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Respiratory Tract Aspergillosis in the Sputum of Patients Suspected of Tuberculosis in Fako Division-Cameroon

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Abstract Respiratory tract aspergillosis refers to fungi infections of the respiratory tract caused by *Aspergillus species*. Respiratory tract aspergillosis has clinical and radiological characteristics which are very similar to tuberculosis thereby making the disease easily misdiagnosed and mistreated as tuberculosis. This prompted us to investigate the prevalence of respiratory tract *Aspergillus sp.* in the sputum of patients suspected of pulmonary tuberculosis and to study the anti-fungal susceptibility of the isolated *Aspergillus* strains. Two hundred sputa samples were studied for *Aspergillus sp. and M. tuberculosis*. Direct microscopy and fungal culture was done on two sets of Sabouraud Dextrose agar. Analysis for Acid-Fast Bacilli (AFB) was done by the Auramine-phenol fluorochrome technique. *Aspergillus sp* were isolated from 30(15%) patients; A. *fumigatus* was isolated in 10 (5%) patients while *A. niger, A. flavus,* and *A. terreus* were isolated from 9 (4.5%), 6 (3%) and 5 (2.5%) patients respectively. *M. tuberculosis* was found in 27(13.5%) and a co-infection of 9(4.5%) was observed.Using the broth micro dilution technique, the minimum inhibitory concentrations (MICs) for *Aspergillus sp* for nystatin, itraconazole and amphotericin B ranged between 0.12- >16 µg/ml, 0.06- >16 µg/ml and 0.12- 0.5 µg/ml, respectively. All the *Aspergillus terreus* strains were consistently sensitive to itraconazole (MIC >16 µg/ml).

Keywords Aspergillosis, Tuberculosis, Diagnosis, Prevalence, Sensitivity Test

1. Introduction

Opportunistic respiratory mycoses have a cosmopolitan distribution. They comprise a large group of fungal diseases, the etiologic agents of which are usually potential pathogens in the immune-compromised or debilitated patients. Species of *Candida* and *Aspergillus* are classical examples of opportunistic pathogenic fungi. Data on worldwide incidence and prevalence of respiratory mycoses is fragmentary.

Mycobacterium tuberculosis (MTB), the causative agent of pulmonary tuberculosis is one of the major micro organisms that infect this site. It is a small aerobic non-motile bacillus which according to Jasmer *et al.,* (2002)[1] infects one third of the world's population. The proportion of people who become sick with tuberculosis each year is stable or falling worldwide but, because of population growth, the absolute number of new cases is still increasing[2].

One cannot over-emphasize the point that the incidence and prevalence of respiratory fungal infections in Cameroon and many other developing countries has remained largely unexplored and neglected[3]. This has led to a widespread erroneous impression about their true public health importance. Given its devastating effects like other respiratory infections, this neglect has to be seen as a major call for concern. As reported by the Centers for Disease Control and Prevention (CDC), the mortality associated with Invasive Respiratory Aspergillosis (IRA) has increased by 35.7% since 1980 with the mortality of untreated IRA being nearly 100%[4].

Furthermore in Fako, Cameroon, little or no work has been done on pulmonary aspergillosis. Cameroon has an enlarging population of patients with AIDS and various immune-deficiencies. Since opportunistic mycoses (aspergillosis) are a serious threat to such patients, it is anticipated that these infections may break out in epidemic proportions under suitable circumstances. It is therefore for these above reasons that this study was instigated with main objective to investigate the prevalence of respiratory tract *Aspergillus sp.* in sputum of patients suspected of Tuberculosis in Fako Division - Cameroon.

2. Materials and Methods

2.1. Study Area

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The study was carried out in three major towns (Limbe, Buea, and Tiko) within Fako division in the South West region of Cameroon. The climate is equatorial, hot and humid, with rainfalls ranging from 2.500 mm to 10,000 mm. Temperature ranges from 22°C to 29°C with a mean temperature of 25°C.

2.2. Study Design and Duration

The study was a cross-sectional one. Specimen collection and analysis was carried out for three months (22th November 2010 to 26th February 2011) with analysis done at the MAFLEKUMEN Higher Institute for Health Sciences Teaching and Research Laboratory in Tiko.

2.3. Study Subjects

Participants who presented with pulmonary symptoms analogous to infection with *Mycobacterium tuberculosis* such as bronchiectasis, recurrent infections (with fever and malaise, dyspnoea, anorexia weight loss, and chest pain) and whose chest radiograph revealed features similar to tuberculosis were recruited into the study. Informed consent was obtained from each participant prior to specimen collection.

