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Genotypic analysis of secreted aspartyl proteinases in vaginal *Candida albicans* isolates

Análise genotípica de aspartil proteases secretórias em isolados vaginais de Candida albicans

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ABSTRACT

Introduction: *Candida albicans* is the most common etiologic agent of fungal vaginitis. These yeasts produce secreted aspartyl proteinases encoded by a family of 10 genes (*SAP1-10*). **Objective:** The purpose of this study was to analyze the presence of genes *SAP1-7* in vulvovaginal *C. albicans*. **Materials and method:** The study included 26 *C. albicans* vaginal isolates. Detection of aspartyl proteases genes (*SAP1-7*) was performed by polymerase chain reaction (PCR). **Results:** The most frequent gene in *C. albicans* isolated from colonization was *SAP6* (93.33%), and from infection, *SAP7* (100%). We observed a statistical difference ($p = 0.049$) in *SAP1* gene frequency between isolates from vulvovaginal colonization and infection. **Conclusion:** High frequency of *SAP* genes was observed in vulvovaginal *C. albicans*. The results suggest *SAP1* participation in vulvovaginal candidiasis infection.

Key words: vulvovaginal candidiasis; *Candida albicans*; virulence factors.

INTRODUCTION

Vulvovaginal candidiasis (VVC) is an endogenous infection caused by yeasts of the genus *Candida*. The disease commonly develops when conditions exist favoring fungal growth, such as alterations in normal microbiota or lowered host immune response[1-5]. The species of *Candida* are microorganisms commonly found in the microbiota of the gastrointestinal and urogenital tracts without causing disease, but when the balance between fungus and host is disturbed, there is an increase in colonization, and the fungus invades tissues, initiating the infectious process[6, 7].

VVC is one of the most frequent diagnoses in clinical practice, with a rising incidence, becoming the second most common genital infection in the United States and in Brazil[1, 8, 9]. Among vulvovaginitides, VVC accounts for 39% of the cases, just behind bacterial vaginosis. *Candida* species can be found in up to 50% of healthy women without causing symptoms; however, around 70%-75% of women in childbearing age develop at least one episode of fungal vulvovaginitis during life. Among these, 50% will present two or more episodes, and approximately 6%-9% are likely to present recurrent VVC (RVVC), characterized by the presence of four or more symptomatic episodes in a year[10-14].

Candida albicans is the most prevalent etiologic agent in VVC, accounting for 85%-90% of the cases. The virulence of this species is due to several mechanisms and abilities inherent in the fungus, as yeast-to-hypha transition, phenotypic switching, expression of adhesins and invasins on the cell surface, biofilm formation, and secretion of hydrolytic enzymes[3, 11, 15-17].

The most studied hydrolases related to *Candida* virulence are proteases, phospholipases, and lipases. Secreted aspartyl proteinases (Sap) form a family of 10 isoenzymes (Sap1-10) that participate in the infection process by degrading several host cell proteins, such as immunoglobulins, proteins of the complement system and extracellular matrix, contributing to tissue damage and the resulting invasion by the microorganism[18-21]. These enzymes play different roles depending on the environmental stimuli and are involved in

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the host inflammatory response to fungus. The activation of these proteins is a well-regulated process at specific time points, what increases the infection potential of *C. albicans*[6, 16, 22, 23]. Among the *SAP* gene family, the most frequently studied in the literature and associated with pathogenicity of *C. albicans* are *SAP1-7* genes. The objective of this study was to evaluate the presence of these genes in vulvovaginal *C. albicans* cell lines.

