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Validation of integral system yeast plus for rapid identification and determination of antifungal susceptibility profile of clinically important *Candida* species

Mitra Barati[1,2], Mona Ghazanfari[3], Fatemeh Bazrafshan[4], Koosha Farhadi[5], Mehri Naghdalipour[1], Zeynab Fakhri[2], Azam Fattahi[6,1]*

[1] Research Center of Pediatric Infectious Diseases, Institute of Immunology and Infectious Diseases, Iran University of Medical Sciences, Tehran, Iran
[2] Department of Infectious Disease, Rasul-e Akram Hospital, Iran University of Medical Sciences, Tehran, Iran
[3] Department of Medical Mycology, School of Medicine, Mazandaran University of Medical Sciences, Tehran, Iran
[4] Department of Medical Mycology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran
[5] School of Medicine, Medical University of Tehran, Tehran, Iran
[6] Center for Research and Training in Skin Disease and Leprosy, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Precise identification of microorganisms involved in candidiasis together with antifungal susceptibility evaluation could help clinicians to prescribe appropriate medicine, especially in patients with critical conditions. The present study has been conducted to evaluate discriminatory power of Integral System Yeast Plus (ISYP) for rapid identification and determination of antifungal susceptibility of clinically important *Candida* species (sp.). Validation of ISYP results was performed using molecular and Broth Micro Dilution (BMD) assays. Referring to the present results, it can be said that ISYP was to some extent successful for identification of *C. albicans* isolates. Major misidentification was observed in cases of non *C. albicans* sp. (NAC) in comparison with sequencing results**.** The relatively unsatisfied outcome of ISYP performance was correlated to antifungals susceptibility assay as well. It is noteworthy to emphasize the potential advantages of ISYP for simultaneous identification of *Candida* sp. together with antifungal susceptibility evaluation that brings hope to the defeat of severe complications. But an essential defect was observed in both identification and antifungal susceptibility tests.

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Keywords
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Corresponding to:
Azam Fattahi,
Center for Research and Training in Skin Disease and Leprosy, Tehran University of Medical Sciences, Tehran, Iran, &
Research Center of Pediatric Infectious Diseases, Institute of Immunology and Infectious Diseases, Iran University of Medical Sciences, Tehran, Iran

Email:
afattahi@sina.tums.ac.ir

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INTRODUCTION

Candida sp. are the causative agents in 80% of nosocomial fungal infections [1], and considered as the fourth most frequently isolates that result in nosocomial bloodstream infections [2]. The incidence of systemic infections caused by *Candida* sp. has been increasing steadily through the course of the last decades [3]. Although *Candida albicans* is the most common fungal pathogens involved in the nosocomial candidiasis, currently with the emerge of non *C. albicans* sp. (NAC), the epidemiology of the infection is changing [3]. This tendency results from the growth in population of immunocompromised patients, brought about by both the establishment of Acquired Immunodeficiency Syndrome (AIDS) pandemic and the constant development of medical techniques, particularly

involving oncology or transplantation patients [4]. Furthermore, the widespread use of antifungal drug emergence of new pathogens and misidentification of fungal agents may lead to poorer clinical outcomes. It is an alarm for clinician and public health authorities. This point highlights the importance of susceptibility testing to select appropriate antifungal drugs [5].

Early diagnosis and treatment of infected patients is required to prevent the disease spread and occurrence of severe complications. It is noteworthy to mention that high degree of morphological similarity between several *Candida* sp. makes identification mistakes inevitable [6]. Regarding the global epidemiology variation of candidiasis along with emerging less susceptible isolates, it is necessary to employ a precise, easy to use, rapid and cost-effective approach to identify Candida sp. and evaluate the ability of currently available antifungal agents against it [4]. Recently, the diagnosis has become fast and easy using newer diagnostic techniques especially molecular methods but regarding to high cost of equipment required for these techniques, they are not accessible for most clinical laboratories [5]. Therefore, precise identification of microorganisms involved in candidiasis together with antifungal susceptibility evaluation could help clinicians to prescribe appropriate medicine, especially in patients with critical conditions. These test results also make it possible to track any changes in *Candida* sp. resistance to antifungals. Because of the importance of precise identification of *Candida* sp. and its susceptibility profile, the present study has been conducted to evaluate discriminatory power of Integral System Yeast Plus for rapid identification and determination of antifungal susceptibility of clinically important *Candida* sp.

MATERIALS AND METHODS

Samples

A collection of 100 clinically *Candida* sp. including *C. albicans*, non *albicans Candida* (NAC) isolates from urine samples were enrolled in the experimental study. All clinical isolates were identified previously as *C. albicans*, NAC by CHROM Agar Candida medium (CHROM agar, France) and were stored in the culture collection of Medical Mycology Laboratory, Rasoul -e- Akram Hospital, Iran University of Medical Sciences, Tehran, Iran.

