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Original Article

Microbiology Section

Evaluation of Five Phenotypic Tests in the Identification of *Candida* Species

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ABSTRACT

Introduction: Rapid and precise identification of *Candida* species is essential for the proper treatment of *Candida* infections. Several phenotypic and molecular methods are being used in clinical microbiology laboratories worldwide for the speciation of *Candida* isolates.

Aim: To evaluate the performance of five phenotypic tests in the identification of *Candida* species.

Materials and Methods: Five phenotypic tests (CHROMagar *Candida*, tetrazolium reduction medium, Candifast, Sugar assimilation and fermentation) were used for the identification of *Candida* species. Clinical *Candida* isolates along with reference strains of *Candida* species were used in the study. The phenotypic test results were compared with PCR-RFLP results to evaluate the performance of the phenotypic tests.

Results: All the *Candida* isolates (100%) were correctly identified to species level by CHROMagar *Candida*, Tetrazolium reduction medium and Candifast. However, 94.8% and 92.3% of the *Candida* isolates were identified by sugar fermentation test and assimilation test respectively. The time required to identify the *Candida* isolates varied with the five phenotypic tests (less than 24 hours for Candifast and maximum of 48-96 hours for sugar assimilation and fermentation).

Conclusions: All the five phenotypic tests showed good correlation in the identification of *Candida* species. However, the time required for identification varied with the different phenotypic tests used, which may have important implications in life threatening infections like candidemia.

Keywords: Assimilation, Chromagar, Candifast, Fermentation

INTRODUCTION

Fungal infections caused by *Candida* species is one of the important causes of hospital acquired infections in immunocompromised patients [1]. Aetiology for the emergence of *Candida* species as important nosocomial pathogens includes - prolonged use of antimicrobial agents, steroid therapy, malignancy, indwelling catheters, total parenteral nutrition etc [2,3]. Many *Candida* species have been involved in causing human infections and disease till the date [2]. Changes in medicine since the 1960's have been partly responsible for the emergence of new species of *Candida* as potential pathogens [4]. It is also a reflection of the novel methods available to mycologists in identifying rare species of yeast. Five *Candida* species are responsible for a majority (>90%) of the invasive infections- *Candida* albicans, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata* and *Candida krusei*. In addition, the number of new *Candida* species isolated from clinical samples increasing continuously every year. The major reason for this could be the use of various commercially available identification methods by clinical microbiology laboratories worldwide to supplement the conventional methods of identification. The rapid increase in the number of immunocompromised patients worldwide in view of the HIV (human immunodeficiency virus) epidemic and increasing numbers of organ transplantations and malignancies could also be responsible for the isolation of uncommon *Candida* species which were previously considered "non-pathogenic" [2].

Confirmation of infections by *Candida* species requires laboratory isolation and identification. Various phenotypic and molecular methods are used by clinical microbiology laboratories and research laboratories worldwide for the identification of *Candida* species. Along with conventional tests like germ tube test, sugar assimilation and fermentation, commercial methods like Candifast, Fungichrom and API 20C Aux system are being increasingly used in clinical microbiology laboratories worldwide for the speciation of *Candida* isolates [5]. Molecular methods like PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) and Taqman-based real time PCR are found to have a very high sensitivity and specificity in the identification of *Candida* species [6,7].

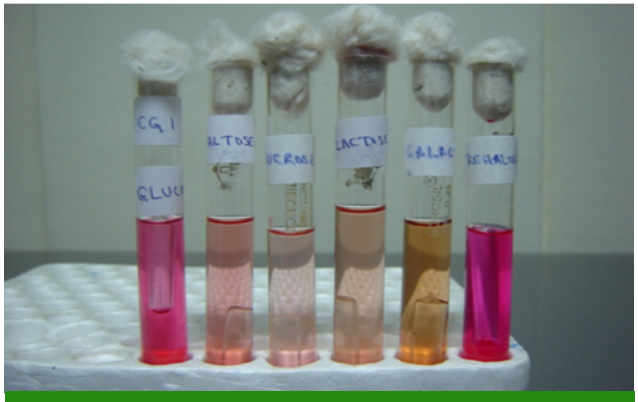
The aim of this study was to evaluate the performance of five phenotypic tests in the identification of clinically significant *Candida* isolates. The phenotypic tests used were Chromagar *Candida*, Tetrazolium reduction medium, Candifast, Sugar assimilation and fermentation.

