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DETECTION OF MYCOBACTERIA IN RAW COW MILK SOLD IN BWARI AREA COUNCIL, ABUJA FCT

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ABSTRACT

Bovine tuberculosis (bTB) is an important zoonotic disease worldwide and hence it is of great public health significance. It is present in most developing countries where surveillance and control activities are often inadequate or unavailable. This study was designed to detect mycobacteria in raw milk of cows using ZN-stain, PCR, and cultural techniques to determine the prevalence of bTB in Bwari area council of FCT Abuja. Out of the 145 raw milk sampled, 6.89% tested positive by ZN-stain and culture while 1.3% were positive by PCR. The herd prevalence per satellite town based on ZN-stain technique was 8.89%, 10.0%, 3.33% and 5.00% for Bwari, Dei-Dei, Kubuwa and Ushafa respectively. While by cultural method, the prevalence was 2.22%, 10.00%, and 5.00% for Bwari, Dei-Dei, Kubuwa and Ushafa respectively. PCR revealed the prevalence of Mycobacterium species for Bwari and Dei-Dei as 2.22% and 3.33% respectively. Detection of Mycobacteria in raw (unpasteurized) pose a serious public health risk to raw milk consumers in Bwari area council.

***Key words:* Raw milk, bTB, ZN stain, PCR, Culture.**

INTRODUCTION

Cow milk is an important source of protein and other nutrients in most communities in Northern Nigeria (Abubakar, 2007). However, it can be contaminated by pathogenic agents such as *Mycobacterium* species, the causative agent of tuberculosis (TB). As such the possibility exists of transmission of TB and other mycobacterial infections from an infected cow to humans as a result of consumption of contaminated milk and milk products (Ayele *et al.,* 2004; Kazwala *et al.,* 2001.). Bovine tuberculosis (bTB) is an important zoonosis worldwide and mostly prevalent in developing countries where surveillance and control activities are often inadequate or unavailable (Cosivi *et al.,* 1988). *Mycobacterium bovis*(*M. bovis)* the causative agent s is a member of the *M. tuberculosis* complex(MTBC) a group that includes; *Mycobacterium tuberculosis, M africanum, M. bovis* (the Bacillus Calmette-Guérin strain) *M. microti, M. canettii, M. caprae, M. pinnipedii, M. suricattae and M. mungi* (Michel *et al.,* 2010).

A systematic literature review revealed only a few reported incidences of zoonotic TB from Africa (Thoen *et al.,* 1989) most of which are from research studies on pre-existing *Mycobacterium* collections on limited clinical setting from Egypt, Nigeria, Zaire , Madagascar and Zambia (Cadmus *et al.,* 2004). Documented cases of bovine tuberculosis in Nigeria through the works of Manley (1929); Alhaji (1976) and Cadmus *et al.* (2004) in studies carried out in various parts of the country using tuberculin test a non-culture based techniques.

This study was undertaken to provide epidemiological information that could serve as baseline data for bovine TB surveillance in the Federal Capital Territory (FCT), using combination of Ziehl neelsen (ZN stain) , Culture and Polymarase chain reaction (PCR) techniques to enhance detection rate. Although, bTB testing in cattle using Purified Protein Derivatives (PPD) provides assessment at herd level only, knowing the strain circulating in any particular environment is also essential for effective control measures.

MATERIALS AND METHODS

Study area

Bwari area council (BAC) is located along coordinate 7° 8” E and 9° 24” N. It is one of the six area councils of the Federal capital territory (FCT), Abuja, with an area of 914 Km^2 and a population of 227,216 according to the 2006 census.

Sample size determination

The required sample size for this study was determined using the formula by Thrusfield, (2007). Expected prevalence of 14% was used as reported by Abubakar *et al.*, (2005). The calculated sample size was 185 milking cows, however, only one hundred and forty five (145) lactating cows were available for sampling.

The survey was conducted in four (4) satellite towns of Bwari area council (Kubuwa, Dei-Dei Bwari and Ushafa) with high population of Fulani pastoralist settlement. Herd selection was based on herdsmen willingness to participate.

Sample collection and processing

Milk samples were collected by cattle owners into 50ml sterile Falcon® tubes as part of their routine milk collection in presence of the researcher and immediately transported to Zankli Hospital TB research laboratory Abuja in ice box(4°C). The Samples were processed by mixing several times using a vortex machine and 1ml transferred aseptically into sterile eppendorf tubes and labeled with the corresponding Lab number. All the aliquot samples were stored at -20 °C while the samples in falcon® tubes were stored at -80 °C until required.

