DETERMINE PHOSPHOLIPASE AND ASPARTIC PROTEINASES ACTIVITY AS VIRULENCE FACTORS IN CANDIDA SPECIES ISOLATED FROM VAGINAL INFECTION IN KIRKUK CITY

M.Sc. Bari Lateef Mohammed*, Pro. Dr. Kalil I. Bander2, Dr. Thekra A. Hamad3

1Department of Biology, Science College, Kirkuk University.
2Department of Biology, Science College, Tikrit University.
3Department of Microbiology in Medicine College, Tikrit University.

ABSTRACT
Vaginal candidiasis is a common complaint among women of different age groups in any society whether or not they are sexually active. Although it is both treatable and mild, when left untreated, The study was set to detect Candida organisms in women vaginal. A prospective study of women vaginal swabs collected from Gynecological Clinic of Azadi teaching Hospital and some privet clinical Kirkuk, Iraq, and a total of two hundred (200) women swab were analysed for microscopy and culture, May 2014 and April 2015. Data on epidemiologic indices were collected from the patients, using structured interviewer- administered questionnaires. fifty- seven positive cultures were obtained, constituting 28.5% (n = 57) in a total of 200 women vaginal samples . A total of 28.5% ( n= 57) isolates were detected which comprises four different Candida species, namely Candida albicans, C. glabrata, C. krusei and C. kefyr respectively as well as isolated Cryptococcus lorynti with frequency of occurrence of 23%, 2%, 1.5%, 0.5% and 1.5% respectively. The extracellular phospholipases and aspartic proteinase of Candida species are considered to play a significant role in the pathogenesis of human infections. Therefore eighteen clinical isolates of Candida species from human in vaginal infection of this study was to evaluate enzymatic activities in vitro were screened for phospholipase production in vitro (using an egg-yolk-agar medium) and aspartic proteinase vitro (using an bovine serum albumin BSA). eighteen isolates were tested by the phospholipase activity from the vaginal samples were C. albicans (10), C. glabrata (4), C. kefyr (3), C. krusei (1). The isolates tested demonstrated low varying degrees of
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In this study showed Phospholipase activity was detected in all of isolates and only minor differences between isolates, The highest enzyme activity detected of C. glabrata (15.65) then C. albicans (11.45), C. krusei (12.00) and the lowest enzyme activity detected of C. kefyr (11) According to these results, there was no statistically significant difference in the mean values of the phospholipase activities of the Candida species (p ≤ 0.05). and the isolates tested demonstrated high varying degrees of Aspartic proteinase activity (Pz value: 0.000000-24.00). In this study showed Aspartic proteinase activity was not detected in all of isolates, The highest enzyme activity detected of C. albicans (12.64) then C. glabrata (3.50), the lowest C. krusei (1.33) and the absence enzyme activity detected of C. kefyr (0) According to these results, there was statistically significant difference in the mean values of the Aspartic proteinase activities of the Candida species (P ≤ 0.05).

KEYWORDS: Candida species, namely Candida albicans, C. glabrata, C. krusei and C. kefyr.

INTRODUCTION

vaginal candidiasis (VC) remains one of the most common infections of the female genital tract. It is considered an important public health problem affecting millions of women worldwide every year. It has been estimated that up to 75% of women will have at least one episode of candidiasis during their lives, with 40% to 50% experiencing chronic recurring episodes (Kelen et al.). vaginal candidiasis is defined as signs and symptoms of inflammation in the presence of Candida spp. and in the absence of other infectious etiology. Over a decade ago, VC was classified into uncomplicated and complicated cases, a classification that has been internationally accepted and adapted (Pappas et al. 2009, Workowski and Berman 2006, Sobel et al. 1998). The common causative agent of vaginal candidiasis is Candida albicans, and occasionally are C. glabrata, C. parapsilosis, C. krusei, C. pseudotropicalis, C. tropicalis, and C. dubliniensis (Arora and Arora 2008, Isibor et al. 2011). Candida albicans, the most virulent of the Candida species, can cause severe oral infections in even mildly immunocompromised hosts. Attributes that putatively contribute to C. albicans virulence include adhesion, hyphal formation, phenotypic switching, (Monod and Borg-von Zepelin, 2002, Cutler 1991), secretion of extracellular enzymes and dimorphism, have been studied by many investigators (Calderone & Fonzi, 2001). Two types of secreted enzyme seem to be the most important: phospholipases and secreted aspartyl proteinases (Ghannoum, 2000; De...
Bernardis et al., 2001). In particular, the secretion of extracellular phospholipases is considered a key attribute that aids invasion of the host mucosal epithelia (Leidich et al., 1998). The phospholipases in general catalyse the hydrolysis of phospholipids, which are major components of all cell membranes (Banno et al., 1985; Salyers & Witt, 1994). The secreted aspartic proteinases (Saps) are responsible for the adhesion, tissue damage and invasion of host immune responses. Their proteolytic activity has been associated with tissue invasion (Hube B, Naglik 2011). Candida infection in the vagina can cause a smelly, thick, white–yellow discharge that might be accompanied by itching, burning and swelling. It can also make walking, urinating or sex very painful. Since the symptoms of vaginal candidiasis are not specific, diagnosis cannot be made solely on clinical basis and should be confirmed by laboratory diagnosis (Sobel 2007).

