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RESEARCH ARTICLE

CANDIDA SPECIATION USING CHROM AGAR AND TO EVALUATE SUSCEPTIBILITY OF CANDIDA SPECIES TO ANTIFUNGAL DRUGS

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ABSTRACT

Candida species colonize the mucosal surfaces of all humans soon after birth. They are the most common cause of fungal infections which includes simple mucocutaneous to severe invasive infections. Apart from candida albicans, most common candida species causing infections in humans are candida tropicalis, candida parapsilosis, candida krusei, candidadubliniensis. Though C.albicans dominates all candidial isolates recovered from yeast infections, more recently Non Albicans Candida (NAC) species have been recovered with increasing frequency. The incidence of opportunistic fungal infections such as candidiasis has considerably increased in recent years. It is important to rapidly and reliably identify C.albicans as well as other Candida speciesin routine clinicalmicrobiology practice. CHROMagar is a differential medium which is developed to produce rapid yeast identification. This medium contains substrates that react with enzymes secreted by microorganisms producing colonies with various degree of pigmentation. Since molecular techniques are too expensive using of CHROM agar for species differentiation would be of benefit for easy, cost effective and rapid speciation. Previous studies identifying candida to species level have shown that there is increased incidence of NAC species in these patients. The antifungal susceptibility of fuconazole has been altered in C.albicans and NAC with increased resistance to fluconazole. Hence an attempt was made to identify Candida at species level and evaluate their susceptibility to different antifungal drugs.

INTRODUCTION

Candida species colonize the mucosal surfaces of all humans soon after birth. They are the most common cause of fungal infections which includes simple mucocutaneous to severe invasive infections. Apart from candida albicans, most common candida species causing infections in humans are candida tropicalis, candida parapsilosis, candida krusei, candida dubliniensis.[1] Though C.albicans dominates all candidial isolates recovered from yeast infections, more recently Non Albicans Candida (NAC) species have been recovered with increasing frequency.[2] While Candida can be isolated from 30-50 % of the oral cavities of healthy adults, making it a constituent of the normal oral flora, clinical oral candidiasis rarely occurs in healthy patients.[3] Candida infections particularly, oral candidiasis has been frequently recognized in diabetic patients, which can be due to their increased glucose in their oral fluids and their immune dysfunction.[4] Oral health is an important component of overall health status in HIV infection. Even common dental diseases such as caries and periodontal disease have greater impact on patients with HIV infection. In contrast, oral candidiasis constitutes the most common sign in HIV patients. [5]CHROMagar is a differential medium which is developed to produce rapid yeast identification. This medium contains substrates that react with enzymes secreted by microorganisms producing colonies with various degree of pigmentation.[2]Previous studies identifying candida to species level have shown that there is increased incidence of NAC species in these patients. The antifungal susceptibility of fuconazole has been altered in C.albicans and NAC with increased resistance to fluconazole.

Since molecular techniques are too expensive using of CHROM agar for species differentiation would be of benefit for easy, cost effective and rapid speciation.[6]

MATERIALS AND METHOD

The subjects included in this study were randomly selected from patients who were referred to the Department of Oral Pathology and Microbiology and at other tertiary health centers for various investigations. The consent for this study was obtained from them after necessary instructions.

The subjects included in this study were categorized into 4 different groups. 30 patients were included in each group.

Group I included 30 known type II diabetes patients with fasting blood glucose levels above 126 mg/dl. It included patients on medication as well as those without medication.

Group II included 30 hospital admitted patients having stay more than 1 week for any chronic infections, surgery or cardiovascular problems and ICU patients.

Group III included 30 known HIV patients, it included patients on medication as well as those without medication.

Group IV/ control group included 30 healthy volunteers/patients with minor dental problems, good oral hygiene and no systemic disorders.

Any habits like tobacco chewing, smoking etc were excluded. Pregnant females and under 18 years subjects were not included in this study.

Protocol for inoculation of Salivary Sample on culture media

The salivary samples from different groups were inoculated on Sabouraud Dextrose agar and CHROM agar plate.

Method - Allow media to warm to room temperature, and the agar surface to dry before inoculating. Inoculate and streak the specimen as soon as possible after collection. Streak the sample with a sterile loop (fig.7) CHROM agar and Sabouraud agar plates simultaneously. Incubate plates at 37 degree Celsius for 48 hours in incubator. Examine plates for typical colonial growth and color.

