Genotypic Identification of *Mycobacterium* species in Suspected TB Patients at Damaturu Specialist Hospital, Yobe State, Nigeria

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Abstract: Pathogenic *Mycobacterium* species continue to be public health threat especially in developing countries like Nigeria. This study aimed to identify pathogenic *Mycobacterium species* in suspected human TB patients at Damaturu Specialist Hospital in Yobe State, Nigeria. A total of 391 sputum samples were examined and tested using standard methods and positive isolates were further subjected to SD-bioline Ag MPT64 test and molecular genotype MTBC for Mycobacterium species identification. The result revealed that 186 samples were positive and 133 (71.51%) were identified as *M. tuberculosis,* 51(27.42%) were identified as *M. africanum* and 2(1.17%) were identified as *M. bovis.* The study revealed that there was no significant difference ($p>0.05$) in the occurrence of Mycobacterium species among the studied subjects in relation to their gender. However, it is worth noting that 74 *M. tuberculosis,* 30 *M. africanum* and 2 *M. bovis* isolates were isolated from males while the remaining 59 *M. tuberculosis,* 21 *M. africanum* isolates were from females. The study highlighted the significance of tuberculosis in suspected TB patients and its public health implications and calls for prompt action towards controlling the disease in Damaturu Specialist Hospital, Yobe State and Nigeria in general.

Keywords: Genotypic, Humans, Identification, Mycobacterium.

INTRODUCTION

Tuberculosis (TB) is a chronic infectious and contagious disease of domestic animals, wild animals and humans (Radostits *et al.*, 2013). It is characterized by the formation of granulomas in tissues especially in the lungs, lymph nodes, intestines, liver and kidneys (Shitaye *et al.*, 2007). It is caused by pathogenic members of the genus *Mycobacterium* which are commonly known as members of *Mycobacterium tuberculosis* complex (*Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium bovis sbs caprae, Mycobacterium microti* and *Mycobacterium cannetti* (Collins and Grange, 1983; Pefeiffer, 2003).

Tuberculosis is widely distributed throughout the world with serious effect on human and animals and is also of significant public health importance (O'Reilly and Daborn, 1995). The disease in humans is known by several common names such as wasting disease, consumption, white plague and pearl disease (Abubakar *et al.*, 2021).

Human infection with *M. bovis* is a recognized public health hazard in developing and industrial nations (Grange and Yates, 1994). The advent of milk pasteurization and eradication programs has reduced the level of human *M. bovis* in industrialized nations, but sporadic cases still occur when individuals come in contact with infected livestock (Pfeiffer, 2003), captive wildlife (Ibrahim *et al.*, 2020) or contaminated animal carcasses (Danbirni, 2016). Humans are susceptible to *M. bovis,* and there are numerous instances of human infection resulting from contact with infected animals. Recently there has been increased interest among public health officials, in drug-resistant strains of *M. tuberculosis, M. bovis* and *M. avium,* because several of such strains have been isolated from HIV/AIDS infected and immunocompromized humans

(Abubakar et *al.*, 2021). Infection with *M. bovis* causes pulmonary and extrapulmonary disease (Abubakar, 2007). Contact with infected animals is a source of *M. bovis* infection for humans and is a recognized hazard for abattoir workers, veterinarians and livestock handlers (Ibrahim, 2016).

Despite this expression of political will to control human TB in Nigeria, and a clearly articulated national TB policy, there was no sufficient financial support necessary for the effective implementation of the policy (Anon, 2006). Effective TB control is also affected by inadequate trained personnel and political instability, poor health infrastructure and facilities, poor referral system, poor procurement and supply management system and poor donor coordination/management locally and internationally. Most importantly also, diagnosis of tuberculosis stops at the smear level, hence the species involved in causing the disease are not known thereby making it difficult to study outbreaks, trace the routes of transmission and also identify the species and strains of *Mycobacterium* involved. Another major setback is the inability of the national TB control program to recognize the significance of *M. bovis*, which are a major public health problem and the general lack of collaboration between human and veterinary medical personnel in this regard. Generally, there has been total lack of synergy and harmony of laws regulating the control of human and bovine tuberculosis in the country (Abubakar, 2010). In 2013 the World Health Organization in its policy statement indicated that effort should be made to reach the 3million new TB case that are missed every year by the health system 70% of which live India, South Africa, Bangladesh, Pakistan, china, DR Congo, Mozambique, Nigeria, Ethiopia, phliphines and Myanmar (WHO, 2013b) Thus, case identification is one of most important factor that determines the successful implementation of TB control strategies or otherwise (Aminu and Tukur, 2016). The use of molecular techniques for epidemiological studies has not been fully exploited in Nigeria; as such there is limited data on the species of *Mycobacterium* responsible for both human and animal tuberculosis (Cadmus *et al*., 2006, Abubakar *et al.,* 2011). In view of this, it is therefore justified that this study be embarked upon from suspected TB patients using genotypic identification of Mycobacterium species at Damaturu Specialist Hospital, Yobe State, Nigeria.