2.4. Specimen Collection

Two hundred (200) participants were recruited into the study. The participants were from three (3) major centers in Fako division as follows; 100 from Limbe Regional Hospital (LRH), 75 from the CDC Central and Cottage Hospital Tiko (TCC) and 25 from Buea Regional Hospital Annex (BRA).

None of the participant in this study had been placed on antifungal therapy. 3 successive morning samples were collected into sterile wide neck universal sputum mugs from males, females, and children ranging from the age group of 8 to 80 years who presented with symptoms similar to tuberculosis. These patients were appropriately instructed to collect each sputum sample prior to mouth washing.

2.5. Specimen Processing

2.5.1. AFB analysis

Specimens for acid fast bacilli (AFB) analysis were analyzed on the same day of collection using the Auramine-phenol fluorochrome staining technique for AFB[5]. Prior to this staining method the sodium hypochlorite technique was used to concentrate the bacilli[6]. The presence of white-yellow rods glowing against a dark background was reported as AFB positive.

2.5.2. Mycological analysis

Direct microscopy: With the use of a Pasteur's pipette few drops of 10% potassium hydroxide were placed on the centre of a clean glass slide and using a sterile wire loop this was mixed with a portion of the sputum. The preparation was flattened under a cover slip and examined with magnification x40 objective for the presence of hyphal fragments.

Culture: Irrespective of the outcome of the direct sputum microscopy, all samples were cultured given that the full characterization of mycotic agents is achieved through culture.

A heavy loop full of each specimen was transferred in to 5ml of mycological peptone water and later incubated aerobically for 24hrs at 27˚C prior to inoculation on pre-dried Sabouraud dextrose agar (SDA) plates, the mixture was re-suspended. Inoculation was done by seeding at the center of the plate using sterile wire loop after which the plates were incubated aerobically at 27°C for 48Hrs, incubation continued for 21days at room temperature. The *Aspergillus* species isolated in the culture were identified as per standard methods[7].

Cultural Identification: After appropriate incubation, the growth form, rate of growth-surface and reversed coloration on Sabouraud agar plates were noted. Pure isolates were obtained by sub-culturing on new plates and colonies growing out of the inoculation area were regarded as contaminants.

Microscopy: Needle mount staining technique was performed .The *Aspergillus* species were identified with the various morphological features associated with the characteristic sporing head according to Collier *et al.,* (1998)[8] while yeasts were identified by their smell, pseudomycellia and budding cells. Further investigations included gram stain, Indian ink stain, germ tube test, urease test, chlamidospore formation, Fermentation reaction and Sugar assimilation.

2.6. Antifungal Sensitivity Test

Antifungal susceptibility to amphotericin B, itraconazole and nystatin was tested, and Minimum Inhibitory Concentrations (MICs) were determined for each fungal isolate.

The broth micro-dilution method[9] was used to test each isolate against two fold serial dilutions of antifungal agents (0.03 to 16 mg/liter). This method was adopted from the proposed reference method described by the National Committee for Clinical and Laboratory Standards (NCCLS) for in vitro susceptibility testing of yeasts[10]. MIC was defined as 75% inhibition after 24 h of incubation of *Aspergillus sp.* MIC 50 and 90%, MICs at which 50 and 90% of the isolates studied, respectively, were inhibited.

2.7. Ethical Consideration

This study was conducted with the ethical approval of the University of Buea, the authorities of the Limbe Provincial Hospital, the Buea Regional Hospital Annex, the Tiko C.D.C. Central and Cottage Hospitals. Informed written consent was obtained from each study participant and all personal information about the participants was treated as confidential.

3. Results

A total of 200 fresh sputa samples were collected from three different sources; Limbe Regional Hospital (LRH), CDC Central and Cottage Hospital Tiko (TCC) and Buea Regional Hospital Annex (BRA). These samples were ex-

amined for the presence of *Mycobacterium tuberculosis* and *Aspergillus species*.

3.1. Study Population Characteristics

In this study, out of the three sample sources, 50% of the participants came from Limbe. The ages of participants ranged from 7-80years with a mean age of 34years, the female population (54.5%) was higher than the male population (44.5%) (Table 1).

Table 1. Age and sex distribution of patients

Age group (years)	Total	Total	Total sampled
	Male	Female	
0-10	2	6	8(4%)
11-20	10	11	21(10.5%)
21-30	24	44	68(34%)
31-40	27	22	49(24.5%)
41-50	17	13	30(15%)
51-60	5	3	8(4%)
61-70	4	8	12(6%)
71-80	2	2	4(2%)
TOTAL	91	109	200

3.2. Detection of *Aspergillus sp* by Direct Microscopy and Culture

Aspergillus species was found in 27(11.5%) of the 200 samples by direct sputum microscopy and 30(15%) of the 200 samples by culture. All 27(100%) samples that were positive for *Aspergillus sp* by direct sputum microscopy were found to be positive with culture. Examination by direct microscopy failed to detect 3 samples which were later found to be positive with culture. Direct microscopy is therefore 90% sensitive in detecting *Aspergillus sp* in sputum smears.

