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ANTIBIOTIC EFFECTS ON BACTERIAL PROFILE IN OSTEONECROSIS OF THE JAW

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Abstract

OBJECTIVE—Oral infection is considered to play a critical role in the pathogenesis of bisphosphonate-related osteonecrosis of the jaw (BRONJ) and antibiotic therapy has become a mainstay of BRONJ therapy. This study was aimed to investigate the effect of antibiotics on bacterial diversity in BRONJ tissues.

MATERIALS AND METHODS—The bacterial profile from soft tissues associated with the BRONJ lesion was determined using 16S rRNA-based denaturing gradient gel electrophoresis (DGGE) and sequencing. Twenty BRONJ subjects classified as stage 0 to 2 were enrolled in this study and patient groups were divided into an antibiotic cohort (n=10) treated with systemic antibiotic and a non-antibiotic cohort (n=10) with no prior antibiotic therapy.

RESULTS—The DGGE fingerprints indicated no significant differences in bacterial diversity of BRONJ tissue samples. Patients on antibiotics had higher relative abundance of phylum *Firmicutes* with bacterial species, *Streptococcus intermedius, Lactobacillus gasseri*, *Mogibacterium timidum* and *Solobacterium moorei* whereas patients without antibiotics had greater amounts of *Parvimonas micra*, and *S. anginosus.* Thirty percent of bacterial populations were uncultured (yet-to be cultured) phylotypes.

CONCLUSION—This study using limited sample size indicated that oral antibiotic therapy may have a limited efficacy on the bacterial population associated with BRONJ lesions.

Keywords

BRONJ; antibiotics; DGGE; microbial diversity

Introduction

Bisphosphonate related osteonecrosis of the jaw (BRONJ) is most frequently defined by current or previous treatment with bisphosphonates (BPs), exposed bone in the maxillofacial region for more than 8 weeks and no history of radiation therapy to the jaws (Ruggiero et al.,

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2009). These drugs are widely used for the prevention and treatment of osteoporosis (Kamel, 2007) and skeletal complications associated with metastatic cancer, breast cancer and multiple myeloma (Coleman, 2008). These cancer patients are immune-compromised and have reduced regenerative capability due to cancer treatment whereas osteoporotic patients have disturbed level of bone remodeling (Ruggiero, 2011). These osteoporotic patients often suffer from other chronic diseases and have increased risk of developing opportunistic infections which may relate to reduce bone mineral density and oral alveolar bone loss, premature teeth loss and increased severity of periodontal diseases (Kyrgidis et al., 2011). A causal relationship between BPs and BRONJ has not been well understood (Dodson, 2009) and current information on incidence and prevalence is inconclusive (Gliklich & Wilson, 2009). Therefore, understanding the underlying pathogenesis and risk are of paramount importance. The general risk factors may be classified as local (e.g., poor oral hygiene, dental extraction, infection), systemic (e.g., cancer, metastasis, chemotherapy, smoking, obesity and malnutrition) and drug-related (e.g., duration of BP therapy, steroid use) (Gebara & Moubayed, 2009, Ruggiero et al., 2009, Wessel et al., 2008).

Common dilemmas arise because discontinuing BP therapy prior to dental procedures does not prevent or improve BRONJ (Krueger et al., 2007, Diel et al., 2007) and poor oral hygiene or periodontal disease may not correlate with risk assessment (Estilo et al., 2008). Patient management is further complicated because recommendations have focused on avoiding dental extractions (Allam et al., 2011, Fleisher et al., 2008) while BRONJ may be triggered by infection(Hoefert & Eufinger, 2011, Kaplan et al., 2009, Kumar et al., 2010, Abraham et al., 2009, Wong et al., 2010). Effective treatment regimens for BRONJ have not been well established (Migliorati et al., 2005). While nonsurgical conservative therapy such as oral rinses, antibiotics, analgesics, and discontinuing BP therapy have been suggested (Van den Wyngaert et al., 2007), patients may not respond to these approaches (Hoff et al., 2008, Williamson, 2010, Otto et al., 2009).

Several possible mechanisms of BRONJ pathogenesis have been suggested which includes ischemia, reduced bone turnover, toxicity to bone, toxicity to soft tissue, microcracks, inflammation and infection (Hoefert et al., 2010, Kumar et al., 2010, Lesclous et al., 2009, Reid, 2009). Oral infection and inflammation are believed to play a critical role in the pathogenesis of BRONJ. According to Reid, (2009), bone necrosis in patients taking BPs transpires as a result of pre-existing dental infection that is odontogenic or periodontal and eventually spreads to the bone culminating into bisphosphonate-related ONJ (BRONJ). Sedghizadeh et al., (2008) have reported that microbial biofilms may play a role in the etiopathogenesis of 65% to 80% of the chronic human infections.

More than 750 species are reported to inhabit the human oral cavity (Jenkinson & Lamont, 2005) and most of the oral microbiota are organized in complex multispecies biofilms attached to the surfaces of teeth and oral soft tissues (Smoot et al., 2005). A biofilm is a complex community of sessile microbes attached to a substrate (Donlan & Costerton, 2002, Kuramitsu et al., 2007, Sedghizadeh et al., 2009). As reports of histological assessments of BRONJ become more numerous, evidence links specific microbial infection to BRONJ and *Actinomyces* representing phylum *Actinobacteria* is a common finding (Kaplan et al., 2009, Kumar et al., 2010, Naik & Russo, 2009). Confirmation of the infectional theory of the condition might result in more rational treatment with antibiotics, with a special reference to the efficient system of application of antibiotics to the hypovascular and hypocellular bone (Kos & Luczak, 2009). The use of oral antimicrobial rinses in combination with oral systemic antibiotic therapy; penicillin, metronidazole, quinolones, clindamycin, doxycycline, erythromycin is indicated for Stages I and II of BRONJ (Ruggiero et al., 2009, Vescovi & Nammour, 2010). Here we report the effect of antibiotic administered for treating infection after the onset of BRONJ. Using 16S rDNA molecular technique we determined

and compared the changes in the bacterial population in soft tissues of BRONJ lesion collected from: an antibiotic cohort treated with antibiotic and non-antibiotic cohort with no prior antibiotic treatment.

