

Detection of *Mycobacterium tuberculosis* Complex in Lung Specimen of Slaughtered Cattle and Goats By a DNA Based multiplex-Polymerase Chain Reaction and Ziehl-Neelsen Methods In Jos, Nigeria.

Chukwu Isioma Doris^{1*}, Chukwu Otuh Okoh Chukwu², Kandakai – Olukemi Yvonne³, Owolodun Olajide⁴, Nwosuh Chika⁵, Agada Godwin Ojonugwa¹, Audu Benshak John⁴ and Chollom Solomon Chuwang⁵

¹ Central Diagnostics Laboratory, National Veterinary Research Institute, Vom Nigeria

² Federal College of Veterinary and Medical Laboratory Technology, Vom, Nigeria

³ Department of Medical Microbiology, University of Jos, Nigeria

⁴ Applied Molecular Biology Division, National Veterinary Research Institute, Vom,

⁵ Viral Vaccine Production Department, National Veterinary Research Institute, Vom

ABSTRACT

Aims : Determine percentage positive by the Ziehl-Neelsen (ZN) stain; detect members of *Mycobacterium tuberculosis* complex using a multiplex PCR (M-PCR); compare number of positive samples detected by ZN stain and M-PCR; determine prevalence rates of *Mycobacterium tuberculosis* complex between the two animal species studied and estimate the rates of detecting agents of tuberculosis using Ziehl-Neelsen (ZN) technique and a Multiplex-Polymerase Chain Reaction (M-PCR) in lung samples of slaughtered cattle and goats in the study area.

Place and Duration of Study:- Samples were analyzed at the Central Diagnostic Laboratory and Molecular Biology Departments of the National Veterinary Research Institute Vom. This work was carried out between July-October 2010.

Results : Study Design : Experimental

Methodology:- Our PCR amplified the 245 base pair (bp) fragment which is specific for the *Mycobacterium tuberculosis* complex while ZN exploited the acid-fast nature of the organisms. We examined one hundred lung samples, fifty each from cattle and goats, fifty for each.

Results:- Out of the fifty (50) lung samples from cattle, 15 (30%) were positive by ZN while 9 (18%) were positive by PCR. Among the 35 ZN negative lung samples from cattle that were negative by ZN smear microscopy, one (1) was positive by M-PCR. All the 50 lung samples from goats were negative by the two diagnostic tools used in this study. Results obtained in this study showed 0% prevalence of MBTC in goats and 18% prevalence in cattle.

Conclusion:- The study showed a high prevalence of tuberculosis among cattle sampled in the area of study, as such, preventive and curative measures have to be stepped up in controlling the zoonosis.

Keywords: Cattle; Goats; m-PCR; *Mycobacterium tuberculosis* complex; Ziehl-Neelsen.

1. INTRODUCTION

Members of the *Mycobacterium tuberculosis* complex (MTBC) are slow-growing, aerobic intracellular and acid-fast bacilli that belong to the family; Mycobacteriaceae. These bacteria are the causative agent of tuberculosis in humans and animals. [1]. *Mycobacterium bovis* is closely related to the species *Mycobacterium tuberculosis* (aetiological agent of tuberculosis in human). *Mycobacterium bovis* can also cross the species barrier and cause tuberculosis (TB) in humans [2]. Due to their close relationship, the *Mycobacterium* species causing tuberculosis form the *M. tuberculosis* complex (MTBC) which includes; *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. bovis* Bacillus Calmette-Guerin (BCG), *M. caprae*, *M. pinnipedii*, and *M. canettii* [3]. These bacteria are closely related and their relatedness, can be verified by the high degree of similarity at the DNA level (DNA relatedness of 85 to 100%) [4].

Despite the genetic close relationship that exists among members of the *M. tuberculosis* complex, they differ in their epidemiology and in their importance for tuberculosis in humans. *M. tuberculosis* is the major cause of tuberculosis and, according to the latest figures of the World Health Organization (WHO), infects more than one-third of the world's population [5]. *Mycobacterium tuberculosis* usually infects humans, but it can also infect animals which have contact with infected humans. *M. africanum* is predominantly isolated from different parts of Africa. In certain regions, *M. africanum* represents up to 60% of the isolates obtained from patients with pulmonary tuberculosis. *M. microti* causes tuberculosis mainly in small rodents like voles [4]. However, to date, its importance for TB in human has remained unclear [4]. *M. canettii* can cause TB in humans, but so far only a few *M. canettii* strains have been isolated and its epidemiological contribution to human TB is uncertain [6].

The host range of the *Mycobacterium tuberculosis* complex is broad and it can cause TB in domestic animals like cattle and goats, wild animals and humans [7]. TB in domestic animals remains an important disease in many countries of the world, causing significant economic losses. TB, limits live stock production and affects their quality. It is transmitted to humans through aerosol route, consumption of contaminated milk, improperly cooked meat from infected animals and contaminated dairy products. Infected milk and milk products have been found to be the sources of primary tuberculosis in children and infants, mainly extra pulmonary forms: (miliary, meningeal or lymphatic nodes-localized) [8].

