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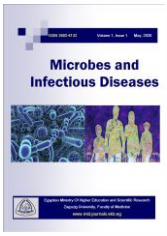

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Original article

Screening for anti-fungal resistance in *Candida* species using chromogenic agar dilution

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ABSTRACT

Background: The wide use of azoles for the prevention and treatment of candidiasis is due to the high morbidity and mortality associated with this nosocomial infection. Consequently, drug resistance has emerged among *Candida* species. The current study aimed to compare between the VITEK-2 and the chromogenic media as regards the identification of *Candida* species and antifungal susceptibility testing (AFST) using agar dilution of different antifungals with multiple concentrations. **Methods:** This cross-sectional study was conducted on 160 *Candida* isolates collected from different specimens submitted to the Central Microbiology Laboratory of Ain Shams University Hospitals from February 2020 to February 2021. The VITEK-2 system and Brilliance *Candida* agar media were used for identifying the isolates to species level. Then, AFST was performed by the VITEK-2 system and agar dilution method on Brilliance *Candida* agar media. Fluconazole, Voriconazole and Micafungin dilutions were prepared as per the Clinical and Laboratory Standards Institute (CLSI). **Results:** Regarding the identification of *Candida* species, there was an almost perfect agreement (KAPPA= 0.9) between the VITEK-2 and Brilliance *Candida* agar. *C. tropicalis* was the most common species, with predominance among urine specimens. AFST using fluconazole showed 88.5% susceptibility, 7.6% susceptible dose dependent (SDD) and 3.8% resistance. While for Voriconazole, 95.0% were susceptible, 4.4% were intermediate, and 0.6% were resistant. Concerning Micafungin, 98.7% and 1.3% were susceptible and resistant, respectively. **Conclusion:** The chromogenic media proved to be a reliable and cost-effective method for identification of common *candida* spp. and for identification of mixed *candida* infections. AFST using agar dilution on chromogenic media showed high accuracy when used with fluconazole.
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Introduction

Infections by *Candida* are presenting international intimidation to patients' health, especially those who are critically ill, receiving immunosuppressive remedies, or undergoing medical intervention [1].

Candida species are the fourth most frequently isolated type from nosocomial bloodstream infections, causing life-threatening diseases [2]. The most commonly found *Candida* species is *Candida albicans (C. albicans)* [3]. Owing to the increased usage of antifungal agents

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and immune inhibitors, non-albicans *Candida* (NAC) species have emerged leading to rising morbidity and deaths [4].

Although antifungal resistance is not as prevalent as antibacterial resistance, the overuse of ineffective, or unnecessary, antifungal therapy is a driving force for the emergence of antifungal resistance in *Candida* species just as it is for bacteria [3].

It is now clear that the in vitro patterns of antifungal susceptibility vary substantially among different *Candida* spp. and the Infectious Diseases Society of America (IDSA) and the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) guidelines for the treatment of candidiasis recommend that species-level identification be used as a surrogate for antifungal susceptibility testing for selecting empirical antifungal therapy [1].

It is becoming inevitable to identify the yeast recovered from clinical samples up to the species level. The routine yeast identification procedures, other than germ tube test, are labour intensive and some may take 72 hours to two weeks. To facilitate rapid identification, several chromogenic substrate-containing culture media have been developed. These special media carry the potential to improve the identification of yeast, especially in mixed cultures [5].

Other methods for yeast identification include biochemical methods such as VITEK, API, MicroScan and others. Several new, however expensive, approaches to yeast identification have greatly improved the timeliness and accuracy of *Candida* species identification. These include peptide nucleic acid (PNA)-fluorescent in situ hybridization (FISH), sequence-based identification, and Matrix Assisted Laser Desorption/Ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) [1].

Current methods for identification of *Candida* azole resistance include disc diffusion, micro broth dilution and E test assays [6]. These assays can be time-consuming and require the isolation of individual strains of *Candida*. With rising resistance rates against fluconazole, it is imperative to rapidly perform antifungal susceptibility testing of *Candida* to attain the most convenient patient treatment [7].

