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Research Article

A STUDY OF FUNGAL INFECTIONS IN CLINICALLY SUSPECTED TUBERCULOSIS PATIENTS

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ABSTRACT

Objectives: The purpose of the current study is to create awareness among the clinicians on the role of fungal etiological agents in suspected tuberculosis (TB) cases. This would help in preventing unnecessary use of anti-tubercular drugs and in decreasing drug-resistant TB cases.

Methods: Prospective study was performed in the clinical microbiology laboratory of Annapoorana Medical College and Hospital, Salem, Tamil Nadu, from January 2013 to February 2017 subsequently getting approval from the institutional ethics committee. 464 sputum samples were collected from both inpatients and outpatients of numerous departments. Signs and symptoms of clinically suspected TB patients were noted for all cases.

Statistical Analysis: Categorical variables were summarized by percentages (%). The Fishers exact test (2 × 2) analysis was done.

Results: A total of 119 fungal isolates were isolated from the culture. The highest number of fungal isolates were of *Candida* species (n=61) (30.5%) followed by *Cryptococcus neoformans* n=38 (19%) and *Histoplasma capsulatum* n=20 (10%). Amongst the used anti-fungals, Fluconazole was the most effective drug for all the isolated fungi followed by Itraconazole, Amphotericin B, Voriconazole, and Nystatin.

Conclusion: Our study findings indicate the significance of considering fungal infections as a prospect however treating disseminated granulomatous infections, even in immune-competent cases, particularly if the reaction to the TB therapy is insufficient.

Keywords: Respiratory tract infections, Fungal infections, Clinically suspected tuberculosis cases, Anti-tubercular drugs.

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INTRODUCTION

Respiratory tract Infections seem to be the most commonly described among social contagions, in that Lower Respiratory Tract Infections are reported to be with 90% [1]. Upper Respiratory Tract Infections are frequently produced by viruses than bacteria and fungi nevertheless lower respiratory infections were found to be universally triggered by bacteria and fewer by fungi and viruses. These disorders cause seven million demise per annum [2]. HIV epidemic was even exacerbated by morbidity and mortality because of lower respiratory infections reported with 70% of infections in AIDS cases [3]. To differentiate Tuberculosis (TB) from other lower respiratory infections such as bacterial pneumonia seems to be significant scientific task in emerging nations, and failure to distinguish TB from other lower respiratory infections may outcome in poorer health outcomes which may cause in high mortality rate [4]. TB is the further most dreaded well-being problem in emerging nations. The occurrence of bacterial co-infections along with the progress of anti-microbial resistance complicates the TB therapy progression [5]. Numerous studies carried out universal document that the potent pathogens of respiratory tract infections are *Streptococcus pneumoniae*, *Haemophilus influenzae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus* spp., *Moraxella catarrhalis*, *Streptococcus pyogenes* and some other enteric Gram-negative rods such as *Salmonella choleraesuis, Citrobacter koseri* [6,7]. Furthermost of these microbes are the usual flora of the human respiratory system. Thus, it is clear that utmost of the interval, the infection originated from normal flora and secondary infection from additional invader microbes [8]. The main objective of this study is to create awareness among clinicians on the role of fungal etiological agents in suspected TB cases. This would help in preventing unnecessary use of anti-tubercular drugs and in decreasing drug-resistant TB cases. Also to demonstrate acid-fast bacteria using direct Ziehl–Neelsen stain (AFB staining) in clinically suspected TB patients, to perform different concentration tests on clinically suspected direct ZN stain negative sputum samples, identify the concentration technique that can be comparable to culture, to isolate fungal organisms from the direct AFB (ZN staining), concentration and culture negative suspected cases of TB and to explore any association between isolated organisms with the baseline characteristics of clinically suspected TB patients.

