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Detection and Quantification of *Mycobacterium tuberculosis* Complex Bacilli in Slaughtered Cattle, at Yola, Adamawa State, Nigeria

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Abstract

Bovine tuberculosis (bTB) is a neglected zoonotic disease of major public health and economic importance in Nigeria. This study determined the prevalence of *Mycobacterium tuberculosis* complex (MTBC) among slaughtered cattle at Yola Modern Abattoir, Adamawa State, using liquid and solid culture methods. A cross-sectional abattoir-based study was conducted in which 190 bovine lung samples with lesions suggestive of tuberculosis were collected. Samples were processed using Petroff's decontamination method and cultured in the Mycobacteria Growth Indicator Tube (MGIT 960 BACTEC system) and on Lowenstein–Jensen (LJ) media supplemented with glycerol and pyruvate. MTBC-positive cultures were confirmed using the SD Bioline™ TB Ag MPT64 assay.

Out of 190 samples examined, 85(44.7%), were posistive for MTBC by TB-MBLA, 73 (38.4%) were positive for MTBC by liquid culture, while 45 (23.7%) were positive by solid culture. TB-MBLA, showed the highest detection rate followed by Liquid culture and the least being solidculture method (χ^2 = 19.34; p = 0.000). Based on TB-MBLA results, the apparent prevalence of bovine tuberculosis was 44.7%.

The high prevalence observed confirms the endemicity of bovine tuberculosis among cattle slaughtered in Yola and indicates a substantial increase compared with earlier reports from the area. The practice of selling carcasses after removal of affected organs poses a significant zoonotic risk to abattoir workers and consumers. Strengthening meat inspection, implementing active surveillance, and adopting effective national bTB control strategies are urgently needed to reduce transmission and protect public health.

Keywords: *Mycobacterium tuberculosis* complex, bovine tuberculosis, Mycobacterium growth indication tube, BACTEC 960, Lowenstein-Jensen medium, TB-MBLA.

INTRODUCTION

Bovine tuberculosis is a chronic contagious bacterial disease of livestock, and occasionally other species of mammals, resulting from infection with *Mycobacterium tuberculosis* complex species. The bacteria associated with the disease may lie dormant in an infected animals for years without causing clinical signs or progressive disease symptoms. It can reactivate during periods of stress or in older animals. When disease becomes progressive, it generally results in enlarged lesions which may be found in a variety of tissues including lymph nodes of the head and thorax, lung, spleen, and liver [1].

Bovine tuberculosis (BTB) is a serious public health and neglected zoonotic disease responsible for 147,000 human cases and 12,500 deaths annually [2] and the aetiologic agents of tuberculosis in mammals are members of the Mycobacterium tuberculosis complex. They consist of the closely related species *M. tuberculosis*, *M. bovis*, *M. caprae M. africanum*, *M. microti*, "*M. canetti,*" and *M. Pinnipedii, M. mungi, M. orygis and M. suricattae* [3]. *M. bovis* is the most common species reported to cause bovine TB in cattle and buffalo. In addition to *M. bovis*, other members of MTBC such as *M. tuberculosis M. caprae, M. orygis, M. pinnipedii* and *M. microti,* have also been reported to cause TB in cattle [4].

Cattle and buffaloes are susceptible to *M. bovis* [5] The disease can be spread to humans primarily by ingestion of viable bacilli of the bacteria in raw milk, improperly cooked meat and meat products of infected animals and poor handling of contaminated meat/ visceral of slaughtered infected animals. Animals get infected by inhalation of respiratory droplets from infected animals when they cough due to close proximity, ingestion of viable MTBC bacilli in contaminated soil during grazing and from contaminated water, milk and feed [6]. The disease is a critical public health burden and causes severe economic losses due to impairment of animal health, production losses, costs of eradication programs, and trade restrictions [4]. Annual agricultural losses due to bovine TB have been estimated to be approximately 3 billion United States Dollars worldwide [7].

