical models are imperative for developing these interventions. Although much information has been gained from studies of periodontitis in various animals including mice, rats, and dogs, the most faithful model of human disease remains the non-human primate [4–11]. Previous studies have demonstrated that non-human primates are closely similar to humans in terms of clinical presentation of disease [12], periodontal structures, host immune responses and to a limited extent the composition of the oral microbiome [4,13,14]. The microbiology of periodontitis in non-human primates has mainly been evaluated using closed end technologies that targeted putative pathogens, but did not comprehensively characterize the entire oral microbiome [14,15]. Furthermore, most studies have been limited to younger animals with ligature-induced versus naturally occurring disease.

In the present study, we characterized naturally occurring periodontitis in a population of older non-human primates (*Macaca mulatta*), from the clinical
and microbiological perspectives. Microbiological analyses employed next-generation 16S rRNA sequencing in order to provide a systematic analysis of the oral microbiome in this model system.

Materials and methods

Animals and ethics statement

Rhesus monkeys (Macaca mulatta) in a breeding colony at the FIOCRUZ Primate Research Center in Manguinhos (Rio de Janeiro, Brazil) were used for all studies. Their care and maintenance have been described previously [16]. This research complied with all relevant national (CONCEA: Brazilian Government Council for Control of Animal Experimentation) and international guidelines for care and use of animals in research. All the procedures involving monkeys conformed to the recommendations of the Weatherall report for the use of non-human primates in research (http://www.acmedsci.ac.uk/images/project/nhpdownl.pdf). Animal experimentation was approved by the Institutional Ethical Review Board (CEUA-FIOCRUZ, resolution #P-55/12–6). To minimize suffering before interventions, including sampling or clinical procedures, animals were anaesthetized with ketamine hydrochloride 10 mg kg\(^{-1}\) (Cetamin, Synthec Vet, São Paulo, Brazil) (62.5 mg/kg)/xylazine (12.5 mg/kg), and midazolam 0.10 mg kg\(^{-1}\) (Dormonid, Farma-Roche, São Paulo, Brazil), injected intramuscularly.

Clinical characteristics of the M. mulatta population

The animals were outbred older adults of both sexes that did not receive regular oral hygiene interventions, all of which had at least mild periodontitis. To characterize the status of animals with respect to naturally occurring periodontitis, 40 animals were assessed by clinical examination. Periodontal measurements were recorded, including: pocket depth (PD) and bleeding on probing (BOP) at six sites per tooth (mesial/buccal, buccal, distal/buccal, distal/lingual, lingual, mesial/lingual). Sixteen posterior teeth per animal were evaluated including the first and second premolars and first and second molars. PD measurements (mm) were made by a trained periodontist using a North Carolina periodontal probe (UNC-15, Hu-Friedy, Chicago, IL); BOP was recorded as a dichotomous measure. A total of 3,720 sites were evaluated.

Clinical and demographic data (Table 1) entry was error-proofed by an investigator (A.P.V.C.), and transferred to an Excel spreadsheet. PD and percentage of sites with BOP were averaged within each animal and then across groups. Animals were categorized into mild and moderate-severe periodontitis groups, based on the presence of more than two sites with PPD \(\geq 5\) mm (Table 2).

### Table 1. Clinical characteristics of the M. mulatta colony.

| Age (yr) | Mean ± SD | Range/animal | Percentage of animals |
|----------|-----------|--------------|-----------------------|
| Bleeding on probing (sites) | 14.1 ± 4.3 | 7 – 22 | 100 |
| Pocket depth (mm) | 51.2 ± 15.6 | 9.4 – 74.2 | 100 |
| Sites (%) 1–3 mm | 4.8 ± 0.4 | 1 – 10 | 100 |
| 4 mm | 7.1 ± 4.2 | 2.1 – 18.8 | 100 |
| 5 mm | 1.8 ± 3.2 | 0 – 14.6 | 37.5 |
| 6 mm | 0.8 ± 1.7 | 0 – 7.3 | 27.5 |
| 7–10 mm | 1.0 ± 0.7 | 0 – 3.3 | 32.5 |

### Table 2. Clinical parameters of mild vs moderate-severe periodontitis.

| Group | \(n\) | Mean PD (mm) | BOP (%) | Age |
|-------|-----|-------------|--------|-----|
| Mild | 26 | 2.30 ± 0.15 | 44.5 ± 13.8 | 142 ± 3.6 |
| Moderate-severe | 14 | 2.93 ± 0.31* | 63.6 ± 9.8 | 13.9 ± 5.5 |

\(p < 0.0001\) vs mild (t test).

