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Role of microbiological culture and polymerase chain reaction (PCR) of actinomyces in medication-related osteonecrosis of the jaw (MRONJ)

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ABSTRACT

We hypothesized that local infection plays a critical role in the pathogenesis of medication-related osteonecrosis of the jaw (MRONJ). Recent developments in molecular methods have revolutionized new approaches for the rapid detection of microorganisms including those difficult to culture. The aim of our study is to identify the bacterial profiles in MRONJ by microbiological culture and polymerase chain reactions (PCR). A retrospective analysis was performed on MRONJ patients from 2008 to 2014. The bacterial profile from MRONJ bone samples was determined using microbiological culture and PCR. Ninety five patients fulfilled the inclusion criteria with mean age of 69.85 ± 8.71 years. A female predilection was detected. The mandible was more commonly affected than maxilla. Tooth extraction was the frequent triggering factor. Breast cancer was the primary cause for administration and intravenous bisphosphonates were the most commonly administrated antiresorptive drugs. The majority of patients were classified as stage 2. Posterior teeth were most commonly affected. Based on bone culture results, the most common microorganism were both actinomyces and mixed flora. PCR confirmed the presence of actinomyces in 55 patients. Our data suggest that PCR might be an innovative method for detection of microorganisms difficult to culture using traditional microbiological techniques.

1. Introduction

Medication-related osteonecrosis of the jaw (MRONJ) is a potentially devastating complication of anti-resorptive drugs used globally to treat bone disorders as osteoporosis, skeletal complications associated with osseous metastasis and multiple myeloma (Licata, 2005; Peer and Khamaisi, 2015). Nowadays, the pathophysiology of MRONJ is not clearly understood. Numerous theories have been proposed, neither of which can provide an adequate explanation of the disease. MRONJ was perceived as a type of avascular necrosis due altered bone turnover or direct toxicity to the soft tissue, infection, inflammation, inhibition of angiogenesis or suppression of innate or acquired immunity have been identified as possible explanations of the disease process (Mitsimponas et al., 2014).

Bacterial infection to the maxillofacial region has been suggested as key factor for the pathogenesis and progression of MRONJ (Otto et al., 2010a, 2010b). The oral cavity comprises of more than 750 bacterial species existing as mixed biofilm communities (Pushalkar et al., 2014). The mandible and maxilla are covered by thin layer of mucosa in close proximity to the external environment. After invasive dental procedures, oral trauma or soft tissue infection, microbial biofilms in the mouth and saliva gain access to the exposed jaw bone and play a significant role in the necrosis of the bone, inhibition of oral wound healing and facilitating bacterial colonization on bone surface (Sedghizadeh et al., 2012; Li et al., 2015). Actinomyces were regularly found in MRONJ suggesting a

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latent role of infection in the pathogenesis (Hansen et al., 2006, 2007; Lazarovici et al., 2009). Actinomyces are filamentous gram-positive anaerobic bacteria that usually can be found in calculus, periodontal pockets, carious lesions and oral mucosal surfaces, in addition to the upper respiratory, gastrointestinal tracts and vagina. They are common saprophyte bacteria of low virulence in nature causing no disease as long as they stay on the surface of the mucosa but in certain conditions where the integrity of the mucosal barrier is compromised, the bacteria may be pathogenic and gain access to the oral tissues or jawbones initiating a prolonged chronic inflammatory process, creating a tumour-like mass, tissue destruction, osteolysis and multiple sinus tracts (Hall, 2008; Kaplan et al., 2009; Norouzi et al., 2013).

MRONJ lesions are usually colonized by oral bacteria and the use of systemic antibiotics failed to restrict the bacterial colonization and effective healing of the lesion. It is important to identify the bacterial species colonizing jaw bone associated with the disease to delineate the pathogenesis. Moreover, it is not well understood whether the bacteria involved in MRONJ is similar or different to other biofilm associated bone infections in the oral cavity (Ji et al., 2012). Recently, bone abnormalities were studied by various modalities but none proved to be reliable in describing the infectious nature of the disease. Recent advances using biomolecular profiling to describe MRONJ flora have decreased this gap (Hinson et al., 2014).

Here, we identify the bacterial profiles that colonize MRONJ bone samples determined by culture approaches and polymerase chain reactions (PCR) with clinical features of patients. This line of investigation could provide rationale in the future for MRONJ therapeutics and targeted antimicrobial therapy.

2. Patients and Methods

This is a retrospective study of MRONJ patients treated at the Department of Oral and Maxillofacial Surgery, Ludwig-Maximillians-Universität Clinic, Munich from January 2008 to December 2014. Inclusion criteria were based on the American association of oral and maxillofacial surgery (AAOMS) Position paper (Ruggiero et al., 2014). Patients missing clinical, radiographic or follow-up data were excluded or if they had a history of head and neck radiation. Appropriate Institutional Review Board approval was obtained.

