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1 **ORIGINAL ARTICLE**
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4 # Bacterial diversity detected in osteoradionecrosis

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22 **ABSTRACT**

23 Direct microscopy, culture based studies and DNA-DNA hybridization have previously
24 demonstrated an association between microorganisms and osteoradionecrosis. The
25 purpose of our study was to use culture independent molecular techniques to detect
26 bacteria in necrotic bone lesions of the mandible after radiotherapy. Bacterial DNA was
27 extracted from six deep medullary specimens from resected mandibles, including one
28 sample of a relapse. 16S rRNA genes were PCR amplified, cloned, transformed into
29 *Escherichia coli* and sequenced to determine species identity and closest relatives. From
30 the analysis of 438 clones, 59 predominant species were detected, of which 27% have not
31 been cultivated. The predominant species detected from radionecrotic mandibles were
32 *Campylobacter gracilis, Streptococcus intermedius*, *Peptostreptocooccus* sp. oral clone
33 FG014, Uncultured bacterium clone RL178, *Fusobacterium nucleatum,* and *Prevotella*
34 spp.. The analysis demonstrated intersubject variability of the bacteria present in
35 osteoradionecrosis. In contrast to the diverse bacterial profile detected in primary
36 infection, only a few members of the oral indigenous flora were identified from a case of
37 relapse. Detection of all members of the complex bacterial flora associated with
38 osteoradionecrosis seems to be necessary to better understand the pathogenesis and to
39 improve the therapeutic approach of the infection.

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44 **INTRODUCTION**

45 Osteoradionecrosis (ORN) is a complex late complication and calculated risk to
46 conventional radiation therapy used in treatment of cancer in the craniofacial skeleton.
47 Adverse effects of radiation therapy affect normal tissue cells and the vascular system of
48 bone, cartilage and soft tissue. Although, the complexity of the disease still leaves the
49 definition, the etiology, and the pathogenesis of ORN unclear. (Hansen et al. 2005, Nason
50 et al. 2007) ORN was earlier attributed to secondary infection in the traumatized
51 irradiated tissue, with following non-healing wounds and exposed bone (Meyer 1970,
52 Rankow 1971). Marx stated in 1983 that infection associated with ORN was only
53 superficial and secondary, and that the microorganisms found in resections would be
54 surface contaminants. The statement was based on a study that failed to describe
55 microorganisms in the medullary parts of the resections (6). Støre et al. demonstrated
56 presence of a diverse microbiota of the medullar parts of mandible both visualized by
57 transmission electron microscopy (TEM) (7) and detected by DNA-DNA hybridization in
58 a checkerboard assay (8). Specimens were obtained with sterile trepan burrs collecting
59 bicortical cylinders from areas covered by mucoperiost. A diverse microbiota of bacteria
60 and yeast was exposed. The detection of anaerobes indicates that infection might play an
61 important role in the pathogenesis of ORN (7, 8). Hansen et al. (2006, 2006, 2006, 2007)
62 has demonstrated an association between *Actinomyces* and infected osteoradionecrosis
63 (IORN) based on analysis of irradiated bone biopsies from a high number of patients with
64 post operative complications. Recently, Nason and Chole (2007) have described
65 formation of biofilm in association with ORN of the temporal bone after external beam
66 radiation. Phylogenetic analysis of complex bacterial communities in biofilms relies on
67 detection of housekeeping genes in bacteria, like 16S rRNA, and include both the
68 cultivable and not-yet cultivable segment of the bacterial flora. Molecular techniques

69 have determined the breadth of microbial diversity of the whole gastrointestinal tract in
70 health and disease, and it is discovered that as much as 50% of the oral- (Aas et al. 2005)
71 and 80% of the intestinal (Eckburg et al. 2005) indigenous bacterial flora consist of not
72 cultivated phylotypes. Detection of all members of the complex bacterial communities is
73 necessary to better understand the role of infection in the pathogenesis of ORN. The aim
74 of this study was to use culture independent molecular techniques to detect bacteria in
75 necrotic bone lesions of the mandible after radiotherapy.

