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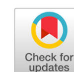
Original Research Article

Evaluation of the four phenotypic methods for the speciation of *Candida* isolates in comparison with molecular method

Reshma Bhaskaran[1,*], Chithra Valsan[1], K A Sathiavathy[1]



[1] *Dept. of Microbiology, Jubilee Mission Medical College and Research Institute, Thrissur, Kerala, India*

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ABSTRACT

Background: The recent change in the epidemiology of Candidiasis with emergence of non albicans *Candida* species as the predominant agents is of serious concern globally. Speciation of *Candida* is very important since many of these species are associated with resistance to the common antifungal agents and often lead to treatment failures. The existing conventional methods for speciation are time consuming and tedious. We attempt to evaluate the performance of the four phenotypic methods for speciation of *Candida* with molecular method.

Materials and Methods: This observational study was conducted on all the clinically significant isolates of *Candida* species obtained from various specimens for a period of 2 years. Speciation was done by phenotypic methods which include conventional germ tube test, chromogenic medium (HiCrome agar), corn meal agar and automated method-Vitek-2 system. Molecular speciation was done by multiplex polymerase chain reaction (PCR). Performance of phenotypic methods is evaluated with results of PCR as gold standard.

Results: During the study period, a total of 80 isolates of *Candida* species from various clinical samples were enrolled in the study. Molecular speciation identified 25 *C.albicans* (31.25%) which was the most common species, followed by 23 *C.tropicalis*(28.75%), 13 *C.parapsilosis* (16.25%), 12 *C.krusei* (15%), 4 *C.pelliculosa* (5%), 2 *C.auris* (2.5%) and one *C.glabrata* (1.25%). Comparing with molecular method, the sensitivity and specificity of chromogenic medium, corn meal agar and Vitek-2 compact system was found to be 91.25% and 100%; 83.75% and 100% and both 100% respectively.

Interpretation and Conclusion: Overall non albicans *Candida* spp predominated in the present study. Among the various phenotypic methods studied performance of HiCrome agar was very good and has the advantage of speciation directly from samples where as that of Vitek 2 Compact was excellent and but requires pure growth. Conventional methods failed to identify many species.

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1. Introduction

Candida species which are normal inhabitants of our body can sometimes cause serious life threatening infections especially in predisposed patients. They are the most common cause of fungal infections. The genus *Candida* comprises of heterogenous group of organisms of which nearly 20 different species are known to cause infections in man.[1] It produces infections that range from nonlife threatening mucocutaneous illness to invasive infections which may involve virtually any organ. Indiscriminate use of antibiotics and prolonged use of indwelling devices in hospitals has led to this increase in *Candida* infections.

C. albicans has been the most common agent of Candidiasis till recently. But the last decade has witnessed a surge in infections with other species collectively called as nonalbicans *Candida*. Infections with *Candidatropicalis*, *Candidaglabrata*, *Candida krusei* and other *Candida* species are emerging as important opportunistic pathogens. The rapid increase in the number of immunocompromised patients worldwide in view of the HIV (human immunodeficiency virus) epidemic and increasing numbers of organ

* *Corresponding author*.
E-mail address: scarletresh@gmail.com (R. Bhaskaran).

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transplantations and malignancies could also be responsible for the isolation of uncommon *Candida* species which were previously considered “non-pathogenic”.[2]

This new trend in epidemiology of *Candida* is associated with significant impact in the management of *Candida* infections because many of these species are resistant to some of the commonly used antifungal agents. Hence speciation of *Candida* is gaining importance in order to start prompt and appropriate antifungal therapy to avoid treatment failures.

Several phenotypic methods are in use for identification of *Candida* species. Some conventional methods like microscopy, colony morphology, germ tube test and corn meal agar for production of chlamydospores and specific characteristics are widely used by many laboratories. Chromogenic media are also available which can help in identification of species looking at the color of the colonies. Automated systems like Vitek 2 compact system also is being used by several laboratories. We attempt to compare the performances of three of the phenotypic methods like germ tube test, Chromogenic medium (HiCrome agar) and automated Vitek 2 compact system with that of the molecular method (Multiplex PCR) for speciation of *Candida* isolates.

2. Materials and Methods

This observational study was conducted in the Department of Microbiology, Jubilee Mission Medical College, Thrissur which is an 1800 bedded tertiary care teaching hospital for a period of 2 years. Institutional ethics committee approval was obtained prior to the study.

