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Research Article

Diversity of Respiratory Yeasts from Suspected Pulmonary Tuberculosis Patients

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Abstract: The synergistic growth promoting association of Candida and Mycobacterium has raised increased concern for studying the various *Candida species* and its significance in pulmonary tuberculosis patients. This study was designed to document the prevalence of TB associated with respiratory candida infections in Dambatta Kano, Nigeria. The study included induced sputum samples from 300 patients with complaints of symptoms suggestive of TB infections. The TB was diagnosed by sputum Ziehl – Neelsen staining technique. *Candida species* were identified using microscopy following growth on SDA medium, germ tube formation, Dalmau Plate Technique (morphology on corn meal agar with tween 80) and specific colour appearance on Chromogenic Candida agar. Of the 300 sputum samples examined, 28(9.3%) were positive to AFB microscopy while 111(37%) yeast mainly belonging to the genus candida. *Candida krusei* was the most common isolates observed in 36(12%) of the patients, followed by *Candida albicans* 28(9.3%), then followed by *Candida tropicalis* 22(7.3%) and *Candida glabrata* with the prevalence of 14(4.7%). The least prevalence of 1(0.3%) each was observed in the genera Geotrichum and Rhodotorula. Mixed infections of *C. tropicalis* and *C. krusei*; *C. krusei* and *C. albicans* with the prevalence of 6(2.0%) and 3(1.0%) were observed. Candida co – infection with the TB was 7(2.3%) in male samples and 4(1.3%) in female samples (P = 0.009359). The prevalence of non – albicans species is increasing possibly due to their apparently greater capacity than *C. albicans* to invade deep tissues of immunocompromised host and inadequate response to anti – tuberculosis drugs.
Keywords: Candida, Geotrichum, Rhodotorula, Tuberculosis, Infection, Dambatta.

INTRODUCTION

Fungi are eukaryotic microorganisms more closely related to humans than bacteria at cellular level [1]. Most species grow as multicellular filaments called hyphae – forming mycelium such as molds; some species also grow as single cells like yeasts [2]. The infections caused by opportunistic fungi are included under new spectrum of fungal pathogens. Such fungi were earlier reported from various plants as pathogens, but now they are known to cause disease in human beings [3]. Fungal infections have dramatically increased in the past two decades as a result of improved diagnostics, high frequency of catheterization, instrumentation and an increasing number of immunosuppressed patients such with Tuberculosis and HIV [4]. The morbidity and mortality rates caused by fungal species such as candida species are relatively higher [5]. In some studies, candidal infections were found to be more prevalent in male tuberculous patients as compared to females. This might be attributable to more exposure of male to external environment and their habit of using some addictive substances [6].

Pulmonary tuberculosis is contagious bacterial infection caused by *Mycobacterium tuberculosis*. The lungs are primarily involved, but the infection also occurs in other organs. Tuberculosis is caused by a group of organisms; infected patients are immunocompromised patients hence leading to mycotic infection of the lungs [7].

Nigeria was listed among the 22 highly burden countries in the world with prevalence rate of 171 per 100,000 population [8]. In 2011, there were an estimated 8.7 million incident cases of TB (range, 8.3 million – 9.0 million) globally, equivalent to 125 cases per 100 000 population (WHO, 2012). Most of the estimated number of cases in 2011 occurred in Asia (59%) and Africa (26%); smaller proportions of cases occurred in the Eastern Mediterranean Region (7.7%), the European Region (4.3%) and the Region of the Americas (3%) [8]. Therefore the objectives of this

study are to determine the prevalence rate of yeast infections in suspected TB patients and demographic characteristics in relation to systemic mycoses.

MATERIALS AND METHODS

Study Site

The site for the current study is Pathology unit, Dambatta General Hospital located at Dambatta Local Government Area, of Kano State, Nigeria. It is located between latitude 12º 25′ 59″ N and Longitude 8º 30′ 55″ E [9].

Study Population

The study population includes patients of all ages and sexes enrolled in Directly Observed Treatment Short course Chemotherapy (DOTS) and patients with suspected pulmonary tuberculosis disease under National Tuberculosis and Leprosy Control Programme (NTBLCP).

Sample Size

This study was carried out between the months of April, 2013 to April, 2014. A total of 300 samples were collected from the subjects who completed consent form and questionnaire. The sample size was calculated according to Henderson and Sundareshy (1982) [10], as follows:

$$n = Z^2pq/ L^2$$

Where n = sample size, Z = Standard normal distribution at 95% confidence interval = 1.96, p = Prevalence in similar work; q = 1 – p; L = Allowable error, taken as 5% = 0.05

Therefore, in this study P =10.59% [11], local prevalence rate of systemic agents.

$$= \frac{(1.96)^2 \times 0.1059 \times (1 - 0.1059)}{(0.05)^2}$$

$$n = 145.4 \approx 145 \text{ Samples}$$

However, in order to improve on the quality of the work, the sample size was doubled and 300 samples were collected.

