Original

Speciation and Characterization of Mycobacterium Tuberculosis Complex Isolated from Multi-Drug Resistance Tuberculosis Patients in Southeastern Nigeria

[1,2,4]Gweba C, [2]Ojule IN, [1]Panwal MT, [3]Girei RS, [1,3]Mamman KE

[1] *Institute of Human Virology, Nigeria*
[2] *University of Port Harcourt, Nigeria*
[3] *Northeast Zonal TB Reference Laboratory, Yola, Nigeria*
[4] *Southeast Zonal TB Reference Laboratory, Umuahia, Nigeria*

Corresponding author: Gweba Cornelius, *Institute of Human Virology, Nigeria, University of Port Harcourt, Nigeria, Southeast Zonal TB Reference Laboratory, Umuahia, Nigeria; gwebacornelius@gmail.com; +2348035697384*

Article history: Received 10 November 2023, Reviewed 21 December 2023, Accepted for publication 23 December 2023

This is an open access journal and articles are distributed under the terms of the Creative Commons Attribution License (Attribution, Non-Commercial, "Share Alike" 4.0) - (*CC* BY-NC-SA 4.0) that allows others to share the work with an acknowledgement of the work's authorship and initial publication in this journal.

How to cite this article:
Gweba C, Ojule IN, Panwal MT, Girei RS, Mamman KE. Speciation and Characterization of Mycobacterium Tuberculosis Complex Isolated from Multi-Drug Resistance Tuberculosis Patients in Southeastern Nigeria. The Nigerian Health Journal 2023; 23(4): 874 - 879. DOI: http://www.doi.org/10.60787/tnhj-707

Abstract

Background: In Nigeria and other low-and-middle-income countries, majority of clinical laboratories use Gene Xpert MTB/RIF assay, Hain lifescience MTBDR*plus* and MTBDR*sl* for rapid diagnosis of both TB and MDR-TB. These tools diagnose TB disease caused by members of *Mycobacterium tuberculosis* complex (MTBC), but do not differentiate among members of the MTBC to the species level. Knowledge of the circulating strains of MTBC in a population is critical for effective TB control measures.

Method: This was a descriptive cross-sectional study amongst confirmed MDR-TB patients who were on MDR-TB treatment regimen from the Southeastern part of Nigeria. DNA material of MDR-TB strains cultivated on BACTEC MGIT 960 instrument were extracted and differentiated into species using Hain lifescience Geno Type MTCB kit for speciation. Interpretation and validation of result was done using Hain lifescience Genoscan.

Result: A total of 96 MTBC isolates were differentiated into species. Of the 96, 58.30% (n=56) were *M. tuberculosis/M. canettii*, 11.45% (n=11) were *M. africanum* and 2.10% (n=2) were *M. bovis*. However, 11.45% (n=11) were high gram-positive bacteria, 14.60% (n=14) were invalid, while 2.10% (n=2) had no evaluable pattern.

Conclusion: Findings from the study shows that *M tuberculosis/M. canettii* and *M. africanum* are the leading causes of MDR-TB amongst the study population. Furthermore, the results of this study indicate that *M. bovis* remains an important cause of tuberculosis particularly amongst groups at risk.

Keywords: *Mycobacterium tuberculosis* complex, Species, MDR-TB, Diagnosis, Nigeria.

Downloaded from www.tnhjph.com

Introduction

Tuberculosis (TB) has been described as an ancient disease whose elimination has been man's desire throughout history.[1] It is a leading cause of death from a single infectious agent.[2] It is estimated that one third of the world's population is infected with TB in both developed and developing countries.[3] In 2020, TB morbidity and mortality rates were 10 million and 1.5 million respectively, this is in addition to the neglected burden of other ongoing morbidity such as respiratory impairment, reduced health related quality of life and psychosocial challenges that TB survivors must endure after completion of treatment.[4] Prior to the 19th century, not much was known about the causative agent of TB until March 24th 1882, when Dr. Robert Koch described the aetiology of TB disease in Berlin and presented *Mycobacterium tuberculosis* as the causative agent.[1] The identification of the causative agent of Tuberculosis by Robert Koch paved the way for additional studies aimed at understanding the aetiology of TB disease which in turn led to more research into development of modern therapy and more effective TB control measures.

