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Virulence Factors and Susceptibility Patterns of Candida Strains Isolated from Patients with Vulvovaginal Candidiasis

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ÖZET

Candida türleri vajinada kolonize olabilmek için çok sayıda virülans faktörü salgılarlar. Bunlardan en önemlisi epitel hücrelerine tutunma ve proteinaz ve fosfolipaz gibi hücre dışı enzimler salgılamalarıdır. Bu çalşmada Candida suşlarının adezyon yetenekleri, enzim üretimleri ve antifungal duyarlılıkları incelenmiştir. Candida albicans suşlarının çoğunun enzim üreticisi olduğu, vajinal epitel hücrelerine adezyon yapabildiği saptanmıştır.

Anahtar kelimeler: Candida, virülans faktörleri, adezyon, proteinaz, fosfolipaz, antifungallere duyarlılık

SUMMARY

Vulvovajinal Kandidozlu Hastalardan İzole Edilen Candida Suşlarının Virülans Faktörleri ve Antifungallere Duyarlılık ları

Candida strains have different virulence factors to colonize vagina and to remain permanent there. The most important factors are the ability to adhere to epithelial cells and to produce the extracellular enzymes such as proteinases and phospholipases. In this study, we investigated the adherence ability, enzyme productions and the susceptibility patterns of the Candida strains. It was shown that the majority of Candida albicans strains were enzyme producers and were also adhesive to vaginal epithelial cells.

Key words: Candida, virulence factors, adhesion, proteinase, phospholipase, antifungal susceptibility

INTRODUCTION

Vulvovaginal candidiasis (VVC) is a significant problem for women of childbearing age. Approximately 75% of all women experience at least one episode of VVC during their life time. Candida albicans, a commensal organism of the gastrointestinal and reproductive tracts, is the causative agent in approximately 85 to 90% of cases of VVC or recurrent VVC (RVVC) (1,2,3). Candida strains possess a number of virulence factors which aid their colonization and permanence in vagina. These include the ability to adhere to host tissue, to undergo a dimorphic transition and to produce the extracellular hydrolytic enzymes such as acid proteinases and phospholipases. It has been shown that these enzymes play a role in the pathogenicity of C.albicans and may assist adherence to and penetration of cell membranes (4). Proteinases hydrolise peptide bonds and phospholipases hydrolise phospholipids (5,6).

Our purpose, in this report is to analyse the production of proteinase and phospholipase, to show the adherence ability and susceptibility patterns of Candida strains isolated from a group of women presenting with vaginitis.

MATERIALS AND METHODS

Clinical isolates

Vaginal swabs were taken from 70 women (aged between 15-49 years) presenting with symptoms of vaginitis. Ten women as healthy controls were non-pregnant, nondiabetic, and > 19 years of age and asymptomatic for vaginitis. Swabs were streaked onto Sabouraud Dextrose Agar (SDA) and incubated at 37°C for 48 h.

Identification Yeast isolates were identified by using the ID32 C strips (bioMerieux, France). The identity of C.albicans was confirmed by polimerase chain reaction (PCR) method using C.albicans specific primers recommended by Miyakawa et al (7).

The DNA was extracted from all the isolates by this method with little modifications of the protocol. The PCR was designed to amplify regions of the EO3 gene of C. albicans with primers; 5'-CAC CAA CTC GAC CAG TAG GC-3' and 5'-CGG GTG GTC TAT ATT GAG AT-3'.

Adherence assay

The adherence ability of the yeast isolates was determined by the method of Kimura and Pearsall (8). Yeast cells were grown to the stationary phase in yeast extract peptone dextrose (YEPD) broth (2% glucose (Difco), 2% bactopeptone (Oxoid), 1% yeast extract (Difco)) at 370C and 200 rpm in orbital incubator for a night. Vaginal epithelial cells (VECs) were suspended in phosphate buffered saline (PBS), washed and mixed with Candida strains (yeast to VEC ratio: 50 to 1, final volume 2ml). This mixture was incubated at 30°C for four hours, and every two hours 100ml amounts were smeared onto microscope slides and were left to dry in open air. The same mixture was incubated for an additional two hour after the supplementation of 10ml glucose and 10ml iodine from the stock solutions of them in 10% concentration. Slides were heat fixed and stained with 0.5% crystal violet solution. The number of yeasts adhering and non-adhering to a total of 100 VECs was determined in each assay.

