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Alpha Defensins Genes and Vulvovaginal Candidiasis: A Study of Cases

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Abstract

Objective: To evaluate the alpha-defensin (α-DF) genes polymorphism in women with vulvovaginal candidiasis and recurrence. Methods: This observational study included clinical vaginal secretion samples collected over four years from 88 women, ranging in age from 18 to 65 years, from medical centers of São Paulo and Mogi das Cruzes, Brazil. Thirty-six of these women were asymptomatic (control group) and 52 presented clinical condition compatible with vulvovaginitis (38 primary or episodic as non-recurrent forms, and 14 recurrent vulvovaginal candidiasis). A portion of each sample was plated on Sabouraud dextrose agar with chloramphenicol and grown on CHROMagar *Candida* for presumptive characterization. The identification of the species was obtained by sequencing of the ITS1 region of rDNA. α-DF genes were amplified for subsequent evaluation of polymorphisms by endonuclease restriction assay. Results: From 88 samples were isolated 60 *Candida albicans* and 28 *non-albicans Candida* spp. Resistant *C. albicans* strains and non-*albicans Candida* spp. were more prevalent in recurrence. In all groups, the number of resistant non-*albicans Candida* spp. was most high than susceptible strains. α-DF1, α-DF3 and α-DF1/α-DF3 genotypes were found in 32 (36.4%), 17 (19.3%), 6 (6.8%) vaginal samples, respectively. About 33 samples were not amplified. Recurrence and severe disease were more observed in homozygous population. Conclusions: Non-*albicans Candida* spp. and homozygotic α-DF genotipes (α-DF1 and α-DF3) were more related with severe clinical signs and recurrence. Further studies about vulvovaginal candidiasis and α-DF genes are necessary to access the more comprehen-

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sive role of defensins in clinical manifestations.

Keywords

Alpha-Defensin Genes, *Candida albicans*, Non-*Albicans Candida*, Vulvovaginal Candidiasis, Recurrent Vulvovaginal Candidiasis

1. Introduction

Vulvovaginitis (VV) is the main reason for a third of all gynecological medical visits, and the most common gynecologic disease in pregnant or non-pregnant women [1]. *Candida* spp. is the second most important agent in these infections [2]-[4].

Candida albicans is responsible for 80% - 92% of all vulvovaginal candidiasis cases (VVC) [5]-[7]. However, the frequency of other species as causative agents for this disease is increasing, particularly those with different susceptibility profiles to antifungal agents, possibly because of the indiscriminate use or misuse of these drugs, which may result in recurrent infections [8]-[11].

Recurrent vulvovaginal candidiasis (RVVC) is defined by the occurrence of three or more VVC recorded episodes, with clinical diagnosis and culture confirmation, in the course of one year [12] [13]. Chronic stress [14], changes in hormone levels [12], use of oral contraceptives [15], use of intrauterine devices, and virulence factors of the infectious agents have been associated with the development of the disease [16] [17].

Defensins (DF) are important antimicrobial polypeptides that act in host defense [18] [19]. Leukocytes and epithelial cells expressed DF as component of innate immunity [18] [20]. DF polymorphisms may be involved in susceptibility to infection by *Candida* spp. or entrainment of the agent [21] [22].

Fluids from patients with genital infections show high levels of alpha-DF (α-DF) modulating the innate immunity expressed in the mucosal surface. In addition to the presence of polymorphisms in the α-DF gene, other factors such as the age, hormonal influence, cervicovaginal mucus, and innate immune defense can be related to the recurrence of VVC [23]-[29].

The goal of this study was to evaluate the presence of α-DF genes in women with or without clinical condition of candidiasis.

2. Materials and Methods

In two medical centers to São Paulo and one of Mogi das Cruzes, Brazil, 88 women, between 18 and 65 years old, were selected to participate in the study; 52 presented VVC (38 cases of non-recurrent and 14 of recurrent vaginitis) and 36 were asymptomatic (control group). Each patient with gynecological complaints was subjected to a thorough clinical examination. The vaginitis cases were classified as primary (one single episode), repeated (2 episodes/year), and recurrent (≥3 episodes/year). The exclusion criteria for the study were pregnancy, diabetes mellitus, HIV infection, immune-suppression, treatment with corticosteroids, antimicrobials, and hormone replacement, and use of an intrauterine device (IUD), vaginal douches, or spermicidal products. Samples of vaginal secretion were collected from all participants on the outside surface of the cervix and posterior vaginal fornix. Patients with vaginitis and clinical manifestations of leukorrhea, pruritus, dysuria, edema, erythema, burning and vulvar pain were examined and their signs and symptoms were recorded in individual form and received arbitrated scores according to the following scale and clinical presentation of the two studied groups: 0 = absent, 1 = mild, 2 = moderate, 3 = severe. They were considered patients with mild clinical frames those obtained 0 - 6 points, moderate 7 to 13 points, and severe more than 13 points. For the treatments of VVC patients was employed weekly 150 mg of oral fluconazole single-dose, nystatin vaginal cream 2% or a combination of them. To refractory and recurrence cases, these treatments were carried for prolonged periods generally superior to 3 months. Institutional review board approval was obtained from Ethics Committee of the Federal University of São Paulo n° 1719/05, and that the participants gave informed consent.

