ISSN 2515-8260 Volume 09, Issue 02, 2022

Speciation of candida isolates from clinical samples: a comparison of two carbohydrate assimilation methods for efficacy, speed and accuracy with special reference to the sensitivity to commonly used antifungal agents

[1]Dr. Amith Joshi, [2]Dr. Suma A, [3]Dr. Sughosh Kulkarni, [4]Dr Deepa Patil [5]Dr. RD Kulkarni

[1]Assistant Professor, Department of Microbiology, S Nijalingappa Medical College, Bagalkote, Karnataka, India
[2,4]Associate Professor, Department of Microbiology, SDM Medical College, Dharwad, Karnataka, India
[3]Assistant Professor, Department of Microbiology, SDM Medical College, Dharwad, Karnataka, India
[5]Professor & Head, Department of Microbiology, SDM Medical College, Dharwad, Karnataka, India

Corresponding Author: Dr. Amith Joshi

Abstract

Candida is ubiquitous in nature and is found on inanimate objects, in foods and on animals. They are commensals of humans. Most Candida infections are endogenous in origin. They inhabit the gastrointestinal tract including the mouth and oropharynx, the female genital tract, and the skin. Health care workers do show carriage on the skin. The transformation of Candida from a commensal to a pathogen depends on the decreased host resistance, local ecology or increased virulence of the organism. The samples received for routine culture and sensitivity like, pus, sputum, Urine, blood, vaginal swab, endotracheal aspirates, endotracheal tube tips, CVC tips, IJC tips, Lumbar drain tip and sterile body fluids like CSF, pleural fluid, ascitic fluid etc. at the Microbiology laboratory were subjected to standard culture and sensitivity tests. The AUX method could not detect *C. kefyr* identified on WB method. Instead *C. kefyr* was identified as *C. tropicalis*. Similarly one of the isolates of *C. parapsilosis* was identified as C. tropicalis by WB method. None of the species of Candida except *C. krusei* were resistant to fluconazole. While a majority of the *C. krusei* isolates (78.1%) were resistant to fluconazole, there were no resistant strains observed for voriconazole in any of the 112 isolates.

Keywords: Candida isolates, agar with indicator method, auxanographic method

ISSN 2515-8260 Volume 09, Issue 02, 2022

Introduction

The word Candida is derived from Latin word 'Candidus' means glowing white. Candida, generally called as yeast, belongs to phylum 'Fungi imperfecti' and Family 'Cryptococcaeceae'. The genus Candida comprises around 200 species of which approximately 20 species have been associated with human infections [1]. This number is not immutable, as newer species are likely to be added and older reasserted because of the technological advances [2].

Candida is ubiquitous in nature and is found on inanimate objects, in foods and on animals. They are commensals of humans. Most Candida infections are endogenous in origin. They inhabit the gastrointestinal tract including the mouth and oropharynx, the female genital tract, and the skin. Health care workers do show carriage on the skin [1]. The transformation of Candida from a commensal to a pathogen depends on the decreased host resistance, local ecology or increased virulence of the organism.

The infection by Candida species is, by far, the most common fungal infection seen in humans[3].Around 10% to 20% or more blood stream infections are caused by Candida and are especially frequent in neonatal blood cultures [1]. They are amongst the most common pathogens to cause nosocomial infections over the last four decades. They are also commonly isolated from urine samples [1].

Of late a number of Candida species are gaining clinical importance. From 1970 to 1990, the non-albicans Candida (NAC) accounted for 10-30 % of all the Candida isolated from clinical specimens, this proportion reached 40-65% in the last two decades [4]. A good number of NAC are known to possess intrinsic or acquired drug resistance e.g. C. krusei and C. glabrata are intrinsically resistant to commonly used antifungal agents like fluconazole [5]. Therefore, speciation of Candida is becoming increasingly important to choose the antifungal agent for the therapy. Characterization to species level helps to identify those strains which might be intrinsically resistant to some of the antifungal agents, and thus provide evidence based options to the clinicians and would positively influence the treatment outcome [6].

The conventional identification protocol is time consuming. It is therefore, essential to establish a speedier and accurate method for identification of Candida species. This study intends to compare two carbohydrate assimilation tests, which are definitive tests for speciation of Candida for speed and accuracy.

