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ORIGINAL RESEARCH ARTICLE

Evaluation of Anticandidial effect of Antifungal drugs against *Candida* Isolates

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ABSTRACT

In the present study, we obtained 150 *Candida* strains which were originally isolated from various clinical specimens from different health institutions and diagnostic laboratories. To identify the various *Candida* species from the 150 *Candida* strains we applied three different techniques. We could able to identify the different *Candida* species from the 150 *Candida* strains which were originally isolated from various clinical specimens ie, *Candida albicans* (75%), *Candida dubliniensis* (15%), *Candida tropicalis* (9%), *Candida kruseii* (8%). Among all the *Candida* species *Candida albicans* was the predominant (75%) *Candida* species identified followed by *Candida dubliniensis* (15%), *Candida tropicalis* (9%) and *Candida kruseii* (8%). Among the 4 different *Candida* morphotypes, *Candida albicans* yielded 3 different *Candida* morphotypes which 254, 534 and 554. *Candida* morphotypes 554 was found to be the predominant *Candida* morphotypes we could able to record and also associated with vaginitis among non diabetic women. All 4 different *Candida* morphotypes were subjected to the antifungal susceptibility test to 6 different antifungal drugs ie, ketoconazole, nystatin, fluconazole, clotrimazole, amphotericin-B and itraconazole by disc diffusion technique. All these 4 different *Candida* morphotypes had shown different types of sensitivity and resistant pattern towards these 6 antifungal drugs, among which we good able to record the *Candida* morphotypes 254 & 554 had shown the maximum resistant towards the antifungal drug clotrimazole. Finally from our short term research on "The *Candida* morphotypes associated with certain clinical infections and its antifungal susceptibility pattern". We could able to note and conclude that certain *Candida* morphotypes are associated with certain infection and also showing significant percentage of antifungal resistance. To find out more details, this field of research need future extensive research and we can suggest that the future study related to this field can be under taken by the other researchers.

Key words: *Candida*, Antifungal agents, Anticandidal effect and Susceptibility.

1. INTRODUCTION

Candida albicans, a form of yeast is a diploid fungus and a causal agent of opportunistic oral and genital infections in humans [1]. *C. albicans* is commensal and is among the gut flora. Candidiasis, also known as "thrush", is a common condition which is usually easily cured in people who are not immunocompromised. To infect host tissue, the usual unicellular yeast like form of *C. albicans* reacts to environmental cues and switches into an invasive and multicellular filamentous forms [2]. *Candida albicans* and related species live as benign commensals in one or more body locations in a majority of healthy individuals and it is responsible for the two different types of the fungal infections both the superficial as well the deep/ systemic fungal infections and affecting both immuno compromised and immuno competent hosts.

The potential impact of the phenomenon of phenotypic switching on the reproducibility of these typing methods was discussed. It was concluded that many of the available typing methods have not been adequately assessed by their developers and that several have only poor discriminatory power or reproducibility [3].

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Morphotyping is a phenotypic method for strain differentiation of yeast cultures based on the comparison of appearance of their surfaces and fringes. Until recently, morphotyping, a method evaluating fringe and surface characteristics of streak colonies grown on malt agar, has been recommended as a simple and unexpensive typing method for *Candida albicans* isolates [4]. Correlation of *Candida* morphotypes and its virulence character was analyzed by certain scientists [5, 6].

2. MATERIALS AND METHODS

Source of *Candida* strains

Totally 150 *Candida* strains were isolated from different clinical infections and obtained from five different hospitals and medical institutions/ Laboratories.

Candida sub-culture

The *Candida* strains obtained from the laboratories and the hospitals/institutions were subjected for the subculture to get fresh culture for further testings like speciation, antifungal susceptibility testing and *Candida* morphotyping.

The sub culture was performed on SDA without antibiotics. The single SDA plate was divided into 8 parts and the different *Candida* strains were inoculated on to it and it was incubated at 37°C for one day and these fresh *Candida* culture was used for the testing proceeding. The isolated *Candida* species were identified by the following methods: Colony morphology in Sabouraud's dextrose agar (SDA), Colony morphology on Chrome agar and Germ tube production.

