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Research Article
Methods of Determination of Biofilm Formation by *Candida albicans*

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Abstract

Background: *Candida albicans* is a pathogenic member of the human oral and gastrointestinal microbiota. Biofilms of *C. albicans* form on indwelling devices, such as catheters and heart valves and recent evidence suggests that biofilms also form on the mucosal surfaces of the mouth and vagina. Biofilm infections of prosthetic devices are untreatable by antifungals and infections of the mucosa are frequently difficult to treat and recurrent. **Materials and Methods:** The patients (18 men and 32 women) suffering from various diseases were considered for the present study. Samples were collected and the *Candida* species were isolated using Hi Chrome *Candida* differential agar, sugar assimilation studies were done. Further biofilm formation was detected by tube method, congo red agar method and germ tube test. **Results:** *Candida* species vary in their distribution based on the age and gender of the patients suffering with various diseases. Biofilm production was maximum by *C. tropicalis* (36%) followed by *C. albicans* (20%). **Conclusion:** The ability of *Candida* species to form drug resistant biofilms is an important factor in their contribution to human disease. Biofilms represent a niche for microorganisms where they are protected from both the host immune system and antimicrobial therapies. Biofilm growth serves as an increasing source of clinical infections.

Key words: *Candida* spp., biofilms, antimicrobial resistance, infections, immune compromising

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The incidence and prevalence of invasive fungal infections have increased since the 1980s, especially in the large population of immuno-compromised patients and or those hospitalized with serious underlying diseases[1,2]. *Candida* species belong to the normal microbiota of an individual's mucosal oral cavity, gastrointestinal tract and vagina[3] and are responsible for various clinical manifestations from muco cutaneous overgrowth to bloodstream infections[4]. These yeasts are commensal in healthy humans and may cause systemic infection in immune compromised situations due to their great adaptability to different host niches. The genus is composed of a heterogeneous group of organisms and more than 17 different *Candida* species are known to be aetiological agents of human infection; however, more than 90% of invasive infections are caused by *Candida albicans, Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei*[5]. The expanding population of immuno-compromised patients that use intravenous catheters, total parenteral nutrition, invasive procedures and the increasing use of broad-spectrum antibiotics, cytotoxic chemotherapies and transplantation are factors that contribute to the increase of these infections[6]. The pathogenicity of *Candida* species is attributed to certain virulence factors, such as the ability to evade host defences, adherence and biofilm formation (on host tissue and on medical devices) and the production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin[7]. Currently, an increase in the number of yeasts that are resistant to antifungal drugs is recognized worldwide; therefore, the use of *in vitro* laboratory tests may aid the doctor in choosing an appropriate therapy[8]. The ability of *Candida* species to form drug-resistant biofilms is an important factor in their contribution to human disease. As in the vast majority of microbial biofilms[9], sessile cells within *C. albicans* biofilms are less susceptible to antimicrobial agents than are planktonic cells[10]. The progression of drug resistance within *Candida* biofilms has been associated with a parallel increase in the maturation process[11]. Furthermore, some studies have also shown that biofilms of *Candida* develop statically in the presence of a minimal matrix and exhibit the same level of resistance to drugs (fluconazole and amphotericin B) as cells grown in a shaker and exhibiting large amounts of matrix[11,12]. The increase in resistant strains necessitates a search for new targets for new antifungal agents. In the present study are focused on the current epidemiology, pathogenicity, biofilm formation and resistance of *Candida* species on various prosthetic medical devices are done.

MATERIALS AND METHODS

Identification of *Candida* and speciation

Patients: The present study was conducted on 50 patients of various disease conditions (18 men and 32 women aged between 16-90 years) admitted in the various department of NRI General Hospital, over a period of 6 months. A well documented history containing general and detailed information of the patient, associated risk factors, urinary symptoms and signs was filed for every patient. Catheterized urine samples and different invasive catheters were collected aseptically for microscopic and macroscopic examination and all the samples were processed for fungal culture.

Sample collection: Samples were collected with all aseptic precautions using sterile swabs (in housemade) invasive catheters. The swabs and catheters were dispensed in a test tube containing 5 mL of sterile saline. The samples were inoculated on HiCrome *Candida* differential agar obtained from Hi-Media, Mumbai. From the same swab a smear was made and gram stained. Germ tube test was done using the same broth to identify the albicans group. Those tested positive were inoculated on corn-meal agar for chlamydospore production. The remaining broth was kept at room temperature to exhaust the carbohydrate reserves in the isolates. After 24 h, the broth was used to do a lawn culture on yeast nitrogen agar for sugar assimilation test. Growth on the chrom agar was observed with in 24 h in most of the cases. For few isolates the plate had to be incubated for up to 48 h appreciate the growth. Colour of the colonies was noted down and the species were identified based on the manufacturer's instructions and by referring different literatures.