All clinical isolates of *Candida* obtained from patient specimens which satisfied the inclusion and exclusion criteria were enrolled in the study. The various clinical specimens from patients sent by the treating doctors for culture were subjected to standard microbiological methods for culture and identification of the isolate. Blood culture was done in BacT alert (bioMerieux pvt ltd). Blood agar, Mac Conkey agar and Chocolate agar were used for the primary inoculation of the rest of the samples as per the laboratory SOP (standard operative procedures).[3,4] The yeast like colonies grown were confirmed by gram stain and were further subcultured on to Sabouraud’s dextrose agar.

The isolates were subjected to speciation by conventional phenotypic methods which included Germ tube test, chromogenic medium (HiCrome agar by Hi Media laboratories, Mumbai), Corn meal agar (Hi Media laboratories, Mumbai) as well as by automated method by Vitek 2 compact system (bioMerieux Pvt Ltd).

2.1. *Speciation by phenotypic methods*

The isolates were subjected to speciation by conventional phenotypic methods which included

a. Germ tube test: An isolated colony was picked and suspended in human serum in a small test tube and incubated at 37^0C for 2 hours and then examined for the formation of germ tubes under high power magnification.[3,4]

b. Chromogenic medium: The isolates were inoculated by streaking on chromogenic media (HiCrome agar by Hi Media labs) and incubated at 37 ^{0}C for 48 hours. The plates are then examined for growth and color of colonies. Species of the isolate tested was identified as per the manufacturer’s instructions as follows:

Colony morphology of *Candida*species on HiCrome *Candida* differential *agar*.

1. *Candida albicans*: light green coloured smooth colonies.
2. *Candida tropicalis*: blue to metallic blue coloured raised colonies.
3. *Candida glabrata*: cream to white smooth colonies.
4. *Candida krusei*: purple fuzzy colonies.

All isolates with doubtful morphology or color were considered as unidentified.

c. Corn meal agar (Hi Media laboratories): The isolates were inoculated by furrowing the medium and incubated at 28^0C for 2-5 days on corn meal agar. This was followed by microscopic examination for the presences of chlamydospores and other characteristic features of different species as described in standard mycology textbooks.[3,4]

d. Automated method: speciation was done by Vitek 2 compact system (bioMerieux pvt ltd) also using ID-YST cards.

The instructions by the manufacturers were strictly followed during the preparation and use of all commercial media as well as during all the automated procedures.

Speciation by molecular method (Multiplex PCR):

All these isolates were subjected to multiplex PCR also using the primers and conditions as follows:

2.2. *DNA isolation*

We used colony PCR method from the fresh culture of the test isolates on Sabouraud’s dextrose agar. Single colony was picked with a sterile toothpick and suspended in 500μl sterile nuclease free water in PCR tubes and subjected to repeated boiling and freezing at -80^0C for breaking the cell wall.

PCR amplification: We did multiplex PCR for each isolate using the yeast specific primers and species specific primers described in earlier studies (Table 1).[5,6] The amplification was performed in a 25 μl volume consisting of 2x PCR buffer, 0.6875 μl of each primers, 10 μl of DNA template and remaining volume consisting of sterilized water. PCR was carried out in a thermocycler under the cycling conditions shown in Table 2.

Statistical analysis was done calculating percentages for frequency. Performance of the phenotypic tests was assessed by deriving sensitivity and specificity using PCR as gold standard.

3. Results

Eighty isolates of *Candida* which appeared to be clinically significant and satisfying the inclusion and exclusion criteria were enrolled in the study during the two years period.

Fifty of these isolates were from males (62.5%) and 30 isolates from females (37.5%). Maximum (34/50) number of samples belonged to the age group above 60 years followed by neonates (23), 31-60 (17) and less than 30 years (6) with the median age of study subjects calculated to be 54.

Among the 80 *Candida* isolates 39 were from blood samples, 19 from urine samples, 10 from pus and exudates and 12 from other body fluids

3.1. Speciation of the isolates

The molecular speciation (Figure 1) of the 80 isolates identified 25 (31.25%) isolates as *C.albicans* and the rest 55 isolates as non albicans *Candida* species as shown in Table 3. The non albicans *Candida* included 23(28.75%) *C.tropicalis*, 13 (16.25%) *C.parapsilosis*, 12(15%) *C.krusei*, 4 (5%) *C.pelliculosa*, 2(2.5%) *C.auris* and 1(1.25%) *C.glabrata*. *C.pelliculosa* and *C.auris* isolates were identified in mycology department of All India Institute of Medical Sciences, New Delhi since primers for these species were not included in our study and hence could not be identified.

