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ORIGINAL ARTICLE

Isolation and Molecular Characterization of *Mycobacterium Africanum* from the Sputum of Butchers in a Municipal Abattoir in Ibadan, Oyo State

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SUMMARY

Tuberculosis (TB) caused by the *Mycobacterium tuberculosis* complex (MTC) remains a major public health concern due to its high rate of person to person transfer as well as a high level of morbidity and mortality. The risk factors for transmission of zoonotic TB to humans are close physical contact with cattle, consumption of unpasteurised milk and milk products and unhealthy meat processing by butchers are common in developing countries like Nigeria. However, the circulating MTC among the occupationally exposed are unknown therefore the need to determine the prevalence of tuberculosis and to characterize the mycobacterial species in them. A cross-sectional study was conducted among butchers, cattle traders and herders in Bodija Municipal Abattoir, Akinyele International Cattle Market and some herds respectively. Using systematic random sampling, 93 sputum samples were collected and analyzed by culture, *Mycobacterium* Genus Typing as well as Deletion Typing (Multiplex Polymerase Chain Reaction (PCR)). Of the 93 sputa collected, two (2.2%) were positive for mycobacteria by culture which were confirmed to be *Mycobacterium africanum* by molecular characterization. These bacilli were isolated from two butchers; one of which had the habit of eating raw meat and cherish '*wara'* (a local soft cheese made from milk). The isolation of *M. africanum* from butchers in this study raises public health concern on the contamination of the meat processed as well as highlights its importance in the epidemiology of tuberculosis in Nigeria.

INTRODUCTION

Tuberculosis caused by the *Mycobacterium tuberculosis* complex (MTC) a highly related group of organisms is of very important global health concern (WHO, 2017). It is a leading cause of morbidity and mortality in developing countries (Zaman, 2010). The disease; though curable and preventable remains a major public health concern due to its high risk of person to person transfer as well as high level of indisposition and death (Mahojan, 2015). Species of the *Mycobacterium tuberculosis* complex traditionally consist of *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium microti*, and *Mycobacterium bovis* (Mostoswy *et al*., 2004 and Bouakaze *et al*., 2010).

The disease in humans is caused by *M. tuberculosis* while *M. bovis* is the causative agent of zoonotic TB (Cadmus *et al*., 2006). However, *M. africanum* is equally important in the epidemiology of tuberculosis in both humans and cattle (Niemann *et al.*, 2002). It has been isolated from milk of trade cattle in addition to tuberculous lesions of cattle in Ibadan in south western Nigeria (Cadmus *et al*., 2008) and from fresh milk of pastoral cattle in north central and south western Nigeria (Cadmus *et al*., 2010 and Agada *et al*., 2014). A study conducted a decade ago in Ibadan revealed that 13% of TB in humans was caused by *M. africanum* and *M. bovis* (Cadmus *et al*., 2006).

The members of MTC are closely related genetically and are hard to distinguish from each other by biochemical characteristics. However, discrimination by spoligotyping (Viana-Niero *et al*., 2001), PCR based regions of difference (RD) (Brosch *et al*., 2002; Warren *et al*., 2004) and gyrB polymorphism (Richter *et al*., 2004) have been recommended for the differentiation of the members of MTC.

Nigeria, with a population of about 186 million in 2016 (WHO, 2017), is among the six countries contributing 60% of TB cases worldwide with the highest burden of TB in Africa (WHO, 2016) as well as one of the top three (India, 25%; Indonesia, 16% and Nigeria, 8%) of the ten countries accounting for 76% of the total reported cases in the world (WHO, 2017). The degree of zoonotic transmission of tuberculosis is not well known among those occupationally exposed even though cultural habits and practices which facilitate transmission from cattle to humans abound. These include: close interaction between farmers and livestock; fattening of cattle and rearing of small ruminants in the backyards; non-wearing of protective clothing and processing of the carcass and offal by butchers with bear hands; food consumption habits and the crowding of cattle and humans in the cattle markets (Ayele, *et al*., 2004, Cadmus *et al*., 2006, Abubakar 2007 and Rodwell *et al*., 2008). Despite all these, there is insufficient information about the prevalence of the disease and the species responsible among the high risk individuals in Nigeria. This study therefore aimed to determine the prevalence as well as identify the species of Mycobacteria responsible for tuberculosis (TB) amongst the most occupationally exposed group in Oyo State using culture and Multiplex PCR: *Mycobacterium* Genus and Deletion Typing.

