ORIGINAL ARTICLE

Species Identification of *Candida* Isolates from Critically Ill Patients by Rapid Commercial and Genotypic Methods

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ABSTRACT

Background: *Rapid, reliable Candida species (spp.) identification is crucial because it enables prompt and effective antifungal treatment. Unfortunately this is difficult due to a lack of knowledge, adequate reagents, and equipments, particularly in countries with limited resources.* ***Objectives:*** *The aim of this study was to assess the effectiveness of rapid commercial methods in identifying Candida spp. isolated from critically ill patients.* ***Methodology:*** *Various clinical specimens were collected from critically ill patients admitted to the Intensive Care Units of Mansoura University Hospitals, and processed by numerous mycological techniques. Gram stained, and lactophenol cotton blue stained films, as well, germ tube test, and corn meal agar with 1% Tween 80 were used to identify the isolated Candida spp. Three commercial methods were used for spp. identification; CHROMagar™ Candida medium, API 20 C AUX and Vitek-2 Compact automated system. In addition, all Candida isolates were subjected to species-specific multiplex PCR as the reference method.* ***Results:*** *A total of 52 Candida strains were identified where C. albicans was the predominant spp. (53.8%) followed by C. parapsilolsis (17.3%), C. glabrata (13.5%), C. dubliniensis (5.8%), C. tropicalis (5.8%), C. auris (1.9%) and C. krusei (1.9%). Both CHROMagar™ Candida medium and Vitek-2 Compact automated system exhibited high sensitivity and specificity rates in identification of various Candida spp. as compared to multiplex PCR.* ***Conclusion:*** *This study demonstrated a good performance of both CHROMagar™ Candida medium and Vitek-2 Compact automated system; however, CHROMagar™ Candida medium may be an easier and more cost-effective choice to identify Candida spp., mainly in areas with restricted resources.*

INTRODUCTION

Candida species (spp.) are part of the commensal microbiota of skin, gastrointestinal tract, and respiratory mucosa; however, they can cause opportunistic infections with impaired immune system[1]. *Candida* has become a major nosocomial pathogen among critically ill immunocompetent and immunocompromised patients for a variety of reasons, such as increased numbers of bone marrow and solid organ recipients, neutropenia, malignancy, overuse of broad spectrum antibiotics and prior surgery. Other risk factors for nosocomial candidiasis include; prolonged stay in the intensive care units (ICUs), total parenteral nutrition and the use of central lines and other invasive devices[2].

The most prevalent *Candida* infections among ICUs patients are bloodstream infections, surgical site infections, respiratory and urinary tract infections. Candidemia is potentially fatal, and is linked to high morbidity and fatality rates[3]. Various studies stated that *Candida albicans (C. albicans)* is still the commonest *Candida* spp. recovered from invasive candidiasis (IC), despite the fact that isolation rates of non-albicans *Candida* (NAC) spp. displayed resistance to typical antifungal medications have dramatically increased recently[4]. Among NAC strains associated with IC are *C. parapsilosis, C. glabrata, C. tropicalis, C. kefyr, C. guilliermondii,* and *C. Krusei*[5]. The development of more advanced diagnostic techniques has aided in the discovery of novel NAC species, such as *C. auris*, which was isolated from numerous nosocomial *Candida* infections worldwide[6].

Because of the heterogeneity in the epidemiology of *Candida* infections and the use of novel antifungal agents with diverse spectrums, therapeutic decisions made by physicians may no longer be based solely on the classification of *Candida* as either *C. albicans* or NAC[7]. Therefore, prompt and accurate identification of *Candida* spp. is essential for the efficient treatment of *Candida* infections. Clinical microbiology and research laboratories use a variety of techniques to detect *Candida* spp[8].