MATERIALS AND METHODS

Study Area

Yobe State is located in North eastern part of Nigeria. A mainly agricultural state, it was created on August 27, 1991. Yobe state was carved out of present-day Borno state, with an area of about 45,502 $km^2$and lies within latitude 11-12^{0}N and longitude 10-13^{0}E and lies within the savannah region of Nigeria. According to the 2006 census, the population of the state is estimated to be 2,532,395. The state borders the Nigerian states of Bauchi, Borno, Gombe, and Jigawa. It borders the Diffa Region and the Zindar Region to the north in The Republic of Niger. Because the state lies mainly in the dry belt, the state is dry and hot for most of the year, except in the southern part of the state which has a milder climate (Gambo *et a*l.,2010).

Ethical Consideration

Approval was obtained from the Yobe State Hospital Management Boards of the Hospital ethics committee prior to the study.

Human Sputum Collection

Non-probability sampling technique was used (Judgemental/purposive) in which Three Hundred and Ninety One (391) Sputum samples were collected from clinical suspected TB patients that attended Damaturu Specialist human hospitals in Yobe State. Three sputum samples were collected from each patient i.e. 1st spot, overnight and 2nd spot as recommended by the International Union Against Tuberculosis and Lung Diseases (IUATLD) (Ipuge, Riedaer and Enarson 1996). Samples were collected in sterile screw capped containers with CetylPyridnium Chloride (CPC) as a preservatives and decontaminants, transported to the laboratory in ice parks for processing.

Laboratory Processing of Human Sputum (Culture)

Sputum samples were preserved and decontaminated using the CetylPyridnium Chloride (CPC) before inoculating on to LJ-slopes using standard method (Van Embden *et al.* 1993). 10ml of sputum (depending on the quantity available), 15ml sterile saline was added and the mixture was then allowed to stand for 15mins at room temperature with occasional shaking before centrifuging at 3000g for 15mins. The supernatant was poured off and 20ml sterile saline was added to re-suspend the sediment before centrifuging again for another 15mins. The supernatant was decanted and the sediments inoculated onto 2 slants of Lowenstein-Jensen media (glycerol and pyruvate enriched) before incubating at 37°c for a minimum of 8 weeks.

Primary Isolation

Cultures were examined weekly for colonies with a hand lens; the growth time and colonial characteristic were noted. A representative colony was smeared and stained by the Zeil Nelsen stain technique for presence of acid-fast bacilli (AFB) and cellular morphology was noted.

Acid-Fast /Zeihl-Neelsen (ZN) Stain

Zeihl-Neelsen staining was carried out using standard protocol as described by ((Anonymous 1970; Corner 1988, Abubakar, 2016) to detect acid-fast bacilli from the suspected TB patients samples collected.

An impression smear was made using new, clean and labelled grease-free slide and the slide was air-dried and heat fixed by passing it through a flame (Over a bursen-bunner) with the specimen side up. This is to fix the specimen to the slide and preserve the bacterial morphology. The slide was then flooded with carbolfuschin and then steamed gently with the flame from underside. It was then rinsed off with water and decolorized with 5% acid alcohol until the red colour is gone. The slide was rinsed again with water and counter-stained with methylene blue. Additional rinsing with water was applied to remove excess colour and air dried. It was then examined under a microscope with oil emersion lens at x 100 to look for acid fast bacilli (AFB). The bacilli appeared red, straight or slightly curved rods occurring either singly or in groups while non-acid-fast microorganisms stained blue.

SD-BiolineTB Ag MPT64

This is rapid immunochromatographic identification test for the *Mycobacterium tuberculosis* complex that uses mouse monoclonal anti-MPT64. This test kits can be easily used for rapid identification of the *Mycobacterium tuberculosis* complex in combination with culture system based on liquid or solid media without any technical complexity in clinical laboratories.

Test procedure

3 – 4 colonies were suspended in 200ul of extraction of buffer prior to test. Removed the test device from the foil pouch and placed it on a flat, dry surface. 100ul of suspended solid cultures in buffer was added in to the sample well. As the test begins to work a purple colour moved across the result window in the centre of the test device. The result was interpreted in 15 minute after sample application

Interpretation of the test result

A colour band appeared in the left section of the result window which indicated that the test was working properly. This band was the control line (C).The right section of the result window indicated the test result (T). The presence of only one purple band within the result window indicated a negative result. The presence of two purple bands ('T' band and 'C' band) within the result window indicated a positive result. If the purple band colour was not visible within 15 minutes after performing the test, the result was considered invalid. It was recommended that the specimen be re-tested. The test procedures were all conducted according to the Manufacturers Instructions.