Materials and methods

Subjects and specimen collection

A total of 20 patients (16 females and 4 males) undergoing BRONJ treatment at New York University College of Dentistry were recruited for this study. The age range was 49 – 84 years, mean 67.05 years. The study was approved by the Institutional Review Board of New York University for human subjects and subjects agreed to participate with their informed consent. These subjects were patients with breast cancer, renal/rectal cancer, multiple myeloma on intravenous BPs (n=14) and those with osteoporosis on oral BPs (n=6). BRONJ subjects were classified as either stage 0, I or II as defined by Ruggiero et al., (2009) and divided into two cohorts: an antibiotic cohort (n=10) treated with systemic antibiotic therapy and non-antibiotic cohort (n=10) with no prior antibiotic therapy (Table 1). For antibiotic cohort, we selected BRONJ subject population on a range of antibiotics. The subjects treated with systemic antibiotics were administered either one of these; tetracycline, doxycycline, ciprofloxacin or amoxicillin for two weeks (Table 1). Specimens were collected from soft tissues associated with the BRONJ lesion and transported in sterile vials containing Tris-EDTA buffer and stored at −20°C.

DNA extraction from BRONJ tissues

Each specimen was suspended in 500 μl of sterile phosphate-buffered saline (PBS), vortexed for 30 sec and sonicated for 5 and 10 sec respectively. Further, pretreated with Proteinase K (2.5 μg/ml) with overnight incubation at 55°C, and if required, homogenized with sterile disposable pestle and vortexed (Hooper et al., 2007). Bacterial genomic DNA was extracted by modified Epicentre protocol (Epicentre Biotechnologies, Madison, WI) and purified by phenol-chloroform extraction (Li et al., 2007, Pushalkar et al., 2011). Qualitative and quantitative analysis of samples was done by NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). The samples were stored at −20°C till analyzed further. The DNA concentration was adjusted to 10 ng/μl for all experiments.

16S rDNA amplification

16S rDNA genes from bacterial DNA were amplified with universal eubacterial primers, forward primer 8f (5′-AGTTGATCCTGGCTCAG-3′) and reverse primer 1492r (5′-ACCTTGTTACGACTT-3′) (Lane, 1991) followed by nested PCR targeting V4-V 5 hypervariable region with primers prbac 1 (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGA-CTACGTGCCAGCAGCC-3′) with 40-nucleotide GC clamp at 5′ end of bac1 (Sheffield et al., 1989) and prbac2 (5′-GGACTACCAGGGTATCTAATCC-3′) (Rupf et al., 1999). Each PCR reaction mix (50 μl) consisted of 1 μl DNA, 5 U Taq DNA polymerase (Invitogen, Carlsbad, CA), 5 μl 10x PCR buffer, 4 μl dNTP mixture (2.5 mM each) and 50 pmole of each primer. The reactions were performed at 95°C for 5 min, followed by 30 thermal cycles of 95°C (1 min), 52°C (1 min), and 72°C (1 min) with final elongation at 72°C (5 min). These PCR products were template for nested PCR. The nested PCR consisted of preheating at 94°C (3 min) with 35 cycles each at 94°C (30 sec), 63°C (40 sec), and 72°C (1 min) and final extension at 72°C for 7 min.

Denaturing gradient gel electrophoresis (DGGE) assay

PCR products from nested PCR were resolved on 40% to 60% linear DNA denaturing gradient polyacrylamide gel (8.0% w/v) for sequence differentiation. DGGE gel was loaded with 30 μl each of samples alongside standard species-specific DGGE reference markers (Li et al., 2005, Pushalkar et al., 2011) using DCode system (BioRad, Hercules, CA). Electrophoresis was carried out for 16 hr at 58°C and 60V in 1x Tris-acetate-EDTA (TAE) buffer, pH 8.5 and stained in ethidium bromide solution (0.5 μg/ml) for 15 min. Gel images were documented using Alpha Imager 3300 system (Alpha Innotech Corporation, San Leandro, CA).

Construction of dendrogram and cluster analysis

DGGE gels were analyzed with Fingerprinting II Informatix Software (BioRad) and statistically interpreted (Li et al., 2007, Li et al., 2006, Pushalkar et al., 2011). The gels were normalized by standard DGGE markers and the background subtracted using mathematical algorithms. The similarity calculated by Dice coefficient and dendrogram constructed by Ward analysis. The Shannon diversity index was calculated by Mann-Whitney U test. Statistical analysis was performed using SPSS software v. 17.0 (SPSS inc., Chicago, IL).

Cloning and sequencing

PCR products from ten samples were ligated to pCR4-TOPO vector and transformed into *E. coli* DH5α cells using TOPO-TA cloning kit according to the manufacturer's instructions (Invitrogen). From each sample 48 to 96 clones were picked. The partial sequencing of 768 clones was performed with average Phred 20 read length of 600–700 bp (Beckman Coulter Genomics, Beverly, MA) using BigDye Terminator v3.1 and M13 forward sequencing primer. Sequences were analyzed on ABI PRISM 3730*xl* coupled with Agencourt Clean SEQ dye terminator removal for generation of long high quality Sanger sequencing reads. The sequences were trimmed for elimination of vector sequences and adjusted for quality values.

