

Hemolytic Activities of the Candida Species in Liquid Medium

Candida Türlerinin Hemolitik Aktivitelerinin Sıvı Besiyerinde Araştırılması

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Abstract

Objective. The aim of this study was to evaluate the in vitro hemolytic activities of 107 Candida strains isolated from different clinical samples in liquid medium, and to examine the impact of glucose on this activity.

Materials and Methods. A total of 107 Candida isolates representing seven species (*C. albicans*, n=28; *C. glabrata*, n=23; *C. tropicalis*, n=17; *C. parapsilosis*, n=16; *C. kefyri*, n=14; *C. krusei*, n=5; *C. guilliermondii*, n=4) were included in the study. The hemolytic activities of the strains were tested on two different Sabouraud dextrose liquid media (SDB) containing 7% defibrinated human blood, one of which is supplemented with 3% glucose and the other without glucose. Cultures were evaluated at the end of a 48-hour incubation. The hemolysis in the media was detected spectrophotometrically by measuring the amount of released hemoglobin and compared with a standard hemolysate which was prepared prior to testing. The degree of hemolysis (percentage value) by an individual strain was calculated according to the following formula below: (Absorbance of supernatant media at 540 nm / Absorbance of standard hemolysate at 540 nm X 100).

Results. In the liquid medium without glucose, strains generally produced hemolysis at low levels. The degree of hemolysis produced by all species increased noticeably in the liquid medium with glucose. Strains of *C. albicans* and *C. kefyri* had demonstrated significant hemolytic activity, whereas others had lower activity. *C. parapsilosis* exerted very little hemolytic activity in the medium with glucose and showed no activity in the medium without glucose.

Conclusion. The hemolytic activities of most Candida species was found to be higher in the human blood-enriched SDB medium containing 3% additive glucose than in the one free from additives. This result indicates that increased blood glucose concentration may contribute to increased hemolytic activity in Candida species, and it suggests a parallel with possible pathogenesis of Candida in patients with diabetes mellitus.

Keywords: Candida species, Hemolytic activity, Human blood sabouraud dextrose broth

Özet

Amaç. Bu çalışmanın amacı değişik klinik örneklerden izole edilen 107 Candida kökeninin hemolitik aktivitelerini ve glikozun bu aktivite üzerindeki etkisini araştırmaktır.

Gereç ve Yöntem. Çalışmada, 7 farklı türden (*C. Albicans*, n=28; *C. Glabrata*, n=23; *C. Tropicalis*, n=17; *C. Parapsilosis*, n=16; *C. Kefyri*, n=14; *C. Krusei*, n=5; *C. Guilliermondii*, n=4) toplam 107 kökenin in-vitro hemolitik aktivitesi araştırıldı. Çalışmada kullanılan kökenlerin hemolitik aktivitesi, %3 glikoz ilaveli insan kanlı Sabouraud Dextrose Broth (SDB) ve glikozsuz insan kanlı SDB olmak üzere iki farklı besiyerinde 48 saatlik inkubasyon süresi sonunda test edildi. Bu besiyerlerinde oluşan hemoliz, ortama salınan hemoglobin miktarının spektrofotometrik olarak ölçülüp standart hemolizat ile karşılaştırılması yöntemiyle belirlendi. Hemoliz yüzdesi her bir köken için (besiyeri süpernatantının 540 nm dalga boyundaki absorbansı / Standart hemolizatın 540 nm dalga boyundaki absorbansı X 100) formülü ile hesaplandı.

Bulgular. Çalışmada kullanılan Candida türlerinin hemoliz yüzdesi glikozsuz besiyerinde düşük oranda tespit edildi. *C. albicans* ve *C. kefyri* türlerinin hemolitik aktivitesi her iki besiyerinde diğer türlere göre yüksek bulundu. *C. parapsilosis* kökenleri %3 glikoz ilaveli SDB besiyerinde düşük aktivite gösterirken glikozsuz SDB besiyerinde hemolitik aktivite göstermedi.

Sonuç. Candida türlerinin hemolitik aktiviteleri %3 glikoz ilaveli SDB besiyerinde glikozsuz besiyerine göre daha yüksek bulundu. Kandaki yüksek glikoz oranının Candida türlerinin hemolitik aktivitelerinin artmasına neden olabileceği sonucuna varıldı.