Genotype® MTBC for Molecular Identification of *Mycobacterium tuberculosis* Complex Species

The GenoType MTBC assay (HainLifescience GmbH, Nehren Germany) a new DNA strip assay for the rapid molecular identification of members of the *M. tuberculosis* complex. The GenoType MTBC assay is based on MTBC specific 23S ribosomal DNA fragment, gyrB NDA sequence polymorphisms and RD1 deletion. The assay was performed according to the manufacturer's instructions and as described by (Richter *et al.,* 2003, Romero *et al.,* 2007) that involved four technical steps; DNA Extraction by Chemical Method, Amplification Reaction, Hybridization and Evaluation and Interpretation of Result.

1. **DNA Extraction**

DNA extraction procedure with genolyse was done according to manufacturer's specifications (HainLifescienceGmbH, Nehren, Germany,2015), WHO (2008) and Aminu and Tukur (2016) Using a sterile graduated pipette 0.5 ml (500 µl) of the decontaminated sputum sample was transferred into microcentrifuge tube. This was done for all the samples, after which the tubes were closed and centrifuged for 15 minutes at 10000xg. The supernatant was discarded and 100µl lysis buffer (ALYS) was added and re-suspended by vortexing gently for 30 sec. The tubes were arranged in a floater inside the BSC II and incubated for 5minutes in a water bath at 950C. Then, 100µl Neutralisation buffer (A-NB) was added and vortexed for 30 seconds and the tubes were centrifuged at maximum speed (10,000xg). The heavier debris formed the pellet and the lighter DNA (free from impurities) was suspended in the supernatant which was transferred into clean labeled micro-centrifuge tubes for further use.

2. **PCR amplification of the extracted DNA**

The master mix preparation was done according to manufacturer's specification (HainLifescienceGmbH, Nehren, Germany, 2015), WHO (2008) and Aminu and Tukur (2016). The master mix was made up of 10µl of the AM-A and 35µl of AM-B Reagent which was placed in a PCR tube labeled with sample number and mixed very well.This was prepared inside dead air box in a clean DNA free room. Then 5µl of each sample (containing the extracted DNA from above) was added to the corresponding tube containing the master mix and then mixed gently by pipetting up and down a few times. The PCR tubes were then placed in a 30 cycle (10 + 20) thermal cycler program for amplification. After amplification the DNA contained in the amplicons were denatured in the TwinCubator® which was pre-warmed to 45 oC and 20µl of denaturation solution (NaOH) was added to each labeled well of the TwinCubator® tray followed by the addition of 20µl of the amplicons respectively. The mixture was mixed gently by pipetting up and down five times and then incubated at room temperature for 5mins.

3. Hybridization and Identification

Hybridization and detection procedures were carried out according to according manufacturer's specification (HainLifescienceGmbH, Nehren, Germany, 2015), WHO (2008) and Aminu and Tukur (2016). After denaturation of the amplicons, 1ml of the pre-warmed hybridization buffer (HYB) was carefully added to the wells using a pipette and thoroughly mixed. The tray was placed on the TwinCubator® and labeled strips were added to each well ensuring that the strips were completely covered by the liquid and incubated at 45oC for 20mins. After incubation, the HYB buffer was aspirated completely from each well and 1ml of the pre-warmed red stringent wash buffer (STR) was then dispensed into the tray. After 10 minutes incubation at 45 oC in the TwinCubator®, STR buffer was aspirated and was washed off with 1 ml of Rinse solution (RIN) for 1 minute. Then 1ml of the Conjugate (CON) solution was dispensed into each well and incubated for 20 minute on the TwinCubator®. The strips were washed twice with 1 ml of Rinse solution (RIN) for 1 minute in the TwinCubator®.

Then sterile distilled water was added and a 1 minute wash performed on the TwinCubator® to wash off the RIN solution after which the distilled water was completely decanted. One (1) ml of the Substrate solution was then dispensed into each well and incubated for 10 minutes on the TwinCubator® after which the Substrate solution was aspirated and the strips washed twice with sterile distilled water. A pair of clean tweezers was used to remove the strips from the TwinCubator® tray and placed onto absorbent paper. The developed strips were partially dried and transferred to the GenoType® MTBC score sheet for interpretation.

Data analysis

Data generated for the study were analysed using percentages and Chi-square ($\chi 2$) was used to compare of infection in various categories. A value of $p \leq 0.05$ was considered significant.