Inclusion Criteria

All patients suspected of Tuberculosis who presented the symptoms of the pulmonary disease, attending the DOTS Clinic and referred to the laboratory for AFB microscopy were enrolled in the study.

Ethical Clearance/Consent Form/Questionnaire

Ethical approval was obtained from Kano State Hospital Management Board's Ethical Committee, Nigeria. Consent form was given to each participant to sign indicating intention to participate in the research. Questionnaire was administered seeking information on demographic characteristics and other possible risk factors to the disease.

Sample Collection

A total of three hundred (300) TB suspects who consented and completed questionnaire were sampled. Early morning sputum samples were collected for TB diagnosis in the laboratory with the assistance of experienced Medical laboratory scientists. The sputum was expectorated from lower respiratory tract and collected in sterile screw capped containers to avoid contamination from external sources in the following order as described by Brooks *et al.* [12].

Collection Procedure

Patients were asked to produce the samples in an open air space away from other people to avoid aerosol spread. The patients were instructed to inhale deeply 3 to 4 times before coughing out from the chest. The sputum produced was carefully spit into the container without contaminating the outside of the container. The lid of the container was screwed tightly before being processed, with utmost care not wrapping the container with the laboratory request form [13].

Direct mount (Wet Mount)

Direct mount was used to determine the size and shape of yeasts cells and conidia of the mould incriminating agents. It also served in observing the presence of other structures such as pseudohyphae, chlamydospores, nucleated yeast cells, blastoconidia, macroconidia, microconidia, etc using lactophenol cotton blue (LPCB) [14].

Cultural Method of Isolation

Sputum specimens were cultured according to the procedure of John [14]. This was achieved by streaking 0.01 cm^3, using sterile inoculation loop on to the surfaces of SDA containing streptomycin (50mg/ cm^3) and Corn meal agar. Duplicate cultures of SDA and Corn Meal agar media were incubated at room temperature as well as at 37^0C for 24 – 48 hours.

Germ Tube Test

A small portion of 18 – 72 hours old isolated colony of the yeast in 0.5cm^3 of human serum was suspended in the test tube. The above procedure was repeated with a known culture of *Candida albicans* ATCC ® 10231 (Positive control) and *Candida krusei* ATCC ® 6258 (Negative control). The tubes were incubated at 37º C for 3 hours. A drop of the yeast suspension was placed on the glass slide on covered with a clean cover slip. Controls were first read and then the test for the presence or absence of germ tubes under the microscope using × 40 objective [14].

Interpretation

Presence of Germ Tube: Indicated Positive result
Absence of Germ Tube: Indicated Negative result

Dalmau Plate Technique

A small portion of the yeast colony was streaked using sterile inoculating wire to make three parallel cuts

½ apart into the agar by holding the wire at an angle of about 45º. A sterile cover slip was placed on the surface of the agar to cover the inoculation streaks and subsequently incubated at room temperature for 24 – 48 hours. The plate was examined directly through the cover slip under the microscope by removing the lid using × 40 Objective. The above procedure was repeated with a known culture of *Candida albicans* ATCC ® 10231 and *Candida krusei* ATCC ® 6258 as Positive controls while *Escherichia coli* ATCC ® 25922 as Negative control [14].

Inoculation on Chromogenic Candida agar

The agar was first dried after being stored in the refrigerator using hot air oven and was brought to room temperature before inoculation onto the surface of the plate and incubated at 37º C for 48 hours.

Interpretation

Candida albicans: Green Colour
Candida krusei: Pink – Brown Colour
Candida tropicalis: Dark Blue Colour
Candida glabrata: White – Mauve Colour

Staining Procedure

A new clean and unscratched slide was used and proper laboratory serial number was given. Sputum was spread on the slide using clean applicator and allowed to air dry for 15 – 30 minutes. The smear slide was passed over a flame for 3 – 5 times for 3 seconds each time. Carbolfuchsin was added to cover the entire slide and was heated with carbolfuchsin on it until vapour rises. It was allowed to stay for 5 minutes. The slide was gently rinsed with tap water until all free carbolfuchsin stain is washed away. Acid alcohol was applied for 1 – 3 minutes after the smear dried, it was rinsed gently. The slide was then rinsed gently with tap water and tilted to drain off the water. Methylene Blue was added on the slide and kept for 30 seconds. It was then rinsed carefully with tap water and allowed to dry. The slide was examined under the microscope using Oil Immersion Objective. The result obtained was based on graded guide lines of WHO Report, 2007 [15].

