

ISOLATION OF FUNGUS FROM SPUTUM SAMPLES OF CHRONIC RESPIRATORY DISEASE PATIENTS: A HOSPITAL-BASED STUDY IN TAMIL NADU, INDIA

Lalithambigai J¹, Prabha Thangaraj², Saraswathi R³, Sivakumar K⁴

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Corresponding Author:

Dr. Lalithambigai J,

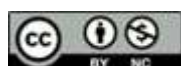
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¹Associate Professor, Department of Microbiology, Trichy SRM Medical College Hospital and Research Centre, Trichy, Tamilnadu, India.

²Associate Professor, Department of Community Medicine, Trichy SRM Medical College Hospital and Research Centre, Trichy, Tamilnadu, India.

³Professor, Department of Microbiology, Trichy SRM Medical College Hospital and Research Centre, Trichy, Tamilnadu, India.

⁴Professor and HOD, Department of Medicine, Trichy SRM Medical College Hospital and Research Centre, Trichy, Tamilnadu, India.

ABSTRACT

Background: Understanding the burden of fungal diseases is a big challenge since these infections are usually difficult to diagnose and they manifest with nonspecific symptoms and are not routinely suspected by physician. **Objective:** To assess the burden of fungal infection among the sputum samples of patients with chronic respiratory disease. Drug susceptibility pattern for fungal infection was also evaluated. **Materials and Methods:** A hospital-based study was done among 1659 chronic respiratory disease (CRD) sputum samples. These samples were examined using direct microscopic method-gram staining, potassium hydroxide. Out of 1659 samples, 143 were shown fungal elements which was inoculated into the Sabouraud's dextrose agar. The colony morphology and microscopic features were identified and reported. All *Candida* isolated were subjected to Congo red agar test, germ tube test, HiCrome *Candida* Differential agar, cornmeal agar and antifungal susceptibility testing as per the standard protocol. **Results:** Fungal growth was detected among 143 out of 1659 samples i.e. 8.62% (95% CI: 7.26-9.97). Mean age of CRD patients with fungal isolates in their sputum was 69.7 ± 11.43 years. Among the fungal type, *Candida species* was the highest i.e. 81.8% followed by *Aspergillus* (16.1%), *Mucor* (1.4%) and *Rhizopus* (0.6%). Amphotericin and Voriconazole were sensitive to all species, Fluconazole and Itraconazole has a sensitivity of 67.2% and 75.9% respectively. **Conclusion:** The burden of fungal infection among the elders with CRD patients was high. Hence, screening for fungal infection on routine basis for these high-risk patients could be included in the management protocol. Amphotericin and Voriconazole should be the preferred drug of choice in the treatment of fungal infection among CRD patients.

INTRODUCTION

Respiratory tract infection (RTI) is a general term used to describe infections that can occur in any part of the respiratory system. Common symptoms of RTIs include coughing, sore throat, and congestion. In some cases, additional symptoms may be present such as fever, body aches, and difficulty breathing. They are caused by viruses, bacteria, fungi, and other organisms. Among the various lung infections, chronic respiratory diseases is one of the most common chronic diseases with high morbidity and mortality; which is fourth leading cause of death in the world and it is expected to rise to the third place by 2020.^[1]

Chronic respiratory diseases (CRD) are a group of conditions characterized by long-term inflammation and/or narrowing of the airways. It includes asthma, chronic obstructive pulmonary disease (COPD), emphysema, bronchitis, interstitial lung disease, occupational lung disease, tuberculosis, and lung cancer. Within a chronic infection site, the microbial community engages in diverse inter-species interactions, encompassing a delicate interplay of competition and cooperation. However, this intricate dynamic often culminates in detrimental consequences for the patients.^[2]

In lung infections, the number of microbes that interact can vary depending on several factors, including the specific infection, the individual's immune system, and the overall microbial ecosystem in the respiratory tract.^[3] Among the microbes, fungal

infections of the lungs have increased dramatically over the past two decades in a variety of fungal infections. The specific diagnosis of fungal pneumonia is gaining importance in view of the variety of treatment strategies and the high mortality associated with acute invasive fungal infections. Unfortunately, fungal respiratory infections are always difficult to diagnose because the difficulty increases when samples are taken to diagnose fungal lung infections when they occur.^[4] An earlier investigation revealed that critically ill patient's respiratory samples taken by bronchoalveolar lavage, endotracheal aspirate, or protected specimen brushing could include *Candida* infestation.