MATERIALS AND METHOD

Studied microorganisms

Twenty-six *C. albicans* cell lines were isolated from the vaginal mucosa of asymptomatic (n = 15) and symptomatic (n = 11) women with VVC. The yeasts were obtained from vaginal swabs as described by Goulart *et al.* (2016)[24]. The clinical samples were collected from women seen at basic health-care units in the municipality of Rondonópolis (MT), Brazil, regardless VVC symptoms. Participants were divided into two groups: 1) symptomatic patients, characterized by the presence of curd-like vaginal discharge, pruritus, edema and erythema of vulva and vagina; and 2) asymptomatic patients, which did not present the mentioned characteristics. Yeasts were stored at Sabouraud agar at 4°C. Previously, microorganisms were grown in Sabouraud broth at 37°C, under agitation [200 revolutions per minute (rpm)] for 24 h. Then the culture was centrifuged, the supernatant was discharged, and deoxyribonucleic acid (DNA) was extracted from the cell sediment with a kit (Nucleo Spin Tissue, Macherey-Nagel GmbH & Co. KG, Duren, Germany), following the instructions by the manufacturer. The yeast species was determined by the species-specific polymerase chain reaction (PCR) based on the protocol of Liguori *et al.* (2010)[25], with modifications.

Identification of *SAP1-7* genes

Genes were identified by PCR based on the method proposed by Bassyouni *et al.* (2015)[26], with some modifications. Reactions were performed at a final volume of 25 µl, containing approximately 20 ng of DNA, 12.5 µl of GoTaq Hot Start Green Master Mix (Promega, Madison, Wisconsin, USA) and 0.75 µl (20 pmol/µl) of each specific primer. Amplification conditions for *SAP1*, *3*, *4* and *7* genes were: initial denaturation at 94°C for 3 minutes, 30 denaturation cycles at 94°C for 30 seconds, annealing at 46°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 10 minutes. PCR for *SAP2*, *5* and *6* genes was standardized from denaturation at 92°C for 3 minutes, 30 denaturation cycles at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 10 minutes. Amplification products were analyzed on 1% agarose gel electrophoresis containing DNA stain (Promega, Madison, Wisconsin, USA) and visualized under ultraviolet light. The oligonucleotide sequences used in the study are described in **Table 1**.

TABLE 1 – Oligonucleotide sequences used in PCR assays for investigation of virulence genes

Primer	Oligonucleotide sequence	Size (*pb*)
SAP 1	Forward 5'-TCA ATC AAT TTA CTC TTC CAT TTC TAA CA-3' Reverse 5'-CCA GTA GCA TTA ACA GGA GTT TTA ATG ACA-3'	161
SAP 2	Forward 5'-AAC AAC AAC CCA CTA GAC ATC ACC-3' Reverse 5'-TGA CCA TTA GTA ACT GGG AAT GCT TTA GGA-3'	178
SAP 3	Forward 5'-CCT TCT CTA AAA TTA TGG ATT GGA AC-3' Reverse 5'-TTG ATT TCA CCT TGG GGA CCA GTA ACA TTT-3'	231
SAP 4	Forward 5'-TTA TTT TTA GAT ATT GAG CCC ACA GAA A-3' Reverse 5'-GCC AGT GTC AAC AAT AAC GCT AAG TT-3'	171
SAP 5	Forward 5'-AGA ATT TCC CGT CGA TGA GAC TGG T-3' Reverse 5'-CAA ATT TTG GGA AGT GCG GGA AGA-3'	277
SAP 6	Forward 5'-CCC GTT TTG AAA TTA AAT ATG CTG ATG G-3' Reverse 5'-GTC GTA AGG AGT TCT GGT AGC TTC G-3'	187
SAP 7	Forward 5'-GAA ATG CAA AGA GTA TTA GAG TTA TTA C-3' Reverse 5'-GAA TGA TTT GGT TTA CAT CAT CTT CAA CTG-3'	196

PCR: polymerase chain reaction.

Statistical analysis

Data were recorded in Excel 2016 spreadsheets and assessed in the statistical software Epi-info 7.2.0. Data analysis was carried out by descriptive statistics and non-parametric Fisher exact test, adopting a 5% significance level. We evaluated the correlation of the presence of *SAP1-7* genes with the infection process and vulvovaginal colonization.