Interpretation

Acid – fast cells: Reddish – purple Colour
Non – acid-fast cells: Blue Colour

In the case of culture for mould and yeasts, positive results of mycological examinations were accepted only if the direct examination was positive and if the two parallel media (SDA and Corn Meal Agar) culture growths of the same fungus specimen were observed and subsequently confirmed by microscopy and Chromogenic Agar in the case of yeast. Cases failing these criteria were regarded as negative and were not included in the study.

Statistical Analysis

The data generated in this study was analyzed for statistical significant difference in the association between demographical characteristics (i.e. age, sex), using Pearson Chi – Square with the aid of Open Epi version 2.2.1.

RESULTS

Out of 300 sputum samples of Tuberculosis suspects involved in the study, 111(37%) yields yeasts mainly from the genus Candida with Geotrichum and Rhodotorula each occurred only once 1(0.3%) (Table 1).

Table 1: Occurrence of Clinical Yeast Isolates based on Age (n=300)

Age Group (Years)	*C. albicans*	*C. krusei*	*C. tropicalis*	*C. glabrata*	*Geotrichum spp*	*Rhodotorula spp*
0 – 5	0	0	0	0	0	0
6 – 10	0	0	0	0	0	0
11 – 15	1	1	2	1	0	0
16 – 20	8	3	2	2	1	0
21 – 25	0	4	3	1	0	0
26 – 30	7	7	2	3	0	0
31 – 35	0	5	3	2	0	0
36 – 40	3	1	3	0	0	0
41 – 45	2	1	1	1	0	0
46 – 50	2	4	2	0	0	1
51 – 55	2	1	0	2	0	0
56 – 60	1	4	1	1	0	0
61 – 65	0	1	1	0	0	0
66 – 70	1	3	2	0	0	0
71 – 75	0	0	0	0	0	0
76 – 80	1	1	0	1	0	0
Total/Percentage	28(9.3%)	36(12%)	22(7.3%)	14(4.7%)	1(0.3%)	1(0.3%)

Yeast infection occurred with 57(19%) males while females had 54(18%) (Table 3). The age specific prevalence of yeast showed that the age group 26 – 30 had the highest prevalence of 19(6.3%). The least prevalence of 2(0.6%) occurred within the age group of 61 – 65 (Table 1). Furthermore, 9(3.0%) of the patients were found to have mixed infections of candida species with the highest prevalence of 3 within the age group 26 – 30 (Table 2). The total prevalence rate of the tuberculosis infection was 28(9.3%) (Table 3). The sex specific prevalence showed that males had the highest TB infection of 22(7.3%) than females with 6(2.0%). With the respect to co – infection based on sex, it was observed that there was co – infection between AFB and yeast infections with total prevalence rate of 11 with 7(63.6%) and 4(36.4%) for males and females respectively. The relation between AFB and yeast was found to be statistically significant with the p – value < 0.01 (0.009359) (Table 3).

Table 2: Mixed growth of *Candida species* based on Age (n=300)

Age Group (Years)	*C. krusei* and *C. tropicalis*	*C. albicans* and *C. krusei*
0 – 5	0	0
6 – 10	0	0
11 – 15	0	0
16 – 20	0	0
21 – 25	1	0
26 – 30	1	2
31 – 35	1	0
36 – 40	1	0
41 – 45	1	0
46 – 50	0	1
51 – 55	0	0
56 – 60	0	0
61 – 65	0	0
66 – 70	1	0
71 – 75	0	0
76 – 80	0	0
Total	6(2.0%)	3(1.0%)

Table 3: Occurrence of Acid FastBacilli, Yeasts and their co – infections based on Sex

Sex (n=300)	AFB	Yeast	AFB and Yeast
Males	22(7.3%)	57(19%)	7(2.3%)
Females	6(2.0%)	54(18%)	4(1.3%)
Total	28(9.3%)	111(37%)	11(3.6%)
P – value	0.009359		
Chi Square (X^2)	6.753		