Sugar assimilation tests: The saline containing the clinical sample was incubated at room temperature for about 24 h to exhaust the carbohydrate reserves so that the sugar supplemented will be utilized properly and this rules out false negative results. Yeast extract agar (Hi-Media, Mumbai) was prepared following the manufacturer's instructions. A lawn culture of the pre-incubated broth was made on the yeast extract agar plate and the sugar disks-glucose, maltose, sucrose, lactose, melibiose, xylose, trehalose, raffinose, cellobiose were put and incubated for 24-72 h. Most of the isolates showed increased growth around the sugars they

used except for few which required incubation for up to a week. The results were noted and tabulated. For sugar assimilation test, the sugar disks were obtained from Hi-Media, Mumbai.

Detection of biofilm formation

Tube method: A qualitative assessment of biofilm formation was done as described by Christensen *et al.*[13]. The TSB_{glu} (10 mL) was inoculated with a loop ful of microorganism from overnight culture plates and incubated for 24 h at 37°C. The tubes were decanted and washed with PBS (pH 7.2) and dried. Dried tubes were stained with 0.1% crystal violet. Excess stain was washed with deionized water. Tubes were then dried in inverted position for biofilm formation.

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Experiments were performed in triplicate and repeated for three times.

Congo red agar method: An alternative method described the screening of isolates for biofilm formation is congo red agar containing Brain Heart Infusion (BHI) broth supplemented with 5% sucrose and congo red. The medium was composed of BHI (37 g L^{-1}), sucrose (50 g L^{-1}), agar (20 g L^{-1}) and congo red stain (0.8 g L^{-1}). Congo red was prepared separately as concentrated aqueous solution and autoclaved at 121°C for 15 min and added separately when the agar has cooled to 55°C. Plates were inoculated and incubated aerobically at 37°C for 2-3 days.

Positive result was indicated by black colonies with a dry crystalline consistency. A non biofilm producer usually remains pink. The experiments were performed in triplicate and repeated for three times.

Germ tube test: Using a sterile loop, a small portion of a pure colony of *C. albicans* was inoculation in to sterile test tubes containing 0.5 mL of each of the test sera. The resulting mixture was incubated aerobically at 37°C for not more than 2 h. About 0.5 mL of human serum in a separate sterile test tube was inoculated a small portion of a pure colony of yeast and incubated in a 10% CO_2 jar for not more than 2 h. At 10 min intervals, a drop of the yeast-serum mixture was placed on a clean microscope slide, covered with a cover slip and examined microscopically, using the x10 and x40 objective lenses. The appearance of small filaments projecting from the cell surface confirmed formation of germ tubes. The earliest time of such germ tubes production was noted for each test serum[14].

RESULTS

The results obtained show that the patients between the age groups of 41-50 has the highest number of isolates 14 (28%) next followed by the age group of 31-40 with the number of isolates 10 (20%) (Table 1). The results in Table 2 show that the highest percentage of female patients suffering from *C. tropicalis* with 28% next followed by *C. albicans* with 16% were observed.

The results show that more patients suffering from *C. tropicalis* next followed by *C. albicans* observed and studied in different cases like in immunosupression malignancy patiences with *C. tropicalis* (16%) next followed by *C. albicans* (8%). In case of ventilator and pregnanacy patients, with *C. tropicalis* (12%) next followed by *C. albicans* (6%). In case of septicaemia patients suffering from *C. tropicalis* (8%) followed by *C. albicans* (4%) were observed (Table 3).

Candida spp., were incubated by using the tube method containing TSB_{glu} in the test tube for various time periods. After 12 h of incubation the layer of *Candida* growth was very thin and transparent, after 24 h of incubation the layer becomes very thin. After 48 h of incubation the layer becomes

Table 1: Age wise distribution

Ages	No. of isolates	Percentage
10	1	2
11-20	5	10
21-30	10	20
31-40	10	20
41-50	14	28
51-60	7	14
61-70	3	6
71-above	0	0
Total	50	100

Table 2: Gender based distribution

	Male		Female	
Species	No.	%	No.	%
C. albicans	4	8	8	16
C. tropicalis	10	20	14	28
C. dubliniensis	1	2	3	6
C. guilliermondii	1	2	3	6
C. famata	1	2	2	4
C. kefyr	0	0	1	2
C. lusitanine	0	0	1	2
C. krusei	1	2	0	0
Total	18	36	32	64