Previous research has shown that *Candida* is the leading cause of lung infection, particularly among the elder patients, although multiple fungi can be responsible.^[5] Major risk factors for high *Candida* isolation rates in lower respiratory tract infection are COPD, smoking, tuberculosis, malnutrition, malignant tumors, diabetes, HIV infection, and long-term use of antibiotics. Among them, the rate of *Candida* isolation was highest in COPD patients.^[6]

Pathogenesis of Candidiasis relies upon on the expression of virulence elements like germ tube formation, adhesions, phenotypic switching, biofilm formation, and the manufacturing of hydrolytic enzymes. Majority of *Candida* isolates produce the biofilm formation genetically resistant to antifungal agents including amphotericin B and fluconazole. The most external layers of *Candida* cells are essential for the adherence to host surface, thereby playing a pivotal role in the pathophysiology of Candidiasis.^[7] Therefore, infections caused by them are very difficult to treat; biofilm is considered a risk factor that increases the mortality rate in Candidiasis in critically ill patients.^[8] In this situation, very important to determining the drug susceptibility on *Candida* isolates. The resistance gene is responsible for the causation of antibiotic resistance, and the relationship between antibiotic resistance and COPD is still poorly understood.

Thus, this study was conducted to isolate fungus in sputum samples of CRD patients and to test drug susceptibility of anti-fungal agents.

MATERIALS AND METHODS

A hospital based cross sectional study was carried out among sputum sample received in the Department of Microbiology, Tertiary care hospital, Trichy from August 2019 to July 2021. The study included all sputum samples of chronic respiratory illness excluding TB patients. A total 1659 Chronic respiratory disease patient's sputum sample were examined by using Gram staining for preliminary identification and by using a 10%potassium hydroxide (KOH) mount for visualization of fungal elements. Out of 1659 sputum samples, 143 samples were showed fungal elements. These 143 samples were then cultured on 2 culture bottles; one bottle was

subjected to Sabouraud's dextrose agar (SDA) supplemented with cycloheximide (0.5 g/L) and gentamicin (0.05 g/L) to prevent bacterial contamination for candida growth. Second bottle were subjected to SDA supplemented with gentamicin (0.05 g/L) for rapid grower fungus (molds). The cultures were incubated at dual temperatures of 25°C and 37°C for 24-48 hours to optimize growth conditions. Following initial growth, isolates underwent a systematic identification process.^[9]

- a. Rapid growers' fungus (molds) was tested for colony morphology and examined by using microscopy method with LPCB mount (Lacto Phenol Cotton Blue) to identify the Rapid growers (*Aspergillus spp.* *Mucor spp.*)
 - b. The white small pasty colonies with yeasty odor were performed with gram staining and to identify the morphology of *Candida* (Yeast). Once confirmed for candida isolates by colony morphology in agar plate and gram staining method, then proceeded with Congo red agar test (for determination of biofilm), Germ tube test and species identification method by using HiCrome *Candida* Differential agar, Corn-Meal Agar (Dalmau technique). After Species confirmation, all candida isolates were examined for Antifungal susceptibility test by using the disk diffusion method following Clinical and Laboratory Standards Institute (CLSI) M44, document guidelines.
- **Congo Red Agar Test: (Determination of biofilm formation):** The medium used in this study consisted of the following components per liter: brain heart infusion [BHI] at a concentration of 37g, glucose at 80g, agar no.1 at 10g, and Congo red stain at 0.8g. The Congo red stain was prepared separately as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes. It was then added to the agar once it had cooled down to 55°C. After preparation, the plates were inoculated and incubated aerobically at 37°C for a period of 24 to 48 hours. Positive results were indicated by the presence of dark red colonies. Weak biofilm producers usually remained pink, though occasional darkening at the centers of colonies was observed. Biofilm negative strains produced white or very light pink-colored colonies. *Candida albicans* ATCC 90028 and *C. parapsilosis* ATCC 96142 served as controls for biofilm production.^[10,11]
 - **Germ tube Test:** The Germ Tube Test (Reynolds-Braude phenomenon) was performed by inoculating a small colony of the yeast in 0.5 mL of human serum and incubating at 37°C for two to three hours. Microscopic examination was conducted to observe the formation of germ tubes, which appear as long, thin, filamentous extensions from the yeast cells without