Yeast specific identification and antifungal susceptibility assay using rapid commercial kit

Specific identification and four antifungal susceptibilities patterns were obtained using Integral System Yeast Plus (ISYP) commercial kit (Liofilchem, Italy), according to the manufacturer's instructions [5]. Briefly, *Candida* conidial suspension was prepared from 24h fresh culture on Sabouraud dextrose agar (Merck, Germany) at 35°C. The cell density was adjusted to 0.5 McFarland standard and inoculated to 12 microwells containing dried sugar. Well thirteen contained chromogenic substrate, then the suspension was subjected to the dilute and released into subjected antifungal wells. The final concentrations of the tested antifungals were as follows: nystatin (1.25 µg/mL), amphotericin B (2.0 µg/mL), 5-fluorocytosine (16.0 µg/mL), econazole (2.0 µg/mL), ketoconazole (0.5 µg/mL), clotrimazole (1.0 µg/mL), miconazole (2.0 µg/mL), itraconazole (1.0 µg/mL), voriconazole (2.0 µg/mL), fluconazole (64.0 µg/mL). The well twenty-four was considered as indicator. The wells, except well thirteen, were covered with a drop of vaseline oil. The microplates were incubated at 36 ± 1°C for 48 h and the obtained results were interpreted based on color change of the wells. The characteristic pattern of sugar assimilation was checked, then a specific code was calculated and the species name were found in the codebook. The *C. albicans* ATCC 24433, *C. glabrata* CBS 138, and *C. krusei* ATCC 6258 were used as quality controls.

Validation of Integral System Yeast Plus (ISYP) results

Definite identification of *Candida* sp. was confirmed by PCR sequencing technique, based on amplification of ITS1-5.8SrDNA-ITS2 region [7].

DNA extraction was performed using Qiagen DNA tissue kit (Germany). PCR was carried out with a PCR reaction mixture including 1 µl of extracted DNA, 10 µl of Taq DNA Polymerase Master Mix RED (Ampliqon), 1 µl of each ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) oligonucleotide primers, in a total volume of 25µl.

PCR cycling parameters were 94°C for 5 min, 35 cycles of denaturation for 1min at 95°C, annealing for 1min at 56°C, an extension for 90 secs at 72°C, with a final extension of 7 min at 72°C. PCR products were visualized by 1.5% agarose gel electrophoresis in TBE buffer and stained with safe stain (Waltham, USA). All amplified products were sequenced by ITS primers (Macrogen, South Korea). The molecular identification of *C. albicans* complex, *C. parapsilosis* complex, and *C. glabrata* complex was performed by sequencing HWP1 [8] and ITS primers, respectively. The sequences were then compared with GenBank.

To ensure accuracy of ISYP susceptibility test, the obtained antifungals susceptibility results of four most routine antifungals amphotericin B, voriconazole, itraconazole, fluconazole were verified by Broth Micro Dilution (BMD) according to Clinical and Laboratory Standards Institute (CLSI) M27-A3 [9].

Statistical analysis

The statistical analysis was performed by Statistical Package for Social Sciences (SPSS) 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

All primary identified *Candida* isolates on CHROM agar were precisely differentiated using ISYP commercial kit and confirmed with PCR sequencing. Comparative distribution of *Candida* strains at species level based on these approaches are listed in Table 1.

Table 1. Comparative distribution of *Candida* sp using ISYP and PCR sequencing

ISYP (n:%)	*PCR Sequencing*
C. albicans (n: 50.6)	**C. albicans* (n:50.6), Acn:MG818805-31
C. tropicalis (n:6)	*C. tropicalis* (n:27), Acn:MG818777-800
C. parapsilosis (n:4.5)	*C. parapsilosis* (n:5.6), Acn:MG825322-26
C. krusei (ND)	*C. krusei* (2.2), Acn:MG818803-4
C. glabrata (ND)	*C. glabrata* (2.2), Acn: MG818801-2

ND: Not Determined, * some of the isolates were deposited in the international public GenBank, Acn: Accession number

Table 2. A head-to-head comparison of four antifungal susceptibilities based on ISYP, BMD

Species	*Antifungals*	*ISYP*			*BMD%*		
		S	*I*	*R*	*S*	*I*	*R*
C. albicans	AMB	77.77	6.66	22.2	98	0	2
	VCZ	84	8	8	92	8	0
	ICZ	65	25	10	77.82	16	6.8
	FCZ	84	16	0	94	6	0
C. tropicalis	AMB	45. 45	16.66	18.25	83.33	8.33	16.66
	VCZ	41.66	41.66	16.16	70.80	20	9.2
	ICZ	33.33	20.84	16.16	87	5	8
	FCZ	41.67	33.33	35	85	3	12
C. parapsilosis	AMB	80	20	0	90	8	2
	VCZ	100	0	0	100	0	0
	ICZ	100	0	0	90	10	0
	FCZ	60	40	0	94	0.6	0
C. krusei	AMB	100	0	0	100	0	0
	VCZ	50	50	0	100	0	0
	ICZ	50	50	0	0	100	0
	FCZ	0	100	0	0	60	40
C. glabrata	AMB	100	0	0	100	0	0
	VCZ	100	0	0	100	0	0
	ICZ	0	100	0	0	50	50
	FCZ	0	50	50	0	0	100