1.2. Aim of Study
1. The aim of this study was to determine the frequency of Candida species isolation from women with vaginitis in Azadi Hospital and some private clinical in Kirkuk city.
2. Determine phospholipases and proteinase production by the isolated of Candida species.

MATERIALS AND METHOD

Respondents
Patients
The study was conducted on 200 female patients attending the Obstetrics and Gynecological Clinic of Azadi Hospital and some private clinical, Kirkuk, Iraq, from May 2014 and April 2015. All women were complaining of symptoms of vaginitis (Discharge that looks like cottage cheese, Itching, pruritus, Redness and burning (especially during urination), Sex may feel painful or dry, Males may experience an itchy rash on the penis, vulvar burning, urinary complaints and dyspareunia) After careful history taking and complete physical examination. Information about age, Women with severe medical disorders, taking oral contraceptive pills, had taken a course of antibiotics or corticosteroids within the preceding 7 days or had vaginal douching during the previous 48 hours were excluded. Written informed consent was obtained from each participant.

Sampling
The patient was placed in lithotomy position and vaginal exposure was done through introduction of a non-lubricated sterile Cusco speculum. Double high vaginal swabs (HVS)
were collected using sterile cotton tipped swabs and they were sent to the laboratory without delay.

**Microscopic examination**

One swab sample was examined microscopically (40x) after 10% KOH preparation to detect the presence of budding yeast cells and pseudohyphae of Candida species. Yeast isolation was performed on Sabouraud’s dextrose agar (SDA) supplemented with 0.05 g/L Chloramphenicol and CHROMagar Candida to detect mixed infections with more than one species of *Candida*. After 48 hours incubation at 37°C, cultures were examined for pasty, creamy and smooth white colonies of yeasts which were further identified.

**Yeast identification**

**Yeast identification was done to each positive growth on SDA as follows**

**a) Cultivation on the selective medium (CHROMagar Candida)**

CHROMagar Candida is a selective fungal medium that includes chromogenic substances allowing for quick identification of several different *Candida spp.* based on a colour change. It can be used for identification of individual non-albicans species, as well as *C. albicans*, if germ tube test was not characteristic. After incubation in the thermostat (48 hours at 37°C), identification of yeast was preformed based on a colony colour. Using this method, we were able to identify the following individual non-albicans species: *C. glabrata* (dark pink colonies, wet), *C. tropicalis* (blue colonies, wet), *C. krusei* (light pink colonies, dry), and *C. albicans* (green colonies, wet), which also facilitates the detection of mixed infections with more than one species of *Candida* (Odds., and Bernaerts. 1994; Pfaffer. *et al.* 1996). The method is based on the differential release of chromogenic breakdown products from various substrates by Candida species following differential exoenzyme activity (Baker1967).

**Germ Tube Test:** This method was used as a presumptive test for identification of *Candida albicans*. Procedure of (Baker *et al*. 1967) was used in carrying out the test. A single colony of the test yeast cells from a pure culture was inoculated in human serum and incubated at 37°C for 2 - 4 hours. A drop of the incubated serum was placed on a microscope slide and covered with a cover slip. The wet mounts were examined under the microscope for the presence of germ tube using the 40× objective (Dalmau morphology method) (Baker *et al*. 1967). The isolates were classified as either germ tube positive or germ tube negative.
Chlamydospores Formation Test
All Candida isolates were tested for the production of chlamydospores in corn meal agar with Tween 80 (Baker1967). The isolates were inoculated in cornmeal agar. The test involved streaking and stabbing the media with a 48 hour old yeast colony and, covered with sterile cover slip and incubated at 25°C for 72 hours. Chlamydospore production was examined after staining with lactophenol cotton blue (Dalmau morphology method) (Baker1967). The isolates were categorized as chlamydospore positive or negative. The test was used as a presumptive confirmatory test for the identification of Candida albicans.