Materials and Methods

Study Area

This study was conducted in Akinyele International Cattle Market, Bodija Municipal Abattoir as well as some herds' locations at Wasimi in Iwajowa, Igangan in Ibarapa North, Igana in Kajola and Ijaye in Akinyele LGAs of Oyo State.

Akinyele International Cattle Market is the main trading point for cattle brought from northern Nigeria and other parts of Africa to Oyo State. The site is a center of livestock market activity; characterized by overcrowding which can aid the transmission of zoonotic BTB from cattle to humans by means of aerosol.

Bodija Municipal abattoir is a major abattoir that services Ibadan Municipality where an average of 250 cattle is slaughtered daily. Again, due to the unregulated crowd control, the abattoir is often overcrowded by both butchers and the general public. In addition, the butchers wear minimal protective clothing while dressing carcasses as well as use bear hands to process offals from carcasses including diseased ones. Resulting from these, the opportunities for infection with zoonotic BTB therefore abound through aerosol spread, skin infection and in some cases by ingestion due to the habit of eating while processing infected carcasses.

Study Design

This was a cross-sectional study.

Sampling Technique

Multi-stage sampling was used. Purposive sampling was used to determine the sampling site while systematic random sampling was used to sample the livestock workers.

Eligibility Criteria

Livestock workers aged 18yrs and above working in Bodija Municipal Abattoir, Akinyele International Cattle Market and in herds in Wasimi, Igana, Igangan and Ijaye.

Sample Size

Based on an earlier report of 5% prevalence of *M. bovis* infection amongst humans in Nigeria by Ofukwu, (2006); the estimated sample size was 73 individuals. However, 93 sputum samples were collected from individuals willing to participate in the study, 40 samples were collected from butchers and another 40 from traders at the cattle market and 13 from herders. The study objective was explained to them and their due consent was fully taken.

Ethical Permission

Ethical clearance for the study was the University of Ibadan/University College Hospital Ethics Committee (UI/EC/11/0238).

Sputum Collection and Processing

The participants were provided with sterile plastic universal bottles into which they voided sputum samples. The samples were transported to the laboratory and stored in a fridge at 4ºC until processing. They were then processed according to Beaton Dickson digestion and decontamination procedure (BD, Sparks, MD, USA). Using a sterile, 50 ml centrifuge tube with a screw cap, equal amounts of specimen and activated NALC (N-acetyl-L-cysteine)-NaOH of 5 ml each were added. The centrifuge tube was capped and mixed on a vortex-type mixer until the specimen was liquefied. The mixture was allowed to stand at room temperature for 15 min with occasional gentle shaking. Prepared phosphate buffer was added to the 15 ml mark on the centrifuge tube and mixed, followed by centrifugation for 15 to 20 min at 3000 x g. The supernatant was carefully decanted, and 2 ml of phosphate buffer of pH 6.8 was added to re-suspend the sediment. The suspension was smeared on the slide for Zeihl Neelsen staining and microscopy while some were then inoculated onto 2 Lowenstein-Jensen slopes (one with pyruvate and the other with glycerol) and incubated at 37ºC for at least 6 weeks.

Identification

Identification was done by observation of growth on the L-J glycerol and pyruvate media based on the criteria for distinguishing *M. tuberculosis* and *M. bovis* (Kubica *et al*., 2006).

Molecular identification

All strains of the mycobacteria obtained were subjected to further characterisation using two-step multiplex polymerase chain reaction (PCR) technique based on genus and deletion typing for the confirmation of their identity.

***Mycobacterium* Genus Typing**

The genus typing was carried out according to the methods of Wilton and Cousins (1992) and Standard Operating Procedure CBU0247 (2005). The composition of the PCR mixture (25 µl)

was: HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) (10 µl), Mycgen-R 100um (0.3 µl), Mycgen –F 100um (0.3 µl), Mycar –R 100um (0.3 µl), Mycint –F 100um (0.3 µl), TB1-F 100um (0.3 µl), TB1-R 100um (0.3 µl), sterile water (6.2 µl) and DNA (isolate) (2.0 µl) (isolates has been heat killed at 80^0C for 1 hour). DNA Ladder, loading dye, Agarose, 10x TAE Running buffer and Ethidium Bromide were used for the Gel Electrophoresis.