Several studies took the advantage of the capability of chromogenic media for identification and used it in combination with fluconazole and other antifungal agents to provide a simple and cost-effective agar dilution method for antifungal susceptibility testing [7-9].

This study aimed to compare between the VITEK-2 and the chromogenic media as regards the identification of *Candida* species and antifungal susceptibility testing using agar dilution of different antifungals with multiple concentrations. These concentrations will differentiate susceptible from SDD and resistant *Candida* isolates versus VITEK-2 **(**bioMérieux, France**)** (Reference Method).

Methods

Study design and study population

This cross-sectional study was conducted on 160 *Candida* isolates collected from specimens of clinically relevant patients with *Candida* infection from different sites. Specimens were submitted to the Central Microbiology Laboratory of Ain Shams University Hospitals from different departments from February 2020 to February 2021. This study was approved by the Ethical Committee of the Faculty of Medicine, Ain Shams University. (Ethical approval number: FMASU MD 93/2020, FWA 000017585).

All isolates were subjected to the following:

1. Germ tube test performed according to **(Leber, 2016)** [10].
2. Culture on chromogenic Brilliance *Candida* agar **(Oxoid, UK)** for identification.
3. Performance of antifungal susceptibility testing using chromogenic agar dilution method by using the following antifungal drugs with the following specified concentrations (according to ***CLSI M60, 2020)*** [11]:
   A. Fluconazole (2 µg /mL, 4 µg /mL, 8 µg /mL).
   B. Voriconazole (0.12 µg /mL, 0.25 µg /mL, 0.5 µg /mL, 1 µg /mL).
   C. Micafungin (0.25 µg /mL, 0.5 µg /mL, 1 µg /mL) for *C. albicans, C. tropicalis,* and *C. krusei*, and (2 µg /mL, 4 µg /mL, 8 µg /mL) for *C. parapsilosis* and *C. guilliermondii.*
4. Performance of *Candida* species identification and antifungal susceptibility testing using the automated system

VITEK-2 compact **(bioMérieux, France)** (Reference method).

VITEK 2 identification and antifungal susceptibility testing

The YST ID and AST-YS08 VITEK 2C cards were used according to manufacturers' instructions for the identification and antifungal susceptibility testing of *Candida spp.* AST-YS08 cards contain the following antifungals: amphotericin B, caspofungin, fluconazole, flucytosine, micafungin, and voriconazole. **Table 1** summarizes the breakpoints used for the antifungal drugs [11,12].

Chromogenic media for identification and antifungal susceptibility testing by agar dilution method

Identification

Brilliance *Candida* Agar **(Oxoid, UK)** was prepared and interpreted according to manufacturers' instructions. *C. tropicalis* appears as dark blue colonies. Both *C. albicans* and *C. dubliniensis* colonies give green color. *C. krusei* is typically dry, irregular pink-brown colonies. *C. glabrata, C. kefyr, C. parapsilosis* and *Candida lusitaniae* appear as a variety of beige/brown/yellow colors, due to the mixture of natural pigmentation and some alkaline phosphatase activity.

Antifungal susceptibility testing by agar dilution method

Preparation of stock solution

A stock solution for each of Voriconazole, Micafungin, and Fluconazole **(Sigma-Aldrich, USA)** was prepared to reach a final concentration of 10 mg/mL for voriconazole and micafungin, and 2 mg/ml for fluconazole.

Preparation of working solution

A fresh working solution was prepared for each antifungal as shown in **Table 2**.

Preparation of inoculum:

The inoculum was prepared by picking five *Candida* colonies (approximately 1mm in diameter). The colonies were suspended in 5 mL of 8.5 % sterile saline and density was adjusted to a 0.5 McFarland standard. A working suspension is made by taking 1 mL of the stock suspension and diluting it in 9 mL saline to dilute it 1:10 and further taking 1 mL of the diluted suspension and adding it in 9 mL saline to dilute it another 1:10 dilution to produce a working suspension of 5 x 10^2 to 2.5 x 10^3 cells per mL as recommended by CLSI, M27A3 [13].