RapID Yeast Plus System “Remel Co.USA”
The RapID Yeast Plus System consists of 18 wells containing the following tests: utilization of glucose, maltose, sucrose, trehalose, and raffinose; hydrolysis of fatty acid ester; p-nitrophenyl-N-acetyl-b,D-galactosaminide; p-nitrophenylal, D-glucoside; p-nitrophenyl-b,D-glucoside; o-nitrophenyl-b,D-galactoside, p-nitrophenyl- a,D-galactoside; p-nitrophenyl-b,D-fucoside; p-nitrophenyl phosphate; p-nitrophenyl phosphorylcholine; urea; proline b-naphthylamide; histidine b-naphthylamide; and leucylglycyl b-naphthylamide. Procedure for Panels were inoculated according to the manufacturer’s instructions RapID Yeast Plus System is a qualitative micromethod that uses conventional and chromogenic substrates for identification of medically important yeasts, yeast-like fungi, and similar organisms isolated from human clinical specimens (Tasna et al. 1996).

RESULT AND DISCUSSION
Results of microscopic examination From total number samples of test 25.5% (51/200) showed positive using the microscopic examination, while 74.5% (149/20) of microscopic findings were determined as negative, negative microscopic findings (Figure 1) were more than positive microscopic examination.

Detection of exoenzymes production
This study was performed in order to evaluate any possible differences in the proteinase activities of different infectious and colonization strains of C. albicans isolates, and to compare the 2 methods that were used in the in vitro experiments to measure proteinase activity.
Preparation of inocula (Júnior et al. 2011)

The inocula of yeast cells were prepared from stock cultures and incubated for 18 hours at 37°C in YEPD medium and turbidity was visually adjusted to that of a 0.5 McFarland standard producing a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells per ml.

Plate method

Phospholipase (Pl) activity was evaluated using a test medium prepared with Sabouraud dextrose agar (SDA) supplemented with 3% glucose, 1M NaCl, 0.005 M CaCl2 and 8% sterile egg yolk emulsion (Price 1982). Pl activity produces a dense zone of precipitation around the inoculum.

Proteinase activities of the strains were evaluated according to the suggestions of Cassone et al. 1987, with slight modifications. Some isolates were evaluated for their ability to secrete aspartyl proteinase on solid medium containing bovine serum albumin (BSA). Briefly, the yeast were precultured in YEPD medium (2% glucose, 1% yeast extract, and 2% Bacto peptone) and induced to secrete proteinases onto the BSA agar. The medium, containing 1.17% yeast carbon base, 0.01% yeast extract and 0.2% BSA was adjusted to a pH of 5.0, sterilized by filtration, and added to a stock solution of autoclaved (2%) agar. Filter paper disks, with a diameter of 6 mm, were dipped into a suspension of yeast culture at a density of 10^6 yeast mL⁻¹ in YEPD medium and applied to the plate. A maximum of 4 disks were used for each 90-mm-diameter plate. The plates were incubated at 28 °C for 7 days. The plates were observed each day for increased opacity around the disks, caused by growing fungi. The opacity caused by precipitated albumin was observed for subsequent clearing due to hydrolysis by the acid proteinases of the fungi. (Price et al. 1982; De Bernardis et al. 1999).

RESULTS OF ISOLATION

Two hundred (200) of women with symptoms of vaginal candidiasis visiting the antenatal Gynecological Clinic of Azadi Hospital, participated in this study. The results growth of Candida species on Sabourauds agar and CHROMagar Candida showed 28.5% (57/200) positive cultures and 71.5 (143/200) negative cultures tested for vaginal candidiasis infection in the laboratory as shown in Figure 2. This finding is agree with studies recorded in Baghdad AL-Hachami, (2001). The high prevalence of vaginal candidiasis among women may be due to inadequate knowledge, poor personal hygiene, limited diagnostic facilities, poor dietary habits, shortage of effective treatment, increased levels of estrogens and corticoids, wearing of tight-fitting synthetic underclothing, prolonged use of antibiotics which kill the good and
beneficial bacteria (Mikolajczyk et al. 2006, Limia and Lantero 2004). The number of positive women in relation to the total population involved in the study was calculated to give the percentage prevalence of vaginal candidiasis among women attending gynecological Clinic of Azadi Hospital teaching.