Respiratory droplet is the transmission vehicle of pulmonary tuberculosis in exposed adults especially among slaughter – house and meat processing industry workers [9]. Animal and human TB, emerging or re-emerging and caused by species of the *M. tuberculosis* complex, are wide-spread and affect the animal industries and human health in Africa [10]. Most human tuberculosis due to *M. bovis*, occur in young individuals due to ingestion of contaminated milk or through breaks in the skin. As a result of this, cervical lymphadenopathy, intestinal lesions, chronic skin tuberculosis (Lupus vulgaris), and other non-pulmonary forms are very common [11].

TB caused by *M. bovis*, is a multi-host infection with slow progression of disease in the individual. Tuberculosis is a major opportunistic infection in human Immunodeficiency Virus (HIV)-infected persons. The epidemic HIV infection in Nigeria, in which TB in animals and the environmental conditions, enhancing zoonotic transmission, could make animal TB infection, a serious public health threat to persons particularly at risk [10-11].

In Nigeria, bovine tuberculosis is an endemic bacterial disease of cattle, judging from the reported prevalence from various abattoirs in the country [12]. Nigeria ranks fifth among the 22 high TB burden countries in the world. One of the available reports in Nigeria of diagnosis of bovine TB by bacteria culture from abattoir specimens of lung samples and associated lymph nodes, showed that *M. bovis* was isolated in 62.5% of cases, *M. tuberculosis* in 14.0% and *M. avium* and unclassified *Mycobacteria* in 9.5% [11].

A report from Sabon Gari Local Government Area of Kaduna State, showed that, out of 32 nomadic Bunaji cows, with a clinical history of persistent cough, emaciation, and superficial lymphadenopathy, using a rapid bovine TB antibody test kit, revealed that, 4 were revealed as positive for antibodies to *M. bovis* using a rapid bovine TB antibody test kit, and were culled to avoid further spread of the diseases [13]. Another study carried out in four abattoirs in North – Eastern Nigeria, two from Borno State in Maiduguri and Damboa, and two from Yobe State in Damaturu and Gashua, showed prominent TB lesions in lungs and associated lymph nodes in slaughtered cattle [12].

Documentation of Bovine TB in goats in Nigeria was documented reported by Ojo [18], on the basis of gross lesions without culture confirmation. Live stock owners in Nigeria normally graze cattle and goats together, and this practice poses a high risk for transmission of bovine TB among these animals [14]. This practice is especially a threat to goats in Nigeria because several reports have described bovine TB in cattle in Nigeria [15]. However, reports of diagnosis in goats in Nigeria are lacking.

Molecular epidemiologic techniques such as deletion typing and spoligotyping have been used to characterize members of the *M. tuberculosis* complex and to provide information on transmission of mycobacterial diseases between animals and human [16]. However, because of limited resources and lack of expertise, these techniques are not commonly used in most developing countries such as Nigeria, where TB is endemic [17].

In Nigeria, as in most resource-limited countries, the Ziehl-Neelsen's (ZN) smear microscopy method is used in clinical laboratories for the confirmation of clinical TB, and for detection of TB agents (acid fast bacilli) in biological samples. It is not very specific and sensitive because it requires 10^3 to 10^4 cells per ml of specimen such as sputum or tissue homogenate and detects only acid fast bacilli [18]. This makes the ZN method less dependable than the other methods such as the DNA based PCR method.

Among these methods, the culture method is still the gold standard as isolates can be used to study the morphology, physiology and biochemical characteristics of the organism. It is also more specific and sensitive than ZN microscopy. Nevertheless, despite being the gold standard, most routine laboratories do not culture for *M. tuberculosis* complex. This may be as a result of the fastidious nature of the organism, the cumbersome nature of its manipulation in the laboratory as well as the slow growth rate (3 – 8 weeks) when cultured. However, the PCR methods have proven to be more rapid, highly specific and more dependable for diagnosis than other methods. Moreover, in recent studies, the spoligotyping method has shown to be useful for the identification of species of the *M. tuberculosis* complex.

Reports on the use of PCR for the detection of MTBC in biological samples date over a decade [19]. Most of the studies, however, were performed in non-endemic western countries with only a few [20] from some endemic nations of sub-Saharan Africa [21]. The IS6110 sequence is specific for the MTBC [22]. The sequence, which undergoes several inverted repeats in the bacterial chromosome, has been cloned and used for detection and for molecular epidemiological studies of the MTBC group [20].

Justification and Scope of Study

Little information is available on the use of ZN and M-PCR for specific and efficient detection of members of *M. tuberculosis* complex in lung samples of slaughtered cattle and goats from the Jos central abattoir, Plateau State, Nigeria. This present study compared the rates of detection of TB agents(*Mycobacterium tuberculosis* complex) (MTBC) by ZN microscopy and IS6110 DNA based m- PCR assay, in lung specimens from slaughtered cattle and goats at the Jos central abattoir, Plateau state, Nigeria. It also attempted to establish the prevalence of TB caused by MTBC group in cattle above goats in the study area.

2. MATERIAL AND METHODS

2.1 Study Area

Lung samples of slaughtered cattle and goats were collected from the central abattoir in Jos, Plateau State, Nigeria. The sample analysis was carried out at the Central Diagnostic Laboratory and Molecular Biology Departments of the National Veterinary Research Institute, Vom.

2.2 Sample Size

The sample size was determined using the formula:

$$N = \frac{Z^2 \times P \times q}{d^2} \quad (\text{Benneth et al., 1999})$$

Where: Z = 95% confidence limit
N = Minimum sample size
P = Local Prevalence
q = 1 – P
d = Tolerable error (5%)

The local prevalence of bovine tuberculosis in Jos, Plateau State was 15.4%.