Phylogenetic analysis

All the trimmed sequences were verified manually for vector sequences using EMBOSS pairwise alignment algorithms (Larkin et al., 2007). The resulting 748 trimmed sequences were aligned using Greengenes Nast aligner (DeSantis et al., 2006a) and chimeric sequences detected by greengenes - Bellerophon chimera check program (DeSantis et al., 2006b). The sequences ≥300 bases with similarity score S_ab score ≥0.8 (Pei et al., 2004, Vickerman et al., 2007) were assembled for phylogenetic affiliations and analyzed by Sequence Match program of Ribosomal Database Project (RDP, update 10) (Cole et al., 2009). The sequences with <0.8 S_ab score were considered unknown sequences. The resulting sequences were binned into two BRONJ libraries, with and without antibiotics and analyzed. In analysis representation, the term '*species*' would refer to *named and unnamed cultured bacterial species* and the term '*phylotypes*' would refer to *uncultured species*.

Species diversity and richness estimators

Clone library size was evaluated by an online program (http://www.aslo.org/lomethods/free/2004/0114a.html), and the output data were treated by the method described by (Kemp & Aller, 2004). Estimation and comparison of species richness in BRONJ tissue specimens with and without antibiotics were performed using EstimateS 8.2 (Colwell, 2009). Rarefaction analysis, diversity estimation and abundance model were generated by PAST version 2.01 (Hammer et al., 2001). The Shannon-Weaver index of diversity (*H*′) in the two sample groups was compared by the Mann-Whitney U test.

Good's coverage was calculated as [1 − (n/N)], where n is the number of species constituted by singleton and N is the total number of clones (Good, 1953).

Results

Analysis of DGGE profile from clinical samples

The overall diversity in bacterial community structures was assessed in 20 BRONJ tissue samples, 10 each with and without antibiotics by PCR-DGGE fingerprints. The gels were normalized using species-specific markers. The DGGE profile of 16S rDNA from BRONJ samples in both the groups showed approximately 18 to 33 bands each indicative of one or more species (Figure 1). Several high and low intensity bands were identical in both the groups but few of the bands were unique in each group. However, there were no significant differences in the total number of bands, mean 25.4±5.6 SD in group with antibiotics as compared to mean 23.4±4.5 SD in those without antibiotics.

The dendrogram of cluster analysis with Dice coefficient showed clusters comprising mixed population of samples with and without antibiotics (Figure 2). The % similarity between two given samples approximately ranged from 75% to 92%. The Shannon-Weaver diversity index (H') of groups, with and without antibiotics was mean 3.20±0.212 SD and mean 3.12±0.172 SD respectively. The Mann-Whitney U test was used to compare the Shannon diversity index of two sample groups (Figure 3). The *p*-value, p=0.426 was two-tailed and no significant differences were displayed in the species diversity among the BRONJ cohorts with and without antibiotics.

Bacterial diversity

Based on the results of the DGGE profile, we selected 10 BRONJ tissue samples, 5 each from with and without antibiotics patients for cloning and sequencing. The resulting 748 sequences from the total 768 bacterial clones were analyzed. A total of 70 (9.1%) sequences were eliminated based on sequence length cutoff limit <300 bases (5.6%) and chimeric (3.5%) sequences. About 678 (88.3%) sequences with sequence length 300–900 bases were analyzed for phylogenetic affiliations. The sequences (26%) with <0.8 S_ab score were assigned as sequences belonging to unknown species. Altogether 478 (62%) sequences were detected that belonged to 244 (32%) cultured species and 234 (30%) uncultured phylotypes. The number of observed species were 84 (69.0% Gram+, 31.0% Gram-) and 68 (75.0% Gram+, 25.0% Gram-) in BRONJ tissues with and without antibiotics respectively.

Bacterial species/phylotypes in the two groups were distributed into 9 phyla and 44 genera. The phyla represented were *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *Synergistetes* and *TM7* (Figure 4a). The phylum *TM7* was uniquely present while other 2 phyla, *Chloroflexi* and *Cyanobacteria*, were absent in samples without antibiotics. The phylum *Firmicutes* was found elevated in subjects on antibiotics. The predominant phylum *Firmicutes* was represented by 22 genera (Figure 4b) with 1 unclassified genus under its cluster. *Parvimonas* and *Streptococcus* represented a larger portion of the bacterial community in both BRONJ groups. The genus, *Eubacterium* (5.46%) and *Pseudoramibacte*r (3.83%) was found to be more prevalent in samples without antibiotics whereas *Lactobacillus* (8.14%), *Peptostreptococcus* (6.44%), *Mogibacterium* (5.08%), *Solobacterium* (4.41%) and *Dialister* (3.73%) in samples with antibiotics. *Veillonella* (2.03%) was detected only in antibiotic-administered group and *Gemella* (2.19%) only in group without antibiotics.

The genera from other phyla (Figure 4c), to name a few, exclusive to antibiotic group were *Olsenella* (0.68%) and *Pyramidobacter* (0.68%) while *Paludibacter* (1.64%) and *TM7* (2.73%) in group without antibiotic. The colonization of bacteria characterizing *Atopobium*

(7.1%), *Fusobacterium* (3.83%), *Actinomyces* (3.28%) and *Bifidobacterium* (2.19%) were higher in the BRONJ group without antibiotics. Some genera usually found in environmental samples were found in either of the group *viz. Desulfobulbus*, *Streptophyta* and *Thermomonas*.

At species-level, *Streptococcus intermedius*, *Lactobacillus gasseri*, *Mogibacterium timidum* and *Solobacterium moorei* did show largely in patients treated with antibiotics whereas *Parvimonas micra*, *Streptococcus anginosus* and *Atopobium rimae* in patients not treated with antibiotics. Some unnamed cultured species and uncultured phylotypes also represented the two groups (Table 2).