Standard conventional methods, based on morphological characters, have been used to identify various *Candida* spp. such as the germ tube test which can differentiate between germ tube-positive spp.

including *C. albicans*, *C. stellatoidae*, and *C. dubliniensis* from the other germ tube-negative spp., and chlamydospores production on cornmeal agar which takes 24−72 h[9]. Besides, biochemical reactions as sugar assimilation and fermentation have been used to differentiate between *Candida* spp. These detection methods are tiresome and time-consuming[10]. Several commercial diagnostic techniques are now available as chromogenic media, API 20 C and automated Vitek-2 Compact system which can help in precise identification of *Candida* spp[11].

CHROMagar™ *Candida* medium is a selective differential chromogenic medium used to isolate and ostensibly identify distinct *Candida* spp. based on their color[12]. Another commercial approach for differentiating between *Candida* spp. is the API 20 C system. It is composed of 20 cupules that allow for the execution of 19 assimilation tests. In this method, yeast cells will only proliferate if they can use the dehydrated substrate as their sole supply of carbon[13].

Various infections, including *Candida*, can be identified and tested for antimicrobial sensitivity using the fully automated Vitek-2 Compact system. It is a quick method that can in 18 h identify microbes down to the species level, compared to 48–72 h in other approaches, by using Vitek-2 cards that allow species identification by comparing the resulting biochemical profile to a large database[14]. In addition to these techniques, DNA-based approaches also aid in the differentiation of distinct *Candida* spp. with high sensitivity and specificity [15].

This study intended to (i) investigate the distribution of various *Candida* spp. among clinical samples collected from critically ill patients (ii) compare the effectiveness of three commercial methods for identifying *Candida* spp., including CHROMagar™ *Candida* medium, API 20 C AUX system, and Vitek-2 Compact automated system with species-specific PCR.

METHODOLOGY

Ethical approval statement

The institutional research board at Mansoura Faculty of Medicine, Egypt gave the study its approval and assigned it the protocol number R.22.09.1814.

Study design and samples collection:

This prospective study was carried out during the period extended from July 2021 to June 2022. Various clinical specimens were collected from severely ill patients, with suspected candidiasis, who were admitted to the ICUs at Mansoura University Hospitals, Egypt. Samples were transferred to the Mycology Lab, Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Egypt within 2 h after collection.

Samples processing and mycological examination:

All clinical samples were prepared for the isolation of *Candida* strains following established mycological protocols. First, *Candida* growth was obtained by streaking of samples on Sabouraud's dextrose agar (SDA) plates supplemented with chloramphenicol (50µg/ml) (Oxoid, UK), then were incubated aerobically at 37˚C for 24 h. Gram stained, and lactophenol cotton blue stained films, in addition to, germ tube tests, and subculture on corn meal agar (Oxoid, UK) with 1% Tween 80 (Sigma, Aldrich) were used to identify the isolated *Candida*[16].

Identification of *Candida* species by rapid commercial methods:

CHROMagar™ Candida medium

The isolated *Candida* colonies were sub-cultured on CHROMagar™ *Candida* medium (CHROMagar, France). Following incubation of the plates for 48 h at 37°C, the growth was interpreted per manufacturer's instructions[17].

API 20 C AUX system

The API 20 C AUX system (Bio-Merieux, France) was also used for differentiation of *Candida* spp. The cupules turbidity was identified after incubation at 37°C for 24−72 h by using the analytical profile index and reading them as compared to the growth control (0 cupule)[18].

Vitek-2 Compact automated system

Moreover, the Vitek-2 Compact automated system (Bio-Mérieux, France) was used to identify *Candida* spp. *Candida* suspensions of 1.8–2.2 McFarland standard were loaded onto the identification cards and incubated for 18 h. Following incubation, the results shown on the cards were compared with an identification database[14].

Reference strains *C. albicans* (ATCC 24433), *C. krusei* (ATCC 6258), *C. tropicalis* (0750), *C. glabrata* (ATCC 90030) and *C. parapsilosis* (ATCC 22019) were used as quality control. *Candida* isolates were stored in medium containing 20% glycerol at −80°C for further molecular identification.

