Original Article

Spectrum of Pulmonary Fungal Pathogens, Associated Risk Factors, and Anti-fungal Susceptibility Pattern among Persons with Presumptive Tuberculosis at Gombe, Nigeria

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Abstract

Background: Pulmonary mycosis (PM) poses a great diagnostic challenge due to the lack of pathognomonic and radiological features, especially in the absence of mycology laboratory tests. This study was aimed to isolate, phenotypically identify, determine the prevalence of pulmonary fungal pathogens and antifungal susceptibility pattern of isolates of presumptive tuberculosis (PTB) patients attending Federal Teaching Hospital (FTH) Gombe, Nigeria. **Methods:** After ethical approval, three consecutive early morning sputa were collected from 216 participants with presumptive of PTB attending FTH Gombe, between May 2, 2017 and May 30, 2018. Samples were processed using standard mycological staining, microscopy, sugar biochemistry, and antifungal susceptibility test protocols. Sociodemographic variables and risk factors of pulmonary fungal infection were assessed through structured questionnaires. Pulmonary fungal infection was defined by the positive culture in at least two sputa. PTB was defined by Genexpert® nested polymerase chain reaction. **Results:** Of the 216 participants, 19.9% had PTB and 73.6% had pulmonary fungal pathogens. Among the isolated pulmonary fungal pathogens, Aspergillus fumigatus made the highest occurrence, while 6.5% had PTB-fungal co-infection. No significant association existed between the prevalence of PM with age and sex of participants ($P > 0.05$). Cigarette smoking (adjusted odds ratio [aOR] = 15.9 [95% confidence interval (CI): 0.9–268.8]), prolong antibiotic use (aOR = 77.9 [95% CI: 4.7–1283]) and possession of domestic pet (aOR = 77.9 [95% CI: 4.7–1283]) were significant risk factors of PM ($P < 0.05$). *Penicillium citrinum*, *Mucor* spp. and *Aspergillus flavus* are more susceptible to voriconazole, and *Candida albicans* was found to be more susceptible to Nystatin. Of the 159 fungal isolates, 92.5% were resistant to fluconazole. **Conclusion:** Findings from this study revealed high level pulmonary fungal pathogens, especially among PTB patients. A majority of fungal isolates were resistant to fluconazole. It's recommended that persons should do away with or minimize risk factors for pulmonary fungal pathogens identified in this study.

Keywords: Antifungal resistance, fungal pathogens; pulmonary infection, tuberculosis

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Introduction

Pulmonary mycosis (PM) is an infectious process in the lungs caused by one or more opportunistic fungi.[1] Fungal colonization and infection occur following the inhalation of spores or by the reactivation of a latent infection.[1] Hematogenous dissemination frequently occurs, especially in an immunocompromised host.[2] Mycosis is due to endemic and opportunistic fungi in immunocompetent and immunodeficient persons, respectively.[2] Case mortality associated with PM

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can be as high as 90% in immunodeficient persons. Although, people who are immunocompetent generally respond well to antifungal drugs.[3] Fungal infections in lung often pose a great diagnostic challenge due to the absence of characteristic pathognomonic and radiological features. In Nigeria and most sub-Saharan Africa countries, PM is further compounded by co-infections with pulmonary tuberculosis and inadequate diagnostic mycology services.[4]

In the last decade, mycosis became a significant healthcare concern due to the rampant use of broad-spectrum antimicrobials, prolong use of immunosuppressive drugs, increased cases of critically ill, and immunodeficient persons such as HIV/AIDS.[5]

Pulmonary fungal pathogens are associated with 58%–81% of morbidity and mortality among people living with HIV.[6] Pulmonary fungal infected individuals present with features similar to those of tuberculosis (presumptive tuberculosis [PTB]), and this makes both diagnosis and management difficult.[7] Even though the prevention of pulmonary mycoses would offer a seemingly better approach in high-risk population, this is complicated by the virtue that fungi are ubiquitous, which propagates their rapid spread. This could constitute misdiagnosis and delayed therapeutic intervention, leading to poor prognosis. In addition, fungal pathogens cases treated with anti-tuberculosis chemotherapy will have poor clinical outcomes, as these drugs have no effect on pulmonary fungal pathogens.[8]

