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Investigating Hemolytic Activity of *Candida* Isolates with Two Different Methods¶

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The hemolytic activity of *Candida* isolates with agar and microplate methods were investigated and compared efficiency of these methods to assess relationship between hyphal formation and hemolysis.

Key Words: *Candida* isolates, Hemolytic activity, Hemolytic index, Hemolysis percentage, Pathogenesis.

INTRODUCTION

Existing widespread in nature, *Candida* isolates are the most frequent cause of fungal infections and are encountered as normal flora of the skin and mucosa of the human body. The increase in the incidence of fungal infections has become quite significant in recent years in parallel to the increase in wide-spectrum antibiotic usage, organ transplantation, HIV infection, invasive catheter applications and immune deficiency due to immunosuppressive drug usage[1-3]. Increases in mortality and morbidity and in the nosocomial fungus infections have led to studies for the investigation of virulence factors. *Candida* isolates produce various enzymes such as protease, lipase, phospholipase, esterase and phosphatase which play a role in disseminated candidiasis[1,2,4].

Microorganisms disintegrate heme in order to utilize the iron required by their hemolytic activities[5,6]. Although the relationship between hemolysis formation and the pathogenicity of *Candida* isolates is not clear and reported as one of the virulence factors[4,7]. The first study on the hemolytic activity of *Candida* isolates has been performed by Manns *et al.*[8] who detected that ferritin, hemin and hemoglobin especially increased the growth of the *C. albicans* and stated that the hyphae form of *C. albicans* resulted in higher rates of hemolysis of sheep erythrocytes opsonized with complement than the yeast form. They also detected that hyphae and yeast forms of *C. albicans* had lower rates of hemolysis on erythrocytes not opsonized with complement compared

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to opsonized erythrocytes. They showed that *C. albicans* has hemolytic activity in Saboraud dextrose agar medium enriched with glucose and blood[8]. There is a limited number of studies on the hemolytic activities of the *Candida* isolates in Turkey and these usually employ the method of determining the hemolytic index[9-13].

The aim of this study was to determine the *in vitro* hemolytic activity of the *Candida* isolates isolated from various clinical samples by using two different methods.

EXPERIMENTAL

A total of 217 *Candida* isolates isolated from various clinical samples (urine, blood, wound, bronchoalveolar lavage, sputum, peritoneal fluid, catheter) during January 2005-July 2006 were examined. The isolates were determined by examining germ tube formation[14] and using API ID 32C (bioMerieux, France). The *in vitro* hemolytic activities of the isolates were determined according to the method of Luo *et al.*[4] by detecting the hemolysis indices in Saboraud dextrose agar (SDA) medium (Oxoid, England), pH adjusted to 5.6, containing 7 % human blood and 3 % glucose and were determined according to the method of Manns *et al.*[8] by detecting the hemolysis percentage measuring absorbance in the 96-well straight based microplates. *C. albicans* ATCC 32354 was used as the control isolates.

Inoculum suspensions with 24 h old cultures of *Candida* spp. on SDA were adjusted by spectrophotometer at 530 nm to match 10^8 cells/mL in 0.9 % NaCl solution. Ten µL was taken from the prepared yeast suspension and planted in the prepared SDA medium containing human blood so that it would form a radius of 5 mm. The plates were evaluated regarding hemolytic activity after 24 and 48 h of incubation at 37 ºC. The proportion of hemolysis radius to the colony radius was determined as the hemolytic index and a value higher than 1 was considered as the existence of hemolysis[4]. *Streptococcus pneumoniae* (ATCC 49619) was used for α-hemolysis and *Streptococcus pyogenes* (ATCC 13615) for β-hemolysis in order to determine the hemolysis zones by growing them under the same conditions.