Species richness and diversity

The richness estimators, Chao1 and ACE, diversity indices, evenness and rank-abundance were determined for independent and combined groups (Table 3). Chao 1 and ACE were elevated in library with antibiotics. Also, the S_{Chao1} and S_{ACE} of the two sample groups reached an asymptote (Figure 5) suggesting that the libraries of both BRONJ tissues with and without antibiotics were large enough to yield optimal stable phylotype richness estimate. Shannon-Weaver and Simpson diversity indices revealed no significant differences among the two BRONJ libraries with and without antibiotics which was also evident from the Mann-Whitney U test of Shannon diversity ($P>0.05$). The evenness was higher in BRONJ group without antibiotics indicating relative species abundance. The two sample groups exhibited similar profile of abundance model with long tail representing rare species as shown in Figure 6. Good's coverage was 89% and 84% of BRONJ tissues with and without antibiotics, respectively suggesting 11 and 16 additional species/phylotypes would be recognized if about 100 more clones would be screened.

Discussion

There are many hypotheses for BRONJ pathogenesis (Allen & Burr, 2009); the manifestation of necrotic bone resulting from bisphosphonate-induced remodeling suppression that allow accumulation of nonviable osteocytes, direct cytotoxic effect of bisphosphonates on osteocytes, bisphosphonates antiangiogenic effects and role of oral bacteria. Despite infection being present in BRONJ patients, there is no clear data as to whether infection plays role in the pathophysiology (Allen & Burr, 2009). The present studies using 16S rRNA culture-independent molecular methods determined the bacterial profile of BRONJ and demonstrated that there were no significant differences in bacterial diversity (culturable and non-culturable) of BRONJ tissues from patients treated with and without antibiotics. To our knowledge, this is the first report to address the effect of antibiotics on bacterial colonization (*in vivo* biofilms) associated with BRONJ. Our results indicated that species/phylotypes affiliated to genera *Parvimonas* and *Peptostreptococcus* were more prevalent in antibiotic-administered group whereas *Fusobacterium*, *Atopobium* and *Streptococcus* predominantly existed in group without antibiotics indicating changes in the composition (relative distribution) of biofilm in two groups. *S. intermedius* and *M. timidum*, which are associated with periodontitis (Rocas & Siqueira, 2008, Rochford, 1980), were elevated in patients on antibiotics while *S. anginosus* and *P. micra* levels were higher in those without antibiotics. *S. anginosus* has been shown to be strongly associated with abscess formation (Gray, 2005) and *P. micra* is frequently related to polymicrobial infections in humans (Ota-Tsuzuki & Mayer, 2010). Each group had its unique species, for instance, *S. moorei*, a species that has been identified as a contributor to halitosis (Kazor et al., 2003), was solely confined to necrotic tissues with antibiotics.

Lactobacillus was found in both the groups but more prevalent in subjects on antibiotics. *Actinomyces* and *Veillonella* species were observed in the subjects treated with antibiotics

unlike in those without antibiotics. However we did not observe high abundance of *Actinomyces* in BRONJ samples. In previous studies using histomorphometric analysis of oral mucosa and jawbones have shown that *Actinomyces* is associated with BRONJ (Kaplan et al., 2009). Naik & Russo, (2009) reported presence of the actinomyces-like organisms from affected bone in most of the cases studied, however, most of these assumptions are based on microscopic observations. In our study we used molecular 16S rRNA techniques to identify bacterial species/phylotypes that represented both culturables and non-culturables and did not observe high abundance of *Actinomyces*. However as compared to non-antibiotics group we observed elevated number of *Actinomyces* in our antibiotic cohort and this may be due to its fastidious nature and not due to its association with BRONJ (Kaplan, 2010). A high level of correlation has been observed between *Veillonella* and the three species, *Lactobacilli*, *mutans streptococci* and *Actinomyces* spp. which ferment carbohydrates to lactate and as a consequence, *Veillonella* concentrations increased due to lactate consumption (Arif et al., 2008). Our results suggested that biofilms in each cohort had a distinct combination of species/phylotypes, segregating and repopulating leading to compositional changes but maintaining functional similarity of acid production that may lead to demineralization of tooth tissues, as observed in dental caries biofilms (Arif et al., 2008, Diaz et al., 2006). It was also observed that some cultured and uncultured bacterial species/phylotypes present in one group were either reduced in numbers or lost in other group as evident from the DGGE fingerprints. The existence of uncultured microflora alongwith cultured bacterial flora indicated their interdependency on nutritional and signaling interactions or community networking (Wade, 2002). Most of the mucosal infections involves biofilms developed on natural tissues (skin, mucosa, endothelial epithelia, teeth, bones) (Lazar & Chifiriuc, 2010). The histopathologic examination have indicated that edentulous jaw contains regions of necrotic bone and microbial biofilm formation even after one year of tooth extraction and mucosal healing which may contribute to BRONJ formation (Kassolis et al., 2010). Similar observations of persist lesion for a variable period of time were reported in the case of chronic suppurative osteomyelitis (Ertas et al., 2004).

There are numerous factors that prevent collection of tissue from homogenous patient populations such as the low incidence of BRONJ, cancer biology and varying chemotherapy. Our study using limited sample size indicated that although there were no significant differences in the total number of bacteria in the two groups as distinguished by DGGE profile and phylogenetic studies, there were differences in species diversity between antibiotic and non antibiotic groups. This finding suggests an alteration in the microbial population, but not enough to reduce or eliminate infection. This may be attributed to either compromised vascular supply of necrotic tissue (Allen & Burr, 2009) or biofilm formation. Indeed, doxycycline is more effective when applied locally compared to systemic administration (Goldie, 2009). Histopathological and scanning electron microscopic studies have indicated that bacterial biofilm in BRONJ is more diverse as compared to osteomyelitis of the jaw and had co-aggregation between *Actinomyces* and coccal forms (Kumar et al., 2010, Sedghizadeh et al., 2008, Sedghizadeh et al., 2009). Moreover, BP effect on bone remodeling due to osteoblast and osteoclast differentiation and deficient antimicrobial response may reduce function and number of cells from the phagocytic lineage, monocytes and macrophages leading to development of local infection (Pazianas, 2011) and also affecting deeper structure of bone (Sedghizadeh et al., 2008). It is known that several species of bacteria could cause alveolar bone destruction mediated by their products (e.g. lipopolysaccharides) (Bertoldo et al., 2007, Nair et al., 1996, Pazianas, 2011). The plausible basis for BRONJ development is also the increased bacterial adhesion to the bisphosphonate covered bone (Allen & Burr, 2009, Kos, 2011, Kos & Luczak, 2009).