Polymerase chain reaction (PCR)

Genomic DNA isolation from Milk sample (phenol chloroform method)

The phenol chloroform method was used for mycobacterial DNA isolation as described by Del Portillo *et al.* (1991) and Chomczynki and Sacchi (1987).

Primer sequences.

Sequences of primers specific for *Mycobacterium bovis* were used as shown in Table 1.

Table 1: Primer Sequence

Primer	Direction of primer sequence	Amplicon size(bp)
Forward	(5'CCCGCTGATGCAAGTGCC3')	500
Reverse	(5'CCCGCACATCCCAACACC3')	

PCR primer oligonucleotide sequences used to amplify the 285- 300 bp *M. bovis* DNA fragment (Romero *et al.*, 1999). Source Bioneer ®.

Primers L1 and L2 (Bioneer®) were optimized by varying the reaction temperatures and timing in 20µl reaction mixture containing 3µl of BCG Pasteur DNA as positive control , Bioneer® Hot stat premix (Bioproducts ,USA), a lyomphilised mixture of Taq polymerase, reaction buffer, dNTPs(dATP,dGTP, dCTP, dTTP) , magnesium chloride ($MgCl_2$) and 13µl and 2µl of distilled water and primers L1 and L2 each respectively. All reactions were carried out in Perkin Elmer thermo cycler (Perkin Elmer Cetus) programmed for 35 cycles .The conditions that yielded bands corresponding to 285-300bp were obtained as:

Denaturation 94 °C for 1min
Annealing 70 °C for 30 sec
Extension 72 °C for 30 sec
Final Extension 72 °C for 15min
} 40 cycles

Amplification

Using the optimized PCR condition above, amplification of all the extracted genomic DNA samples was carried out in 50µl reaction volumes. Each amplification contained 10 µl of extracted genomic DNA in reaction buffer {containing Tris-HCL (pH 8.3), 50 mM KCL, 1.3mM $MgCl_2$ and 0.001 % gelatin}, 2.5U Taq polymerase, 0.2 Mm of each deoxynucleoside triphosphate (dNTP) and 75 pmol of each primer. The amplification protocol entailed an initial denaturation step (for 5 minutes at 95°C) followed by a second denaturation for 1minute at 95°C, annealing at 70 °C for 30 seconds, and extension steps at 72°C for 30 seconds . Samples were subjected to 40 cycles before a final 15-min extension at 72°C. Amplified products were stained with ethidium bromide (EtBr) and visualized with ultraviolet light (UV) illumination.

Gel electrophoresis

After the amplification, 20µl of the PCR mix was loaded on to 1% agrose gel as described by CDC, (2012) and stained with 0.5ug/ml ethidium bromide. Ten micro liter (10µl) of standard molecular marker (Ladder) was loaded along side with samples. The gel was subjected to electrophoresis at 150 volts for 20 minutes. Finally, DNA bands were viewed using Bio-Rad® gel viewing system that scans and photo document images on a computer system (Plate 1).

Culture of Mycobacteria on Lowenstein-media

Lowenstein-Jensen medium with glycerol and with (4%) private instead of glycerol (to enhance *M. bovis* growth) were used. Decontamination was carried out using BBL

Mycoprep® Phosphate according the manufacturer's instructions.

Ziehl-Neelsen staining (ZN stain)

Smear preparation

The sediment above (20ul) was used to prepare smears and stained by ZN stain method as described by World health organization (WHO, 1998) TB manual. The stained smears were examined for Acid fast bacteria.

Identification of mycobacterial Isolates

Morphology and growth rate.

Mycobacteria are slow growing organisms. Presumptive identification of colonies that become visible from 3-6 weeks of inoculation on LJ appearing as white ,small round and wrinkled surface with irregular thin margins(dysgonic) growing on LJ.(Guerreo *et al.*,1997).

ZN stain

All suspected colonies were stained with ZN as above. Positive AFB colonies were further tested with SD-bioline® TB AgMPT64 to rule out Non tuberculosis *Mycobacterium* (NTM).

SD-bioline® TB AgMPT64 Test.