The genus *Mycobacterium* is made up of 163 species and additional 13 subspecies as described in the list of approved names of bacterial species.[5] Human and animal TB cases are known to be caused by closely related *Mycobacterium species* which are collectively referred to as *Mycobacteria tuberculosis* complex (MTBC). Strict human pathogens among the complex include *M. tuberculosis*, *M. africanum* and *M. canettii,* while animal adapted pathogens are *M. pinnipedii* (sea lions and seals), *M. microti* (rodents), *M. bovis* (cattle and sheep), M. *caprae* (goats), *M. mungi* (mongoose), *M. suricattae* (meerkat) and *M. orygis* (antelope). *M. canettii* is considered to be an environmental microbe which has been implicated in causing opportunistic infections in human. *M. canettii* is however, regarded as a member of MTBC based on its nucleotide identity.[6,5,1] Despite the host specificity observed amongst MTBC, occasional cross-species infection has been reported with some members of MTBC having global distribution while others are geographically restricted to a specific human population.[1] Hence, identification and differentiation of the causative organisms of Tuberculosis at the species level will guide epidemiological interventions towards TB eradication.

Study objective

In Nigeria and other low and middle-income countries, majority of clinical laboratories use GeneXpert MTB/RIF assay, Hain lifescience MTBDR*plus* and MTBDR*s* tools for rapid diagnosis of both drug sensitive and drug resistant tuberculosis. These test tools diagnose TB disease caused by members of MTBC, but do not differentiate between the MTBC into different species. The objectives of this study were to cultivate and characterize the different species of MTBC from sputum samples of MDR-TB patients from South-Eastern Nigeria. Diagnosing and characterizing TB disease to the species level will help to identify the predominant MTBC species implicated in causing TB disease in that locality. Characterizing the species will be invaluable in laboratory and clinical decisions ultimately geared towards better management and preventive measures to halt the spread of TB in the community.

Method

Study Design

This was a descriptive cross-sectional study. It involved characterization of MTBC species that were isolated from sputum samples submitted by MDR-TB patients from January to December 2020. As part of standard of care and treatment monitoring, MDR-TB patients are required to submit sputum samples monthly for TB culture.

Study Population

Study participants were confirmed MDR-TB patients who were on approved MDR-TB treatment regimen at different months of treatment. Participants were from the Southeastern part of Nigeria which comprises of Abia, Anambra, Ebonyi, Enugu and Imo states. Their sputum samples are usually sent to the Southeast Zonal TB Reference Laboratory, located at Specialist Hospital Amachara Umauhia, Abia state, Nigeria.

Ethical Consideration

Ethical approval for the study was granted by the ethics and research committee of Southeast Zonal TB Reference Laboratory, Specialist Hospital Amachara.

Data collection procedure

Decontamination of Sputum

Sputum samples submitted by patients for monthly treatment monitoring were processed for TB culture using N-Acetyl-L-cysteine and Sodium Hydroxide (NALC and NaOH) decontamination method under strict biosafety measures in accordance with Nigerian National TB standard Operating Procedures for Laboratory.[7] An equal volume of NALC-NaOH solution (4% sodium hydroxide) was added to sputum

Downloaded from www.tnhjph.com
Print ISSN: 0189-9287 Online ISSN: 2992-345X

sample contained in a 50mls falcon tube, it was placed in a vortex mixer and allowed to stand for 15 minutes. Sterile phosphate buffer saline was added to the solution to make up to 45mls. The solution was capped well, arranged in centrifuge cups, and centrifuged at 4°C for 15 minutes at 3000rpm. After centrifugation, falcon tubes were removed gently from the cups, the supernatant was discarded, and the sediments were re-suspended using 2mls of phosphate buffer saline. The re-suspended sediments were inoculated into MGIT tubes and incubated on BACTEC MGIT 960 instrument.