Quick speciation will help evidence based antibiotic therapy. This will lead to better therapeutic response and will limit unnecessary use of commonly used antifungal agents. Judicious use of antifungal agents will be important in the spread of resistant strains in the long run.

Methodology

The samples received for routine culture and sensitivity like, pus, sputum, Urine, blood, vaginal swab, endotracheal aspirates, endotracheal tube tips, CVC tips, IJC tips, Lumbar drain tip and sterile body fluids like CSF, pleural fluid, ascitic fluid etc. at the Microbiology laboratory were subjected to standard culture and sensitivity tests.

All the blood samples were subjected to automated blood culture system, BACTAlert3D. All the other samples were subjected to conventional culture. Gram stained smears were prepared for all the exudates. The exudates were inoculated on Chocolate agar and MacConkey's agar. The urine samples were subjected to semi Quantitative culture method using Cysteine Lactose Electrolyte Deficient (CLED) agar and MacConkey's agar. Blood cultures flagging positive for growth were sub cultured on Chocolate and MacConkey's agar. The culture plates were incubated at 37 ^{o}C aerobically for 24 hours and plates showing scanty or no growth was further incubated for another 24 hours. In the routine processing the colonies showing yeast cells on Gram stain were further tested as follows-

Gram stain was repeated for confirmation of Candida.

Germ tube test was done for detecting production of true hyphae (Germ tube)

ISSN 2515-8260 Volume 09, Issue 02, 2022

The colonies were then sub cultured on Sabouraud's dextrose agar (SDA) slants. The slants were checked for purity of growth and then the colonies from SDA were inoculated on Corn Meal agar (CMA) according to the Delmau's method [6] for morphological identification.
Further the colonies from SDA were subjected for two carbohydrate assimilation tests and antifungal susceptibility testing.
The following two methods for carbohydrate assimilation were followed:

Agar with indicator method (Modified Wickerham and Burton method, 1948)

Two similar looking colonies from the pure culture on SDA slants were picked and sub cultured on Nutrient agar (NA) slants. After overnight incubation, the colonies from NA slant were again subcultured on a fresh NA slant and incubated overnight to obtain a second generation of glucose free yeast growth. 2-mm loop full of this 2nd generation pure yeast culture was suspended in 9 ml sterile distilled water. The tubes were vortexed to obtain uniform yeast suspension. Each carbohydrate assimilation slant was inoculated with 0.1 ml yeast suspension. The slants were incubated at 25 ^{o}C in air and examined everyday up to 15 days for growth and change in color from purple to yellow.
Assimilation was considered positive when the medium turned to yellow in color from purple and there was growth on the slants.
Assimilations were considered negative when there was no observable growth and no color change in the slants after 15 days of incubation.

Auxanographic method (Hazen and Howell, 2003)

2 ml sterile saline was added to the second generation of yeast growth on glucose free NA slants. The resulting yeast suspension was pipetted into a sterile test tube to which 1.5 ml of yeast nitrogen base was added. This new suspension was poured along with 13.5 ml of freshly prepared basal medium (autoclaved and cooled to 50 ^{o}C) in a sterile glass petri plate. The plates were mixed thoroughly by twirling and let it solidify. After the medium was set, sterile 6 mm filter paper disks were charged with 2 mm loop full of sterile carbohydrate solution. The disks were lifted carefully with the help of the toothless forceps and were placed in pre designated (and labeled) places over the agar medium. A total of 13 different sugar disks were placed in a 90 mm diameter petri plate with the media [8]. The plates were subsequently incubated for 25 ^{o}C for 24 hours and then observed for assimilation.
The assimilation was considered positive for a carbohydrate when there was development of an observable opaque zone around that particular carbohydrate disk.
The assimilations were considered as negative when there was no observable opacity around the disks.

Results

Table 1: Species identified-WB* method

Species	Total	%
C. albicans	29	25.9
C. glabrata	4	3.6
C. guilliermondii	7	6.3
C. kefyr	1	0.9
C. krusei	32	28.6
C. lusitaniae	1	0.9
C. parapsilosis	2	1.8
C. tropicalis	36	32.1
Total	112	100.0

*WB-Modified Wickerham-Burton.