MATERIALS AND METHODS

This prospective study was conducted at a University Teaching Hospital in Chennai, India over a one year period (November 2008-October 2009). Ethical clearance for the study was obtained from the institutional ethics committee. All the isolates of *Candida* species from blood stream infections (BSI) of intensive care unit (ICU) patients during the one year period were considered in the prospective study. A case of candidemia was defined as a patient with at least one blood culture positive for *Candida* species after at least 48 hours of admission into ICU. Hence, all the ICU patients developing candidemia after 48 hours of admission were included in the study. Non-ICU patients who had candidemia were not considered in this study.

Following universal precautions, blood samples were collected from the patients. Automated blood culture system (VersaTREK) was used for blood culture. In samples showing positive growth in VersaTREK after incubation, a gram stain was performed to look for yeast cells. The sample was then sub cultured on Sabouraud dextrose agar (SDA) for isolation and speciation. Out of 5976 ICU patients whose blood samples were received during the one year study period, 39 cases of candidemia (0.65%) were diagnosed. All the 39 strains of *Candida* species were included in the study. ATCC (American Type Culture Collection) strains of *C. albicans* (90028), *C. tropicalis* (750), *C. parapsilosis* (90018), *C. krusei* (6258) and NCCPF (National Culture Collection of Pathogenic Fungi) strain of *C. glabrata* were used as standard strains. Germ tube test was done first for the *Candida* isolates to differentiate between *C. albicans* and non-albicans *Candida* spp. Five phenotypic tests (sugar fermentation test, sugar assimilation test, tetrazolium reduction medium, CHROMagar *Candida* medium and Candifast) were used to identify the 39 *Candida* isolates to species level. The phenotypic test results were compared with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) results to evaluate the performance of the phenotypic tests in the identification of *Candida* isolates. PCR was used to amplify the ITS-1 and ITS-2 regions of *Candida* species using the universal primers ITS-1 and ITS-4. The amplified product was then digested using Msp I restriction enzyme by RFLP [6,8]. The five phenotypic tests are described below.

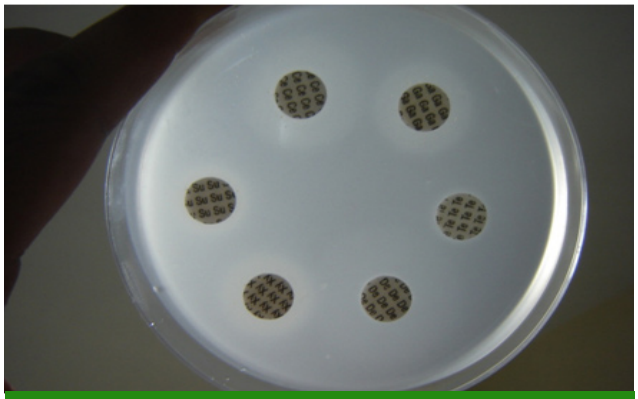
i. Sugar Fermentation Test: Liquid media was prepared containing peptone (1%), sodium chloride (0.5%) with Andrade's indicator. Filter sterilized sugars were added at the concentration of 2% to the medium. The solution was then poured into test tubes containing Durham's tube and sterilized by autoclaving. The sugars used were glucose, maltose, lactose, sucrose, galactose and trehalose. A set of these six sugars was used for the identification of each *Candida* isolate. Each tube was inoculated with 0.1 ml of the inoculum. The tubes were incubated at 25^{0}C upto one week and examined at every 24 hours interval for the production of acid (pink colour) and gas (in Durham's tube). Production of gas in the tube was taken as fermentation positive while only acid production was taken as carbohydrate assimilation [Table/Fig-1].



