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Research Article

Co-infectivity of HIV and Atypical Mycobacteria in Nsukka Local Government Area of Enugu State

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Abstract

Background: The incidence of tuberculosis (TB) in the world is increasing. Due to the increasing level of immunocompromised individuals resulting from diseases like HIV/AIDS, other nontuberculous mycobacteria (NTM) are beginning to thrive, causing tuberculous infections. However, information on the contribution of nontuberculous mycobacteria (NTM) to mycobacterial infections in Africa is scarce due to several factors. Thus, this study is on the co-infectivity of HIV and Atypical Mycobacteria in Nsukka L.G.A.

Materials and Methods: Two hundred cases (100 HIV-negative and 100 HIV-positive patients) (46.5% males and 53.5% females) were identified. The age ranged between 15 and 71 with a mean age of 37.5 years. HIV antibodies were screened using two test kits: the Determine (preliminary test) and the Uni-Gold (consistency test). CD4+ count was determined using cytometry. Acid-fast bacilli (AFB) were detected using sputum smear microscopy. AFB positive samples were subjected to nested PCR for species identification. T-test was employed to check for statistical significance between the mean prevalence in test and control groups and CD4 count of HIV single infection and co-infection with TB. Correlation analysis was used to check for the relationship between the demographic characteristics and the disease distribution.

Results: A preponderance of HIV infection was observed among the 21-50 age group (72.5%) with an overall HIV prevalence of 19.4%. The highest AFB prevalence of 26.6% was observed among patients aged 21-30 years, with an overall prevalence of 24%. About 79.1% of TB infections occurred at CD4 count less than 400 cells/µl. Nested PCR showed 97(78.9%) *M. tuberculosis*, 14(11.4%) *M. bovis*, and 10(8.1%) NTM. The NTM identified was *M. avium* complex. The prevalence rate of TB/HIV co-infection was 24(24%), of which 14(53.8%) were *M. tuberculosis*, 5(20.8%) were *M. bovis*, and 3(12.5%) were NTM. The highest NTM prevalence of 66.7% was observed among patients aged 21-30 years in the HIV positive group while the highest prevalence of 42.8% was observed among 41-50 years in the HIV negative group. TB co-infection was significantly associated with CD4+ cell count ($P < 0.05$). Respectively, rural settlers (RR = 1.40, P = 0.002) and those with lower education (RR = 3.17, P = 0.01) were at higher risk of TB co-infection with HIV.

Conclusion: The study underscores the role of nontuberculous AFB in pulmonary tuberculosis especially in HIV patients, and is suggestive of the implication of therapies without discrimination between TB and NTM. Molecular screening assays with rapid detection of NTM infections should be a priority for strengthening the public health response.

Keywords: Tuberculosis; Atypical Mycobacteria; Acid Fast Bacilli; Co-Infection; HIV; Nontuberculous Mycobacteria

Abbreviations

AFB: Acid-fast Bacilli; AIDS: Acquired immunodeficiency Syndrome; CD: Cluster of Differentiation; DOTS: Directly Observed Treatment Short-course; HAART: Highly active antiretroviral Treatment; HIV: Human Immunodeficiency Virus; MAC: *Mycobacterium avium* complex; MOTT: mycobacteria other than tubercle bacilli; MTB: *Mycobacterium tuberculosis;* NTM: Nontuberculous mycobacteria; PCR: Polymerase Chain Reaction; PTB: Pulmonary Tuberculosis; TB: Tuberculosis; WHO: World Health Organization

Introduction

Tuberculosis (TB) is an infectious disease that primarily affects the lungs causing pulmonary tuberculosis but can also affect the meninges, intestine, bones, lymph nodes, skin, and other tissues of the body. Worldwide, tuberculosis causes 2.9 million deaths annually [1]. An estimated two billion persons are currently infected with *Mycobacterium tuberculosis* and other *Mycobacterium* species. The rates of increase are even greater in developing countries, primarily because of increased immigration of people from regions of high endemicity, declining socio-economic conditions in densely populated cities, and the increasing number of the human immunodeficiency virus (HIV) infected individuals [2]. The total number of tuberculosis cases in the world is increasing and the HIV epidemic is implicated for this increased incidence. An estimated three million persons with tuberculosis worldwide also have AIDS. Over 125 *Mycobacterium* species have been characterized and identified [3]. There are numerous species of *Mycobacterium* and because of recently developed molecular methods, more are being recognized [4], although regional variation in species isolation has been shown [3]. Historically *M. tuberculosis* and *M. leprae* have caused the preponderance of human disease. However, in recent years, other mycobacteria have become more widely appreciated as potential pathogens. Most other mycobacteria are present in the environment as saprophytes. These organisms in the past have been called atypical mycobacteria, the term first coined by Pinner [5] but species have been described with different nomenclatures such as anonymous, nontuberculous mycobacteria (NTM), environmental, opportunistic, and mycobacteria other than tubercle bacilli (MOTT). While there has not been an international consensus on the nomenclature, the American Thoracic Society (ATS) has endorsed the name NTM [6]. The frequency of pulmonary disease from nontuberculous mycobacteria (NTM) is reportedly on the rise in Europe, North America, Asia, and Southern Africa [7]. In sub-Saharan Africa, information on the extent of the burden of pulmonary disease from nontuberculous mycobacteria (NTM) is lacking due to limitations in tools for mycobacterial species identification. However, studies conducted as far back as the late 1950s and early 1960s using traditional tools for identifying mycobacterial groups based on certain characteristics like the speed of growth and morphology, have reported the isolation of NTM from both tuberculosis patients and the general public in some African countries including Nigeria [8,9]. They have been increasingly recognized to cause pulmonary and non-pulmonary infections [5], which is in part explained by the increase in the number of susceptible/immunocompromised individuals such as those suffering from acquired immunodeficiency syndrome (AIDS) and is also due to better recognition of their role through more sensitive and specific techniques). *Mycobacterium* infections are frequent opportunistic pathogens associated with the acquired immunodeficiency syndrome (AIDS). Its relative virulence and potential for person-to-person transmission distinguish *Mycobacterium tuberculosis*. Persons infected with the human immunodeficiency virus (HIV) are particularly susceptible to tuberculosis, either by the reactivation of latent infection or by primary infection with rapid progression to active disease [11]. In addition, disseminated infections with *Mycobacterium avium* complex are increasingly common in advanced human immunodeficiency virus [HIV] infection and cause substantial morbidity [12]. Persons with HIV infection and CD4 lymphocyte count less than 100 cells/mm^3 have a probability of 10% to 20% per year of developing *M. avium* complex disease or bacteremia [13]. Indeed, NTM are being identified as causative agents of human disease with increasing frequency. They were not traditionally considered a threat to public health, as person-to-person transmission rarely occurs, yet these organisms can produce serious morbidity. In addition, cases of mycobacterial disease are becoming more difficult to diagnose or treat, especially when fastidious NTM or drug-resistant strains are involved [6]. The diseases caused by these mycobacteria have varied manifestations but have been broadly grouped as mycobacterioses [14]. In immunocompromised individuals, the infections due to NTM have been observed to be an important cause of morbidity and mortality in western countries [6].