Genotypic detection of *Candida* spp. by species-specific multiplex PCR:

The multiplex PCR technique was used to identify the study isolates using universal primers that target the internal transcribed spacer region (ITS) and *Candida* species-specific primers (Sigma, Aldrich) as shown in table 1[19–23].

Candida DNA was extracted from fresh *Candida* subculture on SDA using Zymo DNA mini kit (Zymo, USA) according to the manufacturers' instructions. The PCR reaction was conducted in 50 µl reaction mix containing 25 µl of 2X GoTaq Green Master Mix (Willowfort, UK), 5 µl of genomic DNA, 1 µl from each universal and species-specific forward and reverse primers and 2 µl DNase free water. The PCR conditions were as follows: initial denaturation step for 5 min at

96°C, forty cycles of: denaturation at 94º C for 30 sec, annealing at 58 ºC for 30 sec, extension at 72°C for 30 sec, followed by a final extension step for 15 min at 72ºC[24].

Utilizing the UV transilluminator (FBTIV-88, Fisher, USA), the amplified PCR products were detected by using 2% agarose gel electrophoresis. Based on the size of the fragments in contrast to a 100 bp DNA marker (Lonza Rockland Inc., USA), *Candida* spp. identification was made [25].

Table 1. Primers used for identification of *Candida* species

***Candida* species**	**Targeted gene**	**Primer sequences (5' to 3')**	**Amplicon size (bp)**	**Reference**
Universal primers	*ITS1* *ITS2*	TCC GTA GGT GAA CCT GCG G GCT GCG TTC TTC ATC GAT GC	–	19
C. albicans	*CalbF* *CalbR*	TGGTAAGGCGGGATCGCTT GGTCAAAGTTTGAAGATATAC	446	20
C. dubliniensis	*CdubF* *CdubR*	AAACTTGTCACGAGATTATTTTT AAAGTTTGAAGAATAAAATGGC	217	20
C. glabrata	*CglaF* *CglaR*	TCACTTTCAACTGCTTTCGC TGCGAGTCATGGGCGGAA	482	21
C. parapsilolsis	*PparF* *CparR*	GCGGAAGGATCATTACAGAATG CTGGCAGGCCCCATATAG	229	21
C. tropicalis	*CtroF* *CtroR*	AGACACGACTTTTTCGCATTTTTC TTCCACAGCTTCAACCAATGCAA	218	22
C. krusei	*CkF* *CkR*	TGTGGAATATAGCATATAGTCGACA CAACTCTGCGCACGCGCAAGAT	182	22
C. auris	*CauF* *CauR*	CGCACATTGCGCCTTGGGGTA GTAGTCCTACCTGATTTGAGGCGAC	215	23
C. lusitaniae	*ClusF* *ClusR*	GCGATACGTAGTATGACTTGCAGACG CAGCGGGTAGTCCTACCTGA	203	23

Note: bp, base pair.

Statistical Analysis

The statistical package of SPSS version 23 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Values were expressed as numbers and percentages. Mean ± standard deviation (SD) were used to express parametric data. Sensitivity and specificity of the various testing procedures were assessed by using species-specific multiplex PCR as the gold standard method.

RESULTS

During our study, 52 non-repetitive *Candida* strains were recovered, by both phenotypic and genotypic techniques, from different clinical samples. Out of these 52 isolates, 28 (53.8%) were *C. albicans* and 24 (46.2%) were NAC. Various *Candida* spp. were illustrated in table 2. Among the study participants, 19 (36.5%) were females while 33 (63.5%) were males with an age range from 26–70 years (Mean: 46.7, SD: 10.0).

