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ORIGINAL ARTICLE/ARTICLE ORIGINAL

Phospholipase and proteinase activities of *Candida* spp. isolates from vulvovaginitis in Iran

Activités phospholipase et protéinase d'isolats de Candida spp de vulvo-vaginite en Iran

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KEYWORDS
Candida;
Virulence factors;
Vulvovaginal candidiasis

Summary
Objective of the study. — This study aims to characterize phospholipase and proteinase activities of *Candida* isolates from 82 vulvovaginal candidiasis (VVC) and to study the relationship of these activities with vulvovaginitis.
Methods. — Totally 82 *Candida* isolates from vagina samples of VVC patients were randomly collected over the period between September and December 2014 from hospitalized patients at the general hospitals of Lorestan province, Iran. Isolates were previously identified by conventional mycological methods. The phospholipase and proteinase activities were evaluated by Egg yolk agar, Tween 80 opacity medium and agar plate methods.
Results. — The most common *Candida* species was identified *Candida albicans* ($n = 34$, 41.5%), followed by *Candida famata* ($n = 13$, 15.8%), *Candida tropicalis* ($n = 11$, 13.4%), and *Candida parapsilosis* ($n = 9$, 11%). The most phospholipase activity was observed in *Candida colliculosa* (40%), followed by *C. famata* (38.5%), and *Candida krusei* (33.3%). The findings revealed that the correlation between phospholipase production by *Candida* spp. and the presence of VVC was not found to be statistically significant ($P = 0.91$). All *Candida* spp. exhibited considerable proteinase activity; so that 100% of *C. colliculosa*, *C. parapsilosis*, *Candida kefyr*, and *Candida intermedia*

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isolates produced high proteinase activity with Pz 4+ scores. There was a significant correlation between proteinase production by *Candida* spp. and the presence of VVC ($P = 0.009$).
Conclusion. — The obtained findings revealed that *Candida* spp. isolates may produce both virulence factors, phospholipase and proteinase. Although the phospholipase production was only observed in < 40% of the isolates; however there was a significant association between proteinase production by *Candida* spp. and VVC.

MOTS CLÉS
Candida ;
Facteurs de virulence ;
Candidose vulvo-vaginale

Résumé
Objectif de l'étude. — Cette étude vise à caractériser les activités de phospholipase et protéases de *Candida* isolé de 82 candidoses vulvo-vaginales et à étudier la relation de ces activités avec la vulvo-vaginite.
Méthodes. — Au total, 82 *Candida* isolés à partir d'échantillons de patientes avec une vulvo-vaginite ont été prélevés de manière aléatoire sur la période entre septembre et décembre 2014 chez des patients hospitalisés dans les hôpitaux généraux de la province du Lorestan, en Iran. Les isolats ont été préalablement identifiés par des méthodes classiques de mycologie. Les activités phospholipase et protéase ont été évaluées par le jaune d'œuf agar, l'opacité du milieu au Tween 80 moyen et les méthodes de gélose en plaque.
Résultats. — L'espèce de *Candida* le plus souvent isolée a été *Candida albicans* ($n = 34$, 41,5 %), suivie par *Candida famata* ($n = 13$, 15,8 %), *Candida tropicalis* ($n = 11$, 13,4 %), et *Candida parapsilosis* ($n = 9$, 11 %). L'activité la plus phospholipasique a été observée chez *Candida colliculosa* (40 %), suivie par *C. famata* (38,5 %), et *Candida krusei* (33,3 %). Les résultats ont révélé que la corrélation entre la production de phospholipase par *Candida* spp. et la présence de VVC n'a pas été statistiquement significative ($p = 0,91$). Tous les *Candida* spp. présentaient une activité de proteinase considérable ; 100 % des *C. colliculosa*, *C. parapsilosis*, *Candida kefyr*, *Candida intermedia* produisaient une grande activité proteinase avec Pz scores 4+. Il y avait une corrélation significative entre la production de proteinase par *Candida* spp. et la présence de VVC ($p = 0,009$).
Conclusion. — Les résultats obtenus ont révélé que *Candida* spp. peuvent produire des facteurs de virulence: phospholipase et protéinase. Bien que la production de phospholipase n'a été observée que dans < 40 % des isolats, il y avait une association significative entre la production de protéinase par *Candida* spp. et VVC.