The global pooled prevalence of BTB in cattle was estimated at 13.12%; Africa had 10.3%, Asia at 13.8%, Europe had 17.8%, North America 33.6%, and South America at 20.5% (In 2018, the WHO reported a global

estimate of human cases at 143,000 and 12,300 deaths due to zoonotic tuberculosis (zTB) caused by *M. bovis* [7].

M. bovis is the leading cause of TB in cattle, Buffalo and other mammals. *M. bovis* comprises *M. bovis* subsp. *bovis*, *M. bovis* subsp. *caprae*, and the *M. bovis*-derived BCG vaccine strain [8]. Both subspecies of *M. bovis* are reported to infect humans, wildlife (deer, wild boars, antelopes and buffalo), domestic animals (pigs, cats and dogs) and livestock cattle, sheep and goats) [9].

Nigeria is the most populated country in Africa with a projected population of 216,783,381 people in 2022,[9] and has about 19.5 million cattle and an unknown population of wildlife ruminants [10]. These factors demonstrate opportunities for easy transmission and spread of BTB, evidenced by the country ranking fourth with tuberculosis in the world and first in Africa [1,11].The constant uncontrolled transborder influx of herders and cattle from neighboring States and countries without adequate and complete inspection, limited access to veterinary services, poor diagnostic facilities, and inadequately trained manpower have immensely contributed to the endemicity of the disease in Nigeria [12].

In Nigeria, overall pooled BTB prevalence in animals is 7.0%; cattle (8.0%), goats (0.47%), sheep (0.27%), camels (13.0%), and wildlife (13.0%) [13]. Over 2 million US Dollars have been lost as a result of the effect of bovine Tb in Nigeria. This is a threat to the animal industry which generates about 94% of animal protein and contributes about 21% of National Gross Domestic Product (GDP) [14].

Bovine tuberculosis (BTB) is a neglected zoonotic disease of public health importance and responsible for high morbidity and mortality among humans in Africa.

The infection of cattle in developed countries has been controlled, using the 'Test and slaughter' policy and vaccination but complete elimination of the disease is complicated by persistent infection in reservoir wild animals such as European badgers in United Kingdom, white-tailed deer in America and Possums in New Zealand [15]. But in Africa especially Nigeria, the 'test and slaughter' policy has not been implemented. Bovine tuberculosis (bTB) in Nigeria is currently controlled through abattoir monitoring. However, Nigeria lacks a national control strategy and active surveillance programs [1].

MATERIALS AND METHODS

Study Area

The study area for this research was the Yola modern abattoir in Yola, Adamawa State, Nigeria. The Yola modern abattoir, is situated between Jimeta and Yola town of Adamawa State (North Eastern Nigeria). The abattoir is owned by the Adamawa state Government, and managed by the Ministry of Livestock and Nomadic Resettlement. The abattoir is the major source of meat for the people of Yola and its environs. It lies between latitude 9° 14 N of the equator and longitude 12° 14 E of the Greenwich-meridian [16].

Sample size

The sample size was determined using the formula proposed by (Araoye, 2003) using the 9.8% prevalence rate reported by Ibrahim et al. [17] in Yola, Adamawa State

$$N = \frac{Z^2(pq)}{d^2}$$

Where: N= Minimum sample size

Z= Standard error at 95% confidence interval= 1.96

p=Local Prevalence (9.8%) = 0.098

q=1 $-p$ = 0.902

d=degree of accuracy (5%) = 0.05

N = $[(1.96)^2 \times 0.098 \times 0.902]/0.0025$

N = 136

The minimum sample size for this study was 136 bovine lung samples from slaughtered cattle. But 190 bovine lung samples with suggestive Tb lesions were collected in Yola modern abattoir, Yola, Adamawa State.