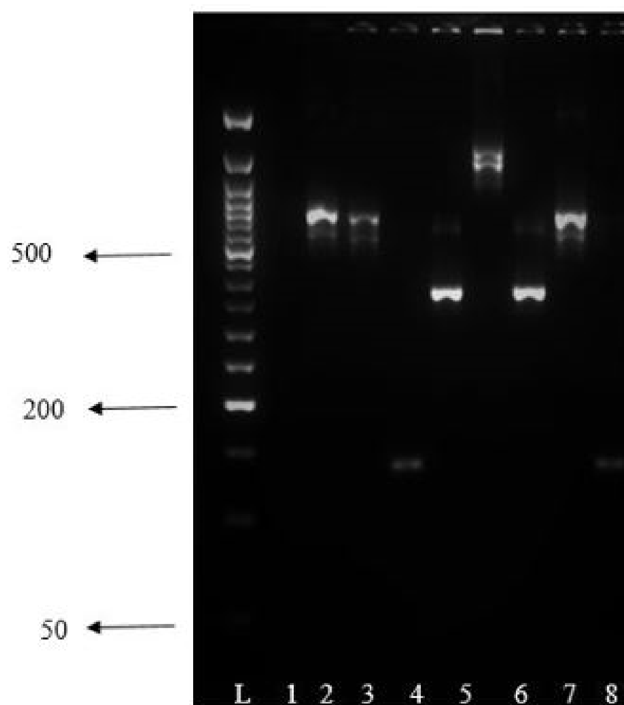

Fig. 1: Agrose gel showing the results obtained for multiplex PCR of isolated yeast genomic DNA. Lanes: (L) 50-bp DNA ladder, (1) Negative control, (2*)* *C.albicans*, (30, (8*)* *C.tropicalis*, (4) *C.krusei*, (5), (7) *C.parapsilosis*, (6) *C.glabrata*

3.2. Quality control

Quality control tests was carried out with standard ATCC strains of *C.albicans* (ATCC 10231) and *C.tropicalis* (ATCC 750) along with 8 bacterial strains and 2 fungal strains which were: E.coli, Klebsiella, Salmonella, Shigella, Pseudomonas, Staphylococcus, Streptococcus, Cryptococcus and Geotrichum species. None of the negative controls have shown any bands in PCR.

The distribution of the *Candida* species with respect to the clinical specimens is shown in Table 4. The 25 isolates of *C. albicans* were isolated from 7 pus samples, 5 blood, 4 urine and 9 other body fluids. Non albicans *Candida* species were recovered from 34 blood samples, 15 urine samples, 3 pus and 3 body fluids other than blood and urine.

3.3. Speciation by phenotypic methods

Germ tube test: Among the 80 isolates, 22 (27.25%) isolates were Germ tube test positive and 58 (72.5%) of them were negative. These 22 isolates were identified as *C albicans* by multiplex PCR also. Since of the isolates identified by PCR could not be detected by germ tube test the sensitivity and specificity of this method was 88% and 100% with respect to PCR results.

Chromogenic medium: Among the 80 isolates, HiCrome *Candida* agar could differentiate 25(31.25%) as *C.albicans*, 23 (28.75%) as *C.tropicalis*, 13 (16.25%) as *C.parapsilosis* and 12 (15%) as *C.krusei*. Seven isolates (8.75%) could not be speciated by this method as they did not produce any specific color or was doubtful (Table 5). These isolates were identified as *C.pelliculosa, C.auris* and *C. glabrata* by PCR.

Corn meal agar: Out of the total 80, 67(83.75%) *Candida* isolates could be speciated, in which 23(28.75%) were identified as *C.albicans*, 23(28.75%) were *C.tropicalis*, 12 (15%) *C.krusei* and 9(11.25%) *C.parapsilosis*. Speciation could not be done for 13(16.25%) isolates (Table 5).