Clinical data relevant to the study were extracted and entered into an excel datasheet with a detailed history concerning: age, gender, location and teeth involved in the lesion, primary cause of the disease, comorbidities, clinical presentation, MRONJ clinical staging, type of anti-resorptive drug, route of administration and pathological/microbiological findings of bone samples. Bone samples were obtained from bone resection surgeries and were sent for microbiological investigations and PCR. Due to high likelihood of false positive culture from environmental exposure, we considered only at least strongly positive culture result (+2) as positive culture. One bone sample from each MRONJ patient was cut into fragments and prepared for microbiological analysis as described below.

Bone samples have been introduced in classical bacterial diagnostics. For this, aerobic cultures were prepared on Columbia blood-agar, MacConckey-agar and Columbia-CAN-agar, anaerobic cultures on Schaedler-agar and Schaedler-KV-agar (all agar plates from BD, Heidelberg, Germany). Besides, the swabs were cultivated in thioglycolate broth. All aerobe cultures have been read after 24 h, 48 h and 72 h, the anaerobic cultures after 2, 5 and 7 days. The bacterial counts have been enumerated semi-quantitative and bacterial colonies were objected to MALDI-TOF MS for further species identification.

Samples were evaluated by the use of Microflex LT mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) in linear positive-ion mode across the *m/z* range of 2000 to 20,000 Da. Each spot was measured by using 240 laser shots at 60 Hz in groups of 40 shots per sampling area of the spot. Spectra were analysed by using MALDI Biotyper software (v 3.1 – Build 65). Sample preparation included either the "direct transfer method", the "Extended Direct Transfer method (EDT)" or the "ethanol/formic acid extract method" as previously described (Schulthess et al., 2014). Resulting spectra were compared against reference spectra using Bruker MALDI-TOF Biotyper software to obtain identification with a confidence score. For most isolates, the MSP (Main Spectral Projection) reference spectra were those contained in the Bruker database of 2013 (database version V 3.3.1.2) containing 364 genera, 2185 species and 4613 individual MSP. Results with score values > 2 were considered as correct species identification, results displaying values of 1.5 ≤ and ≤2 were accepted as correct genus identification.

Identification of bacteria by sequencing of 16S rDNA has been performed as described previously with some modifications (Wragg et al., 2014). In brief, crude bacterial lysates were prepared directly from culture plates by suspending bacteria from a clonal culture in 100 μl of RT-PCR grade water (approximately McFarland Standard 2.0) and placed in a hot block at 100 °C for 10 min. A ~800 bp-fragment of 16S rDNA was amplified using the universal primer pair FD1 5′-AGAGTTTGATCCTGGCTCAG-3′ and 800r 5′-GAGTACCAGGGTATCTAATCC-3′. Resulting PCR amplicons were sequenced using the same primers and standard sequencing methods. Data from both strands was aligned in SeqMan (DNASTAR Lasergene 8 Suite) to generate a contig of around 800 bp. The consensus sequences were then used to compare with online databases (NCBI BLAST—http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project (http://rdp.cme.msu.edu/). Identification criteria of 99% sequence identity for identification to species level were applied (Drancourt et al., 2000) where matches had to be to the species type strain. The identities of type strains, as well as accession numbers in NCBI for equivalent 16S rDNA sequences, are available at http://www.bacterio.cict.fr/for all validly published bacterial species.

Statistical analysis

Descriptive statistics were computed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA).

Results are expressed as mean values including standard error of the mean and range. Means were compared by statistical testing (Student's t-test), where $P < 0.05$ was considered to be significant.

3. Results

A total of 150 patients were diagnosed with MRONJ from 2008 to 2014. However, 95 patients satisfied the inclusion criteria and form the basis of this study. Flow chart of the number of patients included in the study are illustrated in (Fig. 1). The mean age of the patients was 69.9 ± 8.7 years; with a male to female ratio of 1:1.4 (39 males and 56 females). Breast cancer was the primary cause for the administration of antiresorptive drugs (n = 35; 36.8%), followed by prostate cancer (n = 24; 25.3%) and osteoporosis (n = 13; 13.7%) in addition to multiple myeloma (n = 10; 10.5%), lung cancer (n = 4; 4.2%) and finally other cancers (n = 9; 9.5%). The relevant comorbidities identified included: diabetes mellitus (n = 17; 17.9%), cardiovascular diseases (n = 29; 30.5%), chemotherapy (n = 57; 60%), irradiation other than head and neck (n = 51; 53.7%), steroid intake (n = 28; 29.5%), anti-angiogenic drugs (n = 2; 2.1%) and smoking (n = 28; 29.5%). The most commonly administrated antiresorptive drugs (ARD) were bisphosphonates (BPs) in 85 patients (89.5%) of which,