The reaction mixture was then heated in a programmed Thermal cycler (MyGene Series Peltier Model MG 96) amplification was initiated by incubation at 95^0C for 15 min for enzyme activation, followed by 45 cycles at 95^0C for 1 min for denaturation, 61^0C for 1 min for annealing and 72^0C for 1 min for extension. After the last cycle, the samples were incubated at 72^0C for 10 minutes. PCR products were then separated by electrophoretically separated using 1.5% agarose gel and 10xTAE running buffer at 10 V/cm for 2 h. Ethidium bromide at ratio 1:5, 100bp ladder and orange 6x loading dye were used in the gel electrophoresis.

Deletion Typing

This was carried out as described by Warren *et al*. (2006). The reagents used for the PCR reaction include Q-Buffer, 10xBuffer, 25mMgcl_2, 2.5mMdNTPs, and the primers which include RD1A, RD1B, RD1C, RD4A, RD4B, RD4C, RD9A, RD9B, RD9C,RD12A,RD12B and RD12C. All these with HotStarTag, isolate DNA and distilled water were added together and mixed for the running of the PCR reaction.

Primer Design

Primers were designed in silico, according to the previously described DNA sequence of the region of difference (Brosch *et al.,* 2002 and Mostowy *et al*., 2004). Primer set 1 included RD1, RD4, RD9 and RD12 primers and primer set 2 included $RD1^{mic}$ and $RD2^{seal}$ primers.

PCR Amplification

Each PCR reaction contained 1µl DNA template, 5 µl Q-buffer, 2.5 µl 10 Xbuffer, 2 µl 25 mM $MgCl_2$, 4 µl 10 mM dNTPs, 0.5 µl of each primer (50 pmol/µl), 0.125 µl HotStarTag DNA polymerase (Qiagen, Hilden, Germany) and was made up to 25 µl with water. Amplification was initiated by incubation at 95^0C for 15 min followed by 45 cycles at 94^0C for 1 min, 62^0C for 1 min and 72^0C for 1 min. After the last cycle, the samples were incubated at 72^0C for 10 min. PCR amplification products were electrophoretically fractionated in 3.0% agarose in 1Xtbe pH 8.3 at 6V/cm for 4 hours and visualised by staining with ethidium bromide.

RESULTS

Results of Acid-Fast (ZN) stain and Culture of the sputum samples collected cattle traders, butchers and herders in Oyo State.

For acid-fast stain, of the ninety three (93) sputum samples collected, only eight (8, 8.6%) were positive for acid-fast bacilli. Also, out of the 93 sputa collected, only two (2.2%) was positive on culture while 91 (97.8%) were negative and the two positive samples were from butchers (Table 1).

Result of Genus and Deletion typing of the Strains Isolated

The two acid fast bacilli isolated when characterized by genus typing for confirmation were identified as *Mycobacterium* as well as being members of the *Mycobacterium tuberculosis* complex (Plate 1). They were identified to be *Mycobacterium africanum* on further characterization by the deletion typing (Plate 2), thus giving a prevalence of 2.15%.

Table 1. Results of ZN stain and culture of the sputum samples collected from cattle traders, butchers and herdsmen.

Occupation	Total No	Result of ZN		Result of culture	
		No (%) Positive	No (%) Negative	No (%) positive	No (%) negative
Cattle traders	40	3 (7.5)	37 (92.5)	0	40 (100.0)
Butchers	40	3 (7.5)	37 (92.5)	2 (5.0)	38 (95.0)
Herdsmen	13	2 (15.4)	11 (82.6)	0	13 (100.0)
Total	93	8 (8.6)	85 (91.4)	2 (2.2)	91 (97.8)