MTBC Isolates and Extraction of DNA
BACTEC MGIT 960 instrument signals positive tubes, which were flagged out of the instrument and follow-up investigations were performed on the positive MGIT tubes to determine the presence of Acid-Fast Bacilli (AFB). To mitigate the risk of bias arising from over representation of patients with multiple positive culture, one positive MGIT tube was selected for patients with more than one positive culture. Following AFB confirmatory test using Ziehl Neelsen staining techniques, *M. tuberculosis* DNA was chemically extracted from AFB positive MGIT tubes using Hain Lifescience Geno Lyse extraction kit in accordance with manufacturer's instruction. 1ml of liquid medium from positive MGIT tube was transferred into a 1.5ml crew cap tube and centrifuged at 10000xg for 15 minutes. The supernatant was discarded, and the pellet re-suspended in 100µl lysis buffer by vortexing. The solution was incubated at 95°C for 5 minutes and 100µl neutralization buffer was added to the solution, centrifuged for 5 minutes at 10,000xg and the extracted DNA contained in the supernatant was transferred to a new 1.5ml crew cap tube.

DNA Amplification
The Hain Lifescience Geno Type MTBC series was used for speciation of *M. tuberculosis* complex. The reagent which contains polymerase and primes included in amplification mix (AM) AM-A and AM-B was prepared in a clean room free of DNases. Each reaction tube contains 10µl of AM-A and 35µl AM-B to which 5µl of DNA was added in a separate room resulting in reaction volume of 50µl. Amplification was performed using amplification protocol of 1 cycle of 15 minutes of denaturation at 95°C, then 10 cycles of 30 seconds at 95°C and 2 minutes at 65°C, additional 20 cycles consisting of 25 seconds at 95°C, 40 seconds at 50°C and 40 seconds at 70°C, and finally, an extension of 8 minutes at 70°C. Applied biosystems (Thermo Fisher Scientific) 2720 Thermal cycler was used for the amplification reaction.

Reverse Hybridization and Interpretation
Hain Lifescience equipment and hybridization reagents were used for the detection of amplicons generated from the amplification reaction. Hybridization commenced with mixing 20µl of amplified product with 20µl of denaturation reagent in a separate well of a plastic tray and allowed to stay for 5 minutes. This was followed by the manual addition of 1 ml of pre-warmed hybridization buffer, followed by placement of membrane strips in their respective wells and allowed to be completely covered by the hybridization buffer. The reaction tray was placed on shaking twincubator for an incubation period of 30 minutes at 45°C. After the incubation, hybridization buffer was completely aspirated followed by addition of 1 ml of pre-warmed stringent wash and was incubated for 15 minutes at 45°C in a shaking twincubator. The stringent wash was removed at the end of the incubation, strips were washed with 1 ml rinse solution, followed by the addition of 1 ml diluted conjugate solution, and incubated for 30 minutes at 25°C. At the end of incubation, strips were washed twice with 1ml rinse solution and 1ml distilled water and then 1ml diluted substrate solution was added and incubated for 5 minutes away from light. The added substrate was converted into a dye which became visible as a colored precipitate on the membrane strips. The reaction was stop by rinsing with distilled water and the strips were allowed to dry. Hain Lifescience GenoScan was used for interpretation and evaluation of the strips.

Results
A total of 130 MGIT tubes which were flagged positive by BACTEC MGIT 960 instrument were confirmed using AFB smear microscopy. One positive tube was selected for patients with more than one positive MGIT tubes. DNA materials were extracted from 96 isolates which were used for speciation. Of the 96 isolates, 56 (58.30%) were *M. tuberculosis/M. canettii,* 11 (11.45%) were *M. africanum,* 2 (2.10%) were *M. bovis,* 11 (11.45%) were high gram-positive bacteria, 14 (14.60%) were invalid and 2 (2.10%) have "No evaluable pattern" in the current Hain Lifescience package insert of the Geno Type MTBC series.