Key: AFB: Acid Fast Bacilli

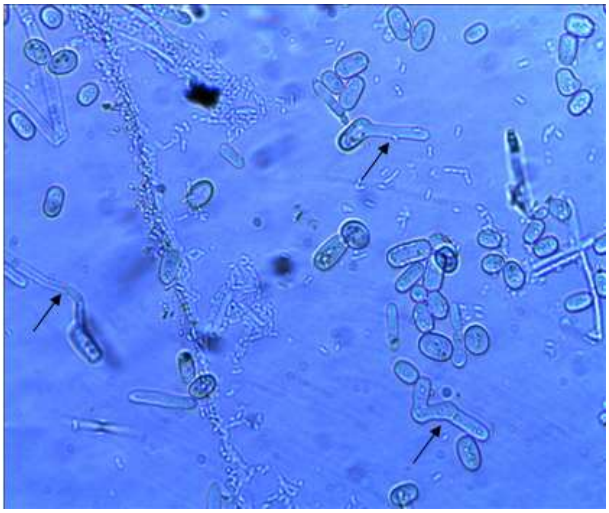

Fig-1: *Candida albicans:* When incubated at 37°C in 0.5 ml of serum for 3 hours, C .albicans rapidly forms elongated hyphae called germ tubes.

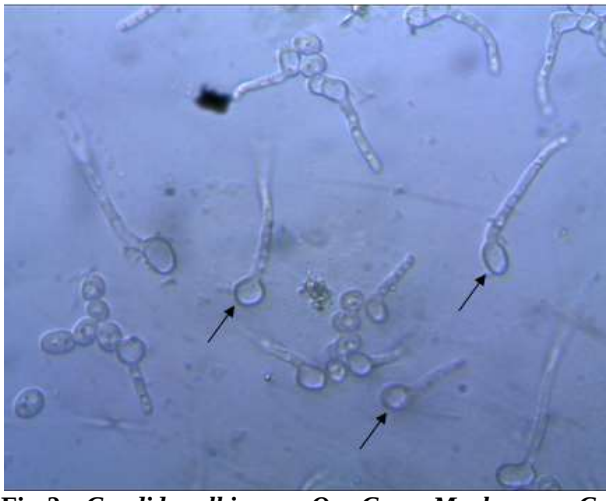

Fig-2: *Candida albicans: On Corn Meal agar, C. albicans forms thick-walled chlamydoconidia, which differentiate it from other* Candida species.

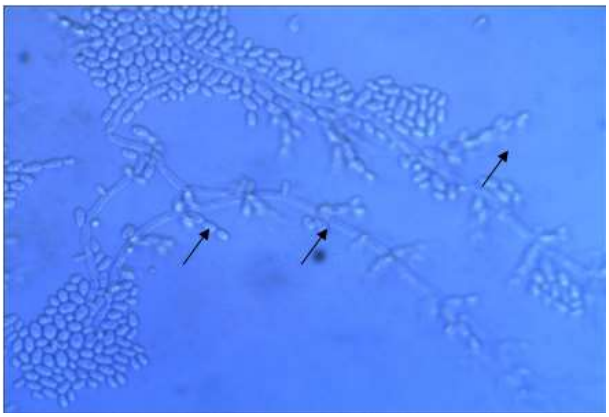

Fig-3:*Candida krusei* grown on Corn Meal Agar showing blastoconidia and pseudohyphae incubated at 24ºC using Dalmau Plate Technique using × 40 objective.

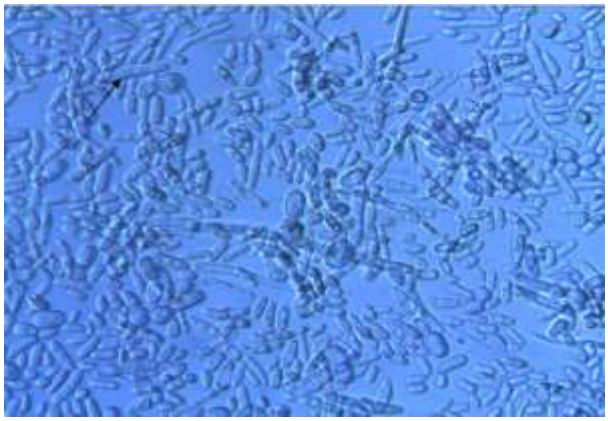

Fig-4: Wet mount of *Candida krusei* showing round and elongated yeast cells using×40 objective, grown on Saboraud's Dextrose Agar at 37°C.