Interpretation

Interpretation was done according to growth on the plates and correlated with the **CLSI** [11] as shown in **Table 3**.

Statistical analysis

The collected data were revised, coded, tabulated and introduced to a PC using Statistical package for Social Science **(IBM SPSS Statistics for Windows, Version 25.0).** Data were presented and suitable analysis was done according to the type of data obtained for each parameter.

Descriptive statistics

Frequency and percentage for non-numerical data.

Analytical statistics

1- Fisher's exact test: was used to examine the relationship between two qualitative variables when the expected count is less than 5 in more than 20% of cells

2- **Cohen's Kappa statistics:** to compute the measure of agreement between two investigational methods Cohen's Kappa's < 0.20 is defined as poor interobserver agreement; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, good; and 0.81 to 1.00, very good or almost perfect

P- value: level of significance

-P>0.05: Non-significant (NS).

-P< 0.05: Significant (S).

-P<0.01: Highly significant (HS).

Results

This study included 160 *Candida* isolates from clinically relevant patients with *Candida* infection from different body sites. Samples were urine (102, 63.7%), blood (30, 18.8%), sputum (19, 11.9%) and pus (9, 5.6%). The patients were 85 females (53.1%) and 75 males (46.9%).

According to the identification results of the Brilliance *Candida* agar media**,** *C. tropicalis* showed the highest isolation rate with 74 isolates (46.25 %), followed by *C. albicans* with 58 isolates (36.25 %), yellow/beige colonies that could be *C. parapsilosis/ C. glabarata/ C. kefyr, C. lusitaniae, C. krusei* with three isolates (1.9 %), and one undetermined species with a percentage of 0.6 %.

As regards the identification results of the VITEK 2 system, *C. tropicalis* showed the highest isolation rate with 73 isolates (45.6 %), followed by *C. albicans* with 57 isolates (35.6 %), *C. parapsilosis* with 20 isolates (12.5 %), *C. krusei* with three isolates (1.9 %), *C. kefyr* and *C. glabarata*

with two isolates each (1.3 %), *and C. lusitaniae, C. guilliermondii* and *C. ciferrii* with one isolate each (0.6 %).

Regarding the results of the identification of *Candida* species, there was a highly significant, very good (almost perfect) agreement (KAPPA= 0.9) between the VITEK 2 system and the Brilliance *Candida* agar media.

The sensitivity of Brilliance *Candida* agar media for identification, in our study, was found to be 100% for *C. albicans, C. krusei and C. tropicalis*.

As regards the antifungal susceptibility testing by agar dilution method using the Brilliance *Candida* agar media, the results obtained for Fluconazole, Voriconazole and Micafungin were as follows: for Fluconazole, susceptible, SDD and resistant strains were 139 (88.5%), 12 (7.6%) and 6 (3.8%) respectively. For Voriconazole, 150 (93.8%), 6 (3.8%), and 4 (2.5%) strains were susceptible, intermediate, and resistant, respectively. Finally, 154 (98.7%) strains were susceptible, and 2 (1.3%) strains were intermediate for Micafungin.

Regarding the antifungal susceptibility testing done by the VITEK-2 system, the results obtained for Fluconazole, Voriconazole and Micafungin were as follows: for Fluconazole, susceptible, resistant and SDD strains were 134 (89.9%), 10 (6.7%) and 5 (3.4%) respectively. Out of the 160 *Candida* strains, 152 (95.0%), 7 (4.4%) and 1 (0.6%) were susceptible, intermediate, and resistant for Voriconazole, respectively. Finally, 154 (98.7%) and 2 (1.3%) were susceptible and resistant strains for Micafungin, respectively.

