



Differentiation and Characterization of *Mycobacterium tuberculosis* Complex Causing Pulmonary Tuberculosis across North Central Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author BA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AC and LL managed the analyses of the study. Authors KO and LA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To differentiate and Characterize *Mycobacterium tuberculosis* complex causing pulmonary tuberculosis across North Central Nigeria

Study Design: This was a simple descriptive health-based study that involved clinically suspected tuberculosis patients who were referred to two selected General Hospitals for diagnosis in each of the states across North Central Nigeria.

Place and Duration of Study: This study was carried out in the North Central zone of Nigeria and it included all the seven States across North Central Nigeria using two General Hospitals per state. The study included 371 positive sputum samples drawn from 2800 suspected pulmonary TB patients between 2017 and 2018.

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Methodology: A total of 371 GeneXpert positive sputa for TB were decontaminated using N Acetyl Cysteine and Sodium Hydroxide under a level 3 Bio-safety cabinet and the resulting sediment was cultured on Lowenstein- Jensen (LJ) media containing glycerol and pyruvate at 37°C in a slanted position, SD- Bioline (TB Ag MPT 64) for the differentiation of MTBC from NTM was carried out using the isolates from LJ culture. Evaluation of speciation was done using Line Probe Assay to determine the predominant species of MTBC. All the protocols used in this study followed the manufacturer's manual strictly.

Results: A total of 371 decontaminated positive GeneXpert sputa derived from 2800 suspected PTB participants were cultured on Lowenstein- Jensen (LJ) medium and 302(81.40%) was found positive while 69(18.60%) were found negative. Out of the culture positive isolates, 288 (95.36%) isolates were detected on SD-BIOLINE TB Ag MPT 64 ® assay for MTBC and 14 (4.64%) as NTM. Of the 288 MTBC, three different species were identified; 272 (94.64%) were *M. tuberculosis*/*M. Canetti*, 7 (2.43%) were *M. africanum* and 9(3.13) showed a no MTBC reaction band on all the samples that were analysed.

Conclusion: Differentiations of MTBC from NTM has help to re-confirm that not all symptoms of pulmonary infection are caused by MTBC but NTM are implicated due to their distribution in the environment, however, molecular characterisation method has narrow our findings down to *M. tuberculosis*/*M. canettii* as the predominant specie of MTBC circulating in the region, although, *M. africaum* was also detected and these two species of MTBC are the leading cause of pulmonary tuberculosis across all the North Central state of Nigeria.

Keywords: *Mycobacterium tuberculosis*; speciation; SD-bioline; pulmonary tuberculosis; North Central; predominant.

1. INTRODUCTION

Mycobacterium tuberculosis complex (MTBC) are the causative agent of Tuberculosis infection [1]. It typically affects the lungs (pulmonary TB) but can also affect other parts of the body (extrapulmonary TB). Infection is usually by inhalation of respiratory droplets, coughed up or sneezed out by a person with active pulmonary tuberculosis (PTB) [1,2]. It can also be transmitted by injecting bacteria from hands and contaminated utensils [3,4].

TB was named among the top 10 causes of death and the major problematic disease agent ranking above HIV/AIDS and several are infected every year all around the world [5].

It was estimated in 2017 that TB caused about 1.3 million deaths (range, 1.2–1.4 million) among HIV-negative people and 300 000 deaths (range, 266 000–335 000) was recorded for HIV-positive people. Worldwide [6], about 10.0 million people (range, 9.0–11.1 million) were estimated to developed TB disease as well as 5.8 million men, 3.2 million women and 1.0 million children [6]. These was recorded in all countries and age groups, although, 90% were adults (aged ≥15 years), 9% were living with HIV (72% in Africa). India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%) comprise the major burden of infection [6].

