Differential Proteinase Patterns among *Candida albicans* Strains Isolated from Root Canal and Lingual Dorsum: Possible Roles in Periapical Disease

Tatiana Teixeira Miranda, MSc, PhD,[*] *Cristina R. Vianna, MSc, PhD,*[†]
Leonardo Rodrigues, MSc, PhD,[*] *Carlos Augusto Rosa, MSc, PhD,*[*] *and Ary Corrêa, Jr, MSc, PhD*[*]

Abstract

Introduction: Proteinases play pivotal roles in *Candida albicans* infections. Although the yeast can colonize the pulpal environment, there is no information about the enzymatic profile of this organism. This *in vitro* study aimed to determine the proteolysis levels and to investigate differences in the expression of aspartyl proteinase genes (Sap 1, Sap 2, and Sap 4) among various root canal strains and clinical isolates from the lingual dorsum. **Methods:** The extracellular proteinase activity of 104 *C. albicans* samples isolated from the lingual dorsum and from necrotic root canals was measured with respect to bovine serum albumin degradation after 5 days of incubation at 37°C. We used reverse-transcription polymerase chain reaction, a highly sensitive method, to detect messenger RNA transcripts of aspartyl proteinase genes (Sap 1, Sap 2, and Sap 4). The *C. albicans* strain SC 5314 was used as a positive control for both experiments because it is recognized as being highly proteolytic. All tests were performed in triplicate. **Results:** Regardless of the isolation site, all *C. albicans* strains produced an opaque precipitation halo around the colonies, indicating some proteinase activity. However, the production of proteinase on the plates was significantly greater ($P < .05$) by the endodontic samples. Sap 2 was the most commonly expressed gene in all samples. Among the root canal samples, the detection of Sap 1 transcripts was always associated with the expression of Sap 2 and Sap 4. Sap 4 gene expression was detected in all root canal samples. The simultaneous expression of the 3 investigated Sap genes (Sap 1, Sap 2, and Sap 4) was more common in strains isolated from the lingual dorsum (50%) than in those isolated from root canals (29.4%). **Conclusions:** The increased proteolytic activity as well as the distinct pattern of Sap expression observed among the root canal samples may suggest a pathogenic role for *C. albicans* in endodontic infections. *(J Endod 2015;41:841–845)*

Key Words

Aspartyl proteinases, *Candida albicans*, root canal, virulence factors

Candida albicans is a commensal yeast that colonizes human mucosal surfaces such as the vaginal and gastrointestinal tracts. However, this yeast can cause both superficial and life-threatening systemic infections in a compromised host. This duality of *C. albicans* is directly related to the expression of several virulence factors, among which secretory aspartyl proteinases (Saps), encoded by a family of 10 genes, have long been recognized as a major cause of yeast virulence (1, 2).

Saps 1 to 10 range between 35 and 50 kd in size and account for all the extracellular proteolytic activity of *C. albicans*. Sap genes are differentially regulated depending on the surrounding environment. The expression of Saps 1–3 has been mainly observed in the yeast phase, whereas Saps 4–6 are expressed in the hyphal phase. Sap 7 expression has been detected in mouse models but not under any *in vitro* conditions. Sap 8 is transiently expressed in yeast and epithelial models. Sap 9 and Sap 10, which encode glycosylphosphatidylinositol-anchoring domains, are expressed under many conditions and are believed to maintain cell wall integrity through the post-translational processing of cell wall proteins. All types of Sap genes are suggested to contribute to various virulence processes, such as adhesion, invasion, degradation of host proteins, and macrophage escape. Therefore, it has been suggested that the virulence of *C. albicans* strains correlates with the level of proteolysis activity as well as with the number of Sap genes (3–6).

Previous studies have reported the presence of *C. albicans* in 7%–26% of infected root canals (7–11). Its presence has been commonly associated with persistent cases of apical periodontitis (12, 13). Although many studies have sought to investigate the frequency of yeasts in root canal infections, the role of *Candida* species in the endodontic environment at the onset of periapical disease remains unclear.