76

77 **MATERIALS AND METHODS**

78 **Subjects**

79 Seven specimens from six consecutive cases of mandibular ORN and one specimen of a subject
80 with relapse were included in the study. All subjects recruited for the study required a full
81 segmental resection larger than 5 cm during the course of treatment. The participants received a
82 conservative treatment regime of local wound care and continuous treatment with tetracycline
83 (doxycycline 100 mg daily). In cases of clinical exacerbation this dose was doubled, or a limited
84 supplementary regime of clindamycin, 150 mg three times daily, was prescribed.

85

86 **Bone samples**

87 All resections were made from the mandibular body region. Deep medullary bone specimens
88 were obtained using sterile trepan burrs (3 mm wide), collecting bicortical cylinders from areas
89 previously covered by mucoperiost, and at a minimum distance of 3 cm from any surface exposed
90 by oro-cutaneous fistulas. The cortical segments were removed from the specimens and the
91 marrow part was placed in a dental transport medium (Anaerobe Systems, Morgan Hill, CA,
92 USA) for further analysis.

93

94

95 **DNA extraction**

96 The marrow part of small bone cylinders was grinded on liquid nitrogen to fine powder in a
97 sterile mortar in a laminar airflow cabinet. The powder was suspended in 50 mM Tris HCl and
98 stored at -20°C. DNA was extracted from the bone tissue samples using the ChargeSwitch
99 Forensic DNA Purification Kit (Invitrogen, San Diego, CA) according to the protocol of the
100 manufacturer.

101

102 **Amplification of 16S rRNA genes**

103 The 16S rRNA genes were amplified under standardized conditions using a universal forward
104 primer (5´-GAG AGT TTG ATY MTG GCT CAG-3´) and a universal reverse primer (5´-GAA
105 GGA GGT GWT CCA RCC GCA -3´) (Paster 2001). PCR was performed with the GeneAmp
106 PCR systems 9700 (ABI, Foster City, CA). One microliters of DNA template were added to a
107 reaction mixture (final volume, 50 µl) containing 20 pmol of each primer, 40 nmol of
108 deoxytriphosphates, and 1 U of Platinum *Taq* polymerase (Invitrogen). In a hot-start protocol, the
109 samples were preheated at 95°C for 4 min, followed by amplification under the following
110 conditions: 95°C for 45 s, 60°C for 45 s, and 72°C for 1.5 min, with an additional 15 s for each
111 cycle. A total of 30 cycles were performed, which was followed by a final elongation step at
112 72°C for 15 min. The results of the PCR amplification were examined by electrophoresis in a 1%
113 agarose gel. DNA was stained with ethidium bromide and visualized under short-wavelenght UV
114 light.

115

116 **Cloning procedures**

117 Cloning of PCR-amplified DNA was performed with the TOPO TA cloning kit (Invitrogen)
118 according to instructions of the manufacturer. Briefly, transformation was done with competent
119 *Escherichia coli* TOP10 cells. The transformed cells were plated onto Luria-Bertani agar plates
120 supplemented with kanamycin (50 µg/ml), and the plates were incubated overnight at 37°C.
121 Colonies were transferred to 70 µl of 10 mM TrisHCl. Correct sizes of the inserts were

122 determined in a PCR with an M13 forward primer and an M13 reverse primer (Invitrogen). Prior
123 to sequencing of the fragments, the PCR-amplified 16S rRNA fragments were purified and
124 concentrated with use of the QIAquick PCR purification kit (Qiagen, Valencia, CA).
125
126 **16S rRNA gene sequencing**
127 Purified DNA from the PCR was sequenced with an ABI Prism cycle sequencing kit (BigDye
128 Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase FS, GeneAmp PCR systems
129 2700 and 9700; ABI). The protocol and primers used for sequencing have been described
130 previously (Paster 2001). The sequencing reactions were run on an ABI 3730 DNA sequencer
131 (ABI).
132
133 **16S rRNA gene sequencing and data analysis of unrecognized inserts**
134 The number of sequenced clones per sample ranged from 45 to 84, with an average of 62.6
135 clones. A sequence of approximately 500 bases was obtained first to determine identity or
136 approximate phylogenetic position. For identification of closest relatives, the sequences of the
137 inserts were compared to the 16S rRNA gene sequences of over 100,000 sequences in the
138 Ribosomal Database Project (Cole et al 2005), more than 188,000 sequences in the Greengenes
139 databases (http://greengenes.lbl.gov/) and NCBI GenBank databases
140 (http://www.ncbi.nlm.nih.gov/). The similarity matrices were corrected for multiple base changes
141 at single positions by the method of Jukes and Cantor (1969). Similarity matrices were
142 constructed from the aligned sequences by using only those sequence positions for which data
143 were available for 90% of the strains tested. Phylogenetic trees were constructed by the neighbor-
144 joining method of Saitou and Nei (1987). TREECON, a software package for the Microsoft
145 Windows environment, was used for the construction and drawing of evolutionary trees (Van de
146 Peer 1994). We are aware of the potential creation of 16S rRNA chimera molecules assembled
147 during the PCR (Liesack 1991). Chimeric sequences were identified by using the Chimera Check
148 program in the Ribosomal Database Project, by treeing analysis, or by base signature analysis.