In WHO list of 30 high TB burden countries, 22 of those countries accounted for 87% of TB cases recorded around the world while Only 6% of the global cases were indicated in European Region and Region of the Americas having (3%) each [5]. Nigeria currently ranks 7th in the world among the 30 countries with the highest burden of TB, TB/HIV, multi drug resistant TB. and 2nd in Africa after South Africa. The burden of tuberculosis is particularly high amongst newly diagnosed HIV-infected children [7].

The severity of national epidemics varies widely among countries. In 2017, there were fewer than 10 new cases per 100 000 populations in most high-income countries, 150–400 in most of the 30 high TB burden countries, and above 500 in a few countries including Mozambique, the Philippines and South Africa [5]. However, adequate TB surveillance programs depend on the capacity to quickly diagnose new cases, to provide an appropriate therapy, and to correctly detect outbreaks in the community, in order to implement proper TB control and elimination strategies [8,9].

The use of molecular techniques to characterize *M. tuberculosis* complex and the concomitant accumulation of epidemiological data has increased the possibility of tracing the transmission routes of *M. tuberculosis* complex outbreaks. [10]. Although, one of the biggest

concerns in TB control is the dispersion of strains with resistance to first-line antibiotics [11], hence, Knowledge of circulating species is required, especially in developing countries. Thus, identification of species circulating in a certain geographic region using molecular tools can contribute to the prospect of improving or developing new vaccines and drugs that can address the issue of resistant strains [12].

Pulmonary Tuberculosis is caused by members of *Mycobacterium tuberculosis* complex (MTBC), However, there are non-tuberculous mycobacteria (NTM) that present the same clinical manifestation as MTBC which makes it difficult to rely on these clinical manifestations for diagnosis for PTB, hence there is need for molecular based differentiation and speciation. Therefore, the aim of this study is to differentiate and characterize *mycobacterium tuberculosis* complex causing pulmonary tuberculosis across North Central, Nigeria

2. MATERIALS AND METHODS

2.1 Study Area and Study Population

This study was carried out using two general hospitals each across the seven states in North Central Nigeria. The North central consists of the seven states situated geographically in the middle belt region of Nigeria, spanning from the west, around the confluence of River Niger and River Benue. It comprises of Niger, Kogi, Benue, Plateau, Nassarawa, Kwara and FCT- Abuja [13]. The study included 371 positive sputa samples drawn from 2800 suspected pulmonary TB patients between 2017 and 2018 using simple random sampling method. High TB patient flow, existence of better diagnostic facilities, DOTS Centers and skilled human resource were the major reasons for selecting the specified health facilities in the seven states.

2.2 Study Design

The study was a health institution-based, descriptive cross-sectional and the sample size was determined using Taro Yamane formula [14] at 95% confidence level.

$$n = N / (1 + N(e)^2)$$

n= sample size required.

N = number of people in the population.

e = allowable error (%) or the level of precision.

For Nasarawa State;

N= population= 1869377

(e)= 0.005

$$n = N / (1 + N(e)^2)$$

$$n = 1869377 / 1 + 1869377 \times (0.02)^2$$

$$n = 1869377 / 1 + 4673.4425$$

$$n = 1869377 / 4674.4425$$

$$n = 399.9$$

n is approximated to 400

The total from the calculation of each states was approximated to 400 and 200 were assign to each general hospital per state.

2.3 Sample Collection

Clinical examination of patients with suspected pulmonary TB was performed by the attending physicians and two consecutive 3-5 ml spot and early morning sputum samples were collected from the participants in a dry, clean, transparent, leak proof, and open-mouthed container for diagnosis of MTB/RIF using GeneXpert while a separate sputum was collected alongside in case of confirmed MTBC positive. After diagnosis, positive TB participant were referred to DOTS centers for treatment as provided by the National TB Control Program guideline in collaboration with World Health Organizations [15,16]. GeneXpert Positive sputum samples were stored at 2-8°C in the fridge and then transported in a cold box (at +4°C) to Zankli TB Research laboratory, Bingham University, Karu, within a week of collection for cultured.