constriction at the point of origin, characteristic of *C. albicans* and *C. dubliniensis*.^[12]

- **Species Identification Test:** All *Candida* isolates were cultured on HiCrome *Candida* Differential Agar (HiMedia Laboratories, Mumbai) and incubated at 37°C for 48 hours. Species identification was based on distinctive colony colors and morphology: light green for *C. albicans*, metallic blue for *C. tropicalis*, purple fuzzy colonies for *C. krusei*, and cream to white for *C. glabrata*. Confirmation of species identification was performed using the cornmeal agar (Dalmau technique), where morphological characteristics such as blastoconidia, pseudohyphae, and chlamydo spores were observed after incubation at 25°C for 48-72 hours.^[13,14]
- **Antifungal susceptibility testing (AFT) for *Candida* Isolates:** AFT was conducted using the disk diffusion method following Clinical and Laboratory Standards Institute (CLSI) M44, document guidelines. Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/mL methylene blue was used as the culture medium. The inoculum was prepared by suspending colonies in sterile saline to achieve a turbidity equivalent to 0.5 McFarland standard. The suspension was spread evenly on the agar surface, and antifungal disks were placed with appropriate spacing. The plates were incubated at 35°C for 24 hours for *C. albicans*, *C. tropicalis*, and *C. krusei*, and 48 hours for *C. glabrata*. Zone diameters were measured to the nearest millimeter using a calibrated scale and interpreted according to CLSI guidelines. Quality control was performed using *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 reference strains. The following antifungal agents were tested: Fluconazole (10 µg), Voriconazole (1µg), Itraconazole (10 µg), and Amphotericin B (100 units).^[15]

Ethical consideration: Institutional ethics clearance was obtained prior to the study from the institution review board (IEC no: 636/TSRMMCH&RC/ME-1/2019-IEC No: 016) dated on 17.7.2019. Patient

identifying details such as name and address was anonymized.

Statistical analysis: Data entry was done in MS Excel 2021 and analysis using SPSS (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp., United States). Continuous variable was expressed in mean and standard deviation while discrete data in frequency and percentage. The burden of fungal infection was expressed in proportion with 95% confidence interval.

RESULTS

A total of 1659 samples were examined during the study period of which 143 were tested positive for fungal growth i.e. 8.62% (95% CI: 7.26-9.97). The mean age of patients with CRD and having fungal isolates in their sputum was 69.7 ± 11.43 years with majority aged between 40 to 60 years and 81.8% were males.

Around 60.1% of the samples had chronic obstructive pulmonary disease (COPD) patients followed by chronic cough for evaluation (16.8%) and interstitial lung disease (9.1%). (Table 1)

Disease specific fungal species distribution is given in table 2. Overall, *candida species* was the most common fungal infection identified (i.e. 81.8%) followed by 16.1% *Aspergillus*, *Mucor* (1.4%) and *Rhizopus* (0.6%). The type and subtype of fungal infection based on the respiratory infection is given in Table 2.

