Actinomyces israelii in radicular cysts: a molecular study

Nathália Rodrigues Gomes, DDS, Marina Gonçalves Diniz, BSc, PhD, Thais dos Santos Fontes Pereira, DDS, MSc, Carlos Estrela, DDS, PhD, Luiz de Macedo Farias, DDS, PhD, Bruno Augusto Benevenuto de Andrade, DDS, PhD, Carolina Cavaliéri Gomes, DDS, PhD, and Ricardo Santiago Gomez, DDS, PhD
Universidade Federal University de Minas Gerais, Belo Horizonte, Brazil

Objective. To investigate whether the microscopic filamentous aggregates observed in radicular cysts are associated with the molecular identification of Actinomyces israelii. Moreover, to verify whether this bacterium can be detected in radicular cyst specimens not presenting aggregates.

Study Design. Microscopic colonies suggestive of Actinomyces were found in 8 out of 279 radicular cyst samples (case group). The case and control groups (n = 12; samples without filamentous colonies) were submitted to the semi-nested polymerase chain reaction to test the presence of A israelii. DNA sequencing was performed to validate polymerase chain reaction results.

Results. Two and 3 samples in the case and control groups, respectively, did not present a functional genomic DNA template and were excluded from the study. A israelii was identified in all samples of the case group and in 3 out of 9 samples of the control group.

Conclusions. Although A israelii is more commonly identified in radicular cysts presenting filamentous aggregates, it also appears to be detected in radicular cysts without this microscopic finding. (Oral Surg Oral Med Oral Pathol Oral Radiol 2017;123:586-590)

The genus Actinomyces consists of a heterogeneous group of gram-positive facultative anaerobic bacteria, usually found in symbiosis with the human microbiota, especially in dental plaque.1 In the event of disruption of the mucosa barrier, these microorganisms can cause infection in almost any anatomic site, most commonly affecting the cervicofacial, thoracic, abdominopelvic, and cerebral regions.2 Cervicofacial actinomycosis comprises about 55% of all cases3-5 and includes, among others, intraoral and periapical forms.6 A large number of Actinomyces species has been described, and Actinomyces israelii is considered the major causative agent of human actinomycosis.7

In any of its forms, the diagnosis of actinomycosis is based on clinical manifestations together with microbiologic exams and histopathology. Histopathologic examination of actinomycosis may reveal the presence of masses of branching gram-positive filament aggregates surrounded by neutrophils, known as the Splendore-Hoeppli phenomenon.8 However, direct identification and/or isolation of the infecting organism from a clinical specimen are necessary for a definitive diagnosis. The identification of Actinomyces species must be performed by culture, which requires ideal growth conditions and is not always a reliable and efficient method.9 The most sensitive technique for bacteria identification is the 16S rRNA gene sequencing.10 However, this is not without limitations, such as the difficulty of extraction of viable DNA and with elevated costs.3

A israelii represents commensal bacteria of the oral cavity isolated in the tonsils, dental plaque, periodontal pockets, and carious lesions.11 Infection of A israelii was reported in periapical inflammatory lesions12-17 and exemplifies a representative extraradicular infection, given the ability of the A israelii to sustain inflammation even after orthograde root canal treatment.11

Radicular cysts are the most common periapical cyst of the jaws and arise from a long-standing inflammatory process surrounding the root apex, originating from the epithelial cell rests of Malassez proliferation.18 The Splendore-Hoeppli phenomenon was observed in histologic exams of periapical lesions, including radicular cysts,2 and has been diagnosed as periapical
actinomycosis when associated with an inflammatory reaction. This study verified whether the microscopic filamentous aggregates observed in radicular cysts are related to the molecular identification of A. israelii. In addition, we determine whether this bacterium can also be detected in specimens of radicular cysts not presenting aggregates.

METHODS AND MATERIALS

Histopathological analysis and sample selection

Hematoxylin and eosin stained slides of all radicular cysts diagnosed between 2008 and 2014 at the Oral Pathology Service (School of Dentistry of Federal University de Minas Gerais, Brazil) were retrieved and reviewed. Figure 1 shows a schematic diagram outlining the experimental protocol. From 279 cases examined, 8 had the characteristic Splendore-Hoeppli phenomenon suggestive of Actinomyces. Twelve samples of radicular cysts with no histopathologic signs of bacterial deposits were randomly selected and composed the control group. The 20 selected samples were from both male and female patients between 8 and 63 years of age (mean [SD], 36 ± 16.8 years). The local ethics committee approved this study, and the authors have followed the guidelines of the Declaration of Helsinki.