All samples were plated on Sabouraud Dextrose Agar and CHROMagar Candida® (Difco, USA), incubated at 37˚C for 48 hours. The phenotypic identification was accessed by micro- and macro-morphological evaluations and biochemical profiling. The molecular identification was performed by sequencing the ITS1 region of rDNA.

Azole-resistance or susceptible profile of yeasts strains was evaluated by Etest (AB BIODISK, Solna, Sweden). Ketoconazole and fluconazole strips were used for direct antifungal susceptibility testing.

DNA extraction from the vaginal samples was performed with the Amersham-Pharmacia GFX® kit, and according to the manufacturer's instructions. Polymorphisms were assessed in PCR fragments of α-DF genes amplified with the primers DEF F (5'-CAG CGG ACA TCC CAG AAG TGG-3') and DEF R (5'-GCG TTT TGG TAC GTG TAT CC-3') according to [30]. The beta-globin gene was used as the PCR reaction control and amplified using the primers GH20 (5'-GAA GAG CCA AGG ACA GGT AC-3') and PC04 (5'-CTT CAA CAT CCA CGT TCA CC-3'). PCR amplified fragments were digested with the restriction enzyme HAE III (New England Biolabs Inc., Beverly, MA) for 4 hours at 37˚C in a water bath, to assess the presence of α-DF 1 and α-DF 3.

3. Results

The homozygous and heterozygous patterns of the α-DF genes, after digestion with HAE III, are presented in **Figure 1**. The presence of the α-DF1 polymorphic allele was evidenced by two fragments with 650 bp and 300 bp, after digestion with HAE III. Heterozygous patients (α-DF1/α-DF3 genotype), carriers of one wild type allele and one polymorphic allele, showed a restriction pattern with three fragments, of 950, 650, and 300 bp. The wild type homozygous genotype (of rare incidence) showed only one fragment with 950 bp. Lanes 01, 03, 04, and 06 show the polymorphic homozygous pattern that represents the α-DF1 genotype. Lanes 02, 05, and 07 show the heterozygous pattern that represents the α-DF1/α-DF3 genotype. Lane 09 shows the wild type pattern that represents the α-DF3 genotype. Lane 08 shows negative α-DF gene PCR amplification.

The identification of the species found in the control group and patients with VVC and recurrent forms is shown in **Table 1**. *Candida albicans* was identified in 60 out of 88 isolates. High number of resistant species was observed in group of non-*albicans Candida* spp. (57.1%), while *C. albicans* resistance rate was 20%. Resistant species were more associated to recurrence (RVVC). In this study, we also observed that the treatment failure of VVC refractory or relapsed was mostly related with the decreased susceptibility to antifungal agents and the presence of fluconazole-resistant Candida isolates.

A high percentage of homozygous polymorphisms in α-DF genes were observed in 10 out of 14 patients with RVVC (71.4%) compared to the non-recurrent (5 out of 38% - 13.2%) or control groups (8 out of 36% - 22.2%). In cases of non-RVVC we observed clinical manifestations predominantly mild or moderate in 37 (90%) patients, severe symptoms and signs in only one woman (3%) (**Table 2**). Vaginal discharge, pruritus, and erythema were the main manifestations of VVC non-recurrent group (78.9%), being edema observed in 11 cases (28.9%). Seven patients (18.4%) presented only mild or moderate pruritus, edema, and erythema. All RVVC cases had vaginal discharge, pruritus, erythema, edema, vulvovaginal burning sensation and pain. Five RVVC patients (36%) had severe symptoms (verified more in homozygotes). The presence of heterozygosis or homozygosis in α-DF genes may also be associated to the occurrence of *C. albicans* compared to non-*albicans Candida* spp. In

