

ORIGINAL ARTICLE

Oral microbiota and host innate immune response in bisphosphonate-related osteonecrosis of the jaw

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Bacterial biofilms have emerged as potential critical triggers in the pathogenesis of bisphosphonate (BP)-related osteonecrosis of the jaw (ONJ) or BRONJ. BRONJ lesions have shown to be heavily colonized by oral bacteria, most of these difficult to cultivate and presents many clinical challenges. The purpose of this study was to characterize the bacterial diversity in BRONJ lesions and to determine host immune response. We examined tissue specimens from three cohorts ($n=30$); patients with periodontal disease without a history of BP therapy (Control, $n=10$), patients with periodontal disease having history of BP therapy but without ONJ (BP, $n=5$) and patients with BRONJ (BRONJ, $n=15$). Denaturing gradient gel electrophoresis of polymerase chain reaction (PCR)-amplified 16S rRNA gene fragments revealed less bacterial diversity in BRONJ than BP and Control cohorts. Sequence analysis detected six phyla with predominant affiliation to *Firmicutes* in BRONJ (71.6%), BP (70.3%) and Control (59.1%). Significant differences ($P<0.05$) in genera were observed, between Control/BP, Control/BRONJ and BP/BRONJ cohorts. Enzyme-linked immunosorbent assay (ELISA) results indicated that the levels of myeloperoxidase were significantly lower, whereas interleukin-6 and tumor necrosis factor- α levels were moderately elevated in BRONJ patients as compared to Controls. PCR array showed significant changes in BRONJ patients with downregulation of host genes, such as nucleotide-binding oligomerization domain containing protein 2, and cathepsin G, the key modulators for antibacterial response and upregulation of secretory leukocyte protease inhibitor, proteinase 3 and conserved helix-loop-helix ubiquitous kinase. The results suggest that colonization of unique bacterial communities coupled with deficient innate immune response is likely to impact the pathogenesis of ONJ.

International Journal of Oral Science (2014) 6, 219–226; doi:10.1038/ijos.2014.46; published 8 August 2014

Keywords: bisphosphonates; denaturing gradient gel electrophoresis; host response; innate immunity; oral microbiota; osteonecrosis of the jaw

INTRODUCTION

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is most frequently defined as current or previous treatment with a bisphosphonate, exposed bone in the maxillofacial region for more than 8 weeks and no history of radiation therapy to the jaws.¹ BP drugs, most extensively, the potent nitrogen containing bisphosphonates are used for the treatment of osteoporosis and skeletal complications in cancer and multiple myeloma.² The risk factors include drug therapy (e.g., antiresorptive therapy, chemotherapy),³ local factors (such as infection, dental extraction and poor oral hygiene)^{1,4} and/or systemic comorbidities.⁵ Patient management is further complicated as the recommendations have focused on avoiding dental extractions,^{5–6} while other reports suggest that ONJ may be triggered by infection.⁷ Hence, BRONJ remains a significant concern and may have broader health impact due to exponential increase in the number of reported cases, and incidences more common with nitrogen-containing BPs, which aptly will rise over coming years.^{8–10} Understanding the risk and

pathogenesis of BRONJ has been confounded by occurrence of osteonecrosis associated with non-bisphosphonate antiresorptive therapy and chemotherapy.^{10–15}

There are many hypotheses for the pathogenesis of BRONJ that can be broadly classified; the *inside-out* theory describing bone changes and the *outside-in* theory describing effects on bone by surrounding tissues. Proposed mechanisms involved in BRONJ pathogenesis could include ischemia, reduced bone turnover, anti-angiogenic effect, BP toxicity to bone, BP toxicity to soft tissue, microcracks, inflammation, compromised immune response and infection.^{16–23}

The oral cavity comprises of more than 750 bacterial species existing as mixed biofilm communities.^{19–20,24} The presence of bacterial biofilms, most commonly, *Actinomyces* at BRONJ sites, has been demonstrated by histomorphometric and histological studies.^{6,10,25–29} Our previous studies, for the first time, using 16S rRNA sequencing, have shown the presence of polymicrobial communities, both cultivable and uncultivable, in a broader perspective in the soft tissues¹⁹ and

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Accepted on 22 April 2014

jaw bone²⁴ sites of BRONJ lesions which otherwise, could go undetected by histomorphometric or histopathological analyses. Nevertheless, the ubiquitous influence of bacterial biofilms at the site of BRONJ lesions may impact the pathogenesis of BRONJ.

The purpose of this study was (i) to characterize the bacterial diversity in BRONJ lesions using 16S rRNA-based approaches; and (ii) to determine the host antibacterial immune response using tissue-based enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) arrays. We hypothesize that BRONJ is associated with diminished immune response.

MATERIALS AND METHODS

Subjects and specimen collection

A total of 30 patients, 73% female and 27% male, with a mean age of (62.2±15.4) years, undergoing oral surgery treatment at New York University College of Dentistry, were recruited for this study. The study was approved by the Institutional Review Board of New York University and subjects agreed to participate by signing informed consent. This study had three patient cohorts: patients with BRONJ (BRONJ group, $n=15$); patients with periodontal disease who had a history of BP therapy (BP group, $n=5$) and patients with periodontal disease (Control group, $n=10$) (Supplementary Table S1). BRONJ lesions were classified into stages I–III as described by Ruggiero *et al.*¹ About 67% of BRONJ lesions were spontaneous while the remaining occurred after dental extractions. All the cancer patients had a history of chemotherapy. The BRONJ subjects selected for microbiome sequencing study were not on antibiotics for about 3 months prior to sample collection, to preclude the bias of antibiotic effects on bacterial colonization in BRONJ lesions. Each of the soft tissue samples associated with the necrotic bone in BRONJ lesions and of the periodontium from the Control and BP groups were collected in two sterile plastic tubes and transported on dry ice. The sample tubes with N-tris(hydroxymethyl)-amino methane (Tris)-ethylenediaminetetraacetic acid (EDTA) buffer were stored at $-20\text{ }^{\circ}\text{C}$ while the other having RPMI with gentamycin were processed as described below (see the section on ‘Tissue-based ELISA’).

