

Study of haemolytic, esterase activities and germ tube formation in *Candida albicans* from patients with vaginitis and urinary infections

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ABSTRACT

Introduction: The opportunistic fungus *Candida albicans* is a normal flora of human. Due to the increase in the number of immunocompromised patients, infections due to the *Candida* species have considerably increased in the recent years. Extracellular hydrolytic enzymes appear to play an essential role in candidal overgrowth. Dimorphism, adhesions, and the production of hydrolytic enzymes are suggested as *C. albicans* virulence factors and also germ tube formation of *C. albicans* among vaginitis and urinary infections. **Materials and Methods:** One hundred fifty-one *C. albicans* isolates including isolates from patients with vaginitis and urinary infections, and oral cavity were used. All isolated were assessed for germ tube formation in human serum. The haemolytic activity was tested with Sabouraud dextrose agar plate including sheep blood SDA and 3% glucose. Esterase activity was tested with Tween 80 medium and incubated at 37°C. **Results:** The data analysing germ tube formation confirmed significance differences in 0.5 hour and one hour among various sources of isolates. All *C. albicans* isolates from different sources displayed the haemolytic activity after 24 hours. Ninety-five percent of vaginal and urinary isolates demonstrated positive esterase activity. **Conclusions:** These data provide evidence of extracellular enzyme activity and germ tube formation in *Candida* isolates.

Keywords: *Candida albicans*, hemolytic activity, esterase activity, germ tube

INTRODUCTION

The occurrence of invasive fungal infections has drastically increased in the last few years.

¹⁻⁴ *Candida albicans* is the most commonly

isolated fungal pathogen in humans. This fungus is a ubiquitous microflora of human but is also an opportunistic pathogen which can cause both superficial and systemic infections. *C. albicans* causes a major morbidity and mortality, particularly in immunocompromised patients. *C. albicans* is the most commonly isolated microorganism, and the fourth

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important microorganism responsible for infections of bloodstream.⁵ *C. albicans* possess several factors which could be involved in the process of invasion. Dimorphism, adhesions, and the production of hydrolytic enzymes are suggested as *C. albicans* virulence factors. There are many studies on hydrolytic enzymes of *C. albicans* such as proteases, lipases and phospholipases.⁶⁻¹⁰ However little is known about the haemolytic activity of *C. albicans*. In fact, many microorganisms use haemin or haemoglobin as a source of iron for growth in the host.

Rapid recognition of *Candida* isolates in the clinical laboratory is important as the incidence of candidiasis by *C. albicans* is increasing. *C. albicans* displays the capability to develop as yeast form or a mycelial form in response to various factors in the environment.

Germ tube formation is a characteristic morphology in *C. albicans*. Production of germ tube is a rapid technique for identification of *C. albicans*. The formation of germ tube was first reported in 1956 by Reynolds and Braude.¹¹ They reported that yeast cells transform into germ tube in fluid mediums such as blood, plasma, sera and cerebrospinal fluid (CSF). The formation of mycelium in infected tissues may be an important virulence factor for *C. albicans* to adhere to host epithelial tissues. Germ tube is induced from yeast form and transformed into mycelial form.

Different species of *Candida* were suggested to have lipolytic activity. Several species of *Candida* secrete lipolytic enzymes such as phospholipases and esterases. The

esterase activities of *Candida* species have been revealed with the application of the Tween 80 opacity test with various Tween compounds. *C. albicans* secrete an extracellular esterase in medium containing Tween 80 as the sole source of carbon.

The objective of this study was to test the haemolytic, esterase activities and germ tube formation of *C. albicans* isolates from patients with vaginitis and urinary infections.

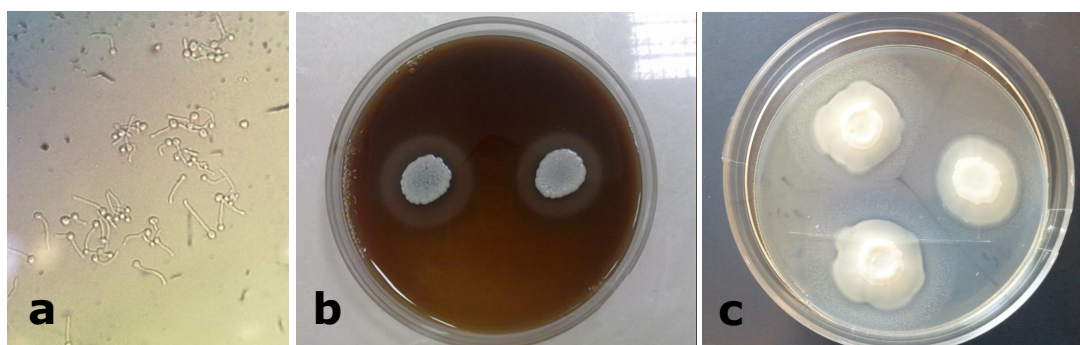
MATERIALS AND METHODS

Organisms: A total of 151 isolates of *C. albicans* were used, including 60 isolates from vaginitis and urinary infections each, 30 isolates of normal flora from oral cavity and standard strain of ATCC 10231.