Table 2: Distribution of *Candida albicans* and non-albicans *Candida* isolates among the study participants

***Candida* species (n=52)**	**No.**	**%**
C. albicans	28	53.8
Non-albicans *Candida*	24	46.2
C. parapsilolsis	9	17.3
C. glabrata	7	13.5
C. dubliniensis	3	5.8
C. tropicalis	3	5.8
C. krusei	1	1.9
C. auris	1	1.9

Among the isolated *Candida*, 19 (36.5%) showed evidence of germ tube production, while 33 (63.5%) were negative (figure 1). The 19 positive strains were further confirmed as *C. albicans* by multiplex PCR. On corn meal agar with 1% Tween 80, 22 strains (42.3%), and 3 strains (5.8%) were identified as *C. albicans* and *C. dubliniensis*, respectively with typical chlamydospores formation (figure 2). As opposed to that, 27 (51.9%) *Candida* strains couldn't be speciated by the corn meal agar.

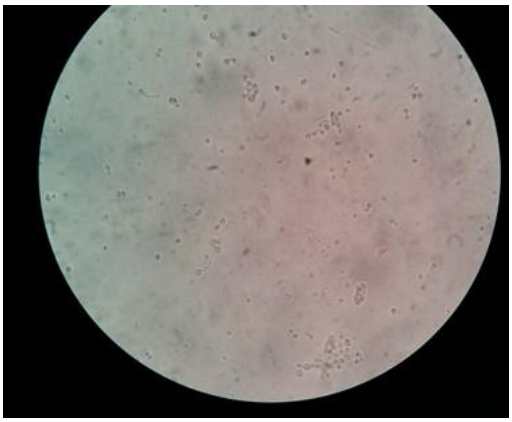

Fig. 1: Positive germ tube formation of *Candida* isolates in fresh human serum at 37°C for 4 h (40x).

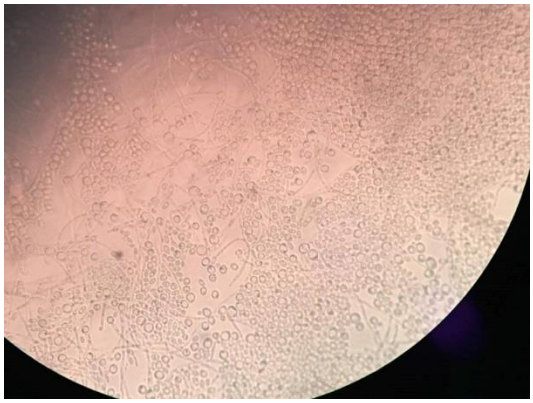

Fig. 2: Subculture of *Candida* species on corn meal agar with 1% Tween 80 showing yeast cells, pseudohyphae and chlamydospores (40x).

Additionally to these conventional methods, we used three commercial procedures for the species detection of *Candida* isolates: CHROMagar™ *Candida* medium, API 20 C AUX and Vitek-2 Compact automated system. By subculture of *Candida* growth on CHROMagar™ *Candida* medium, different color degrees of *Candida* colonies were observed as shown in (figure 3). This chromogenic media identified 28 (53.8%) strains as *C. albicans*, 4 (7.7%) as *C. glabrata*, 3 (5.8%) as *C. dubliniensis*, 3 (5.8%) as *C. tropicalis* and 1 (1.9%) as *C. krusei*. By this diagnostic test, 13 (25.0%) strains couldn't be differentiated as their colors were doubtful. These 13 isolates were furthered identified as *C. parapsilolsis*, *C. glabrata* and *C. auris* by multiplex PCR.