[Table/Fig-1]: Sugar fermentation test positive for *C. glabrata*

ii. Sugar Assimilation Test: A 24-48 hours old culture was taken and yeast suspension was prepared in 2 ml of yeast nitrogen base (YNB) by adding a heavy inoculum. 18 ml of molten agar, cooled to 45^{0}C, was taken and the yeast suspension was added to it and mixed well. The molten agar along with the yeast suspension was poured into a 90 mm petri plate and allowed to set at room temperature until the agar surface solidified. Sugar discs obtained commercially from Hi media, Mumbai were placed onto the surface of the agar plate. The sugar discs used were glucose, galactose, xylose, sucrose, trehalose and cellobiose. Then the plate was incubated at 37^{0}C for 3-4 days. The presence of growth around the disc was considered as assimilation positive for that particular carbohydrate [Table/Fig-2]. Growth around glucose disc was recorded first which served as positive control (viability of yeast).

iii. Tetrazolium Reduction Medium: Tetrazolium reduction medium (TRM) was used to differentiate various *Candida*



[Table/Fig-2]: Sugar assimilation test positive for *C. albicans*

species like *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, *C. pseudotropicalis*. Tetrazolium is reduced in different gradients by various species of *Candida* to produce different colours depending on the species [Table/Fig-3]. The yeast colonies were inoculated onto the tetrazolium reduction medium and incubated at 37^{0}C for 24-48 hours after which the colour produced by the isolates was noted [Table/Fig-4].

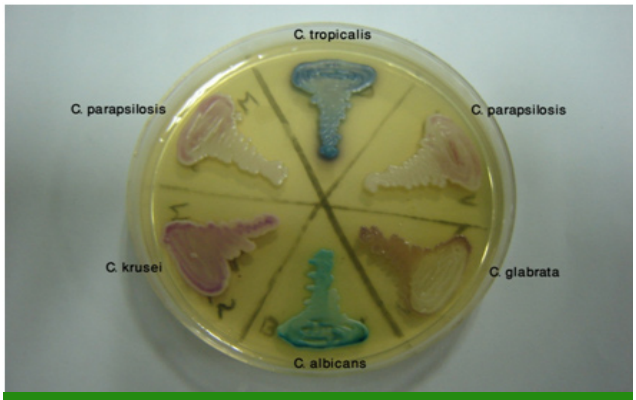
S. No.	*Candida* species	Colour on TRM
1	*C. albicans*	Cream (glistening)
2	*C. tropicalis*	Dark, maroon red
3	*C. parapsilosis*	Rose pink
4	*C. glabrata*	Pale pink
5	*C. krusei*	Pink (dry)

[Table/Fig-3]: Speciation of *Candida* isolates using Tetrazolium reduction medium

[Table/Fig-4]: Tetrazolium reduction medium showing different *Candida* species

iv. CHROMagar Candida Medium: The CHROMagar *Candida* (Paris, France) is a selective and differential chromogenic medium that is useful for the identification of various *Candida* species. This medium is based on direct detection of specific enzymatic activities by adding multiple chemical dyes i.e substrates of fluorochromes to media. Due to the chromogenic substrates added in the medium, the *Candida* colonies of various species produce different colours, thus allowing the direct identification of these *Candida* species on the isolation plate [Table/Fig-5]. Colonies from the various *Candida* isolates were inoculated onto the CHROMagar *Candida* medium and incubated at 37^{0}C for 48-72 hours after which the colours were noted [Table/Fig-6].

v. Candifast: The CANDIFAST kit (INTERNATIONAL MICROBIO, France) allows the identification of the various pathogenic *Candida* species as well as the testing of their resistance to various antifungal agents [Table/Fig-7]. The identification of the yeast is based on the fermentation of seven sugars (glucose, galactose, trehalose, maltose, cellobiose, raffinose, lactose) which is visualized by a colour change of the indicator to yellow or yellowish-orange due to acidification of the medium. The indicator used is phenol red.



[Table/Fig-5]: Identification of different *Candida* species by CHROMagar (Paris, France)

S. No	*Candida* species	Colour on CHROMagar *Candida*
1	*C. albicans*	Light green
2	*C. glabrata*	Purple
3	*C. krusei*	Pink
4	*C. parapsilosis*	Cream to pale pink
5	*C. tropicalis*	Steel blue