Therefore,

$$N = \frac{0.95^2 \times 0.154 \times 0.846}{0.05^2} = 47$$

One hundred (100) lung samples were collected (50 each from slaughtered cattle and goats).

2.3 Sample Collection and Preparation

Lung samples were collected from slaughtered cattle and goats into sterile universal containers and taken to the Laboratory for detection of acid fast bacilli and *Mycobacterium tuberculosis* complex by ZN stain microscopy and multiplex PCR respectively. All samples were collected between September and December 2010.

Lung samples were homogenized using sterile sea-sand, mortar and pestle. 1 ml of phosphate buffer saline was added to about 2 g of lung sample when homogenizing. The homogenate of each lung sample was strained into 1.5 ml micro-centrifuge tubes, corked and stored at – 80°C until ready for use. Smears were made from lung homogenates and stained by Ziehl-Neelsen method and observed under the light microscope (x100) for detection of acid-fast bacilli. The stored homogenates were further used for detection of *M. tuberculosis* complex by the m-PCR assay.

2.3.1 DNA Extraction

Total DNA was extracted from 95 µL of homogenate based on the protocol provided by the manufacturer of the kit used (Zymo Research Genomic DNA™ – Tissue Mini – Prep, (Catalog: Numbers, D3005 and D3051), which was purchased from Inqaba Biotech Company in Pretoria South Africa). The protocol was as below:

1. To a tissue sample homogenate of 95µl, 95µl of 2x digestion buffer and 10 of proteinase K were added and mixed properly by vortexing in a 1.5ml centrifuge tube.
2. It was incubated at 55°C for 3 hours on a heating block.
3. 700µl Genomic lysis buffer was added to the tube and mixed thoroughly by vortexing and centrifuged at 10,000 Xg for one minute to remove insoluble debris.
4. The supernatant was transferred to a Zymo-Spin tm IIC column in a collection tube and centrifuged at 10,000 Xg for one minute.
5. 200µl of DNA pre-wash buffer was added to the spin column in a new collection tube and centrifuged at 10,00Xg for a minute.
6. 400µl of g DNA wash buffer was added to the spin column and centrifuged at 10,000Xg for one minute.
7. The spin column was transferred to a clean micro-centrifuge tube. 30µl DNA elution buffer was added to the spin column and incubated for 5 minutes at room temperature then, centrifuged at 14,000 Xg for 30 seconds for the elution of the DNA. This was repeated for all the lung samples.

2.3.2 DNA Amplification

A multiplex PCR assay was employed, to detect the presence of MTBC in the lung samples, in order to establish Tb in the animals by using the INS1+INS2 primers for the 245_bp fragments and the JB21+JB22 primers for *M. bovis*, to know if the Tb detected was caused by *M. bovis*. The m-PCR was performed in a 50_µl reaction volume comprised of 26.5_µl nuclease-free water (Fermentas®), 5_µl dNTPs (10_mM), 1_µl each of JB21 and JB 22 primers for 500_bp (JB21: (5'-TCGTCCGCTGATGCAAGTGC-3') and JB22: (5'-CGTCCGCTGACCTC AAGAAG-3'), INS1+INS2 primers for 245_bp (INS1, 5'-INS1 (5'-CGTGAGGGCATCGAGGTGGC-3') and INS2, (INS2 (5'-GCGTAGGCGTCGGTGACAAA-3'); [22], 0.5_µl taq DNA polymerase and 5_µl extracted DNA. The total reaction mixture was submitted to an initial denaturation at 94°C for 5 minutes, then to 30 cycles of denaturation at 94°C for 1 minute, annealing at 68°C for 1 minute, and elongation at 72°C for 7 minutes and cooling at 4°C for as long as there was time.

Amplicons and 100 DNA base pair (Fermentas®) molecular markers were loaded in 2% agarose gel stained with ethidium bromide (10_µg/ml (Promega®) with 6x loading dye (Fermentas®) and electrophoresed at 100_Volts for 45_minutes. Two hundred and forty five based pair (bp) target DNA fragments, specific for MTBC were viewed under ultraviolet illumination. An in house *Mycobacterium tuberculosis* complex positive lung sample was used as a positive control, while all the constituents of the PCR master mix without the DNA extracts, was used as the negative control. The m-PCR products were snapped and recorded.

3. RESULTS AND DISCUSSION

The incidence of bovine tuberculosis is currently increasing in many parts of the world, especially in the third world countries, where the standard of living is poor, such as in Asia and Africa [1]. In this study, one hundred lung samples were tested for *Mycobacterium tuberculosis* complex group by Ziehl-Neelsen stain microscopy and m-PCR methods. From the 100 lung samples processed, 50 were from slaughtered cattle and goats. Out of the 50

Formatted: Normal, Justified, Space After: 0 pt, No bullets or numbering

Comment [D1]: Please confirm

lung samples from cattle, 15 (30%) were positive for Acid fast bacilli (AFB) by Ziehl-Neelsen stain microscopy, while 35 (70%) were negative. Also, out of the 50 lung samples from slaughtered goats, 0 (0%) was positive for AFB by ZN stain microscopy. All lung samples collected from both animals had lesions suggestive of Tb.