Persistent cough for more than three weeks is a common symptom of pulmonary disorders caused by a wide range of pathogens, including Fungi, Bacteria, Parasites, and even viruses. Although active PM may denote advanced immunosuppression, it may also act as a co-factor in facilitating clinical tuberculosis.[9]

Pulmonary fungal pathogens are life-threatening infections in the majority of immunocompromised patients, and the diagnosis and treatment of pulmonary mycoses are not included in the management of people with PTB and such studies have not hitherto hence, the need to carry out this study.[4] In view of the clinical burden of pulmonary fungal pathogens, this study sought to isolate, phenotypically characterize and determine the prevalence of pulmonary mycotic agents and determine the antifungal susceptibility pattern of fungi from patients presumptive of pulmonary Tuberculosis attending Federal Teaching Hospital (FTH) Gombe, Nigeria.

Methods

Study area

The FTHG is located within the city of Gombe, the capital of Gombe state. It is one of the 36 states of the Federal Republic of Nigeria. It is located in the center of North-East of the country. It has a population of about 2,857,042 and an area of 20,265 km^2. The temperature averages 30°C with an annual rainfall of 1200 mm. The predominant occupations of its people are agriculture and livestock rearing.

Ethical consideration

Ethical clearance was obtained from the ethical and human research committee of FTHG. Informed consent was obtained from all participating participants in accordance with the standard of human experimentation and with the Helsinki Declaration of 1975, as revised in 2000. This was done through informed consent forms duly completed by all participants.

Study design

This was a hospital-based, cross-sectional study conducted between May 2, 2017 and May 30, 2018.

Sample size and population

The minimum sample size of this study was calculated using the Fischer expression for cross-sectional studies. Using a previous prevalence of 13.5% pulmonary fungal infection,[10] a minimum sample size of 179 was calculated. However, the sample size was increased to 216 participants. Sputum samples were collected from 216 persons with presumptive PTB.

Participants' selection criteria

Individuals who present with clinical symptoms of pulmonary tuberculosis who were HIV seronegative, no history of diabetes mellitus, and other immunosuppressive diseases were enrolled. Apparently healthy individuals without any clinical symptoms of pulmonary tuberculosis or who with HIV seropositive results, or those with a history of diabetes mellitus and other immunosuppressive diseases were excluded.

Study participants

Participants were enrolled the general outpatients' clinics of FTHG and referred to the Department of Medical Microbiology and Immunology of FTHG for the microscopic examination of sputum samples for acid-fast bacilli were recruited into the study. Signed informed consent was obtained from each patient before sample collection. All participants aged between 15 and 50 years were screened, and their HIV status was confirmed by the World Health Organization HIV testing algorithm. In addition, they were all tested for Mycobacterium tuberculosis infection using GeneXpert® nested polymerase chain reaction equipment.

Samples collection

Three consecutive early morning sputum samples were obtained from the participants in a sterile plain wide neck and leak-proof container for fungal analysis.

Data collection, informed consent, and ethical consideration

The study was explained to the enrolled participants, and they gave their written informed consent. Family relatives gave approval for the participants who do not have formal education. A structured questionnaire was used to obtain sociodemographic variables from these participants in accordance with the Declaration of Helsinki. Parents/guardians filled questionnaires on behalf of their children. Those who did not consent to participate were excluded from this study. Ethical approval (NHREC/25/10/2013) was obtained

from the Ethical Research Committee of the FTH, Gombe State, Nigeria. Data generated were anonymously analyzed throughout the study.