As regards the antifungal susceptibility testing done for all the studied *Candida* strains by VITEK-2 susceptibility testing and agar dilution method using the Brilliance *Candida* Agar media, there was a highly significant, good agreement (KAPPA= 0.638) between both methods regarding Fluconazole susceptibility testing as shown in **Table 4**.

Regarding Voriconazole and Micafungin, there was a poor agreement between both methods with (KAPPA= 0.07) and (KAPPA= 0.004), respectively as shown in **Tables 5, 6**.

Regarding antifungal susceptibility testing done for each *Candida* spp. using the VITEK-2 system, the results are shown in **Table 7**.

Concerning *C. krusei*, the isolated three strains, were susceptible for both Voriconazole and Micafungin. The two isolates of *C. glabrata*, were also tested only for Voriconazole and Micafungin, and were susceptible for both. The two isolates of *C. kefyr*, and the one isolate of *C. ciferrii* were tested only for voriconazole and were susceptible. Lastly, the sole isolate of C. guilliermondii was tested for Voriconazole and Micafungin and was resistant for both.

Table 1. Species-specific breakpoints for antifungal drugs used in the VITEK 2 AST card (AST-YS08)

Organism/Antibiotic		**S**	**R**	**Source**
Candida	Voriconazole (AST-YS08)	≤1	≥4	FDA
C. albicans	Fluconazole (AST-YS08)	≤ 2	≥ 8	CLSI
	Micafungin (AST-YS08)	≤ 0.25	≥ 1	CLSI
C. glabarta	Micafungin (AST-YS08)	≤ 0.25	≥ 1	CLSI
C. guilliermondii	Micafungin (AST-YS08)	≤ 2	≥ 8	CLSI
C. krusei	Micafungin (AST-YS08)	≤ 0.25	≥ 1	CLSI
C. parapsilosis	Fluconazole (AST-YS08)	≤ 2	≥ 8	CLSI
	Micafungin (AST-YS08)	≤ 2	≥ 8	CLSI
C. tropicalis	Fluconazole (AST-YS08)	≤ 2	≥ 8	CLSI
	Micafungin (AST-YS08)	≤ 0.25	≥ 1	CLSI

(Quoted from CLSI M60 and FDA STIC Website)

Table 2. Summary for different antifungal concentrations of working solutions and final working concentrations reached using specific volumes

Antifungal drugs	**Concentration of working solution**	**Volume to be taken from working solution**	**Final concentration**	**Final volume**
Voriconazole	25 µg /mL	0.6 mL	0.12 µg /mL	125 mL
		1.25 mL	0.25 µg /mL	
		2.5 mL	0.5 µg /mL	
		5 mL	1 µg /mL	
Micafungin	500 µg /mL (1^{st} working solution)	0.5 mL	2 µg /mL	125 mL
		1 mL	4 µg /mL	
		2 mL	8 µg /mL	
	25 µg /mL (2^{nd} working solution)	1.25 mL	0.25 µg /mL	125 mL
		2.5 mL	0.5 µg /mL	
		5 mL	1 µg /mL	
Fluconazole	100 µg /mL	2.5 mL	2 µg /mL	125 mL
		5 mL	4 µg /mL	
		10 mL	8 µg /mL	

Table 3. Minimal inhibitory concentration breakpoints for in vitro broth dilution susceptibility testing of *Candida spp*.

Antifungal agent	Species	MIC breakpoints and interpretive categories, µg /mL			
		S	I	SDD	R
Fluconazole	*C. albicans*	≤ 2	-	4	≥ 8
	C.glabarta	-	-	≤32	≥64
	C.krusei	-	-	-	-
	C.parapsilosis	≤ 2	-	4	≥ 8
	C.tropicalis	≤ 2	-	4	≥ 8
Voriconazole	*C. albicans*	≤ 0.12	0.25-0.5	-	≥ 1
	C.glabarta	-	-	-	-
	C.krusei	≤ 0.5	1	-	≥ 2
	C.parapsilosis	≤ 0.12	0.25-0.5	-	≥ 1
	C.tropicalis	≤ 0.12	0.25-0.5	-	≥ 1
Micafungin	*C. albicans*	≤ 0.25	0.5	-	≥ 1
	C.glabarta	≤ 0.06	0.12	-	≥ 0.25
	C.guilliermondii	≤ 2	4	-	≥ 8
	C.krusei	≤ 0.25	0.5	-	≥ 1
	C.parapsilosis	≤ 2	4	-	≥ 8
	C.tropicalis	≤ 0.25	0.5	-	≥ 1