Downloaded from www.tnhjph.com
Print ISSN: 0189-9287 Online ISSN: 2992-345X

Table 1: Proportion of MTBC species

Mycobacterium species	No.	Percent (%)
M. tuberculosis/M. canettii	56	58.30
M. africanum	11	11.45
M. bovis	2	2.10
High gram-positive bacteria	11	11.45
Invalid	14	14.60
No evaluable pattern	2	2.10
Total	**96**	**100**

Discussion

The main objective of this study was to characterize MTBC strains cultivated from MDR-TB patients from southeastern Nigeria into species. Findings from the study shows that more than half of the study population were found to be infected with *Mycobacterium tuberculosis/M. canetti*, while slightly more than one tenth of the study population were found to be infected with *Mycobacterium africanum* and *Mycobacterium bovis* was found to be responsible for MDR-TB in relatively small fraction of the study population. Finding from this study agrees with the known global distribution of *M. tuberculosis*.[8] *M. canettii* was tied to *M. tuberculosis* in this study because both have identical biochemical attributes as well as 16s rRNA sequence. Also, the molecular method used in this study is not sensitive enough to differentiate between *M. tuberculosis* and *M. canettii*. One account described *M. canettii* as an ancestor of *M. tuberculosis*, although it was discovered much later than *M. tuberculosis* in 1965. It has been described as an emerging pathogen especially in the Horn of Africa where clinicians have correlated its signs and symptoms with lymphatic and abdominal TB.[9] A history of a visit or residence in countries in the Horn of Africa should alert clinicians to consider *M. canettii* in making a diagnosis of TB. In year 2022, study conducted amongst MDR-TB patients in Plateau State, North central Nigeria, reported that over 90% of the study population had sputum that were positive for *M. tuberculosis* species.[10]

Also, another study in North Central Nigeria that employed the same molecular technique as the one used in this study, however among drug susceptible patients, reported that 94.4% of their study population were infected with *M. tuberculosis/M. canetti.*[11] Hence, findings from previous studies and this current study suggests that *M. tuberculosis* is the predominant species responsible for TB disease in both drug susceptible and MDR-TB patients in Southeast and North Central Nigeria. Different studies which utilized various molecular techniques such as whole genome sequencing, mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) and spoligotyping have been conducted to investigate the phylogeographic identity of MTBC in West Africa. Findings shows that *M. tuberculosis* is the dominant species circulating in the region. A Cameroon lineage of *M. tuberculosis* has been identified to be the commonest *M. tuberculosis* strain circulating in the West African region. The reason for this is not far-fetched. The cross border migration which is facilitated by the Economic Community of West African States Treaty, a multilateral agreement signed by the member states to increase the ease of doing business, has through economic activities, facilitated the circulation of the MTBC lineage in the region.[9,1]

M. africanum was found to be responsible for MDR-TB in 11.45% of the study population. This species is most commonly found in West Africa and some scientists identified its special genetic adaptation to West African populations.[7,10] A study in North Central Nigeria, reported *M. africanum* to be responsible for drug susceptible TB in 3.1% of their study population.[11] With geographical restriction and adaptation to West Africa, *M. africanum* accounts for about 40% of human TB cases in the West African region,[12] yet active research on this clinically important mycobacterial specie is still inadequate. Both epidemiologically and clinically, *M. africanum* is distinct from *M. tuberculosis* in several respects viz; it is slow growing taking up to 10 weeks or more to develop colonies against 3 to 4 weeks for *M. tuberculosis*.[12] This knowledge gives the impetus to laboratory personnel in West Africa to always allow TB culture to stand for up to 10 weeks or more to allow for colony formation before they make any conclusion. *M. africanum* is noted to be less pathogenic when compared with *M. tuberculosis,* and is less likely to progress to clinical disease in an immune-competent individual, making it an important opportunistic infection in people living with HIV.[13] The association between *M. africanum* and TB/HIV co-infection and its prevalence in West Africa presents mixed benefits. On one hand, *M. africanum* is less pathogenic, less infective and clinically less debilitating.[1] On the other hand, the reduced sensitivity of *M. africanum* to some rapid TB diagnostic kits (MPT64 based diagnostics) often leads to false negative results[14] which negatively impacts on the global fight against TB. Therefore, having 11% of the study population to be infected by *M. africanum,* coupled with the strain's reduced sensitivity to some TB diagnostic tools, calls for

Downloaded from www.tnhjph.com
Print ISSN: 0189-9287 Online ISSN: 2992-345X

due diligence to be exercised by clinical laboratory personnel to ensure that valid and accurate results are dispatched. To this end, we recommend that TB clinical laboratories, particularly TB culture laboratories in West African region, do additional follow-up investigations on positive TB cultures with negative rapid identification result (MPT64 based), whose morphological characteristics (on solid medium or appearance in liquid medium) suggests being MTBC. Rapid TB molecular diagnosis using Xpert MTB/Rif assay or MTBDR*plus* would be necessary in such instances to ensure valid and accurate results. On the other hand, manufacturers of rapid TB identification kits should as a matter of urgency, improve their products with a view to addressing the reduced sensitivity being experienced in the diagnosis of *M. africanum*.