3.3. Prevalence of *Aspergillus sp.* and Other Fungal Isolates

The 30 (15%) of *Aspergillus* species comprised of 10(5%) *Aspergillus fumigatus*; 09(4.5%) *Aspergillus niger;* 06(03%) *Aspergillus flavus* and 5(2.5%) *Aspergillus terreus*. Other fungal species recorded included; *Penicillium species* 3(1.5%) and *Histoplasma species* 1(0.5%). Nine species of yeast organisms were equally isolated. Among these were *Candida species* 55 (27.5%) comprising of 12 (6%) *C. albicans,* 21 (10.5%) *C. tropicalis,* 15 (7.5%) *C. krusei,* 3 (1.5%) *C. guilliermondii,* 2 (1%) *C. pseudotropicalis,* 1(0.5%) *C. parapsilosis and* 1(0.5%) *C. stellatoidea.* Others included *Cryptococcus species* 6(3%) and *Torulopsis species* 1 (0.5%).

3.4. Prevalence of *Aspergillus sp.* According to Gender and Age

30(15%; 95% CI: 10.1≤15.0≤20.0) of the 200 participants were infected with *Aspergillus sp*. There was no significant difference between the rate of infection in males and females (P = 1.0000), with the highest prevalence found in the age group 61 to 70 years (41.67%), while none of those under ten years of age were infected (Table 2).

3.5. Prevalence of Mycobacterium Tuberculosis

A prevalence of 13.5% (95% CI: 8.8≤13.5≤18.0) was observed with no statistically significant difference in the infection among males and females (P = 0.5452). The highest incidence 9(33.33%) was found in patients aged 31-40 years (Table 3).

Table 2. Prevalence (%) of *Aspergillus sp* by Age and Gender

Age group (years)	Total Sampled	Male (N=91)	Female (N=109)	Total
0-10	8	-	-	0(0.00)
11-20	21	-	2	2(9.52)
21-30	68	3	11	14(20.59)
31-40	49	2	2	4(8.16)
41-50	30	3	1	4(13.33)
51-60	8	-	-	0 (0.00)
61-70	12	5	-	5(41.67)
71-80	4	1	-	1(25)
TOTAL	200	14(15.4)	16(14.7)	30(15.00)

Table 3. Prevalence of *M. tuberculosis* by age and gender

Age group (years)	№ of Samples collected	Males (N=91)	Females (N=109)	Total (%)
0-10	8	-	-	0(00.0)
11-20	21	2	1	3(14.3)
21-30	68	4	5	9(13.2)
31-40	49	4	4	8(16.3)
41-50	30	3	2	5(16.7)
51-60	8	1	-	1(12.5)
61-70	12	-	-	0(00.0)
71-80	4	-	1	1(25.0)
TOTAL	200	14(15.4%)	13(11.9%)	27(13.5%)

3.5. Co-infection of Aspergillus Species - Mycobacterium Tuberculosis

There were 9(4.5%) patients infected with both infections. Among the 9 patients who were infected with *Aspergillus sp* and TB, 6 were infected with *A. fumigatus*, 2 with *A. niger* and 1 with *A. flavus*. None of the patients was infected with *A. terreus.*

3.6. Antifungal Sensitivity

Mean Inhibition Concentrations (MICs) (μg/ml) for nystatin, itraconazole and amphotericin B ranged between 0.12- >16 μg/ml, 0.06 - >16 μg/ml and 0.12- 0.5 μg/ml, respectively. All the *Aspergillus terreus* strains were consistently resistant to Amphotericin B (MIC >16 μg/ml) (Table 4).

4. Discussion

Pulmonary Aspergillosis is fairly common in Cameroon and could be seen as an important emerging disease. However, its diagnosis is usually missed due to lack of pathognomonic clinical features and paucity of diagnostic myco-

logical laboratories[11].

Preliminary detection of *Aspergillus sp* was done by the direct examination of sputum before culturing. The results obtained by direct microscopy, 27(13.5%), were comparable to those obtained by culture, 30 (15%). All samples that were positive with direct microscopy were positive with culture as well. This therefore implies that direct microscopy is very sensitive (90%) in the detection of *Aspergillus Sp* in sputum samples. This is an indication that hospitals in Cameroon should not only rely on mycology research institutions to diagnose *Aspergillus* infections in patients who present with symptoms. Diagnosis of this infection can be done by a microbiologist using a simple light microscope with results available within a short time and the result will be similar to that of culture.