(CLSI, 2020)

S: Susceptible, I: Intermediate, SDD: Susceptible dose dependent, R: Resistant

Table 4. Agreement between VITEK-2 and Brilliance *Candida* agar dilution as regards antifungal susceptibility testing using Fluconazole

		Fluconazole susceptibility by VITEK-2 system						**KAPPA**	**P-value (sig)**
		Susceptible		**Resistant**		**SDD**			
		N	**%**	**N**	**%**	**N**	**%**		
Fluconazole susceptibility by Brilliance *Candida* agar dilution method	**Resistant**	0	0.0%	5	50.0%	1	20.0%	0.638	0.001(HS)
	SDD	5	3.7%	3	30.0%	4	80.0%		
	Susceptible	129	96.3%	2	20.0%	0	0.0%		

Table 5. Agreement between VITEK-2 and Brilliance *Candida* agar dilution as regards antifungal susceptibility testing using Voriconazole

		Voriconazole susceptibility by VITEK-2 system						**kappa**	**P-value (sig)**
		Susceptible		**Intermediate**		**Resistant**			
		N	%	N	%	N	%		
Voriconazole susceptibility by Brilliance *Candida* Agar dilution method	**Resistant**	4	2.6%	0	0.0%	0	0.0%	0.07	0.27(NS)
	Intermediate	5	3.3%	1	14.3%	0	0.0%		
	Susceptible	143	94.1%	6	85.7%	1	100.0%		

Table 6. Agreement between VITEK-2 and Brilliance *Candida* agar dilution as regard susceptibility testing using Micafungin

		Micafungin susceptibility by VITEK-2 system				**kappa**	**P-value (sig)**
		Susceptible		**Resistant**			
		N	**%**	**N**	**%**		
Micafungin susceptibility by Brilliance *Candida* Agar dilution method	**Intermediate**	2	1.3%	0	0.0%	0.004	0.9 (NS)
	Susceptible	152	98.7%	1	100.0%		

Table 7. Antifungal susceptibility testing for *C. albicans, C. tropicalis* and *C. parapsilosis* using Fluconazole, Voriconazole and Micafungin by the VITEK-2 system

***Candida* spp. (no.)**	**Antifungal drug**	**Susceptibility results using VITEK-2 system**	**No. of isolates**	**%**
***C. albicans* (57)**	**Fluconazole**	**Susceptible (S)**	56	98.2%
		Resistant (R)	1	1.8%
	Voriconazole	**Susceptible (S)**	57	100%
	Micafungin	**Susceptible (S)**	57	100%
***C. tropicalis* (73)**	**Fluconazole***	**Susceptible (S)**	67	91.8%
		Susceptible dose dependent (SDD)	2	2.7%
		Resistant (R)	3	4.1%
	Voriconazole	**Susceptible (S)**	73	100%
	Micafungin*	**Susceptible (S)**	72	98.6%
***C. parapsilosis* (20)**	**Fluconazole**	**Susceptible (S)**	11	55%
		Susceptible dose dependent (SDD)	3	15%
		Resistant (R)	6	30%
	Voriconazole	**Susceptible (S)**	13	65%
		Intermediate (I)	7	35%
	Micafungin	**Susceptible (S)**	20	100%

*One C. tropicalis isolate was terminated by the VITEK-2 system for both Fluconazole and Micafungin and no result was obtained for both.