Using the formula $= \frac{O}{P} \times 100$

where: $O = \text{The number of individuals with the disease.}$

$P = \text{Total number of individuals in the population involved in the study at the study period.}$

**Distribution frequency of isolates in vaginal infection**

Four *Candida* species were isolated from the women, namely, *C. albicans*, *C. glabrata*, *C. krusei* and *C. kefyre* with percentage occurrence of 23%, 2%, 1.5%, and 0.5% respectively as well as isolated *Cryptococcus lorynti* 1.5%. *C. albicans* had the highest occurrence *C. glabrata* found next after *C. albicans* with 1.5%, *C. krusei* and *C. kefyre* in this study which is comparable with the reports of (Esmaeilzadeh et al. 2009 and Jombo et al. 2011). (Fig. 14). This is similar to the reports of (Shivan and Saldanha 2011) in India and (Rad et al. 2012) in Iran. The high occurrence rate (23%) of *C. albicans* observed in this study is an indication that it is a leading causative agent of the reproductive tract yeast infections in women of child bearing age as also observed (Isibor 2011). These results are comparable to Kalkanci et al. (2012) who reported similar results. This may be due to its virulent factors which include dimorphism and phenotypic switching. *Candida albicans* produces protease and phosphatase which enhance its attachment to human epithelium. It can also be deduced that the high incidence rate of *C. albicans* could be due to increased physiological changes, estrogen and rich glycogen content of the vaginal mucosa thereby providing an adequate supply of utilizable sugar that favor its growth during pregnancy(Wise, et al. 2007). However, Wise et al 2007. and Trofa et al. 2008, reported a low occurrence of *C. albicans* in New York. The low occurrence of *C. albicans* reported by Wise et al. 2007, and Trofa et al. 2008 may be as a result of good personal hygiene, appropriate nutrition, adequate diagnostic facilities and treatment.

**Etiological Agent That Isolated in This Study**

*C. albicans*: *C. albicans* cultures in the Laboratory on Sabourauds (SDA) agar Colonies was white to cream colored smooth, glabrous, on CHROMagar Colonies was green, wet Figure 3. microscopic examination Spherical to sub spherical budding blastoconidia Figure 4. Germ tube positive Figure 5. Dalmau Plate Culture on Cornmeal and Tween 80 Agar: branched
pseudo hyphae with dense blastoconidia. Spherical chlamydoospores, mostly terminal Figure 6,7.

*C. glabrata*: *C. glabrata* cultures in the Laboratory on Sabourauds (SDA) agar Colonies was Small, white, shiny and smooth, on CHROMagar Colonies was dark pink colonies, wet Figure 8, microscopic examination Small, raised, smooth with an entire periphery Figure 9; Dalmau Plate Culture on Cornmeal and Tween 80 Agar: negative. Spherical chlamydoospores, mostly terminal. Germ tube negative.

*C. krusei*: *C. krusei* cultures in the Laboratory on Sabourauds (SDA) agar Colonies was Flat, dry, spreading, ground-glass appearance; variants dull and no separated, glabrous, on CHROMagar Colonies was light pink colonies, dry Figure 10. Microscopic examination Large, flat, dry with a delicate feathery periphery Figure 11. Dalmau Plate Culture on Cornmeal and Tween 80 Agar: negative. Germ tube negative.

*C. kefyre*: *C. kefyre* cultures in the Laboratory on Sabourauds (SDA) agar Colonies was white to cream colored smooth, glabrous, on CHROMagar Colonies was white, wet Figure 12. Microscopic examination Spherical to sub spherical budding blastoconidia Figure 13. Dalmau Plate Culture on Cornmeal and Tween 80 Agar: negative. Germ tube negative.

**Extracellular phospholipase production by Candida species**
eighteen isolates were tested by the phospholipase activity from the vaginal samples were *C. albicans* (10), *C. glabrata* (4), *C. kefyre* (3), *C. krusei* (1). The isolates tested demonstrated low varying degrees of phospholipase activity (Pz value: 4.60-20.00). In this study showed Phospholipase activity was detected in all of isolates and only minor differences between isolates, The highest enzyme activity detected of *C. glabrata* (15.65) then *C. albicans* (11.45), *C. krusei* (12.00) and the lowest enzyme activity detected of *C. kefyre* (11) According to these results, there was no statistically significant difference in the mean values of the phospholipase activities of the *Candida* species (*p* ≤ 0.05).

The findings from the present study partly agrees with findings from similar result by those of Kantarcioğlu & Yucel (2002). their study was on the differences in phospholipase and proteinase production between different *Candida* species, it can be concluded from their data that *Candida* isolates that originated from the respiratory tract showed the highest mean production of both phospholipase and proteinase. Furthermore, compared to other sources
(oral cavity, urogenital system and blood), this site of infection showed the highest number of positive isolates in the phospholipase assay. Price et al. (1982) found that blood isolates were the highest producers of phospholipase, and this finding partly agrees with findings are related to those of earlier reports by Samaranayake et al. (1984) and Kothavade & Panthaki (1998), which mention relatively high numbers of phospholipase producers among clinical oral C. albicans isolates (79 and 89 %, respectively) using an identical plate assay. This finding is different with studies recorded in Baghdad AL-Hachami, (2001). In a study conducted in Turkey, it was reported that proteinases and phospholipases can act as virulence factors that contribute to host tissue invasion by digesting proteins such as hemoglobin, keratin, and collagen, and can also degrade cell membranes. Cerikcioğlu et al. (2004)

Table 1. Phospholipase activity(mm) of colonization of Candida species measured by the plate method.