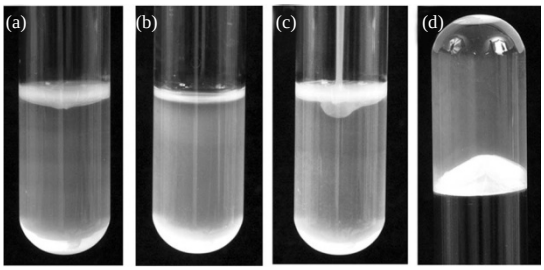

Fig. 1(a-d): Tubes showing (a) 12 h after incubation, (b) 24 h after incubation, (c) 48 h a biofilm network is formed on surface of liquid culture and (d) Inverted test tube with biofilm

Fig. 2: Tubes showing various degrees of biofilm production, +1 shows after 24 h of aerobic incubation, +2 shows after 48 h of aerobic incubation

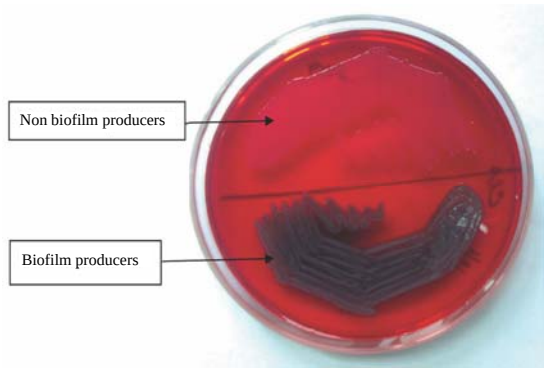

Fig. 3: Biofilm producing and non biofilm producing *Candida*

more thick and results in the formation of biofilm network on the surface of liquid culture which proves when the test tube is in inverted position the thick biofilm stops the free follow of culture liquid (Fig. 1).

Table 3: Disease wise distribution of *Candida* species

	Immunosupression malignancy		Ventilator		Pregnancy		Septicemia	
Isolates	No.	%	No.	%	No.	%	No.	%
C. albicans	4	8	3	6	3	6	2	4
C. tropicalis	8	16	6	12	6	12	4	8
C. dubliniensis	2	4	2	4	0	0	0	0
C. guilliermondii	1	2	1	2	2	4	0	0
C. famata	1	2	1	2	1	2	0	0
C. kefyr	1	2	0	0	0	0	0	0
C. lusitanine	0	0	1	2	0	0	0	0
C. krusei	0	0	1	2	0	0	0	0
Total	17	34	15	30	12	24	6	12

Table 4: Biofilm producing *Candida* spp., biofilm production was maximum by *C. tropicalis* (36%) followed by *C. albicans* (20%)

	Biofilm producing		Non biofilm producing	
Isolates	No.	%	No.	%
C. albicans	10	20	2	4
C. tropicalis	18	36	6	12
C. dubliniensis	4	8	0	0
C. guilliermondii	1	2	3	6
C. famata	2	4	1	2
C. kefyr	0	0	1	2
C. lusitanine	1	2	0	0
C. krusei	1	2	0	0
Total	37	74	13	26

Various degrees of biofilm production were observed at different time intervals of test tube aerobic incubation results in +1 in 24 h of incubation and +2 in 48 h of aerobic incubation (Fig. 2).

About 6 (12%) isolates have been isolated, among them *C. albicans* is more and followed by sputum 5 (10%), vaginal candidiasis is seen more in sexually active young females may be hormonal fluctuation may lowered the local immune response hence they may have recurrent candidiasis, followed by bronchial washing 3 (6%) and last is pus 1 (2%) among all the *Candida* strains isolated 37 (74%) are found to be biofilm producing organism and 13 (26%) are non biofilm producing *Candida* (Table 4).

On congo red agar method *Candida* produce black coloured colonies which show biofilm producing organisms (Fig. 3). After 24 h of incubation biofilm formation is less when compared with 48 h (Fig. 4), a surface pellicle is formed on the surface of liquid cultures suggests the strong biofilm producing organisms.

DISCUSSION

Several *Candida* species are commensal and colonize the skin and mucosal surfaces of humans. Critically ill or otherwise immune compromised patients are more prone to develop