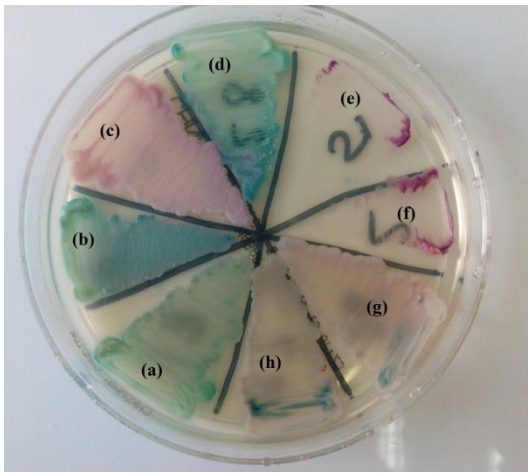

Fig. 3: Different *Candida* species on CHROMagar™ *Candida* medium at 37 °C for 48 hours (a): *C. albicans*; (b) and (d): *C. dubliniensis*; (c): *C. Krusei*; (e) and (f): *C. tropicalis*; (g) and (h): *C. glabrata*.

In our study, the API 20 C AUX system classified the *Candida* strains into 25 (48.1%) *C. albicans*, 5 (9.6%) *C. parapsilolsis*, 3 (5.8%) *C. dubliniensis*, 3(5.8%) *C. glabrata*, 2 (3.8%) *C. tropicalis* and 1 (1.9%) *C. krusei*. On the other hand, this diagnostic technique was unable to differentiate between a total of 13 (25.0%) strains. Notably, the Vitek 2 automated system was able to identify all the isolated 52 *Candida* strains, and among them, 28 (53.8%) were *C. albicans*, 9 (17.3%) were *C. parapsilolsis*, 7 (13.5%) were *C. glabrata*, 3 (5.8%) were *C. dubliniensis*, 3 (5.8%) were *C. tropicalis*, 1 (1.9%) was *C. auris* and 1 (1.9%) was *C. krusei*. These results were confirmed by multiplex PCR.

Molecular examination of the 52 *Candida* strains by multiplex PCR revealed the following spp.; *C. albicans* (28, 53.8%), *C. parapsilolsis* (9, 17.3%), *C. glabrata* (7, 13.5%), *C. dubliniensis* (3, 5.8%), *C. tropicalis* (3, 5.8%), and each of *C. auris* and *C. krusei* was (1, 1.9%).

The results of conventional and rapid commercial methods were compared with multiplex PCR which is the reference standard (figure 4). The CHROMagar™ *Candida* medium and Vitek-2 Compact automated system exhibited high specificity and sensitivity rates in identification of *Candida* spp. The performances of various diagnostic methods were illustrated in table 3.