The distribution of NTM and the incidence of disease caused by them are perhaps not fully understood in most parts of the world. NTM are widely distributed in nature and have been isolated from natural water, tap water, soil, water used in showers, and surgical solutions [15]. In the United States, most isolates were *M. avium*, *M. kansasii,* and *M. fortuitum* [16]. There have also been some reports from Japan [17], the UK [18], and India [19]. They are considered opportunistic pathogens, and several species which include *M. avium* Complex [MAC], *M. intracellulare*, *M. kansasii*, *M. paratuberculosis*, *M. scrofulaceum*, *M. fortuitum*, *M. chelonae*, and *M. ulcerans* are associated with human disease which is typically pulmonary, skin/soft tissue, lymphatic, or disseminated in presentation. It is a common observation that environmental mycobacteria cause disease in individuals who offer some opportunity due to altered local

or systemic immunity [5,6,10]. NTM causes pulmonary diseases that are similar to *M. tuberculosis* and is often chronic and occurs in older women or those with underlying lung disease [20]. They also cause skin/soft-tissue infections of varying severity in both sporadic and epidemic forms.

Although it has been shown that most culture-positive Mycobacteria are *M. tuberculosis* in regions where tuberculosis is highly prevalent, NTM isolates have been increasing gradually. There are several reasons for this increase in disease due to NTM, including improved diagnostic techniques, increased recognition of NTM in immunocompetent patients, increasing life expectancy and increased numbers of, particularly, older women, increased incidence of NTM associated with HIV/AIDS, and other forms of immune compromise and altered host defense [21]. These organisms trigger diseases and true infections and thus can be important clinically [22]. The prevalence of NTM and NTM-associated hospitalization has been on the increase in several industrialized countries [23]. Some of the countries have reported an NTM prevalence rate as high as 50% among cultured mycobacteria [23]. However, there is a paucity of data from developing countries largely due to a lack of laboratory infrastructure for culture and species identification. In Africa, the contribution of NTM to such disease has been examined on a small scale only [24]. In Nigeria, NTM have been reported in several places [25].

Diagnosis and treatment of TB patients in most Sub-Saharan African countries including Nigeria are based solely on the results of microscopic smear positivity. As such, all sputum smear-positive diagnosed patients are indiscriminately placed on Directly Observed Treatment Short-course [DOTS], the current international TB treatment strategy. Consequently, many of the pulmonary diseases caused by NTM are not identified but rather treated with conventional anti-TB which eventually fails because the majority of the NTM are resistant to conventional anti-TB treatment [26]. The implication is that NTM is inappropriately managed with first-line antituberculous drugs [27], worsening the patient's condition and raising the risk of drug resistance.

Nigeria has one of the highest burdens for TB in the world and remains a major target in the global control of the disease. In 2011 an estimated 280,000 cases of TB [68% incident cases] were reported from Nigeria which corresponds to a prevalence rate of 280 per 100,000 population according to the WHO global tuberculosis report of 2012. The in-country prevalence of pulmonary TB due to species other than *Mycobacterium tuberculosis* like *Mycobacterium bovis* and *Mycobacterium africanum* is reportedly on the rise [25]. However, this evidence is inconclusive, and data are insufficient on the prevalence of other mycobacterial species including those associated with HIV patients, raising questions about the importance of the different species of *Mycobacterium* causing tuberculosis [TB] in Nigeria. Thus, this research is aimed at studying the prevalence of atypical mycobacteria among HIV-seropositive individuals.

Materials and Methods

Sample collection for HIV screening and CD4+ cell count

Patient's whole blood was collected into vacutainer brand blood collection tube containing EDTA. The collection tube was filled to 2/3 to ensure acceptable blood-to-anticoagulant ratio.

Sample collection for sputum microscopy

Sputum samples were collected from patients suspected of having pulmonary tuberculosis. The chances of detecting Acid-fast bacilli (AFB) are greater with three samples than with one or two. Consequently, three sputum samples were collected from each patient as follows; patient provides an on-the-spot sputum under supervision and then asked to bring early-morning sputum the next day. On submission, patient provides another on-the-spot sputum sample under supervision.

Screening for HIV1 and 2 antibodies

HIV screening was performed, according to manufacturer's instruction, using two HIV test kits: Abbot Determine HIV-1 and 2 kit (Abbot Japan Co ltd. Tokyo, Japan) used for preliminary test and Uni-Gold which was used to check for consistency. About 50µl of patient's blood (plasma) was applied to appropriately labeled sample pads. After 15 minutes of sample application, the results were read. The inherent quality control of the kit validates the results. Two visible red lines occurring in the region labeled control and test represents HIV seropositive reaction while a single red colour in the region of control validates the test kit. Absence of red line in the test region represents HIV seronegative reaction.

Determination of CD4+ count

Flow-Cytrometric method used by Alexander [28] was employed in the $CD4^+$ counting. Specimens collected in sterile vacutainer (anticoagulant required for the FDA-approved TRIO procedure) were stained with the TRIO reagents. 100 µl of EDTA-treated blood with 10 µl of antibody were incubated for 20 to 30 min at room temperature and in the dark. All specimens were analyzed on the Ortho Cytoronabsolute cytometer. At least 2,000 cells in the lymphocyte gate were counted. The two-color tubes were gated on lymphocytes based on light scatter, and the percentage of cells staining for both CD3 and $CD4^+$ was determined. This value was multiplied by the absolute lymphocyte count determined from EDTA-containing tubes analyzed on a Sysmex NE 8000 hematology analyzer (Toa, Inc., Chicago, Ill.) to yield an absolute $CD4^+$ lymphocyte count. The TRIO tubes were analyzed by using Ortho's ImmunoCount II software. Appropriate counting times for absolute numbers were determined with Ortho Validation beads. Lymphocytes were detected by both light scatter and immunoscatter gates. Immunoscatter gates use an immunofluorescence marker and right-angle scatter to define a population. Isotype controls were used for both the two- and three-color methods to set positive-negative discriminators at a 2% background level. The TRIO panel is validated by using four levels of quality control (QC), including tube-to-tube consistency, Immunosum (lymphosum), T sum, and background fluorescence. We had five specimens initially fail a QC parameter; four were corrected by regating, and one was reanalyzed by using new tubes and was probably due to a pipetting error. The TRIO panel does require accurate, precise pipetting for all QC parameters to be acceptable.