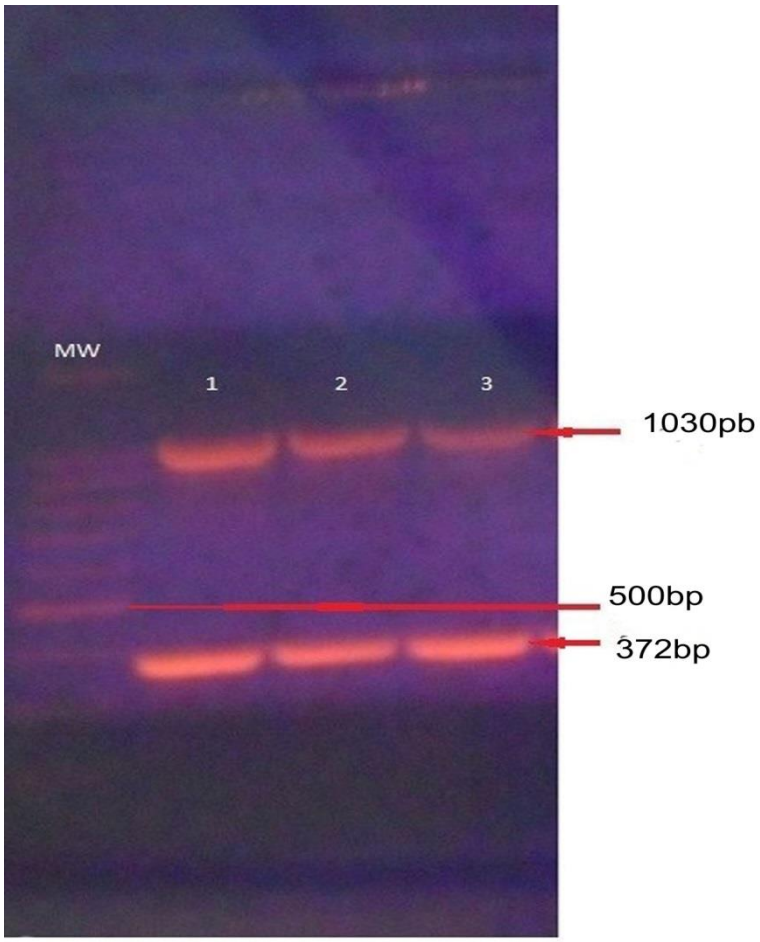

Figure 1. Electrophoresis seperaton of PCR product of multiplex genus typing of MPB70 gene of mycobacteria from butchers in Ibadan Nigeria. MW= 100bp Ladder; lane 1 and 2= isolates from butchers, lane 3= *M. tuberculosis*

DISCUSSION

This study was undertaken to determine the prevalence of zoonotic tuberculosis among butchers; one of the major stakeholders in the beef industry in Nigeria. There have been studies on the prevalence of TB in cattle traders (Adesokan *et al*., 2012) and on pastoralist (Ibrahim *et al*., 2012 and Damina *et al*., 2011) and on butchers and cattle traders in Nigeria (Cadmus *et al*., 2018) reflecting varied prevalence.

The result of this study indicate a prevalence of 2.2% among the most exposed group comprising cattle traders, butchers and herdsmen in Oyo State and 5% amongst the butchers. This does confirm the report by Adesokan *et al*., (2012) and Cadmus *et al*., 2018 of the prevalence of tuberculosis in livestock workers in Oyo State southwestern Nigeria. In contrast to other findings (Kiros, 1998, Pavlik *et al*., 2003, Ayele *et al*., 2004, Adesokan *et al*., 2012 and Cadmus *et al*., 2018) neither *M. tuberculosis* nor *M. bovis* was isolated but *M. africanum*. The isolation of *M. africanum* from butchers in this study may