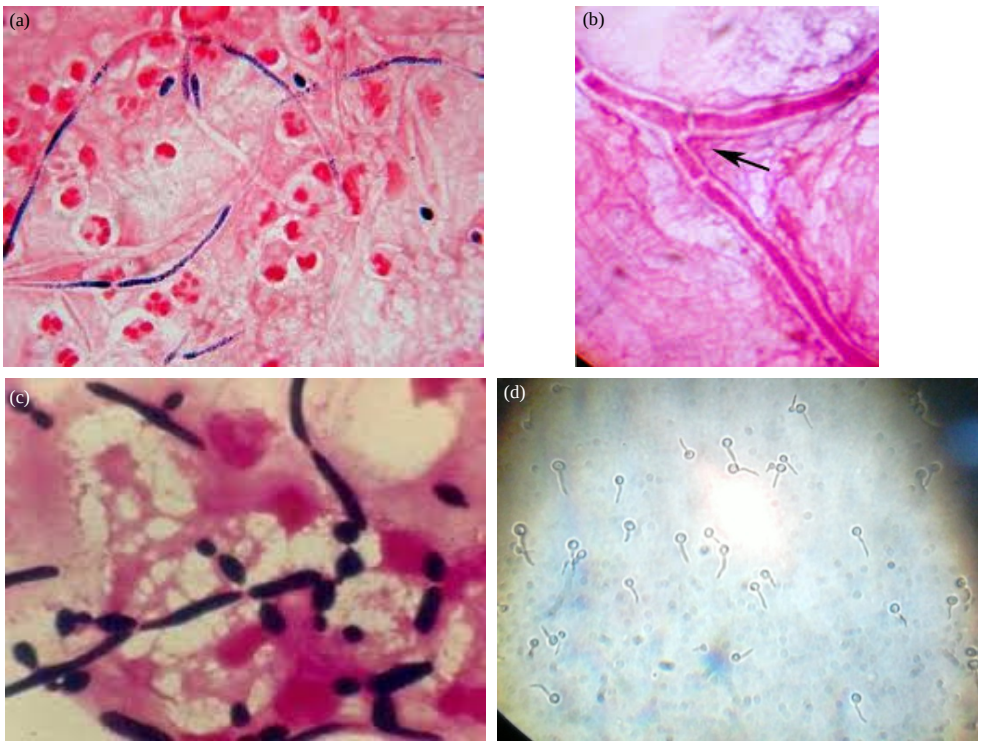

Fig. 4(a-d): Images of Gram stained smear shows candidiasis, (a) Gram stained smear of *Candida*, (b) *Candida* with pseudohypae in vaginal swab, (c) Gram positive budding yeast in candidiasis and (d) Germ tube test for *C. albicans*

both superficial and life-threatening *Candida* infections[15]. *Candida* infections also constitute the most common fungal infections in AIDS patients[15,16]. These patients predominantly develop oropharyngeal candidiasis, which can lead to malnutrition and interfere with the absorption of medication. *Candida albicans* is the predominant cause of invasive fungal infections[17] and represents a serious public health challenge with increasing medical and economic importance due to the high mortality rates and increased costs of care and duration of hospitalization[18,19]. Although *C. albicans* is the most prevalent species involved in invasive fungal infections, the incidence of infections due to non-albicans species is increasing. In a study with 2019 patients at major North American medical centres, a predominance of non-albicans species was observed; although *C. albicans* was the most frequently isolated species, it was followed by *C. glabrata* and other non-*C. albicans* species. This change in epidemiology could be associated with severe immune suppression or illness, prematurity, exposure to broad-spectrum antibiotics and older patients[17]. In European countries, an analysis showed that more than half of the cases of candidaemia were caused by *C. albicans* and the incidence rates for non-albicans *Candida*emia infections were 14% each for *C. glabrata* and *C. parapsilosis*, 7% for *C. tropicalis* and 2% for *C. krusei*[20]. Changes in the epidemiology have also been observed in Latin American countries. In Chile, the prevalence of *C. albicans* has changed and a progressive increase of non-albicans infection has been observed; *C. parapsilosis* was the most frequent species, followed by *C. tropicalis* and *C. glabrata*. All isolates were susceptible to amphotericin B; however, 50% of the *C. glabrata* isolates were resistant to fluconazole[21]. According to the Brazilian Network Candidaemia Study, *C. albicans* accounted for 40.9% of cases in Brazil, followed by *C. tropicalis* (20.9%), *C. parapsilosis* (20.5%) and *C. glabrata* (4.9%)[22,23]. Other species have been isolated in healthy people and patients. *Candida dubliniensis* was usually found in combination with other yeast species, especially *C. albicans*[24]. A high prevalence of *C. dubliniensis* in the oral cavities of HIV-infected and AIDS patients has also been reported[25,26].

By using models of *C. albicans* biofilms, several studies have shown uniform resistance of the organisms in the biofilm to a wide spectrum of conventional antifungal agents including resistance to the new triazoles[27], which have been shown to be fungicidal with extended activity against many azole-resistant organisms[28]. Therefore, biofilm-associated infections are difficult to treat, which emphasizes the need to develop antimicrobial drugs that show activity against

biofilm-associated organisms and specifically target biofilm-associated infections. All the biofilm producing organisms are isolated from catheters and various invasive procedures.

CONCLUSION

In the present study candidiasis has been characterised from various patients admitted in different wards for long time i.e., more than 3 days. Some of them are catheterized with urinary catheters where a huge number of *Candida* is isolated because of the biofilm formation on the invasive prosthetic devices. In the present study urine and urinary catheters isolates are 30 which is 60% and most of them found to be biofilm producing organisms and followed by blood in which high mortality rate is seen.

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