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Study of biofilm production and its correlation with antifungal resistance among Candida species isolated from suspected cases of Tuberculosis

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Abstract

Background: Production of the biofilm may be considered as a virulence factor because it shows resistance towards many antimicrobial agents. *Candida albicans* is the most commonly isolated fungal pathogen and cause severe secondary infections in immuno-compromised population, including tuberculosis patients. This study was aimed to speciate *Candida* isolates, production of biofilm and its antifungal susceptibility pattern.

Materials and Methods: Totally 178 sputum specimens were screened by RNTCP Unit were included in the study. The clinical isolates of candida were identified by using conventional methods and their ability to produce biofilm formation was detected by the tube method. Antifungal susceptibility testing was carried out by direct susceptibility method using CHROM agar.

Results and Conclusion: Out of 178 sputum samples, *Candida albicans* 26 (72.2%) was the predominant species isolated. Strong biofilm production was seen with *Candida albicans* (47.2%), *Candida parapsilosis* (8.3%) and *C.kruseii* (5.5%) whereas *C.dubliniensis* (2.7%) was found to be weak biofilm producer. The biofilm positivity was found more with C.*albicans* (47.2%) as compared to *Non-albicans Candida* (18.1%).Antifungal resistance by *Candida* strains for Itraconazole, Nystatin, and Amphotericin B about 33.3%, 19.4% and 8.3% respectively.

Keywords: Sabouraud's Dextrose Agar (SDA), Non-Albicans Candida (NAC),Antifungal Resistance, Medical Devices.

Introduction

Many microorganisms grow in complex communities on specific substrates in their ecologic environments rather than as single free-living organisms. These substrate-attached communities are frequently referred to as biofilms.Since biofilm, they serve as nidus for disease and are associated with high-level antibiotic resistance of the microorganisms.[(1)]Tuberculosis (TB) causes significant morbidity and mortality throughout the world, particularly in developing countries in Asia and Africa[(1,2)] Nine to eighty percentages of pulmonary tuberculosis patients are infected by *Candida* species.[(1,3,4)] Options of the antifungal drugs available for the treatment of systemic and invasive Candidiasis are restricted to polyenes, allylamines, azoles and echinocandins class of molecules.[(2)] A variety of infections are caused by biofilm ranging from common urinary tract infections, catheter infections, ear infections, dental plaque to more life threatening infections such as endocarditis and severe candidaemia in immune-suppression & ICU patients.[(1,2)]

Biofilm formation is a complex developmental and genetically controlled phenomenon with three basic stages (reviewed by Nobile and Mitchell).

- Attachment and yeast cell colonization of substrate
- Yeast cell growth and proliferation forming a basal layer of yeast cells
- Pseudohyphae, hyphal extension, and concomitant production of an extracellular matrix[(3,4,21)]

C.albicans can form extensive biofilms on medically implanted, indwelling devices such as catheters, shunts, stents, prosthesis, cardiac implants, endotracheal tubes. Since *C.albicans* is a human commensal, it can frequently come into contact with indwelling medical devices, attach, develop a biofilm and cause severe infections which is responsible for development for high level antifungal resistance.[(5-7)]

The biofilm induced anti-fungal resistance represents major therapeutic challenge to clinicians. It has also been shown that yeast in biofilms are resistant to azoles and standard polyenes.This has direct effects on clinical management of these kinds of candida infections as antifungal activity in biofilms is altered compared to free-living yeast cells.[(7-9)]

Materials and Methods

A total of 178 Sputum samples were collected from patients suspected for tuberculosis attended the RNTCP clinics at Shri Siddhartha Medical College, Hospital & Research Centre, Tumkur, were included in this study during the period of May 2015- December 2015.The patients symptoms present with persistent fever & cough, prolonged treatment with broad spectrum antibiotic therapy, immune-compromised state and other risk factors were also included in our study.

Specimen Collection: Two sputum samples, spot sample (i.e. at the time when patient was examined) and the next day early morning sample were collected in a separate sterile container from patients suspected for tuberculosis attended the RNTCP clinics at Shri Siddhartha Medical College, Hospital & Research Centre. Sputum smears were stained with Auramine-Rhodamine stain and examined under flourescent microscopy for the presence of acid fast bacilli.

Candida isolation: The sputum samples were inoculated onto Blood agar, MacConkey's agar and SDA supplemented with 0.05g/L Chloramphenicol. After 48hrs of incubation at 37ºC, growth on SDA were examined for yeasts colonies. The isolates were further identified by conventional methods.[10] Culture on CHROM agar (Hi-media, Mumbai) was also used for identification of the species. Isolates were maintained on SDA slopes. For biofilm analysis, isolates were plated on SDA and fresh cultures were used.

Biofilm formation: Biofilm formation was assessed by visual method described by Yigit et al[11]The adherent biofilm layer was scored visually as either negative or weak positive(1+), moderate positive (2+) or strong positive (3+). Staphylococcus epidermidis ATCC 35984(Hi-media, Mumbai) served as positive control.