In vitro antifungal drug susceptibility testing

The isolated and identified *Candida* strains from the various clinical specimens were included to determine the *in vitro* anticantidal activity of certain antifungal drugs. The following antifungal drugs were included in our study i.e., Ketoconazole (Kt), Nystatin (Ns), Fluconazole (Fu), Clotrimazole (Cc), Amphotericin - B and Itraconazole (It). The test was performed by the routine Disc diffusion technique.

Disk diffusion method

The commercially available following antifungal discs i.e., Clotrimazole, Ketaconazole, Fluconazole, Nystatin and Amphotericin – B were used in this study.

Inoculum preparation

Candida species/strains inoculum was prepared by suspending several colonies of 24 hrs grown cultures on Sabouraud's Dextrose Agar (SDA) in phosphate buffered saline pH 7.2. Inoculum standardization was done by using standard procedure in which the fungal suspensions were adjusted to the 0.5 McFarland standards, equals to 10^8 cells/ml.

Inoculation and testing

Each SDA plate was inoculated with the standard inoculum suspensions by soaking a swab and rotating it over the agar plate. The antifungal disks were placed over the inoculated agar. After 48 hours of incubation at 37°C, zone of inhibition of growth was measured and recorded.

Candida Morphotyping

The morphotyping technique of Phongpaichit *et al.* [7] was used to differentiate the species and the strains of *Candida* isolates, isolated from the different types of clinical infections. Yeast cells from 24 hrs culture which were grown on 2% (W-V) Malt agar at 25 - 30ºC and were suspended in 5 ml of sterile distilled water in screw capped glass bottles. That was adjusted to a Mac Farland's opacity No.4 turbidity (i.e., approximate 10^7 - 10^8 cells/ml^{-1}). Six percent Malt extract agar with the addition of 2% Oxoid noble agar was autoclaved and distributed in 18 - 20 ml quantities and were dispensed into 9 cm disposable petridishes.

A loopful of yeast suspension was inoculated onto malt agar. The plates were then incubated aerobically in a BOD incubator at 30ºC for 10 days in the dark and cultures were marphotyped according to their surface characteristics. A series of defining colony fringe characteristics were studied according to Hunter *et al.* [8].

The morphotype code used was as follows

Fringe characteristics

The Morphotyping code used was (1) fringe characteristics of the colony streak designated as absent (0), discontinuous < 20% of streak Margin (1), discontinuous 20-50% of margins (2) discontinuous >50% of margins (3), continuous at periphery or strands consciously fan shaped (5), continuous filament growth (7).

Width characteristics

The width characteristics of the colony streak designated as absent (0), <2 mm (2), 2 – 5 mm (3), >5 mm (5).

Texture of the colony

The texture of the colony designated as absent (0), very course (1), coarse (2), intermediate (3), fine (4). Thus for example, a morphotype code of 532 indicates (5) a continuous peripheral fringe is present with evidence of fan shaped configurations; (3) the width of the fringe measured from the outer margin of the central

streak colony to the margin of the fringe is greater than 6 mm (2) the texture of the fringe is considered coarse.To confirm the accuracy of the marphotyping method used the colony morphology was compared with the standard strain and colony photographs and morphotype code followed by Oliver *et al.* [9]. All different types of *Candida* morphotypes and their results were recorded.

3. RESULTS AND DISCUSSION

The results of different *Candida* morphotypes and the predominant *Candida* morphotypes associated with the different types of clinical specimens and its antifungal susceptibility pattern to various antifungal drugs are presented here in this chapter.

Sub-culture of *Candida* Strains

Totally 150 *Candida* strains has been collected from various institutions and were subjected for the presents study. The details and source and distribution the origin of the *Candida* strains were verified with the respected institution (Visva diagnostic & research laboratory). The *Candida* strains which were originally isolated from various types of clinical infections (**Table 1 & 2**). On sub-culture, they yielded good growth after overnight incubation at 37^0C.