[Table/Fig-6]: Speciation of *Candida* isolates using CHROMagar

[Table/Fig-7]: Candifast kit for identification of *Candida* species

RESULTS

A total of 39 isolates of *Candida* were obtained from blood stream infections of ICU patients during the 1 year period. Out of the 39 *Candida* isolates, 4 were positive by germ tube test. 5 phenotypic tests (sugar fermentation test, sugar assimilation test, tetrazolium reduction medium, CHROMagar *Candida* medium and Candifast) were then used to speciate the *Candida* isolates. The results of the phenotypic tests were compared with the PCR-RFLP results. All the 4 isolates positive by germ tube test were found to be *C. albicans* by PCR-RFLP. The rest 35 isolates of non-*C. albicans* were identified by PCR-RFLP as follows: 29 *C. tropicalis*, 3 *C. parapsilosis*, 2 *C. krusei* and 1 *C. glabrata*. Tetrazolium reduction medium, CHRO Magar *Candida* and Candifast were able to identify all

the 39 *Candida* isolates (100%) to species level. There was complete concordance in the results for speciation of *Candida* isolates by the above mentioned 3 methods with PCR-RFLP. Fermentation test and Sugar assimilation test were able to correctly identify 37 (94.8%) and 36 (92.3%) of the 39 *Candida* isolates respectively. 1 isolate each of *C. parapsilosis* and *C. glabrata* was not identified by the fermentation and sugar assimilation tests. In addition, 1 isolate of *C. tropicalis* was not correctly identified by the sugar assimilation test. [Table/Fig-8] shows the comparative performance of the 5 phenotype tests in the identification of *Candida* species.

	C. tropicalis	*C. albicans*	*C. parapsilosis*	*C. krusei*	*C. glabrata*	**Total**
PCR-RFLP	29	4	3	2	1	39
Tetrazolium Reduction Medium	29	4	3	2	1	39
CHROMagar *Candida*	29	4	3	2	1	39
Candifast	29	4	3	2	1	39
Sugar Fermentation	29	4	2	2	-	37
Sugar Assimilation	28	4	2	2	-	36

[Table/Fig-8]: Total of isolates of *Candida* identified by PCR-RFLP and 5 phenotypic tests

The time taken to speciate the 39 *Candida* isolates using the five phenotypic tests was different. Candifast was able to identify all the *Candida* isolates within 24 hours of inoculation. The time taken by tetrazolium reduction medium and CHROMagar *Candida* varied from 24-48 hours. However, the time taken by sugar fermentation and assimilation tests varied from 48-96 hours.

DISCUSSION

In our study, the most common *Candida* species during the 1 year study period was *C. tropicalis* 74.4% (29/39) while *C. albicans* was isolated only from 10.3% (4/39) of the candidemia cases. There are several other studies from India which have reported this shift from *C. albicans* to non-albicans *Candida* (especially *C. tropicalis*) as the major cause of candidemia in the last few decades [9]. One of the major causes for the emergence of non-albicans *Candida* species, especially *C. tropicalis*, is the increase in the use of fluconazole in developing countries [9]. *C. tropicalis* has been found to be the most common *Candida* species causing candidemia in studies from south India by Shivprakasha et al., and Adhikary et al., (35.6% and 39.7% respectively) [10,11]. Other studies on candidemia from north India by Kothari et al., and Xess et al., have also found *C. tropicalis* to the commonest cause of candidemia [12,13].

Several phenotypic methods including germ tube test, sugar assimilation and fermentation, tetrazolium reduction medium, CHROMagar, etc, have been developed for the speciation of *Candida* isolates. Many studies have evaluated the performance of various other commercially available methods for speciation of *Candida* isolates. Many of these identification systems like Candifast, Fungichrom and API 20C Aux systems are being used in laboratories all over the world and have shown varying degrees of efficacy [5]. In our study, five phenotypic tests were used to identify *Candida* isolates from blood to the species level. The phenotypic results were compared with the PCR-RFLP results. A number of studies have found PCR-RFLP using ITS 1 and ITS 4 primers and restriction enzyme to be a rapid, easy and reliable method for the identification of medically important *Candida* species [6,8,14].