ISSN 2515-8260 Volume 09, Issue 02, 2022

Table 2: Species distribution across various samples (WB* method)

Species	Blood	Urine	Sputum	Pus	ET	Peritoneal fluid	Nasal swabs	CSF	Total
C. albicans	5	11	10	1	2	0	0		29
C. glabrata	0	4	0	0	0	0	0	0	4
C. guilliermondii	3	3	0	0	0	0	1	0	7
C. kefyr	0	0	1	0	0	0	0	0	1
C. krusei	29	1	1	0	0	0	0	1	32
C. lusitaniae	0	0	0	1	0	0	0	0	1
C. parapsilosis	0	2	0	0	0	0	0	0	2
C. tropicalis	9	22	3	1	0	1	0	0	36
Total	46 (41.1%)	43 (37.3%)	15 (13.4%)	3	2	1	1	1	112

*WB-Modified Wickerham-Burton.

Table 3: Species diversity from samples

Sample	No. of species isolated
Urine	6
Blood	4
Sputum	4
Pus	3

Maximum diversity of the samples were from urine, suggesting improper collection of the samples.

Table 4: Speciation by auxanographic method

Auxanographic method	Isolates	Percentage
C. albicans	29	25.89
C. tropicalis	38	33.92
C. krusei	32	28.5
C. glabrata	4	3.57
C. guilliermondii	7	6.2
C. parapsilosis	1	0.7
C. lusitaniae	1	0.8

Table 5: Comparison of speciation by two methods

Species	WB#	Aux*
C. albicans	29	29
C. tropicalis	36	38
C. krusei	32	32
C. guilliermondii	7	7
C. glabrata	4	4
C. parapsilosis	2	1
C. lusitaniae	1	1
C. kefyr	1	0
Total	112	112

#-Wickerham-Burton method; *Auxanographic method.

The AUX method could not detect *C. kefyr* identified on WB method. Instead *C. kefyr* was identified as *C. tropicalis*. Similarly one of the isolates of *C. parapsilosis* was identified as C. tropicalis by WB method.

ISSN 2515-8260 Volume 09, Issue 02, 2022

Table 6: Agreement between two methods for speciation

Species	**WB**	**AUX**	**Kappa values**
C. albicans	29	29	1
C. tropicalis	36	38	0.98
C. krusei	32	32	1
C. guilliermondii	7	7	1
C. parapsilosis	2	1	1
C. glabrata	4	4	1
C. lusitaniae	1	1	1
C. kefyr	1	0	0.7

Table 7: Time taken for identification-WB method#

Time taken (in days)	**WB Method**		**AUX method**	
	Number of isolates	**Percentage**	**Number of isolates**	**Percentage**
3	0	0	111	99.1
4	13	11.5	1	0.9
5	64	57.1	-	-
6	32	28.6	-	-
7	3	2.7	-	-
Mean days	5.5	-	3.5	-
Total	112	100	112	100

WB method-Modified Wickerham Burton method.

A mean difference of 2 was observed in the time taken by both the methods for identification which was significant considering the need for rapid speciation of the isolates.

Table 8: Sensitivity & specificity of auxanographic method compared to WB method

Parameter	**Albicans**	**Krusei**	**Tropicalis**	**Guilliermondii**	**Glabrata**	**Parapsilosis**
Sensitivity	100	100	100	100	100	50
Specificity	100	100	97.36	100	100	100
PPV	100	100	94.73	100	100	100
NPV	100	100	100	100	100	99

Table 9: Drug susceptibility of predominant Candida species

Drug	***C albicans* (*n* = 29)**			***C krusei* (*n* = 32)**			***C tropicalis* (*n* = 36)**		
	S (%)	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**
Fluconazole	29 (100)	0	0	3 (9.4)	4 (12.5)	25 (78.1)	34 (94.4)	2 (5.6)	0
Voriconazole	29 (100)	0	0	32 (100)	0	0	36 (100)	0	0

None of the species of Candida except *C. krusei* were resistant to fluconazole. While a majority of the *C. krusei* isolates (78.1%) were resistant to fluconazole, there were no resistant strains observed for voriconazole in any of the 112 isolates.