The regulation of Sap secretion and Sap gene expression has been extensively evaluated in different *C. albicans* strains (14–16). Nevertheless, there is no information about whether *C. albicans* isolated from endodontic infections expresses distinct patterns of Sap genes and whether it secretes different amounts of Saps. Thus, we hypothesized root canal strains would also have the ability to secrete proteolytic enzymes as reported for isolates from other yeast infections, which would contribute to colonization and survival in endodontic harsh ecologic conditions. The production of aspartyl proteases would increase their pathogenic potential.

This *in vitro* study aimed to determine the proteolysis levels and to investigate differences in the expression of aspartyl proteinase genes among various root canal strains

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From the *Department of Microbiology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte; and †Department of General Education, Federal Center of Technological Education, Varginha, Minas Gerais, Brazil.

Address requests for reprints to Dr Tatiana Teixeira Miranda, Department of Microbiology, Institute of Biological Sciences, Federal University of Minas Gerais, Avenida Antonio Carlos 6627, Pampulha, CEP31270-901, Belo Horizonte, MG, Brazil. E-mail address: microtati@yahoo.com.br

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and clinical isolates from the lingual dorsum. Sap 1, Sap 2, and Sap 4 genes were selected as a members of 2 Sap subfamilies, Sap 1–3 and Sap 4–6, respectively, which differ in their biological properties and potential roles in different types of *C. albicans* infections.

Materials and Methods

Clinical Material

Patients seeking treatment at the Endodontic Department of the Federal University of Minas Gerais, Belo Horizonte, Brazil, were selected. A detailed medical and dental history was obtained from each patient. Patients who had received antibiotic and antifungal treatment during the previous 6 months or who had a systemic disease were excluded. The Ethical Committee in Research of the Federal University of Minas Gerais approved the protocol describing the specimen collection for this investigation, and all patients were informed of the method, objectives, and risks of the procedures. Patients who met the inclusion criteria and agreed to take part in the experiment were asked to sign the written informed consent form.

Sampling Procedure and Yeast Isolation

A total of 168 patients without pain (94 women and 74 men) from 20–65 years of age were selected. One hundred eighty-four teeth with intact pulp chambers and no cracks and therefore with no direct oral pulpal communication but presenting with caries, pulpal necrosis, and radiographic evidence of apical periodontitis were selected from the group and microbiological samples collected. Pulpal necrosis was confirmed through heat, cold, and electric pulp sensibility tests. Based on the periodontal evaluation, the study population was considered healthy. Normally, a single tooth was sampled from each patient; however, in 16 patients, 2 teeth were sampled. For the statistic analysis of yeast frequency, 16 teeth were randomly excluded; therefore, each patient contributed only 1 tooth. All the teeth were subject to the initial standard endodontic procedure in which a rubber dam was used for the complete isolation of the target tooth from the oral cavity and the root canal was accessed using a sterilized dental drill without irrigation. Decontamination of the crown and the surgical field was accomplished by the sequential application of 30% hydrogen peroxide, 5% iodine dye, and 5% sodium thiosulfate solutions; all solutions were left in place for 1 minute. The aseptic status of the crown was confirmed by rubbing sterilized swabs along the crown and then using the swabs to streak modified Sabouraud agar (2% glucose, 1% peptone, 0.5% yeast extract, 2% agar, and 100 mg/L chloramphenicol). After intracoronal access, the canals were irrigated with sterile saline to remove debris. Samples were collected from root canals flooded with sterile absorbent paper cones that remained inside the root canal for 1 minute (8). The samples were collected aseptically in triplicate. In each case, a single root canal was sampled in order to restrict the microbial evaluation to a single ecologic environment. In multirooted teeth with periapical lesions, only the widest root canal was selected. The paper points were transferred immediately aseptically to tubes containing 4 mL modified Sabouraud broth with 100 mg/L chloramphenicol. After sampling, the teeth were restored temporarily using a standard cement and a base of zinc oxide. The tubes were immediately transported to the laboratory and left at room temperature for 48 hours. After incubation, aliquots of 0.1 mL were transferred to modified Sabouraud agar with 100 mg/L chloramphenicol, and the plates stood for 5 days at room temperature.