Bacterial DNA extraction and PCR amplification

Randomly selected 15 tissue samples, five from each group, were suspended in 500 μL of sterile phosphate-buffered saline, vortexed for 30 s and subsequently sonicated for 5 and 10 s, respectively. For digestion, Proteinase K (2.5 $\mu\text{g}\cdot\text{mL}^{-1}$) was added and incubated overnight at 55 $^{\circ}\text{C}$. Bacterial genomic DNA from the samples was purified as described previously¹⁹ and stored at $-20\text{ }^{\circ}\text{C}$ till further analysis.

For cloning, DNA samples (20 $\text{ng}\cdot\mu\text{L}^{-1}$) were amplified with universal primer set, 8F and 1492R and for DGGE assay, nested PCR targeting V4–V5 16S region was performed using eubacterial primers, prbac1 with 40-nucleotide GC clamp at 5' end and prbac2 as described previously.^{24,30–31}

Denaturing gradient gel electrophoresis assay

The nested PCR amplified products were analyzed for sequence polymorphism on 40%–60% linear DNA denaturing gradient polyacrylamide gel, 0.8 $\text{g}\cdot\text{L}^{-1}$ alongside species-specific markers using DCode system (Bio-Rad, Hercules, CA, USA) for 16 h at 58 $^{\circ}\text{C}$ and 60 V in 1× Tris-acetate-EDTA buffer, pH 8.5.^{19,30} The gels were stained with ethidium bromide solution (0.5 $\mu\text{g}\cdot\text{mL}^{-1}$) for 15 min and the images digitally captured using Alpha Imager 3300 system (Alpha Innotech San Leandro, CA, USA).

Cluster and statistical analyses of denaturing gradient gel electrophoresis microbial profiles

Denaturing gradient gel electrophoresis (DGGE) fingerprints were analyzed with Fingerprinting II Informatix Software (Bio-Rad,

Hercules, CA, USA) and interpreted statistically.³⁰ The gels were normalized with standard DGGE markers and background subtracted using mathematical algorithms based on spectral analysis of overall densitometric curves. The similarity index was calculated by Dice coefficient and dendrogram constructed from average matrix by Ward analysis. The differences were determined using Mann–Whitney *U* test and Chi-square test. Statistical analysis was performed using SPSS software version 17.0 (SPSS, Chicago, IL, USA).

16S rRNA cloning and sequence analysis

PCR amplified products were ligated to pCR4-TOPO vector and transformed into *E. coli* TOP10 cells using TOPO-TA cloning kit according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). From each sample, 48 to 96 clones were picked and sequenced.¹⁹ The sequences were aligned and analyzed as described earlier.³¹ Chimeras were eliminated by greengenes chimera check program.³² Sequences with 350 to 900 bases were identified against 16S rRNA reference dataset of Human Oral Microbiome Database (version 10.1).³³ The assigned phylogenetic threshold for sequences with $\geq 98\%$ similarity was till species level, while those with $< 98\%$ similarity were classified till genus level. Three libraries, namely Control, BP and BRONJ were constructed for clonal analysis. Chi-square test was used to compare phylogenetic differences between two libraries. The terminologies, ‘species’ refers to named cultivated species and unnamed cultivated taxon, whereas ‘*phylotype*’ refers to uncultivable or yet-uncultured species.

Species diversity and richness estimation

The richness estimators, Chao1 and abundance-based coverage estimator (ACE) and rarefaction curves, rank abundance and diversity indices, Shannon (*H'*) and Simpson ($1-D$) as well as Good's percent coverage were computed as described previously.^{19,31}

Tissue-based ELISA

Twelve BRONJ and 5 Control tissue specimens were examined for the levels of myeloperoxidase (MPO), tumor necrosis factor (TNF)-alpha and interleukin-6 (IL-6), important indicators of antimicrobial response. The samples were processed (Supplementary Information: ‘Materials and methods’) and the supernatants from the lysates were stored at $-80\text{ }^{\circ}\text{C}$. Further, ELISA was performed according to manufacturer's (R&D Systems, Minneapolis, MN, USA) instructions (Supplementary Information: ‘Materials and methods’). Each data point is the mean of triplicate measurements of each factor. Long dash is the mean for each group. The two groups were compared using unpaired two-tailed *t*-test.

SuperArray screening

The tissue samples from a subset patient population with BRONJ ($n=3$) and no BRONJ controls without BP ($n=2$) were used to study the innate immune mechanisms to bacterial pathogens using Human Antibacterial Response RT² Profiler PCR Array System (PAHS-148Z; SABiosciences, Qiagen, Valencia, CA, USA) (Supplementary Information: ‘Materials and methods’). Data analysis was performed using the web portal <http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>. The positive value signifies upregulation, whereas the negative value indicates downregulation of genes.

RESULTS

To decipher the relationship between bacterial colonization and host antibacterial response in BRONJ patients, we evaluated the total bacterial profile (cultivable and uncultivable) based on 16S rRNA gene assays. Subsequently, a subset of the same tissue samples was selected

to determine the expression of genes involved in antibacterial response using ELISA and further confirmed with PCR arrays.