METHODS

A prospective study was performed in the clinical microbiology laboratory of Annapoorana Medical College and Hospital, Salem, Tamil Nadu, from January 2013 to February 2017 after obtaining the institutional ethical committee clearance. 464 sputum samples were collected from both inpatients and outpatients of several departments. Unexplained weight loss, loss of appetite, night sweats, fever, and coughing for longer than 3 weeks, hemoptysis (coughing up blood), and Chest pain are the signs and symptoms of clinically suspected TB cases. Age, sex, weight loss, occupation, HIV status, cough for more than 2 weeks, chest X-ray findings, history of diabetes, hypertension, frequency of respiratory infections, antibiotic usage, and steroid usage information were collected.

Clinically suspected TB patients with a cough of more than 2 weeks were included in the study. Conditions such as identified smear-positive pulmonary TB cases, cases undergoing anti-TB therapy, HIV-positive cases, if the sample volume is inadequate and salivary specimen which is not representative of the lower respiratory tract were excluded from the study.

Clinically suspected TB patient's sputum samples were exposed to direct ZN stain. The AFB negative sputum samples were treated by concentration methods and were also cultured on Lowenstein-Jensen medium (LJ medium). After concentration, smears were studied under oil immersion for acid-fast bacilli by conventional ZN staining technique. The direct ZN stain negative, concentration negative, L.J culture-negative samples were cultured onto different media for the isolation of bacteria and fungi.

Direct ZN stain negative sputum samples were thoroughly mixed and divided into parts to perform different concentration methods namely N-acetyl-L-cysteine-NaOH (NALC) method, bleach method, bleach sedimentation, pot method/phenol ammonium sulfate basic fuchsin method, and C18-carboxypropylbetaine (CB-18) method/Zwitterionic Detergent.

Fungal culture

For fungal isolation, the sputum samples were homogenized, inoculated onto Sabouraud's dextrose agar with and without antibiotics and incubated at ambient temperature and at 37°C for 3 weeks before considering no growth. The Yeast Phosphate Agar (HiMedia) media were also used for the selective isolation of Histoplasma as it is difficult to isolate organisms from sputum. Antifungal susceptibility test was executed by disc diffusion technique 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 pharmaceuticals, Mumbai.

Statistical analysis

Categorical variables were summarized by percentages (%). The Fisher's exact test (2 × 2) analysis was done.

RESULTS

To rule out TB using direct Ziehl-Neelsen stain (AFB staining) in clinically suspected TB cases. A total of 464 clinically suspected TB cases were enrolled in the study. The sputum samples from the cases were collected and exposed to direct ZN stain for the detection of AFB. Out of n=464 microbiological sputum samples, n=185 were found to be positive for acid-fast bacilli by direct ZN staining method among which, males were n=103 and females were n=82 (Table 1).

The remaining n=279 samples were negative by direct staining technique among which males were n=159 and females were found to be n=120. The age group was between 50–70 years. The RNTCP grading, distribution of age, and sex with direct ZN stain positive and negative sputum samples were shown in (Tables 2-4). RNTCP grade was 3+ in 92 (49.7%) cases, 2+ in 26 (14.0%) cases, 1+ in 67 (36.3%) cases. The lowest age group was among 21–30 years and the highest age group was greater than 80 years in direct ZN stain-positive cases.

A significant proliferation in the percentage of AFB positivity was perceived after performing concentration procedures among direct ZN negative cases. Among the performed concentration procedures, CB-18 was observed to be more sensitive. A total of 69 samples were significantly positive for AFB after performing the concentration method by CB-18 out of 279 direct ZN-negative sputum samples (Tables 5 and 6).

In our study, when compared to NALC-NaOH method (n=48 [17.2%]), CB-18 (n=69 [24.7%]) had significantly increased the positivity out of 279 direct ZN stain negative sputum specimens.

The direct ZN stain AFB-negative sputum samples were subjected to the concentration method and were also cultured on LJ medium. LJ culture had increased the positivity from n=69 (24.7%) (Concentration method positive) to n=79 (28.3%) (Tables 7 and 8). Although our results showed more patients gave culture positivity for M. tuberculosis than smear positivity by CB-18 method, the difference was established to be statistically not significant. In concentration and LJ-negative patients, the predominant age group was between 60 and 80 years.