In our study, Tetrazolium reductionmedium, CHROMagar *Candida* and Candifast were found to identify all the 39 *Candida* isolates (100%) to species level. Fermentation and sugar assimilation tests were able to identify 94.8% and 92.3% of the *Candida* isolates respectively. Fermentation and sugar assimilation tests are conventional methods for the identification of *Candida* species which have been in common use and are reported to have varying degrees of sensitivity and specificity [15]. Tetrazolium reduction medium is known since more than four decades to be an efficient medium for the identification of *Candida* isolates to the species level. Tetrazolium is reduced in different gradients by different species of *Candida* to produce colonies with specific colours for different *Candida* species. In a study by Denny et al from London in 1968, tetrazolium reduction medium was found to be a rapid, relatively accurate and simple means of differentiating *Candida* species from other yeasts [16]. It was one of the earliest studies to evaluate the efficacy of tetrazolium reduction medium in differentiating *Candida* species.

CHROMagar *Candida* is a commercially available chromogen-based culture medium which has shown a high degree of accuracy in the identification of various *Candida* species and also facilitates the detection and identification of yeasts from mixed cultures [17]. In a study by Pfaller et al., CHOMagar *Candida* was found to accurately identify over 95% of stock and clinical isolates of *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata* [18]. Another study by Odds et al., found that the sensitivity and specificity of CHROMagar *Candida* for the presumptive identification of *C. albicans*, *C. krusei*, and *C. tropicalis* exceeded 99% for all three species [17]. In addition to the common species of *Candida* isolated in busy clinical microbiology laboratories, CHROMagar *Candida* has been found to be useful in the identification of rare *Candida*

species like *Candida inconspicua*, *Candida lipolytica*, *Candida lusitaniae*, *Candida norvegensis*, *Candida rugosa* etc [19]. CHROMagar has also been used for detection and identification of *Candida* species directly from clinical specimens [20]. Another advantage of using CHROMagar is that it can differentiate between *C. albicans* (light green colonies) from *C. dubliniensis* (dark green colonies) which is not possible with the other four phenotypic tests discussed [21].

Many studies have evaluated the performance of various commercially available methods for the speciation of *Candida* isolates. Commercially available identification systems like Candifast, Fungichrom and API 20C Aux systems are being used in clinical microbiology laboratories all over the world [5]. In this study, Candifast was able to identify all the 39 *Candida* isolates to the species level. In a study by Gundes et al., Candifast was able to correctly identify 82.7% of all the clinically significant yeasts [5]. Along with identification of *Candida* isolates, Candifast can be used for the determination of anti-fungal susceptibility pattern of *Candida* isolates which is an added advantage [22]. There are seven antifungal agents (amphotericin B, fluconazole, nystatin, flucytosine, econazole, ketoconazole, and miconazole) for which susceptibility pattern of *Candida* isolates can be determined using Candifast.

One of the limitations of these phenotypic tests is that they cannot differentiate between *C. parapsilosis*, *Candida* metapsilosis and *Candida orthopsilosis*. *C. parapsilosis* is an emerging cause of nosocomial infections and previously comprised of a complex of three genetically different groups (group I, II, III). But presently, these three groups have been renamed as distinct species (*C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*) based on their identification by different molecular methods like PCR, randomly amplified polymorphic DNA (RAPD) analysis etc [23].

In this study, we observed variability in the time required for the identification of *Candida* isolates using various phenotypic methods. Whereas Candifast was the fastest among the phenotypic methods (all isolates identified within 24 hours of inoculation), CHROMagar *Candida* and Tetrazolium reduction medium took 24-48 hours for the speciation of *Candida* isolates. The maximum time (48-96 hours) was taken by fermentation and Sugar assimilation tests. This has important implications in serious and life threatening infections like candidemia where rapid identification and antifungal susceptibility results are extremely important in determining the correct therapeutic measures to be taken [22]. With the emergence of fluconazole resistant non-albicans *Candida* species like *C. glabrata* and *C. krusei* as causes of candidemia in several countries worldwide, rapid speciation of *Candida* isolates has become all the more important [2].

LIMITATION

One limitation of the study was the fewer number of samples used for the comparison. In future, a study involving a larger number of samples will be helpful in further validating the findings of this study.

CONCLUSION

In the present study, a good correlation between five phenotypic tests (CHROMagar, tetrazolium reduction medium, Candifast, Sugar assimilation and Fermentation) in the identification of *Candida* isolates was found. However, the time required for speciation of the *Candida* isolates varied among the five phenotypic tests used, which may have significant implications for treatment in serious life threatening *Candida* infections like candidemia.

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