Table 1: Place and distribution of the *Candida* strains

S. No	Place /Institution	*Candida* Strains
1	Visva Diagnostic & Research Laboratory, Chidambaram	30
2	Rajah Muthiah Medical College and Hospital, Chidambaram	30
3	Rajah Muthiah Dental College & Hospital, Chidambaram	17
4	Jubilee mission medical college and hospital Thrisure – Kerala	45
5	Mahathma Gandhi Medical College and Research Institute, Pondicherry	28
	Total	**150**

Table 2: Clinical Sources of the *Candida* Strains

S. No	Clinical Sources	*Candida* Strains No
1	Vaginal specimens from non diabetic cases	40
2	Vaginal Specimens from diabetic cases	18
3	Pus	07
4	Sputum	30
5	Oral Swabs	35
6	Indwelling medical devices	20

The predominant clinical specimen and *Candida*

Among the various clinical specimens, vaginal specimens (24%) yielded the maximum number of *Candida* followed by oral swab sample (23%) (**Fig 1**).

Candida Species Identification

Based on the following tests i.e, colony morphology on SDA Germ tube test and Chrome agar culture. The following *Candida* species has been identified. All the tests employed for the identification of the *Candida* species had been 100% given good results and matched with each other.

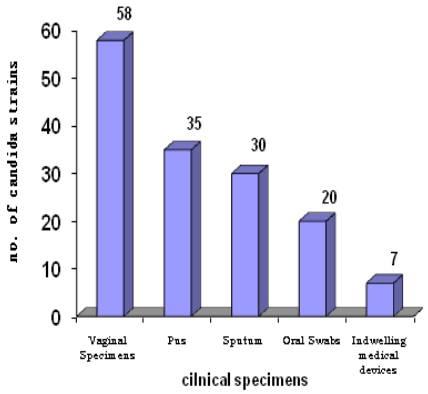

Fig 1: Predominant clinical specimen and *Candida*

Table 3: *Candida* species identified

S. No	Clinical Sources		*Candida* Strains No.	*Candida* species			
				C. albicans	*C. dulbruski*	*C. tropicalis*	*C. kruzei*
1	Vaginal specimens from non diabetic cases	n=125	40	36	01	01	-
2	Vaginal Specimens from diabetic cases	n=75	18	12	05	03	03
3	Pus	n=30	07	05	-	01	-
4	Sputum	n=175	30	26	05	04	01
5	Oral Swabs	n=200	35	18	03	02	03
6	Indwelling medical devices	n=150	20	15	02	02	02
	Total	**755**	**150**	**112**	**16**	**13**	**09**

Table 4: *Candida* Morphotypes and Antifungal resistance

S. No	*Candida* Morphotypes	Resistance to			
		No	Clotrimazole	Fluconazole	Ketoconazole
1	254	09	08	07	05
2	534	46	26	25	10
3	554	68	45	23	16
4	334	27	17	14	12
Total		150	96	69	43

On Chrome agar 4 types of *Candida* species produced colonies with different colors they are *Candida albicans* - light green. *Candida dubliniensis* dark-green. *Candida tropicalis* - pink and *Candida kruseii* - violet colour growth they produced. Among 150 *Candida* strains subjected for these species identification, 112 *Candida* strains has been identified as *Candida albicans* (75%) followed by *Candida dubliniensis* (15%),

Candida tropicalis (9%) followed by *Candida kruseii* (8%).

The predominant *Candida* species identified

Candida albicans (75%) was the predominant *Candida* species identified from the *Candida* strains isolated from various clinical specimens (Table - 3).

Antifungal Susceptibility test result

Pertaining to the disc diffusion test performed, all the *Candida* species had shown different types of results with the applied antifungals (Table - 4). Each *Candida* strains had given different types of sensitivity pattern towards each antifungal drug. Totally 40 % of *Candida* strains had shown resistant towards one or more antifungal drugs.