Antifungal Susceptibility Testing: Antifungal susceptibility test was performed by disc diffusion method with commercially available antifungal discs- Amphotericin B 100 units, Fluconazole 25 mcg, Nystatin 100 units, Voriconazole 1 mcg and Itraconazole 10 mcg all were supplied by Hi- Media, Mumbai.[12]

Direct susceptibility testing: The isolates were inoculated directly onto CHROM agar (Hi- Media) to identify *Candida* to species level and to predict the susceptibility to various antifungal agents had been noted. A sterile nontoxic cotton swab dipped in the standard inoculum and streaked the entire agar plate. Then apply the discs- Amphotericin B 100 units, Fluconazole 25 mcg, Nystatin 100 units, Voriconazole 1 mcg and Itraconazole 10 mcg all were supplied by Hi-Media, Mumbai.

Using aseptic technique with a distance of at least 24 mm, incubate the plates at 37ºc for 24-48 hrs. If it showed insufficient growth; read only after 48 hrs. The zone of inhibition around the discs were noted and recorded.[13] Quality control was performed using *Candida albicans* ATCC 90028 (Hi-media, Mumbai) as reference strain.

Results

Out of the total 178 patients with suspected tuberculosis, 29 patients (16.29%) who were positive for Tuberculosis, whereas 149(83.7%) patients were negative for Tuberculosis. Among the 178 patients with suspected pulmonary tuberculosis, *Candida spp* were isolated from 36 (20.2%) patients. *Candida albicans* was the most common isolate 26 (72.2%), followed by *C. parapsilosis 6* (16.6%) *C.dubliniensis 2*(5.5%) and *C.krusei.2* (5.5%) (Table 1) (Fig. 1).

Among the 36 isolates subjected for biofilm production, 23 (63.8%) were positive along with the standard strains. Strong biofilm production (3+) was seen in 11 strains, moderate (2+) was seen in 6 strains and weak (1+) in 6 strains, while 13 (36.1%) did not produce biofilms. *C.albicans, C. parapsilosis* and *C. krusei* were found to be strong biofilm producers whereas C.*dubliniensis* was identified as weak producers (Table 2) (Fig. 2).

Candida albicans were highly susceptible to Voriconazole (72.2%), Fluconazole (72.2%). Amphotericin B was the next effective drug showed intermediate sensitive with 63.8% susceptibility followed by Nystatin 52.7% and Itraconazole 38.8%. *Non-albicans candida* (NAC) were highly susceptible to Voriconazole (27.7%), Fluconazole (22.2%). Amphotericin B was the next effective drug showed 5.5% susceptibility. Nystatin showed intermediate sensitive with 18.1% followed by Itraconazole 11.1% which showed dose dependent susceptibility (Fig. 3) (Table 3).

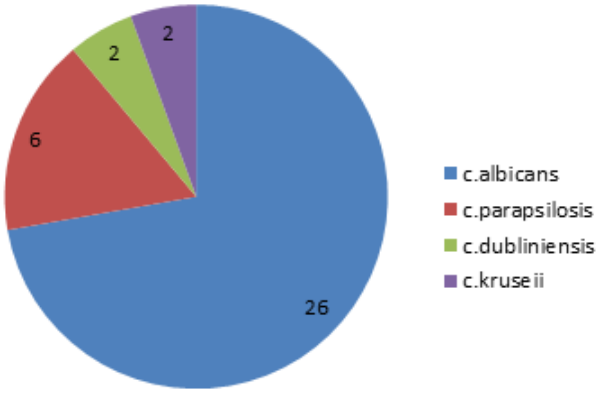

Fig. 1: Distribution of various *Candida spp*. from patients with tuberculosis

Table1: Candida species isolated from the sputum of Tuberculosis patients

Tuberculosis status	*Candida* spp isolated	*Candida spp* not isolated
Sputum AFB positive	4	25
Sputum AFB negative	32	119

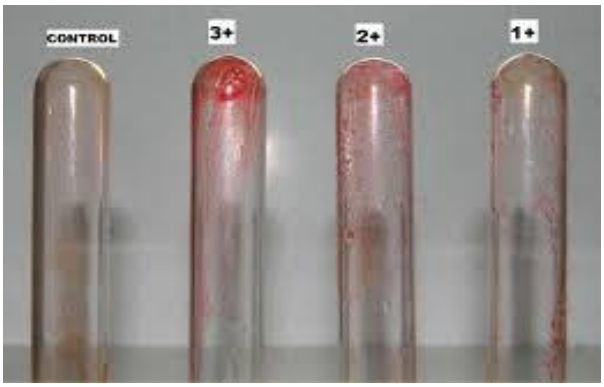

Fig. 2: Grading of biofilm formation in candida species

Table 2: Results of biofilm production: Species wise distribution

Candida spp	1+	2+	3+	Negative
C.albicans	6	3	8	9
C.parapsilosis	-	1	2	3
C.kruseii	-	2	-	-
C.dubliniensis	-	-	1	1
Total	6	6	11	13