Laboratory analysis

Microscopic examination

Potassium hydroxide mount, Gram staining for detection of yeast and fungi, and Giemsa staining for detection of cyst and trophozoite of Pneumocystis jirovecii as previously described by Ochei and Kolhatkar.[11]

Isolation of fungal species

Isolation of molds

The sputum samples were cultured on sabouraud dextrose agar (SDA) containing Chloramphenicol and incubated at ambient temperature and 37°C for 7–14 days as described by Baker *et al*.[12] A loopful of sputum was stricken on the surface of the sterile SDA using a sterile wire loop as employed by John.[13]

Isolation of yeast

The sputum samples were cultured on SDA containing Chloramphenicol (Art. No. 06-118 CASE) and incubated at ambient temperature and at 37°C for 7–14 days as described by Baker *et al*.[12] A loopful of sputum was striken on the surface of the sterile medium using a sterile wire loop as employed by John.[13]

Characterization of isolates

The fungal isolates were characterized using standard mycological procedures; these include colony morphology, physiological, and biochemical test. Molds were identified based on colony morphology, i.e., rate of growth, surface texture, and pigmentation. The yeast-like colonies were identified based on germ tube production, chlamydospores formation on cornmeal agar, sugar fermentation tests, and temperature studies using malt extract agar.[11]

Characterization of yeasts isolates on cornmeal agar (Dalmau plate technique)

This involves the use of cornmeal agar with tween 80 to differentiate yeast by their structural differences in terms of production of pseudohyphae, blastochonidia, and chlamydospores on microscopic examination.[8] Morphological features such as hyphae, pseudohyphae, blastospores, ascospores, chlamydospores, basidiospores, or sporangia were noted.

Sugar assimilation and fermentation test

Six sugars (glucose, maltose, sucrose, lactose, xylose, and inositol) were utilized for these purposes. Production of gas in the tube was taken as fermentation positive.

Temperature for isolation and growth

This method determines the ability of *Candida* spp. to grow at an elevated temperature. *Candida albicans* and *Candida tropicalis* were stricken on the surface of malt extract slant were inoculated and incubated at 37°C and 25°C. The tubes were examined every day for up to 7 days for the presence of growth**;** Growth in both tubes indicates that the *Candida* spp. has the ability to grow at 37°C.[11]

Molds were identified based on their colony morphology (macroscopic features) such as surface topography, surface texture, pigmentation macro- and micromorphology (microscopic features).[11]

Anti-fungal susceptibility testing

Agar based disc diffusion method was used Clinical and Laboratory Standards Institute.

Inoculum preparation

Sterile swab dipped into sterile tween 80 was used to pick the pure colony of yeast. This was then suspended in 3–4 ml of sterile normal saline and vortexed. The turbidity was of the suspension was adjusted to 0.5 Mcfarland standard. Similarly, the inoculum was prepared for mold by swabbing the pure colony and then suspended in 3–4 ml of sterile normal saline and vortexed. The turbidity was of the suspension was adjusted to 0.5 McFarland standard.[14] Mueller Hinton agar plate was prepared. Modified (as per CLSI for antifungal susceptibility tests). Commercially prepared disc (voriconazole 1 mcg, nystatin 100 units, fluconazole 25 mcg) were aseptically applied onto the agar plate. The disc was deposited at least 24 mm apart. The plates were incubated at 37°C and examined after 24 h of incubation, zone diameter of each drug was interpreted through criteria published by the clinical and laboratory standard institute.[14]

Statistical analysis

In the analysis of results, the data obtained were entered in Microsoft Excel sheet, and analysis was conducted by using the Statistical Package for the Social Sciences (SPSS) software (IBM, California, USA) version 20.0. Chi-square was used to determine the association between two categorical variables. Values of $P \leq 0.05$ at 95% confidence intervals were considered statistically significant.

Results

Prevalence of pulmonary fungal isolates

In this study, we reported 73.6% prevalence of pulmonary fungal pathogens, of which *Aspergillus fumigatus* made the highest occurrence [Table 1]. The prevalence of pulmonary tuberculosis was 19.9% in the study population. The prevalence of PTB-fungal co-infection was 6.5% of participants [Figure 1].