The age and sex distribution of concentration and LJ-negative patients were tabulated in Table 9.

Among the total number of direct AFB (ZN staining) negative, concentration and culture negative TB suspected cases (n=200), a total of n=81 bacterial and n=119 fungal isolates were obtained by culture.

A total of n=119 fungal isolates were isolated from the culture. The highest number of fungal isolates were of *Candida* species (n=61) (30.5%) followed by *C. neoformans* n=38 (19%) and *H. capsulatum* n=20 (10%).

Table 1: Direct ZN staining of sputum samples

Total No. of sputum samples	AFB+ve	AFB -ve
n=464	**n=185**	**n=279**
Percentage	41%	59%

Table 2: RNTCP grading in AFB-positive cases

RNTCP grade	No: of cases n=185 (%)
3+	92 (49.7)
2+	26 (14.0)
1+	67 (36.3)
Scanty	Nil

Table 3: Age and sex distribution of patients with Direct ZN stain positive (n=185)

Age (Years)	Male (55.7%) (n=103)	Female (44.3%) (n=82)	Total (n=185) (%)
21–30	19 (18.4)	13 (15.8)	32 (17.2)
31–40	14 (13.5)	11 (13.4)	25 (13.5)
41–50	27 (26.6)	19 (23.5)	46 (25)
51–60	22 (21.3)	17 (20.7)	39 (21)
61–70	11 (10.6)	8 (9.7)	19 (10.3)
71–80	8 (7.7)	10 (12.1)	18 (9.7)
>80	2 (1.9)	4 (4.8)	6 (3.3)
Total	103	82	185

Table 4: Age and sex distribution of patients with Direct ZN stain negative (n=279)

Age (Years)	Male (57%) (n=159)	Female (43%) (n=120)	Total (n=279) (%)
21–30	10 (6.3)	19 (16)	29 (10.4)
31–40	19 (12)	10 (8.3)	29 (10.4)
41–50	21 (13.2)	21 (17.5)	42 (15.2)
51–60	32 (20.1)	20 (16.5)	52 (18.6)
61–70	37 (23.2)	30 (25)	67 (24)
71–80	29 (18.2)	13 (11)	42 (15)
>80	11 (7)	7 (5.7)	18 (6.4)
Total	159	120	279

Table 5: Concentration methods in AFB-negative samples

Direct ZN stain-VE sputum samples	Concentration methods				
	CB-18	NALC	Bleach	Bleach sediment	POT
n=279	***n=69**	***n=48**	***n=44**	***n=10**	***n=11**
% of positivity	24.7	17.2	15.7	3.5	3.9

*n-indicates number of direct ZN stain negative sputum samples showing acid-fast bacilli after the concentration methods

Table 6: Comparison between direct AFB staining and concentration method CB-18 using Fisher's exact test

Types of AFB staining (n=279)	Positive	Negative
Direct AFB	0	279
Concentration	69	210
p-value	<0.01**	

**p value of<0.01 is considered as highly significant

The results of different fungal species and their antifungal drug resistance patterns are tabulated in Table 10.

The antifungal sensitivity of histoplasma species was shown in Table 11. According to the findings of our study drug sensitivity pattern of *H. capsulatum* isolates were as follows: Amphotercin 85%, flucanozole 65%, itraconzole 58%, voriconozole 56%, and nystatin 45%.Out of the used antifungals flucanozole, itraconzole, and amphotercin were effective for the isolates. In the current study, we found that amongst the used anti-fungals, fluconazole was the most effective drug for all the isolated fungi followed by itraconazole, amphotericin b, voriconazole, and nystatin.