2.4 Decontamination of Sputum

All direct sputum samples from patients that were positive for GeneXpert were subjected to treatment using N Acetyl Cysteine and Sodium Hydroxide (NALC and NAOH) method under the biosafety cabinet in accordance with Centre for Disease Control (CDC) guidelines for public health mycobacteriology [17]. Decontamination of sputum was done by adding 1 ml of 4% Sodium hydroxide (NaOH) to 1 ml of sputum to make 2 ml of solution inside the falcon tubes, the caps were tightened and mixed well using a Vortex machine inside the biosafety cabinet and allowed to stay for 15 minutes. Two milliliters of sterile water were added to 2 ml of the solution, to make up to 4 ml. These (sputum + NaOH + sterile water) were centrifuged at 300 rpm for 15

minutes. The falcon tubes were gently removed from the centrifuge and the supernatant were discarded and the sediment was stored for culture.

2.5 Sputum Culture for MTB

The sediments obtained were inoculated onto Lowenstein- Jensen (LJ) media containing glycerol which favors the growth of *M. tuberculosis* and a separate LJ media containing pyruvate which favors the growth of *M. bovis* were used for each Sputum sample. The inoculated media were then incubated at 37°C in slanted position for 1 week and upright position for 4–5 weeks. The growth of the bacteria was read every week until the 8 weeks of culture. Cultures were considered positive if they showed a growth on Lowenstein Jensen medium [18].

2.6 SD-Bioline for the Differentiation of MTBC from NTM

Colonies from L.J media were emulsified in about 200µml of sterile buffered saline, then 100µml of the suspension added into the sample wells of TB Ag MPT 64 (SD Bio-line) cassette and allowed to stay for 15 minutes before being read. Positive result were indicated by the presence of only two-color band (one control band and one test band). The presence of only one control band within the result window indicated a negative result. Faint color band was recorded as a weak positive and the sample retested. If test results were negative, isolates were considered NTM species [19].

2.7 Line Probe Assay (Geno Type MTBC)

2.7.1 DNA extraction

The DNA of colonies on L J media that were differentiated into MTBC were extracted using Geno-Lyse kit (Hain Life-science GmbH, Nehren, Germany) base on manufacturer's instructions. Extracted DNA was processed for Line Probe Assay for detection of MTB complex.

2.7.2 DNA amplification

Amplification mixture (45 µl) was prepared in DNA free room, including 5 µl extracted DNA (20-100 ng DNA) in the reaction mixture contained 35 µl primer nucleotide mix, 5 µl 10 × polymerase incubation buffer for HotStar Taq (QIAGEN, Hilden, Germany), 2 µl 25 mM MgCl

solution, 0.2 µl HotStar Taq and 3 µl water (biology grade water). Amplification was carried out in a thermal cycler (MJ Research, PTC-100 Thermal Cycler, GMI, Inc, USA), which involved 1 cycles of denaturation solution (DEN) at 95°C for 5 minutes, annealing of primers at 95°C for 30 s, 2 minutes at 58°C for 10 cycles, then 20 cycles at 95°C for 25 s, 53°C for 40 s and 70°C for 40 s and final primer extension at 70°C, 8 minutes for 1 cycle.