Anahtar Kelimeler: Candida türleri, Hemolitik aktivite, İnsan kanlı sabouraud dekstroz broth

Introduction

Candida is arguably the archetypal opportunistic human pathogen, having been identified as early as the era of Hippocrates. Today, we know that Candida is a commensal fungus that harmlessly inhabits various niches of the healthy human body [1].

The AIDS epidemic, improved life-sustaining therapies and aggressive anticancer therapies have contributed to the rise in the number of severely immunocompromised patients. This has led to an increase in oral and systemic fungal infections. Several factors, such as adherence, persistence, dimorphism and/or germ tube formation, phenotypic switching, interference with the host defense system, synergism with bacteria and the production of hydrolases (such as lipases and proteases like secreted aspartyl proteinase, phospholipase, esterases, and phosphatases) have been proposed as virulence factors for Candida spp [2-6].

Hemolysin is another putative virulence factor thought to contribute to candidal pathogenesis. In particular, the secretion of hemolysin - followed by iron acquisition - facilitates hyphal invasion in disseminated candidiasis [7-9]. While there have been a number of detailed studies on some of hydrolytic enzymes, such as proteases, lipases and phospholipases, papers on the hemolytic activity of Candida spp are few. A complement-mediated hemolysis induced by *C. albicans* was first reported by Manns et al. [8] and Luo et al. [7], who studied 80 Candida isolates representing 14 species and reported that *C. albicans* and others showed alpha and beta hemolysis. This was the first study to demonstrate the variable expression profiles of hemolysins in different Candida species with a blood plate assay using a sheep blood SDA medium.

In the present study, we aimed to investigate the hemolytic activity of Candida species isolated from different clinical specimens in two different liquid media.

Materials and Methods

Yeast Strains:

A total of 107 Candida isolates were tested for hemolytic activity. They were recovered from different clinical specimens and consisted of 28 *C. albicans*, 23 *C. glabrata*, 17 *C. tropicalis*, 16 *C. parapsilosis*, 14 *C. kefyr*, 5 *C. krusei* and 4 *C. guilliermondii*. Of the test organisms, 40 were isolated from the bloodstream, 35 from the oral cavities and pharyngeal swabs, 22 from urine, 6 from vaginal swabs and 4 from sputum. The distribution of the strains by their species and origin are shown in Table 1. Four reference strains (*C. albicans* ATCC 26555, *C. albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. parapsilosis* ATCC 90018) were also included in the study. In addition to the yeast strains, one of each strain of *Streptococcus pyogenes* and *Streptococcus sanguis* (which induce beta and alpha hemolysis, respectively) were used as positive controls.

Primary isolation of the yeasts from pathological samples (except the blood samples) was done on Sabouraud dextrose agar (SDA) supplemented with 1% chloramphenicol, and CHROMagar Candida. The blood samples were inoculated into aerobic media and processed using the BACTEC blood culture system (Becton Dickinson, Sparks, MD). All blood cultures were subcul-

tured on SDA and CHROMagar Candida. The yeast isolates were subsequently identified by a germ tube test, the development of blastospores, chlamydoconidia and pseudohyphae, and assimilation tests by employing the commercial kit API 20C AUX system (Bio-Merieux, France).

Assessment of Hemolytic Activity:

The hemolytic activities of the strains were measured on two different Sabouraud dextrose liquid media (SDB) (Difco, USA) containing 7% defibrinated human blood, one of which was supplemented with 3% glucose (SDBwG) and the other without glucose (SDBwoG).

Suspensions equal to McFarland 2 turbidity from the pure culture of the yeasts colonies on Sabouraud dextrose agar were prepared. Ten microliters of this suspension were inoculated into 2 ml of SDBwG and SDBwoG media separately. The tubes were incubated at 37°C in 5% CO₂ for 48 h. At the end of the incubation, tubes were centrifuged at 1800 g and at 4°C for 10 minutes in order to separate non-hemolyzed erythrocytes. The released hemoglobin in the supernatants was then quantified by spectroscopic analysis using the Cyanmethemoglobin method as follows: Ten microliters of supernatants were transferred to sterile polyethylene tubes, and five milliliters of Drabkin's reagent (KCN, K₃Fe (CN)₆, NaHCO₃, KH₂PO₄, TritonX-100) were added. The absorbance of the mixture was measured at 540 nm. On the other hand, standard hemolysate suspension was prepared in order to compare the hemoglobin content of this standard with those of the test samples. One hundred microliters of defibrinated human blood was mixed with 1430 microliters cold water (final dilution of blood is 7%), and incubated at 4°C for 30 minutes in order to lyse the erythrocytes. The hemoglobin content of this standard was then determined using the same procedure as above. The degree of the hemolysis (percentage value) by an individual strains was calculated according to the formula below: (Absorbance of test tubes / absorbance of standard hemolysate) x 100 [10-12].