Research Design

A cross -sectional design was carried out to determine the present prevalence of bovine Tb in the study area, by collecting lung samples with suggestive Tb lesions from slaughtered cattle

Sample Collection

Specimens of lungs, with suspected Tb lesions, were collected into sterile plastic containers from slaughtered cattle at the Yola modern abattoir. The specimens were transported in ice box to the National Veterinary Research Institute, Vom and stored at -80^{0}C until ready for use.

Sample Preparation for culture

Approximately 5g each of lung sample was cut using a clean, pair of forceps and scissors and transferred into Falcon tubes containing 5ml of sterile phosphate buffer saline (PBS) and homogenized in a biosafety cabinet. Using Petroff's standard method, equal volumes of 4% sodium hydroxide and tissue homogenates were mixed vigorously to decontaminate.The suspensions were centrifuged at 3000g for 15 minutes, the supernatant was discarded and the sediment was re-suspended in 5ml of sterile phosphate buffered saline. Suspension was centrifuged at 3000g for 10 minutes, supernatant was discarded and sediment was used for liquid and solid cultures.

Liquid culture in MGIT 960 Bactec system (MGIT)

MGIT tubes were inoculated with 0.5 ml of the processed specimen and slotted into the MGIT 960 instrument. The tubes were incubated at 37°C and were monitored automatically every 60 minutes for increase in fluorescence for 8 weeks.

Any sample, which was identified as positive, was removed from the instrument.

Furthermore, a brain heart infusion agar plate was inoculated to determine non-Mycobacterium isolates which grew after 24-48 hours incubation at 37^{0}C.

Solid culture on Lowenstein-Jensen (LJ) medium

LJ medium was prepared in duplicates (one with glycerol and the other with pyruvate). Each decontaminated sample was inoculated into LJ medium with glycerol and another with pyruvate (Glycerol supports the growth of some MTBC such as *M. tuberculosis*, while pyruvate supports the growth of *M. bovis.*

Confirmation test for MTBC Positive Cultures

The SD Bioline™ TB Ag MPT64 kit was used to confirm the growth of MTBC in both liquid and solid media. The Bioline TB MPT 64 assay, is an immune-photocromatographic identification test for the presence of MTBC in culture fluids. It was used to confirm presence of MTBC based on the manufacturer's instructions.

The SD bioline TB AgMTP64 kit has been successfully used to differentiate MTBC from non-MT C species [18].

A drop of the specimen was placed on the specimen placing area on the test plate.

The colloidal gold-labelled MPT64 antibody 'A' dissolves and forms an immune complex with the MPT 64 antigens in the specimen. The immune complex migrates through the developing area by capillary action and it is captured by the anti-MPT64 antibody 'B', fixed in the reading area 'T' test. The resultant complex forms a purple-red line of colloidal gold in the reading area 'T'. This visually indicates the presence of MPT 64 antigen in the specimen.

Sample preparation for MTBC Bacilli Detection and Quantification

The Tuberculosis Molecular Bacterial Load Assay (TB-MBLA), which is a real time PCR, was used to detect *Mycobacterium tuberculosis* complex and to quantify the bacilli load in each sample. Approximately 5g of bovine lung samples with TB lesions, were cut out from the lung of each slaughtered animal into sterile universal bottles. These were first homogenized in falcon tubes containing 4ml of guanidine thiocyanate (GTC) to deactivate the organisms and expose the tubercle bacilli, in a biosafety cabinet within the biosafety laboratory class 3 facility at the National Veterinary Research Institute Vom, and stored in 15ml falcon tubes at -80˚C ultra- low freezer until ready for analysis.

RNA Extraction and DNA removal from Tissues

RNA was extracted and DNA removed from all the bovine lung samples using the protocol provided in the MP Biomedicals RNApro blue RNA extraction kit. This was done in a class II biosafety cabinet to avoid the inhalation of 2-mecapto-ethanol, during the early stages of RNA extraction. DNA was removed from all the samples by DNase treatment with the Invitrogen Turbo DNA-free kit, Ambion AM1907, as described by the protocol provided in the kit.