Diversity and abundance of specific oral bacteria in BRONJ

The total bacterial diversity in the three cohorts, five each from Control, BP and BRONJ groups, was differentiated using DGGE fingerprints (Figure 1). The intensity profiles were compared based on band position, after gel normalization with species-specific marker. Each band represented one or more bacterial species. The number of bands observed in tissue samples from three different cohorts ranged from 31 to 36 (mean: 32.8 ± 2.5) in Control, 28 to 30 (mean: 29 ± 0.7) in BP and 25 to 34 (mean: 29.6 ± 3.2) in BRONJ cohort (Figure 1a).

The non-parametric Mann–Whitney *U* test for testing equality of means indicated significant intergroup differences ($P < 0.05$), between Control/BP cohorts ($P = 0.044$) and Control/BRONJ cohorts ($P = 0.007$). Cluster analysis of DGGE fingerprints of the three cohorts was performed using Dice coefficient and the dendrogram revealed three separate clusters each representing Control, BP and BRONJ cohorts (Figure 1b). The above results suggest that DGGE bacterial fingerprinting can be used as a preliminary rapid assessment tool for identifying patient at a higher risk of BRONJ.

We further examined 14 tissue samples, five each from Control and BRONJ cohorts and four from BP cohort for phylogenetic affiliations by cloning and sequencing. From a total of 887 sequences, 389