149 Species identification of chimeras was obtained, but the sequences were not examined for
150 phylogenetic analysis. Published nucleotide sequences are available for electronic retrieval in the
151 EMBL (http://www.ebi.ac.uk/embl/), GenBank (http://www.ncbi.nlm.nih.gov/Genbank/), and
152 DDBJ (http://www.ddbj.nig.ac.jp/) nucleotide sequence databases under accession numbers
153 included in Figure 1.
154

155 **RESULTS AND DISCUSSION**

156 Scanning electron microscopy (Støre 2005) demonstrated presence of bacteria in marrow
157 spaces of the mandible and the following study based on DNA-DNA hybridization (Støre
158 2005) from our group detected the cultivable bacterial flora present in ORN specimens.
159 In this study, eight specimens from six subjects with ORN were selected for further
160 analysis with culture independent molecular techniques to detect both cultivated and not
161 cultivated bacteria. From a total of 438 clones, a diverse bacterial flora of 59 taxa was
162 detected from eight specimens of ORN. Six phyla were represented in the diverse
163 bacterial flora, including *Firmicutes, Actinobacteria*, *Proteobacteria*, *Fusobacteria*,
164 *Spirochaetes* and *Bacteroides*, of which 27% of the bacteria present have not been
165 cultivated.

166 The introduction of molecular techniques and the opportunity of using housekeeping
167 genes, like 16S rRNA, to detect and identify microbes has manifested the human
168 microbiota as a biofilm of complex microbial communities. In the last decade, culture
169 independent techniques have underlined the need to include all members of the bacterial
170 flora to understand the role of bacteria in health and disease. Despite the known
171 challenges related to DNA extraction from bone tissue, Fenollar et al. (2006)
172 demonstrated bacterial diversity, including not cultivated phylotypes, from an impressing
173 large set of 525 samples of infected bones and joints. The bone and joint samples were

174 collected from the whole body (Fenollar et al. 2006). Their results confirmed findings of
175 previous studies (Støre et al. 2005, Lewis et al. 1978) suggesting that anaerobes are
176 underestimated and may play a central role in polymicrobial infections in bone. From the
177 data of the ORN specimens in the present study we also recognize a predominance of
178 anaerobes (Figure 1). In accordance to our data, Fenollar et al. (2006) also detected in
179 their study several human pathogens not previously reported or rare in bone and joint
180 infections, like *Streptococcus anginosus*, *Peptostreptococcus micros*, *Peptoniphilus*
181 *lacrimalis*, *Porphyromonas asaccharolytica*, and *Prevotella buccalis* (Figure 1).

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233 determine the usefulness of PCR amplification and sequencing of the 16S rRNA
234 gene for diagnosis of bone and joint infections. *J Clin Microbiol* 2006;**44**:1018-1028.