Growth and preparation of organism: All the yeast isolates were cultured in Sabouraud dextrose agar (Merck, Germany) and incubated at 37°C for 24 hours. The *C. albicans* isolates were confirmed on the basis of germ tube formation on serum, clamidospore formation on corn meal agar medium and growth on CHROMagar *Candida* medium for colony color. The isolates were grown on 45°C for differentiation between *C. albicans* and *C. dubliniensis*.

Germ tube formation test: A small part of *C. albicans* colony was transferred to 0.5 ml human serum and incubated at 37°C for 3 hours. The germ tube formation was observed by a light microscope.

Analyses of germ tube formation: The *C. albicans* isolates were prepared with growing on Sabouraud dextrose agar for 24 hours at



Figs. 1: a) Germ tube formation by *C. albicans* on human serum at 37°C after 3 hours, b) Haemolytic activity of *C. albicans* on sheep blood Sabouraud-dextrose agar supplemented with 3% glucose at 37°C after 48 hours, and c) Esterase activity of *C. albicans* on Tween 80 medium at 30°C after 10 days.

37°C. Yeast cells were diluted to 15×10^9 cells/ml in sterile physiological solution. Aliquots of 100 µl of yeast solution were taken and added to 0.5 ml human serum, and incubated at 37°C for 3 hours. The total number of yeast cells (cells/ml) and germ tube formation were counted with a Neubauer chamber. The germ tube formation (Fig. 1a) was considered as a percentage of cells which formed the germ tube from the total number of yeast cells.

Haemolytic activity

Preparation of Sheep blood SDA: This medium was prepared by adding 5 ml sheep blood to 100 ml of SDA supplemented with 3% glucose (Merck, Germany).

Assessment of haemolytic activity: The haemolytic activity was assessed with plate assay technique described by Luo *et al.*¹² *C. albicans* isolates were streaked out onto SDA and incubated at 37°C for 24 hours. A yeast suspension was prepared, sized 1.5×10 cells per milliliter, with McFarland standard $\neq$ 0.5.

sion were spot on sheep blood SDA with 3% glucose. The plates were kept at 37°C in 5% CO₂ for 48 hours. The presence of a characteristic clear halo around the inoculums spot indicated a positive hemolytic activity of beta (complete).

Esterase assay: Esterase activity was tested with the method described by Price *et al.*¹³ All *C. albicans* isolates were inoculated onto SDA and incubated at 37°C for 24 hours. The Tween 80 medium was prepared with 10.0 g peptone, 5.0 g sodium chloride, 0.1 g calcium chloride, 15 g agar and 1000 ml distilled water. The medium was autoclaved, it was cooled about 50°C, and 5 ml of autoclaved Tween 80 was added and dispensed into Petri dishes. *C. albicans* samples were transferred into the Tween 80 medium with use of a microbiological loop. The inoculated agar Petri dishes were kept at 30°C and observed daily for 10 days (Fig. 1c). All experiments were performed in duplicate. Detection of esterase activity on the test medium was performed with observing halos of precipitation around the inoculated site.

Ten microliters of the yeast suspen-

Statistical analysis: Statistical analyses

Table 1: Germ tube formation of *C. albicans* isolates from various sources.

Sources	Time (h)		0.5		1		1.5		2		2.5		3	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Urinary infection	7	11.7	24	40	38	63.3	48	80	55	91.7	60	100		
Vaginitis	1	1.7	15	25	35	58.3	46	76.7	57	95	60	100		
Oral cavity	0	0	5	16.7	9	30	20	66.7	28	93.3	30	100		
ATCC 10231	-	-	-	-	-	-	1	-	-	-	-	-	-	-

Table 2: Analysis of *C. albicans* isolates according to percentage of germ tube formation for each isolate.