Sputum smear preparation and ziehl-neelsen staining for acid-fast bacilli (AFB)

Grease free slides were labeled on frosted end with a pencil according to worksheet. Sputum smears were made on the slides using applicator stick. A small concentric movement was applied to make the smear. The smear was allowed to air dry for at least 1 hour and then fixed by passing the smear 3 times through a blue flame. The smears were then stained using standard Ziehl Neelsen staining technique. All the three sputum samples from each TB suspect were examined and recorded accordingly. Result as indicated in the table 1 below were then used to categorize patients.

Categorizing patients as smear-positive or negative requires results from more than one smear. Patients with pulmonary symptoms were classified using the guide below (Table 2) [29].

Table 1: Interpretation of Ziehl-Nelseen results.

Number of AFB Found	Records	Report
No. AFB in at least 100 fields	0	Negative
1-9 AFB in 100 fields	Actual AFB counts	Actual AFB count
10-99 AFB in 100 fields	1+	1+
1-10 AFB per field in at least 50 fields	2+	2+
>10 AFB per field in at least 20 fields	3+	3+

Table 2: Classification of the sputum smears.

Smear positive	Intermediate	Smear negative
At least 2 smears examined and both positive, i.e. reported 1-9 per 100 fields (scanty) or greater	Several possibilities, e.g. Only one smear examined (whatever the grading) 3 smears examined but only one reported positive In either of these situations, either further sputum smear or a CXR are required before a patient can be classified.	At least two smears reported 0 (negative)

DNA extraction for *Mycobacterium* testing

About 500 µl of sputum was transferred into a clean 10 ml tube and 500 µl of sputum lysis buffer added. The mixture was vortexed and incubated for 25 min at room temperature with shaking, and the volume adjusted to 10 ml with sterile distilled water. The mixture was centrifuged at 6000 rpm for 30 min and the supernatant discarded using a 200 µl pipette. The pellet was re-suspended in 500 µl of ATL tissue lysis buffer and vortexed gently, 200 µl of the re-suspended sample was transferred to a new micro-centrifuge tube and 20 µl of Proteinase K added, mixed gently by vortexing and incubated at 56°C for 10 min.

DNA extraction for PCR

The micro-centrifuge tube (from above) was briefly centrifuged to remove drops from the inside of the lid and 200µl absolute ethanol (molecular grade) was added to the sample and mixed again by pulse-vortexing for 15 seconds. After mixing, the 1.5 ml micro-centrifuge tube was again briefly centrifuged to remove drops from the inside of the lid. The mixture was carefully applied to the

QIAamp Mini Spin column (in a 2 ml collection tube) without wetting the rim. It was allowed to stand for 3 min and then centrifuged at 10000 rpm for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate discarded. Carefully, the QIAamp Mini spin column was opened and 500 µl Buffer AW1 added without wetting the rim, the cap closed and centrifuged at 10,000 rpm for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate discarded. Carefully, the QIAamp Mini spin column opened and 500 µl Buffer AW2 added without wetting the rim, the cap closed and centrifuged at full speed (14,000 rpm) for 3 min. The QIAamp Mini spin column was placed in a 2 ml collection tube and the old collection tube discarded with the filtrate. The spin column was centrifuged again at full speed (14,000 rpm) for 1 min. The spin columns were turned upside down onto a clean tissue paper and allowed to air-dry for 5 min (to remove residual ethanol). The QIAamp Mini spin column was placed into a clean 1.5 ml micro-centrifuge tube. 60 µl Buffer AE was added and incubated at room temperature for 5 min, and then centrifuged at 8000 rpm. DNA yield and purity was checked by adding 5 µl of DNA elute into a cuvette and adding 95 µl of AE buffer and then read against AE blank using the Eppendorf Biophotometer. The A_{260}/ A_{280} ratio should be 1.7 to 2.0. DNA samples were stored at -20°C until required for analysis.

Detection of *Mycobacterium* species by Nested PCR

The mycobacterial DNA was identified using the method as described by Wu., *et al.* [30]. Samples used included; DNA samples extracted from sputum lysate, two positive controls, two negative controls, water and negative control genomic DNA in each run.

For the 1st round of PCR for HSP 65, the tubes were labelled appropriately. About 10 µl of the AmpliTaq Gold PCR Master mix (Applied Biosystems) was dispensed into each PCR tube. 5 µl of HSP-65 Outer primer mix was added into each tube. 5 µl of genomic DNA was added. The tubes were briefly spun using the minifuge. All tubes were transferred to the thermal cycler and the programme 'tb-hsp' was run in Paul.

For the 1st round of PCR for IS6110, the tubes were labelled appropriately. About 10 µl of the AmpliTaq Gold PCR Master mix (Applied Biosystems) was dispensed into each PCR tube. 5 µl of IS6110 Outer primer mix was added into each tube. 5 µl of genomic DNA was added. The tubes were briefly spun using the minifuge. All tubes were transferred to the thermal cycler and the programme 'tb-hsp' was run in Paul or Barnabas.

For the 2nd round of PCR for HSP 65 inner, the PCR tubes were labelled appropriately. About 10 µl of the AmpliTaq Gold PCR Master mix (Applied Biosystems) was dispensed into each PCR tube. 5 µl of HSP-65 Inner primer mix was added into each tube. 5 µl of 1st round PCR product was added. The tubes were briefly spun using the minifuge. Care was taken in handling the tubes to avoid generating or releasing aerosols. All tubes were transferred to the thermal cycler and the programme 'tb-hsp' was run in Paul or Barnabas.

For the 2nd round of PCR for IS6110 Inner, tubes were labelled appropriately. About 10 µl of the AmpliTaq Gold PCR Master mix (Applied Biosystems) was dispensed into each PCR tube. 5 µl of IS6110 Inner primer mix was added into each tube. 5 µl of 1st round PCR product was added. The tubes were briefly spun using the minifuge. All tubes were transferred to the thermal cycler and the programme 'tb-hsp' was run in Paul or Barnabas.

Agarose electrophoresis

While the PCR was running, a 2.0% agarose gel (2 g of agarose in 100 ml of 1x TAE buffer or 0.5 TBE buffer) was prepared, adding 10 µl of Ethidium Bromide to decontaminate the gels after electrophoresis. The gel was poured and allowed to solidify. The products were transferred by mixing 10 µl of second round PCR product with 2 µl of 6x loading buffer and loaded into the gel immersed in 1x TAE buffer. The order in which samples and controls were loaded was noted on a worksheet. A 50 bp DNA ladder was included on the first lane. The electrophoresis was run at 100V for 40 minutes, checking quality of separation with the UV trans-illuminator.