Suspensions of the *Candida* isolates, of which fresh cultures were obtained in the SDA medium, were prepared with the microplate method so that there would be 2×10^8 cell/mL in the RPMI 1640 broth and incubated at 37 ºC, with 5 % CO_2 in the shaker (150 rpm) for 18 h. The formation of hyphal cells was confirmed by microscopy. The obtained suspensions were put into eppendorf tubes and washed three times with Ca^{2+}, Mg^{2+} free sterile phosphate buffer saline (PBS) and centrifuged and then transformed into suspension again with sterile PBS. An erythrocyte suspension (10^7 cells/mL) was prepared to detect hemolytic activity and added onto the yeast suspension and incubated at 37 ºC for 2 h after centrifugation. The supernatant parts of the suspension in the tubes were transferred into the wells in the microplate and optical densities were measured at 405 nm by spectrophotometry (Biotek, ELx800, USA). The percentages of optical density the other wells were calculated according to the optical density of the well in which the erythrocyte suspension was incubated with distilled water prepared as the positive control[8].

Four isolates were included in the statistical analysis considering the numbers of the isolates (*C. albicans, C. tropicalis, C. glabrata, C. parapsilosis*).

The Kruskal-Wallis, chi-square and Pearson correlation tests (to compare the hemolytic indices and percentages between isolates) were used for statistical analysis.

RESULTS AND DISCUSSION

Of the 217 *Candida* isolates included in the study, 126 were identified as *C. albicans*, 29 as *C. tropicalis*, 19 as *C. glabrata*, 16 as *C. parapsilosis*, 5 as *C. kefyr*, 5 as *C. lusitaniae*, 4 as *C. famata*, 3 as *C. sake*, 3 as *C. norvagensis*, 3 as *C. pelliculosa*, 2 as *C. krusei*, 1 as *C. utilis* and 1 as *C. globosa*. α-Hemolysis was detected in 76.9 % of all isolates after 24 h of incubation with the agar method while β-hemolysis was detected in 57.6 % and α-hemolysis in 19.3 % of the isolates after 48 h of incubation. However, hemolysis could not be detected in the 23.1 % of the isolates (γ-hemolysis). Distribution of the hemolysis types after 48 h of incubation according to the isolates has been presented in Table-1.

TABLE-1
DISTRIBUTION OF THE HEMOLYSIS TYPE DETERMINED WITH THE AGAR METHOD BY *Candida* ISOLATES

Candida isolates	n	β-Hemolysis		α-Hemolysis		γ-Hemolysis	
		n	%*	n	%*	n	%*
C. albicans	126	96	76.2	15	11.9	15	11.9
C. tropicalis	29	16	55.2	9	31.0	4	13.8
C. glabrata	19	9	47.4	8	42.1	2	10.5
C. parapsilosis	16	3	18.8	3	18.8	10	62.5
*C. kefyr***	5	–	–	–	–	5	–
*C. lusitaniae***	5	–	–	–	–	5	–
*C. famata***	4	–	–	1	–	3	–
*C. sake***	3	–	–	1	–	2	–
*C. norvegensis***	3	–	–	1	–	2	–
*C. pelliculosa***	3	–	–	2	–	1	–
*C. krusei***	2	–	–	2	–	–	–
*C. globosa***	1	1	–	–	–	–	–
*C. utilis***	1	–	–	–	–	1	–

*The percentages are line percentage. **The percentage has not been calculated.

When evaluated in terms of hemolytic index, the hemolytic index of *C. parapsilosis* was found to be lower than the other isolates ($p < 0.05$) (Table-2), but in present studies, no difference between the isolates in terms of hemolysis percentages ($p > 0.05$) is observed. A moderate correlation was detected between the hemolytic index and hemolysis percentage for all the isolates ($r = 0.262$, $p = 0.000$, Pearson correlation test). The most powerful correlation was found in the *C. parapsilosis* isolates ($r = 0.678$, $p = 0.04$, Pearson correlation test). An average level of correlation was found for *C. albicans* ($r = 0.31$, $p = 0.03$, Pearson correlation test).