Discussion

In the present study, *C. tropicalis* was the predominant isolated species (45.6 %). This was followed by *Candida albicans* (35.6 %), *C. parapsilosis* (12.5 %), *C. krusei* (1.9 %), *C. kefyr* and *C. glabarata* (1.3 %), *and C. lusitaniae, C. guilliermondii* and *C. ciferrii* with (0.6 %) each. This was in accordance with the results by other studies where *C. tropicalis* was the predominant species recovered with (35.3%, 46.5%, 54.5%, and 38.6%), respectively [14, 5, 15, 16].

As regards the identification of *Candida* species by the VITEK-2 system and Brilliance *Candida* agar, both methods were in an almost perfect agreement (KAPPA= 0.9). According to our study, only three isolates showed discrepant results. The first isolate was identified as *C. ciferrii* by the VITEK-2 system (low discrimination), but the chromogenic media identified it as *C. albicans* with a positive germ tube test. *C. ciferrii* is a rare kind of opportunistic pathogenic fungus that was first identified in 1962. It is now part of a microbial complex called *Stephanoascus ciferrii* complex which is composed of *C. ciferrii*, *Candida allociferrii*, and *Candida mucifera*. Usually, the VITEK-2 yeast identification system just identifies the microorganism as *S. ciferrii* complex, and it does not identify the complex at species level [17].

The second isolate was identified as *C. guilliermondii* by the VITEK-2 system (88% probability), while the chromogenic media yielded beige-coloured colonies that could not be definitely recognized. The third isolate was a *C. parapsilosis* by the VITEK-2 (94% probability), although identified on the chromogenic media as a mixed *Candida* infection of *C. albicans* and *C. tropicalis*. These results highlight that the chromogenic media is advantageous in identifying samples of mixed *Candida* infection. However, it faces difficulty in discriminating some rarer types of *Candida* such as *C. guilliermondii* and *C. ciferrii*.

The sensitivity of Brilliance *Candida* agar media for identification, in our study, was found to be 100% for *C. albicans, C. krusei and C. tropicalis*.

In a Turkish study**,** the sensitivity ratio of CHROMagar *Candida* and VITEK-2 was found to be 96.2% and 90.7% of all 54 tested *Candida* strains, respectively. One *C. albicans* and one *C. glabrata* isolates were misidentified as *C. parapsilosis* by CHROMagar *Candida*. Two *C. parapsilosis* and three *C. albicans* isolates were misidentified by VITEK-2 [18].

As regards the distribution of different types of *Candida* spp. among the various isolation sites in our study, it was isolated most frequently from urine (63.7%). 50% of *C. tropicalis* isolates were recovered from urine specimens.

These results were comparable with an Indian, where most of their isolates were obtained from urine samples (63.9%) and were predominantly *C. tropicalis* [15].

As regards the antifungal susceptibility results, concerning Fluconazole susceptibility testing, our data show that 88.5% of the 160 *Candida* isolates were susceptible, 7.6% were SDD, and 3.8% were resistant. Regarding *C. tropicalis, C. albicans and C.* parapsilosis their detailed susceptibility for Fluconazole, Voriconazole and Micafungin were as mentioned in **Table 7**.

Our results agreed with ***Arastehfar et al.***, who reported that among their 64 *C. tropicalis* strains, 82.8% were susceptible to Fluconazole, 10% were SDD, and 6.25% were resistant [19].

Regarding *C. albicans*, our results agreed well with ***Chadwick and his co-workers*** who reported that out of the 1187 tested *C. albicans* isolates, 98.4% were susceptible to Fluconazole, and 0.8% were resistant. On the other hand, out of 23 isolated *C. tropicalis* strains 43.5% were susceptible to Fluconazole, 21.7% were SDD, and 34.8% were resistant. They explained their high level of Fluconazole resistance with *C. tropicalis* to the misinterpretation of the trailing effect as true resistance. As for *C. parapsilosis*, results were comparable where out of the 15 isolates, 13.3% were SDD to Fluconazole. On the other hand, they reported higher susceptibility to Fluconazole (86.7%), and no resistant *C. parapsilosis* isolates were found [7].