RESULTS

By means of PCR method, it was possible to detect the presence of *SAP1-7* genes in clinical isolates of *C. albicans*. After molecular analysis of *C. albicans* isolated in women with symptoms of VVC, the presence of *SAP1* gene was identified in 90.9% (10/11), *SAP2* in 63.63% (7/11), *SAP3* in 45.45% (5/11), *SAP4* in 90.9% (10/11), *SAP5* in 72.72% (8/11), *SAP6* in 72.72% (8/11), and *SAP7* in 100% (11/11) of the samples. The frequency of virulence genes in *C. albicans* from colonization was 53.33% (8/15) for *SAP1*, 60% (9/15) for *SAP2*, 46.66% (7/15) for *SAP3*, 73.33% (11/15) for *SAP4*, 86.66% (13/15) for *SAP5*, 93.33% (14/15) for *SAP6* and 73.33% (11/15) for *SAP7* (**Table 2**).

TABLE 2 – Frequency of *SAP1-7* genes in *C. albicans* isolated from vulvovaginal infection and colonization

Genes	Infection (n = 11) n (%)	Colonization (n = 15) n (%)	Total (n = 26) n (%)	p-value
SAP1	10 (90.9)	8 (53.33)	18 (69.23)	0.049
SAP2	7 (63.63)	9 (60)	16 (61.54)	0.588
SAP3	5 (45.45)	7 (46.66)	12 (45.15)	0.631
SAP4	10 (90.9)	11 (73.33)	21 (80.77)	0.273
SAP5	8 (72.72)	13 (86.66)	21 (80.77)	0.345
SAP6	8 (72.72)	14 (93.33)	22 (84.61)	0.187
SAP7	11 (100)	11 (73.33)	22 (84.61)	0.091

When comparing the frequency of genes encoding aspartyl proteases (*SAP1-7*) among *C. albicans* isolated in asymptomatic and symptomatic women with VVC, we found a statistical difference just for *SAP1* gene (p = 0.049), with this being more prevalent in isolates associated with the infectious process. Although genes *SAP2-7* present distribution profiles distinct among the groups, this difference did not represent statistical difference. These pieces of data are shown in Table 2.

The results revealed genetic variability for the members of the *SAP* family in the studied cell lines, with 19 different genotypic patterns being found; just three isolates presented all the studied genes (**Table 3**).

TABLE 3 – Genotypic patterns identified in vaginal isolates of *C. albicans*

Pattern	Genotype	n (%)
1	*SAP1-7*	3 (16)
2	*SAP1/SAP2/SAP4/SAP5/SAP6/SAP7*	3 (16)
3	*SAP1/SAP3/SAP4/SAP5/SAP6/SAP7*	2 (10)
4	*SAP2/SAP4/SAP5/SAP6*	2 (10)
5	*SAP2/SAP5/SAP6/SAP7*	2 (10)
6	*SAP1/SAP2/SAP3/SAP4/SAP5/SAP7*	1 (5)
7	*SAP1/SAP2/SAP3/SAP5/SAP6/SAP7*	1 (5)
8	*SAP1/SAP2/SAP3/SAP4/SAP6/SAP7*	1 (5)
9	*SAP3/SAP4/SAP5/SAP6/SAP7*	1 (5)
10	*SAP1/SAP3/SAP4/SAP6/SAP7*	1 (5)
11	*SAP1/SAP2/SAP4/SAP6/SAP7*	1 (5)
12	*SAP1/SAP2/SAP3/SAP4/SAP7*	1 (5)
13	*SAP1/SAP4/SAP5/SAP6/SAP7*	1 (5)
14	*SAP2/SAP3/SAP4/SAP5/SAP6*	1 (5)
15	*SAP4/SAP5/SAP6/SAP7*	1 (5)
16	*SAP1/SAP5/SAP6/SAP7*	1 (5)
17	*SAP1/SAP4/SAP5/SAP7*	1 (5)
18	*SAP1/SAP4/SAP7*	1 (5)
19	*SAP5/SAP6*	1 (5)

DISCUSSION

SAP genes greatly stand out in the pathogenesis of candidiasis, once they encode proteins able to degrade collagen, keratin, and peptides found in the surface of mucosas, ensuring, thus, an important and efficient proteolytic system to *C. albicans*, from the colonization process to active infection[20, 23, 27-30].