TABLE-2
HEMOLYTIC INDEX VALUES OF THE *Candida* ISOLATES

Candida isolates	Hemolytic index (mean ± standard deviation)
C. albicans	1.48 ± 0.58
C. tropicalis	1.46 ± 0.62
C. glabrata	1.46 ± 0.58
C. parapsilosis	0.63 ± 0.84

When the characteristics of hyphae formation of the *Candida* isolates were examined, 54.7 % of the 190 isolates included in the statistical evaluation were found to form hyphae (Table-3).

TABLE-3
DISTRIBUTION OF THE *Candida* ISOLATES ACCORDING TO THEIR HYPHAE FORMATION CHARACTERISTICS

Candida isolates	n	Isolates forming hypae		Isolates not forming hypae	
		n	%	n	%
C. albicans	126	72	57.1	54	42.9
C. tropicalis	29	19	65.5	10	34.5
C. glabrata	19	–	–	19	100.0
C. parapsilosis	16	7	43.8	9	56.3

There was a moderately significant correlation between the hemolytic index and percentages of hemolysis of the isolates that did not form hyphae ($r = 0.326$, $p = 0.002$, Pearson correlation test). *C. tropicalis* isolates formed higher rates of hyphae compared to the others, while *C. glabrata* didn't have such a characteristic ($p > 0.05$). Of the non-hemolytic isolates, 80.6 % did not form hyphae also and there was a significant difference with the hemolytic isolates (chi-square = 19.084, $p = 0.000$). Isolates forming hyphae were found to have higher rates of α-hemolysis compared to the other isolates ($p < 0.05$).

Virulence factors of the pathogen in addition to an improper host factor play a role in candidasis pathogenesis. Protease, phospholipase, biofilm formation, germ tube formation and yeast-hyphae dimorphism are among these virulence factors[1,2]. However, *Candida* isolates have also been detected to have the capacity to form one or more hemolysins[6,15]. Metin *et al.*[12] investigated the hemolytic activities of the *Candida* isolates with the agar method and observed differences between the isolates with microscopic examination of the preparations between a slide and cover-slip compared to the subjective evaluation of the plates. The erythrocyte content was the same as for normal medium in the microscopic preparation examination of the *C. parapsilosis* isolates that subjectively displayed hemolysis in the medium. On the other hand, there were no erythrocytes in the microscopic preparations obtained from the α-hemolysis areas with *C. tropicalis* isolates and the hemolysis was consistent with β-hemolysis[12]. Studies have demonstrated that only the hyphae form of *C. albicans* produces hemolysin[5,15]. In present study, 89.5 % of *C. glabrata* isolates

do not form hyphae and form hemolysis with 47.4 % β-hemolysis and this result is consistent with other studies[4].

Luo *et al.*[4] investigated the *in vitro* hemolytic activities of 14 different *Candida* isolates in a total of 80 isolates by modifying the disc method previously described by Manns *et al.*[8] and reported that they detected both α (in the first 24 h) and β-hemolysis (after 48 h) in *C. albicans, C. dubliniensis, C. kefyr, C, krusei, C. zeylanoides, C. glabrata, C. tropicalis* and *C. lusitaniae*, but only α-hemolysis (after 48 h) in *C. famata, C. guillermondii, C. rugosa* and *C. utilis* and no hemolytic activity in *C. parapsilosis* and *C. pelliculosa* isolates. They put forward the hypothesis that the erythrocytes are disintegrated with a two-stage mechanism as a result of two different kinds of hemolysis seen in the same isolates. They suggest that partial damage occurs due to α-hemolytic factor formation of the young *Candida* colonies in the first stage while metabolic end products of the first stage can induce secretion of β-hemolysin which causes the hemoglobin to be disintegrated completely in the second stage. While hemolysin plays an important role in the pathogenesis of the bacterial infections formed by *Streptococcus* and *Staphylococcus*, its role in *Candida* infections has not been completely defined[4]. It was suggested by Watanabe *et al.*[6] that the cell wall mannoprotein of *C. albicans* may be β-hemolysin. There may be changes in the mannoprotein structure of different isolates as they are found in the cell wall of all *Candida* isolates and thus different rates of hemolysis can be found in the same isolates.