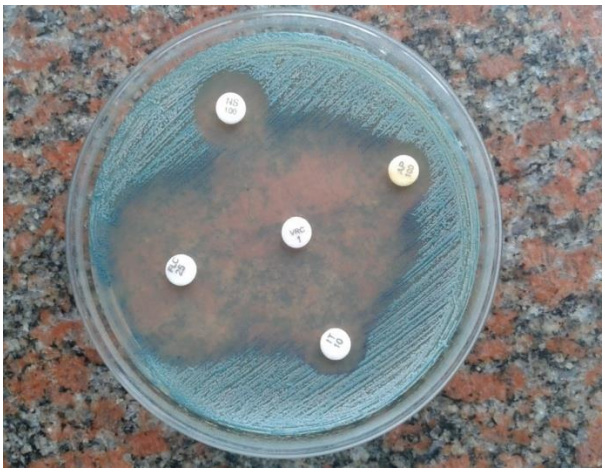

Fig. 3: Antifungal resistance of Candida species

Table 3: Antifungal drug resistance pattern of Candida strains in percentage

Candida strains	AmphotericinB			Nystatin			Fluconazole			Itraconazole			Voriconazole		
	S	IS	R	S	IS	R	S	IS	R	S	IS	R	S	IS	R
C.albicans(26)	2.7	61.1	8.3	-	52.7	19.4	72.2	-	-	5.5	33.3	33.3	72.2	-	-
C.parapsilosis(6)	2.7	8.3	5.5	-	11.1	5.5	18.1	-	-	-	2.7	13.8	18.1	-	-
C.dubliniensis(2)	2.7	2.7	-	-	2.7	2.7	5.5	-	-	-	5.5	-	5.5	-	-
C.kruseii(2)	-	5.5	-	-	2.7	2.7	2.7	-	2.7	-	2.7	2.7	5.5	-	-

Discussion

Tuberculosis is well recognized for its wide range of clinical spectrum, chronicity and sequlae. Respiratory fungal infections are one of the emerging conditions complicating pulmonary tuberculosis. Weak immune status, destruction of lung tissues and lesions formed due to TB are the predisposing factors for fungal infections.[13] The increasing incidence of HIV-1 infection, use of therapeutic modalities, advanced life support, organ transplantation, and implantation of prosthetic devices have expanded the incidence of *Candida* infections.[14-16]

Respiratory candida infections involving the lungs or bronchial system appear predominantly in patients with underlying primary diseases and usually the result of aspiration in contrast secondary Candida pneumonia seen in patients with hematogenous *Candidiasis*.[15,16]

In the present study, biofilm production was found to occur most frequently in *C.albicans.* This finding is similar to an earlier report suggested that pathogenic *C.albicans* were more likely to produce biofilms than among NAC such as *C.parapsilosis*.[17] In the present study 63.8% of the *Candida* isolates tested were found to be biofilm producers. This finding is in concordance with studies conducted by Muni et al 2012 (64%)[18] and Mohandas et al 2011 (73%).[19,20]

Strong biofilm production was seen with *Candida albicans* (47.2%), *Candida parapsilosis* (8.3%) and *C.kruseii*(5.5%) whereas *C.dubliniensis* (2.7%) was found to be weak biofilm producer. The biofilm positivity was found more with C.*albicans*(47.2%) as compared to *Non-albicans candida* (18.1%).

In this study we present evidence that candida isolates inoculated directly onto CHROM agar allows the rapid identification as well as determination of susceptibility pattern for the majority of *Candida* isolates encountered in the clinical laboratory.

The antifungal susceptibility results showed *Candida albicans* were highly susceptible to Voriconazole (72.2%) and Fluconazole (72.2%). Amphotericin B was the next effective drug showed intermediate sensitive with 63.8% susceptibility followed by Nystatin 52.7% and Itraconazole 38.8%. *Non-albicans candida* (NAC) were highly susceptible to Voriconazole (27.7%), Fluconazole (22.2%). Quite high level percentage (33.3%) of the resistance towards the antifungal drug Itraconazole was observed from all the

Candida species followed by Nystatin drug (19.4%) and Amphotericin B (8.3%).

From our study we found that the significant percentage (12.3%) of the resistant *Candida* strains have been isolated from the sputum of the TB clinic attendees with both the pulmonary TB cases and patients with other respiratory tract infections without TB.

Conclusion

This study noted that biofilm formation as an important trait exhibited by *Candida species.* This ability to form biofilms is linked with the ability of the organisms to attach, colonise and subsequently cause infection and resistance to anti-fungal agents. Therefore, screening of pulmonary tuberculosis patient for *Candida* infection should be routinely practiced along with anti-fungal sensitivity testing for Non-Albicans *Candida* isolates.

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