Other studies found different findings. ***Yesudhason and Mohanram*** observed a high level of resistance to Fluconazole (37.7%) among their tested 61 *C. tropicalis* isolates [15]. ***Wiebusch and his colleagues*** reported that 54.16% of their 24 *C. albicans* isolates were susceptible to Fluconazole, and 45.83% were resistant [20].

Fekkar et al. also reported a high susceptibility rate among their 283 *C. parapsilosis* isolates, with 90.8% susceptible isolates and 9.2% resistant strains [21]. The high resistance rate in our results concerning *C. parapsilosis* with Fluconazole,

could be attributed to the high prescription rate of Fluconazole among our patients since it is the cheapest and most affordable antifungal drug in our country.

As for Voriconazole, out of our 160 *Candida* isolates, 95.0% were susceptible, 4.4% were intermediate, and 0.6% were resistant. All our isolated 73 *C. tropicalis* strains and 57 *C. albicans* isolates were susceptible to Voriconazole. As for *C. paraspilosis*, out of the 20 isolates, 65% were susceptible to Voriconazole.

Our results disagreed with the other studies. ***Arastehfar et al.*** reported a higher resistance to Voriconazole among their 64 *C. tropicalis* isolates, where 10.9% of them were resistant, and 28.1% were intermediate to Voriconazole [19]. ***Wiebusch and his colleagues,*** in Brazil, also observed a higher resistance to Voriconazole. Out of their 24 *C. albicans* isolates, 54.16% were susceptible and 45.83% were resistant [20]. In a study by ***Xiao and his co-workers,*** they showed that 99.2% of their 392 isolated *C. parapsilosis* strains were susceptible to Voriconazole [22].

Concerning Micafungin in this study, out of the 156 *Candida* isolates, 98.7% and 1.3% were susceptible and resistant to Micafungin, respectively. Out of the 73 *C. tropicalis* strains, 98.6% were susceptible to Micafungin. All our 57 *C. albicans* and 20 *C. parapsilosis* isolates were susceptible for Micafungin.

Similarly, ***Arastehfar et al.*** reported that out of the 64 *C. tropicalis* strains, 3.1% were resistant to Micafungin [19].

For *C. albicans* isolates and *C. parapsilosis*, there were no sufficient data to compare our results with. However, the low resistance rates for Voriconazole and Micafungin results throughout our isolates could be the result of their limited use in our country as they are mostly limited to oncology patients and their very expensive price.

In our study, the three *C. krusei* isolates (100%) were susceptible to both Voriconazole and Micafungin. But they were resistant to Fluconazole. In the three years surveillance by ***Xiao and his colleagues,*** all 40 *C. krusei* isolates were (100%) susceptible to voriconazole and 99% were susceptible to Micafungin [22].

Our two *C. glabrata* isolates were susceptible to Voriconazole and Micafungin. ***Xiao and his colleagues*** found that all their 258 *C. glabrata* isolates were susceptible to Micafungin. 14.3% of *C. glabrata* isolates were Fluconazole resistant, and 11.6% were cross-resistant to Fluconazole and Voriconazole [22].

Our sole isolate *C. guilliermondii* was resistant to both Voriconazole and Micafungin. ***Hirayama and his co-workers*** revealed that all tested 27 *C. guilliermondii* isolates were susceptible to Micafungin [23].

There were no available breakpoints provided by the CLSI concerning *C. kefyr* and *C. ciferrii* susceptibility testing. In our study, the two *C. kefyr* isolates and the single *C. ciferrii* isolate were only tested for Voriconazole and were presumably susceptible.

In a 5-year Iranian study, 43 out of their tested 44 *C. kefyr* isolates were susceptible to Voriconazole [24]. Concerning *C. ciferrii*, there were no available data to compare our results with.