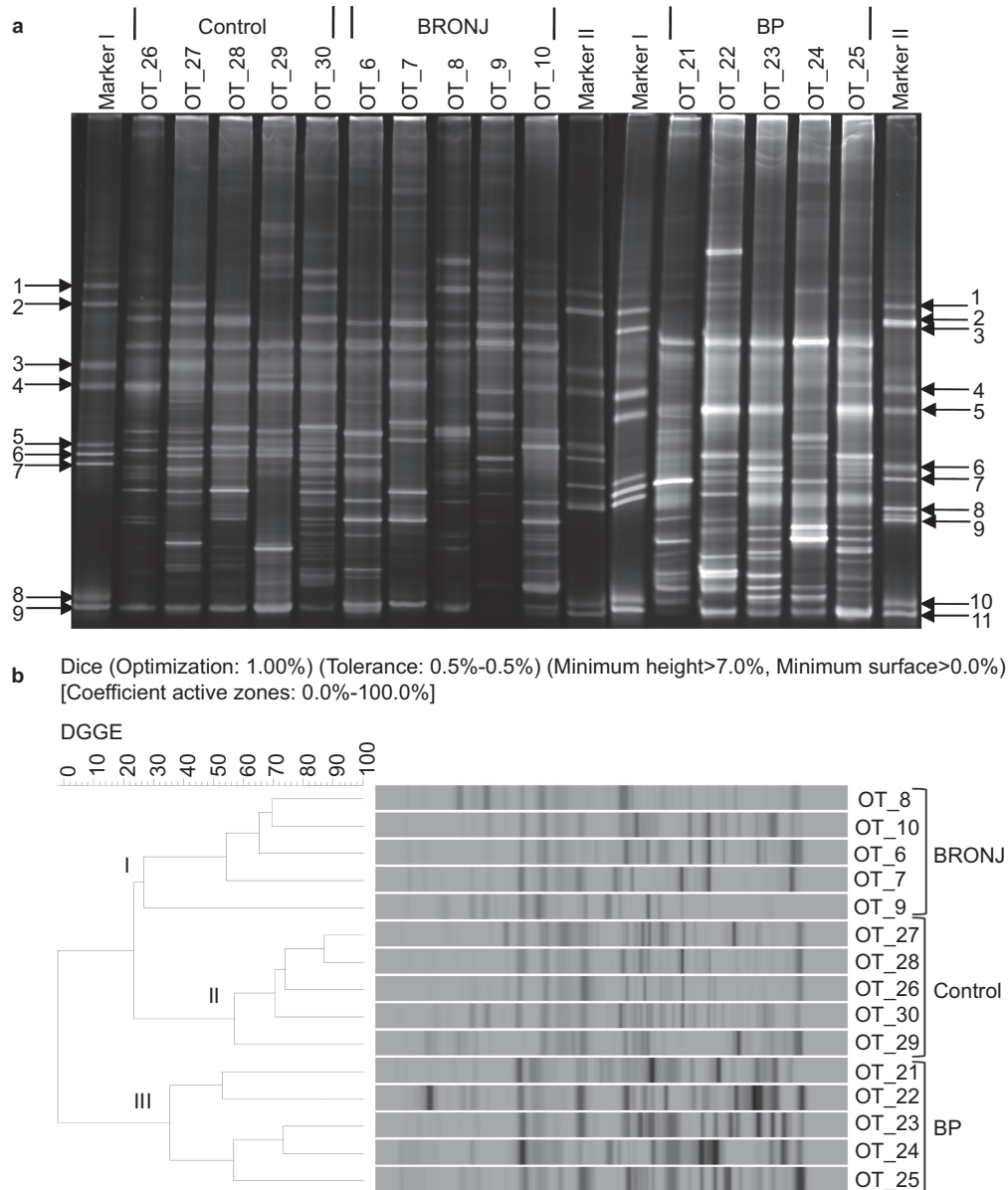


Figure 1 Bacterial diversity in the tissues of Control, BP and BRONJ cohorts. (a) Band intensity profiles of DGGE gels. Marker I and II: DGGE reference markers corresponding to 16S rRNA gene fragments of the given bacterial species [Marker I: 1. *F. nucleatum* subsp. *vincenti* (ATCC 49256); 2. *F. nucleatum* subsp. *nucleatum* (ATCC 25586); 3. *S. sanguinis* (ATCC 10556); 4. *S. oralis* (ATCC 35037); 5. *S. salivarius* (ATCC 7073); 6. *S. mutans* (UA 159); 7. *L. paracasei* (ATCC 25598); 8. *A. odontolyticus* (ATCC 17929); 9. *A. naeslundii* (ATCC 12104). Marker II: 1. *F. nucleatum* subsp. *vincenti* (ATCC 49256); 2. *F. nucleatum* subsp. *nucleatum* (ATCC 25586); 3. *B. forsythus* (ATCC 43037); 4. *S. sanguinis* (ATCC 10556); 5. *S. oralis* (ATCC 35037); 6. *V. parvula* (ATCC 17745); 7. *P. intermedia* (ATCC 25611); 8. *A. actinomycetemcomitans* (ATCC 43717); 9. *P. gingivalis* (ATCC 33277); 10. *A. odontolyticus* (ATCC 17929); 11. *A. naeslundii* (ATCC 12104)]. (b) Cluster analysis by Dice coefficient of the bacterial fingerprints. BP, bisphosphonate; BRONJ, bisphosphonate-related osteonecrosis of the jaw; DGGE, denaturing gradient gel electrophoresis.

sequences were characterized. Based on sequence length cutoff of <350 bases, 498 (~56%) sequences and 2% chimeras were eliminated. The phylogenetic affiliations for 371 (42%) sequences of 350–900 bases were assigned by Human Oral Microbiome Database. Thirty sequences (3%) with <98% similarity were considered as unclassified sequences. Of 341 (39%) sequences with >98% similarity, 312 sequences (36%) showed homology to cultivable species and 29 (3%) to uncultured phylotypes.

Bacterial diversity in all the three cohorts was characterized into six phyla represented by *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Proteobacteria* and *TM7* (Figure 2a). The species of phylum *Firmicutes* were highly prevalent in all the three cohorts but elevated in BRONJ subjects (71%). Also, *Fusobacteria* was predominant in BRONJ cohort. BP cohort showed the presence of *Actinobacteria* in higher numbers as compared to Control and BRONJ. Phyla, *Proteobacteria*, *Bacteroidetes* and *TM7* had higher prevalence in Control than in BP and BRONJ. Significant differences in percentage relative distribution at phylum level were observed between Control/BRONJ cohorts (Chi-square test, $P<0.05$).

In total, 11 classes, 15 order, 29 families and 48 genera were identified (Supplementary Figures S1–S3 and Figure 2b). There were significant differences in these phylogenies between Control/BP, BP/BRONJ and

Control/BRONJ cohorts (Chi-square test, $P<0.05$). Class *Mollicutes* was present in BP and BRONJ cohorts while absent in Control cohort, while *Gammaproteobacteria* was exclusive to BRONJ cohort. Genus *Streptococcus* was highly prevalent in all the three cohorts. The predominant genera in the Control group were *Veillonella* (19.7%), *Peptostreptococcus* (8.6%), *Lactobacillus* (7.3%), *TM7[G-1]* (6.3%), *Actinomyces* (4.4%), *Mogibacterium* (3.9%), *Porphyromonas* (3.6%) and *Scardovia* (1.8%). However, in BP cohort, *Atopobium* (8.7%), *Rothia* (6.3%), *Pseudoramibacter* (4.2%), *Shuttleworthia* (4.2%), *Afipia* (3.1%), *Bacteroidetes[G-8]* (3.1%), *Delftia* (3.1%), *Veillonellaceae[G-1]* (2.5%), *Solobacterium* (1.5%) and *TM7[G-5]* (1.5%) were observed. Genera with higher frequency in BRONJ cohort were *Parvimonas* (18.3%), *Fusobacterium* (4%), *Eubacterium[11][G-6]* (3.1%), *Gemella* (2.1%), *Leptotrichia* (1.7%) and *Selenomonas* (1%). *Dialister* (2.8%) and *Bulleidia* (1.1%) were present in BP and BRONJ cohorts but predominant in BRONJ patients. Genera exclusive to BRONJ were *Xanthomonas* (3.8%), *Lachnospiraceae[G-7]* (2.1%), *Eubacterium[11][G-1]* (2.1%), *Bifidobacterium* (2%) and *Bacteroidetes[G-2]* (1.7%). A relative dysbiosis was observed in gram-positive and gram-negative bacteria in the three cohorts (Figure 2c). Gram-negative microbiota was higher in Control (30.8%) than in BRONJ (17.2%) and BP (15.8%), whereas gram-positive predominate the BP and BRONJ cohorts.