Only positive samples for HSP65 PCR were run for Nar 1 digestion. Restriction enzyme (RE) mix was prepared using 10x RE buffer (3 µl), Ultrapure Water (1.5 µl), and Nar I enzyme (0.5 µl) per sample. 15 µl of the PCR product was transferred into 1.5 ml tube and 5 µl of the RE mix was added. It was mixed by repeated pipetting. The tube was sealed with Clingfilm (nesofilm), spun briefly and incubated at 37°C overnight. Products were run on 3.0% agarose gel. Positive samples for *M. tuberculosis* yield two fragments

of 70 and 72 bp. Positive 123 bp insertion sequence IS6110 is also consistent with *M. tuberculosis* infection.

Results

Patients suspected of having pulmonary tuberculosis, that are seeking medical attention at Nsukka Local Government Health Department, District Hospital Nsukka and Bishop Shanahan Hospital Nsukka, Enugu State between June and December, 2013 were enrolled in the study. The study was conducted at the DOTS Laboratory, Nsukka Local Government Health Department. Two hundred cases (100 HIV negative and 100 HIV positive patients) were identified out of which 46.5% were male while 53.5% were female. The age ranged between 15 and 71 with mean age of 37.5 years.

Age distribution of HIV positive and negative patients

The result in table 3 shows the age specific distribution of HIV seropositive patients from the surveyed population. From the result, the 31-40 years age group had the highest HIV prevalence rate of 28.7%, followed by the 21-30 years age group with 23.4% prevalence. The least prevalence of 8.0% was seen in patients aged 60 years and above. Overall, 19.4% of the surveyed population was HIV positive.

Table 3: Age distribution of HIV Positive and Negative Patients.

Key: +ve= Positive; –ve= Negative

Age	No Screened	HIV +ve No. (%)	HIV –ve No. (%)
≤20	62	10 (16.1)	52 (83.9)
21-30	111	26 (23.4)	85 (76.6)
31-40	94	27 (28.7)	67 (71.3)
41-50	103	21 (20.4)	82 (79.6)
51-60	71	10 (14.1)	61 (85.9)
>60	75	6 (8.0)	69 (92.0)
TOTAL	516	100 (19.4)	416 (80.6)

CD4+ cell count of the HIV seropositive patients

The mean CD4 lymphocyte cell count was 282 cells/µl, 31 patients had CD4 count <200 cells/µl, 37 had CD4 count levels between 200 and 399 cells/µl, 14 had between 400 and 599 cells/µl while 18 patients had CD4 count ≥600 cells/µl. Out of the 24 patients that had positive sputum smears, 45.8% had $CD4^+$ cell count less than 200 cells/µl, 33.3% had CD4 count levels between 200 and 399 cells/µl, 8.3% had CD4 count between 400 and 599 cells/µl while 12.5% had CD4 count ≥600 cells/µl. The association between severe immune-suppression (CD4 count <200 cells/µl) and positive smear was significant (P = 0.018 < 0.05) (Table 4).

Table 4: CD4+ cell count of the HIV seropositive patients.

$Cd4^+$ range (cells/µl)	No. Screened	No. Co-infected with TB No. (%)	No. Not Co-infected No. (%)
<200	31	11 (45.8)	20 (26.3)
200-399	37	8 (33.3)	29 (38.2)
400-599	14	2 (8.3)	12 (15.8)
≥600	18	3 (12.5)	15 (19.7)
TOTAL	100	24 (100%)	76 (100%)

Age specific distribution of TB in HIV positive patients

The result of the prevalence of TB in the various age groups of the HIV seropositive patients is presents in table 5. The result showed that out of the 100 samples, 24% was positive for AFB. The highest prevalence of TB/HIV co-infection (30.8%) was seen in the 21-30 years age group, followed closely by the 51-60 years age group with 30.0% prevalence. The least prevalence was seen in patients aged 60 years and above with 16.7% prevalence.

Age specific distribution of TB in HIV negative patients

The result of the prevalence of TB in the various age groups of the HIV seronegative patients is presented in table 6. The result showed that patients aged 21-30 years had the highest prevalence rate of 26.6%, closely followed by patients aged 20 years or less with 25.5% prevalence while patients aged 51-60 years had the least prevalence rate of 17.7%. Overall, 24% of the surveyed population was AFB positive.

Table 6: Age specific distribution of TB in HIV negative patients.

Key: AFB = Acid Fast Bacilli; +ve = Positive; -ve = Negative.

Age	No. Screened	AFB +ve No. (%)	AFB -ve No. (%)
≤20	47	12 (25.5)	35 (74.5)
21-30	94	25 (26.6)	69 (73.4)
31-40	91	23 (25.3)	68 (74.7)
41-50	89	22 (24.7)	67 (75.3)
51-60	62	11 (17.7)	51 (82.3)
>60	33	7 (21.2)	26 (78.9)
TOTAL	416	100 (24.0)	316 (76.0)

Species of Mycobacterium characterized from sputa of the subjects

Smear positive sputum samples were sent to Safety Molecular Pathology Laboratory Enugu. There, identification of *Mycobacterium* species was carried out directly from the sputum specimen using nested-PCR. The DNA was amplified and subjected to Nar1 enzyme digestion. A total of 124 samples were analyzed, out of which 97 (78.9%) were *Mycobacterium tuberculosis*, 14 (11.4%) were *M. bovis*, 10 (8.1%) were NTM and 3 (1.5%) was found not to be members of the genus *Mycobacterium* (Table 7). The NTM identified was *Mycobacterium avium* complex (MAC) (Figure 1).

Species	Test	Control	Total
	Prevalence (%)	Prevalence (%)	Prevalence (%)
M. tuberculosis	14 (58.3)	83 (83.0)	97 (78.9)
M. bovis	5 (20.8)	9 (9.0)	14 (11.4)
M. avium	3 (12.5)	7 (7.0)	10 (8.1)
TOTAL*	22 (91.7)	99 (99.0)	121 (97.6)

Table 7: Species of *Mycobacterium* characterized from sputa of the subjects.

* 3 Samples were inconclusive (not *Mycobacterium*).

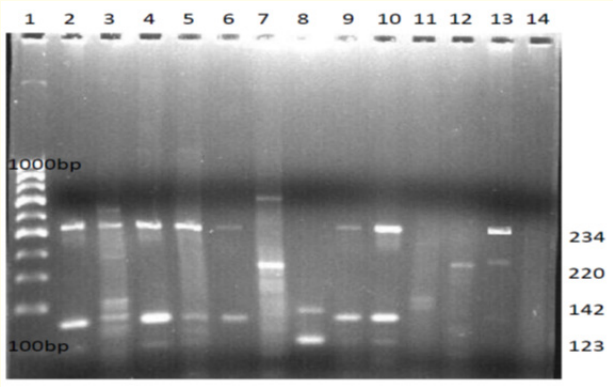

Figure 1: PCR amplification of 123bp, 142bp, 220bp and 234 regions of the clinical specimens of M. tuberculosis and nontuberculous mycobacteria on 3% agarose gel. Lane 1: 100bp Molecular Marker; Lane 2-12: Clinical specimens (2, 4, 6: M. avium); (3, 5, 7, 9, 10: M. tuberculosis); (8: M. bovis); (11, 12: Inconclusive); Lane 13: Positive Control; Lane 14: Negative Control.