In order to obtain DNA- free RNA from the bovine lung samples, they were treated with a master mix of 10 µl DNA buffer and 2 µl of DNase enzyme. The master mix for DNA removal, contained 10µl of DNA buffer multiplied by the number of samples, plus 2µl of DNase enzyme, multiplied by the number of samples plus 2 µl extra of both DNA buffer and DNase enzyme to make up for volume loss during pipetting/ pipetting errors. Each sample, was expected to have 11 µl volume of the DNA buffer (10 µl) and (1 µl) DNase enzyme mixture. (DNA-free RNA, was obtained from each sample, by following the protocol provided in the Invitrogen Turbo DNA kit).

Primers and TaqMan Probes 5' – 3' Sequences

Primer and probes (Table 1) were supplied by VITAL BACTERIA Ltd University of St. Andrews, Scotland UK. The kit consists of the information which were followed for dissolving the primer and probes to obtain stock solutions.

Before the RT-qPCR, the master mix for the number of intended reactions, were prepared. This is a solution of PCR reagents sufficient for all samples and standards to be amplified in a run. Each RNA sample and standard were amplified in duplicates.

Table 1: Primers and Probe sequences for TB-MBLA Assay

Name	Sequence	Reference
MTB 16s forward	GTGATCTGCCCTGCACTTC	(Zainabadi et al., 2022)
MTB 16s reverse	ATCCCACACCGCTAAAGCG	
IC MMtmRNA F	CGTCATCCTGGCTAGTTC	
IC MMtmRNA R	CTACGGCATTCCCTCAAG	
Mtb 16s probe	FAM-AGGACCACGGGATGCATGTCTTGT-BHQ1	
IC MMtmRNA probe	HEX-AGT CCG CTA TGT CTC TGC TCG-BHQ1	

Preparation of standard samples for standard curve

The standard curve was imported into the amplification curves, generated from the thermocycler by TB-MBLA-RT-qPCR, using Rotor Gene Q, to translate quantification cycles into estimated colony forming unit per gram or per millilitres of the samples which helps to capture the cycle threshold values (CT values) as described by Sabiiti and colleagues [19].

To prepare the standard samples, the Vital bacteria MTB standard and external control (EC) RNA standards, were thawed on ice, then seven (7) RNase- free- tubes were labelled with appropriate 10fold dilution from tube 1-7 which is (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}). That is, 7

tubes for MTB standard and 7 tubes for external control RNA extracts control standards (Both were supplied in the Vital bacterial kits). Ten microlitres(10µl) was added to the first tube and mixed by vortex. A one in ten dilution was carried out from tube 1 to tube 7, changing pipette tips between tubes. This dilution was done for external control (EC) RNA standard.

To construct the standard curve for MBLA, the MTBC standard and external control standards were mixed to be at concentrations of 10^8 -10^7 CFU/ml. To achieve these dilutions, two sets of seven 1.5ml RNase- free tubes, were labelled with appropriate names and dilution numbers (1-7). One set of dilutions was prepared for MTB standard and the second set for external control standard. These standards were provided in the Vitabacteria MTB kit, which contains the MTB and EC standards, the Primers and probes for the yellow and green channels. The kit was prepared by the University of Saint Andrews, Scotland UK. These standards, were diluted decimally (1:10) to create a series of standard dilutions.