Phospholipase assay

Phospholipase secretion was measured by the method described by Price et al (9), using SDA plates (6.5% SDA (Oxoid), 5.84% NaCl (Difco), 0.55%CaCl2 (Difco), 8% egg-yolk emulsion (Sigma)). Isolates were grown to the stationary phase in Sabouraud liquid medium (Oxoid). Amounts of 10ml of suspension at a density of 1x108 cells/ml in PBS, were transferred onto SDA plates. After an incubation at 370C for 72h, phospholipase activity was determined as the ratio of the colony diameter to the diameter of the colony plus the zone of precipitation (Pz), using the formula; Pz= B/A (A= Diameter of colony+zone of precipitation, B=diameter of colony).

Proteinase assay

All isolates were tested for their ability to grow and to produce clear zone of hydrolysis in bovine serum albumin (BSA) agar (1% bactoagar (Difco), 0.1% KH2PO4 (Difco), 0.5% MgSO4 (Difco), 1% glucose (Oxoid), 0.16% BSA (Sigma)). Cultures were grown to the stationary phase overnight in Sabouraud liquid medium (Oxoid). Cells were suspended at a density of 1x108 cells/ml in PBS. Amounts of 10ml were placed onto test medium. After an incubation at 37oC for 5 days, the plates were fixed with 20% tricholoroacetic acid and stained with amido black (90% methanol, 10% acetic acid), destained with 15% acetic acid, and the clear zones were measured. Serum aspartly proteinase (SAP) activity was scored as follows: -when no visible clarification of the agar was present, 1+ when a visible clear zone was observed (1 to 2 mm in diameter), and 2+ when agar clarification largely exceeded (by 3 to 5 mm) (10).

Antifungal susceptibility testing

The broth microdilution method was performed as described in National Comittee for Clinical Laboratory Standards (NCCLS) document M27-A (11) with RPMI 1640. Activities of itraconazole (Janssen Pharmacentica), ketoconazole (Janssen Pharmacentica), fluconazole (Pfizer), terbinafine (Novartis) and amphotericin B (Sigma) against Candida spp. were studied. Interpretive guidelines for terbinafine and amphotericin B were not documented in the M27-A. For this reason, in our study susceptibility results were presented as geometric mean values. C.krusei ATCC 6258 and C.parapsilosis 22019 were used as quality control strains.

RESULTS

Candida spp. were isolated from 21 (20 women with vaginitis and 1 carrier) of 80 samples examined (70 women with vaginitis and 10 healthy controls). Twenty (28,5%) out of the 70 samples with vaginitis proved culture positive for yeasts. All of the 20 isolates were examined. A single yeast species was isolated in each of the 20 patients in the culture positive population. C.albicans was isolated from 13 (65%), C.glabrata from 3 (15%), C.krusei from 3 (15%) and C.inconspicua from one (5%). One C.albicans was isolated from the group of asymptomatic controls (10%).

Adherence of Candida strains varied considerably

among the different species. Figure I shows the percentage of adhesion presented as the number of yeasts adherent to 100 VECs. Clinical C.albicans isolates showed adhesion as 28 yeasts adherent to 100 VECs (28%) after 2h incubation. One strain of three C.glabrata isolates showed 4% adherence while the other two C.glabrata were non-adherent. Only one C.krusei strain showed 25% adherence. C.inconspicua showed no adherence ability. C.albicans isolated from the 10 healthy control showed 12% adherence. The ratio of the mean number of adherent yeasts to 100 VEC was 46.8 for C.albicans isolates, when the incubation was prolonged to 4h. There was statistically significant difference between 2h and 4h results ($p<0.05$). After the glucose supplementation, the ratio of yeasts to VEC was 59.4 to 100 ($p<0.05$) and after the iodine supplementation it was 35.6 to 100 ($p>0.05$).

The majority of C.albicans strains (84.6%) produced phospholipase while the other tested species of Candida genus such as C.glabrata, C.krusei and C.inconspicua were all phospholipase non-producing strains (Figure II and Picture I). C.albicans isolated from the healthy control was phospholipase negative. All isolates of C.albicans expressed an enzymatically active SAP. However, no production was observed in the non-albicans Candida species (Picture II). C.albicans isolated from the healthy control was proteinase negative.