Prevalence of Mycobacterium species in HIV positive and negative patients

The result in table 8 shows the distribution of the species in test and control populations. Of the 24 smear positive samples from the test group assayed, 14 (58.3%) were identified as *M. tuberculosis*, 5 (20.8%) were *M. bovis* and 3 (12.5%) were NTM. In the control group, 83% were *M. tuberculosis*, 9% were *M. bovis* and 7% were NTM. From the result, all the cases of TB in patients aged ≤20 years in test group were caused by *M. tuberculosis* while in control group, 16.7% of TB were caused by NTM.

Percentage age distribution of the species in HIV positive and negative patients

The result in table 9 shows the percentage age distribution of the species in HIV positive (test) and negative (control) patients. *M. tuberculosis* was most prevalent among HIV patients aged 21-30 years in both test and control population with prevalence rate of 35.7% and 25.3% respectively. Highest prevalence rate of *M. bovis* (40%) in the test population was seen in the 31-40 years age group while in the control population, both age groups of 31-40 years and 51-60 years showed the highest prevalence rate of 33.3% each. For NTM, 66.7% prevalence was seen in the 21-30 years age group of the test population while 42.8% prevalence was seen in the 41-50 years age group of the control population.

Demographic characteristics of the population

Demographic characteristics of the patients were obtained from the hospital records. In the test population, 47% of the patients were male while 53% were female. Rural dwellers constituted 60% of the population while 40% reside in the urban/sub-urban areas. Educational status shows that 24% attended or were still in higher institution while 76% were in secondary school and lower or uneducated. Based on occupation, artisans and traders were grouped as artisans. Similarly, employees of both public and private establishments were grouped as civil servants. From the result, 41% were farmers, 38% artisans, 10% civil servants and 11% students (Table 10). In the control population, 46% were male while 54% were female. Rural dwellers constituted 57% while 43% were urban dwellers. Educational status shows that 27% attended or were still in higher institution while 73% were in secondary school and lower or uneducated. Based on occupation, 38% of the population were farmers, 36% were artisans, civil servants and students were 13% each (Table 11).

Age	Test				Control			
	No. Screened	MTB (%)	MB (%)	NTM (%)	No. Screened	MTB (%)	MB (%)	NTM (%)
≤20	2	2 (100)	0 (0)	0 (0)	12	10 (83.3)	0 (0)	2 (16.7)
21-30	8	5 (62.5)	1 (12.5)	2 (25)	25	21 (84.0)	2 (8.0)	1 (4.0)
31-40	6	3 (50.0)	2 (33.3)	0 (0)	23	20 (87.0)	3 (13.0)	0 (0)
41-50	4	2 (50.0)	1(25.0)	1 (25)	22	19 (86.4)	0 (0)	3 (13.6)
51-60	3	2 (66.7)	0 (0)	0 (0)	11	7 (63.6)	3 (27.3)	1(9.1)
>60	1	0 (0)	1 (100)	0 (0)	7	6 (85.7)	1 (14.3)	0 (0)
TOTAL	24	14	5	3	100	83	9	7

Table 8: Prevalence of *Mycobacterium* in HIV positive and negative patients.

Key: MTB = *Mycobacterium tuberculosis;* MB = *Mycobacterium bovis;* NTM = Nontuberculous Mycobacteria.

Age	MTB		MB		NTM	
	Test No (%)	Control No (%)	Test No (%)	Control No (%)	Test No (%)	Control No (%)
≤20	2 (14.3)	10 (12)	0 (0)	0 (0)	0 (0)	2 (28.6)
21-30	5 (35.7)	21 (25.3)	1 (20.0)	2 (22.2)	2 (66.7)	1 (14.3)
31-40	3 (21.4)	20 (24.1)	2 (40.0)	3 (33.3)	0 (0)	0 (0)
41-50	2 (14.3)	19 (23.0)	1 (20.0)	0 (0)	1 (33.3)	3 (42.8)
51-60	2 (14.3)	7 (8.4)	0 (0)	3 (33.3)	0 (0)	1 (14.3)
>60	0 (0)	6 (7.2)	1 (20.0)	1 (11.2)	0 (0)	0 (0)
TOTAL	14 (100)	83 (100)	5 (100)	9 (100)	3 (100)	7 (100)

Table 9: Percentage age distribution of the species in HIV positive and negative patients.

Key: MTB= *Mycobacterium tuberculosis*; MB= *Mycobacterium bovis*; NTM= Nontuberculous Mycobacteria

Age		Sex		TB Prevalence	Settlement		Education		Occupation			
	No Screened	M (%)	F (%)	No. (%)	R (%)	U (%)	H (%)	L (%)	F (%)	A (%)	C (%)	S (%)
≤20	10	6 (60.0)	4 (40.0)	2 (20.0)	6 (60.0)	4 (40.0)	3 (30.0)	7 (70.0)	0 (0)	2 (20.0)	0 (0)	8 80.0)
21-30	26	15 (58.0)	11 (42.0)	8 (30.8)	11(42.0)	15 (58.0)	7 (27.0)	19 (73.0)	10 (38.0)	11 (42.0)	2 (8.0)	3 (12.0)
31-40	27	7 (26.0)	20 (74.0)	6 (22.2)	17 (63.0)	10 (37.0)	6 (22.0)	21 (78.0)	13 (48.0)	12 (44.0)	2 (8.0)	0 (0)
41-50	21	13 (62.0)	8 (38.0)	4 (19.0)	13 (62.0)	8 (38.0)	6 (29.0)	15 (71.0)	9 (43.0)	7 (33.0)	5 (24.0)	0 (0)
51-60	10	4 (40.0)	6 (60.0)	3 (30.0)	8 (80.0)	2 (20.0)	2 (20.0)	8 (80.0)	3 (30.0)	6 (60.0)	1 (10.0)	0 (0)
>60	6	2 (33.0)	4 (67.0)	1 (16.7)	5 (83.0)	1 (17.0)	0 (0)	6 (100.0)	6 (100.0)	0 (0)	0 (0)	0 (0)
OVERALL (%)	100	47	53	24	60	40	24	76	41	38	10	11

Table 10: Demographic characteristics of the test population.

Key: M = Male; F = Female; MTB = *M. tuberculosis*; MB = *M. bovis*; NTM = Nontuberculous Mycobateria; R = Rural; U = Urban; H = Higher; L = Lower; F = Farmer; A = Artisan; C = Civil Servant; S = Student.