***Candida* Morphotyping**

Among 150 *Candida* strains applied for morphotyping technique totally 4 different *Candida* Morphotypes has been identified (Fig 4.1,5,6,7 & 8). Of 122 *Candida albicans*, 3 different *Candida* morphotypes has been obtained and they were identified as *Candida* morphotype 534,554 and 334 among which the *Candida* morphotype 554 has been identified as the predominant *Candida* morphotypes obtained from *Candida albicans*. Except *Candida albicans*, all other *Candida* species subjected to morphotypes yielded less than 3 morphotypes (254,534 and 334), in detail, *Candida tropicalis* given 2 different morphotypes (254 and 334) *Candida kruseii* (334) and *Candida dubliniensis* had given only one morphotype i.e., 534.

Clinical infections and predominant *Candida* morphotype

In addition to *Candida* morphotype 334, *Candida* morphotype 534 was predominant and associated with vaginal specimen among the non diabetic women were as the *Candida* morphotype 554 was predominant in oral swab and sputum and the *Candida* morphotype 254 was the predominant *Candida* morphotype associated with the indwelling devices infection.

Different *Candida* morphotypes and its antifungal susceptibility pattern

The different *Candida* morphotypes identified had shown different types of antifungal susceptibility to Ketoconazole–(kt), Nystatin-(NS), Fluconazole-(Fu), Clotrimazole-(cc), Amphotericin-(B) and Itraconazole-(It). Among the four different *Candida* morphotypes, the *Candida* morphotypes 254 and 554 had shown maximum resistance to the antifungal drugs.

4. DISCUSSION

Candida albicans and related species live as benign commensals in one or more body locations in a majority of healthy individuals and it is responsible for the two different types of the fungal infections both the superficial as well the deep/ systemic fungal infections and affecting both immunocompromised and immunocompetent hosts. As opportunistic pathogens, they are poised to overgrow cavities and penetrate tissue in response to an alteration in host physiology that presumably compromises the immune functions that normally suppress their growth.

Morphotyping is a phenotypic method for strain differentiation of yeast cultures based on the comparison of appearance of their surfaces and fringes. For its simplicity and cost-effectiveness, it is recommended as an alternative tool to genotyping of *Candida albicans*. This advantage as well as easiness to perform and low costs but markedly lower reproducibility in comparison with molecular genetic techniques makes it an optimal typing method for first-line use. Evaluation of morphological diversity of strains by this method can be further utilized in the studies of virulence, switching phenomenon or antifungal resistance.

Morphotyping is extremely simple and cheap to perform, requiring only malt agar plates. Moreover, special morphological markers, like discontinuous fringes, have been found to be associated with virulent strains and an increased risk of death in the case of deep infection. Unfortunately, reproducibility of typing schemes based on colony morphology has already been reported to be affected by phenotypic variation [10], but a better *in vitro* reproducibility has been claimed for Hunter's scheme [11]. Hunter's scheme clusters Phongpaichit's morphotypes in a lower number, simplifying plates reading.

Based on these above mentioned informations, we selected the present study topic on "The *Candida* morphotypes associated with certain clinical infections and its antifungal susceptibility pattern". With the help of Visva diagnostic and research laboratory, we could able to obtaine 150 *Candida* strains and on enquiry, we came to know the details of the *Candida* strains, and they all were originally isolated from various clinical specimens.

All 150 *Candida* strains had given good growth on SDA, by subculture (24 hrs incubation) and subjected to the speciation all strains were found

to be identified as *Candida albicans* (112), *Candida dubliniensis* (16), *Candida tropicalis* (13) and *Candida kruseii* (9). Compare to all clinical specimens, from where these *Candida* strains has been isolated, vaginal specimens were found to yield more number of *Candida* strains. From this we came to understand that the *Candida* association and its relationship with infectious status or as colonizer.

Even among the vaginal specimens (200), the vaginal specimens collected from the diabetic women (75), yielded more number of *Candida* (24 %) compare to the vaginal specimens collected from the non diabetic women (125) which yielded (32%). This is due the hyper glycemic condition of the vaginal secretion of the diabetic women favouring the growth of *Candida*, which is elsewhere mentioned by many researchers [12, 13]. Totally, 150 *Candida strains* of four different species *Candida albicans, Candida dubliniensis, Candida tropicalis* and *Candida kruseii),* yielded 4 different *Candida* morphotypes i.e., 254, 534, 554, 334. Our study results gaining the support of the following. Many authors did study on the morphotyping of the *Candida* isolated from the clinical specimens and they found particular *Candida* morphotypes and their association with particular specimens/infections.