The colonies were identified on CHROM agar plates using color characteristics specific for each species as per the standards given by the manufacturer's instructions. The number of colonies was calculated using conventional method which includes a click counter and a pen.

Protocol for antifungal drug susceptibility testing

Color specific colonies inoculated on CHROM agar are isolated using a sterile loop and then are spread on Mueller Hinton agar medium by streak method. Antifungal drug discs are then placed on the Mueller Hinton agar plate and incubated at 37 degree Celsius for 48 hours to observe if any zone of inhibition is developed around the discs or not.

RESULTS

All the subjects were investigated for oral candidal carriage and candidal status. No patients showed evidence of oral candidal infection on clinical examination. The study comprised of total 120 cases that were divided into three groups. The Candidal colonies were grown on CHROM agar and Sabouraud agar simultaneously, identification of different species was done according to their color characteristics of colonies observed on CHROM agar medium.

Appearance of Candida species on CHROM agar were as follows:

- C.albicans - blue green
- C.tropicalis-dark blue gray centre with pink halo
- C.krusei-pink large rough spreading colonies with pale edge
- C.parapsilisis-pale cream colored colonies
- On Sabouraud dextrose agar pale white color colonies were observed.

Candida albicansspecies seen in different groups:

In the present study the mean number of colonies of Candida albicans observed on CHROM agar medium in diabetic subjects is 26.37 ± 28.24 (Table1). For Non-albicans Candida species the count is 6.20 ± 17.20 (Table 2). This suggests that Candida albicans were more frequently isolated species in Type II Diabetes Mellitus subjects than NAC. In hospital admitted subjects the mean number of colonies of Candida albicans is 17.50 ± 11.72 (table1) and for NAC the mean is 5.90 ± 4.38 (table2). From these findings it can be inferred that C.albicans is more prevalent in these subjects as compared to NAC. In HIV infected patients the mean number of colonies of NAC is 33.87 ± 21.90 (table2) and mean number of colonies of C.albicans is 16.10 ± 12.42 (table1), which indicates that NAC are more frequently isolated in these subjects than C.albicans. Based on these findings, it can be inferred that C.albicans were more frequently observed in Type II Diabetes Mellitus and Hospital admitted patients while NAC were more in HIV subjects. On comparison of C.albicans growth amongst different groups, a statistically significant growth was found in diseased groups than normal ($p<0.005$) (table1). On comparison of NAC amongst different groups, NAC showed statistically significant growth in HIV group than Diabetes, hospitalized and normal subjects ($p<0.005$) (table2).

When CHROM agar was compared with Sabouraud agar medium the mean number of colonies was higher in CHROM agar medium in all groups which suggests that CHROM agar is more efficient that Sabouraud agar in identification of Candida species. However the results obtained were statistically non-significant ($p>0.005$) (Table 3).

In our study the different Candida species identified were C.albicans, C. Tropicalis, C. Krusei, and C. Parapsilis. These species were tested with different antifungal drugs such as Nystatin, Fluconazole, Clotrimazole, Ketoconazole, Itraconazole and Amphotericin B. It was found that both C. albicans and NAC are sensitive to Nystatin and intermediate sensitivity for Amphotericin. B. C. albicans and C. Tropicalis showed resistance for Fluconazole.

However, C. Krusei and C. parapsilis showed intermediate sensitivity for Fluconazole. C. tropicalis and C.krusei were also found to be sensitive to Ketaconazole and Itraconazole. (Table1). The differences between the four groups were analyzed for differences in the mean numbers of candida spp. and mean number of colonies seen using Kruskall-Wallis test (non-parametric ANOVA). Post-hocpair-wise individual comparisons were done using Mann-Whitney 'U' test.

Comparisons were also done for individual study groups (within group) for the mean number of colonies seen on Sabouraud agar and CHROM agar using Mann-Whitney 'U' test. For all the tests, a P- value of 0.05 or less was considered statistically significant.

The mean number of colonies of candida albicansspecies observed in different groups were found to be statistically significant ($p<0.005$).