Comparatively, 9 (18%) lung samples from cattle were positive for *Mycobacterium tuberculosis* complex group by multiplex – polymerase chain reaction, amplifying the 245 base pair fragment (Figure 1). However, there was no amplification of the 500 bp fragment, which is supposed to be specific for *M. bovis* (Figures 1, 2, and 3). All the lung samples from slaughtered goats were negative for both AFB by ZN stain microscopy and MTBC using an m-PCR assay.

In this study, all the lung samples from slaughtered goats were both negative for AFB by ZN-smear microscopy and by the M-PCR respectively. Among the 35 lung samples from cattle negative by ZN smear microscopy, only one (1) was positive by M-PCR, amplifying the 245 bp fragment.

Statistically, the chi-square analysis at 95% confidence limit, showed a high infection of Tb in cattle and absence in goats by both ZN stain microscopy and m-PCR assay, (Tables 1 and 2). With a chi-square value of $\chi^2=17.647$ it means that, there is a significant rate of infection in cattle than in goats (Table 1).

Table 1: Detection of Acid fast Bacilli in Lung Samples from Slaughtered Cattle And Goats Using Ziehl-Neelsen Stain Microscopy

Type of Animal	Number of lung samples tested	Number (%) positive for Acid fast bacilli
Cattle	50	15 (30)
Goat	50	0 (0)
Total	100	15 (30)

$$\chi^2 = 17.647 \quad DF = 1 \quad P = .05$$

Comment [D2]: one is sufficient as an example

Comment [D3]: one is sufficient as an example

Formatted: Indent: Left: 0", Space Before: 0 pt

Formatted: Indent: Left: 0", Space Before: 0 pt

Formatted: Indent: Left: 0", Space Before: 0 pt

Formatted: Indent: Left: 0", Space Before: 0 pt

Comment [D4]: delete



Fig 1. Agarose Gel Electrophoresis of IS6110 m-PCR for Detecting *M. tuberculosis* Complex from Lung Samples of Slaughtered Cattle—
 Lane A: 100bp DNA ladder (Fermentas). Lane 1: m-PCR amplification of 245bp fragment diagnostic of MTBC. Lane 7: Negative control. Lane 8: Positive control

Formatted: Font: (Default) Arial

Formatted: Centered

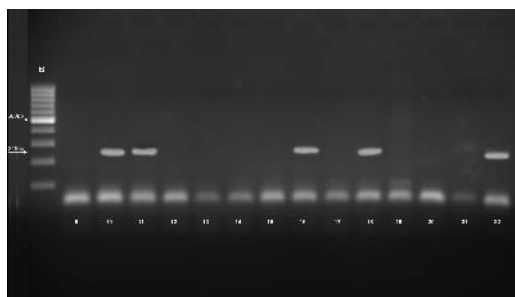
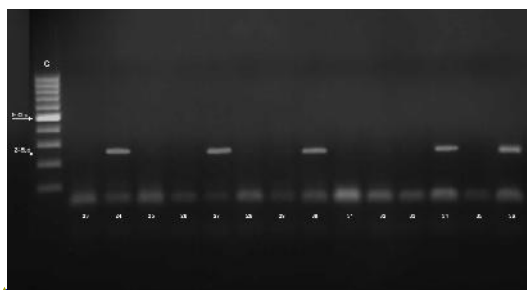


Fig 12. Agarose Gel Electrophoresis of IS6110 m-PCR for Detecting *M. tuberculosis* Complex from Lung Samples of Cattle

Lane 8: 100bp DNA ladder (Fermentas®). Lane: 10, 11, 16 and 18: m-PCR amplification of the 245bp fragment diagnostic for MTBC. Lane 21: Negative control. Lane: 22: Positive control.

Formatted: Centered

271



Formatted: Font: (Default) Arial

Formatted: Centered

272

273

274

275

276

277

278

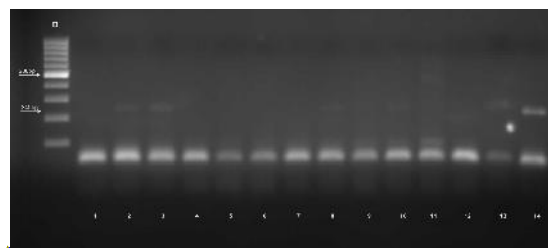
279

Fig 3. Agarose Gel Electrophoresis of IS6110 M-PCR for Detecting *M. tuberculosis* Complex From Lung Samples Of Cattle

Lane C: 100bp DNA ladder (Fermentas).Lanes 24, 27, 30 and 34: m-PCR amplification of the 245bp fragment diagnostic for MTBC.

Lane 35: Negative control, Lane 36: Positive control.

280



Formatted: Font: (Default) Arial

Formatted: Centered

281

282

283

284 **Fig 4. Agarose Gel Electrophoresis of IS6110 m-PCR for Detecting *M. tuberculosis***

285 **Complex from Lung Samples of Slaughtered Goats**

286 *Lanes 1-12, showed no amplification of both 245bp and 500bp fragments. Lane 13: Negative control.*

287 *Lane 14: Positive control.*

288

289

Table 2: Detection of the MTBC group in lung samples from Cattle and Goats using m-PCR

Type of Animal	No Tested	M-PCR	
		No (%) Positive	No (%) Negative
Cattle	50	9 (18)	41 (82.0)
Goat	50	0 (0)	50 (100)
Total	100	9 (18)	91 (91)

$\chi^2 = 9.890$ — $DF = 1$ — $P = .05$

Formatted: Left, None, Don't keep with next, Don't keep lines together

Formatted: Left, None, Space After: 0 pt, Don't keep with next, Don't keep lines together

The incidence of bovine tuberculosis has been observed to be increasing in many parts of the world, especially in third world countries where, the standard of living is poor, such as in Asia and Africa [1]. In this study, one hundred lung samples were tested for *Mycobacterium tuberculosis* complex group, 50 lung samples each were collected from cattle and goats respectively. Two methods were used for the detection of MTBC groups in the lung samples: Ziehl-Neelsen stain microscopy and m-PCR methods.