Table 10: Sensitivity of all the isolates

Species	**Fluconazole**		**Voriconazole**	
	S	**R**	**S**	**R**
C. albicans	29	0	29	0
C. tropicalis	36	0	36	0

ISSN 2515-8260 Volume 09, Issue 02, 2022

C. krusei	7	25	32	0
C. guilliermondii	7	0	7	0
C. parapsilosis	2	0	2	0
C. glabrata	4	0	4	0
C. lusitaniae	1	0	1	0
C. kefyr	1	0	1	0

Sensitive strains and intermediate sensitive strains are counted together.

Discussion

The Candida isolates were subjected to two methods of speciation and the efficacy of the methods was compared later on. Modified Wickerham Burton (Tube) method (WB) and Auxanographic (plate method) (AUX) were run in parallel for the identification of all the isolates. If there was disagreement between the assimilation results in both methods, the WB method results were considered for final identification. A few isolates belonging to different species have been externally confirmed to validate our results.

The most predominant species isolated was *C. tropicalis* (36/112; 32%) followed by *C. krusei* (32/112; 29%) and *C. albicans* (29/112; 26%). Other species isolated include *C. guilliermondii* (6%), *C. glabrata* (3%), *C. parapsilosis* (2%) and *C. lusitaniae* (1%).

The predominance varied when sample wise isolation of Candida species was analyzed. Most isolation was made from blood and urine accounting for 41.1% and 38.3% respectively. *C. krusei* was the predominant species (29/46) from blood samples and was isolated only once from CSF, urine and sputum each. *C. tropicalis* and *C. albicans* were the predominant isolates from urine (22/43 & 11/43 respectively), whereas *C. albicans* was the most common species in sputum (10/15).This data suggests that different species of Candida might have predilection for different sites in the body. For example *C. glabrata* constitutes approximately 10- 20% of all candiduria. The reason even though is not correctly known, Fisher *et al.* (2011) suggested that *C. glabrata* due to its high resistance to azole drugs and the ability to form chronic mild inflammation in the glomerulus helps in colonization of the urinary tract mucosal surfaces [7]. It was interesting to note that in our study all the isolates of *C. glabrata* were from urine only. Other Candida species such as C. *parapsilosis* has predilection for catheters and other medical devices due to its ability to form biofilm and other virulence traits as demonstrated in a study by Kojic *et al.* (2003) [8].

C. krusei is another emerging NAC species. Studies by Prasad *et al.* (2013) and many other studies have demonstrated the increased colonization of *C. krusei* in pediatric population as opposed to neonates and adults [9]. However in our study almost all the isolates of *C. Kruse* were from neonates (30/32). Samaranayake *et al.* (1994) suggested that *C. krusei* is the most hydrophobic of all the Candida species [10] and this could explain the reason for their higher isolation from blood in patients with in-dwelling catheters. *C. albicans* is a colonizer of the oral and vaginal mucosa; this could explain the higher prevalence of *C. albicans* in the respiratory samples as opposed to others as reported by Mohandas *et al.* (2011) [11, 12].

Highest number of species isolated was from urine samples giving 6 different species from a total of 43 isolates. It was followed by blood and sputum-accounting for four species each and pus giving three species. Genital mucosa is commonly colonized with Candida species. Isolation of diverse species from urine samples could have reasons like collection contamination, catheter colonization etc. This underlines the importance of utmost care in collection of samples. If the collection is reliable; then only, the laboratory will be able to opine on the isolate, as colony count does not help to decide the pathogenic nature of the isolate.

Of the 112 isolates, 110 matched precisely in the assimilation reaction by both the methods. One strain was identified as *C. kefyr* by WB method and the differentiation was based on assimilation of raffinose. This sugar was a consistently not assimilated in AUX method but was always assimilated by WB method. One isolate of *C. tropicalis* was differentiated by lack