The finding that there were no significant differences in bacterial diversity (culturable and non-culturable) of BRONJ tissues from patients treated with and without antibiotics supports surgical treatment options (Carlson & Basile, 2009) and may explain poor success rates when using only antimicrobial therapy (Bamias et al., 2005). This was further supported by case report by Ertas et al., (2004) which highlighted that in chronic suppurative osteomyelitis the infection is localized but is persistent because the infected necrotic area remained as a sequestrum and this chronic lesion persist for a variable period up to many years with intermittent exacerbation and ultimately required surgical intervention. In another case report of chronic osteomyelitis with serious MRSA infection of the mandible demonstrated purulent discharge. The patient failed to recover despite prolonged postoperative treatment and the administration of several antibiotics. Further the treatment protocol involved a multimodal approach with parenteral and local antibiotics treatment and necessary surgical interventions (Tuzuner-Oncul et al., 2009). Conservative management including the judicious use of antibiotics may be ineffective with progressive infection and bone destruction (Hoff et al., 2008, Williamson, 2010) may needing alternative preoperative and surgical strategies in BRONJ (Hoefert & Eufinger, 2011, Montefusco et al., 2008, Pautke et al., 2011). Moreover, microbial cultures have not been helpful in directing therapy because specific pathogens have not been identified (Fantasia, 2009). Our study using 16S rDNA molecular technique reflects that the use of systemic antibiotics failed to restrict the bacterial colonization without effective healing of the lesion after the onset of BRONJ. However, this finding requires further evaluation with larger subject population.

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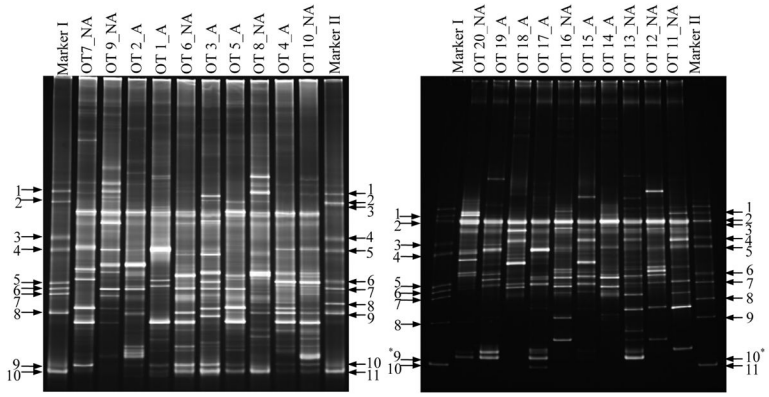

Figure 1.

PCR-DGGE profile of bacterial 16S rDNA gene fragments of BRONJ tissue samples with and without antibiotics. Marker I & II: DGGE reference markers correspond to 16S rRNA gene fragments from quoted specific bacterial species [Marker I: 1. *Fusobacterium nucleatum* subsp. *vincenti* (ATCC 49256); 2. *Fusobacterium nucleatum* subsp. *nucleatum* (ATCC 25586); 3. *Streptococcus sanguinis* (ATCC 10556); 4. *Streptococcus oralis* (ATCC 35037); 5. *Streptococcus salivarus* (ATCC 7073); 6. *Streptococcus mutans* (UA 159); 7. *Lactobacillus paracasei* (ATCC 25598); 8. *Porphyromonas gingivalis* (ATCC 33277); 9. *Actinomyces odontolyticus* (ATCC 17929); 10. *Actinomyces naeslundii* (ATCC 12104), Marker II: *F. nucleatum* subsp. *vincenti* (ATCC 49256); 2. *F. nucleatum* subsp. *nucleatum* (ATCC 25586); 3. *Bacteroides forsythus* (ATCC 43037) (T.f.); 4. *S. sanguinis* (ATCC 10556); 5. *S. oralis* (ATCC 35037); 6. *Veillonella parvula* (ATCC 17745); 7. *Prevotella intermedia* (ATCC 25611); 8. *Aggregatibacter actinomycemcomitans* (ATCC 43717); 9. *P. gingivalis* (ATCC 33277); 10. *A. odontolyticus* (ATCC 17929); 11. *A. naeslundii* (ATCC 12104)]

A - BRONJ tissue samples with antibiotics; NA - BRONJ tissue samples without antibiotics