The results obtained from this study, showed that tuberculosis was present in cattle and absent in goats. This agrees with the results of other authors [11], who reported a 62.5 % prevalence in 1,698 cattle, in the northern region of Nigeria, against a 0.36% prevalence in goats, reported by Ojo [18] in Ibadan, Western Nigeria, who tested 1,387 goats [18]. Ojo [18] isolated four *M. bovis* strains and a *M. tuberculosis* strain from goats. Both the mentioned studies used bacterial culture method for their studies, and samples were from lung and associated lymph nodes. The wide disparity of Tb infection among these animals in these studies cannot be ascertained, since the studies were carried out in different geographic locations and with different herds of animals.

Out of the 50 lung samples from slaughtered cattle, 15 (30%) were positive for Acid fast bacilli (AFB) by Ziehl-Neelsen stain microscopy, while all of the 50 lung samples from slaughtered goats were negative for AFB by both ZN staining and m-PCR methods. This does not agree with the findings of Ojo, [18], where 4 strains of *M. bovis* and 1 strain of *M. tuberculosis* were isolated from goats in Ibadan, Nigeria.

In this study, the results obtained by the m-PCR technique, showed that amplification at the 245bp target region by INS1 and INS2 primers was obtained and that, there was no amplification at the 500bp fragment by the JB21/JB22 primers. This agrees with [23], who reported a 13.3% failure of *M. bovis* isolates to produce the 500bp fragment. This failure was traced to mutations which occurred along the insertion sequence of the organism, leading to alterations of the diagnostic base pair fragments. This resulted to failure of the JB21\JB22 primers to amplify the 500bp fragment diagnostic for *M. bovis*. It is possible that members of the MTBC responsible for TB in the animals tested, lacked the 500bp fragment.

Amplification of the 245 bp fragment by INS1 and INS2 primers is diagnostic for the MTBC-group while amplification of 500 bp fragment is diagnostic for *M. bovis* [24]. PCR using primers JB21 and JB22 has been considered to be highly reliable in identifying *M. bovis*, presenting 100% concordance with the conventional microbiological method. However, the absolute specificity of JB 21 and JB 22 (500 bp) primers to *M. bovis* has been disputed by some studies which reported that 13.3% (4/30) of *M. bovis* isolates failed to produce the 500bp fragment [25]. This failure was traced to the mutations occurred along the insertion sequence of the organism, leading to alterations of the diagnostic base pair fragments. This resulted to failure of the JB21\JB22 primers to amplify the 500bp fragment diagnostic for *M. bovis* [23]. Using specific primers for 1S6110 sequence, the 500bp – negative isolates were identified as belonging to the MTBC groups, causing the authors to suggest that, those isolates may lack the genomic target for JB21 and JB22 primers for 500bp fragment.

With the above limitation that may occur using PCR, it is possible that 18% lung samples which produced amplification of the 245bp fragment only, may lack the genomic target for the JB21 and JB23 primers for 500bp fragment, as suggested by [25].

In this study, out of the 30% positive for tuberculosis by ZN staining, it was observed that but only 18% were positive by multiplex – PCR. Several reasons may be responsible for the differences in results. Some of the isolates may lack the genomic targets for both JB21 and JB22 and INS1/INS2 primers respectively. Alternatively, they may be members of the MTBC

Comment [D5]: please verify the citation, 25 or 23

Comment [D6]: please verify the citation, 25 or 23

requiring the use of Specific primers for confirmation, or environmental or atypical mycobacterium species which do not belong to MTBC.

In this study, only lung samples were collected for analysis and this could be a limitation of the study, since samples from other predilection sites for members of the MTBC were not considered. Although, the lungs are the major sites of infection in pulmonary TB.

Members of the MTBC undergo mutations constantly and as such, primer specificity cannot be relied upon over a long period of time. These mutations can be identified by PCR amplification of target loci and subsequent analysis by PCR-SSCP (Single strand conformation polymorphism), probe hybridization; sequencing etc.; one of such assay is commercially available. While this assay has been found promising and reproducible [26], it is not applicable to a number of strains and correlation of 60% has been observed in some of these studies due to unknown mutations.

Cultivation of MTBC, though not fully proven, is considered the gold standard for the diagnosis of tuberculosis in both humans and animals. However, this gold standard lacks sensitivity and is negative in specimens from majority of paucibacillary cases. This poses a great dilemma for comparing gene amplification methods which are vastly more sensitive, but have danger of false positivity or negativity due to contamination. While PCR is specific for other gene amplification assays, other data should be carefully interpreted and should be correlated with clinical, histological, cytological, biochemical, imaging and therapeutic parameters [26].