Two to three suspected colonies were emulsified using condensation fluid present on the LJ slant in a biosafety cabinate. Condensation fluid (100ul) was added to the sample well. The inoculated cassette were kept un disturbed at room temperature in the biosafety cabinet and were examined at the end of 15 minutes for the presence of pink band in the "Control" and "Test" region.

Interpretation of test result

The appearance of control band confirmed the validity of the test. If the control band was not visible in 15minutes, the sample was considered invalid and sample retested. The presence of only control band in the absence of test band was considered negative test and interpreted as absence of MPT64 antigen, confirming it is non tuberculosis *Mycobacteria* (NTM). Presence of both control and test band indicated a positive result and interpreted as presence of MPT64 antigen, confirming MTBC.

RESULTS AND DISCUSSION

Results of tests for detection of Mycobacteria by ZN-stain, PCR and Culture, showed a positive detection rate by ZN-stain and culture to be 6.89 % each, while that of PCR was 1.3% (Table 2). The test based calculated prevalence per satellite town is as shown in Table 3. Based on ZN technique, prevalence rates of 8.89 %, 10.0 %, 3.33 % and 5.00 % were calculated for Bwari, Dei-Dei, Kubuwa and Ushafa respectively. Detection based on culture prevalence rates were 2.22 %, 10.00 %, and 5.00 % for Bwari, Dei-Dei, Kubuwa and Ushafa respectively. PCR based calculated prevalence for Bwari was 2.2 % and 3.3 % for Dei-Dei.While that of Ushafa and Kubuwa was 0 % each.

Agrose gel electrophoresis of PCR products using *M. bovis* specific primers (oxyR gene) yielded a band of 258bp which is consistent with *M. boivs* (Plate 1)

All LJ slants that showed growth in less than three (3) weeks were disregarded as contaminants. The result is as presented in Table 4. Two of the ten (10) isolates grew on LJ pyruvate and were positive for MTB complex using SD-bioline®MPT64 Rapid test, suggestive of *M. bovis*. The remaining eight (8) grew on LJ glycerol were positive for non MTB complex. The two MTB complex isolates were from Bwari and Dei-Dei (Table 5)

Tuberculosis, caused by *M.* bovis is emerging as the most important disease affecting cattle. It results in a major public health problem when transmitted to humans. The detection of *M. bovis* in milk samples by bacteriological examination results in delayed diagnosis although it has a specificity that approaches 100 %. Ziehl-Neelsen staining of clinical specimens lack sufficient sensitivity and species specificity (Parashar *et al.* 2009). The present study was carried out to detect Mycobacteria in milk using three techniques namely ZN stain, Culture and PCR.

The detection of AFB in raw cow milk from Fulani herds in Bwari Area Council confirms that zoonotic mycobacteria is present in cow milk and may have persisted over time, corroborating the previous findings of Alhaji (1976); Shehu, (1991); Kolo,(1991); Ofukwu *et al.* (2008) and Cadmus *et al.*(2010). In this study ZN-staining technique had the same detection rate (6.89 %) as culture. This is slightly higher than the 6.3 % detection rate reported by Ofukwu *et al.*(2008) and 4.4 % detection rate reported by Alwathani *et al.*(2012) from milk of 105 tuberculin positive cows by ZN stain. The high ZN positivity recorded could be due to the inability of ZN staining technique to differentiate between *M. bovis* and other mycobacteria (i.e. *Mycobacterium* other than *M. bovis*).Thus confirming the conclusion by Parashar *et al.*(2009) and Bermudez *et al.*(2010) , that Ziehl-Neelsen staining lacks sufficient sensitivities and specificities.

The detection rate by culture recorded in this study was 6.8 %, which is similar to that of Aydn *et al.* (2008) of 7.6 %. However, Cadmus *et al.* (2010) and Alwathani *et al.* (2012) reported lower *M. bovis* detection rates of 1.2 % and 2.3 % respectively by culture. Differences in the detection rates between the previous two studies and this study may be due to the different decontamination technique and culture media used and also sample size.

To evaluate the use of the PCR as a diagnostic method for detection of *M. bovis*, *oxyR* gene located within 16-23S rRNA regions which is part of the *AHpc-oxyR* regulon was targeted and amplified. The agrose gel analysis of PCR products depicted the band of *M. bovis* (*oxyR* gene amplicon) in two (2) of the milk samples representing 1.3 % detection rate. In contrast to the present study, Isioma *et al*. (2013) reported a higher *M. bovis* positive detection rate of 30 % in Jos, Plateau state. Reasons for the high detection rate may due to the amplification of 245bp fragment, which is specific for MTBC, primer specification, specimen nature (lung tissues) and sample size used in the study.