Table 7: Comparison of positivity between CB-18 concentration method and LJ culture

Direct ZN stain negative	C18-carboxy propyl betaine positivity for acid-fast bacilli	LJ culture positive
n=279	n=69	n=79
% of positivity	24.7	28.3

Table 8: Comparison between concentration methods and LJ culture using fisher exact test

Methods	Positive	Negative
Concentration	69	210
LJ method	79	200
p-value	>0.05 (Not significant)	

*p value of<0.05 is considered as highly significant

Table 9: Age and sex distribution of concentration and LJ-negative patients (n=200)

Age (Years)	Male (61.5%) (n=123) (%)	Female (38.5%) (n=77) (%)	Total (n=200)(%)
21–30	5 (4.0)	3 (3.8)	8 (4.0)
31–40	11 (8.9)	8 (10.3)	19 (9.5)
41–50	20 (16.2)	14 (18.1)	34 (17.0)
51–60	21 (17.0)	14 (18.1)	35 (17.5)
61–70	29 (23.5)	17 (22)	46 (23)
71–80	27 (21.9)	17 (22)	44 (22)
>80	10 (8.13)	4 (5.1)	14 (7.0)
Total	123	77	200

Table 10: Fungal sps isolated from direct AFB negative, concentration and LJ culture-negative sputum samples

Fungal isolates (n=119)		
***Candida* sps**	***Cryptococcus neoformans***	***Histoplasma capsulatum***
61	38	20
30.5%	19%	10%

DISCUSSION

Drug resistance in TB is rapidly becoming a universal problem and now threatens to overcome progress achieved in TB control. The estimated global annual occurrence of multidrug-resistant TB is almost 4,40,000 cases [9]. Pulmonary histoplasmosis symptoms are comparable to TB infection, presentation with upper respiratory symptoms of cough and fever with connected lymphadenopathy are frequently established as TB. Even a biopsy would give the results of chronic granulomatous inflammation. Appropriate cultures and fungal stains should be done to establish the correct etiology instead of the wrong diagnosis [10]. Antibiotic use without confirming the causative agent may lead to the wrong treatment and might contribute to the progress of drug resistance. With the aid of culture and sensitivity, antibiotic treatment for respiratory infections can be treated more precisely and effectively. The incidence, diagnosis, and clinical severity of pulmonary fungal infections have dramatically increased in recent years in response to a number of factors [11].

In the current study, we have employed 5 different concentration procedures to find out their sensitivity. NALC-NaOH method has been reported as the most widely investigated method to increase the sensitivity modestly compared to direct smear microscopy [12]. Our results were also comparable with the earlier published sensitivities [13]. CB-18 method is based on the use of zwitterionic detergent CB-18 which it acts by alternating the buoyant density of mycobacteria to improve the collection efficiency during centrifugation and also scattering of those mycobacteria that will cord [13]. In our study, NALC-NaOH processing technique significantly yielded 48 (17.2%) AFB positives out of 279 direct ZN negative sputum samples. Ganoza *et al*. also reported the same results which in accordance with our report, showed a significant increase in the sensitivity of AFB smears from 28.6 % (direct ZN stain) to 66.7 % by NALC-NaOH method [14]. Castro *et al*. reported the similar results after examination of NALC-NaOH concentrated sputum when compared with direct smear [15].

Tripathi *et al*. also reported a significant proliferation in positivity of n=24 (16.32%) among 147 direct ZN negative cases after digestion and decontamination by NALC-NaOH method and diagnosed patients received early therapy [16].

In the present study, we observed an yield of 48 (17.2%) and 44 (15.7%) positives out of 279 direct ZN stain negative sputum specimens using NALC-NaOH decontamination and Bleach method respectively. The bleach method was almost analogous to NALC-NaOH processing technique. This finding recommends that the Bleach technique may be used as a substitute of NALC-NaOH processing technique as it is easy and inexpensive technique and does not necessitate any professional to process the sputum. Our observations are comparable to the study reported by Ongkhammy *et al*. in that they havecomparedBleach technique with standard ZN stain and observed a proliferation in smear positivity rate of 33.5% [17]. Similarly, Mindolli *et al*. used 3 specimens from each case, a statistically significant increase in the positivity was perceived with bleach method (n=84) compared to direct ZN stain (n=25) among n=255 specimens [18]. An increase of sputum positivity