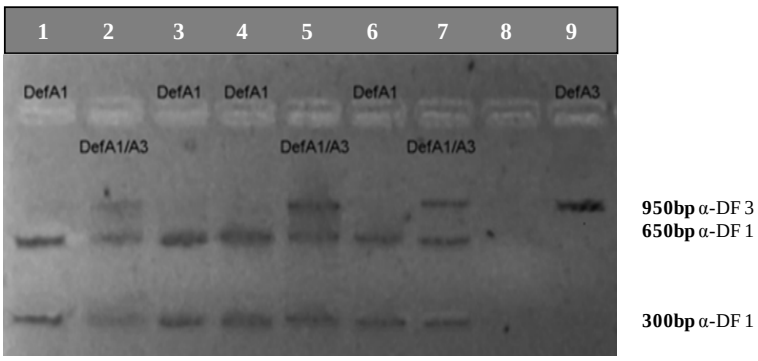

Figure 1. Agarose gel electrophoresis of the alpha-defensin (α-DF) genes PCR fragments digested by endonuclease HAE III. α-DF1 restriction patterns with 650 and 300 bp (DefA1, lanes 1, 3, 4, and 6). α-DF3 fragment with 950 bp (DefA3, lane 9). α-DF1/α-DF3 heterozygous patterns with 950, 650 and 300 bp (DefA1/A3, lanes 2, 3 and 7). No PCR-product after enzymatic restriction (lane 8).

Table 1. Profile of *Candida* isolates from three group samples.

Groups	*C. albicans*		Non-*albicans*		Total
	S	R	S	R	
Asymptomatic	20	5	5	6	36
VVC	27	2	6	3	38
RVVC	1	5	1	7	14
Total	48	12	12	16	88

S: susceptible, R: resistant, VVC: vulvovaginal candidiasis, RVVC: recurrent vulvovaginal candidiasis.

Table 2. Alpha-defensin genes according clinical presentations of the groups.

*α*DF-genes	Asymptomatic	Non-RVVC	RVVC
α-DF1	4	L (3), M (1)	M (5), S (4)
α-DF3	4	L (1)	S (1)
α-DF1/*α*-DF3	14	L (4), M (10)	M (4)
No PCR-product	14	L (3), M (15), S (1)	
Total	36	L (11), M (26), S (1)	M (9), S (5)

L: mild; M: moderate; S: severe.

heterozygosis, *C. albicans* and non-*albicans Candida* spp. rate were 28 (50.9%) and 4 (7.3%), while in homozygosis were 10 (18.2%) and 13 (23.6%), respectively.

4. Discussion

Defensins, important peptides found in polymorphonuclear and epithelial cells, participate in the innate immune response by acting as critical immune adjuvants against pathogenic microorganisms before the acquired immune response is mounted [31]-[35]. In addition to inducing cytokine production, which ensures activity against Gram-positive and Gram-negative bacteria, fungi, and viruses, including non-enveloped viruses such as HIV, *α*-DF immunostimulates T cells, monocytes, and immature dendritic cells [19] [31] [36] [37].

Candida colonization can induce a high expression of defensins [38] [39]. Neutrophils release *α*-DF1 to peri- and extracellular matrix as a important factor against *Candida* [40]. The presence of polymorphisms can act on host-parasite relationships [12] [28] [29]. We observed more high frequencies of *α*-DF homozygote genotypes in the group of recurrent patients than in the non-recurrent and control groups, which suggest that the polymorphism might have an important role in VVC relapses. Although the presence of polymorphisms in *α*-DF genes, which may be considered as a relevant factor for the maintenance of VVC recurrent, other factors such as the hormonal influence, innate immune defense, microbiota, physiological regulation and the species involved in the process should be taken into consideration.

Candida albicans and non-*albicans Candida* spp. have become more resistant to azoles in the last decades, whereas nystatin has shown good results *in vitro* and *in vivo*, including against the fluconazole-resistant yeast strains [41] [42]. Clinical and mycological cure over 90% of cases were provide after short treatments with oral azoles and/or nystatin topical. Oral fluconazole dose (150 mg once-weekly) has been recommended as standard therapy for VVC and recurrence, showing high therapeutic efficacy [43] [44]. Two oral doses of fluconazole 150 mg regimen were effective in the treatment of severe VVC [45]. However, prolonged treatment with triazole and polyenes antifungals, during periods of time between 3 to 12 months may be necessary particularly in RCVV cases [42] [46] [47].

5. Conclusion

Our results suggest that the *α*-DF polymorphisms may be related to the recurrence of VVC and severity of signs

and symptoms especially in homozygotic females. Further investigations on the α-DF genes polymorphism are necessary to better elucidate their rule in pathophysiology of VVC and RVVC, and the possible relationship with symptomatology and therapeutics.

Conflict of Interest

The authors did not report any potential conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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