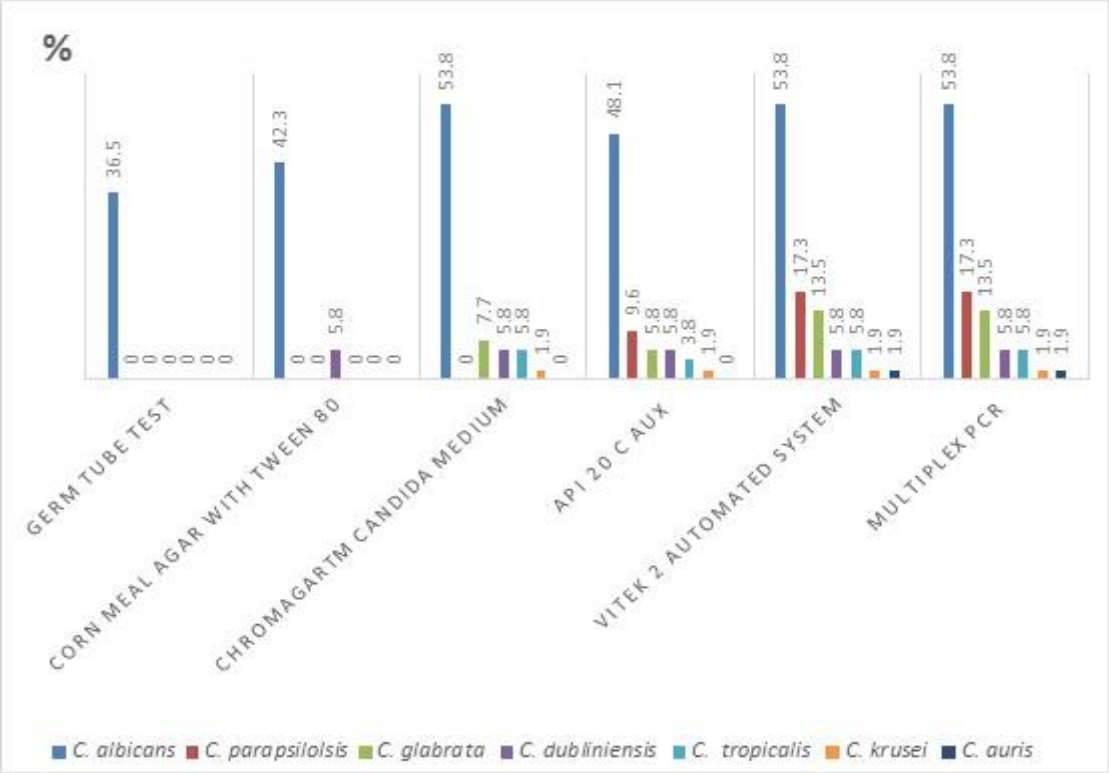

Fig. 4: Identification of *Candida* isolates by different diagnostic methods.

Table 3. Sensitivity and specificity of different methods used for *Candida* species identification in relation to multiplex PCR as the gold standard

Performance	Diagnostic test	*Candida* species (n=52)						
		C. albicans	***C. parapsilolsis***	***C. glabrata***	***C. dubliniensis***	***C. tropicalis***	***C. krusei***	***C. auris***
Sensitivity	Germ tube test	67.9%	–	–	–	–	–	–
	Corn meal agar with Tween 80	78.6%	–	–	100%	–	–	–
	CHROMagar™ *Candida* medium	100%	–	57.1%	100%	100%	100%	–
	API 20 C AUX	89.3%	55.6%	42.9%	100%	66.7%	100%	–
	Vitek-2 Compact automated system	100%	100%	100%	100%	100%	100%	100%
Specificity	Germ tube test	100%	–	–	–	–	–	–
	Corn meal agar with Tween 80	100%	–	–	100%	–	–	–
	CHROMagar™ *Candida* medium	100%	–	100%	100%	100%	100%	–
	API 20 C AUX	100%	100%	100%	100%	100%	100%	–
	Vitek-2 Compact automated system	100%	100%	100%	100%	100%	100%	100%

Regarding the *Candida* spp. distribution among various samples, 26 strains were recovered from blood (15, 57.7% *C. albicans*; 8, 30.8% *C. parapsilolsis*; and 3, 11.5% *C. glabrata*), and 12 from endotracheal aspirates (8, 66.7% *C. albicans*; 2, 16.7% *C. glabrata*; 1, 8.3% for each *C. dubliniensis*, and *C. tropicalis*), followed by 4 from sputum (2, 50.0% *C. albicans*, 1, 25.0% *C. krusei* , and 1, 25.0% *C. dubliniensis*). Overall, 10 strains were isolated from urine specimens (3, 30.0% *C. albicans*; 2, 20.0% for each *C. glabrata*, and *C. tropicalis*; followed by 1, 10.0% for each *C. parapsilolsis*, *C. auris*, and *C. dubliniensis*) (figure 5).