RESULTS

A total of 391 sampleswere examined and tested using culture, acid- fast staining and SD-biolineAg MPT64 in which 186 samples were positive and were subjected to genotypic identification of Mycobacterium species (Table 1).133 (71.51%) were identified as *M. tuberculosis,* 51(27.429%) were identified as *M. africanum* and 2(1.17%) were identified as *M. bovis.* The study revealed that there was no significant difference ($p>0.05$) in the occurrence of Mycobacterium species among the studied subjects in relation to their gender. However, it is worth noting that 74 *M. tuberculosis,* 30 *M. africanum* and 2 *M. bovis* isolates were isolated from males while the remaining 59 *M. tuberculosis,* 21 *M. africanum* isolates were from females.

Table 1: Identification of Tubercle bacilli from suspected TB patients Isolates at Damaturu Specialist Hospital, Yobe State Nigeria

Sex	Isolates(%)	*M. tb*(%)	*M. africanum*(%)	*M. bovis*(%)	(P-Value)
Male	109(58.60)	74(55.64)	30(58.82)	2(100)	(0.137)
Female	77(41.40)	59(44.36)	21(41.18)	0(00)	
Total	**186**	**133**	**51**	**2**	

Percentage = ( )

(P<0.05) regarded as significant

M.tb = Mycobacterium tuberculosis

DISCUSSION

The identification of 186 (47.57%) Mycobacterium species isolates using Molecular Genotyping in this study from suspected TB patients in Damaturu Specialist Hospital, Yobe State is of great epidemiological and public health importance. This study has not shown a significant difference between sex and the Mycobacterium infection. However, it reflects that there are more positive TB cases among males than females observed. Other authors have reported similar findings in their studies (Holmes, Hausler and Nunn 1998; Borgdorff, Nagel, Dye and Nunn 2000; Lawson 2006; Abubakar 2007). This could be argued that males might be more at risk of having TB than females which could be due to the nature of their work which exposes them to more risks especially, in Northern Nigeria where the women are mostly at home unemployed and thereby hardly exposed to risk of infection. Although, most studies agree with this finding, Nwachokor(2000) in a 30 year review of tuberculosis in Ibadan, Nigeria reported that, more females were affected with TB which reached twice the rate

of males. Also the incidence of the disease is higher among age groups 25 and 34 years old, which is the age group often associated with TB and also HIV (Lawson, 2006).

The identification of *M. bovis* from humans sputum is intriguing; while animal-to-human transmission of *M. bovis* has been reported (Ayele *et al.* 2004, Abubvakr, 2016, Danbirni, 2016 and Ibrahim, 2016). Traditional practice exists in the Northern Nigeria that could facilitate the transmission of Tuberculosisbetween animals and humans, during watering and grazing. This practice involves rearing and using animals in close proximity and mixing with their owners thereby giving increased avenues for zoonotic transmission. The habit of people by eating undercooked meat and improperly roasted meat in the form of "suya" and "kilishi" is gaining ground in Nigeria (Abubakar*et al.*, 2021) leading to potential zoonotic transmission of the disease, although, no study was undertaken to isolate the organism in goat "suya" and "kilishi" in the country. However, the possibility of cross-contamination especially from infected animal to the humans cannot be ruled out. This is also of public health importance as animal handlers and consumers of infected animals product stand the risk of getting infected.

Most importantly, the identification of *M. bovsis* from suspected TB patients observed in this study is of serious public health importance. This finding reveals that there is a definite association between human infection and the disease in animals. This can also justify a recommendation that any preventive measure for TB in human population in Nigeria should lean heavily and associated with the same level of measures in animal population.

Similarly, the identification of *M. africanum* species from suspected TB patients is of interest, as while it is virulent for humans but it is genetically related to *M. bovis* and has rarely been isolated from animals. However, Cadmus and colleagues (2007); Abubakr, 2016, Danbirni, 2016 and Ibrahim 2016 reported a similar findings in Nigeria

This study has demonstrated the effectiveness of Genotype MTBC method used in the diagnosis of tuberculosis in humans and animals. However, this might justify saying that the Genotype MTBC is more efficient, accurate and faster at least for field and epidemiological purposes as well as for conducting some PCR based molecular methods like the spoligotyping and VNTR which do not need high quality DNA and also can be conducted directly from clinical samples.This study should be interpreted in the context of its limitations. Because the sources of the location of the patients were unknown, we could not determine whether the organisms were imported from a neighbouring or within the country. In addition, we lacked information on the condition of the patients. However, we have identified *M. tuberculosis*, *M. bovis* and *M. africanum* in suspected TB patients at Damaturu Specialist Hospital, Yobe State, Nigeria.

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