2.7.3 DNA Hybridization

Briefly, 20 µl of Denaturation Solution was dispensed in the corner of each of the wells used and then 20 µl of amplified sample was added by pipetting up and down to mix well and incubated at room temperature for 5 minutes. At least 1 ml of pre-warmed Hybridization Buffer was added carefully to each well followed by gently shaking the tray. A strip was placed in each well using tweezers in a manner to make sure complete flooding of solution over strips. Then tray was placed in shaking TwinCubator and was incubated for 30 minutes at 45°C followed by complete aspiration of Hybridization Buffer. Washing was done using 1 ml of Stringent Wash Solution to each strip and incubated for 15 minutes at 45°C in shaking TwinCubator. The work was being done at room temperature from this step forward with Complete removal of Stringent Wash Solution [32]. Again, each strip was washed once with 1 ml of Rinse Solution for 1 minute on shaking TwinCubator. Then 1 ml of diluted Conjugate was added to each strip and incubated for 30 minutes on shaking TwinCubator. The solution was removed and each strip washed twice for 1 minute with 1 ml of Rinse Solution and once for 1 minute with 1ml of distilled water on shaking TwinCubator. 1 ml of diluted substrate was added to each strip and incubated and protected from light without shaking for 3-20 minutes.

As soon as bands were clearly visible, brief rinsing was done twice with distilled water to stop the reaction. Strips were removed from the tray and dried between two layers of absorbent paper using tweezers. Evaluation and interpretation of the results were done based on the presence and absence of different bands and compared with reference band provided in the kit [20,21].

3. RESULTS

A total of 371 decontaminated positive GeneXpert sputa was culture on Lowenstein-

Jensen (LJ) media and Table 1 showed a total of 302(81.40%) was found positive why 69(18.60%) were found negative. Out of the culture positive isolate, 288 (95.36%) isolates were detected on SD-BIOLINE TB Ag MPT 64 ® assay for MTBC and 14 (4.64%) as NTM. Table 1 and shows the TB Ag MPT 64 (SD Bio-line) test results and percentage of MTBC to NTM respectively. Fig. 1 showed the distribution of MTBC and NTM on histogram.

Amplification and hybridization controls (UC and CC, respectively) was used to verified the test procedures of Geno-Type MTBC assays and it gave unequivocal results and none of the

amplification reactions were inhibited. A higher percentage of test isolates investigated showed the pattern *M. tuberculosis/M. canettii* as the most predominated organisms. The identification results for all isolate were in complete accordance with the interpretation chart provided with the kit. Only those strips, that developed both control bands, UC (Universal Control) and CC (Conjugate Control), were regarded as interpretable. Of the 288 MTBC, three different species were identified, 272 (94.64%) were *M. tuberculosis/M. Canetti*, 7 (2.43%) were *M. africanum* and 9(3.13) showed a no MTBC reaction band across the states in North central Nigeria. Table 2

Table 1. Sputa culture of MTBC isolates and analysis by SD-BIOLINE to differentiate MTBC from NTM across state in North Central, Nigeria

State	Total number of positive sputa by GeneXpert	Total number of culture positive	Total number of culture negative	Total number of NTM	Total number of SD-bioline positive for MTBC
Abuja	44(11.86%)	41(13.58%)	3(4.35%)	0(0%)	41(14.24%)
Benue	64(17.25%)	54(17.88%)	10(14.49%)	4(28.57%)	50(17.36%)
Kogi	46(12.40%)	39(12.91%)	7(10.15%)	1(7.14%)	38(13.19%)
Kwara	57(18.57%)	40(13.25%)	17(24.64%)	2(14.29%)	38(13.19%)
Nasarawa	46(12.40%)	41(13.58%)	5(7.25%)	1(7.14%)	40(13.89%)
Niger	64(17.25%)	51(16.89%)	13(18.84%)	5(35.71%)	46(15.97%)
Plateau	50(13.48%)	36(11.92%)	1(1.45%)	1(7.14%)	35(12.15%)
Total	371(13.25%)	302(81.40%)	69(18.60%)	14(4.64%)	288(95.36%)