ISSN 2515-8260 Volume 09, Issue 02, 2022

of assimilation of cellobiose and was ascribed to *C. parapsilosis* by modified WB method.
Except for one *C. kefyr* and one *C. tropicalis* isolate, there was an excellent agreement in the identification by both the methods. As far as the reliability of identification is concerned both the methods are equally useful.
Speciation of Candida is important for evidence based treatment. When a laboratory provides preliminary identification as *Candida* species, the clinicians usually tend to put the patients on fluconazole therapy. Only after the speciation and sensitivity test the right choice of antibiotic can be made. Here, the speed of identification and sensitivity testing will decide the clinical outcome. Therefore, the speed of identification or time taken for speciation by both the methods was compared. All the 112 strains were subjected to identification by both the methods. An important prerequisite for both the methods is to subculture a pure isolate on glucose free media for two generations. With the WB method, the minimum time taken for identification was four days and maximum was seven says. By AUX method, the minimum time and maximum time needed was three days and four days respectively.
The highlight of the comparison was that 99% of the isolates could be speciated within three days by AUX method whereas no strain could be identified by WB method in three days. The maximum time required for identification by AUX method was 4 days compared to 7 days taken by the WB method. By 4th day, the WB method could identify only 11.5% of the isolates. Thus 88.5% of the isolates took more than 4 days for identification by WB method.
Accuracy of speciation, speed of identification and ease of performance are the most important parameters that need to be compared. As far as accuracy of identification is concerned both the methods are matching optimally. To perform the testing the WB method looks easier but it needs a lot of preparation before actual execution of the test. The AUX method is more complicated to perform; however, it does not need the kind of preparation demanded by the WB method. Therefore, for this parameter of „ease of performance' also both the methods seem to be comparable. The speed however, seems to the deciding factor. Identification of most (99.1%) isolates within 3 days vis-à-vis identification of zero isolates by 3rd day is decisive in making a choice for identification of the species as speciation is imperative for guiding the antibiotic therapy.

Conclusion

Rapid identification and testing is a must for all the Candida encountered in clinical samples and utilization of tests which are rapid, reliable and feasible is pertinent and so is institution of appropriate antifungal therapy based on the evidence of species identified for quality patient care and management.

References

1. Merz W, Hay R. editors. Topley & Wilson's Microbiology & Microbial infections, Medical mycology, 9th ed. Hodder Arnold, ASM Press, 2005, 1658-1690.
2. Murray P. Manual of clinical microbiology. Washington, DC: ASM Press, 2003, 1762-1779.
3. Kavanagh K. Medical mycology. Chichester, England: Wiley, 2007, 8-16.
4. Pfaller M, Diekemam D. Epidemiology of Invasive Candidiasis: A Persistent Public Health Problem. Clinical Microbiology Reviews. 2007;20(1):133-63.
5. Li H, Zhang C, Chen Z, Shi Wand Sun S. A promising approach of overcoming the intrinsic resistance of *Candida krusei* to fluconazole (FLC)-combining tacrolimus with FLC. FEMS Yeast Research. 2014;14(5):808-81.
6. Larone D. Medically important fungi. Washington, D.C.: ASM Press, 2002, 88-180.
7. Fisher J. Candida Urinary Tract Infections-Epidemiology, Pathogenesis, Diagnosis and Treatment: Executive Summary. Clinical Infectious Diseases. 2011;52(6):S429-S432.
8. Kojic E, Darouiche R. Comparison of adherence of Candida albicans and Candida parapsilosis to silicone catheters *in vivo* and *in vitro*. Clin Microbio Infect. 2003;9:684-690.

ISSN 2515-8260 Volume 09, Issue 02, 2022

9. Prasad P, Fisher B, Coffin S, *et al.* Pediatric Risk Factors for Candidemia Secondary to Candida glabrata and Candida krusei Species. Journal of the Pediatric Infectious Diseases Society. 2013;2(3):263-266.
10. Samaranayake Y, Samaranayake L. Candida krusei: biology, epidemiology, pathogenicity and clinical manifestations of an emerging pathogen. Journal of Medical Microbiology. 1994;41(5):295-310.
11. Mohandas V, Ballal M. Distribution of *Candida* Species in Different Clinical Samples and

ISSN 2515-8260 Volume 09, Issue 02, 2022

Their Virulence: Biofilm Formation, Proteinase and Phospholipase Production: A Study on Hospitalized Patients in Southern India. Journal of Global Infectious Diseases. 2011;3(1):4-8.

12. Vazquez J, Peng G, Sobel J, Steele-Moore L, Schuman P, Holloway W and Neaton J. Evolution of Antifungal Susceptibility among *Candida* Species Isolates Recovered from Human Immunodeficiency Virus-Infected Women Receiving Fluconazole Prophylaxis. Clinical Infectious Diseases. 2001;33:1069-75.