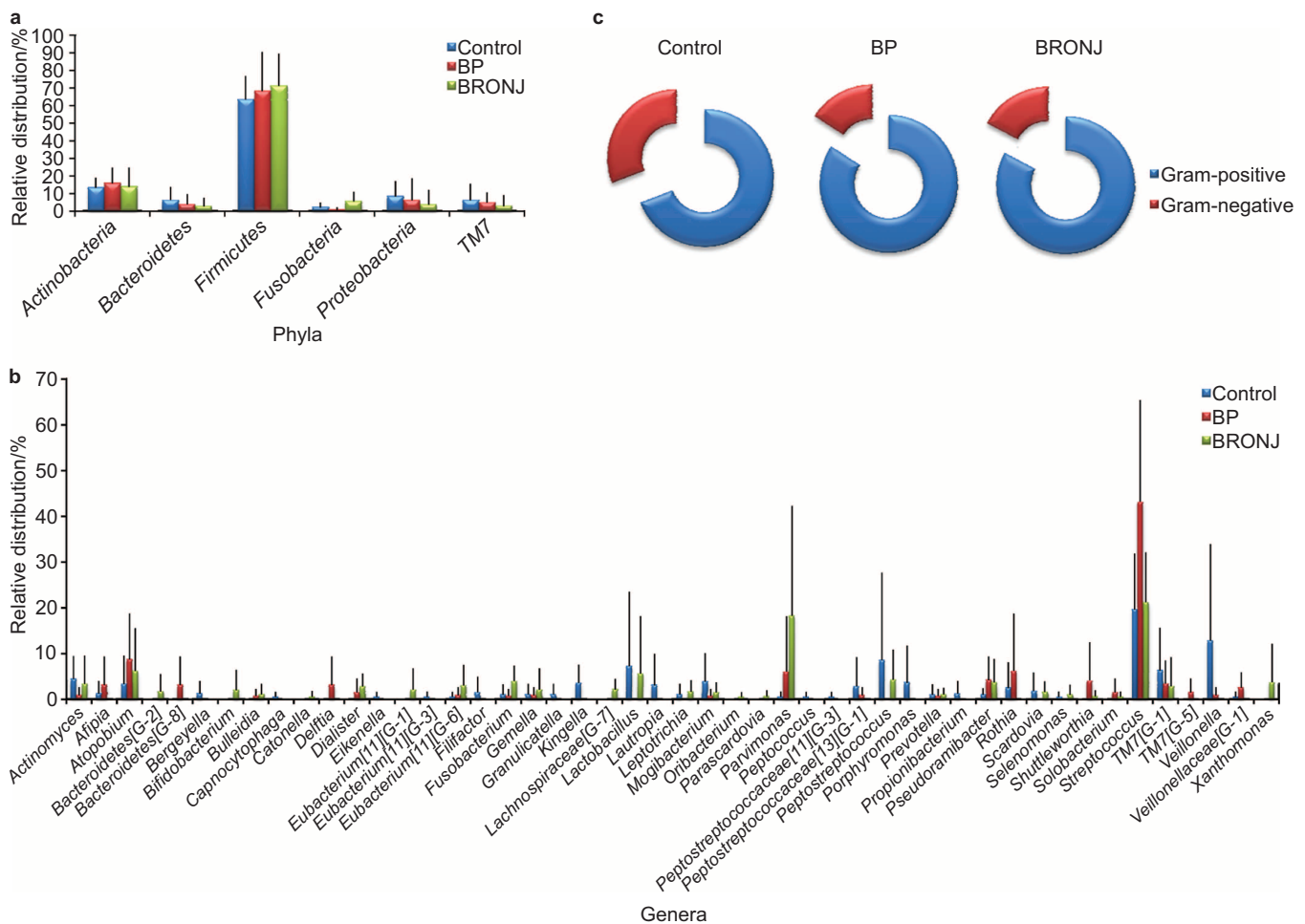


Figure 2 Taxonomic distribution of microbiota in the tissues of Control, BP and BRONJ cohorts. (a) Phylum level [significant differences were observed in relative distribution (%) of phyla between Control/BRONJ cohorts ($P<0.05$, Chi-square test)]; (b) genus level [significant differences were observed in relative distribution (%) of genera between Control/BP, BP/BRONJ and Control/BRONJ cohorts ($P<0.05$, Chi-square test)]; (c) frequency of gram-positive and gram-negative bacteria. BP, bisphosphonate; BRONJ, bisphosphonate-related osteonecrosis of the jaw.

Of the 91 total bacterial species/phylotypes identified in this study, 50 belonged to Control cohort, whereas 39 and 43 were found in BP and BRONJ cohorts respectively. Table 1 depicts some of the predominant, common and exclusive species/phylotypes detected in the three cohorts. *Parvimonas micra*, *Streptococcus anginosus*, *Atopobium rimae*, *Peptostreptococcus stomatis* and *Eubacterium*[11][G-6] *nodatum* were present in higher numbers in BRONJ lesions. *Streptococcus constellatus*, *Bifidobacterium dentium*, *Eubacterium infirmum*, *Selenomonas sputigena* and uncultivable phylotypes, *Actinomyces sp. oral taxon 525* and *Lachnospiraceae*[G-7] *sp. oral taxon 086* were exclusive to BRONJ cohort.

Chao1 and ACE richness estimators, diversity indices and evenness for three cohorts were calculated (Supplementary Table S2). The BRONJ cohort showed less species richness as compared to Control and BP cohorts. Good's coverage was 72%, 61% and 90% for Control,

Table 1 List of bacterial species/phylotypes found common to and exclusive in the tissues of three cohorts of patients, Control, BP and BRONJ

Bacterial species/phylotypes	
Common in cohort(s)	Exclusive in cohort(s)
Control/BP	Control
<i>Streptococcus gordonii</i>	<i>TM7</i> [G-1] <i>sp. oral taxon 353</i> **
<i>Veillonella dispar</i>	<i>Streptococcus sanguinis</i>
<i>Streptococcus sp. oral taxon 071</i> **	<i>Lautropia mirabilis</i>
<i>Rothia dentiocariosa</i>	<i>Veillonella parvula</i>
<i>Streptococcus oralis</i>	<i>Filifactor alocis</i>
<i>TM7</i> [G-1] <i>sp. oral taxon 348</i> **	<i>Actinomyces sp. oral taxon 171</i> *
<i>Streptococcus cristatus</i>	<i>Granulicatella adiacens</i>
<i>Streptococcus pneumoniae</i>	<i>Kingella oralis</i>
<i>Peptostreptococcaceae</i> [XIII][G-1] <i>sp. oral taxon 113</i> **	<i>Porphyromonas sp. oral taxon 395</i> **
	<i>Prevotella sp. oral taxon 472</i> *
Control/BRONJ	BRONJ
<i>Peptostreptococcus stomatis</i>	<i>Streptococcus constellatus</i>
<i>Lactobacillus gasseri</i>	<i>Bifidobacterium dentium</i>
<i>Streptococcus vestibularis</i>	<i>Eubacterium</i> [11][G-1] <i>infirmum</i>
<i>Fusobacterium nucleatum ss vincentii</i>	<i>Actinomyces sp. oral taxon 525</i> **
<i>Scardovia sp. oral taxon 195</i> *	<i>Lachnospiraceae</i> [G-7] <i>sp. oral taxon 086</i> **
<i>Actinomyces israelii</i>	<i>Bacteroidetes</i> [G-2] <i>sp. oral taxon 274</i> *
<i>Lactobacillus fermentum</i>	<i>Selenomonas sputigena</i>
<i>Lactobacillus paracasei</i>	<i>Prevotella denticola</i>
BP/BRONJ	BP
<i>Parvimonas micra</i>	<i>Atopobium sp. oral taxon 199</i> *
<i>Streptococcus anginosus</i>	<i>Veillonellaceae</i> [G-1] <i>sp. oral taxon 155</i> *
<i>Streptococcus intermedius</i>	
<i>Dialister invisus</i>	
<i>Fusobacterium nucleatum ss. animalis</i>	
<i>Streptococcus mutans</i>	
<i>TM7</i> [G-1] <i>sp. oral taxon 346</i> **	
<i>TM7</i> [G-1] <i>sp. oral taxon 349</i> **	
<i>Solobacterium moorei</i>	
Control/BP/BRONJ	
<i>Pseudoramibacter alactolyticus</i>	
<i>Streptococcus mitis</i>	
<i>Mogibacterium timidum</i>	
<i>Atopobium rimae</i>	
<i>Eubacterium</i> [11][G-6] <i>nodatum</i>	
<i>Gemella morbillorum</i>	
<i>Streptococcus sinensis</i>	