Sample collection

After periodontal screening, all sites presenting PD \(\geq 4\) mm, and four to six clinically healthy sites (PD \(\leq 2\) mm) in each animal were selected for microbiome sampling. Supragingival plaque was removed with sterile gauze and discarded, and subgingival plaque was taken from the selected periodontal sites with Gracey curettes (Hu-Friedy), pooled and suspended in TE buffer (50 mM Tris HCL, 1 mM EDTA pH 7.6), placed on ice, and stored at \(-80^\circ C\) until further analysis. Whole genomic bacterial DNA was extracted and purified from the pooled subgingival microbiome samples using the MasterPure™ DNA purification kit (Epicentre®, Madison, WI [17]).

16S rRNA cloning and sequencing

DNA isolated from plaque from all animals was PCR-amplified for the 16S rRNA gene using a universally conserved primer set and a primer set specific for Bacteroidetes taxa. Plaque from PCR positive animals was subjected to definitive taxonomic identification, by cloning, sequencing, and informatics analysis. Four libraries were constructed, two with broad range primers and two with Bacteroidetes-specific primers. 16S rRNA PCR amplification, cloning and sequencing were performed as previously described [17]. More than 80 clones were picked from each library, amplified, and sequenced, yielding 330 partial sequences. Partial sequences of approximately 800 bp each were edited, aligned, and checked for chimeric inserts. Identifications were made using the BLAST tool on the Human Oral Microbiome
Database (HOMD, http://www.homd.org/) and confirmed using BLAST on NCBI, the Ribosomal Database Project (RDP), and an in-house database containing 16S rDNA sequences from the oral cavity of younger macaques [18].

**Next-generation sequencing analysis**

HOMINGS (Human Oral Microbial Identification by Next-Generation Sequencing) employs a ProbeSeq program for species detection with modifications as previously described [19]. Briefly, 50 ng of genomic DNA was used for each initial single round of PCR. Amplification of the 16S rRNA gene (V3–V4 region) was followed by purification and processing using a modified next-generation sequencing method as described [18] (MiSeq, Illumina, Inc., San Diego, CA). Species-specific 16S rRNA-based oligonucleotide probes were used in the BLAST program ProbeSeq for HOMINGS, to identify the frequency of oral bacterial species/taxa and frequency [19]. Partial matches were not considered as a match. Sequences that were not detected by a single species probe were subsequently processed against genus level probes (two or more species within the genus). All hits were accumulated by species/genus by animal. Chimeric sequences were not removed from analyses for this study. In a separate analysis, chimeric sequences ranged from 10 to 15% of the total reads. The relative proportions of detected taxa did not vary significantly (data not shown).

**Statistical analyses**

Differences in the clinical parameters between groups were evaluated by Student’s *t* test. Antibody levels were analysed by Spearman rank correlation tests. HOMINGS hits data (0–300,000 range) were converted into relative proportions, and species/genera total abundance per animal and site (diseased vs healthy) was determined. The Wilcoxon signed-rank test was used to determine the statistical significance of differences found between healthy and diseased sites in all 40 animals, and overall in the mild vs moderate-severe periodontitis subgroups. For this analysis, *p* < 0.05 in combination with an FDR value of 0 was considered statistically significant. To determine how bacterial community composition varied across samples, we compared microbial sequencing profiles for each sample using correspondence analysis (CoA), providing a statistical visualization for picturing the associations between the levels of a two-way contingency table [20]. MeV 4_8_1 [21] was used for statistical software and CoA analysis. Statistical analyses including outlier determinations were performed using XLSTAT-Pro (version 2014.4.06) and/or SAS Enterprise Guide® (version 6.1).

**Results**

**Clinical characteristics of naturally occurring periodontitis in adult macaques**

Forty adult *M. mulatta* monkeys were screened for clinical signs of naturally occurring periodontitis. Animals ranged in age from 7 to 22 years; 68% were females. Most animals (32/40) had a complete posterior dentition, with 96 sites measured per animal (six sites/tooth). Eight of 40 had one or more missing posterior teeth owing to undetermined causes.

As summarized in Table 1, all animals had at least some periodontitis, with numerous sites with probing PDs of 4 mm or greater, considered to represent significant periodontal pathology in this model [22]. A subset of macaques (14/40) had more extensive pathology, with more than two sites with PD ≥ 5 mm (mean ± SD: 9.0 ± 6.3 deep sites/animal), significantly greater mean pocket depth and sites with BOP, consistent with moderate to severe periodontitis (Table 2). Of note, the mild and moderate-severe disease groups were nearly identical in age, suggesting that naturally occurring periodontitis disproportionately affects a subset of susceptible macaques, similar to its clinical prevalence in humans [23].