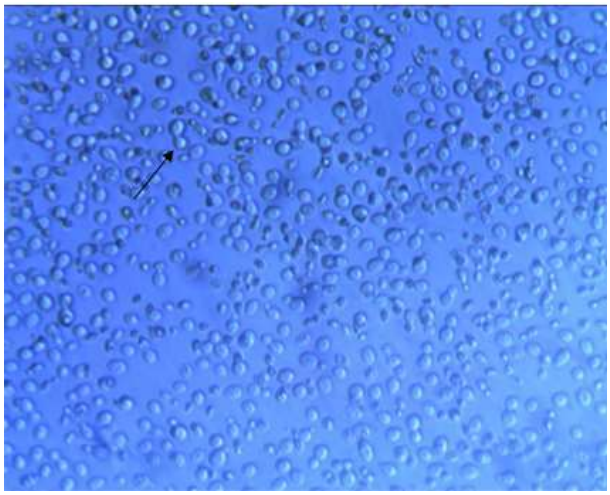

Figure 5: Wet mount of *Rhodotorula specie* grown on Saboraud's Dextrose Agar at 37°C showing round and budding yeast cells using × 40 objective.

DISCUSSION

Fungal infections of lungs are important infective processes [16]. Although active mycosis may be an independent marker of advanced immunosuppresion, it may also act as co – factor in accelerating and amplifying the clinical course of tuberculosis disease [17].

In the present study, the sex – wise distribution of TB infection is sex dependant with males having higher prevalence of 22(7.3%) more than females with the prevalence of 6(2.0%). This agrees with the findings of WHO Report [18], which put the males as more prone to TB infection than females. This might be attributed to more exposure of males to external environment than females. Also agrees with the findings of Elizabeth [19] (Table 1).

Yeast and mould infections are dependent on the sex, although, according to this study, the difference on the occurrences of fungal infections between the two sexes was very low, with the prevalence rates of 14 and 12 for mould infection, while 57(19%) and 54(18%) for yeast infections for males and females respectively. This result was in agreement with that of Bansod and Rai [20], who observed that mould infection was higher in males compared to females as men are more vulnerable to fungal infections than females due to their great exposure to the surrounding (Table 1). This finding disagrees with the finding of Hidalgo and Vazquez [21] in which it depicted that sex is independent of the distribution of the fungal infections.

Table 2 shows sex distribution of AFB and Yeast co – infections with the prevalence rate of 11(3.6%), Males had prevalence of 7(2.3%) in AFB and Yeast co – infection. This study suggests that fungal/TB co – infections is independent of the sex but rather depends largely on the possible risk factors such as fungal colonization, integrity of skin and mucomembrane, prolong duration of antimicrobial therapy, corticosteroids therapy, diabetes mellitus, neutropenia, occupational hazards such as farming. This agrees with the findings of Samonis *et al.* [22].

Table 2 shows four Candida species, Rhodotorula and Geotrichum species isolated. Although according to Pukhlik *et al.* [23], *C. albicans* is common yeast isolated from tuberculosis patients and it is responsible for severe secondary infections in such patients, in this result, *C. albicans* occurred with total prevalence of 28 across the age groups while *C. krusei* had the highest prevalence of 36 which is higher than the remaining candida species. With the regards to high prevalence of non *C. albicans* species, this result agrees with the study carried out in Kuwait, where the data for five years on yeasts isolates suggests that candida species other than *C. albicans* are most frequently associated with blood stream after breaching the mucomembrane barrier [24, 25].

Although, this result shows higher prevalence of *C. albicans* 28(9.3%) over *C. tropicalis* 22(7.3%), it shows the emergence of *C. tropicalis* as new opportunistic pathogen to cause severe invasive disease. It has an apparently greater capacity than *C. albicans* to invade deep tissues of immunocompromised host. This agrees with the findings of Roilides *et al.* (2003) [26]. *C. glabrata* had a prevalence of 14, though low compared to the rest of the candida isolates, but it seems important as other non *C. albicans* are increasing in frequency. This agrees with the findings of Lane [27].

This finding also suggests that these mycotic infections are age dependant as patients below 10 years did not show the presence of the systemic infection and old patients greater than 76 years also show very low prevalence possibly due to low environmental exposure of these age brackets, particularly in secondary infections (Table 1).

Table 2 shows a mixed growth of Candida species based on the age of the subjects. There were mixed

growth of *C. tropicalis* and *C. krusei* with the prevalence of 6(2.0%) while the mixed growth between *C. albicans* and *C. krusei* yielded prevalence of 3(1.0%), possibly as well due to their increasing capacity of invasiveness.

CONCLUSION

In conclusion, pulmonary fungal infections co – exist with tuberculosis and the prevalence rate was low but statistically significant. The prevalence of non – albicans species is increasing possibly due to their apparently greater capacity than *C. albicans* to invade deep tissues of immunocompromised host and inadequate response to anti – tuberculosis drugs

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