Age		Sex		Settlement		Education		Occupation			
	No. Screened	M (%)	F (%)	R (%)	U (%)	H (%)	L (%)	F (%)	A (%)	C (%)	S (%)
≤20	12	7 (58.0)	5 (42.0)	7 (58.0)	5 (42.0)	1 (8.0)	11 (92.0)	1 (8.0)	2 (17.0)	0 (0)	9 (75.0)
21-30	25	13 (52.0)	12 (48.0)	15 (60.0)	10 (40.0)	6 (24.0)	19 (76.0)	7 (28.0)	12 (48.0)	2 (8.0)	4 (16.0)
31-40	23	8 (35.0)	15 (65.0)	10 (43.0)	13 (57.0)	7 (30.0)	16 (70.0)	9 (39.0)	10 (43.0)	4 (18.0)	0 (0)
41-50	22	9 (41.0)	13 (59.0)	13 (59.0)	9 (41.0)	7 (32.0)	15 (68.0)	9 (41.0)	7 (32.0)	6 (27.0)	0 (0)
51-60	11	6 (55.0)	5 (45.0)	7 (64.0)	4 (36.0)	3 (27.0)	8 (73.0)	7 (64.0)	3 (27.0)	1 (9.0)	0 (0)
>60	7	3 (43.0)	4 (57.0)	5 (71.0)	2 .0)29	0 (0)	7 (100.0)	5 (71.0)	2 (29.0)	0 (0)	0 (0)
OVERALL (%)	100	46	54	57	43	24	76	38	36	13	13

Table 11: Demographic characteristics of the control population.

KEY: M = Male; F = Female; R = Rural; U = Urban; H = Higher; L = Lower; F = Farmer; A = Artisan; C = Civil Servant; S = Student.

Attributable and relative risks of the disease in HIV condition

From the result in table 12, the risk of developing TB was higher in HIV negative patients of the 41-50 years age group than in the HIV positive patients, while it was higher in HIV positive than negative in the rest of the age groups. Overall, HIV patients were 1.26 times more at risk of developing the disease than non-HIV patients.

Attributable and relative risks of the disease at different risk factors

Different demographic factors were identified as risk factors for the disease and these include gender (female), age (≤40), rural settlement, lower education and farmers. From the results, the females were 1.59 times at risk of developing the disease than males.

Age	HIV +ve				HIV –ve				RR
	No. screened	AFB +ve A	AFB -ve b	Risk x = a/(a+b)	No screened	AFB +ve c	AFB -ve d	Risk y = c/(c+d)	x/y
≤20	10	2	8	0.20	47	12	35	0.25	0.80
21-30	26	8	18	0.30	94	25	69	0.26	1.15
31-40	27	6	21	0.22	91	23	68	0.25	0.88
41-50	21	4	17	0.19	89	22	67	0.24	0.79
51-60	10	3	7	0.30	62	11	51	0.17	1.76
>60	6	1	5	0.16	33	7	26	0.21	0.76
Total	100	24	76	0.24	416	100	316	0.24	1.00

Table 12: Attributable and relative risks of the disease in HIV condition.

Key: +ve = Positive, - ve = Negative, RR = Relative Risk, a = TB-positive samples in HIV group, b = TB-negative samples in HIV group, a+b = total number tested in HIV group, a/a+b = attributable risk in HIV group, c = TB-positive samples in non HIV group, d = C. TB-negative samples in non HIV group, c+d = total number tested in non HIV group, c/c+d = attributable risk in non HIV group, (a/a+b)/(c/c+d) = attributable risk ratio.

Age wise, patients 40 years or younger were 1.15 times at risk. Patients with lower education and farmers were 3.17 and 3.21 times higher than those with higher education and non-farmers respectively (Table 13).

The distribution of the species with respect to the demographic factors

The distribution of the species with respect to the demographic factors in the test and control groups is presented in figures 2 - 9.

Risk factors	Risk in presence of RF	Risk in absence of RF	Relative risk
F	0.54	0.47	1.15
≤40	0.62	0.39	1.59
RS	0.59	0.42	1.40
L	0.76	0.24	3.17
FAR	0.77	0.24	3.21

Table 13: Attributable and relative risks of the disease at different risk factors.

Key: F = Female; RS = Rural settlement; LE = Lower education; FAR = Farmers; RF = Risk factors.

From the result in test and control groups (Figures 2 and 3), *M. tuberculosis* was more prevalent in female while *M. bovis* and NTM were more prevalent in male. The result in figures 4 and 5 shows that in test population, all the species were more prevalent among rural dwellers. In control population, *M. tuberculosis* and *M. bovis* were more prevalent among rural dwellers while NTM was more in urban dwellers.

Educational status shows that all the species were more prevalent among patients with low literacy level in both test and control populations. There was complete absence of *M. bovis* among patients that acquired higher education in the control population (Figures 6 and 7). Distribution of the species based on occupation shows that in test population, *M. tuberculosis* was more prevalent among artisans while *M. bovis* and NTM were more prevalent among farmers. There was no *M. bovis* among civil servants and students in test population (Figure 8). In control population, there was equal distribution of NTM in artisans, civil servants and students. There was also equal distribution of *M. bovis* in artisans and students, although *M. bovis* was highest in farmers (Figure 9).

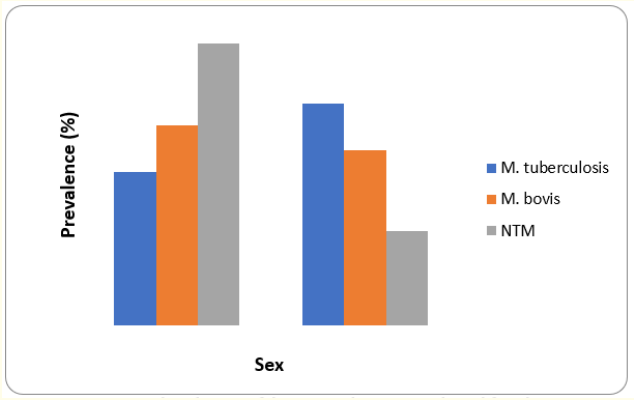

Figure 2: Percentage distribution of the species between male and female in test group.

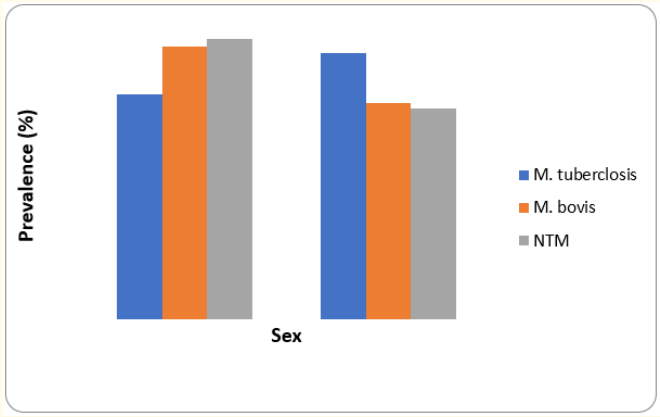

Figure 3: Percentage distribution of the species between male and female in control group.