Non-albicans Candida species seen in different groups:

The mean number of colonies of NAC species seen in Group I is 6.20 ± 17.20(SD) and the frequency of number of Non-albicans Candida species was 6%. The mean number of colonies of Non-albicans Candida species seen in Group II is 5.90± 4.38 (SD) and the frequency of number of Non-albicans Candida species was 6%.

The mean number of colonies of Non-albicans Candida species seen in Group III is33.87 ±21.90 (SD)and the frequency of number of Non-albicans Candida species was 34%.

The mean number of colonies of Non-albicans Candida species seen in Group IV is6.07±11.70 (SD) and the frequency of number of Non-albicans Candida species was 6%.

Mean number of colonies of Non-albicans Candida spp. seen in different groupswas found to be statistically significant ($p< 0.005$).

Comparison between Chrom Agar and Sabouraud Agar

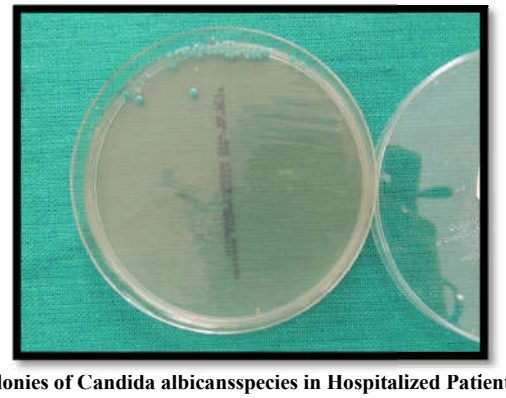
The mean number of colonies seen on Sabouraud agar in Group I is 19.87 ± 10.04 (SD) and on CHROM agar is 25.70 ± 25.44 (SD), the frequency of number of Candida species was 25% on Sabouraud agar and 26% on CHROM agar respectively. The mean number of colonies seen on Sabouraud agar in Group II is 24.13 ± 17.35 (SD) and on CHROM agar is 25.77 ± 18.80 (SD), the frequency of number of Candida species was 25% on Sabouraud agar and 26% on CHROM agar. The mean number of colonies seen on Sabouraud agar in Group III is 43.13 ± 19.35 (SD) and on CHROM agar is 48.37 ± 26.45(SD), the frequency of number of Candida species was 43% on Sabouraud agar and 48% on CHROM agar.

Colonies of Candida albicans species in Diabetes Mellitus Patients on CHROM agar

The mean number of colonies seen on Sabouraud agar in Group IV is 2.87 ± 3.01(SD) and on CHROM agar is 5.23 ± 5.99(SD), the frequency of number of Candida species was 2% on Sabouraud agar and 5% on CHROM agar (Table2, Graph2). The mean of number of colonies found in different groups in Sabouraud agar and CHROM agar was found statisticallynon-significant.

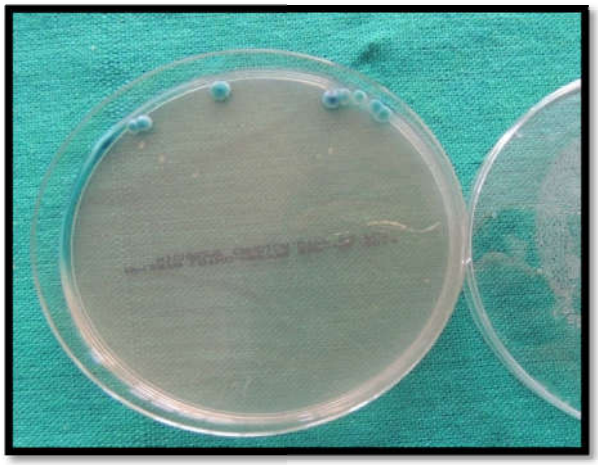
Antifungal Drug Susceptibilty of Different Candida Species to Antifungal Drugs

Different Candidal species were tested with various antifungal drugs to find out the susceptibility of these species to drugs such as nystatin, ketoconazole, clotrimazole, fluconazole, itraconazole and amphotericin B.