BP, bisphosphonate; BRONJ, bisphosphonate-related osteonecrosis of the jaw.
* Unnamed cultured phylotype.
** Currently uncultured phylotype.

BP and BRONJ cohorts, respectively. In case of BRONJ cohort, the decrease in rate of phylotype detection as depicted in the rarefaction curves indicated that majority of the diversity in the libraries have been identified (Supplementary Figure S4). The library size of BP and Control cohorts was insufficient and additional clones are required to be screened for the curve to reach asymptote. Rarefaction curves also showed less diversity in BRONJ cohort than in Control and BP cohorts. This observation was reflected in Shannon-Weaver and Simpson diversity indices of the three libraries (Supplementary Table S2).

Antibacterial host response in BRONJ patients

We hypothesize that the unique bacterial colonization in BRONJ is associated with a compromised antimicrobial response. To test this possibility, tissue-based ELISA performed on BRONJ and Control tissues, revealed that, the mean MPO levels in Controls were significantly higher than in BRONJ ($P=0.02$) (Figure 3a), whereas the levels of IL-6 (Figure 3b) and TNF-alpha (Figure 3c) were similar (no significant differences) in both the groups. This may not be surprising as

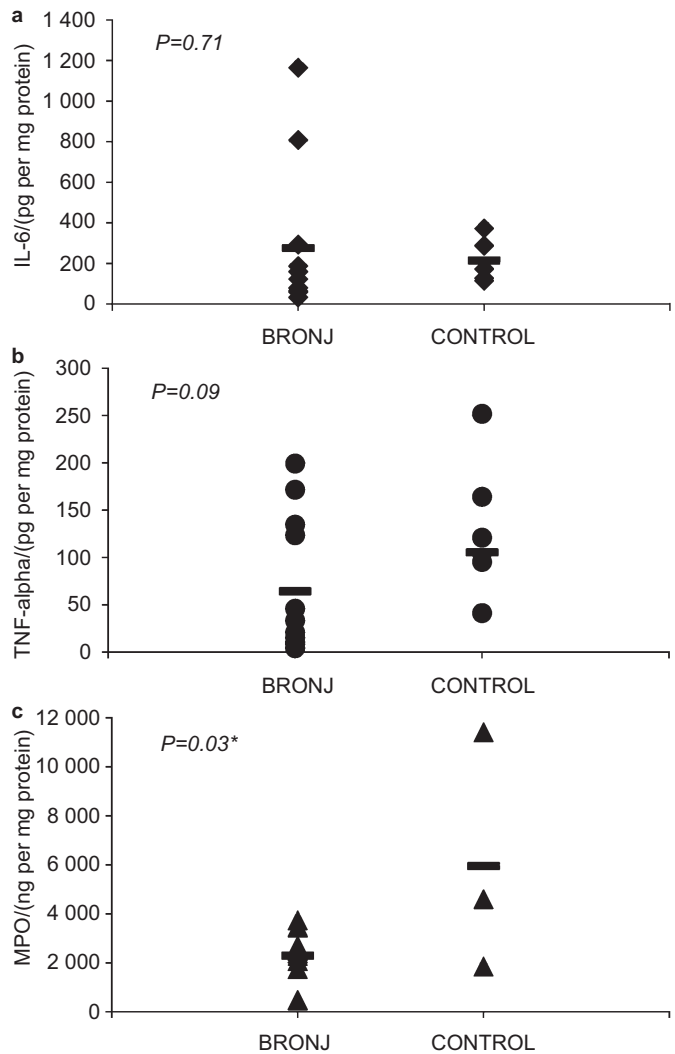


Figure 3 Expression levels of MPO, IL-6 and TNF-alpha, in BRONJ and Control cohorts by ELISA. BRONJ, bisphosphonate-related osteonecrosis of the jaw; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; MPO, myeloperoxidase; TNF, tumor necrosis factor.