Distribution of fungi according to the gender of the patients

Out of the 216 sputum samples collected in the study, males had relatively higher prevalence of fungal isolates, 63.5% than females counterpart, 36.5% [Table 2]. There was no significant association between sex and the prevalence of fungal pathogens ($P = 0.553$).

Distribution of fungi according to the age of the patients

Out of the 216 sputum samples collected in the study, the age group 30–38 years recorded the highest pulmonary fungal pathogens, 44 (27.7%), whereas the age group 12–20

recorded the lowest prevalence of pulmonary fungal pathogens, 16 (10.1%) [Table 3]. There was no significant association between age and prevalence of pulmonary fungal pathogens ($P = 0.986$).

Distribution of fungal isolates by risk factors of pulmonary infections

Based on the risk factors considered in the study, participants with prolonged antibiotic use, cigarette smoking, PTB, and possession of household pets had a significantly higher risk of pulmonary fungal pathogens ($P < 0.05$) [Table 4].

In vitro antifungal susceptibility pattern of pulmonary fungal isolates

Three fungal species *Penicillium citrinum*, *Mucor* spp. and *Aspegillus flavus* are more susceptible to voriconazole and *C. albicans* was found to be more susceptible to Nystatin. A majority of the fungal isolates were resistant to fluconazole [Table 5].

Table 1: Prevalence of pulmonary fungal isolates in the study population (n=159)

Fungal isolates	Frequency (%)
Aspergillus fumigates	69 (43.4)
Candida albicans	28 (17.6)
Aspergillus niger	26 (16.4)
Candida tropicalis	15 (9.4)
Candida guilliermondi	8 (5.0)
Penicillium citrinum	5 (3.1)
Penicillium glabrum	3 (1.9)
Mucor spp.	3 (1.9)
Aspergillus flavus	2 (1.3)
Total	159 (100)

Table 2: Sex distribution of respiratory fungal pathogens

Sex	Number of participants tested	Participants with fungal pathogens, *n* (%)	χ^2	*P*
Male	134	101 (63.5)	0.564	0.553
Female	82	58 (36.5)		
Total	216	159 (100)		

Table 3: Distribution of fungi according to the age of participants

Age (years)	Number of participants studied	*n* (%)	χ^2	*P*
≤20	21	16 (10.1)	0.653	0.986
21-29	39	28 (17.6)		
30-38	59	44 (27.7)		
39-47	37	26 (16.4)		
48-56	23	18 (11.3)		
>56	37	27 (16.9)		
Total	216	159		

Discussion

Findings from this study revealed a prevalence of pulmonary tuberculosis as 19.9%, pulmonary fungal pathogens as 74%, while the prevalence of PTB-fungal pathogen coinfection was 6%. The reason for increased fungal pathogen prevalence could be due to immunosuppression due to tuberculosis and the prolonged use of anti-TB drugs, which promote the overgrowth of the fungus flora, and in turn, aggravate the course of the underlying process in the lung infection.

A. fumigatus was the most most prevalent fungal pathogen, 43.4%. This is similar to a report by Nasir *et al*. and Mwaura and Elizabeth.[4,15] This could have been due to spores of *Aspergillus spp* in the atmosphere, their ability to grow in abundance everywhere, production of small conidia that easily penetrate deep in the alveoli region and grows at 37°C.[16]

A similar report was made by Ved *et al*.[17] However, Chandwani *et al*.[18] reported that *A. niger* was the most common species isolated in their study. These variations could be attributed to variations in geographical locations and ecological niches. *A. fumigatus* has been regarded as the most invasive Aspergillus species due to the high frequency of clinical manifestations (cough, chest pain, fever, hemoptysis, weight loss, and dyspnea) associated with this pathogen as observed in the participants enrolled in this study.