M. bovis was found to be responsible for MDR-TB in 2% of the study population. This finding reiterates the zoonotic nature of TB as a public health concern. In an effort to provide evidence of occupational hazard, a study found 2% infection rate by *M. bovis* amongst livestock workers in Akinyele Cattle Market Ibadan, South Western Nigeria.[15] This constitutes a serious public health threat particularly to butchers and herders whose occupation requires that they maintain close association with cattle, in addition to other unwholesome practices such as herder's consumption of unpasteurized milk and processing of animals without appropriate protective measures. However, a study in North East Nigeria found a high level of awareness about zoonosis, bovine tuberculosis, knowledge of signs and symptoms of TB and use of protective clothing but with low level of practice amongst abattoir workers in Bauchi state.[16] What his finding has shown is that the frequent sensitization activities being carried out by veterinarians and other public health concerns are yielding some positive results. This calls for more action to improve good and healthy practices amongst groups rearing animals as well as those handling the raw flesh. The authors consider the 2% prevalence of *M. bovis* in the study population to be high, given the fact that cattle rearing is not a common practice in Southeastern Nigeria. However, improper handling of raw meat, consumption of poorly cooked meat and products could be some of the reasons for the high *M. bovis* infection in the study population.

Limitation of the study

The Geno Type MTBC series kit used for speciation in this study is not sensitive enough to differentiate between *M. tuberculosis* and *M. canettii*, both of which are genetically identical. Also, the high number of invalid results and few non-evaluable results can be attributed to the inability of the Geno Type MTBC kit to correctly differentiate the species. Therefore, further studies which could employ more sensitive and advanced molecular techniques are advocated.

Conclusion

Study findings shows that *M tuberculosis/M. canettii* and *M. africanum* are the leading causes of MDR-TB in the study population. Furthermore, the results of this study indicate that *M. bovis* remains an important cause of Tuberculosis particularly amongst groups at risk. Further studies should be targeted at understanding the dynamics, mechanism of transmission and identification of clusters of infection.

Declarations

Ethical consideration: Ethical approval for the study was granted by the ethics and research committee of Southeast Zonal TB Reference Laboratory. All laboratory procedures including TB culture and molecular analysis were performed in biosafety level II laboratory under strict compliance with the National TB program biosafety guideline at the Southeast Zonal TB Reference Laboratory, Specialist Hospital Amachara, Umuahia Abia state, Nigeria.

Authors' contribution: Conceptualization (Gweba C), Methodology (Ojule IN), Original draft (Gweba C), review and editing (Panwal MT, Mamman KE and Girei RS), supervision (Ojule IN).

Conflict of interest: Authors have no competing interest.

Funding: This study was made possible by the GF funded care for MDR-TB patients through the Institute of Human Virology Nigeria and National Tuberculosis and Leprosy Control Program at the South East Zonal TB Reference Laboratory.

Acknowledgement: We appreciate the management and the entire staff of Southeast Zonal TB Reference laboratory for their support throughout the study. This study was made possible by the GF funded care for MDR-TB patients through the Institute of Human Virology Nigeria and National Tuberculosis and Leprosy Control Program at the Southeast Zonal TB Reference Laboratory.