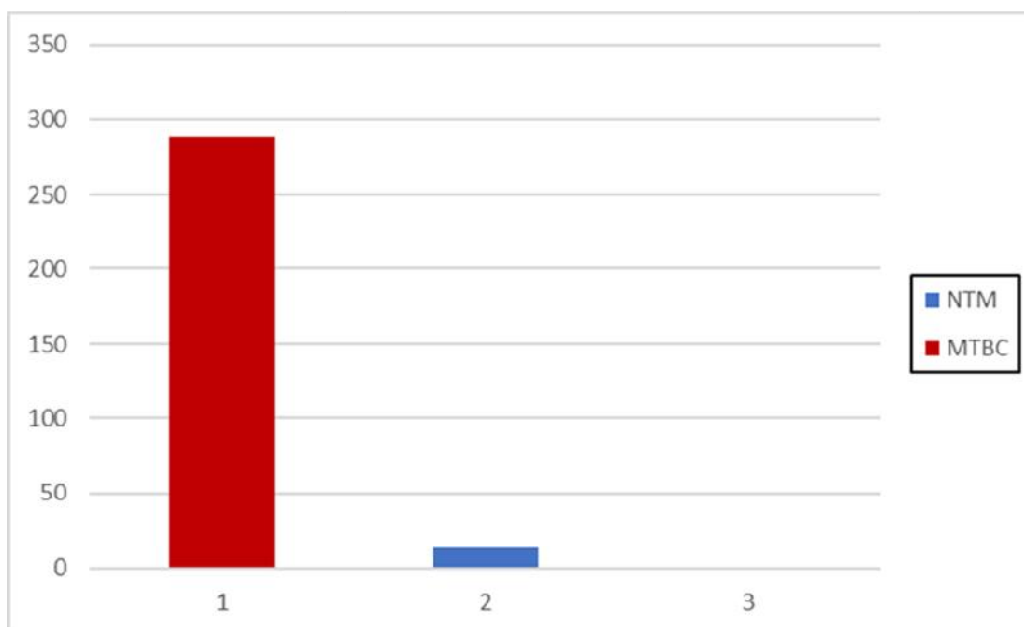


Fig. 1. Distribution of *M. tuberculosis* complex and non-tuberculous in the Population

Table 2. Speciation and Distribution of MTBC Detected Across the States in North Central, Nigeria

State	MTBC positive	<i>M Tuberculosis/ M Canetti</i>	<i>M Africanum</i>	No MTBC Reaction
Abuja	41(14.24%)	37(12.60%)	2(28.57%)	2(22.22%)
Benue	50(17.36%)	48(17.65%)	1(14.29%)	1(11.11%)
Kogi	38(13.19%)	36(13.24%)	1(14.29%)	1(11.11%)
Kwara	38(13.19%)	35(12.87%)	1(14.29%)	2(22.22%)
Nasarawa	40(13.89%)	38(13.97%)	1(14.29%)	1(11.11%)
Niger	46(15.97%)	44(16.18%)	0(0.00%)	2(22.22%)
Plateau	35(12.15%)	34(12.50%)	1(14.29%)	0(0.00%)
Total	288(95.36%)	272(94.44%)	7(2.43%)	9(3.13%)

4. DISCUSSION

Tuberculosis is caused by members of *Mycobacterium tuberculosis* complex (MTBC), which includes *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetti* and *M. microti*. And recently, diseases caused by mycobacteria other than tuberculosis (MOTT), also known as non-tuberculous mycobacteria (NTM) are on the rise. NTM infection has also been found to be one of the causes of opportunistic infection diseases such as pulmonary infection, especially in immunocompromised patients such as human immunodeficiency virus (HIV)-infected patients, patients on immunosuppressant therapy, those with TB and diabetes [22,23]. To isolate and characterize *mycobacterium tuberculosis* causing pulmonary TB, it is therefore imperative to accurately differentiate mycobacteria since NTM are usually found abundant in the environment causing respiratory infection, are resistant to conventional anti-tuberculous drugs, require modified treatment regimens and are often misdiagnosed as multi-resistant tuberculosis (MDR) [24] and “SD BIOLINE TB Ag MPT 64 Rapid ®” was used as simple and rapid immune-chromatographic in differentiating Mycobacteria into *Mycobacterium tuberculosis* (NTM).