C. albicans is the second most prevalent isolate in this study and common in fungal co-infection with pulmonary tuberculosis with a prevalence rate of 17.6% these opportunistic fungi are potential pathogen in the immunocompromised individuals, patients with some preexisting disease and patients with a long history of antibiotics usage.[3]

The prevalence of *fungal* infection of lung ranges between 12.7 and 36% in different studies, which reported *C. albicans* been the most predominant isolate from the sputum of tuberculosis patients.[2,4,9,19] These variations in percentages are mainly attributed to differences in the local prevalence of different species due to different environmental conditions, as well as to the various detection methods employed.

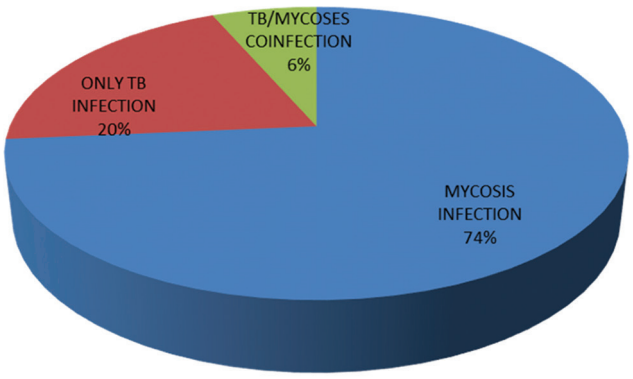

Figure 1: Prevalence of tuberculosis, fungal pathogens and co-infection among study participants

Table 4: Risk factors of pulmonary fungal pathogens

Characteristics	Number of persons tested	Number of positive (%)	aOR (95% CI)	*P*
Presence of PTB				
Yes	43	14 (32.6)	0.1 (0.05-0.21)	<0.0001*
No	173	143 (82.7)		
Total	216	159 (73.6)		
Prolong antibiotic use (≥3 weeks)				
Yes	60	60 (100)	77.9 (4.7-1283)	0.002*
No	156	95 (60.9)		
Total	216	159 (73.6)		
Possession of pets				
Yes	33	33 (100)	30.5 (1.8-505.7)	0.017*
No	183	126 (68.9)		
Total	216	159 (73.6)		
Cigarette smoking				
Yes	19	19 (100)	15.9 (0.9-268.8)	0.05*
No	197	140 (71.1)		
Total	160	159 (73.6)		

PTB: Pulmonary tuberculosis, aOR: Adjusted odds ratio, CI: Confidence interval

Table 5: *In vitro* antifungal susceptibility pattern of pulmonary fungal isolates

Fungal isolates	Antifungal agents					
	Fluconazole (25 mcg)		Voriconazole (1 mcg)		Nystatin (100 mcg)	
	Sensitive, *n* (%)	Resistant, *n* (%)	Sensitive, *n* (%)	Resistant, *n* (%)	Sensitive, *n* (%)	Resistant, *n* (%)
Aspergillus fumigatus	0 (0.0)	69 (100)	30 (43.5)	39 (56.5)	-	-
Candida albicans	0 (0.0)	28 (100)	2 (7.1)	26 (92.9)	17 (60.7)	11 (39.3)
Mucor spp	0 (0.0)	3 (100)	2 (66.7)	1 (33.3)	-	-
Penicillium citrinum	0 (0.0)	5 (100)	4 (80.0)	1 (20.0)	-	-
Candida guilliermondi	0 (0.0)	8 (100)	1 (12.5)	7 (87.5)	3 (37.5)	5 (62.5)
Penicillium glabrum	0 (0.0)	3 (100)	0 (0.0)	3 (100)	-	-
Aspergillus niger	2 (7.7)	24 (92.3)	8 (30.8)	18 (69.2)	-	-
Candida tropicalis	1 (6.7)	14 (93.3)	1 (6.7)	14 (93.3)	2 (13.3)	6 (86.7)
Aspergillus flavus	0 (0.0)	2 (100)	1 (50.0)	1 (50.0)	-	-