In this study, we observed that the most prevalent *SAP* genes in *C. albicans* isolated from vulvovaginal infection and colonization were *SAP7* (100%) and *SAP6* (93.33%), respectively. Monroy-Pérez *et al.* (2013)[31] determined the frequency and the expression of *SAP1-10* genes isolates from women with VVC in Mexico and identified that *SAP4-6* genes were the most frequent (100%), besides observing that all *SAP* genes were expressed in a model of reconstituted human vaginal epithelium (RHVE), suggesting that aspartyl proteases play an important role in the pathogenesis of the infection. Bassyouni *et al.* (2015)[26] investigated the presence of *SAP1-8* genes in *C. albicans* isolated from vaginal mucosa of diabetic and non-diabetic women and identified that *SAP1* and *SAP2* were the most frequently detected genes, followed by *SAP5* in both groups; there was no difference in the distribution of genes between diabetic and non-diabetic women. Kalkanci *et al.* (2005)[32] verified that *SAP1-3* genes were the most prevalent in vaginal isolates of *C. albicans*, with a 92.5% rate, followed by *SAP6*, with 12.5%, and *SAP4-5*, with 7.5%.

The most frequent *SAP* genes in the present research and in other studies are available in **Table 4**.

Predominance of the *SAP6* gene in the studied colonization isolates and *SAP7* in the studied infection isolates suggests the necessity of further studies to evaluate the expression of these genes *in vivo*. The *SAP6* gene participates with the *SAP4-6* subfamily in the development of hyphae, an essential process for the fungus[33, 34] capacity of invasion. Besides, Sap6 protein was associated with integrity maintenance of cell surface and capacity of producing inflammatory response in the host[33, 35, 36]. The *SAP7* gene encodes the most divergent protein within the Sap family and can be preferably associated with infections of human mucosas[36]. The expression of this gene was associated with the initial adaptation of *C. albicans* to human cells of the intestinal tract, while transcripts of *SAP6-7* were associated with tissue damage in the early phase of infection, after 24 h, at a RHVE[37, 38] model.

This study revealed high (90.9%) presence of *SAP4* gene in vaginal cell lines of *C. albicans* involved in infection. The proteins encoded by these genes are associated with adherence of the fungus to human cells and alterations in the morphogenesis of the yeast, playing an important role in biofilm formation. The *SAP4* gene

has also been implicated in evasion of phagocytosis[30, 39, 40]. The expression of *SAP4* genes was demonstrated *in vivo* in the vaginal mucosa of women who were pregnant (40%), post-menopausal (50%), and in childbearing age (33%)[41].

The statistical analysis showed significant difference (p = 0.049) for the *SAP1* gene frequency among the *C. albicans* cell lines isolated from infection (90.9%) and colonization (53.33%). This suggests a probable participation of *SAP1* in the infectious process of VVC and points to a possible aim of further studies to distinguish colonization from active infection. This result can also contribute to researches aimed at developing new diagnostic methods for VVC. Sap1 protein is known to be linked to the capacity to cause lesions in the mucosa and to the development of systemic infections[30]. At a previous work, the expression of *SAP1* in *C. albicans* from patients with VVC was observed in 80% of the studied isolates[42]. The *SAP1-7* genes can be expressed by *C. albicans* in the vaginal mucosa, both in colonization processes and in infections, but a differential expression of these genes is observed when comparing the transcript levels in isolates of carriers and of active VVC, besides a predominance of certain members of the *SAP* family during vaginal infection[43].

A study of genic expression of *C. albicans* revealed that *SAP1*, *SAP3*, and *SAP6-8* genes are related with active VVC. Moreover, *SAP1* and *SAP3* are preferably expressed in the vaginal mucosa, when compared with the oral infection, indicating that the differential expression of genes encoding hydrolytic enzymes varies according to the phase of the disease and the anatomical location[18]. Lian and Liu (2007)[43] evaluated the expression of *SAP1-7* genes in *C. albicans* isolated from asymptomatic women, and women with symptomatic and recurrent VVC. The authors observed that the transcripts for *SAP1* and *SAP3* genes were present only in isolates of VVC and RVVC. Lin *et al*. (2007)[44] determined by real-time PCR (RT-PCR) that *SAP2* and *SAP5* were the most commonly expressed genes in the vaginal mucosa of women with acute VVC. The expression of *SAP* genes in the different studies is shown in **Table 5**.