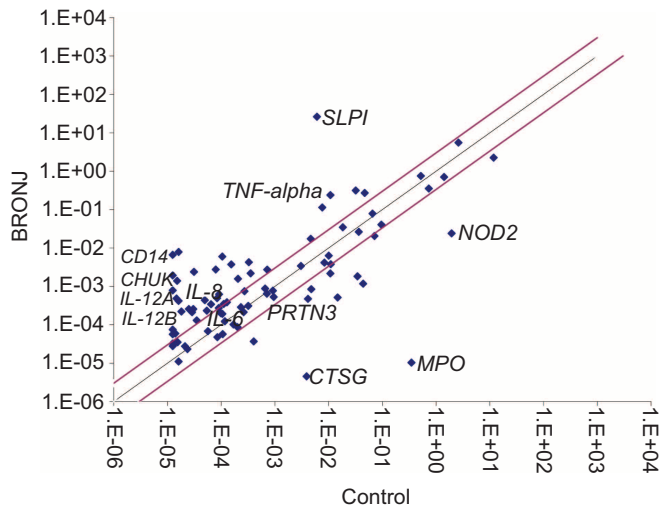


Figure 4 Regulatory profiles of different genes in the tissues of BRONJ and Control patients generated by PCR array. BRONJ, bisphosphonate-related osteonecrosis of the jaw; CD, cluster of differentiation; CHUK, conserved helix-loop-helix ubiquitous kinase; CTSG, cathepsin G; IL, interleukin; MPO, myeloperoxidase; NOD, nucleotide-binding oligomerization domain; PCR, polymerase chain reaction; PRTN, proteinase; SLPI, secretory leukocytes protease inhibitor; TNF, tumor-necrosis factor.

IL-6 production is governed by many cells in response to any inflammation and has little impact on antimicrobial activity.

To determine the expression of various antimicrobial genes, we used 84 different inflammatory response genes PCR Array System (Human Antibacterial Response RT² Profiler). We observed that, of the 84 inflammatory mediators tested, 34 genes were significantly upregulated, whereas 11 genes were significantly downregulated in BRONJ samples (Figure 4). Supplementary Table S3 demonstrates the fold difference in the levels of few selected genes that may represent appreciable response of the host immune system. The expression profile of secretory leukocytes protease inhibitor (SLPI), proteinase 3 (PRTN3), conserved helix-loop-helix ubiquitous kinase (CHUK) and several interleukins was higher, whereas nucleotide-binding oligomerization domain (NOD2), cathepsin G (CTSG) and MPO genes were significantly downregulated in patients with BRONJ (Supplementary Table S3).

Thus, the expression pattern of MPO, IL-6 and TNF-alpha in neutrophil-dominated dental infection (BRONJ and Controls) as observed by ELISA (Figure 3) was consistent with our PCR array analysis results (Figure 4).

DISCUSSION

Microbial infections are hypothesized as one of the factors impacting the pathogenesis of BRONJ. To our knowledge, this is the first report, describing the relationship between microbial colonization and host antibacterial immune response in patients with BRONJ. Complex microbial consortia, specifically in the oral cavity, provide an interface that initiates and perpetuates the infectious attack on host tissues and are resistant to host defenses and antibacterial agents.³⁴⁻³⁵

The DGGE bacterial fingerprints were found to be diverse among Control, BP and BRONJ cohorts and clustered separately signifying the presence of distinct bacterial flora in that particular cohort. The bacterial phyla prevalent in the tissues of BRONJ patients by 16S sequencing, were consistent with our earlier study on bone samples of BRONJ²⁴ as well as with recent study on saliva samples of BRONJ patients.³⁶ Our

result indicated *Streptococcus* to be highly prevalent, and is the only genus, that extensively exhibits intra- and intergeneric co-aggregation, binding to other early colonizers, such as *Actinomyces*, *Capnocytophaga*, *Eikenella*, *Prevotella*, *Propionibacterium* and *Veillonella*, as well as to host molecules. On the other hand, *Fusobacterium*, a gram-negative bacteria found in all the three cohorts, usually act as a bridge between early colonizers and late colonizers, like *Eubacterium* and *Porphyromonas* which may explain its presence at all sites. These late colonizers co-aggregate with *Fusobacterium* but not with one another.³⁷ *Lactobacilli*, *Streptococci* and *Actinomyces* spp. were predominantly found in Control cohort, and are known fermenters of carbohydrates primarily to lactate, showed high correlation to increased *Veillonella* levels, perhaps for its affinity for lactate consumption.³⁸

P. micra, *S. anginosus*, *A. rimae*, *P. stomatis* and *Eubacterium*[11][G-6] *nodatum* dominated the BRONJ lesions. *P. micra*³⁹ and *A. rimae*⁴⁰ are strongly associated with polymicrobial infections mainly endodontic infections or periodontitis in humans and *S. anginosus* with abscess formation.⁴¹ *P. stomatis*, aciduric and weakly saccharolytic bacteria produces fermented products, such as acetic, butyric, isobutyric, isovaleric and isocaproic acids.⁴² *Eubacterium* are major asaccharolytic bacteria present in oral lesions playing a crucial role in root canal and periodontal pockets infections.⁴³⁻⁴⁴ *Dialister invisus* observed in BP and BRONJ cohorts, and *Prevotella denticola*, exclusively found in the mucosal tissues of BRONJ cohort, are known putative endodontic pathogens have been detected in our previous study in the bone samples of BRONJ patients.²⁴ *S. sputigena* was exclusively detected in BRONJ cohort. Species from genera, *Fusobacterium*, *Streptococcus*, *Actinomyces* and *Selenomonas*, found in this study have been previously identified histomorphometrically,²⁸ as well as in our molecular study²⁴ in the bone samples of BRONJ patients.