Automated method: All the 80 isolates of *Candida* species were speciated by Vitek-2 system and among them, 25 (31.25%) were *C.albicans*, 23 (28.75%) were *C.tropicalis,* 13 (16.25%) were *C.parapsilosis*, 12 (15%) *C.krusei*, 4 (5%) *C.pelliculosa*, 2(2.5%) were *C. auris* and one (1.25%) was *C.glabrata.*

Performance of phenotypic methods in comparison with molecular method: Results of different phenotypic methods were statistically analyzed using results of molecular method as gold standard. The sensitivity and specificity of

Table 1: Primers used for identification of *Candida* species5,6

Candida species	Primer name	Sequence (5'-3')	Amplicon size (bp)
Universal primers	UNI1	GTCAAACTTGGTCATTTA TTCTTTTCCTCCGCTTATTG	-
C.albicans	Calb	AGCTGCCGCCAGAGGTCTAA	583/446
C.glabrata	Cgla	TTGTCTGAGCTCGGAGAGAG	929/839
C.krusei	Ckru	CTGGCCGAGCGAACTAGACT	590/169
C.tropicalis	Ctro	GATTTGCTTAATTGCCCCAC	583/507
C.parapsilosis	Cpar	GTCAACCGATTATTTAATAG	570/370
C.guilliermondii	Cgui	TTGGCCTAGAGATAGGTTGG	668/512
C.lusitaniae	Clus	TTCGGAGCAACGCCTAACCG	433/329
C.dublinensis	Cdub	CTCAAACCCCTAGGGTTTGG	591/217

Table 2: PCR program

Reaction	Temperature (°C)	Duration
1. Initial denaturation	94°C	10 minutes
2. Denaturation	94°C	15 seconds
3. Annealing	55°C	30 seconds
4. Extension	65°C	45 seconds
Steps 2,3,4	40 cycles	
On hold	4°C	∞

Table 3: Distribution of *Candida* albicans and non albicans *Candida*

***Candida* species**		**Total**
C.albicans (N=25)		25
Non albicans Candida (N=55)	*C.tropicalis*	23
	C.parapsilosis	13
	C.krusei	12
	C.pelliculosa	4
	C.auris	2
	C.glabrata	1
	Total	80

Table 4: Distribution of *Candida* species in clinical specimens

Candida species N=80	Total	Blood	Urine	Pus	Other body fluids
C.albicans	25	5	4	7	9
C.tropicalis	23	7	13	2	1
C.parapsilosis	13	10	1	1	1
C.krusei	12	11	0	0	1
C.pelliculosa	4	4	0	0	0
C.auris	2	2	0	0	0
C.glabrata	1	0	1	0	0
Total	80	39	19	10	12

Table 5: Speciation of *Candida* isolates by different methods (N-80)

Species	Hicrome agar (%)	Corn meal agar (%)	Vitek-2 System (%)	PCR (%)
C.albicans	25 (31.25%)	23 (28.75%)	25 (31.25%)	25 (31.25%)
C.tropicalis	23 (28.75%)	23 (28.75%)	23(28.75%)	23(28.75%)
C.parapsilosis	13 (16.25%)	9 (11.25%)	13 (16.25%)	13 (16.25%)
C.krusei	12 (15%)	12 (15%)	12 (15%)	12 (15%)
C. pelliculosa	-	-	4 (5%)	4 (5%)
C. *auris*	-	-	2 (2.5%)	2 (2.5%)
C.glabrata	-	-	1 (1.25%)	1 (1.25%)
Unidentified	7(8.75%)	13 (16.25%)	-	-

Table 6: Statistical analysis of different phenotypic methods used for speciation of *Candida* using multiplex PCR as the gold standard

	Germ tube test	HiCrome agar	Corn meal agar	Vitek-2 system
Sensitivity	88%	91.25%	83.75%	100%
Specificity	100%	100%	100%	100%
Positive predictive value	100%	100%	100%	100%
Negative predictive value	94.8%	100%	53.8%	0

different methods are shown in the Table 6.

4. Discussion

The emergence of non albicans *Candida* as an increasing cause of Candidiasis is of serious concern in the recent years. Such infections are often associated with increased mortality and morbidity especially in hospitalized patients because many of these species are associated with either intrinsic or high level resistance to the antifungal agents which are commonly used to treat fungal infections. This emphasizes the need for speciation of the clinical isolates of *Candida* by the clinical laboratories as there is wide variation in the distribution of these species in different geographic locations.

Several methods are available and being used by the clinical laboratories for speciation of *Candida* isolates. These include conventional, kit based commercial, automated methods and molecular methods like PCR. Some of these techniques requires prior isolation of *Candida* whereas other methods can be directly applied to clinical specimens for species identification. Each method has its own merits and demerits. It is of utmost importance that clinical laboratories should adopt a reliable and feasible method for accurate identification of the Candida species to start prompt and appropriate antifungal therapy to save lives.