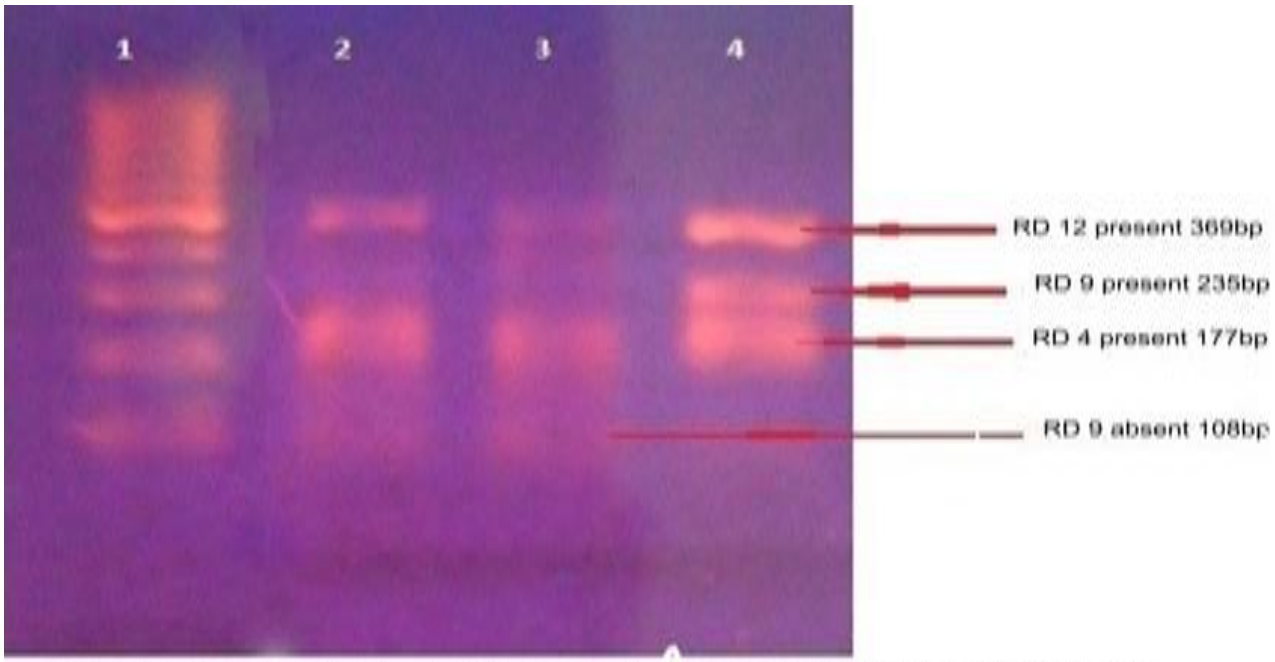

Figure 2. Gel electrophoresis seperation of PCR products by multiplex PCR deletion typing of *M. tuberculosis* complex isolated from butchers in Ibadan, Nigeria. Lane 1= 100bp ladder; Lane 2 and 3= isolates from butchers, RD 4 and RD 12 present, RD 9 absent (*M. africanum);* Lane 4= *M. tuberclosis* H37Rv positive control RD 4, RD 9, and RD 12 present.

indicate occupational exposure since it has been isolated from slaughtered cattle and raw milk from cows awaiting slaughter at the same Bodija Abattoir (Cadmus and Adesokan 2007, Cadmus *et al*., 2008 and Cadmus *et al*., 2006). This organism has also been isolated from fresh milk of pastoral cattle in Oyo and Niger States, Nigeria (Agada *et al*., 2014; Cadmus *et al*., 2010) and is reported to be a common cause of tuberculosis in West Africa (de Jong *et al*., 2010). Though the strains isolated were not genotyped to discover transmission relationships, the habit of consuming uncooked meat, unpasteurised milk and milk products such as cheese (wara) which characterizes these butchers have been documented to be potential risk of mycobacterial transmission (Mfinanga *et al*., 2003, Ayele *et al*., 2004, Cadmus *et al*., 2008 and Hambolu *et al*., 2013).

This study has shown that livestock workers particularly butchers are infected with *M. africanum* in Ibadan. It has been recognised as an important cause of human tuberculosis with varied prevalence in the West African countries affecting almost 50% of all TB cases reported in the region (Zumla *et al*., 2017). It has also been diagnosed in 3% of children who had acid fast bacilli positive stool as well as from adults in Nigeria (Cadmus *et al*., 2006, Cadmus *et al*, 2009 and Adesokan *et al*., 2019).

Finally, a number of important limitations need to be considered. First, the *M. africanum* isolated from the butchers were not spoligotyped to determine if the strains have been isolated from cattle to indicate zoonotic transmission. Secondly, the samples collected were for the diagnosis of active pulmonary infection, clinical parameters that could indicate suspicion of ongoing extra-pulmonary infection were not obtained. And thirdly, the population sampled was small especially the butchers, this is due to their reluctance to participate in the study.

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