Table 4. In vitro susceptibilities of 30 isolates of aspergillus species to Amphotericin B, Itraconazole and Nystatin

Organism, (no. tested)	Antifungal agent	MIC µg/ml		
		Range	50%	90%
A. fumigatus (10)				
	Amphotericin B	0.12-0.25	0.25	0.12
	Itraconazole	0.06->16	0.12	0.25
	Nystatin	0.12->16	>16	0.12
A. niger(9)				
	Amphotericin B	0.12-0.25	0.12	0.25
	Itraconazole	0.06->16	0.25	0.5
	Nystatin	0.12->16	>16	0.12
A. flavus(6)				
	Amphotericin B	0.25-0.50	0.5	0.25
	Itraconazole	0.06->16	0.06	0.12
	Nystatin	0.12->16	0.12	0.25
A. terreus(5)				
	Amphotericin B	8->16	>16	>16
	Itraconazole	0.06->16	0.06	0.06
	Nystatin	0.12->16	0.25	>16

The prevalence of *Aspergillus spp* in the sputum of patients suspected of pulmonary tuberculosis was 15% (95% CI, 10.1 – 20.0). Our findings relating to the prevalence is in accordance with previous published report by Shahid *et al.,* (2007)[12], however, it is much lower than the prevalence reported by Kurhade *et al.* (2002)[13] and Bakare *et al.,* (2003)[14]. No difference was observed in the infection of *Aspergillus species* in males and females (P=0.9048). It was also observed that infection with *Aspergillus sp* was more common in the elderly, 61-70 years of age (41.67%). This finding could be explained by the diminishing immune response to infection with *Aspergillus sp*. The predominant *Aspergillus species* isolated from sputum in the present study was *Aspergillus fumigatus* (5%) and correlates well with results of other similar studies[12, 13, 15]. The frequency of isolation of *A. flavus 6* (3%) in the present study is lower than those obtained by Shahid and Malik, (2002)[7] and Razmpa *et al.,* (2007)[15] who got a prevalence as high as 30% for *A. flavus*. This wide variation in the incidence and frequency of isolation of various *Aspergillus* species colonization may be due to geographical differences[16].

A lower prevalence of *Aspergillus fumigatus* 10(5%) compared to *Candida albicans* 12(6%) was observed in this study. This might be because the occurrence of spores in the lung and sputum samples appears to be affected by their size, as suggested by Mullins and Seaton, (1978)[17]. Thus, *A. fumigatus* with smaller spores may be present more frequently in the lung than in samples of respiratory secretions; however, *C. albicans* occurs with similar frequencies in the lung and sputum samples[17].

A tuberculosis prevalence of 13.5% (95% CI, 8.8 – 18.0) was observed in the study population, which is comparable to the prevalence of infection with *Aspergillus sp* (15%). 9(4.5%) of the patients were positive for both pulmonary *M. tuberculosis* and *Aspergillus sp*. This established relationship was also observed by Sahoo *et al.,* (1988)[18] and a possible justification for this is the fact that tuberculosis remains the most important cause of sub-acute and chronic respiratory morbidity which most often leaves behind a scarred pulmonary parenchyma vulnerable to fungal colonization. Therefore tuberculosis of the lung can be seen as a predisposing factor for colonizing aspergillosis (in cases with aspergilloma)[19].

Itraconazole was found to be very active against *Aspergillus* species (MIC at which 90% of the isolates were inhibited, 0.06 to >16 µg/ml), whereas Amphotericin B was less effective (MIC90%, 0.12 to>16 µg/ml). This finding is in accordance with the work done by Hennequin *et al.,* (1997)[20] and Oakley *et al.,* (1999)[21]. While all isolates of *Aspergillus terreus* were resistant to amphotericin B with a MIC of >16 µg/ml. MICs for nystatin ranged from 0.12 to 16 µg/ml, contrary to the observation of Oakley *et al.,* (1999)[21].

5. Conclusions

In conclusion aspergillosis commonly due to infection with *Aspergillus fumigatus* is common in individuals suspected of pulmonary tuberculosis in Fako Division. This therefore undermines the importance of including aspergillosis as one of the diagnostic criteria in suspected TB infection as well as in cases of post-tuberculosis complications. Aspergillosis is more common in the elderly above 60years which can be accounted for by their diminishing immunity and therefore presents one of the most common fungal infection in this group. Once diagnosed, itraconazole should be the treatment of choice since it was observed to be very effective against all *Aspergillus* isolates.

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