Discussion

In this study, 8.1% of patients who sought clinical treatment for tuberculosis in Nsukka and environs were caused by NTM infection. This is comparable though slightly lower than 9.1% and 11% earlier reported in south west region of Nigeria [21], but much lower than 16% and 15% reported in Cross River State and northern Nigeria respectively [31,32], and 23.1% reported in Jos [25]. NTM and MTB infections share clinical radiographic similarities. They tend to be more common among older age groups, people with preexisting lung conditions, cases of advanced HIV disease and may take long to treat, often with poor outcome compared to MTB

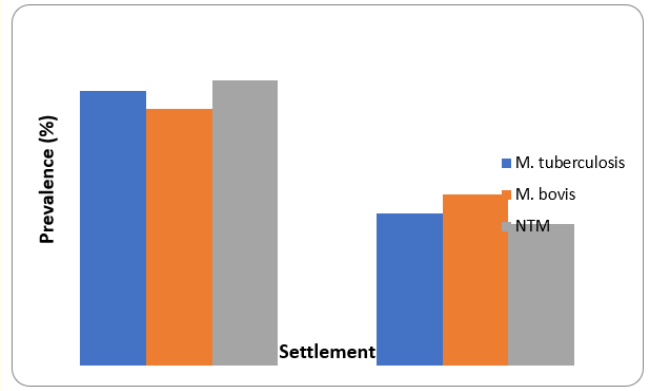

Figure 4: Percentage distribution of the species between urban and rural dwellers in test group.

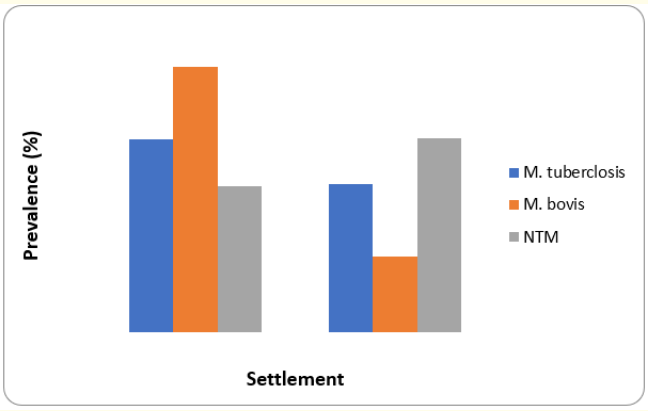

Figure 5: Percentage distribution of the species between urban and rural dwellers in control group.

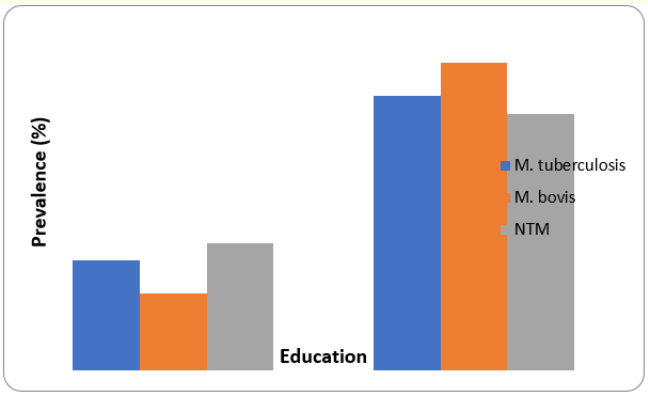

Figure 6: Percentage distribution of the species based on educational status in test group.

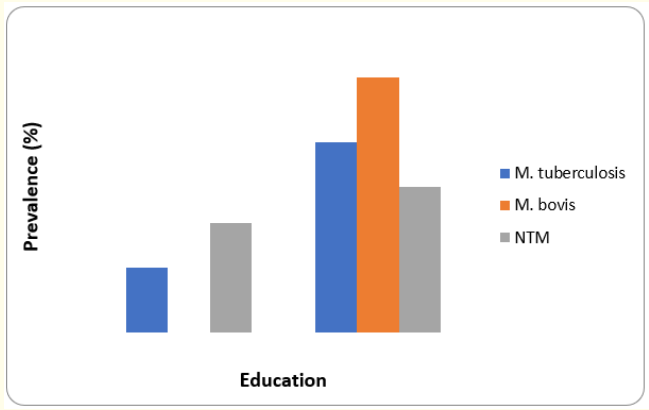

Figure 7: Percentage distribution of the species based on educational status in control group.

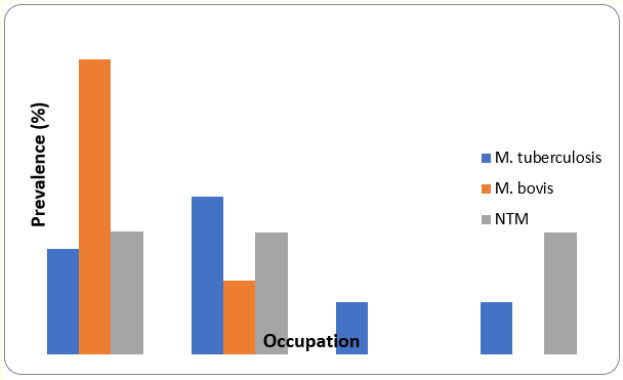

Figure 8: Percentage distribution of the species based on occupation in test group.

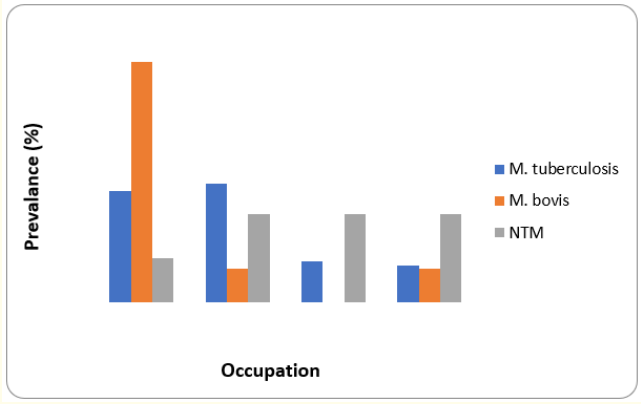

Figure 9: Percentage distribution of the species based on occupation in control group.