In the present study we analyze and evaluate the performance of some of these phenotypic methods comparing the results with molecular method. All the clinically significant isolates of *Candida* obtained from patient samples received in the microbiology laboratory of our centre which is an 1800 teaching hospital for routine culture were included in the study.

During the 2 year study period, we recovered 80 isolates of *Candida* in the microbiology laboratory. These were from 50(62.8%) male patients and 30 (37.1%) females patients having a mean age of 54. Twenty five of the total isolates were *C.albicans* (31.25%) and 55 (68.75%) were nonalbican *Candida* species. The major non albicans species was *C.tropicalis* 23 (28.75%) followed by *C.parapsilosis* 13 (16.25%), C.krusei 12 (15%), C.pelliculosa 4 (5%), *C auris* 2 (2.5%) and *C.glabrata* 1 (1.25%).Thus in the present study though *Candida* albicans is still the commonest single isolate, overall, nonalbicans *Candida* species predominated.

The changing trend in the distribution of *Candida* species and non albicans *Candida* gaining predominance over *C.albicans* has been reported by many authors from India and abroad. Bhattacharjee P had reported *C. albicans* as the commonest species (48.57%) followed by *C.tropicalis* (24.28%) similar to our findings.[7] In a study from North American medical centres, a predominance of non-albicans species was noted; although *C.albicans* was the most frequently isolated species, it was followed by *C.glabrata* and other non- albicans *Candida* species.[8] Some of the factors attributed to this change in epidemiology could be due to severe immunosuppression, comorbidities, prematurity, prolonged antibiotic therapy and indwelling devices etc.

The commonest non albicans *Candida* (NAC) species recovered in our study was *Candida* tropicalis (28.75%) followed by *C.parapsilosis* (16.25%) *C.krusei* (15%) and others which is in agreement with many similar Indian studies also.[9] In a surveillance study conducted in around 39 countries, an increase in the prevalence of *C.tropicalis* (4.6% in 1997 to 7.5% in 2003) and *C.parapsilosis* (4.2% in 1997 to 7.3% in 2003) has been reported, with a rise in isolation rates of rare species like C.guilliermondii, *C.kefyr* and *C.rugosa*.[10] Another study at Mumbai in 2019 has shown highest isolation rates for *C.tropicalis* (10/29) followed by *C.parapsilosis* (8/29), *C.albicans* (3/29) and C.krusei (3/29).[11] A report from Delhi also states *C.tropicalis*, 82 (38.7%) as the most common isolate followed by *C.parapsilosis*, 43 (20.3%) and *C.albicans*, 29 (13.7%).[12]

We also got 4 isolates *C.pelliculosa* also which were recovered from an outbreak in neonatal ICU. *C.pelliculosa* has been identified as an important agent of outbreaks of health care associated infections in critical care units especially neonatal ICUs.[13]

In the present study we evaluated the performance 4 phenotypic methods with that of molecular method for speciation of *Candida* isolates – the germ tube test, growth on corn meal agar, HiCrome agar and Vitek 2 compact system.

Germ tube test which is known as "Reynolds-Braude phenomenon" is the most widely used conventional technique for identification of *Candida* spp. Germ tube is filamentous extension that protrudes from the

blastoconidium that has parallel walls, but no constriction at their point of origin. It can be differentiated from pseudohyphae by the fact that pseudohyphae are constricted at the point of emergence from the blastoconidium.[14] Among *Candida* spp. of medical importance, C. albicans and C. dubliniensis produce germ tubes. In addition to these species, C. africana is also germ tube positive isolate.[15] In our study 22 of the 25 *C.albicans* isolated were identified by this method and has got a sensitivity and specificity of 88% and 100% respectively. As 90% of the *Candida* spp. isolated from clinical specimens that have a germ tube test positive are *C. albicans*, most of laboratories report germ tube positive yeasts as *C. albicans* without further testing. Thus though less common, it may misidentify other species producing germ tubes also as *C.albicans* and is also subjective.