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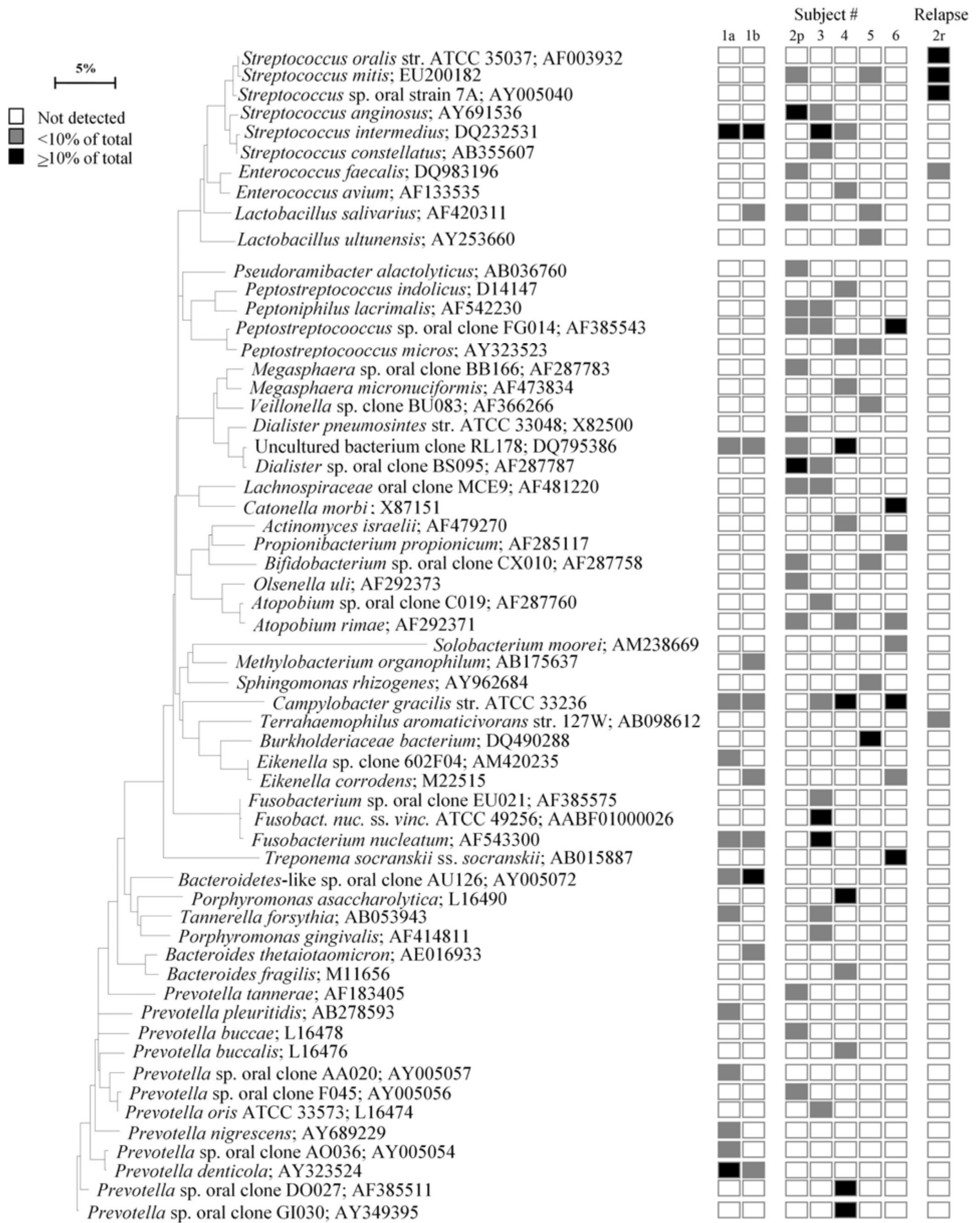
238 **Figure legends**

239 Figure 1. Bacterial profiles of mandibular osteoradionecrosis. Subject #1 represented
240 with two separate specimens (1a and 1b) from one lesion and subject #2 with primary
241 infection (2p) and relapse (2r). Distribution and levels of bacterial species/phylotypes
242 among the six subjects is shown by the columns of boxes to the right of the tree as either
243 not detected (clear box), <10% of the total number of clones assayed (shaded box), or
244 ≥10% of the total number of clones assayed (darkened box). 10% was chosen arbitrarily.
245 GenBank accession numbers are provided. Marker bar represents a 5% difference in
246 nucleotide sequences.



247

248 **Figure 1.**