Sources	Vaginitis	Urinary infection	Oral cavity	ATCC 10231
Range	1.6 - 21.6 (%)	2.4 - 25.9 (%)	1.6 - 19.0 (%)	22.4 (%)
Mean	16.2	17.6	16.5	-

were carried out using SPSS version 17.0. Student's test performed for analysing and differences were considered as significant if $p < 0.05$.

RESULTS

Germ tube formation test: Table 1 shows the results obtained with *C. albicans* isolates for germ tube formation in human serum. After 30 min incubation, the transition rate was 11.7% for urinary infections isolates and 1.7% for vaginitis isolates. Germ tube formation was not observed for normal flora from oral cavity and standard strain of ATCC 10231. When culturing for 2 hours, *C. albicans* generated germ tubes with 80% of urinary infections isolates, 76.7% of vaginitis isolates and 66.7% of normal flora from oral cavity. Data analyses confirmed significance differences in 0.5 hour and 1 hour among various sources of isolates ($p < 0.05$).

Analyses of germ tube formation: For each isolate, the total number of yeast cells (cells/ml) which transited to germ tube was counted after 3 hours incubation in serum.

Germ tube formation was considered as a percentage of cells which formed germ tube for each isolate. The mean number of germ tube formation for urinary, vaginitis and oral cavity isolates was 17.6%, 16.2% and 16.5% respectively (Table 2). Data analyses verified significance differences between urinary and vaginitis isolates ($p < 0.05$) and between urinary and normal flora isolates ($p < 0.05$).

Haemolytic activity: All tested *C. albicans* isolates from different sources displayed the haemolytic activity after 24 hours. The haemolytic zone around each colony was recorded and the haemolytic index was calculated (Table 3). The haemolytic index for vagina and urine isolates was approximately similar. The data showed significance difference be-

Table 3: Haemolytic indexes of *C. albicans* isolates.

Sources	n	haemolytic index (mm)
Vaginitis	60	1.83
Urinary infection	60	1.87
Oral cavity	30	1.68
ATCC 10231	1	1.66

tween urine and oral cavity isolates ($p < 0.05$).

Esterase assay: Esterase activity of *C. albicans* isolates was shown in Table 4. Ninety-five percent of vagina and urine isolates demonstrated positive esterase activity. While the percentage for oral cavity isolates was 86%.

DISCUSSION

C. albicans virulence factor is due to the combination of several factors. Evidence of connection between potential virulence factors is recently explained. The formation of germ tubes is the result of converting yeast to a filamentous growth phase. Furthermore, the pseudohyphae formation occurs with cell division when the cells grown by budding have extended without separation from adjacent cells. In this research, there were significant differences among various sources of isolates after 0.5 hour and 1 hour. A report by Ibrahim et al.¹⁴ revealed that *C. albicans* isolates from blood were able to producing longer germ tube in higher frequency than isolates from commensal sources. The data support this idea that the ability of *C. albicans* to transform from yeast cell to hyphae contributes to the fungus pathogenicity.¹⁵ This morphological transition of *C. albicans* frequently suggests a response of the yeast to environmental conditions changes and allows the yeast to adapt to unusual biological nicks.

The ability of pathogenic microorganisms to obtain elemental iron has been shown.^{16, 17} This ability is important for their survival and helps the organism to create infection in mammalian host. The haemolytic activity factors like the exo-enzymes, help in the characterisation of *C. albicans* isolates.

Table 4: Esterase activities of *C. albicans* isolates in Tween 80 medium.

Sources	n	Positive isolates	
		n	%
Vaginitis	60	57	95
Urinary infection	60	57	95
Oral cavity	30	26	86
ATTC 10231	1	1	-
Total	151	140	-

Several studies on haemolysin activity of *Candida* spp. have been reported.¹⁸⁻²⁰ These researches have significantly improved the knowledge of the iron uptake mechanisms by *C. albicans*. In this study, all *C. albicans* isolates exhibited haemolytic activity. In our study, we showed that the haemolytic activities of *C. albicans* were significantly higher in urine isolates compared to vaginal isolates using sheep blood SDA. Therefore, the haemolytic activity may engage as vital role for infections in *C. albicans*.

Previous investigations by the Tween opacity test have been performed to identify the lipolytic activities of different species of bacteria, yeast and mould. In this study, we measured the esterase activities of *C. albicans* isolates and found that 95% of both urinary and vaginal isolates have esterase activity. The patterns of precipitation resulting from esterase activity were valuable in distinguishing of *C. albicans*.

In conclusion, our data provide evidence of extracellular enzyme activity and germ tube formation in *Candida* isolates. These may be useful in making early diagnosis of *C. albicans* infections. Further studies are required to confirm our findings.

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