Statistical Analysis:

Statistical analyses were performed using Student's t-test (independent samples test) in SPSS, version 11.0.

Results

The mean average of hemolysis percentage by Candida isolates in two different liquid media is presented in Table 2, and the tube images for the expression profiles of hemolysins by *S. sanguis*, *C. albicans*, *C. parapsilosis*, and *C. glabrata* are shown in Figure 1.

As seen in Table 2, the hemolytic activities of *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. kefyr* were higher than in other species. The mean average hemolysis percentage for these species (in SDBwG medium) varied between 40% and 49.6%. *C. krusei*, *C. guilliermondii* and *C. parapsilosis* showed weak hemolytic activity in the same medium; the mean average hemolysis percentage for these species ranged between 6.8% and 28.3%. The hemolytic activities of all the Candida strains in SDBwoG medium were lower than that of SDBwG medium. None of the 16 *C. parapsilosis* isolates exhibited hemolytic activity in SDBwoG medium. The differences between the results obtained from the two media were statistically significant (*C. albicans*; t=2.93, P<0.01, *C. glabrata*; t=3.23, P<0.01, *C. tropicalis*; t=2.25, P<0.05, *C. parapsilosis*; t=2.82, P<0.001). *C. krusei* and *C. guilliermondii*



Fig. 1 — Eolysis profiles of various *Candida* species in human blood SDB (3% glucose). From left to right: SDB without blood, *S. sanguis*, *C. albicans*, *C. parapsilosis* and *C. glabrata*.

were not calculated because sample numbers were low.

Discussion

The ability of pathogenic organisms to acquire elemental iron has been shown to be of critical importance in their survival and helps to establish infection within the mammalian host. Because there is little free iron in the human host, most pathogens acquire iron indirectly from commonly available iron-containing compounds such as hemoglobin. In order to do so, however, the pathogen must be equipped with a mechanism that destroys the heme moiety and enables it to extract the elemental iron. The enzymes involved in this activity are classified as hemolysins [7,8,9,13,14].

Studies on the activity of hemolysin in *Candida* spp are limited [7,8]. A complement-mediated hemolysis induced by *C. albicans* was first reported by Manns et al. [8], but the literature revealed no other reports on the hemolytic activity of the non-*albicans* species of *Candida*. Luo et al. [7] demonstrate that, both qualitatively and quantitatively, the hemolytic activity in a wide spectrum of *Candida* species belong to 14 genera. The

same authors suggested that the hemolysis so induced could be categorized according to conventional microbiologic nomenclature as complete (beta), incomplete (alpha) or no hemolysis (gamma or non). Thus the terms alpha and beta hemolysis used to describe the different patterns of hemolysis in *Candida* spp can only be regarded as descriptive because the exact nature of these variants and their underlying mechanisms are yet to be fully explored [7]. However some authors indicate that there are some genetic attributes that contribute to the hemolytic activity of *Candida* species, and also there is a significant positive correlation between the phenotypic and genotypic expressions of hemolytic activity [15,16].

The modified plate assay with sheep blood SDA medium described in the previous report is simple, reproducible, and sensitive, and is a relatively fast screening method for assessing the hemolytic activity of *Candida* spp [7,8]. Recently, we reported that *Candida* species, including *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. kefyr* and *C. krusei*, exhibit varying ability to produce hemolysin on SDA media containing human, rabbit and sheep blood (supplemented with 3% glucose) [17]. Nevertheless, the literature provides no information about the relative performance of the liquid medium enriched with human blood on the hemolytic activity of *Candida* isolates.