DNA extraction

Formaldehyde-fixed paraffin-embedded samples were microdissected in a series of 10-μm-thick paraffin-embedded tissue sections and digested with proteinase K. Genomic DNA was isolated using a QIAamp DNA formalin-fixed paraffin-embedded tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol and were quantified in a NanoDrop 2000 (Thermo Fisher, Wilmington, DE). A positive control was carried out by polymerase chain reaction (PCR) amplification of the endogenous human gene, β-globin, after a protocol described elsewhere. After the PCR reaction, the products were analyzed by electrophoresis in a 6.5% polyacrylamide gel. Samples that did not show β-globin amplification were excluded from the study to avoid false negative results.

Semi-nested PCR

Semi-nested PCR was performed using 2 primer sets previously described by Hansen et al. (Table I). The first-round primers spanned a specific DNA sequence for A. israelii identification; after that, a second PCR reaction was carried out using the same reverse (R) primer, but a different forward (F) primer, to amplify a smaller target within the first PCR product. In both reactions, the PCR mixture yielded a final volume of 15 μL, containing 2.5 mM of DNTP, 2.5 mM of MgCl₂, 1.5 μL of 10X PCR buffer, 0.6 U of Taq DNA polymerase (Invitrogen, Life Technologies, Inc., Carlsbad, CA), 3 pM of each primer, and 100 ng of template DNA. Thermocycling steps for the first PCR round were as follows: preheating at 94°C for 10 min, 40 cycles of sequential incubations at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and a final extension period at 72°C for 5 min. For the second PCR reaction, the number of cycles was reduced to 30 and the primer annealing temperature was set to 66°C. The final PCR products were subjected to electrophoresis in a 6.5% polyacrylamide gel and visualized after silver staining.

DNA sequencing (Sanger)

The semi-nested PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Amersham, UK) and then sequenced. The PCR product band was cut out from a 1.5% agarose gel with GelRed Nucleic Acid Stain (Biotium, Hayward, CA) immediately after staining of the electrophoresis. The purified product was sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using 10 pM of

Fig. 1. Schematic diagram outlining the experimental protocol.
the F primer (of the second round reaction). Reactions were done in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The sequencing electropherograms were analyzed with the Sequence Scanner Software 2 (Applied Biosystems) and the sequence of the gene 16S rRNA of *A. israelii* from the GenBank (access number: X82450.1) was used as a reference. The Sanger sequencing was performed only on the positive samples of the semi-nested PCR.

**RESULTS**

All radicular cysts with filamentous aggregates suggestive of *Actinomyces* reported proximity with inflammatory infiltrates. Two samples of radicular cyst of the case group and 3 of the control group did not present a functional genomic DNA template based on the results of the β-globin PCR test and were excluded from the molecular study. The semi-nested PCR and Sanger sequencing results are shown in Table II. All of the 6 non-excluded samples with filamentous aggregates (case group) in the hematoxylin and eosin stain revealed a positive result for *A. israelii* using the semi-nested PCR and were excluded from the molecular study. The semi-nested PCR and Sanger sequencing results are shown in Table II.

Table II. Primer sets used in the experiments

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>A <em>israelii</em></td>
<td>1st round</td>
<td>5’AAGTCAACGGGTCTGCTC CGT CGT CCGTCTTG3’</td>
<td>5’TCAAGCCTTGCCAGGCCATC3’</td>
</tr>
<tr>
<td>B- globin</td>
<td>5’TACCTTGCCTCATCTGCCTG3’</td>
<td>5’TCAAGCCTTGCCAGGCCATC3’</td>
<td>170 bp</td>
</tr>
</tbody>
</table>

bp, base pairs.

Three out of 9 samples (cases 8, 12, and 13) from the control group were also positive for *A. israelii* using the semi-nested PCR. One PCR product sample (case 12) was successfully sequenced, and the result was confirmed. The PCR products of the remaining 2 samples (cases 8 and 13) were of low yield after purification of the gel band.

**DISCUSSION**

Actinomycosis is a chronic infection caused by bacteria of the genus *Actinomyces*, with the most common causative species being *A. israelii*. *Actinomyces* colonies have been reported in periapical lesions submitted for histologic examination. Most of the studies use culture or microscopic examination to detect *Actinomyces*. Nevertheless, molecular techniques such as PCR and DNA hybridization have been used to identify bacterial species more successfully. The semi-nested PCR strategy increases the specificity of the reaction and is more sensitive and advantageous when only small amounts of the microorganism’s DNA are available. Additionally, the semi-nested PCR was found to be suitable to detect *A. israelii* in samples from paraffin-embedded tissues. Although the PCR technique does not distinguish secondary contaminations from the targeted infection, it has been widely used to support the diagnosis of various infectious diseases.
Table II. PCR and DNA sequencing results from each sample