Figure I: Adhesion values of Candida strains

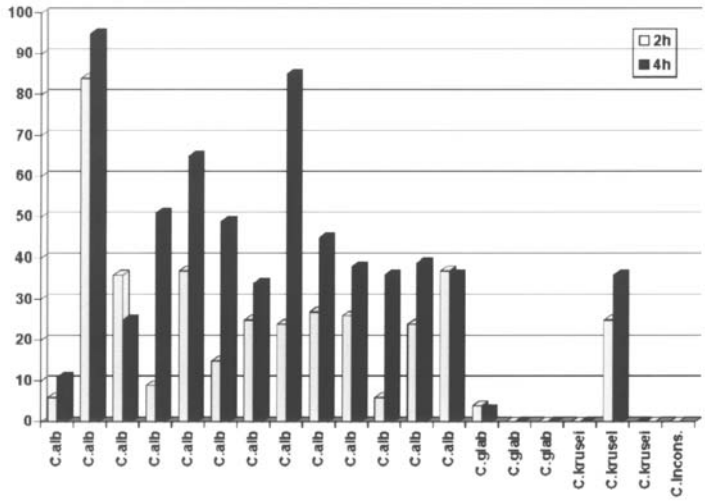
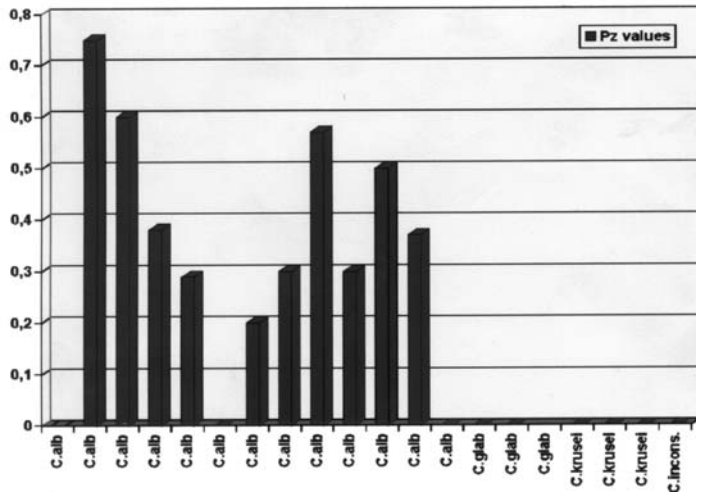
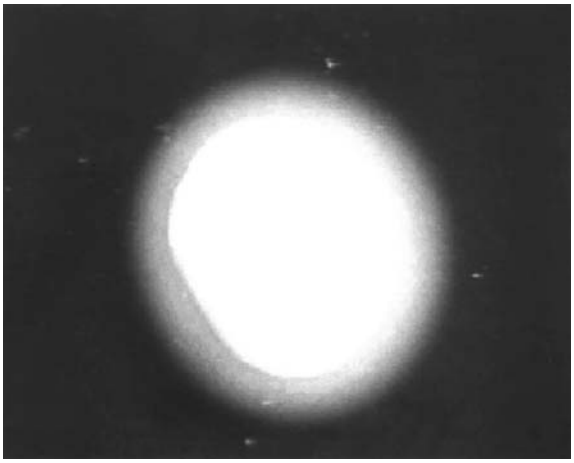


