

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.

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ABSTRACT

Aims :- Determine percentage positive by the Ziehl-Neelsen (ZN) stain ; detect 245 base pair fragment of the IS6110 gene for *Mycobacterium tuberculosis* complex using INS1 and INS2 primers and 500 base pair fragment of the RvD1Rv2031c gene for *M.bovis* using the JB21 and JB22 primers by 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 this group of *Mycobacterium* while ZN exploited the acid-fast nature of the organisms. We examined one hundred lung samples, of cattle and goats, fifty for each.

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

Conclusion - : The study showed a high infection rate 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.

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1. INTRODUCTION

Members of the *Mycobacterium tuberculosis* complex (MTBC) are slow-growing, aerobic intracellular and acid-fast bacilli (AFB) that belong to the family; Mycobacteriaceae[1]. The host range of *Mycobacterium tuberculosis* complex is broad and it can cause tuberculosis (TB) in domestic animals like cattle and goats, wild animals and humans [2]. 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. Respiratory droplet is the transmission vehicle of pulmonary TB in animals and exposed humans, especially among slaughter – house and meat processing industry workers [3]. Animal and human TB, emerging or re-emerging and caused by species of the MTBC, are wide-spread and affect the animal industries and human health in Africa [4]. 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 [4-5].

In most resource-limited countries, such as Nigeria, 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 [6]. 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 – 8weeks) when cultured. However, the PCR methods have proven to be more rapid, highly specific and more dependable for diagnosis than other methods. The 245 base pair fragment of the IS6110 gene sequence is specific for the MTBC [7]. 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 [8]. Moreover, in recent studies, deletion typing and spoligotyping methods have shown to be useful for the identification of species of the *M. tuberculosis* complex.

In Nigeria, bovine tuberculosis is an endemic bacterial disease of cattle, judging from the reported prevalence from various abattoirs in the country [9]. 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% [5]. 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, 4 were revealed as positive using a bovine rapid antibody test kit for antibodies to *M. bovis* and were culled to avoid further spread of the diseases [10]. Bovine TB in goats in Nigeria, was documented 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 [11]. This practice is especially a threat to goats in Nigeria because several reports have described bovine TB in cattle in Nigeria [12]. However, reports of diagnosis in goats in Nigeria are scarce.

77 The aim of this study is to provide information on MTBC infection in cattle and goats from the
78 study area, which hitherto has been very limited. Also, we intend to compare the detection
79 rates of the organisms using the very conventional ZN stain and a more improved
80 technique, M-PCR assay.
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2. Materials 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:

$$N = \frac{Z^2 \times P \times q}{d^2} \quad (\text{Benneth et al., 1991})$$

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. 1ml of phosphate buffer saline was added to about 2g of lung sample when homogenizing. The homogenate of each lung sample was strained into 1.5ml 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 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 95uL of homogenate based on the protocol provided by the manufacturer of the kit (Zymo Research Genomic DNA™ – Tissue Mini – Prep, (Catalog: Numbers, D3005 and D3051), by Inqaba Biotech Company in Pretoria South Africa.

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 245bp 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 (10mM), 1 µl each of JB21 and JB 22 primers for 500bp fragment of the RvD1Rv2031c gene (JB21: 5'-TCGTCCGCTGATGCAAGTGC- 3' and JB22 : 5'-CGTCCGCTGACCTC AAGAAAG-3'), INS1+INS2 primers for 245bp fragment of the IS6110 gene (INS1: 5'-CGTGAGGGCATCGAGGTGGC-3' and INS2 : 5'-GCGTAGGCGTCGGTGACAAA-3') [13]. 0.5 µl taq DNA polymerase and 5µl extracted DNA. The total reaction mixture was submitted to an initial denaturation at 94°C for 5 minutes, then to 30 cycles of denaturaion 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. The INS1/INS2 and the JB21/JB22 primers are meant to amplify the IS6110 and the RvD1-Rv2031c [14].

Amplicons and 100 DNA base pair (Fermentes®) molecular markers were loaded in 2% agarose gel stained with ethidium bromide 10mg/ml (Promega®) with 6x loading dye (Fermentas®) and electrophoresed at 100Volts for 45minutes. 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

Out of the 50 lung samples from cattle, 15 (30%) were positive for (AFB) by ZN stain microscopy but none in goats. All lung samples collected from both animals had lesions suggestive of Tb. MTBC was detected in 9 (18%) of samples from cattle by a M-PCR, amplifying the 245 base pair fragment of the IS6110 gene, (Fig.1) but none from goats. However, there was no amplification of the 500bp fragment of the RvD1Rv2031c gene, which is supposed to be specific for *M. bovis* .[14] The inability of this technique to detect the 500bp fragment in this study, has previous been reported elsewhere [15]. The authors explained that 13.3% (4/30) of *M. bovis* isolates failed to produce the 500bp fragment . This failure was traced to the mutations which occurred along the insertion sequence of the organism, leading to alterations of the base pair fragments, thereby preventing the JB21 and JB22 primers from amplifying the 500bp fragments . Among the 35 lung samples from cattle negative by ZN smear microscopy, only one was positive by M-PCR, amplifying the 245bp fragment.

Statistically, the chi-square analysis at 95% confidence limit, showed a high infection of Tb in cattle and absence in goats by both diagnostic tools, with a chi-square value of $X^2=17.647$.

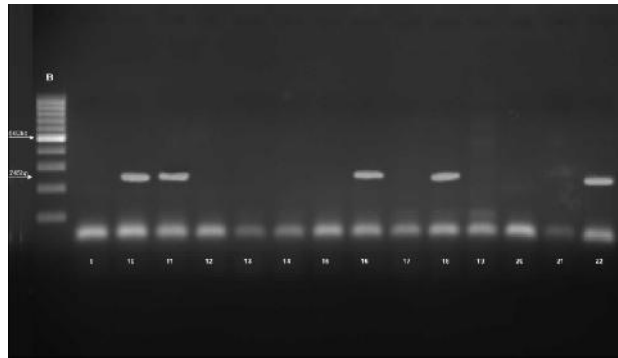


Fig 1. Agarose Gel Electrophoresis of IS6110 m-PCR for Detecting *M. tuberculosis* Complex from Lung Samples of Cattle

Lane B: 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.

The results obtained from this study, agrees with the results of other authors [5], 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 [6] in Ibadan, Western Nigeria, who tested 1,387 goats. Ojo [6] isolated 4 *M. bovis* strains and a *M. tuberculosis* strain from goats. Both the mentioned studies used bacterial culture method, 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.

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.

4. CONCLUSION

The presence of MTBC infections in cattle as observed in this study indicates that the agents are in circulation in the study area. This has therefore placed man and other animals at risk of this zoonosis. Animal handlers including butchers should be advised of the danger of the infections and take appropriate preventive measures. Also, animal products meant for consumption should be properly cooked or pasteurized to avoid infection of unsuspecting consumers.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

Authors' Contributions

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

Chukwu Otuh Okoh Chukwu : Wrote the Introduction

Yvoun Kandakai- Olukemi : Read and corrected the entire manuscript

Olajide Owolodun : 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 Ojonugwa : Did the Ziehl-Neelsen staining for this work

Benshak John Audu : Assisted in DNA extraction and the PCR assay

Solomon Chuwang Chollom : Assisted in writing the results and preparing the manuscript for publication

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