Two sets of seven (7) tubes were arranged based on the dilution factor, 90µl of RNase-free water was added to each tube.10µl of the external control standard was added to 90µl of RNase-free water in the first tube and a 1:10 dilution was done for the seven tubes. This was also repeated for the MTB standards, by adding 10µl of the MTB standard to 90µl of the RNase-free water in the first tube followed by a 1:10 dilution to the 7th tube. All the tubes were mixed by vortex for 5 seconds, changing pipette tips in between each dilution. The dilutions of the EC and MTB standards were run with the Rotor Gene Q machine, to determine the standard curve for the samples to be ran in this study. The standard curve, was imported into the Cycle Threshold values (CT) of each sample to standardize the Tb-MBLA-RT-qRNA assay. The CT value, is inversely proportional to the Mycobacterial load in the samples. The higher the bacterial load, the smaller the CT value. For the TB-MBLA assay, a sample with a CT value of less than 30, is considered positive for MTBC, while a sample of CT value of 30 and above are negative for MTBC. The standard curve, once prepared for a TB-MBLA assay, can be used to determine the Cycle values and the quantity of MTBC viable cells that is, Colony forming unit/millilitres (CFU/ml) for 3-4 months [19].

Master- Mix for Thermocycler Set Up

The ready to go master mix is a solution of PCR reagents sufficient for all samples and standards to be amplified. Each RNA extract from each sample, and its decimal dilution and standards were amplified in duplicates. Into each PCR tubes, 4µl of sample RNA extract was added to 16µl of RT+ master mix solution (containing Reverse transcriptase enzyme) to sum up to 20µl reaction volume. Each sample and the controls, were ran in duplicates in the RT positive wells (RT+ containing the reverse transcriptase enzyme) while the RT negative (RT-) wells, containing 16µl of the RT negative (RT-) master mix and 4µl of the RNA extract neat samples and their control (RNase-free water), were ran in a single reaction and the concentration of MTBC cells is calculated from 16SrRNA present in the sample.

Thermocycler Set Up for Rotor Gene Q

For every run, the reaction tubes were arranged in appropriate rotor and locked with the locking ring to prevent spillage. The rotor was placed inside the Rotor Gene Q instrument, clicked into position and the lid was closed. The machine was switched on and appropriate information of the samples were filled into the Rotor gene Q software, by the operator. The Rotor Gene Q machine, runs for two (2) hours and thirty (30) minutes before a run is completed.

The reaction volume for every run on the Rotor Gene Q, was 20µl and the reaction condition for TB-MBLA requires a hold at 50^0C, for 30 minutes (this is the reverse transcription),95^0C, for 15 minutes (To activate the Taq DNA polymerase) and cycling, at 40 cycles at 94^0C for 45 seconds not acquiring, 60^0C, for 60 seconds, acquiring at green and yellow channels. All parameters were observed according to the description on the manual in the master-mix kit.

Result Interpretation and Quantitative PCR Output Data Analysis

Amplification data for TB-MBLA samples, positive results and internal controls were evaluated at all times. Table 2, illustrates how TB-MBLA results were interpreted relative to the internal control (IC). Results data were generated automatically, after constructing the standard curve and importing it into the amplification curve of the RT-PCR run of the samples, by the Qiagen Rotor Gene Q software.

Table 2: Interpretation of RT-qPCR Results for TB-MBLA

Type	MTB channel	EC channel	Interpretation
Sample	Positive	Positive	Valid
Sample	Positive	Negative	Indeterminate
Sample	Negative	Positive	Valid
Sample	Negative	Negative	Invalid
MTB positive Control	Positive	Negative	Valid
Extraction Control (EC)	Negative	Positive	Valid
DNA control	Negative	Negative	Valid
Negative Control (NTC)	Negative	Negative	Valid

RESULTS

Recovery of MTBC from slaughtered cattle in Yola, Adamawa State using liquid and solid cultures

Out of 190 lung samples collected from Yola modern abattoir, 73 (33.8%) were positive for MTBC by liquid culture (MGIT 960 Bactec system) while 45(23.7%) were positive by solid culture (Lowentein-Jensen medium) (Table 3).