Due to various limitations of false negativity and risk of false positivity, no guidelines for application of gene amplification methods are readily available. American Thoracic Society [27] recommended using PCR as additional evidence in specimens smear positive for AFB with smear negative cases, cautious approach of using gene amplification as one of the evidences would be preferable [28], which should be repeated in cases of doubt. The use of ZN-stain microscopy as a diagnostic tool for tuberculosis is still very much in use in third world countries like Nigeria because it is cheap and affordable.

Mawak and collaborators, [14] reported a 15.4% prevalence of *M.bovis* infection among humans in Jos Plateau State. In their study, they examined human subjects using sputum samples. But in this study, slaughtered cattle and goats (lungs samples) were used and *M.bovis* was not detected, therefore, we cannot compare the results from the two studies because of differences in subjects, methods used and infecting agents identified. It is highly probable that the majority of the MTBC species detected from cattle in this study could be *M .bovis*, but additional work will have to be carried out to establish this.

It can however be concluded that, the prevalence of bovine tuberculosis in cattle in this environment is high and one reason for this high prevalence rate, may probably be attributed to the lack of national and state control strategies. However, other factors may be involved, taking into consideration the limitations of this study.

Moreover, as tuberculin testing of human, cattle and other susceptible animals is not routinely performed, the detection of bovine tuberculosis is restricted mainly to the observation of meat inspectors at the abattoirs. This inspection on the other hand is often not consistently carried out by the inspectorate agency. This no doubt poses a great threat to the unsuspecting consumers. This has economic and public health implications in Jos due to the zoonotic nature of the organism.

The possibility of zoonotic transmission of bovine tuberculosis from animals to humans has been reported by several authors [14] but its degree is not clear because of inadequate

401 follow-up and documentation. However, cultural practices exist that could facilitate
402 transmission between cattle and humans. For example, prior to sale, cattle are fattened in
403 close proximity to farmers' homes. After being sold at markets, cattle are often slaughtered
404 in nearby abattoirs, where the butchers wear minimum protective clothing and process offal
405 from diseased carcasses with their bare hands. The close association between farmers and
406 animals is exemplified by the Fulani herdsmen, who live their entire lives with their animals,
407 offering ample opportunity for zoonotic transmission of infection.

408
409 In the course of this study, it was observed that, animals brought to the Jos main abattoir
410 were not examined or inspected before slaughter and also, meat inspection was not carried
411 out before sale. In order for meat inspection to be an effective means of monitoring the level
412 of bovine tuberculosis in the study area, all predilection tissues and organs should be
413 thoroughly examined during inspection and condemned if contaminated by MTBC species.
414 Major improvements in animal and human health, within the concept of meat consumer
415 protection and eradication of epizootic tuberculosis during the 1960s in developed countries
416 were achieved, when drastic destruction of organs with relevant or suspicious lesions at
417 meat inspection was the main strategy employed [30]. Rigorous meat inspection tracing of
418 TB lesions back to the animal farms, pasteurization of raw milk before consumption,
419 continuous tuberculin testing of human and domestic animals susceptible to TB and prompt
420 treatment of infected humans are complementary to eradication or consistent reduction of
421 zoonotic bovine TB. [30]

422
423 Health education on the prevalence of bovine tuberculosis, the mode of transmission,
424 prevention and control is needed in the study area. This is because, most of the butchers
425 and other abattoir workers often wear little or no protective outfit when slaughtering the
426 animals and handling of their viscera organs. This attitude may be due to lack of proper
427 education on the part of the workers or lack of knowledge of the high prevalence of this
428 important zoonotic disease by the public health workers and their agencies in the Jos central
429 abattoir.

430
431 However, bovine TB and zoonotic TB due to *M. tuberculosis* complex group are poorly
432 investigated and controlled in most African countries including Nigeria [31]. The cattle and
433 goats slaughtered at the abattoir in the study originated from different major cattle rearing
434 areas of the country. Nevertheless, the sources or farms from where the animals were
435 reared before being brought for slaughter at the abattoir were not known.

436
437 The incidence of bovine tuberculosis in cattle herds in Nigeria will continue to increase
438 unless there is a concrete effort by the Nigerian government to formulate a comprehensive
439 policy on the control of bovine tuberculosis as is done in the developed countries for
440 example in Europe. If this is not done, the zoonotic spread to humans as documented by
441 [15] will increase and this may have a very devastating effect on the people when one
442 considers the epidemic of HIV/AIDS in the country.

443 444 4. CONCLUSION

445
446 | The observations from this study have shown that cattle was more infected by- tuberculosis
447 than goats. This was shown by the various tests carried out, for the detection of members of
448 the *Mycobacterium tuberculosis* complex in both slaughtered cattle and goat.

449 By the statistical analysis using Pearson-chi square test, the prevalence of the disease in
450 cattle was significant and had relationship with the animal type. Nevertheless, limitations
451 cannot be ruled out, which makes this work subject to further research. It has been shown
452 that the best technique for detecting the various species of the MTBC is spoligotyping [32].
453 In this study, spoligotyping was not carried out.

Bovine tuberculosis is underestimated, but endemic in slaughtered cattle in Nigeria [15]. The test and segregation, with removal of infected livestock, intensification of slaughter and meat inspection, laboratory analysis of lesions for isolation, and confirmation of isolates by molecular typing for strain differentiation and identification are practical intervention for bovine TB in the study area. They will clarify the epidemiology of animal and human TB including zoonotic bovine TB for effective control to be developed.