Several unnamed cultured and yet-uncultured phylotypes were unique to BP and BRONJ cohorts as seen in Table 1. Interestingly, the cultured phylotype, *Atopobium* sp. *oral taxon* 199 found exclusively in mucosal tissues of BP was also observed in bone samples of BRONJ.²⁴ This suggests that certain oral bacteria primarily adhere and colonize the host tissues⁴⁵, further perpetuating deep to bony surface as a result of BP accumulation onto the jawbone. A recent study showed that the bone BP levels were higher in BRONJ patients as compared to the Controls on BP therapy without BRONJ and the increase was proportional to the duration of BP therapy, type of BP and to age, weight and race of the patient.⁴⁶ The predominance of gram-negative bacteria in the Control cohort is understandable, as it comprised of patients with advanced periodontal disease.⁴⁷⁻⁴⁸ Bacterial biofilms are constantly adjusting to the dynamics of oral microenvironment leading to dysbiosis or restructuring from gram-negative to gram-positive oral biofilms as observed in BP and BRONJ cohorts. The adhesin proteins 'MSCRAMM' mediates the initial attachment of gram-positive bacterial strains to host tissues, and these interactions are critical to establish infection⁴⁹⁻⁵⁰ in BRONJ, early dental implant failure and/or other bony conditions.^{20,24,26,28-29,51-52} It is speculated that the cationic nitrogen-containing domain of BPs interacts with the amino-terminal of these adhesin molecules and hence BRONJ is rare with non-nitrogen BPs. Kassolis et al.⁵³ showed that BRONJ lesions with biofilms can remain in edentulous marrow spaces for more than a year after tooth extraction and mucosal healing. We have shown that systemic antibiotics failed to restrict bacterial colonization or promote effective healing of lesions after the onset of BRONJ.¹⁹ Combinatorial treatments, surgery together with long-term preoperative antibiotic regime have proven to be effective in BRONJ patients than short-term treatment.¹⁸

Protection of the host from pathogenic colonization and invasion requires robust innate immunity. BP affects monocyte migration and macrophage recruitment^{14,23} essential for tissue repair and antimicrobial activity.^{54–55} The bacterial infection elicits the host immune response by stimulating the release of pro-inflammatory cytokines and chemokines. Our results indicated that the mediators of infection and inflammation such as antimicrobial peptides (SLPI, PRTN3, MPO, CTSG), cytokines (IL-12A, IL-12B), inflammatory response (IL-6, IL-8, CD14, NOD2, TNF-alpha) and signal transduction (CHUK) factors were differentially altered. Acute, neutrophil-dominated responses are characterized by high levels of a potent microbicidal factor, MPO,⁵⁶ a clinically important marker of neutrophil activation and also produced by macrophages. Monocytes, macrophages and other cells make IL-6, which protect the host cell from apoptosis. In response to microbial products, monocytes and macrophages release TNF-alpha, which activates many antimicrobial pathways critical for host defense.⁵⁷ In fact, the use of TNF-alpha antagonists makes patients susceptible to infections.⁵⁷ Thus, low MPO and moderate activity of TNF-alpha and IL-6 in the BRONJ lesions is consistent with a diminished host antimicrobial response. Our findings substantiate the role of microbes and their ability to circumvent the host immune response in BRONJ progression. MPO affects tissue directly by releasing reactive oxygen species along with other pro-inflammatory cytokines (TNF-alpha, IL-1, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF)) from macrophages.⁵⁸ NOD2 has direct antibacterial effect and is a sensor for recognizing intracellular pathogens leading to induction of cytokines and antimicrobial peptides.⁵⁹ Also, the upregulated levels of SLPI are indicators of progression of microbial infections. Bacterial lipopolysaccharides induce SLPI production either directly by macrophages or by IL-1 β , TNF-alpha, IL-6 and IL-10.⁶⁰ Besides antibacterial defense, the neutrophil-derived serine proteases PRTN3 have been implicated in inactivation of progranulin, an anti-inflammatory factor and promoting neutrophil activation and inflammation.⁶¹

Multiple factors may contribute to deficient immune response in BRONJ patients, such as the age of the patient, sclerotic bone changes, cancer and chemotherapy. Ten percent of patients undergoing chemotherapy for solid tumors develop infections⁶² and certain types of chemotherapy may exacerbate BRONJ lesions.⁶³ The compromised immune response will lead to the collapse of indigenous commensal oral microbiota and increase the risk of pathogenic infections. As we know that the co-evolution and co-existence between the host and microbiota is mutual, in which the microbiota contributes to host physiological processes and, in turn, the host provides niches and nutrients for microbial survival.⁶⁴ The dysfunction in this complex interplay between the host immune system and the microbiota elicits infections. Favot *et al.*⁶⁵ showed that BP perturbs neutrophils migration with decrease in reactive oxygen species production in patients with BRONJ and in those after post-pamidronate therapy as compared to the controls, thus decreasing the neutrophil recruitment detrimental to fight the ongoing infection.

To conclude, the dysbiosis in oral microbiota and alterations in the mediators of immune response in BRONJ patients may create an environment for colonization of opportunistic pathogens, such as *Parvimonas*, *Peptostreptococcus*, *Fusobacterium*, *Eubacterium*, *Dialister* and *Gemella* on BP-bound bone and promote necrosis. Certain bacterial communities, specifically, *Dialister*, *Prevotella* and *Atopobium* found in this study, were not just confined to soft tissue but have been detected deep into the necrotic bone.^{24,28} Despite the fact that BP effects on bone remodeling process, these bacteria have shown to elicit

inflammatory and acute, neutrophil-dominated response which could further impair this process.⁶⁶ The study has limitation of having small and non-homogenous patient cohort, but with the type of oral disorder, it is difficult to procure homogeneous patient population. The findings provide novel insight into host-microbial interactions in patients with BRONJ. Future study designs with larger sample population are required to validate the involvement of specific microbial species and biofilm characteristics, which may aid in risk assessment and therapeutic management of BRONJ.

ACKNOWLEDGEMENTS

This work was partially supported by NIH grants CA172894, CA180277, DE020891 and New York University Research Funds.

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