Table 3: Detection of MTBC in bovine lung samples by liquid and solid cultures

Culture medium	No of samples	No positive MTBC (%)	X^2	P-value
Solid Medium Lowenstein-Jensen	190	45 (23.65%)	9.636	0.002
Liquid medium MGIT 960	190	73 (38.4%)		

With a P-value of less than 0.05, there is a statistical sinificance between MTBC positives in the two media

MTBC Bacilli concentration from16SrRNA in bovine lung samples

Figure 1, shows the grouped concentration of MTBC bacilli from 16S rRNA in each positive bovine lung sample, calculated automatically by the Qiagen rotor gene software in (CFU/g). The concentration of MTBC cells calculated by the Qiagen rotor gene software from 16S rRNA in each positive sample, in (CFU/g) were grouped as follows and represented on a bar chart (Fig.1) 9.4% of the samples yielded ≤1000 cfu/g, 34.1% yielded 1,001-10,000cfu/g, 23.5% of samples yielded 10,001-100,000cfu/g, 14.1% yielded 100,001-1,000,000cfu/g, 10.6% yielded 1,000,001-10,000,000cfu/g and 8.3% yielded ≥10,000,0001cfu/g (Fig.1).

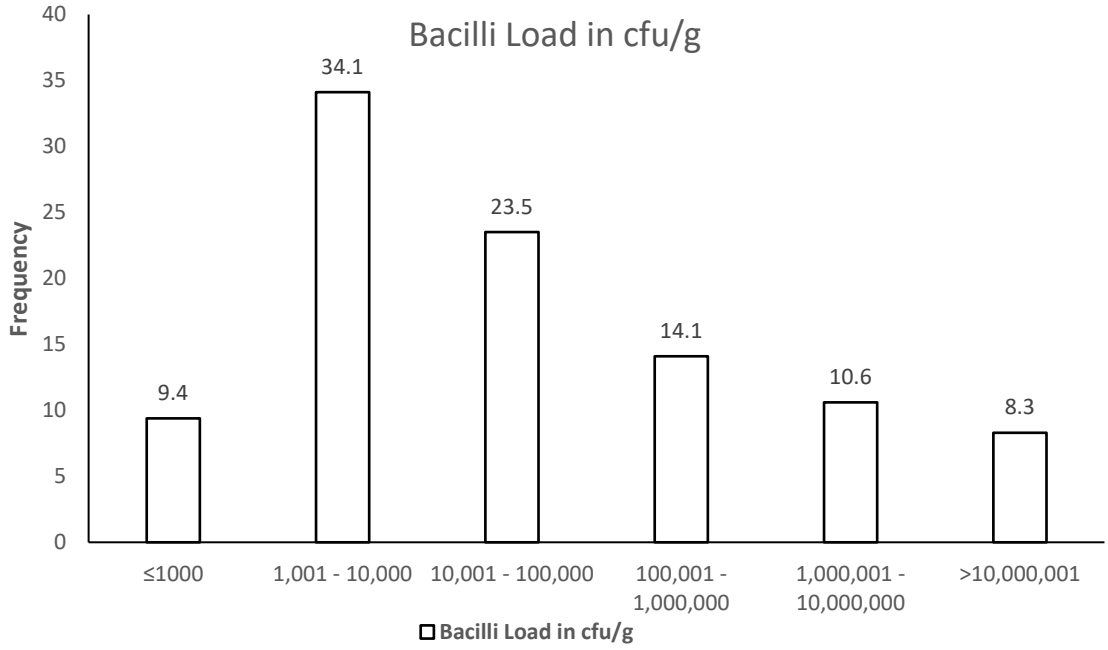

Figure 1: MTBC Bacilli concentration from16SrRNA in bovine lung samples

Detection of Mycobacterium tuberculosis complex Using Three Different Methods

Table 4, shows the comparison of detection rates of MTBC bacilli using liquid culture (MGIT), solid culture (Lowenstein-Jensen) and TB-MBLA. Out of 190 bovine lung samples analyzed in this study, the detection rate of MTBC by liquid medium, was 38%, solid medium was 23.7% and TB-MBLA yielded 44.7%.