In this study, *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. kefyr* exhibited higher hemolytic activities in human blood SDBwG at 48 h, with the mean average for hemolysis percentages as 49.6%, 43.0%, 40.0% and 47.4%; other mean averages include those of *C. krusei* (28.3%) *C. guilliermondii* (21.5%) and *C. parapsilosis* (6.8%) (Table 2). The hemolytic activity of the same strains in human blood SDBwoG was lower than that of the SDBwG medium. *C. parapsilosis* isolates did not exhibit any hemolytic activity in SDBwoG medium. The differences between hemolytic activities of the strains in two different media were statistically significant (*C. albicans*; $t=2.93$, $p<0.01$, *C. glabrata*; $t=3.23$, $p<0.01$, *C. tropicalis*; $t=2.25$, $p<0.05$, *C. parapsilosis*; $t=2.82$, $p<0.001$).

Luo et al. [7] reported that *C. parapsilosis* strains did not exhibit alpha and beta hemolysis on sheep blood Sabouraud dextrose agar. In our previous study, we demonstrated that *C. parapsilosis* strains did not exhibit any hemolytic activities on SDA media enriched with human, sheep and rabbit blood [17]. In the present study, *C. parapsilosis* strains showed low hemolytic activity in human blood SDB with 3% glucose medium, but no activity in the medium without glucose.

Manns et al. [8] defined the conditions under which *C. albicans* can display hemolytic activity, but discovered that hemolysis was non-existent when no glucose was available in the cul-

Table 1. The isolation origin of *Candida* species

<i>Candida</i> species	Blood	Urine	Vaginal swabs	Sputum	Pharyngeal swabs	Oral cavities
<i>C. albicans</i> (n=28)	10	3	5	2	2	6
<i>C. glabrata</i> (n=23)	8	4	-	1	3	7
<i>C. tropicalis</i> (n=17)	9	5	-	-	1	2
<i>C. parapsilosis</i> (n=16)	4	4	1	1	2	4
<i>C. kefyr</i> (n=14)	6	5	-	-	-	3
<i>C. krusei</i> (n=5)	3	1	-	-	-	1
<i>C. guilliermondii</i> (n=4)]	-	-	-	-	1	3
Total (n=107)	40	22	6	4	9	26

Table 2. Mean average hemolysis percentage by Candida isolates in two different liquid media.

Candida species	Human blood SDB without glucose (%)	Human blood SDB with 3% glucose (%)
<i>C.albicans</i> (n:28)	32.9	49.6
<i>C.glabrata</i> (n:23)	27.6	43.0
<i>C.tropicalis</i> (n:17)	27.8	40.0
<i>C.parapsilosis</i> (n:16)	0.0	6.8
<i>C.kefyr</i> (n:14)	40.1	47.4
<i>C.krusei</i> (5)	17.2	28.3
<i>C.guilliermondii</i> (4)	18.1	21.5
<i>C.albicans</i> ATCC 90028*	47.2	61.1
<i>C.albicans</i> ATCC 26555*	42.6	58.1
<i>C.glabrata</i> ATCC 90030*	44.4	47.2
<i>C.parapsilosis</i> ATCC 90018*	0.0	0.0
<i>S.pyogenes</i> *	63.8	83.3
<i>S.sanguis</i> *	44.4	58.3

*single value, not average

ture medium. On the other hand, Luo et al. [7] have tested 80 *Candida* isolates from clinical sources in different geographical locales and detected only alpha, and not any beta, hemolysis in experiments with glucose-free sheep blood agar. Tsang et al. [9] reported that oral *C. albicans* isolates from type 2 diabetes mellitus patients possess significantly higher hemolysin activity than isolates from healthy, non-DM controls. The same authors suggested that an increased blood glucose concentration may contribute, directly or indirectly, to increased hemolysin activity among *C. albicans* isolates in DM patients and, in addition, an increased salivary glucose concentration in DM patients may also influence hemolysin production in *C. albicans* [9]. In our study, the degree of hemolysis produced by all species increased in the liquid medium with glucose. The results of the present study are

consistent with these findings.

Another result of this study is that the hemolytic activity of *Candida* species did not differ by isolation origin. This result is supported by the results of other research [7,17].

In conclusion, we report here that *Candida* spp displayed different hemolytic activity in human blood SDBwG and human blood SDBwoG media. This activity was found to be higher in the SDBwG medium than in the SDBwoG medium. This result suggests that an increased blood glucose concentration may contribute to increased hemolysin activity. However, further studies are needed to understand the nature of the hemolytic factors and their host-pathogen relationships associated with candidal infections.

Conflict interest statement The authors declare that they have no conflict of interest to the publication of this article.

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