Medeiros *et al*. (2017)[45] did not find differences in the expression of virulence factors between *C. albicans* isolated from patients with sporadic VVC and those obtained from patients with RVVC, suggesting that the ability to express virulence factors is important in the pathogenesis of VVC, but it seems not to be crucial for the transition from colonization to infection. We did not identify statistical difference for the presence of *SAP2-7* genes in isolates from infection and vulvovaginal colonization. This suggests that other factors that control the genic fungal expression and characteristics inherent in the host are likely to influence in the infection/colonization process. Also, further studies comprising a larger number of samples must be carried out.

CONCLUSION

C. albicans isolated from symptomatic women with VVC and asymptomatic women presented different patterns of distribution for *SAP1-7* genes, with predominance of the *SAP6* gene in colonization and *SAP7* gene in infection. The *SAP1* gene was associated with the process of vaginal infection, suggesting its participation in the pathogenesis of VVC.

TABLE 4 – Frequency of *SAP* genes in different studies

Studies	Population	Frequency of *SAP* genes
Our study	Women from Rondonópolis with symptomatic VVC	*SAP7* (100%) and *SAP1-4* (90.9%)
Our study	Women from Rondonópolis asymptomatic for VVC	*SAP6* (93.33%) and *SAP5*(86.66)
Monroy-Pérez *et al.*[31]	Women from Mexico with VVC	*SAP4-6* (100%)
Bassyouni *et al.*[26]	Diabetic and non-diabetic women from Egypt	*SAP1*, *SAP2* (100%) and *SAP5* (75%)
Kalkanci *et al.*[32]	Women from Turkey with VVC	*SAP1-3* (92.5%), *SAP6* (12.5%) and *SAP4-5* (7.5%)

VVC: vulvovaginal candidiasis.

TABLE 5 – Expression of *SAP* genes in different studies

Study	Population	Expression of *SAP* genes
Nas *et al.*[41]	Women from Turkey with VVC	Expression of *SAP4*: pregnant (40%), post-menopausal (50%) women, and women in childbearing age (33%)
Lian and Liu[43]	Symptomatic, asymptomatic women, and women with RVVC from China	*SAP1* and *SAP3* were present only in VVC and RVVC isolates
Lin *et al.*[44]	Women from China with acute VVC	*SAP2* and *SAP5* were most commonly expressed in the vaginal mucosa

VVC: vulvovaginal candidiasis; RVVC: recurrent vulvovaginal candidiasis.

RESUMO

Introdução: Candida albicans *é o principal agente etiológico das vaginites fúngicas. Essas leveduras produzem aspartil proteases secretórias que são codificadas por uma família de 10 genes (*SAP1-10*).* ***Objetivo:*** *O objetivo deste estudo foi avaliar a presença dos genes* SAP1-7 *em linhagens vulvovaginais de* C. albicans*.* ***Materiais e método:*** *O estudo incluiu 26 isolados vaginais de* C. albicans*. Os genes de aspartil proteases (*SAP1-7*) foram detectados por reação em cadeia da polimerase (PCR).* ***Resultados:*** *O gene mais frequente em* C. albicans *isolado de colonização foi* SAP6 *(93,33%), e de infecção,* SAP7 *(100%). Foi observada diferença estatística (*p = *0,049) na frequência do gene* SAP1 *entre isolados oriundos de colonização e infecção vulvovaginal.* ***Conclusão:*** *Constatou-se alta frequência dos genes* SAP *em linhagens vaginais de* C. albicans*. Os resultados sugerem uma participação de* SAP1 *no processo infeccioso da candidíase vulvovaginal.*

Unitermos: candidíase vulvovaginal; Candida albicans*; fatores de virulência.*

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