* - light bands not visible in photograph

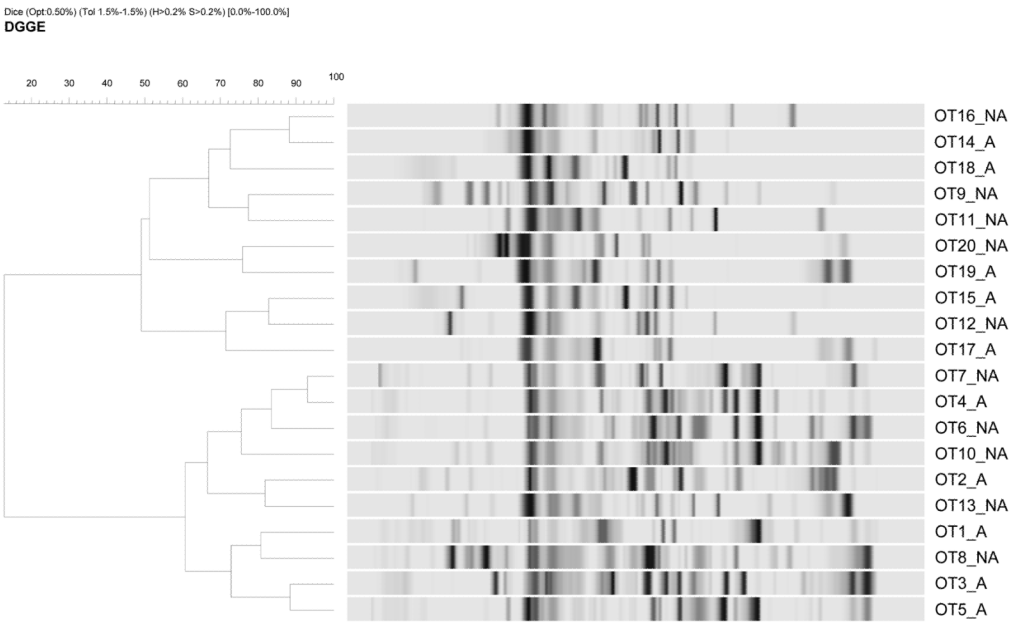

Figure 2.

Dendrogram of DGGE profiles of BRONJ tissue samples with and without antibiotics based on Dice coefficient depicting microbial diversity

A - BRONJ tissue samples with antibiotics; NA - BRONJ tissue samples without antibiotics

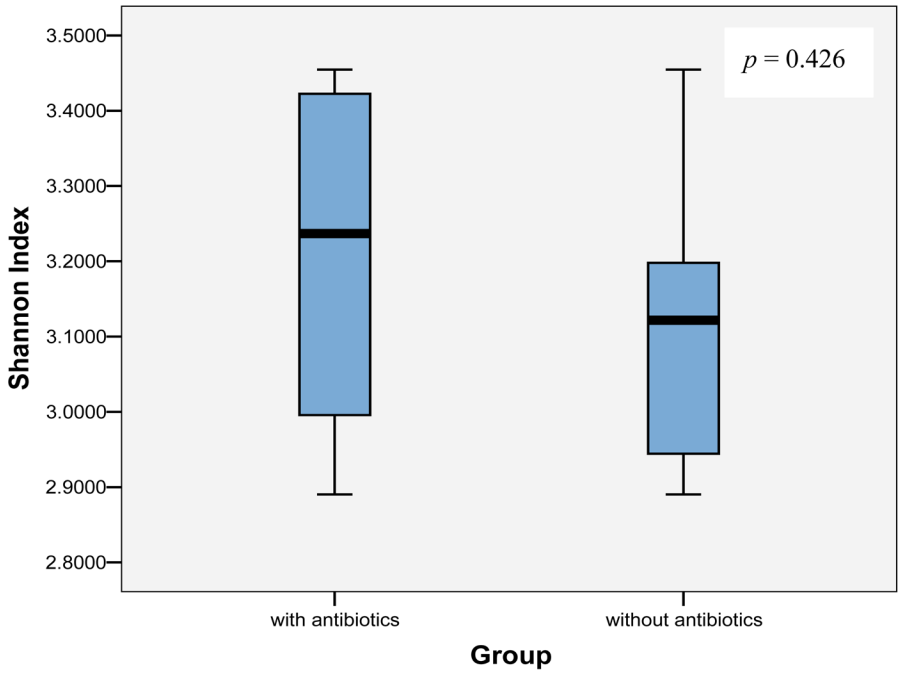

Figure 3.
Shannon index depicting bacterial diversity of BRONJ samples as in subjects with and without antibiotics