<table>
<thead>
<tr>
<th>Case Group</th>
<th>Semi-nested PCR</th>
<th>Result</th>
<th>Sanger</th>
<th>Result verified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>NA</td>
<td>A israelii</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>NA</td>
<td>A israelii</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>A israelii</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>A israelii</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>A israelii</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>A israelii</td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>7</td>
<td>—</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>NA</td>
<td>A israelii</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>—</td>
<td>—</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>—</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>—</td>
<td>—</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>+</td>
<td>A israelii</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>NA</td>
<td>A israelii</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>—</td>
<td>—</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>—</td>
<td>—</td>
<td>Negative</td>
<td></td>
</tr>
</tbody>
</table>

NA, not available due to low concentration of the PCR product purified from the agarose gel.

*The Sanger sequencing was performed only on the positive samples of the semi-nested PCR.

In the present study, the histopathologic analysis of 279 cases of radicular cysts revealed 8 samples with unequivocal evidence of typical *Actinomyces* colonies, indicating a prevalence of 2.8%. This result is in line with previous findings in the literature, where actinomycetes aggregates were found in <5% of all periapical lesions analyzed.2,11,23,24 All cases with Splendore-Hoeppli phenomenon that were investigated by molecular methods confirmed the presence of *A. israelii*. In the retrospective analysis of the clinical records available, no evidence of suppuration, abscess formation, or draining sinus tracts were found.

Interestingly, we also found the presence of *A. israelii* in 3 out of 9 radicular cysts without histologic evidence of the Splendore-Hoeppli phenomenon. A previous study reported that 56% of 129 root canals and abscesses were infected by *Actinomyces*.16 Only 1 study investigated the presence of *Actinomyces* in a radicular cyst using the PCR method. The authors investigated several oral inflammatory lesions, and only 1 radicular cyst reported the presence of *Actinomyces naeslundi*.3 In our study, we performed a molecular investigation for *A. israelii* only. Although this species is the most common cause of actinomycotic infection, other *Actinomyces* species are also sporadically related to the disease.

Although radicular cysts infected by *A. israelii* are not associated with a specific clinical presentation, this finding raises the question of whether there might be a risk of infection of the surrounding tissues, propagation, and development of cervical actinomycosis.2 The association between *Actinomyces* and the progression and persistence of periapical lesions has been explored in the literature. Many studies report that *Actinomyces* are mostly found in persistent infections and extraradicular lesions compared with early stages of periapical infections.12,15,16,22,25 It has been reported that bacteria from this genus have the ability to adhere to intracanal surfaces and create a foundation for secondary colonizers.26

Periapical actinomycosis has a more indolent clinical presentation, and there is no consensus about the clinical meaning of filamentous aggregates of bacteria in the histopathologic exam.19 According to Kaplan et al.,19 true actinomycosis can only be diagnosed when colonies generate an inflammatory response and/or fibrosis in their immediate proximity. In our results, we observed an increased prevalence of *A. israelii* in radicular cysts presenting filamentous aggregates, but some cysts without this histopathologic finding also presented this microorganism. One might speculate that the presence of bacteria forming colonies with or without inflammatory reaction may not have substantial clinical significance when no signs or symptoms of infection are observed. However, there are no consistent data showing that this diagnosis should be excluded in asymptomatic patients with radicular cyst without the Splendore-Hoeppli phenomenon who are positive for *Actinomyces* at molecular investigation. Although the molecular identification of the bacterium does not show the role, if any, of the microorganism in the context of the lesion, further studies are necessary to confirm which of these possibilities is true.

An important limitation of the present study relates to its retrospective nature. Because of the incomplete

Fig. 4. Electropherogram showing DNA analysis of Sanger sequencing from sample 3. The peaks represent each base pair corresponding to the 16S ribosomal RNA gene sequence of A. israelii.

clinical information and evolution data of each patient included in the analysis, the results should be interpreted with caution.

In summary, although A. israelii is more commonly identified in radicular cysts presenting filamentous aggregates at the microscopic exam, it can also be detected in radicular cysts not presenting this feature.

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)/Brazil, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)/Brazil, and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG)/Brazil. RSG, MGD, CE, and CCG are research fellows at CNPq.

REFERENCES


Reprint requests:

Ricardo S. Gomez, DDS, PhD
Department of Oral Surgery and Pathology
School of Dentistry
Universidade Federal de Minas Gerais
Av. Antônio Carlos, 6627
Belo Horizonte
Minas Gerais
31270-901
Brazil
rsgomez@ufmg.br