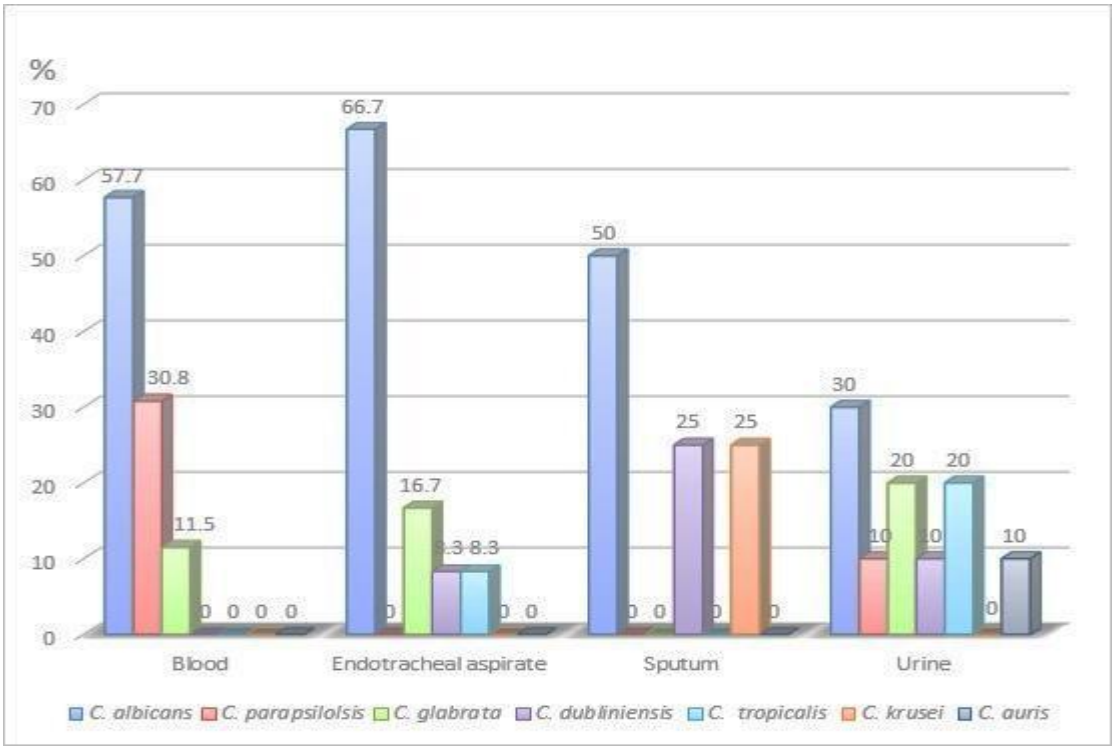

Fig. 5: Distribution of *Candida* species among various clinical specimens

DISCUSSION

Invasive candidiasis is a dangerous and potentially fatal condition with exceedingly high morbidity and fatality rates among individuals with critical illnesses[26]. Non-albicans *Candida* has gained a grave concern worldwide owing to the high proportions of *Candida* infections among severely ill patients caused by NAC. Furthermore, several NAC spp. are linked to increased incidence of inherent resistance to various antifungal medications that are frequently used to treat fungal infections. Therefore, treating infections caused by NAC spp. can be challenging to physicians which could increase mortality rates especially among ICUs patients[27].

Nowadays, a variety of kit-based commercial and molecular approaches for *Candida* spp. identification are available[28]. While some of these methods can detect *Candida* isolates directly from clinical specimens, others necessitate the isolation of *Candida* beforehand. These approaches vary in principle, cost, specificity and sensitivity rates and each has benefits and drawbacks of its own[29]. It is crucial that clinical laboratories implement a method that is accurate and practical for identifying *Candida* spp. so that timely and suitable antifungal therapy can be initiated in order to save lives[30]. However, in low income countries with limited resources, laboratories restrict the diagnosis to *C. albicans* or NAC to reduce the financial burden and most of them usually do not proceed further than the germ tube test[9].