Sterile swabs were used to collect samples from the dorsum of the tongue from each patient during the treatment. The swab was inserted inside the mouth, and the collection tip was rubbed along the tongue. The material was streaked directly on modified Sabouraud agar with 100 mg/L chloramphenicol and incubated for 5 days at room temperature (9). Each patient contributed only 1 tooth and 1 tongue sample.

Yeast Strains

Yeasts were characterized by the standard methods of Yarrow. The taxonomic keys of Kurtzmann and Fell (17) were used for species identification. The polymerase chain reaction (PCR) fingerprint technique was used to confirm identification of all the yeasts.

In total, 115 yeast colonies were obtained. Those yeasts identified as *C. albicans* isolated from the lingual dorsum (n = 70) and necrotic root canals (n = 34) were selected for this study. The *C. albicans* strain SC 5314, previously recognized as a virulent strain (18, 19), was used as a positive control.

Assessment of *C. albicans* Extracellular Proteolytic Activity on Albumin Agar Plates

The extracellular proteinase activity of *C. albicans* isolates was analyzed in terms of bovine serum albumin (BSA) degradation according to a previously described protocol (20). Briefly, 5 μL of each *C. albicans* strain at 10^7 colony formed unit (CFU)/mL was spot inoculated on plates containing 0.15% yeast nitrogen base (Difco, Detroit, MI), 0.2% BSA (Sigma-Aldrich), 2% glucose (Sigma-Aldrich), and 2% agar (Sigma-Aldrich) at a pH of 5.0. Plates were incubated at 37°C for 5 days. Each isolate was tested in triplicate. After incubation, the plates were fixed with 20% trichloroacetic acid (Sigma-Aldrich) and stained with 0.5% amido black (Sigma-Aldrich). Decolorization was performed with acetic acid. The zone of clearance around the colony indicated enzyme production and was recorded as the proteinase zone (P_Z). Using a caliper ruler, the proteinase activity was calculated as the ratio of the diameter of the colony to the diameter of the proteolytic unstained zone and was scored in 3 categories:

Index zero: P_Z = 1 mm (strain without enzymatic activity)
Index 1: 1 mm > Pz > 0.64 mm (strain with some enzymatic activity)
Index 2: Pz ≤ 0.64 mm (strain with high enzymatic activity)

Culture Growth Conditions to Identify Aspartyl Proteinase RNA Transcripts of *C. albicans* Strains

C. albicans strains were initially grown in YPD broth containing 1% yeast extract (Sigma-Aldrich), 1% peptone (Difco), and 2% glucose (Sigma-Aldrich) at 37°C overnight. After this incubation period, 50 mL YPD-grown culture was centrifuged (2000*g* for 10 minutes). The yeast cells were washed twice in phosphate buffered saline (pH = 7.5) and used to inoculate, at a final concentration of 10^5 cells/mL, yeast nitrogen base-BSA medium, which contained 0.17% yeast nitrogen base without amino acids or ammonium sulfate (Difco), 0.2% glucose (Sigma-Aldrich), and 0.1% BSA (Sigma-Aldrich) at a pH of 5.5. Cultures (200 mL) were incubated at 37°C in a rotary shaking incubator (200 rpm). Sap 1, Sap 2, and Sap 4 RNA transcripts were investigated after 48 hours of *C. albicans* growth because a good expression profile of Sap 1, Sap 2, and Sap 4 was detected in *C. albicans* SC5314. Culture aliquots were taken, and the optical density values at 540 nm (Shimadzu Corporation, Kyoto, Japan) did not differ among strains at 48 hours after inoculation. Culture aliquots (35 mL) were transferred to RNA-free centrifuge tubes containing RNAse-free ice-cold water and centrifuged (2000*g* for 5 minutes at 4°C). The supernatant was decanted, and the cells were resuspended in 1 mL RNAse-free ice-cold