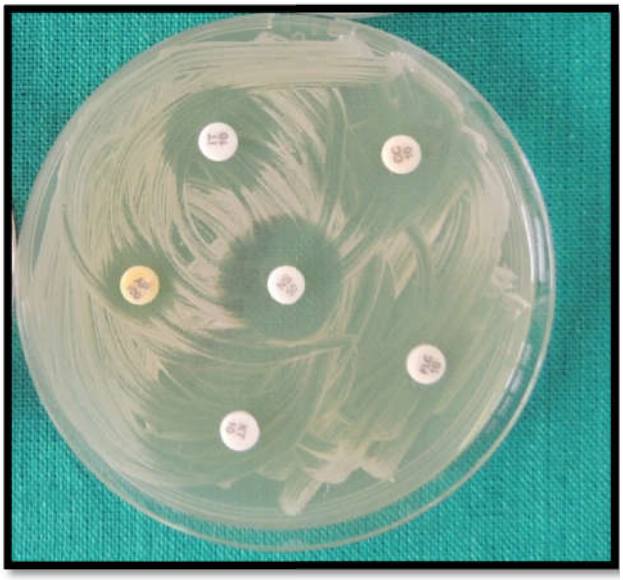
Colonies of Candida albicansspecies in Hospitalized Patients on CHROM agar

The differences between the four groups were analyzed for differences in the mean numbers of candida spp. and mean number of colonies seen using Kruskall-Wallis test (non-parametric ANOVA). Post-hocpair-wise individual comparisons were done using Mann-Whitney 'U' test.



Colonies of Candida albicansspecies in HIV Patients on CHROM agar

Colonies of NAC species in HIV Patients on CHROM agar



Antifungal drug susceptibility on Mueller Hinton agar

Comparisons were also done for individual study groups (within group) for the mean number of colonies seen on Sabouraud agar and CHROM agar using Mann-Whitney 'U'

test. For all the tests, a P- value of 0.05 or less was considered statistically significant.

DISCUSSION

Candida species are present as normal microbiota in the human body, i.e. on skin, mouth, large intestines, urinary and reproductive systems. The common etiology of many fungal infections especially among diabetic, hospital admitted and immunocompromised patients is known to be Candida species.[7]

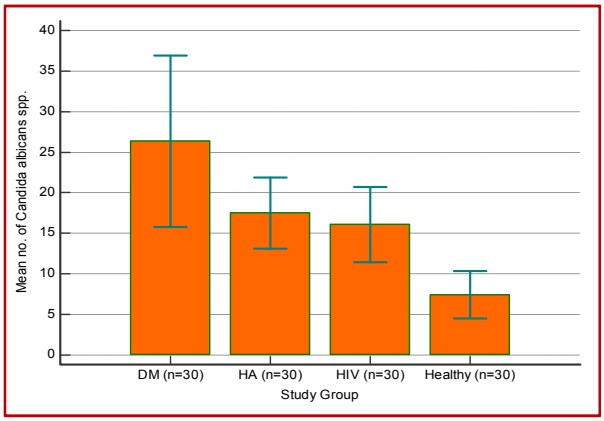
Table 1

Species Identified N=5	NS	FLC	CT	KT	IT	AP
C.Albicans	S	R	R	R	I	I
C.Tropicalis	S	R	I	S	S	I
C.Krusei	S	I	I	S	S	I
C.Parapsilisis	S	I	I	I	I	I

S= sensitive, R= resistant, I= intermediate

Table 2

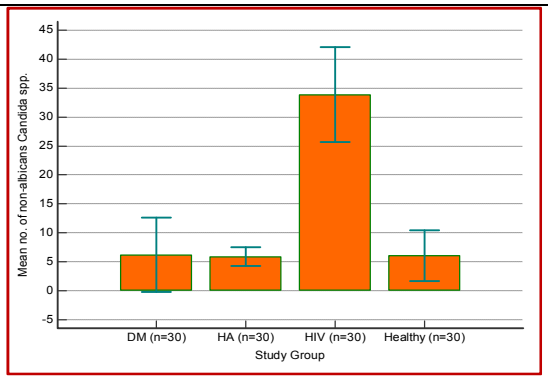
Candida albicans	N	Mean	SD	Median	Mann-Whitney 'U' test P<0.05 (Vs. group)
(1) Diabetes mellitus	30	26.37	28.24	20	4
(2) Hospital admitted	30	17.50	11.72	17	-
(3) HIV positive	30	16.10	12.42	12	-
(4) Healthy control	30	7.43	7.77	6	1
Kruskall-Wallis test	H	22.219			
	P	<0.0001			