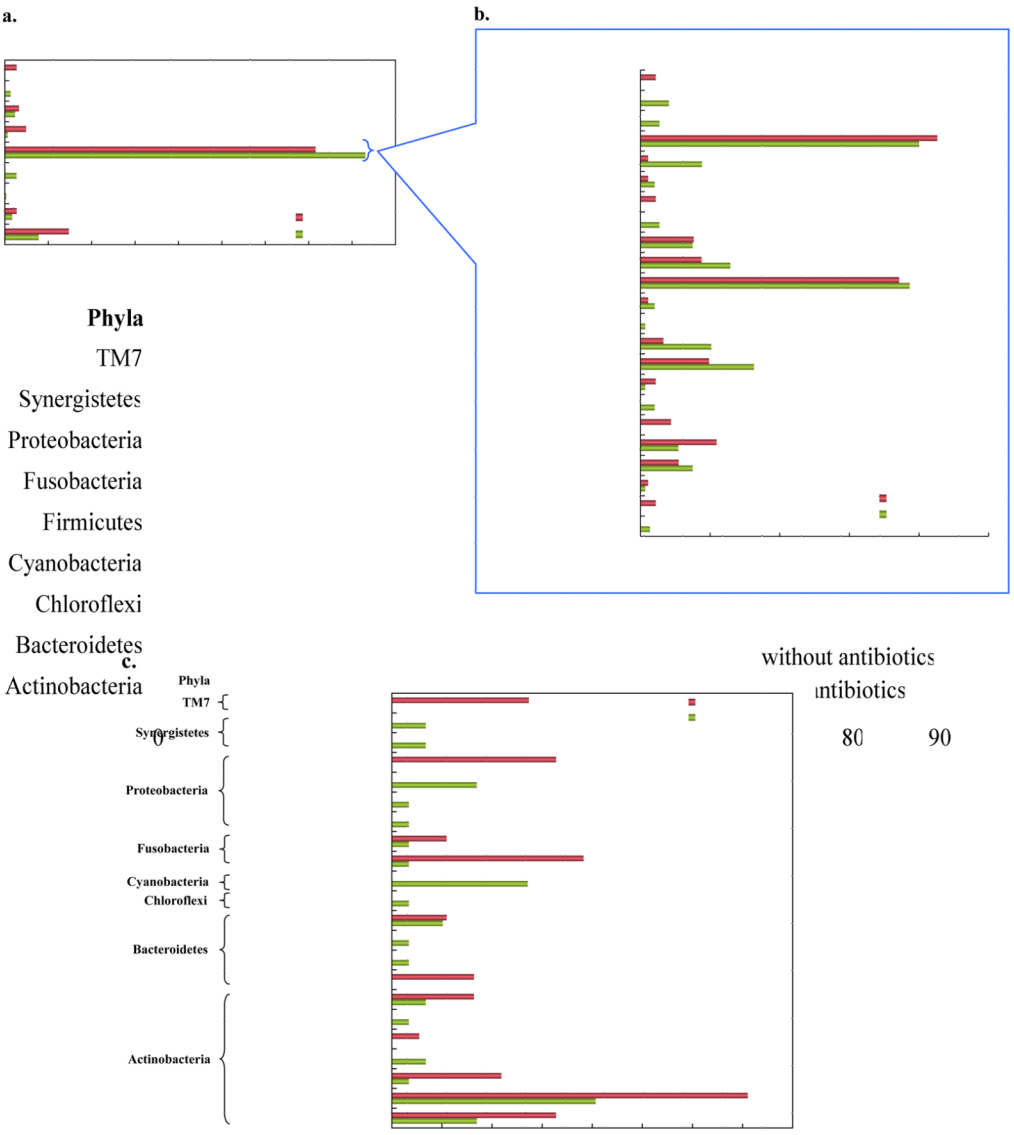

Figure 4.
Relative distribution of (a) phyla; (b) genera in most prevalent phylum Firmicutes; (c) genera in less abundant phyla in BRONJ tissue samples with and without antibiotics

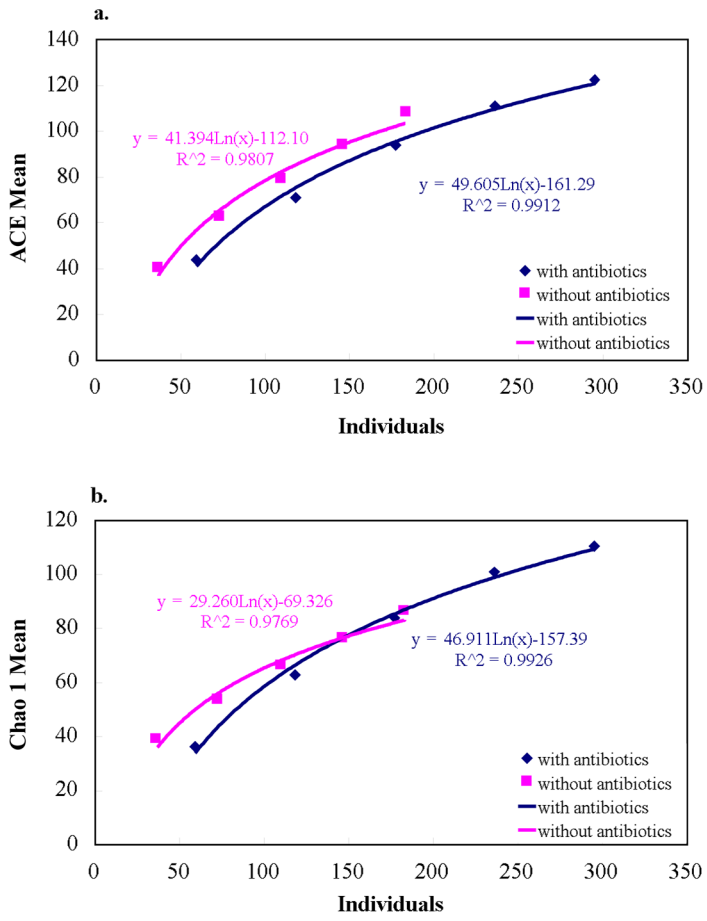

Figure 5.
Comparison of species richness between BRONJ tissue samples with and without antibiotics using estimators (a) ACE; (b) Chao1

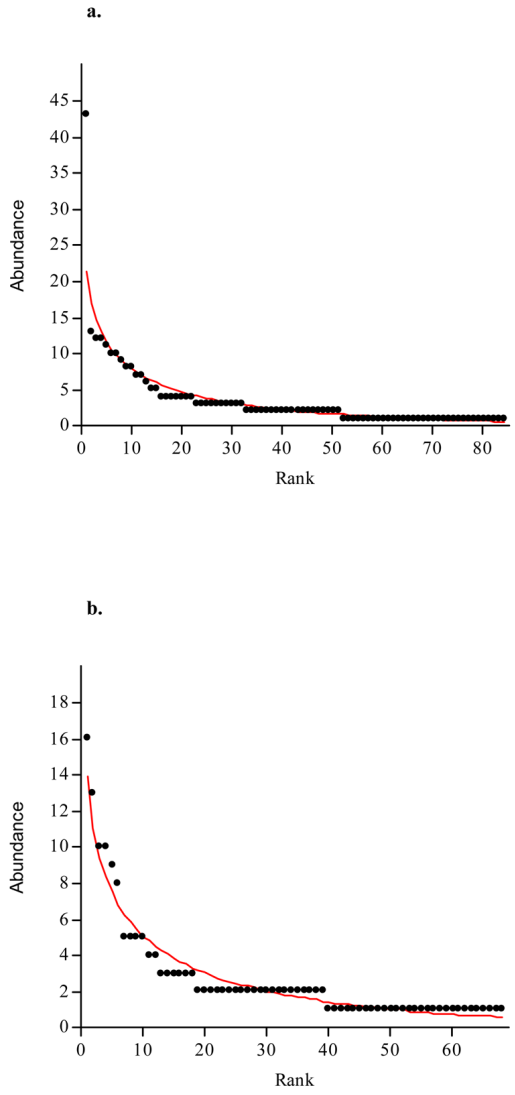

Figure 6.
Rank-abundance curves for BRONJ tissue samples (a) with antibiotics; (b) without antibiotics