A study of describing the morphotypes of 446 strains of *Candida albicans,* isolated from a variety of clinical specimens was reported. By this limited code, 50 different morphotypes were distinguished, the largest group comprising 23% of the population. The simplicity and good discrimination of the method make it a useful typing scheme for *C. albicans*. Discontinuous colonial fringes were associated with strains from oral sites and deep infections. Significantly, 67% of strains from fatal infections were of the discontinuous fringe type, compared to only 11% of strains from other infections. Further associations between morphotype and anatomical source included narrow-coarse fringes in genitourinary isolates [14].

Hunter and Fraser [15] suggested that smooth fringeless colonies are the wild type [16] and so could be the most virulent. Then one should expect to see increased numbers of this morphotype in strains isolated from blood cultures. Correlation of *Candida* morphotypes and its virulence character was analyzed by certain scientists [17]. The other associations of morphotype with anatomical source are also difficult to explain. The increased number of discontinuous fringed strains from oral sites may reflect the variety of the ecological environment within the oral cavity. The variety of niches may favour those strains that can readily adapt from one niche to another. Whether there is any direct relationship between oral strains and strains from deep infections is unclear. Narrow coarse fringe formation in genitourinary isolates may indicate that these strains adhere more firmly to the epithelial cells lining the genitourinary tract.

In our study we could able to record the association of *Candida* morphotype 334 with severe vaginitis in both diabetic and non diabetic women Udhaya *et al.* [18] stated that the *Candida* morphotype 753 was associated with vaginitis cases among the cancer cervix patients and *Candida* morphotype 153 was predominantly isolated from normal healthy women's vaginal specimens. In addition to *Candida* morphotype 334 (fig-6), *Candida* morphotype 534 (Fig-7) was predominant and associated with vaginal specimen among the non diabetic women were as the *Candida* morphotype 554 was predominant in oral swab and sputum and the *Candida* morphotype 254 was the predominant *Candida* morphotype associated with the indwelling devices infection. From this results, it is possible for as to understand the relation between certain morphotype and its associate with particular infection.

A study of Morphotypes of *C. albicans* from HIV-infected patients by Oliver and Reade [19] found the morphotype 724 to be isolated from 52% of ARC (AIDS-related complex) and AIDS patients, whilst this morphotype was not isolated from HIV asymptomatic patients. These two morphotypes (754 and 724) differ only in the width of the fringe which may reflect technical variations, but both had continuous fine fringes. There was an association between the discontinuous fringe morphotype and fatal deep infections. Immune status/immunosuprmetion of the patients and the association of various *C. morphotypes* were studied by Blumberg *et al.* [20].

From our short term research could able to record the antifungal resistance of 4 different *Candida* morphotypes isolated from various infections (Table - 6), from which we could able to observe the antifungal resistance occurring towards 3 antifungal mainly in which clotrimazole drug resistance and is dominants (64%) followed by fluconazole (46%) and ketoconazole (29%). Morphotypes observed in *C. albicans* strains

isolated from the mouth of children with and without Down's syndrome, was analyzed by [21] and 9 different *C. albicans* morphotypes had been isolated form children with Down's syndrome and children without Down's syndrome.

Overall from our study we could able to obtain the *Candida* isolates originally isolated from various clinical specimens and we could able to speciate and identify the four different *Candida* species among which *Candida albicans* stood forward. The four different *Candida* morphotypes ie, 254, 534, 554 and 334 were able to identify from the obtained *Candida* isolates and also able to found out the correlation between the *Candida* morphotypes and the antifungal resistance. Since, there seems to be little available informations about the *Candida* morphotypes, the field of study needs researcher's attention. In order to find out the correlation between different *Candida* morphotypes and its association with clinical and non clinical source, research view and touch is essential to this field of study. Like other research, this field also needs encouragement and involvement. Future elaborative study is also essential to justify or to criticize our results focused here.

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