Introduction

Candidiasis is a fungal infection caused by yeasts that belong to the genus *Candida*. Since the last three decades, *Candida* spp. has emerged as an important cause of health care associated and opportunistic infections [20]. The increased use of intravenous catheters, total parenteral nutrition, broad spectrum antibiotics, and cytotoxic chemotherapy and an increase in the population of immunocompromised patients have contributed to the increase of candidiasis [11]. The clinical spectrum of candidiasis is different ranging from mucocutaneous over growth to systemic infections such as candidemia. Genital/vulvovaginal candidiasis (VVC) is also called a ''yeast infection,'' and it occurs when there is overgrowth of the normal yeast in the vagina. This infection is estimated to be the second most common cause of vaginitis after bacterial vaginosis [25]. Although the most infections are related to *Candida albicans*, other species of *Candida* account for more than 50% of fungal infections [19]. Previously it has been proven that expression of virulence factors like germ tube formation, adhesins, phenotypic switching, biofilm formation and the production of hydrolytic enzymes contribute to the pathogenesis of candidiasis [5,18]. However, expression of virulence factors may vary depending on the infecting species, geographical origin, type of infection, the site and stage of infection, and host reaction [3]. Today, understanding these virulence factors will be a main tool to determination pathogenesis of candidiasis and also will help discovery new antifungal drug targets for improved therapeutic regimens [7]. Several studies have demonstrated that *Candida* species are able to secrete many exoenzymes such as phospholipase, esterase, hemolysin, and proteinase that are necessary to colonize and invade host tissues [9,12,24,28]. In Iran, Pakshir et al. (2013) have demonstrated that among 84 *Candida* isolates from onychomycosis, and oral lichen planus patients, the most of the isolates tested had different enzymatic patterns and *Candida parapsilosis* strains had less phospholipase activity [18]. A review of the available literature has showed there is no information regarding the enzymatic activity of *Candida* species isolated from VVC in Iran. Therefore, this study for the first time aims to investigate the phospholipase and proteinase activities of vaginal isolates of *Candida* and their relationship with the presence of VVC.

Materials and methods

Ethical statement

This study was approved by Ethics Committee of Lorestan University of Medical Sciences. In addition, a written informed consent was obtained from all the participants before sampling.

Clinical isolates

Totally 82 *Candida* isolates from vagina samples of VVC patients were randomly collected over the period between September and December 2014 from hospitalized patients at the general hospitals of Lorestan province, Iran. Vaginal sampling of the participants performed by using a sterile swab by the principle researcher and was cultured simultaneously onto sabouraud dextrose agar (SDA) medium. Standard strains of *C. albicans* (Persian Type Culture Collection, PTCC 5027) and *C. glabrata* (CBS 138) were kindly repapered from Department of Medical Mycology, Iran University of Medical Sciences (Tehran, Iran).

Candida identification

Identity of clinical isolates was confirmed by conventional mycological methods, such as the germ tube test in serum, microscopical morphology and chlamydospore formation in corn meal agar (Oxoid, Basingstoke, UK) supplemented with Tween 80, and carbon source assimilation by means of the commercial kit ID 32C (bioMérieux). When necessary, the identification was confirmed using PCR-RFLP with specific primers of ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) according to the method described elsewhere [15]. The PCR cycling conditions comprised: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. During the second step, PCR products were digested with the restriction enzyme *Hpa*II (Fermentas, Vilnius, Lithuania). Five microliters of each PCR amplicons and 10 μL of RFLP products were separated by gel electrophoresis on 1.5% and 2% agarose gel (containing 0.5 μg/mL ethidium bromide), respectively.