Table 4: Comparison of Detection Rates of *Mycobacterium tuberculosis* complex bacilli Using Three Different Methods

Type of Method	No. Positive for MTBC (%)	χ^2	P-value
Liquid culture	73(38.4)	19.34	0.000
Solid culture	45(23.7)		
TB-MBLA	85(44.7)		

With p-value < 0.05, there is a statistical significance in detection of MTBC by liquid culture, solid culture and TB-MBLA.

DISCUSSION

All the 190 bovine lung samples collected in this study, had lesions suggestive of bovine Tb by physical examination.

Out of the 190 samples inoculated into Mycobacterium growth indication tube (MGIT 960 BACTEC system), 73(38.4%) were positive for *Mycobacterium tuberculosis* complex while solid culture Lowenstein-Jensen medium yielded 45(23.65%) and TB-MBLA detected 85(44.7%).

The detection rates of MTBC, was highest by TB-MBLA, which is a quantitative PCR assay, followed by liquid culture and solid culture methods. This corroborates with previous studies, which have reported highest detection rates by molecular methods followed by liquid culture methods before solid methods [20,21,22].

Using the Chi square statistical method, the results from the three methods used to detect the presence of MTBC species, shows that, with X^2 value of 19.34 and a P-value of 0.000, which is less than 0.05, there was a statistical significance in MTBC detection rates by liquid culture, solid culture and TB-MBLA.

The quantity of *Mycobacterium tuberculosis* complex bacilli load in infected bovine lung samples, was automatically estimated with the Rotor Gene Q software to be between 1,000 and ≥10,000,000 cfu/g. The estimates of bacilli cfu/g of the positive samples, were very high, compared to a study, which reported the generation of bovine tuberculosis pathology in cattle, divided into four groups of twenty (20) animals infected by the intratracheal route with 1,000, 100, 10, or 1 CFU of *M. bovis*, showed that one-half of the animals infected with 1 CFU of *M. bovis*, developed pulmonary pathology typical of bovine tuberculosis and no differences in the severity of pathology were observed for the different *M. bovis* doses [23].

CONCLUSION

From this study the prevalence of bovine tuberculosis in Yola, Adamawa State by liquid media, solid media and TB-MBLA, were 38.4%, 23.8% and 85(44.7%) respectively. This study shows a huge increase in prevalence of bovine TB in Yola modern abattoir, compared to a prevalence rate of 2.36% reported in 2017 by a study conducted using only Ziehl-Neelsen stain as the diagnostic tool [24,1], who reported 6.12% prevalence.

During the course of this study, it was observed that, once affected organs with bovine TB lesion are removed, the rest of the beef is sold to the public which agrees with a report in 2023 [25] This could be a source of transmission to humans especially butchers at the abattoir, who had no protective gloves on, when handling meat.

Having MTBC detection rates of 23.8%, 38.4% and 44.7% by solid (LJ medium), liquid (MGIT 960 BACTEC system) cultures and TB-MBLA methods respectively, a prevalence rate of 44.7% was estimated by using the detection rate value of TB-MBLA method which is a molecular method and the preferred method for TB diagnosis by employing the Rogan& Gladen formular [26.] An estimated bovine TB prevalence of 44.7% shows that, bovine tuberculosis is present in cattle slaughtered at the Yola modern abattoir, Yola, Adamawa state. This study validates the endemicity of bovine tuberculosis in Yola, Adamawa State and calls for the adoption of stricter measures for animal examination before slaughter, to minimize the spread of bovine TB in Yola, Adamawa State.

In this study, the 8.3% of the bovine lung samples, from slaughtered cattle, yielded up to ≥ 10,000,000 cfu/g of MTBC bacilli by the molecular bacterial load assay. This is a strong indication that, that infection will occur in animals or humans, if ingested in raw or poorly cooked meat and/meat products.

Conflict of Interests: There was no conflict of interests.

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