Finally, TB is prevalent in cattle destined for human consumption in Nigeria with serious public health implications. The general public is at risk, and infected individuals can serve as source of infection. Many opportunities exist for the emergence of zoonotic TB and necessitate further study into the modes of transmission and link between human and bovine TB through molecular techniques. Targeted control of infected animal populations, concerted veterinary and medical efforts, active involvement of the populations at risk, and good health systems are essential for effective control.

From the foregoing, the following recommendations can be made:

1. More of such research should be carried out in the study area to see if results obtained will be in concordance with the results obtained from this research to establish the prevalence of bovine tuberculosis among cattle above goats.
2. An all encompassing research can be carried out in the study area, involving more techniques for the identification of the members of *Mycobacterium tuberculosis* complex in domestic animals and animal farmers and handlers in order to establish the prevalence of tuberculosis in both humans and animals in the area.
3. Financial support of research in the study area should be encouraged, to enhance purchase of the necessary biologics required for speciation of the members of the *Mycobacterium tuberculosis* complex, in order to identify the particular species responsible for tuberculosis in the area.
4. Adequate animal inspection before sale and slaughter, should be encouraged and annual tuberculin testing of animals and animal handlers should be carried out to reduce the prevalence of tuberculosis in that area.

ACKNOWLEDGEMENTS

The authors of this research, wish to acknowledge and thank members of staff of the molecular Biology Department, National Veterinary Research Institute Vom, for all the technical assistance they rendered during the course of this work. We also wish to acknowledge Edore Akpokodje for carrying out the statistic analysis of the results obtained in this work and also Tyagnet Elisha for editing and typesetting this work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

Authors' Contributions

Chukwu Isioma Doris : Did the sampling, ran the DNA extraction and wrote the major aspect of the manuscript

Chukwu Otuh Okoh Chukwu : Wrote the Introduction

Kandakai- Olukemi : Read and corrected the entire manuscript

Owolodun Olajide : Prepared the PCR master mix for the m-PCR assay

Chika Nwosuh : Assisted in purchasing the extraction kits and primers used for this work
 Godwin Agada : Did the Ziehl-Neelsen staining for this work
 Benshak Audu : Assisted in DNA extraction and the PCR assay
 Chollom Solomon : Assisted in writing the results and preparing the manuscript for publication

REFERENCES

1. American Thoracic Society (ATS) Rapid diagnostic tests for tuberculosis. What is the appropriate use of American Thoracic Society Workshop: Medical section of the American Lung Association. American Jour. Respiratory Care Medicine. 1997; 155: 1804-1814.
2. Ayele WY, Neill S D, Zinsstag J, Weiss MG, Pavlik I. Bovine Tuberculosis: an old disease but a new threat to Africa. Int'l J. Tuberc. Lung Dis. 2004; 8: 924-937.
3. Cadmus SI, Atsanda NN, Oni SO, Akang EE. Bovine tuberculosis in one cattle herd in Ibadan in Nigeria. J. Vet. Med. 2004; 49:406-12.
4. Cadmus SIB, Adesokan H K. "Causes and implications of bovine organs/offal condemnations in some abattoirs in Western Nigeria.' Tropic. Anim Hlth. and Prod., Vol.41, no. 7, PP. 2009; 1455-1463.
5. Cadmus S I, Palmer S, Okker M, Dale J, Gover K and Smith N . Molecular analysis of; human and bovine tunercule bacilli from a local setting in Nigeria. J. Clin. Microbiol. 2006; 44:29-34.
6. De Kantor I N and Torres P M, . La tuberculosis bovina en la Argentina Y en Otros paises de America Latina. Su importancia Zoonotica. In: R. Cacchione, R. Durlach and Larghi, OP, editors. Temas de zoonosis 5th ed. Asociacion Argentina de zoonosis, Buenos aires. 2008; 23-26
7. Einsenach K D., Cave M D., Bats J H and Crawford JT. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. J Infect Dis. 1990; 161:977-981.
8. Good M. "Bovine tuberculosis eradication in Ireland." Irish Veterinary Journal, 2006; 59 (3) : (*In Press*)
9. Grange JM, Malcolml DY and Isabel N. Guidelines for Speciation within the *Mycobacterium tuberculosis* complex.1996; EMC Zoo 96:4 2nd ed. Accessed 02-August-2007. Available : <http://www.who.qlibdos.who.int/hp/1996/WHO> EMC of)" .1996 World Health Organisation
10. Hellyer TJ, Des Jardin L E, Assaf M K, Bates J H, Cave M D, Einsenach K D. Specificity of IS6110 based amplification assays for *Mycobacterium tuberculosis* complex. J clin microbiol. 1996; 34:2843-2846.
11. Igbokwe I O, Madaki I Y, Danburam S, Ameh J A, Aliyu M M, Nwosu C O. Prevalence of Pulmonary Tuberculosis Lesions in Cattle Slaughtered in Abattoirs in Northern Nigeria. Revue Elev. Medic Vet Pays tropics., 2001; 54 (3-4): 191-195.
12. Kambushi B G, Mbulu R, Mcnerncy R. Tembwe A, Kambashi V, Tihon P and Godfrey-Fausett. Utility of nucleic acid amplification technique for the diagnosis of pulmonary tuberculosis in sub-Saharan Africa. Int J Tuberc Lung Dis. 2001; 5 : 346-369
13. Kubica T, Rusch-Gerdes S and Niemann . *Mycobacterium bovis* subsp. Caprae caused one third of human *M. bovis* strains associated tuberculosis cases Reporsrted in "Germany between 1999 and 2001. J Clin Microbiol 2003; 41:3070-3077.
14. Mawak J D, Gomwalk NE, Bellow CSS and Kandakai- Olukemi. Human pulmonary infections with Bovine and Enivronment (Atypical) mycobacteria in Jos. Ghana Med. J. 2006; 40(4): 132-136.