Preparation of the inoculum

Isolates were grown on SDA plates for 24 h at 37 °C. After the incubation, sufficiently grown microorganisms were inoculated in sterile saline (0.85%), and then standardized according to the turbidity to 5×10^3 CFU (McFarland n° : 0.5) per well in RPMI medium under sterile conditions. Serial dilutions were prepared in 100 μL RPMI medium with an equal amount of the test samples, and 100 μL each microorganism suspension was pipetted into each well and incubated at 37 °C for 24 h [13].

Phospholipase activity

Phospholipase activity of the isolates was carried out using the egg yolk agar plate method as described elsewhere [16]. Briefly, a base medium containing 65 g SDA, 58.4 g NaCl, 5.5 g $CaCl_2$ was dissolved in 980 mL of distilled water. The solution was autoclaved, and after cooling down to 50 °C, the base medium was mixed with 20 mL of sterile egg yolk emulsion (Merck, Germany). Plates were inoculated and incubated at 37 °C, and results were recorded after 6 days. The phospholipase activity of the isolates was interpreted as positive when a precipitation zone was visible around the growth. In addition, the value of phospholipase activity (Pz) was determined as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone, and was scored and categorized as follows: Pz-1 (negative); 0.35—0.5 (high producers, +++); 0.51—0.74 (moderate producers, ++); and 0.75—0.9 (low producers, +). Each isolate was tested in triplicate and the phospholipase activity value was recorded as the average of the three measurements.

Proteinase activity

Proteinase activity of *Candida* isolates were evaluated by their ability to secrete aspartyl proteinase on a solid medium according to the method described by Cassone et al. [4], with some modifications. At first, a solution was prepared by dissolving 11.7 g yeast carbon base (Difco, Sparks, MD, USA), 0.1 g yeast extract and 2 g bovine serum albumin, (Sigma-Aldrich, St Louis, MO, USA) in 200 mL of distilled water. The solution was sterilized by filtration, and added to a previously sterilized stock solution of 16 g of Bacto-Agar (Difco, Sparks, MD, USA) in 800 mL of distilled water. After inoculation, plates were incubated at 37 °C, and results were recorded after 6 days of incubation. Enzyme activity was measured as the diameter of a lytic area around the growth on serum albumine and scored as non-producers (−) when there was no visible lysis, as moderate producers (+) when the width of the halo was 1—2 mm, and highly producers (++) when the width of the halo was more than 2 mm. Each isolate was tested in triplicate, and the enzyme activity value was taken as the average of the three measurements.

Statistical analysis

SPSS Software version 17 (SPSS Inc., Chicago) was used for data entry and statistical analysis.

Chi-square test was used to assess the correlation of proteinase activity with the presence of VVC. Assessment of the normality of data was performed by Explore test. Moreover, *T*-test was used to correlate the Pz values in phospholipase production. Values of $P < 0.05$ were considered statistically significant.

Results

Totally 82 *Candida* isolates from VVC patients were isolates. A total of ten species including *C. albicans*, *Candida tropicalis*, *Candida kefyr*, *Candida colliculosa*, *C. parapsilosis*, *Candida intermedia*, *Candida famata*, *Candida krusei*, *Candida mundia*, *C. glabrata*. The most common *Candida* species was identified *C. albicans* ($n = 34$, 41.5%), followed by *C. famata* ($n = 13$, 15.8%), *C. tropicalis* ($n = 11$, 13.4%), and *C. parapsilosis* ($n = 9$, 11%) (Table 1).