Graph 1

Table 3

Non-Candida albicans	N	Mean	SD	Median	Mann-Whitney 'U' test P<0.05 (Vs. group)
1. Diabetes mellitus	30	6.20	17.20	1	3
2. Hospital admitted	30	5.90	4.38	6	3
3. HIV positive	30	33.87	21.90	28.5	1, 2, 4
4. Healthy control	30	6.07	11.70	2	3
Kruskall-Wallis test	H	55.218			
	P	<0.0001			



Graph 2

With the overuse of antibacterial agents, immunosuppressive agents, cytotoxins and steroids a new category of systemic mycoses has emerged. The emergence of non-albicans Candida species as significant pathogens has been well recognized now, however Candida albicans is by far the most commonly isolated species in humans.

The appropriate treatment for these fungal infections can be initiated based on the identification of the particular candida species.

In the present study the mean number of colonies of Candida albicans observed on CHROM agar medium in diabetic subjects is 26.37 ± 28.24 (Table1). For Non-albicans Candida species the count is 6.20 ± 17.20 (Table 2). This suggests that Candida albicans were more frequently isolated species in Type II Diabetes Mellitus subjects than NAC. Oral epithelia of diabetics favor adhesion, colonization of Candida may be due to the intrinsic qualitative changes on the cell surface receptors modulating Candida adhesion in diabetes subjects. C.albicans adheres to buccal epithelial cells in vitro to a greater degree than other Candida species. [8]

In hospital admitted subjects the mean number of colonies of Candida albicans is 17.50 ± 11.72 (table1) and for NAC the mean is 5.90 ± 4.38 (table2). From these findings it can be inferred that C.albicans is more prevalent in these subjects as compared to NAC. Use of various medical devices like vascular catheters, pacemakers, intubation tubes etc has greatly facilitated the management of hospitalized patients. However use of these artificial devices into various body systems has been accompanied by the ability of yeast to adhere and colonize these devices.[9]

In HIV infected patients the mean number of colonies of NAC is 33.87 ± 21.90 (table2) and mean number of colonies of C.albicans is 16.10 ± 12.42 (table1), which indicates that NAC are more frequently isolated in these subjects than C.albicans. In HIV patients several virulence factors contribute to the pathogenicity of Candida species including the ability to change cell morphology, a capacity to adhere to epithelial cells as well as the potential to secrete extracellular enzymes such as phospholipases and aspartyl protienases. These enzymes are considered to play important role in Candida overgrowth, tissue penetration and subsequent invasion of the host.

Based on these findings, it can be inferred that C.albicans were more frequently observed in Type II Diabetes Mellitus and Hospital admitted patients while NAC were more in HIV s When CHROM agar was compared with Sabouraud agar medium the mean number of colonies was higher in CHROM agar medium in all groups which suggests that CHROM agar is more efficient that Sabouraud agar in identification of Candida species.

In our study the different Candida species identified were C.albicans, C. Tropicalis, C. Krusei, and C. Parapsilis. These species were tested with different antifungal drugs such as Nystatin, Fluconazole, Clotrimazole, Ketoconazole, Itraconazole and Amphotericin B. It was found that both C. albicans and NAC are sensitive to Nystatin and intermediate sensitivity for Amphotericin. B. C. albicans and C. Tropicalis showed resistance for Fluconazole.

However, C. Krusei and C. parapsilis showed intermediate sensitivity for Fluconazole. C. tropicalis and C.krusei were

also found to be sensitive to Ketaconazole and Itraconazole. (Table1).

Fluconazole is widely used as a prophylactic drug because of its high oral bio-availability, minimal drug interaction and minimal side effects. Resistance to fluconazole has been reported by many workers. Candida infections have become very common in recent years and resistance to routine antifungal drug has become a challenge for accurate therapy.[10] Based on these results it can be inferred that antifungal susceptibility testing is important with the advent of newer antifungal drugs.

CONCLUSION

So, it can be concluded that there is increase in Candida species diversity in the oral cavities of diseased subjects. Although C.albicans is more prevalent but NAC species are increasing which cannot be ignored. The presence of isolates showing resistance to antifungal drugs represents a serious problem for the selection of effective antifungal therapy. Therefore, a greater attention should be given to microbiology for accurately identifying different Candida species in mixed cultures and also antifungal drugs susceptibility should be done for effective therapy.

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