Culture isolates were derived from 2800 sputa of suspect TB patients across North central of Nigeria with a total number of 371(13.2%) sputa found positive for GeneXpert in this study. All positive sputum was cultured on LJ media to produce 302(81.40%) positive isolates of which 69(18.60%) were culture negative. The percentage of positivity on LJ media is high as compare to 18.60% non-growth. The percentage of negative could probably be justified base on the fact that Culture still relies on relatively cumbersome and lengthy process starting with the collection of appropriate clinical specimens

and their transport to the laboratory, decontamination of clinical specimens likely to be contaminated by a commensal flora, inoculation and incubation of appropriate media, growth detection and mycobacteria identification. In addition, it could perhaps be due to technical error i.e. competency of the performers.

Among the pulmonary TB patients examined in this current study, 288(95.36%) were found to be MTBC positive and 14 (4.64%) NTM isolates were detected. A close percentage was also recorded in a study conducted by Ibrahim *et al*/ who recorded that 31(77.5%) were characterized as MTBC, while nine (22.5%) were NTM [19]. Since the Identification of MTBC and detection of NTM is based on growth acceleration and colony morphology. The detection of 4.64% NTM in this study could probably be as a result of mix growth of both MTBC and NTM because technically GeneXpert will only pick MTBC especially if it is more in population than NTM, however, culture will show the growth of NTM within the first two weeks of incubation and might probably out grown MTBC species. This could also be the reason why SD- BIOLINE test was negative for MTBC, indicating the presence of NTM. This was also in support with the study done by Shahraki *et al*, who reported that an increase in coexistence detection of NTM species in patients with pulmonary TB with anti-TB therapy can occur at any point after therapy program is completed [24]. A study conducted in Nigeria also support the coinfection of NTM among Tuberculosis suspect in another location [25]. Other reports also indicate that Isolation of NTM as species from clinical specimens were detected from the respiratory tract in TB-endemic countries in addition to MTBC as coinfection [26]. Another reason for the outcome of the presences of NTM in this study could also be attributed to the indices of drug resistance which are also reported in the study. It has been reported that

misdiagnosis of NTM disease as MDR-TB frequently occurred in clinical settings because of similar clinical manifestations, especially in high-MTBC-burden countries and different NTM species exhibited various pathogenicity with antibiotic susceptibility patterns, hence NTM species were most often found to be resistant toward the first-line anti-TB drugs and this was supported by different findings [27,28]. However, NTM detection in this study could probably be interpreted on basis of whether MTBC isolated were causative pathogens of pulmonary infection since colonization or contamination of various NTM species are commonly found in human or animal environment. Therefore, differentiations of MTBC from NTM by molecular method has help to narrow down the type of Mycobacteria circulating in the North Central of Nigeria.

On the basis of Speciation, the commercial Geno-Type MTBC DNA strip assay (Hain Life-science GmbH) used in this study does not differentiate *M. canettii* from *M. tuberculosis* [29], however, the study speciated MTBC isolates obtained from the sputum of suspected pulmonary TB patients across all the states in North Central Nigeria. A total of two hundred and eighty-eight (288) positive MTBC isolates from seven states; Abuja (41), Benue (50), Kogi (38), Kwara (38), Nasarawa (40), Niger (46) and Plateau (35) were used to determine the species of MTBC using Hain Genotype technique. The results obtained were 272(94.44%) of the species of *M. tuberculosis/M. canettii*, 7(2.43%) was detected for *M. africanum* and 9(3.13%) was recorded for Non interpretable strips. No other species of MTBC were identified from the isolates. This study showed that *M. tuberculosis* and *M. canettii* shared the same band strips hence, the commercial Geno-Type MTBC DNA strip assay (Hain Life-science GmbH) used in this study does not differentiate *M. canettii* from *M. tuberculosis* and this is because it has been documented that *M. canettii* may be merely a subspecies of *M. tuberculosis* [30,31]. However, spoligotyping will differentiate the variance of the subspecies genotype. This study recorded *M. tuberculosis/M. canettii* as the highest predominant species of MTBC circulating in the North central zone and it is the leading cause of pulmonary tuberculosis. *M. tuberculosis/M. canettii* showed (94.44%) frequency while *M. africanum* showed (3.1%). Previous studies also reported *Mycobacterium tuberculosis* as the predominate species from the mycobacterial family around the world especially in sub-Saharan Africa. In Kenya