The difference in AFST compared to other studies may be attributed to the geographical distribution of *Candida* spp., together with the difference in species distribution among body sites. Also, the genotypic variability in *Candida* spp. and previous exposure to antifungal drugs played an important role in these discrepancies [25].

In the current study, concerning the Fluconazole susceptibility results using the agar dilution method by Brilliance *Candida* agar media, they were in good agreement (KAPPA= 0.63) with the VITEK-2 system. However, there was a poor agreement (KAPPA= 0.07) between the antifungal susceptibility testing for Voriconazole using the two methods. Also, susceptibility testing results done for Micafungin using both methods were in poor agreement (KAPPA= 0.004).

Other investigators performed AFST on chromogenic media using disc diffusion methods, which failed to separate susceptible dose-dependent isolates [26, 27]. Others added antifungals to the CHROMagar but could only identify highly susceptible and highly resistant isolates. These studies were promising but required multiple plates and failed to identify the SDD isolates [8, 9, 28, 29].

Chadwich et al. added Fluconazole with different concentrations to chromogenic media and could identify susceptible, SDD, and resistant isolates [7].

In this study, the Brilliance *Candida* agar media demonstrated 100% sensitivity in speciation

of the three major *Candida* species, i.e., *C. albicans*, *C. tropicalis*, and *C. krusei*, which were easily identified on the plates. However, there was some difficulty in the discrimination of several rarer *Candida* species, such as *C. parapsilosis*, *C.kefyr*, *C.glabarata* and *C.lusitaniae*, as they appear as a variety of beige/brown/yellow colours.

During our work, the cost for the identification and AFST using the chromogenic media costed approximately 2.4 U.S. dollars per sample, while they costed approximately 10.7 U.S. dollars per sample using the VITEK-2 system. The Brilliance *Candida* agar media also demonstrated a considerable advantage in its ability to test several samples on the same plate. An advantage that could not be provided by the VITEK-2 system. These advantages are very cost-effective in the laboratory. Moreover, mixed populations of *Candida* species were identified easily. This advantage reduces the risk of misidentification and is very time saving for critically ill patients.

Number of limitations affected our work. Some biochemical reactions, needed as a further step for species confirmation by the VITEK-2 system, were unavailable in our laboratory, and the same for the Brilliance *Candida* agar media. Also, antifungal susceptibility provided by the YS-08 cards did not include Fluconazole data for some species such as *C. glabrata, C. kefyr, C. ciferrii* and *C. guilliermondii.* Also, it did not contain Micafungin data for *C. kefyr* and *C. ciferrii.* This is probably due to the lack of data by the CLSI, 2020 for interpreting the rarer *Candida* species.

The ability of the VITEK-2 to detect resistance of Voriconazole with *C. albicans, C. krusei, C. parapsilosis, C. tropicalis, C. lusitaniae, C. guilliermondii* and Micafungin with all *Candida* spp is unknown. Also, an alternative method of testing is required to be performed prior to reporting of results of Fluconazole with: *C. glabarta and C. kefyr* (According to manufacturer's instructions).

Another problem faced by the VITEK-2 was the unexpected termination of samples. This was due to the unacceptable growth in the control well of the VITEK-2 automated susceptibility testing card. This problem, though not frequent, will cost the patient extra valuable time for being repeated, putting an additional financial burden on the laboratory.

Conclusion

There is a shift in the distribution of the *Candida* spp. towards the non-albicans *Candida* with the predominance of *C. tropicalis* among our hospitalized patients. This mandates the need for species identification to properly perform antifungal AFST as recommended by the CLSI.

The chromogenic media proved to be a reliable and cost-effective method for identification of common *candida* spp. and for identification of mixed *candida* infections.

AFST using agar dilution on chromogenic media showed high accuracy when used with Fluconazole. Fluconazole showed a high rate of resistance reaching 30% among *C. parapsilosis* isolates, which suggests the need for alternative empirical therapy. On the other hand, micafungin showed no resistance with almost all tested *Candida* spp.

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Conflicts of interest

The authors declare that they do not have any conflict of interest.

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