AMB: Amphotericin B, VCZ: Voriconazole, ICZ: Itraconazole, FCZ: Fluconazole

A complete overlap was observed between ISYP and PCR sequencing in identifying *C. albicans* species, but they were different in cases of NAC in which 11.22% of agreement was observed between the two assays. The partial and complete nucleotide sequences of all isolates were confirmed with PCR and have been deposited in Gen Bank.

All *C. albicans*, *C. parapsilosis*, and *C. glabrata* complex strains were identified as *C. albicans*, *C. parapsilosis*, and *C. glabrata* with HWP1 and ITS primers, respectively. The results from the employed susceptibility tests are presented in Table 2.

DISCUSSION

Referring to the present results, it can be said that ISYP was to some extent successful for identification of *C. albicans* isolates. Major misidentification was observed in cases of NAC when compared to sequencing results and in most cases, four digit codes were incompatible with the codes mentioned in the codebook. Present finding is consistent with Haleim et al [10] and Bicmen et al [11] studies on assessing performance of ISYP. It must be mentioned that this ready to use commercial kit failed to accurately discriminate NAC. Recently, successful outcome of ISYP to identify spectrum *Candida* sp. has been reported in patients with chest infection. Although these findings reflected discriminatory power of this rapid commercial kit to differentiated *albicans* and non-*albicans Candida* sp. [12].

An interesting fact that observed in cases of misidentification of all tasted strains were related to dulcitol. The present and past finding suggest that the result of the identification would be correct when excluding dulcitol substrates. In contrast to Szweda et al's [5] investigations, the present findings confirm ISYP system success to assimilation xylose. The reason for the observed differences can be attributed to probable contaminations in xylose disc package or through the experiment procedure performance.

The relatively unsatisfied outcome of ISYP performance was correlated to antifungals susceptibility assay as well. Significant disagreement between ISYP and BMD has been reported previously [5, 11]. In line with previous investigations, the present findings revealed that no definite burden exists between interpretation sensitivity and intermediate results as well as interpretation of intermediate and resistant results with regard to deficient color changes, which makes interpretation by visual monitoring difficult. Subsequently, isolates were classified into susceptible groups according to CLSI criteria with ISYP remaining in the intermediate group. Similar classification was seen in case of isolates that are recorded as resistant according to ISYP. Also, one of the other possible scenarios about

different results between the two tests origin age from a single concentration of each antifungal coated into ISYP wells. In fact, considerable differences exist between the results of the two minimum inhibitory concentration (MIC) methods.

Based on ISYP results, all isolates were found to have the highest sensitivity (100%) to both of econazole and flucytosine. In the case of nystatin, 20.90 % of the tested strains showed MIC of 1.25 and were introduced as resistant isolates while 51.81% were failed to growth at the same concentration of drug and were exhibited sensitivity to nystatin. It has been well established that *C. albicans* is intrinsically sensitive to a broad range of antifungal classes and resistance must be acquired [13-15]. In concordance with this finding, results from ISYP in this study show that most of *C. albicans* is susceptible to all antifungal agents that were used here. The effectiveness of nystatin for topical treatment of cutaneous and mucosal fungal infections caused by *C. albicans* was reported before [16,17]. In contrast to the reported findings, ISYP results indicated that *C. albicans* isolates have the lowest sensitivity to nystatin.

Excellent susceptibility to clotrimazole and miconazole have also been reported [14,18]. Here, only three strains showed decrease susceptibility to clotrimazole and thirty-seven strains grew at a concentration of 0.5 µg/ml which is known as the intermediate group. The results were nearly similar to miconazole but some differences observed in resistant isolates.

CONCLUSION

Finally, this work demonstrates that overcoming sever complications would be possible if a combination of ISYP (for simultaneous identification of *Candida* sp.) and antifungal susceptibility evaluation are employed. It is noteworthy to mention that ISYP suffers from multiple shortcomings that limit its reliability. For instance, ISYP assay is based on color changes produced by different biochemical reactions and the result interpretation is dependent on a certain code guide. However, our data shows this code system needs to be upgraded since it fails to distinguish between different strains of specific species. For example, precise identification of *C. glabrata* and *C. parapsilosis* from their sibling species *C. orthopsilosis* and *C. metapsilosis* is not possible using ISYP and the accurate identification of these species is highly important because of their different antifungal susceptibilities. Collectively, a comparison between antifungal susceptibility results in the four tested agents by BMD indicated that ISYP results are not reliable.

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CONFLICT OF INTEREST

The authors declare that this research does not have any conflict of interest with anyone or any Institute.

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