Phospholipase activity

Table 1 indicates phospholipase activity of all *Candida* species isolates. Majority of the positive phospholipase isolates had Pz 3+ scores. The most phospholipase activity was observed in *C. colliculosa* (40%), followed by *C. famata* (38.5%), and *C. krusei* (33.3%). Moreover, among *C. albicans* isolates as the most frequent species only 10 isolates (29.4%) exhibited high phospholipase activity and other twenty-four did not present any activity. The findings revealed that the correlation between phospholipase production by *Candida* spp. and the presence of VVC was not found to be statistically significant ($P = 0.91$).

Proteinase activity

As shown in Table 2 all *Candida* spp. exhibited considerable proteinase activity; so that 100% of *C. colliculosa*, *C. parapsilosis*, *C. kefyr*, and *C. intermedia* isolates produced high proteinase activity with Pz 3+ scores. One isolate of *C. albicans* (2.9%) had moderate proteinase activity, whereas the remaining 33 isolates (97.1%) exhibited a high proteinase activity. There was a significant correlation between proteinase production by *Candida* spp. and the presence of VVC ($P = 0.009$).

Discussion

More than 17 different *Candida* spp. are known to be etiological agents of human infections, ranging from superficial candidiasis of the skin and mucosal surfaces to more serious, life-threatening infections. However, more than 90% of invasive infections are caused by *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* [20]. Previously it has been proven that *Candida* isolates exhibited significantly more extracellular enzyme activity than commensal ones [3]. Nowadays, various roles have been ascribed to phospolipase and proteinase activities of clinical isolates of *Candida* spp. [17,26].

In the present study, the most common *Candida* species was identified *C. albicans* (41.5%), followed by *C. famata* (15.8%), *C. tropicalis* (13.4%), and *C. parapsilosis* (11%). Interestingly and unlike to other studies we found *C. famata* as the second most common *Candida* species. Consistent with these findings, Ramos Lde et al. (2015) have demonstrated *C. famata* (18.96%) as the second most common *Candida* species of obtained from individuals with cutaneous candidiasis in Brazil [23]. *C. famata* has been described in human infections, including catheter-related bloodstream infections, peritonitis, acute zonal occult retinopathy and mediastinitis [2]. It is a rare cause of candidiasis, accounting for only 0.2%–2% of isolates collected from antifungal surveillance studies [21]. The high prevalence of *C. famata* in the VVC isolates may indicate the emergence of an uncommon species of *Candida* and competing for the most common cause of candidiasis, *C. albicans*.

Here, we found that *Candida* isolates had phospholipase activity ranging from 27 to 40%, indicating that there was no correlation between phospholipase production by *Candida* spp. and the presence of VVC. Review have reported that *Candida* spp. have 30–100% phospholipase activity depending on the site of isolation or the presence of a *Candida*-associated disease [6]. In line with our results, Kantarcioglu and Yucel (2002) did not observe any difference in enzyme activities of *C. albicans* isolated from healthy people or from distinct anatomical sites [10]. Moreover, Thiele et al. (2008) demonstrated a higher phospholipase activity in oral *Candida* isolates from persons without denture stomatitis [27]. Conversely, Zarei Mahmoudabadi et al. (2010) have shown that the phospholipase activity was detected in all tested isolates with a high level in Pz; so that 100% clinical isolates of *C. albicans* from vaginitis and urine samples demonstrated phospholipase activity [29].

Our results similar to the study conducted by Abaci (2011) reported that all *C. albicans* isolates with the ability to produce the phospholipase activity showed the high level of enzyme activity [1]. We found that phospholipase activity was observed in the only 27.3% *C. tropicalis* isolates ranging from week to potent enzyme activity. Similarly, Galán-Ladero et al. (2010) have demonstrated very low phospholipase activities in *C. tropicalis* isolates from hospitalized patients [8]. In the present study, *C. parapsilosis* and *C. glabrata* isolates exhibited no phospholipase activity; whereas *C. krusei* and *C. famata* showed 33.3 and 38.5% phospholipase activity. Consistent with our findings, Pinto

Table 1 Phospholipase activity of *Candida* isolates from vulvovaginitis.
Activité phospholipase de Candida *isolé de vulvo-vaginite*.