Biofilm was positive among 30 samples of total 116 *candida species*. Of these *C. albicans* had the highest proportion of biofilm i.e. 40.0%. Other species *C. glabrata*, *C. tropicalis* and *C. krusei* had biofilm among 25%, 15.7% and 12.5% respectively. (Table 3)

Anti-fungal Amphotericin and Voriconazole had 100% sensitive for all the *candida species*. Fluconazole had an overall sensitivity of 67.2% with 100% resistance to *C. krusei*. Itraconazole was sensitive to all *candida species* which was least for *C. tropicalis* (65.8%) and maximum for *C. albicans* (84.0%). (Table 4)

Table 1: Demographic details and classification of CRD patients with fungal isolates (N=143)

Variable	Frequency	Percentage
Age		
20-39	3	2.1
40-59	14	9.8
60-79	102	71.3
≥80	24	16.7
Sex		
Male	117	81.8
Female	26	18.2
Diagnosis		
COPD	86	60.1
Asthma	8	5.6
Allergic rhinitis	1	0.7
Interstitial Lung Disease	13	9.1
Occupational lung disease	4	2.8
Chronic cough for evaluation	24	16.8
Chronic cough with pleural effusion	3	2.1
Dyspnoea for evaluation	4	2.8

Table 2: Fungal type and species seen among the different types of CRD (n=143)

Type of CRD	Fungal type and species	Frequency
COPD (n=86)	<i>A. flavus</i>	3
	<i>A. fumigatus</i>	14
	<i>A. niger</i>	2
	<i>C. albicans</i>	31
	<i>C. tropicalis</i>	19
	<i>C. krusei</i>	14
	<i>C. glabrata</i>	2
Asthma (n=8)	<i>Mucor</i>	1
	<i>C. albicans</i>	5
	<i>C. tropicalis</i>	2
Allergic rhinitis (n=1)	<i>C. krusei</i>	1
Interstitial Lung Disease (n=13)	<i>A. flavus</i>	1
	<i>A. fumigatus</i>	2
	<i>C. albicans</i>	4
	<i>C. tropicalis</i>	3
	<i>C. krusei</i>	2
	<i>Mucor</i>	1
Occupational lung disease (n=4)	<i>C. albicans</i>	1
	<i>C. tropicalis</i>	2
	<i>C. krusei</i>	1
Chronic cough for evaluation (n=24)	<i>C. albicans</i>	9
	<i>C. tropicalis</i>	7
	<i>C. krusei</i>	5
	<i>C. glabrata</i>	2
	<i>Rhizopus</i>	1
Chronic cough with pleural effusion (n=3)	<i>C. albicans</i>	1
	<i>C. tropicalis</i>	2
Dyspnoea for evaluation (n=4)	<i>C. tropicalis</i>	3
	<i>Aspergillus fumigatus</i>	1

Table 3: Presence of biofilm among the Candida species (n=116)

Candida species	Biofilm present (%)
<i>C. albicans</i> (n=50)	20 (40.0%)
<i>C. tropicalis</i> (n=38)	6 (15.7%)
<i>C. krusei</i> (n=24)	3 (12.5%)
<i>C. glabrata</i> (n=4)	1 (25.0%)

Table 4: Antifungal susceptibility among the Candida species (n=116)

Candida species	Amphotericin		Voriconazole		Fluconazole		Itraconazole	
	S	R	S	R	S	R	S	R
<i>C. albicans</i> (n=50)	50 (100%)	0 (0%)	50 (100%)	0 (0%)	44 (88.0%)	6 (12.0%)	42 (84.0%)	8 (16.0%)
<i>C. tropicalis</i> (n=38)	38 (100%)	0 (0%)	38 (100%)	0 (0%)	31 (81.6%)	7 (18.4%)	25 (65.8%)	13 (34.2%)
<i>C. krusei</i> (n=24)	24 (100%)	0 (0%)	24 (100%)	0 (0%)	0 (0%)	24 (100%)	18 (75.0%)	6 (25.0%)
<i>C. glabrata</i> (n=4)	4 (100%)	0 (0%)	4 (100%)	0 (0%)	3 (75.0%)	1 (25.0%)	3 (75.0%)	1 (25.0%)
Total (n=116)	116 (100%)	0 (0%)	116 (100%)	0 (0%)	78 (67.2%)	38 (32.7%)	88 (75.9%)	28 (24.1%)

S-Sensitive, R-Resistance

DISCUSSION

In the present study, a total of 1659 sputum samples of CRD patients were collected during the period of August 2019 to July 2021 in a tertiary care hospital. Among them, 143 i.e. 8.62% (95% CI: 7.26-9.97) were found to have fungal infection. CRD patients comprised of COPD, asthma, interstitial lung disease, occupational lung disease, chronic cough for evaluation and others. COPD patients comprised the highest proportion of 60.1% followed by chronic cough for evaluation (16.8%), interstitial lung disease (9.1%), asthma (5.6%), occupational lung disease, and dyspnoea for evaluation was 2.8% each.