The agar method has been used in a limited number of studies investigating the hemolytic activities of *Candida* isolates in Turkey. Aktas *et al.*[9], detected α-hemolysis in all 114 yeast isolates they studied (54 *C. albicans*, 33 *C. tropicalis*, 11 *C. glabrata*, 6 *C. kefyr*, 5 *C. parapsilosis*, 3 *C. guillermondii*, 1 *Geotrichum candidum*, 1 *C. norvagensis*) except for *C. parapsilosis* at 24 h and found β-hemolysis in *C. tropicalis, C. glabrata, C. kefyr* and observed hemolytic activity in *C. parapsilosis*. Ozekinci *et al.*[10], detected hemolysis in 59 (84.2 %) of 70 *Candida* isolates (63 *C. albicans*, 3 *C. pseudotropicalis*, 2 *C. guillermondii*, 2 *C. tropicalis*). They found β-hemolysis in 59 *C. albicans* and *C. guilermondii* isolates and α-hemolysis in 4 *C. albicans* isolates and reported that they observed hemolytic activity in 6 *C. albicans*, 3 *C. pseudotropicalis* and 2 *C. tropicalis* isolates. We found hemolysis in 76.8 % of the 217 *Candida* isolates. While this rate is lower than the other studies, different isolates had different rates of hemolysis. If the number of isolates with few numbers in this study (*C. kefyr, C. lusitaniae, C. famata, C. sake, C. norvagensis, C. pelliculosa, C. krusei, C. globosa, C. utilis*) were to be increased, it would be possible to expose this difference in a better way. Metin *et al.*[12] investigated *in vitro* hemolytic activity in a total of 100 *Candida* isolates consisting of *C. albicans* (59), *C. glabrata* (15), *C. tropicalis* (14), *C. parapsilosis* (7), *C. guillermondii* (2), *C. dubliniensis* (2) and *C. krusei* (1) and detected that all the isolates except the *C. parapsilosis* isolates formed a minimum of one and a maximum of 5 concentric α- and β-hemolysis zones after 48 h. Five *C. parapsilosis* isolates formed hemolysis

at 48 h. Birinci *et al.*[11] used 108 *Candida* isolates (80 *C. albicans*, 10 *C. tropicalis*, 5 *C. glabrata*, 5 *C. guilliermondii*, 4 *C. parapsilosis*, 2 *C. krusei*, 2 *C. kefyr*) in their study and detected α-hemolysis at 24 h and double zone hemolysis with β-hemolysis inside the α-hemolysis at 48 h in *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. kefyr*. They didn't find hemolytic activity in *C. parapsilosis*. They determined the hemolysis indexes of *C. albicans*, *C. tropicalis* and *C. glabrata*, respectively as 1.585 ± 0.234, 1.495 ± 0.184 and 1.493 ± 0.124. The hemolytic indexes detected in present study were found to be consistent with previous results (Table-2)[12]. Cevahir *et al.*[13], on the other hand, detected β-hemolysis in all 86 *C. albicans* isolates.

The role of the characteristics of hemolytic activity different *Candida* isolates regarding pathogenesis will be determined if the mannan structure of the cell wall of different isolates is explored by the help of molecular studies for showing whether it contains different components or not. Determination of hemolytic activity of *Candida* isolates with the hemolytic index is an easy and practical method as it provides results within a short time. The microplate method, on the other hand, can determine even low level of hemolysis but it is time-consuming. The present findings show that the characteristics of hemolysin production of *Candida* isolates are unstable.

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