Candida spp.	Phospholipase activity, *n* (%)				Total
	Negative	+	++	+++	
C. albicans	24 (70.6)	0 (0)	0 (0)	10 (29.4)	34 (100)
C. tropicalis	8 (72.8)	1 (9.1)	0 (0)	2 (18.2)	11 (100)
C. kefyr	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
C. colliculosa	3 (60)	0 (0)	1 (20)	1 (20)	5 (100)
C. parapsilosis	9 (100)	0 (0)	0 (0)	0 (0)	9 (100)
C. intermedia	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
C. famata	8 (61.5)	0 (0)	1 (7.7)	4 (30.8)	13 (100)
C. krusei	2 (66.7)	0 (0)	0 (0)	1 (33.3)	3 (100)
C. mundia	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
C. glabrata	4 (100)	0 (0)	0 (0)	0 (0)	4 (100)

+++: high producers of proteinase; ++: moderate producers of proteinase; +: low producers of proteinase.

Table 2 Proteinase activity of *Candida* isolates from vulvovaginitis.
Activité protéinase de Candida *isolés de vulvo-vaginite*.

Candida spp.	Proteinase activity, *n* (%)				Total
	Negative	+	++	+++	
C. albicans	0 (0)	1 (2.9)	0 (0)	33 (97.1)	34 (100)
C. tropicalis	0 (0)	1 (9.1)	2 (18.2)	8 (72.7)	11 (100)
C. kefyr	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)
C. colliculosa	0 (0)	0 (0)	0 (0)	5 (100)	5 (100)
C. parapsilosis	0 (0)	0 (0)	0 (0)	9 (100)	9 (100)
C. intermedia	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)
C. famata	0 (0)	0 (0)	1 (7.7)	12 (92.3)	13 (100)
C. krusei	0 (0)	1 (33.3)	0 (0)	2 (66.7)	3 (100)
C. mundia	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)
C. glabrata	0 (0)	0 (0)	1 (25)	3 (75)	4 (100)

+++: high producers of proteinase; ++: moderate producers of proteinase; +: low producers of proteinase.

et al. (2008) have demonstrated that *C. albicans* was the only species producing phospholipase enzyme among *Candida* species isolated from patients with denture-related stomatitis, while other *Candida* species such as *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. famata* did not show phospholipase activity [22].

The obtained findings in the current study demonstrated that all *Candida* spp. exhibited considerable proteinase activity so that 100% of *C. colliculosa*, *C. parapsilosis*, *C. kefyr*, and *C. intermedia* isolates produced high proteinase activity with Pz 4+ scores. There was also a significant correlation between proteinase production by *Candida* spp. and the presence of VVC. Previously, Pinto et al. (2008) have indicated that *C. albicans* and *C. parapsilosis* exhibited 90 and 100% proteinase activity, respectively [22]. However, Marcos-Arias et al. (2011) have shown that proteinase production was observed in $< 30\%$ of *Candida* isolates from denture wearers, and it was not related to the presence of denture stomatitis [14]. In the other study conducted by Kantarcioglu and Yucel (2002), protease activity was detected in 57 (95%) of *C. albicans* strains tested and in a few strains of *C. kefyr*, *C. lipolytica*, *C. parapsilosis* and *C. tropicalis* — the remaining isolates were negative [10]. Reviews have also reported that these differences in the enzyme activity of *Candida* isolates might be associated to some factors such as the method used, sample size, geographical origin, type of infection, the site and stage of infection, host reaction, and especially differences in enzyme patterns [3].

Conclusion

The obtained findings revealed that *Candida* spp. isolates may produce virulence factors, phospholipase and proteinase. Although the phospholipase production was only observed in $< 40\%$ of the isolates; however there was a significant association between proteinase production by *Candida* spp. and VVC.

Disclosure of interest

The authors declare that they have no competing interest.

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