Table 11: Antifungal drug resistance pattern of Fungal isolates (n=119)

Anti-fungals	***Candida* sps n=61**						***Histoplasma capsulatum* n=20**		***Cryptococcus neoformans* n=38**	
	***Candida albicans* (n=48) (79%)**		***Candida tropicalis* (n=8) (13%)**		***Candida dublinensis* (n=5) (8%)**					
	S%	**R%**	**S%**	**R%**	**S%**	**R%**	**S%**	**R%**	**S%**	**R%**
Amphotericin B	60	40	60	40	60	40	92	8	85	15
Fluconazole	83	17	83	17	83	17	86	14	65	35
Itraconazole	80	20	80	20	80	20	82	18	58	42
Voriconazole	78	22	78	22	78	22	72	28	56	44
Nystatin	70	30	70	30	70	30	73	27	45	55

by 13% Ängeby *et al*. 15.1% by Matu *et al*., 42.7% by Muhammad *et al*. with bleach method studies respectively [19-21].

In the present study, we have observed that the Bleach sediment decontamination yielded n=10 (3.5%) AFB positive out of 279 direct ZN stain-negative sputum specimens which was not much significant compared to the Bleach centrifugation. This may be because of the lack of centrifugation process. However, other reports by Yassin *et al*. [22] and Bonnet *et al*. [23] have stated that bleach sedimentation had significantly increased the positivity from 17.5% to 26% and 21.7%.

In our study, an outcome of n=11 (3.9%) positives out of 279 direct ZN stain-negative sputum specimens was observed and comparable to the bleach sedimentation method. However, other studies had not found any significant difference in the sensitivity of the two methods [24]. In another study, by comparing NALC and Phenol Ammonium Sulphate methods, the authors found smear quality equally good in both methods; however, the cell count was significantly higher in the PhAS than in the NALC method [25].

In the current study, we found CB-18 as the most sensitive method which had given a higher positivity of 69 (24.7%), followed by NALC-NAOH48 (17.2%), Bleach 44 (15.7%), Pot/Phenol Ammonium Sulfate 11 (3.9%), Bleach sedimentation 10 (3.5%) respectively from 279 Direct ZN stain negative specimens. In our study, we have isolated comparatively more fungal isolates n=119 than bacteria n=81 from the sputum culture. In the present study, we have isolated n=61 (30.5%), *Candida* species from clinically suspected TB patients. Among the isolated *Candida* species, *Candida albicans* (n=48) (79%) predominantly isolated which was followed by *Candida tropicalis* (n=8) (13%) and finally *Candida dublinensis* (n=5) (8%). Our study findings are in agreement with other studies by Yehia and Abdullah, 2012 in which they compared immunocompromised with immunocompetent patients. The authors have isolated a significantly higher percentage of *Candida* isolates n=72 (35.5%) in immune-competent patients [26].

Nevertheless, invasive lung infection by *Candida* species is rare in nonimmunocompromised subjects. The criterion for the diagnosis of pulmonary candidiasis is still controversial [27]. Another similar study isolated 12.2% of *Candida* species from the cases of lower respiratory tract infection. They had stated the *Candida* species is the third most common pathogen isolated from patients with lower respiratory tract infection [28]. In our study, the high number of *Candida* species isolates might be attributed to chrome agar, since this media is essentially very useful to isolate the organisms and differentiate the species as well. Our findings suggest that *Candida* isolates from patient needs to be clinically correlated to rule out the uncertainty. In some cases, it may be better to request repeat cultures of clinical samples to deliver unequivocal evidence of Candidal infection.