We also evaluated the performance of cornmeal agar for speciation of *Candida* isolates. *Candida* albicans and some other species like *C.dublinensis*, few strains of *C.tropicalis* and some saprophytic *Candida* produce chlamydospores on nutritionally deficient media. Chlamydospores are round, refractile and resistant asexual spores. Production of chlamydospores and other features like arrangement of pseudohyphae and other fungal structures on cornmeal agar (Dalmau plate technique) can be used for speciation of *Candida* isolates.[16] In our study, this method had a sensitivity of 83.5% and 100% specificity. This procedure is time consuming and require proper training and expertise for correct identification of these species and also the results are subjective. Also it can be done on the isolate in pure culture only and not directly from the samples.

We also evaluated the results of chromogenic medium (HiCrome agar) for speciation of *Candida* isolates. Several brands of chromogenic media have been developed and are available in the market that help in rapid yeast identification. These media contain chromogenic substrates that react with enzymes secreted by microorganisms producing colonies with various pigmentation. These enzymes are species specific, allowing organisms to be identified to the species level by their colour and colony characteristics.[17] HiCrome *Candida* differential agar (Himedia, Mumbai, India) is one such chromogenic medium which is introduced by Himedia laboratory to differentiate *Candida* species namely *C. albicans*, *C. krusei*, *C. tropicalis*, and *C. glabrata* based on colony color.[18]

C albicans produce light green yellow colonies on Hicrome agar, *C tropicalis* produce blue to metallic blue colonies, *C krusei* produce purple colonies and *C parapsilosis* produce light pink to pink colonies as per the manufacturer, which was seen in our study also and these species were correctly identified. This is in agreement with other authors like Kumar et al., Sukumaran et al. and Rachana Mehta who also have reported the same findings.[17,19,20] But *C.pelliculosa*, *C.auris* and *C.glabrata* could not be identified by this method in our study. Thus the sensitivity and specificity of HiCrome agar in comparison with molecular method in the present study is 91.75% and 100% respectively. A review by Pincus et al. has pointed out that there is lack of consensus for the ability to identify *C.glabrata* using chromogenic medium by many authors.[21] Several other studies have reported good performance of this medium in the speciation of *Candida* isolates.[17,22] Jain et al. has reported that agreement between the chromogenic agar method and conventional methods was 80% for *C.tropicalis*, 100% for *C.rugosa*, 89% for *C.albicans*, and 77% for *C.parapsilosis*.[23] But there are other studies also which reported this method as unreliable compared to PCR-RFLP where some of *C.albicans* were misidentified in HiCrome agar.[24]

This method helps in very good identification of the *Candida* species especially in mixed bacterial and *Candida* colonies by seeing the characteristic color of the colonies produced by individual *Candida* species. The method gives rapid results as direct sample can be inoculated on to the media without the need for primary isolation in another media.

In our study, results of automated method Vitek 2 compact was in 100% agreement with that of molecular method identifying correctly all the isolates as by PCR. Thus both sensitivity and specificity of Vitek 2 in the present study is 100% comparing with PCR. Graf et al. reports that in their study Vitek 2 system performed well for the correct identification with or without additional tests (92.1 and 87.6%, respectively).[25] In a study by Jain et al. and Rajkumari et al. chromogenic agar was found to have better performance than Vitek for identification of *Candida* species.[23,26] Vitek has the advantage of doing simultaneous antifungal susceptibility testing of the isolates also.

We used multiplex PCR for evaluating the results of phenotypic method. Molecular techniques are potentially more sensitive, highly reliable and precise. DNA amplification with universal fungal primers followed by detection using species-specific probes greatly enhances the sensitivity of *Candida* detection.[26] It gives faster results than the conventional methods. Also such techniques can be done on pure culture of the organism as well as clinical specimens. However, high cost and the need for infrastructure and technical limits its use in majority of diagnostic microbiology services. Molecular methods have other advantage also than mere identification of the isolate. These techniques can be employed for detection of mutations associated with antifungal resistance, quantification of fungal load in clinical specimen, antifungal therapy monitoring and pathogenesis of *Candida* infection.[14]

Thus, in the present study we found a predominance of non albicans *Candida* (NAC) species among our isolates. Among the methods for speciation, performance of chromogenic method and automated method by Vitek

2 was superior over conventional phenotypic methods like germ tube test and cornmeal agar method. As prevalence of NAC spp. vary significantly as per country and health-care setups within the country, accurate species identification definitely plays an important role in the formulation of local therapeutic guidelines.

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6. Source of Funding

None.

7. Conflict of Interest

None.

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Author biography

Reshma Bhaskaran Junior Resident

Chithra Valsan Professor

K A Sathiavathy Professor

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