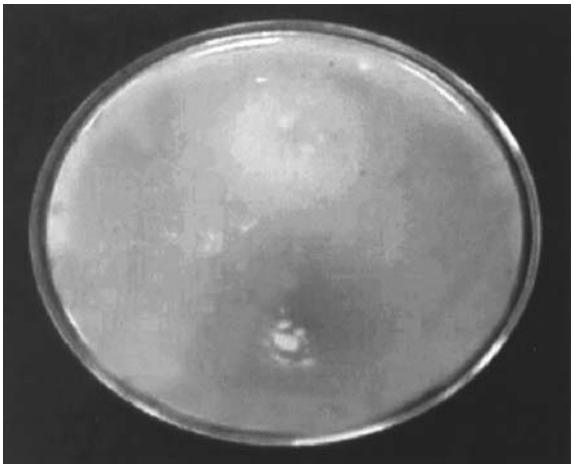
Figure II: Pz values of Candida strains



Picture I: Morphology of phospholipase positive colony



Picture II: Morphology of proteinase positive and negative colonies



Susceptibility results of Candida strains to fluconazole, itraconazole, ketoconazole, ampho-tericin B and terbinafine, were also measured. All the isolates except C.krusei, were sensitive to all the antifungal drugs above. The results obtained from the quality control strains indicated that our test was in the range of 'good quality' according to NCCLS recommendations. MIC ranges were narrow such as; <0.125-8µg/ml for fluconazole, <0.0625-8µg/ml for ketoconazole, 0.0625-8µg/ml for itraconazole, <0.0312-4µg/ml for amphotericin B, <0.0312-8µg/ml for terbinafine. C.krusei strains were evaluated as fluconazole resistant strains, regardless of their MIC results. MIC values for C.krusei strains were as follows: >32-64µg/ml for fluconazole, 8µg/ml for ketoconazole, 2-4µg/ml for itraconazole, 0.5µg/ml for terbinafine, and 0.0625-0.125µg/ml for amphoterisin B.

DISCUSSION

In the present study, we have shown that phospholipase and proteinase production combined with adherence ability promote virulence. The postulate that there is a correlation between extracellular enzymes such as proteinase and phospholipase and virulence in Candida species has been indicated by many authors (6,12,13). The majority of clinical C.albicans strains are proteinase and phospholipase producers. These two enzymes allow modification of the host-cell interaction and assist adherence to epithelial cells (14). The 84% positivity for phospholipases and 100% positivity for proteinases in C.albicans strains included in this study suggest that these enzymes may be the virulence factors for vaginal candidasis. Thus, the pathogenesis of candidal vaginitis may depend on the production of proteinase and phospholipase as a critical central point. This data correlated well with the other studies concerning the extracellular enzymes as pathogenicity determinants of C.albicans (12,14). Proteinases and phospholipases, may account for pathogenicity and such extracellular enzymes remain to be assessed in a category of adhesion molecules. Candidal mannoproteins seem to be the most important adhesins in mediating attachment to host cells (15,16,17,18). We have established that the majority of C.albicans strains were highly adhesive to VECs and produced the two enzymes assayed. C.albicans adheres to VECs in vitro in greater degree than other Candida spp. and this may explain why some species are isolated more frequently from mucosal surfaces (18,19,20). C.krusei, one of the non-albicans Candida strains of our study, showed 25% adhesion; one C.glabrata showed 4% adhesion and C.inconspicua was not adhesive. Yeast and filamentous forms adhered strongly to the exfoliated, keratinized epithelial cells of the vagina. Our in vitro adherence assays also demonstrated that C.albicans strains expresses marked adhesive properties to epithelial cells, to an extent at least equal to that shown by the proteinase-producer strain, and significantly higher than the adherence expressed by a non-proteinase-producer strain such as C.krusei, a negligibly adhesive yeast. Glucose was evaluated as an augmentative factor for the adherence of Candida strains to VECs. It is possible that the accumulation

of glycosylation products in epithelial cells may increase the number of receptors for C.albicans on epithelial surfaces. An important finding in the present study was that, in vitro iodine was not effective as an inhibitory factor against Candida adhesion to epithelial cells. It was thought that curative role of iodine is apart from the inhibition of atherence of yeast cells in the vagina.

C.albicans isolates used in this study were identified according to current taxonomic criteria, including the ability to form germ tubes in serum. All our isolates readily formed germ tubes in serum. Germ tubes may also confer virulent properties to the yeast by favoring increased adherence to the epithelium, with adherence itself being the determinant of pathogenicity (19). The confirmation of C.albicans identity was made by using molecular methods, for a definite determination of the relation between C.albicans and virulence factors cited here. The aim of using PCR for the re-naming of C.albicans strains was to obtain definiteness in their identification.

This work sought to determine the relation between the antifungal susceptibility patterns and virulence factors of Candida strains in vaginitis. All the isolates in this study were susceptible to antifungal agents, except C.krusei isolates, that were resistant to fluconazole in vitro. Althought the majority of the Candida strains were positive for the virulence factors such as adherence and enzyme producing, they were not resistant to antifungal agents. C.krusei strains were resistant but also negative for proteinase and phospholipase enzymes. No correlation was observed in terms of their virulence ability and susceptibility patterns to antifungal agents. The low MICs of terbinafine made us believe that it can be used as an alternative for the treatment of vaginal candidiasis in any case of resistance.

In conclusion, the present study has demonstrated a simple and reproducible method for investigating candidal adhesion in vitro. VVC is a valuable clinical entity for investigating proteinase, phospholipase production and adhesion as pathogenicity factors of C.albicans strains.

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