97.6% were reported for *M. tuberculosis*, 1.7% for *M. africanum* and 0.7% recorded for *M. bovis* [32]. in Cameroun, Niobe-Eyangoh *et al* also reported a similar result with very low prevalence for *M. Bovis* and high proportions of *Mycobacterium tuberculosis* and slight proportion of *M. africanum* [33]. Another research work in Tanzania also supported this present work [34]. Ndadilnasiya *et al* also identified *M. tuberculosis* (89.2%) and *M. africanum* (10.8%) as species of MTBC causing human tuberculosis in Zaria, Nigeria [35]. In North central of Nigeria, a previous work done by Sani *et al* reported that (99.2%) were *Mycobacterium tuberculosis*, (0.4%) recorded for *Mycobacterium africanum* and (0.2%) for *Mycobacterium bovis* [36]. This present study did not record any species of *Mycobacterium bovis*. However, the lack of detection of *Mycobacterium bovis* in this study does not rule out possibility of zoonotic transmission of bovine tuberculosis in the region, as there are various cultural practices such as herdsmen co-existing with their cattle and direct ingestion of unpasteurized milk and milk products, which could serve as vehicle for the transmission of *M. bovis* between cattle and humans. This agreed with findings in southern part of Nigeria that reported 5% occurrence of *M. bovis* [37]. This present study also reported a 9(3.13) Non MTBC reaction band. The reasons for No MTBC interpretable strips may probably be due to reagents not equilibrated to room temperature; addition of insufficient reagents, improper mixing of reagents, addition of reagents in incorrect amounts; improper immersion of strips in the reagents during incubation; improper washing of strips and improper storage, transport or preparation of specimen. Therefore, the Species of MTB complex detected in this study are the primary cause of PTB in humans in North central of Nigeria.

5. CONCLUSION

Differentiations of MTBC from NTM has help to re-confirm that not all pulmonary infection are caused by MTBC but other NTM are implicated due to their distribution in the environment, however, molecular characterization method has narrow our findings down to *M. tuberculosis/M. canettii* as the highest predominant specie of MTBC circulating in the region, although, *M. africanum* was also recorded in this study. These two species of MTBC are the leading cause of pulmonary tuberculosis across all the North Central states of Nigeria.

6. LIMITATION OF THE STUDY

The identification of clustered and strains would have given more knowledge to the genetic diversity or relatedness of *Mycobacterium tuberculosis* complex isolated from pulmonary patients in the North Central of Nigeria. More work should be targeted towards genotyping the species isolated from not central Nigeria.

CONSENT AND ETHICAL APPROVAL

Ethical approval was granted by the ministry of health in all the respective states while informed and written consent was also obtained from the participants and questionnaire were administered with their name and status kept confidential. The following are Ref No from the ministry of health in each state of North Central Nigeria.

Ministry of Health FCT-Abuja FHRCE/2017/01/110/11-12-17 (FCTA/HHSS/HMB/GEN/038/T)

Ministry of Health Benue State- MOH/STA/204/Vol.1/97

Ministry of Health Niger State- STA/495/Vol/136

Ministry of Health Nasarawa state- NHREC/18/06/2017 (DASH/L/ADM/0340)

Ministry of Health Kogi State- MOH/PRS/465/V1/001

Ministry of Health Plateau- MOH/MIS/202/Vol.T/x

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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