Zone diameter in mm. Fluconazole=>19=Susceptible, <14 mm=Resistant. Voriconazole=29-39 mm=Sensitive, <29 mm=Resistant. Nystatin=15-21 mm=Sensitive, <15 mm=Resistant

From the study, the distribution of fungal isolates varies with respect to the gender of the patients. Among the patients with positive fungal isolates, Males were found to be 101 (63.5%) and females 58 (36.5%). According to Hidalgo and Vazquez, colonization rates of *Candida* species are equal in males and females. In another study, fungal pathogens were significantly higher among female patients compared to male patients.[20] The relatively higher colonization rates in male could have been responsible for increased risk among male patients because they mostly engage in outdoor activities that may predispose them to inhalation of fungal spores and conidia.

Participants with pulmonary fungal pathogens were mostly those ≥40 years compared to patients within lower age groups. Indeed, old age is a known risk factor for pulmonary fungal infection probably due to wane immune function as one gets aged. This disagrees with studies conducted by Ogba *et al*.[19] and Ofonime *et al*.[21] who reported age group 20–34 years to be mostly affected by fungal pathogens.

The distribution of fungal isolates on the basis of some risk factors was assessed. Participants who had prolonged antibiotic usage were found to be more vulnerable to have a significantly higher prevalence of pulmonary fungal pathogens ($P < 0.05$). This finding is supported by findings of Spader and Catherine,[22] who reported that that fungal colonization and infections appeared to be related to the prolonged use of broad-spectrum antibiotics.

Cigarette smoking, possession of pets and contact with PTB-infected persons were also risk factors of contacting pulmonary fungal pathogens.[23] Cigarette smoking is one of the major risk factors for the development of most lungs diseases which include pulmonary mycosis which facilitates airway epithelium dysfunction.[24] In our study, participants with a history of cigarette smoking had significantly higher cases and the odds ratio of pulmonary fungal pathogens. This indicates cigarette smoking to be risk factor for pulmonary fungal colonization and infection.[25]

Findings from this study revealed that persons who had domestic pets had higher cases of pulmonary fungal infection than those who do not. Mycotic agents are in most domestic animals and birds discharging a wide range of airborne fungal spores and yeast when these animals sneeze, yawn or cough. This could be detrimental when these animals live in close vicinity to immunocompromised persons. Various disease manifestations such as localized fungal infections to fatal disseminated diseases, as well as allergic responses to inhaled conidia have been attributed to animal-to-man transmission of fungal agents.[26]

In this study, voriconazole and Nystatin had the highest antifungal action, where voriconazole has the best activity against the isolates, nystatin was the second-most effective antifungal drugs. It is believed that these drugs should be considered as the first choice of treatment of pulmonary mycoses. In this study, fluconazole showed the lowest antifungal activity on the isolates tested. Most fungal isolates were resistant to fluconazole. These findings are consistent with the findings of Siakwa *et al*. where they reported similar widespread antifungal resistance to fluconazole but good anti-fungal susceptibility to voriconazole and nystatin.[23] They also noted that fluconazole and nystatin faced significant resistance from the fungal isolates. This may be because fluconazole being a triazole, has some components of SDA that interfere with its antifungal activity.[23] This antifungal resistance could also be due to prolonged singular use of fluconazole for most fungal infections in the study area because of they the readily available antifungal drugs in our setting.

Conclusion

A high prevalence of pulmonary fungal pathogens (74%) was obtained in patients with presumptive pulmonary tuberculosis in this study. Of this, *A. fumigatus* and *C. albicans* were the predominant isolates. This study also demonstrated voriconazole as the best antimycotic agents against fungal isolates. Based on these findings, its recommended that fungal pathogens be investigated in all the presumptive PTB cases to avoid misdiagnosed and unnecessary use of antibacterial chemotherapy.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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