<table>
<thead>
<tr>
<th>Candida spp.</th>
<th>No.</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>10</td>
<td>11.45</td>
<td>5.11</td>
<td>4.60</td>
<td>20.00</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>4</td>
<td>15.65</td>
<td>5.16</td>
<td>12.00</td>
<td>19.30</td>
</tr>
<tr>
<td>C. krusei</td>
<td>3</td>
<td>12.00</td>
<td>4.24</td>
<td>9.00</td>
<td>15.00</td>
</tr>
<tr>
<td>C. kefyr</td>
<td>1</td>
<td>11</td>
<td>0</td>
<td>11.00</td>
<td>11.00</td>
</tr>
</tbody>
</table>

a: Values within columns followed by similar letters no significantly at probability p ≤ 0.05.

Extracellular Aspartic proteinase production by Candida species

Eighteen isolates were tested by the Aspartic proteinase activity from the vaginal samples were C. albicans (10), C. glabrata (4), C. kefyr (3), C. krusei (1). The isolates tested demonstrated high varying degrees of Aspartic proteinase activity (Pz value: 0.000000-24.00). In this study showed Aspartic proteinase activity was not detected in all of isolates, The highest enzyme activity detected of C. albicans (12.64) then C. glabrata (3.50), the lowest C. krusei (1.33) and the absence enzyme activity detected of C. kefyr (0) According to these results, there was statistically significant difference in the mean values of the Aspartic proteinase activities of the Candida species (p > 0.05).

As phospholipases and aspartyl proteinases of C. albicans are considered important virulence factors Basu et al. (2003) the absence or lowered expression of these enzymes may indicate
the less virulent nature of *Candida* species, when compared with *Candida* species with higher expression of these enzymes (Borst and Fluit 2003, Dagdeviren *et al.* 2005). Proteinase production by *C. albicans* depends not only on strain type or type of infection, but also on phenotypic switch type, environmental conditions and even the stage of infection (De Bernardis *et al.*, 2001). Aspartyl proteinases facilitate invasion by degrading proteins at the site of the infection. However, their role as major virulence factors of *C. albicans* is being questioned (Odds 2008).

In this study showed differences in phospholipase and aspartic proteinase production between different *Candida* species. The extracellular proteinases of eukaryotic microbial pathogens have attracted the attention of many laboratories due to their potential role in pathogenesis. In the genus *Candida*, *C. albicans* is the major human pathogen and it has extracellular proteinases. Gokce *et al.* (2007) analyzed the proteinase, phospholipase, and biofilm production of 68 *C. albicans* and 31 nonalbicans *Candida* strains (11 *C. tropicalis*, 8 *C. parapsilosis*, 6 *C. glabrata*, 4 *C. quillermo*ndii, and 2 *C. krusei*) isolated from bloodstream cultures. In total, 61 (89.7%) *C. albicans* strains were detected as proteinase-positive, whereas 8 (25%) nonalbicans *Candida* strains were proteinase positive.

**Table 2. Proteinase activity (mm) of colonization *Candida* species measured by the plate method.**

<table>
<thead>
<tr>
<th><em>Candida</em> spp.</th>
<th>N</th>
<th>Mean ± Std.Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>10</td>
<td>12.64 ± 8.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00</td>
<td>24.00</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>4</td>
<td>3.50 ± 7.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00</td>
<td>14.00</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>3</td>
<td>1.33 ± 2.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00</td>
<td>4.00</td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>1</td>
<td>0 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.000000</td>
<td>0.000000</td>
</tr>
</tbody>
</table>

a, b: Values within columns followed by different letters differ significantly at probability P >0.05.

**We conclude**

that the extracellular proteinases produced by *candidia* spp are important virulence factors associated with this organism. The absence or lowered expression of these enzymes may indicate the less virulent nature of *Candida* spp. when compared with *Candida* species with higher expression of these enzymes, particularly in immunosuppressed patients, and the
degree of virulence and pathogenicity are correlated with the level of secreted proteinases. Determination of these factors might be a helpful tool to inform the clinicians about the possible virulence of the strain.

REFERENCES