[3]. However, in this study, we found that both NTM and MTB infected cases were older in age. This is contrary to earlier report by Aliyu., *et al.* [32], although it agrees in part with their report in that the disease was more common among younger patients with HIV. This difference may be because of the lower number of patients who presented to the clinic in this age category. We did not find any association between NTM and population environment [rural or urban dwellers]. Given the very low sensitivity of the standard of smear diagnostic test and absolute specificity of the newly WHO recommended point-of-care diagnostic [GeneXpert] for the NTM, a mechanism for the routine identification of NTM infections in high burden resource limited areas of the world is urgently needed [33].

In this study, the majority of *Mycobacterium tuberculosis* complex [MTBC] cases were caused by *M. tuberculosis* with few cases caused by *M. bovis*. The 11.4% prevalence of *M. bovis* reported in our study is comparable though lower than recent report in Ibadan [34]. Our finding agrees with recent evidence that strains of *M. bovis* different from the cattle strains infect humans [34]. This further strengthens the speculation on the possibility of human-to-human airborne transmission of bovine tuberculosis and its relative contribution to new infections in humans [35]. This suggests that in addition to ingestion, an inhalational route of transmission from cattle to human may occur with those working with infected livestock on farms or slaughter houses. Closeness of people in the rural areas to domestic and wild animals might be the reason for the high prevalence of *M. bovis* in the rural areas as reported in our study.

The atypical mycobacterium isolated from the sputum specimen was *Mycobacterium avium* complex [MAC]. This organism has been reported in literature as significant NTM that is responsible for PTB-like infections in humans [13]. Currently, many species of NTM are recognized as potential pathogens. With AIDS epidemic, atypical mycobacteria have taken a new importance with the recognition that the *M. avium* complex [MAC] generally infect AIDS patients more so when their CD4+ cell count decreases below 200/mm3. 90% of mycobacterial infections in patients with AIDS involve either MTBC or MAC while the remaining 10% of infections is accounted for by various NTM [36].

Molecular techniques showed that 3 of the 124 smear positive samples were not members of the genus *Mycobacterium*, despite the fact that they were isolated from sputum smear positive patients. It may be that these organisms are *Nocardia* spp. or *Tsukamurella* spp. The incidence of Nocardiosis especially *Nocardia asteroids* complex has been on the increase due to increase in the number of immunocompromised patients during the recent decades [37]. More than 70% of patients with *nocardia* infections are immunocompromised and disseminated nocardiosis is associated with several immunocompromising conditions. *Nocardia* spp. and *Tsukamurella* spp. have also been associated with pulmonary diseases in humans [38]. All three genera [*Mycobacterium*, *Nocardia* and *Tsukamurella*] belong to the same family Actinomycetales with mycolic acid cell walls [39]. More recently, HIV infection has been described as a risk factor for disseminated nocardiosis.

In the present study, 24% of the HIV-seropositive patients had TB. This is much lower than the 40% and 32.8% prevalence of active TB reported among HIV-seropositive patients in the Nigerian cities of Ilorin [40] and Ibadan [41] respectively. However, it is higher than 10.5% reported in Northern Nigeria [42] and much higher than that in the United States [43]. Evidence from areas with high TB and HIV burden indicates a high incidence of TB/HIV co-infection [44]. The differences observed between our centre and other Nigerian centres could be due to selection factors: the other studies were conducted before free antiretroviral drugs were provided in government hospitals. Antiretroviral drugs became free of charge in Nigeria in 2005, before which only those who could afford therapy went to hospitals, sometimes as a last resort, which would have resulted in a pooling of late-stage patients at these hospitals. Several factors have been suggested to contribute to the variation of the smear positivity rate. Among them are the demographic position of the patient and their socio-economic status [45]. It has been observed that TB thrives most in communities in which poverty and destitution abound [46]. The age distribution reveals highest prevalence to be in the age group 31 - 40 yrs. (24.5%) followed by 21 - 30yrs (22.5%), which represent the most productive age group economically and correlates with work done by Onipede., *et al.* [46] at Ile-Ife. This can have a serious negative effect on socio-economic status of a country being the economically productive age group.

Out of the 24 co-infected cases, 11 (45.8%) had CD4 count less than 200 cells/µl This shows that a low CD4 count may increase the chance of developing TB. In the medical literature, there is no clear cutoff for CD4 count above which the risk for TB development is

diminished. However, there is a clear inverse correlation between CD4 count and the risk of opportunistic infections and death [47]. Smear positivity was significantly associated with severe immune-suppression (CD4 count <200 cells/µl) ($P < 0.05$). Markowitz., *et al.* [48] identified 2 major risk factors for TB progression: a positive result on a protein purified derivative test at baseline or during the study, which indicates the importance of the degree of previous or current exposure to *Mycobacterium tuberculosis*, and a low CD4 count, which shows the role of immune-depression in the development of active TB. Development of active TB is often prevented by the host's intact immune system [49], but this is the target of HIV infection. In TB/HIV co-infected patients therefore, there will be a steady deterioration in this protective capacity of the cell-mediated immunity till a critical point at which tubercle bacilli begin to proliferate and cause clinical disease [49].

Educational status shows that all the species were more prevalent among patients with low literacy level in both HIV positive and negative populations. This might be due to increased level of awareness of the risks, signs and symptoms of TB among population with high literacy level. The association of educational status with awareness of TB signs may be attributed to higher literacy. Higher level of educational attainment is often a factor for better family income. Families that have high income are able to purchase household assets like television, radio and Wi-Fi internet, and other communication appliances that increase their knowledge of health-related matters which are of public concern. Similar studies have also supported our observation that literates were more likely to be aware than the illiterates of the signs and symptoms of TB [50].

Conclusion

The data obtained in this study provides some evidence of the role of nontuberculous AFB organisms in pulmonary tuberculosis especially in HIV patients. The comparable ineffectiveness of the standard of smear test in identifying the NTM infection underscores the need for a cheaper, easier, highly sensitive and specific TB screening protocol for effective disease control. The high prevalence of clinical pulmonary tuberculosis due to *M. bovis* linked to HIV co-infection (20.8%) as reported in our study presents novel public health challenge which needs to be considered when planning prevention and treatment of the disease. The challenge therefore in most African countries and especially Nigeria still remains the introduction in a large scale of laboratory procedure for the specific identification of mycobacteria. Introduction of molecular screening assays that include rapid detection of NTM infections in high burden resource limited settings should be a priority for strengthening the public health response.

Conflict of Interest

The authors declare no conflict of interest.

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The authors conducted the study without external funding.

Authors' Contributions

UMD conceived and designed the study. AA collected the data. UMD and AA was responsible for coordination and implementation of study. UMD, AA, SCE and CE conducted data analysis and article writing. All authors read and approved the final manuscript.

Data Availability

Data for this study will made available upon request.

Consent for Publication

All the authors have read and approved submission of this research article.

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