15. Mustapha A S, Abal AT and Chugh TD . Detection of *Mycobacterium tuberculosis* complex and non- tuberculosis mycobacteria by multiplex polymerase chain reaction. EMHJ 1999; 5: 61-70.
16. O'Reilly L M and Daborn C J. "The epidemiology of *Mycobacterium bovis* infections in animals and man: A review" Tuberc. and Lung Dis. 1995; 76, supplement 1,1-16.
17. Office International des Epizooties (O.I.E). "Manual of Diagnostic Tests and Cases for Terrestrial Animals" OIE Terrestrial Manual, Paris, France, 2008; Available : <http://www.oie.int/eng/normes/mmanreal/Asummary.htm>. Accessed: May 2010
18. Ojo SA, A survey of pathological conditions in slaughtered goats at Zaria Slaughter houses. Small ruminant research and development in Africa. Proceedings of the third Biennial Research Network UICC. Kampala, Uganda. 2009; Available : <http://www.fao.org/documents/pub-dett.asp?Pubuid=18823&diang=en>. Accessed August 2010
19. Pavlik I, Machackova M, Ayele WY, Lamka J, Parmova I, Melicharek I, *et al.* Incidence of bovine tuberculosis in domestic animals other than cattle and in wild animals in six central European Countries during 1990-1999. J Vet Med. 2002e; 47: 122-131.
20. Perry B D, Randolph T F, Madermott J J, Sones K R, Thornton PK. editors. Investigation In Animal Health Research to Alleviate Poverty. International Livestock Research Institute, Nairobi, Kenya. Letters. 2002;(1)148-148,
21. Ritcher EL, Weizenegger M, Fahr AM, Rusch-Gerdes S. Usefulness of Genotype MTBC Assay for Differentiating species of the MTBC in cultures obtained from clinical specimens. J Clin Microbiol. 2004; 42:4303-4306.
22. Sechi LA, Dupre I, Leori G, Fadda G and Zanetti S. Distribution of a specific 500 base pair fragment in *M.bovis* isolates from Sardinian Cattle. J. Clin. Microbiol. 2000; 38: 3837 – 3839.
23. Shah DH, Rishendra V, Bakshia CS, Singh KR-. A multiplex-PCR for the differentiation of *M. bovis* and *M. tuberculosis*. FEMS Microbiology Letters. 2002; 214: 39-43.
24. Shah J, Liu J, buxton D, Hendrix A, Robinson L, Radcliffes G. Q-beta preplacase amplified assay for detection of *Mycobacterium tuberculosis* directly from clinical specimens, Journal of Clin. Microbiol. 1995; 33:1435-1441.
25. Sharma M, Sethi S, Mishra B, Sengupta C, Sharma SK-. Rapid detecton of mutations in *spoB* gene of resistant *Mycobacterium tuberculosis* strains by line probe assay. Indian Jour -Medic Res. 2003; 117:76-80.
26. Sola C, Rastogi N, Guitierrez MC, Vincent V, Brosol R, Parsons L, *et al.*, Is *Mycobacterium africanum* subtype II (Uganda II) a genetically well-defined sub-species of the *Mycobacterium tuberculosis* complex J Clin Microbiol. 2002; 41: 1345-1348.
27. Tanya V N, "The contribution of animal and fisheries research to poverty alleviation in cameroon," In proceedings of the 11th Annual conference of Bioscience: Animal Production and Poverty Alleviation In : Mbiapo FT, Etoa FX, *editors*. 2004; The Cameroon Bioscience Society, Yaounde, Cameroon, 10th edit.
28. Thoen CH, Lobue P A, dekantor I N. The importance of *Mycobacterium bovis* as a zoonosis. Veterinary Microbiolgy. 2006 112: (2-4), 339-345.
29. Torrea G, van de Perre P, Ouedraogo M, Zougba A, Sawadogo A, Dingtombda B, *et al.* PCR based detection of *Mycobacterium tuberculosis* complex in urine of HIV-infected and non-infected pulmonary and extra pulmonary patients in Burkina Faso. J Med Microbiol. 2005; 54: 39-44.
30. Van der Zanden A G M, Kremer K, Schouls L M, Caimi K, Cataldi A, Hullernan A, *et al.* Improvement of differentiation and interpretability of spoligotyping for *Mycobacterium tuberculosis* complex isolates by introduction of New Spacer Oligonucleotides. J. Clin. Microbiol. 2002; 40: 4628-4639.

Formatted: Font: Italic

609 31. World Organization for Animal Health (WOAH) Manual of Diagnostic tests and
610 vaccines for terrestrial Animals. Accessed: 29th April, 2008, Available:
611 <http://www.worldorganizationforanimalhealth.org/2008>

612

613