In the current work, 52 *Candida* strains were recovered from samples collected from critically ill patients. Half of these strains were recovered from blood stream infections and 30.8% were recovered from respiratory tract infections. Our results were in line with a previous study which reported that 45.2% of their subjects had candidemia and 25.8% had respiratory infections[31]. Although high prevalence of NAC strains (46.2%) was identified in the present work, *C. albicans* (53.8%) still represented the majority of strains isolated from critically ill patients in our locality. Among the isolated NAC, *C. parapsilolsis* (17.3%) and *C. glabrata* (13.5%) represented the majority of spp. Our results were consistent with a previous study from Egypt that reported high prevalence rates of *C. albicans* among ICU patients followed by NAC[32]. In addition, a former study conducted in Iran has reported that *C. albicans* was the commonest cause of *Candida* infection (62.4%), followed by *C. parapsilolsis* (17.5%) and *C. glabrata* (8.8%)[25]. Besides, another study from Germany declared that 60.9% of their isolates were *C. albicans* followed by *C. glabrata* (19.4%), and *C. parapsilosis* (6.6%)[3]. In contrast to our results, a former study from India reported *C. tropicalis* as the commonest *Candida* spp. followed by *C. albicans*[33]. Such discrepancy may be attributed to geographical

variations and differences in antifungals regimen between countries.

Numerous conventional methods, including the germ tube test and cornmeal agar with 1% tween 80, have been established for the speciation of *Candida*[34]. However, in the present study, most of the *Candida* strains couldn't be differentiated by these methods. Numerous researches have assessed how far the alternative commercially available techniques for identification of *Candida* isolates function[35]. In the present study, three commercial methods (API 20 C AUX, CHROMagar™ *Candida* medium and Vitek-2 Compact automated system) were evaluated for their accuracy in discrimination of *Candida* isolates in respect with multiplex PCR as the reference method.

In the present study, 25.0% *of Candida* isolates couldn't be identified up to species level with API 20 C AUX system. The sensitivity of this test in identifying *C. albicans* was 89.3%, compared to 100% sensitivity and specificity of both CHROMagar™ *Candida* medium and Vitek-2 Compact automated system. This was in line with the findings of another study[36]. Sariguzel *et al.*[14] agreed with our results regarding the CHROMagar™ *Candida* medium and Vitek-2 Compact automated system. In contrast to our results, Malik and his colleagues proved the superiority of API 20 C AUX to CHROMagar™ *Candida* medium in identifying *Candida* spp.[37]

Notably, Vitek-2 Compact automated system and multiplex PCR results were quite consistent in identification of both *C. albicans* and NAC spp. with high sensitivity and specificity rates (100%). However, PCR requires more tools and reagents, so Vitek-2 system may be preferred in localities where resources are available. A preceding study from Turkey agreed with our findings[14].

CHROMagar™ *Candida* medium is a readily accessible chromogen-based culture medium that has been evaluated in the current work. Although, colors of some *Candida* strains were doubtful and some spp. couldn't be identified by CHROMagar™ *Candida* medium, this did not detract from its efficiency in differentiation of various *Candida* spp. This was in line with a foregoing Indian study that conveyed respectable performance of CHROMagar™ *Candida* medium and Vitek-2 Compact automated system as diagnostic techniques[38]. A study performed by Mathavi and his colleagues has demonstrated a high degree of precision of CHROMagar™ *Candida* medium in the discrimination of diverse *Candida* spp.[17] Furthermore, another study documented accurate species identification of *Candida* isolates using CHROMagar™ *Candida* medium[39]. Besides, a prior work by Scharmann *et al.*[12] confirmed the efficacy of this medium in differentiation of *Candida* spp. Moreover, a prior study from Egypt agreed with our results[40].

CONCLUSION

Despite the Vitek-2 Compact automated system's accuracy and benefits in both speciation of *Candida* spp., and determination of antifungal susceptibility, its high cost makes it unsuitable for low-income countries. Similarly, the API 20 C AUX is laborious, time-consuming, and subject to individual errors as it depends on turbidity measurement. On the other side, CHROMagar™ *Candida* medium has the benefits of rapid detection of *Candida* spp., technically straightforward preparation, and cost effectiveness. Therefore, it may be considered a cheaper and accurate alternative for Vitek-2 Compact automated system and molecular techniques in developing countries with low resources.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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