**Comparisons between *M. mulatta* and human microbiomes**

We first evaluated the overall similarity of the macaque oral microbiome to that of humans. Based on sequence analysis of 330 16S rRNA clones from subgingival plaque from seven macaques, 56% of 108 bacterial taxa detected were identical or closely similar to human oral species, including those that are health-associated as well as putative periodontal pathogens (Figure 1). Health-associated species included many *Streptococcus*, *Lactobacillus*, and *Gemella* spp. Putative periodontal pathogens included *Porphyromonas gingivalis*, *Tannerella forsythia*, *Filifactor alocis*, *Parvimonas micra*, several *Treponema* phylotypes, *Fusobacterium* spp., *Dialister invisus*, a *Desulfovibrio* phylotype, and *Aggregatibacter actinomycetemcomitans*. Caries-associated species, namely *Streptococcus mutans* and *Scardovia wiggsiae*, were also present. Forty-eight species were unique to the macaque; however, all were closely related to human counterparts (Tables 3 and 4).

Since *P. gingivalis* is of particular interest as a potential ‘keystone pathogen’ in periodontitis, we amplified DNA from subgingival sites using *P. gingivalis*-specific primers and found that all monkeys tested in a screen (50/50) were positive for this species. Several of these PCR products were subjected to more extensive sequence analysis, which showed that macaque *P. gingivalis* species were
100% identical to human species, based on sequencing of up to 300 bp of the 16S rRNA gene amplicon (data not shown).

**Relationship of the macaque oral microbiome at diseased and healthy sites**

Subgingival plaque sample pools were subjected to next-generation sequencing for the 16S rRNA gene. Samples from 24/40 monkeys gave amplified products by PCR sufficient for comprehensive sequence analysis. A minimum of 50,000 sequences were obtained from each sample, from both diseased and clinically healthy sites in the two disease severity groups (mild and moderate-severe). Sequences were identified *in silico* by reference to the HOMD using probeSeq [19]. Approximately 45% of the sequences were identified at the species level and 21% at the genus level in HOMD. The balance (34%) was not identified by reference to either HOMD or RDP databases.

The results revealed that 125/767 species were significantly different in diseased vs healthy sites in this adult macaque population, representing 16% of the total species/taxa identified. Seventy-eight were more prevalent in diseased sites and 47 were found at higher levels in healthy sites (see Table S1 for the complete list). Notable species higher in diseased sites included: *Atopobium* sp., *Bacteroidetes* sp. oral taxons 280, 281, 365, *D. invisus*, *P. micra*, numerous *Prevotella* spp., *Streptococcus intermedius*, TM7, and several species of *Treponema*. Species higher in health included: *Actinomyces*, *Capnocytophaga*, *Campylobacter*, *Corynebacterium*, *Fusobacterium*, *Leptotrichia*, and *Propionibacterium* spp. Of note, *P. gingivalis* was present in all subgingival samples, but relative proportions were not different in diseased vs healthy sites (mean levels: 0.32% of...
A CoA of the beta diversity of the microbiome based on profiles at diseased vs healthy sites shows a clear and highly significant separation (Figure 2).

Animals were further sub-grouped on the basis of periodontitis severity as detailed above (mild vs moderate-severe), and the microbial profiles at healthy versus diseased sites in these animals analysed. As shown in Figure 3, four distinct clusters were present, albeit with considerable overlap between the microbiomes at both diseased and healthy sites. Comparisons of diseased or healthy sites in the mild vs moderate-severe disease groups did not reveal any statistically significant differences, indicating a high degree of concordance in microbiome composition that was independent of overall disease severity.

**Discussion**

Many animal models of periodontitis have been developed over the past several decades, which have been employed in studies to predict clinical outcomes in humans. For a model to be useful, it must closely resemble the clinical, structural, immunologic, and microbiologic features of the human disease. Whereas there is general agreement that non-human primates meet most of these criteria, there remain...
gaps in knowledge, in particular regarding the composition of the oral microbiome at sites of disease vs health. In the present study, we characterized naturally occurring chronic periodontitis in a colony of *M. mulatta* with respect to these parameters, with particular emphasis on the composition of the oral microbiome.

Our findings demonstrated that *M. mulatta* exhibits essentially all of the clinical features of chronic adult periodontitis, with a range of PDs and widespread inflammation as evidenced by BOP. The oral microbiome at subgingival sites in these animals was closely similar to that found in humans, with 56% of 108 bacterial taxa found to be identical or closely similar to human oral species. The balance of the microbiome consisted of unique macaque species that, nevertheless, were related to human counterparts. Disease-associated species included *P. gingivalis*, *T. forsythia*, *F. alocis*, *P. micra*, *Treponema phylotypes*, *Fusobacterium* spp., *D. invisus*, and several *Treponema* spp. Although several of the consensus ‘red complex’ periodontal pathogens were present in higher numbers at diseased compared with healthy sites, most of the periodontitis-associated organisms we have identified in macaques have recently been associated with human disease [25]. Taxa present in higher numbers in health included: *Actinomyces*, *Capnocytophaga*, *Campylobacter*, *Corynebacterium*, *Fusobacterium*, *Leptotrichia*, and *Propionibacterium*. Taken together, we conclude that the same range of periodontitis- and health-associated micro-organisms were present in the macaque as has been reported in humans.