Table 1

Data of patients with BRONJ

Sample name	Age	Sex	Staging of BRONJ	Type of antibiotic	History	BP	Location
OT 1	69	M	I	Tetracycline	Renal cancer	IV	Mx
OT 2	72	F	I	Tetracycline	Multiple myeloma	IV	Mn
OT 3	81	F	II	Tetracycline	Breast cancer	IV	Mn
OT 4	49	M	I	Ciprofloxacin	Breast cancer	IV	Mx
OT 5	84	F	II	Amoxicillin (discontinued 1 week prior to Tx)	Breast cancer	IV	Non-restorable tooth #18
OT 6	56	F	I		Breast cancer	IV	Mn
OT 7	79	F	I		Osteoporosis	O	Mn
OT 8	72	F	I		Osteoporosis	O	Mn
OT 9	73	F	II		Osteoporosis	O	Mx
OT 10	57	M	I		Rectal cancer	IV	Mx
OT 11	62	F	0		Breast cancer	IV	Mn
OT 12	56	F	0		Breast cancer	IV	Non-restorable tooth #18
OT 13	72	F	II		Osteoporosis	O	Mx
OT 14	53	M	I	Tetracycline	Multiple myeloma	IV	Mn
OT 15	76	F	II	Tetracycline	Colon cancer	IV	Mn
OT 16	71	F	II		Osteoporosis	O	Palatal tori
OT 17	65	F	II	Doxycycline	Multiple myeloma	IV	Mn
OT 18	57	F	I	Doxycycline	Breast cancer	IV	Mn
OT 19	56	F	I	Doxycycline	Breast cancer	IV	Mn
OT 20	72	F	II		Osteoporosis	O	Mn

F-Female; M- Male; IV-Intravenous: O-Oral; Mn-Mandible; Mx-Maxilla

Regimen/Formulation/Dosages: Tetracycline 250 mg QID × 2 weeks; Doxycycline 100 mg BID × 2 weeks; Ciprofloxacin 100 mg × 2 weeks

Table 2

Bacterial species/phylotypes exclusive as well as common in ONJ tissues of patients with and without prophylactic antibiotics

Bacterial species/phylotypes	
with antibiotics	
Abiotrophia defectiva	*Oribacterium sp. oral taxon* 372**
Actinomyces cardiffensis	*Propionibacterium acnes*
Bacteroidales oral clone MCE7_20	*Pyramidobacter piscolens*
Chloroflexi genomosp. P1	*Shuttleworthia satelles*
Desulfobulbus sp. oral taxon 041***	*Solobacterium moorei*
Dialister pneumosintes	*Streptococcus constellatus subsp. constellatus*
Eubacterium sp. oral clone BP1-41	*Streptococcus genomosp*. C3
Firmicutes oral clone AO068**	*Streptococcus gordonii*
Granulicatella adiacens	*Streptococcus milleri*
Johnsonella sp. oral taxon 166***	*Streptococcus pneumoniae*
Lachnospiraceae oral clone MCE9_173***	*Streptococcus sp. oral taxon* 071**
Lactobacillus fermentum	*Veillonella parvula*
Porphyromonas endodontalis	*Veillonella sp. oral clone* VeillA8
Lactobacillus sp.	*Veillonellaceae bacterium oral taxon* 129**
Lactobacillus sp. NEQAS6172	*Veillonellaceae bacterium oral taxon* 132***
Olsenella uli (T)	*Veillonellaceae bacterium oral taxon* 150***
without antibiotics	
Actinomyces dentalis	*Leptotrichia hofstadii* F0254
*Actinomyces sp. oral taxon 525**	*Parascardovia denticolens*
Bacteroidetes bacterium oral taxon 274**	*Peptostreptococcus sp. oral clone* FG014
Eubacterium sp. oral clone EH006	*Streptococcus sp*. 99
Eubacterium sp. oral taxon G32	*Thermomonas hydrothermalis* (T)
Fusobacterium nucleatum	*Thermomonas sp*. FLA17
Gemella morbillorum	*TM7 phylum sp. oral taxon* 346***
Lachnospiraceae bacterium oral taxon 086***	*TM7 phylum sp. oral taxon* 349***
Lactobacillus rhamnosus	
common in with and without antibiotics	
Atopobium rimae	*Parvimonas micra*
Bifidobacterium dentium	*Peptostreptococcus stomatis*
Bulleidia moorei	*Prevotella sp. oral taxon* G57
Dialister invisus	*Pseudoramibacter alactolyticus*
Eubacterium infirmum	*Scardovia sp. oral taxon* 195*
Eubacterium sp. oral clone BU061	*Streptococcus anginosus*
Eubacterium sp. oral strain A35MT	*Streptococcus intermedius*

Bacterial species/phylotypes	
Lactobacillus gasseri	*Streptococcus mitis*
Lactobacillus salivarius	*Streptococcus mutans*
Mogibacterium timidum	

* cultured;

** unnamed cultured phylotype;

*** currently uncultured phylotype

Table 3

Species richness and diversity indices estimators of microflora in ONJ tissues with and without antibiotics

	with antibiotics (n=5)	without antibiotics (n=5)	combined (n=10)
No. of clones	295	183	478
Species/phylotypes (S)	84	68	120
Singletons	33	29	45
Doubletons	19	21	26
Chao1 estimator of species richness	110.40	86.45	156.67
Chao1 standard deviation	12.11	9.05	14.38
ACE estimator of species richness	122.59	108.00	165.31
Shannon’s index for diversity (H)	3.88	3.83	4.17
Simpson’s index for diversity (1-D)	0.96	0.97	0.97
Evenness (e^H/S)	0.58	0.68	0.54
Good’s estimator of coverage (%)	88.80	84.20	90.60

n – number of samples; combined – with and without antibiotics