Jahan et. Al,^[16] in their study also observed 31.5% of COPD to have fungal infected. Elderly patients with COPD have progressive decrease in their lung function due to poor ability in clearing the secretions. The damaged airway pathways make them more prone to repeated infection and making conditions favourable for fungal growth.^[17] (19 in 1) A case series by Johnson,^[18] (6) document 11 patients with history of chronic cough for more than 6 months to have Candida species growth in their sputum culture. The most common species were *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis* and *A. fumigatus* and *A. terreus*. Most of these patients had abnormal chest scan indicating bronchiectasis or atelectasis or

consolidation. Ten out of the 11 cases were taking chronic oral or inhaled steroid. Thus, the study emphasized the need for fungal culture for patients which chronic cough and not getting better with antibacterial.

Our study found more than 70% of CRD patient with fungal growth were aged more than 70 years. Sindhanai et. Al,^[19] also in their study also reported similar findings. Older age is more likely to develop fungal infection for several reasons such as weakened immunity and presence of co-morbidities that can collectively increase the chance of opportunistic infections.^[20]

Males are more prone to fungal infection than female in our study. Others studies by Khandelwal et. Al,^[21] and Sindhanai et. Al,^[19] also reported males to have a higher risk of fungal infection than females. This difference could be attributed to having outdoor occupation, higher burden of smoking, greater exposure to air pollution and mostly the underlying CRD which are predominantly seen among males.

Among the subspecies of fungal growth *Candida* was high (81.8%) with *Aspergillus* constituting 16.1% and others (*Mucor* and *Rhizopus*) less than 2%. Among the subspecies of *Candida*, *C. albicans* was the commonest i.e. 43.1% in our study, followed by *C. tropicalis* (32.7%), *C. krusei* (20.6%) and *C. glabrata* (3.4%). A study by Chiti et.al,^[22] also found similar results in their study with *C. albicans* (53%) was most common followed by *C. tropicalis* (27.8%), *C. glabrata* (13.9%) and *C. krusei* (5.2%). *C. albicans* are the known to cause mucosal and systemic infection. It is often isolated from respiratory tract of patients admitted in ICU or on intubation. The presence of *C. albicans* in the airway reflects colonization and is generally not considered as infection. Pneumonia due to the candida species is generally seen among immunocompromised patients or those with malignancies on treatment.^[23]

Amphotericin and Voriconazole had 100% sensitive for all the *candida species*. Fluconazole and Itraconazole had an overall sensitivity of 67.2% and 75.9% respectively. Chiti et.al,^[22] had also conducted a similar study and found only Voriconazole to have 100% sensitivity to *C. albicans* and *C. krusei* while other anti-fungal were sensitive to approximately 96 to 98% of samples. *C. tropicalis* had showed the maximum resistance ranging between 6.5% to Voriconazole and 25% to Amphotericin. The only antifungal that was sensitive to *C. tropicalis* was Caspofungin. The study concluded that *non-candida albicans* had higher resistance in comparison to *C. albicans* similar to our study.

Considering the findings of the present study, it is recommended that *Candida* isolation in hospitalized CRD patients should be closely and regularly checked, and that each patient's specific requirement for decolonization therapy also should be assessed. More significantly, research is needed to understand the mechanisms through which *Candida* interacts with other pathogens and the human immune system,

how this interaction results in CRD, and how new therapies might be developed.

CONCLUSION

Our study suggests the need for routine screening for fungal infection among CRD patients. Integration of fungal diagnostics of especially among elders with CRD may aid physician to distinguish between fungal superinfection and plan appropriate treatment. Healthcare professionals should be aware of the drug sensitivity of antifungal before its administration in the management of fungal infections among CRD patients.

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