Only one previous study employed next-generation sequencing to characterize the oral microbiome in macaques, albeit in Simian Immunodeficiency Virus (SIV) infection [18]. Prominent taxa in dental plaque in that study included: *Streptococcus* spp., *Lachnospiraceae*, *G. morbillorum*, *Selenomonas*, *Filifactor*, *Peptostreptococcus*, *Clostridiales*, *Capnocytophaga* spp., and *Bacteroidetes* spp. The periodontal status of animals was not assessed; however, *Porphyromonas* and *Treponema* were not found in plaque, and *Tannerella* was rare. The tongue microbiome was considerably altered in SIV infection, with high levels of *G. morbillorum* and reductions in
Streptococcus. This microbial profile is distinct from that observed in the present study, likely owing to the modifying effects of SIV infection, age of the animals, and lack of significant periodontitis in that population. We identified P. gingivalis in all animals at levels that were not significantly higher in diseased vs. healthy sites. This finding suggests that P. gingivalis might not be critical for periodontal pathogenesis in macaques. Indeed, the list of species that have been associated with periodontitis in humans has grown dramatically with the widespread adoption of next-generation sequencing [25]. However, alternative explanations may shed light on this observation. Although ‘healthy’ sites did not exhibit clinical measures of PD > 3 mm or BOP, the amount of plaque was elevated in virtually all sites, since animals did not receive mechanical plaque control, suggesting some degree of incipient inflammation and subclinical disease in most sites. It is also known from human studies that clinically healthy sites in diseased individuals usually harbour higher mean counts of pathogens than healthy sites of non-periodontitis subjects [26]. Another factor may relate to the metabolic activity of the microbiome. Recent studies of the metatranscriptome in progressing human periodontitis have revealed a dramatic lack of concordance between microbial profiles and their transcriptional activity, i.e. which species were metabolically active within the community [27–30]. Signature pathways associated with tissue destruction were identified, including cell motility, lipid A and peptidoglycan biosynthesis, and transport of iron, potassium, and amino acids [30]. Consensus periodontal pathogens T. forsythia and P. gingivalis upregulated various TonB-dependent receptors, peptidases, proteases, aerotolerance genes, iron transport genes, hemolysins, and CRISPR-associated genes. Such results indicate that gene-expression patterns may better define the disease state, compared with the simple composition of the microbiome. Community-wide transcriptomic studies are now needed to identify the most transcriptionally active species in macaques with periodontitis, as well as the pathways they express in disease vs. health.

There were distinct subgroups within the macaque population with respect to disease severity, with about 35% of animals exhibiting moderate-severe periodontitis. Given that the mean ages of animals in these two subgroups were nearly identical, this suggests that there is heterogeneity among macaques with respect to susceptibility (Table 2), a picture previously observed in a retrospective study of this animal model [31]. The microbial challenge in the moderate-severe vs mild disease groups was not significantly different. Hence, the macaque disease can be viewed as representative of the ‘natural history’ of periodontitis in this species, with greater susceptibility likely due mainly to genetic factors. In humans, the summary effect of genetics on periodontitis, based on family and twins studies, has been estimated at approximately 50%, independent of smoking and other behavioural variables [32–34]. Of interest, these susceptibility findings are not dissimilar to studies in adult humans age 30 and older, conducted as part of the National Health and Nutrition Examination Survey (NHANES) [23,35]. As in the present study, NHANES also carried out full mouth examinations of six sites per tooth, to provide a comprehensive disease assessment; the prevalence of moderate and severe disease was found to be 8.9% and 30.0% respectively. The relationship of monkey to human age is approximately 0.3; thus, the population we have evaluated roughly corresponds to a mean age of 42 years in humans.

In summary, the present findings indicate a strong parallel between all of these factors, in naturally occurring periodontitis in M. mulatta, and chronic adult periodontitis in humans. We conclude that M. mulatta therefore represents an excellent model for human periodontitis, and for evaluating therapeutic regimens and agents for human disease.

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Disclosure statement

Drs J. McCluskey and H. Kleanthous are employees of Sanofi-Pasteur. No other conflicts of interest were reported by the authors.

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