Downloaded from www.tnhjph.com

References

1. Asare P, Asante-Poku A, Osei-Wusu S, Otchere ID, Yeboah-Manu D. The Relevance of Genomic Epidemiology for Control of Tuberculosis in West Africa. Front Public Heal. 2021;9:1–17.
2. Sylverken AA, Kwarteng A, Twumasi-Ankrah S, Owusu M, Arthur RA, Dumevi RM, et al. The burden of drug resistance tuberculosis in Ghana; results of the First National Survey. PLoS One [Internet]. 2021;16(6 June):1–14. Available from: http://dx.doi.org/10.1371/journal.pone.0252819
3. Miller MA, Buss P, Roos EO, Hausler G, Dippenaar A, Mitchell E, et al. Fatal tuberculosis in a free-ranging African elephant and one health implications of human pathogens in wildlife. Front Vet Sci. 2019; 6:1–8.
4. Tomeny EM, Nightingale R, Chinoko B, Nikolaidis GF, Madan JJ, Worrall E, et al. TB morbidity estimates overlook the contribution of post-TB disability: Evidence from urban Malawi. BMJ Glob Heal. 2022;7(5):1–12.
5. Živanović I, Vuković D, Dakić I, Savić B. Species of mycobacterium tuberculosis complex and nontuberculous mycobacteria in respiratory specimens from Serbia. Arch Biol Sci. 2014;66(2):553–61.
6. Brites D, Loiseau C, Menardo F, Borrell S, Boniotti MB, Warren R, et al. A New Phylogenetic Framework for the Animal-Adapted Mycobacterium tuberculosis Complex. 2018;9 :1–14.
7. Federal Ministral of Health (FMoH). Department of Public Health. National Tuberculosis and Leprosy Control Programme. National Guidelines
for Culture and Drug Susceptibility Testing. Abuja, Nigeria, December 2015. Available from https://ntblcp.org.ng/resources/national-guidelines-for-culture-and-drug-susceptibility-testing/
8. Cá B, Fonseca KL, Sousa J, Maceiras AR, Machado D, Sanca L, et al. Experimental Evidence for Limited in vivo Virulence of Mycobacterium africanum. Front Microbiol. 2019;10 :1–9.
9. Blouin Y, Cazajous G, Dehan C, Soler C, Vong R, Hassan MO, et al. Progenitor "Mycobacterium canettii" Clone responsible for lymph node tuberculosis epidemic, Djibouti. Emerg Infect Dis. 2014;20(1):21–8.
10. Bakuła Z, Wuyep VB, Bartocha Ł, Vyazovaya A, Ikeh EI, Bielecki J, et al. Molecular snapshot of drug-resistant Mycobacterium tuberculosis strains from the Plateau State, Nigeria. PLoS One [Internet]. 2022;17(5 May):1–13. Available from: http://dx.doi.org/10.1371/journal.pone.0266837
11. Chuku A, Ajide B, Lawson L, Orule K, Adogo L. Differentiation and Characterization of Mycobacterium tuberculosis Complex Causing Pulmonary Tuberculosis across North Central Nigeria. Int J Trop Dis Heal. 2019 :1–10.
12. Osei-wusu S, Otchere ID, Asare P, Ntoumi F, Zumla A, Asante-poku A, et al. International Journal of Infectious Diseases Relevance of genomic diversity of Mycobacterium tuberculosis complex in Africa. Int J Infect Dis [Internet]. 2022;124:S47–9. Available from: https://doi.org/10.1016/j.ijid.2022.03.016
13. Asante-poku A, Otchere ID, Osei-wusu S, Sarpong E, Baddoo A, Forson A, et al. Molecular epidemiology of Mycobacterium africanum in Ghana. BMC Infect Dis [Internet]. 2016;1–8. Available from: http://dx.doi.org/10.1186/s12879-016-1725-6
14. Ofori-anyinam B, Kanuteh F, Agbla SC. Impact of the Mycobaterium africanum West Africa 2 Lineage on TB Diagnostics in West Africa : Decreased Sensitivity of Rapid Identification Tests in The Gambia. 2016;1–12.
15. Adesokan HK, Jenkins AO, Soolingen D Van, Cadmus SIB. Mycobacterium bovis infection in livestock workers in Ibadan , Nigeria : evidence of occupational exposure. 2012;16:1388–92.
16. Sa'idu AS, Okolocha EC, Dzikwi AA, Gamawa AA, Ibrahim S, Kwaga JKP, et al. Public Health Implications and Risk Factors Assessment of Mycobacterium bovis Infections among Abattoir Personnel in Bauchi State, Nigeria. Journal of Veterinary Medicine 2015; Vol. 2015. Article ID 718193. Available from https://doi.org/10.1155/2015/718193

Downloaded from www.tnhjph.com
Print ISSN: 0189-9287 Online ISSN: 2992-345X