Our study isolated n=38 *C. neoformans* from clinically suspected TB patients n=200. Pulmonary cryptococcal infection occurs in both immunosuppressed and immunocompetent populations. The identification of Cryptococcus depends on the presence of a capsule, growth on BHI blood agar at 28°C, and positive urease test. The demonstration of encapsulated yeast cells by nigrosine in sputum specimens should be considered significant for Cryptococcus. Cryptococcus was primarily considered an opportunistic pathogen and is recently being reported as a cause of severe infection in immunocompetent population as well [29].

Pulmonary cryptococcosis is an underdiagnosed entity due to its highly variable clinical presentation [30]. Pulmonary cryptococcosis is frequently reported to be misdiagnosed as pulmonary TB. In an autopsy series n=589 out of n=8421 South African miners who had pulmonary cryptococcosis were misdiagnosed as pulmonary TB [31]. Yehia and Abdullah. isolated Cryptococcus from sputum of lower respiratory tract infection among both immunocompromised and immunocompetent patients [26]. In countries such as India where TB is more prevalent, to diagnose TB purely based upon radiological features might be difficult because of resembling image findings. The most common patterns observed in immunocompetent population are multiple pulmonary nodules or masses with consolidation and lymphadenopathy [32]. A research by Jarvis in a young female with HIV was misdiagnosed as smear-negative pulmonary TB with fatal consequences instead of pulmonary cryptococcosis. This case report stresses the need for appropriate clinical studies and guidelines for the diagnosis of cryptococcosis in a TB endemic country like India [33].

Infection with *H. capsulatum* frequently occurs by the inhalation of micro conidia by the host which gets deposited in the alveoli of the lungs and gets rapidly converted into a parasitic yeast form in tissues. This propagation and conversion can occur before (or) after ingestion by pulmonary macrophages [34]. Histoplasmosis can involve every organ system during the course of dissemination. Clinically pneumonia occurs in those with exposure to a large number of infected spores. Resolution of pneumonia often leaves calcified pulmonary nodules, calcified mediastinal lymph nodes. Chronic respiratory diseases caused by Histoplasma manifest the symptoms, months or years after the primary infection which mimics TB [35]. A case report by George *et al*. in a 70 years old south-Indian lady who was apparently normal and immune competent had stated that the pulmonary histoplasmosis mimics the carcinoma of the lung [35]. Our findings are in agreement with other similar studies by Qureshi *et al*., Yehia and Abdullah, where Histoplasma was isolated in patient with clinically suspected TB cases [10,26].

CONCLUSION

The direct AFB-positive cases revealed that both males and females were affected, 40–60 years. The RNTCP grading had shown that almost 50% of direct AFB positive cases were graded as 3+ followed by 1+ and 2+. CB-18 is more sensitive and almost comparable to LJ culture than other methods. The isolated fungal (*Candida*, *Cryptococcus*, *Histoplasma*) species revealed that these infections might mimic TB by clinical symptoms. Follow-up studies are important to explore the role of etiological agents in diseases and the effectiveness of specific drugs thereby in achieving cure of the suspected TB cases. The major risk factor interpretations for the organisms isolated in our study were as follows: *Candida* – Diabetics with antibiotic exposure >4 times/year, *Cryptococcus*-Poultry workers independent of diabetes, *Histoplasma*-Diabetic poultry workers. Our study findings indicate the importance of considering fungal infections as a possibility while treating disseminated granulomatous infections, even in immune-competent patients, especially if response to the TB treatment is inadequate.

LIMITATIONS

More accurate identification of specific risk factors for fungal infections should open new possibilities to revisit the TB diagnosis, especially in a resource-limited setups where sputum microscopy and radiography are major diagnostic tests for TB. The current study does not direct whether the TB suspected cases had certain lower respiratory tract infections such as pneumonia. This study does not include the follow-up.

AUTHORSHIP CONTRIBUTIONS

Dr. G. Amar Kumar and Dr. J. Visalasree were involved in the literature search, design and data collection, data analysis along with interpretation, manuscript writing and editing and final submission. Both authors read and approved the final manuscript.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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