Table 2: Detection of Mycobacteria in milk samples in Bwari Area council by three different techniques (N=145).

Test Method	Number of Positive	Positive detection Rate (%)
ZN Stain	10	6.8
Culture on LJ media	10	6.8
PCR	2	1.3

Table 3: Herd Prevalence of Zoonotic TB based on three test methods.

Satellite Town	Milk samples collected	Tested Positive			Herd Prevalence (%)		
		ZN STAIN	PCR	CULTURE	ZN	PCR	CULTURE
Bwari	45	4	1	1	8.89	2.22	2.22
Dei-dei	30	3	1	3	10.00	3.33	10.00
Kubuwa	30	1	0	4	3.33	-	13.33
Ushafa	40	2	0	2	5.00	-	5.00
Total	145	10	2	10			

Table 4: Preliminary Identification of suspected Mycobacterial Isolates on LJ media.

Isolate number	LJ medium	ZN Stain Reaction	MPT64 Rapid test	
			MTB Complex	Non MTB
S2-10H10	Pyruvate	+	+	-
S9-10H1	Glycerol	+	-	+
W6-6H11	Glycerol	+	-	+
TH2-10H13	Glycerol	+	-	+
TH2-6H15	Glycerol	+	-	+
SA4-4H20	Pyruvate	+	+	-
W3-4H8	Glycerol	+	-	+
W7-2010	Glycerol	+	-	+
SA1-5H26	Glycerol	+	-	+
S1-7H10	Glycerol	+	-	+

KEY: AFB = Acid fast bacilli, + = Positive, - = Negative
LJ = Lowenstein-Jensen media, ZN= Zeihl-Neelsen stain
MTB = Mycobacterium tuberculosis

Table 5: Distribution of Mycobacterial Isolates and Characterization Using SD bioline®.

Satellite town	Milk samples collected	Number of Isolates	MPT64 Rapid test confirmation	
			MTBC	NON MTBC
Bwari	45	1	1	0
Dei-Dei	30	3	1	2
Kubuwa	30	4	0	4
Ushafa	40	2	0	2
Total	145	10	2	8

KEY: MPT64 =mycobacterium protein 64
MTBC= mycobacterium tuberculosis complex

PCR GEL PRODUCT PICTURE

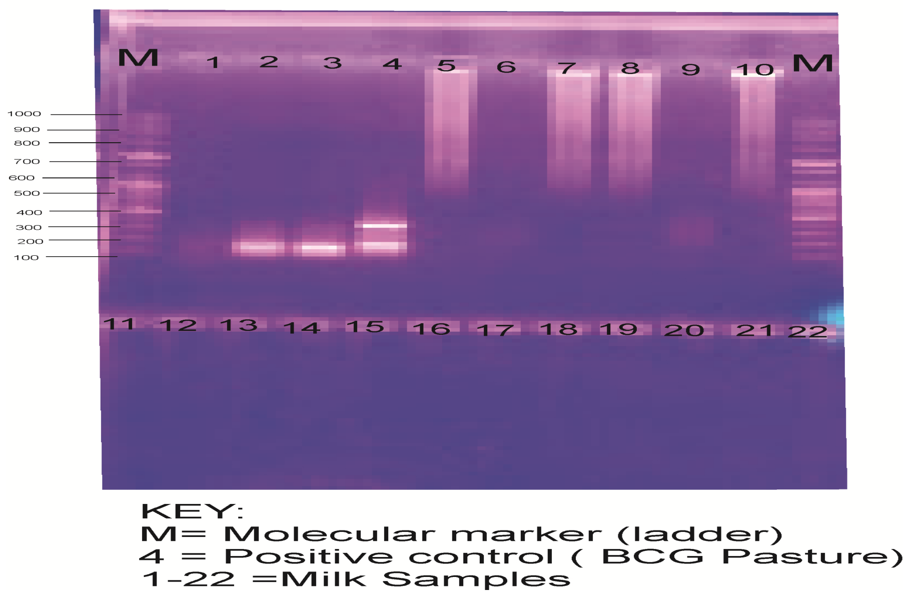

PLATE 1: A two panel agarose gel electrophoresis of PCR amplification of oxyR gene specific for *M.bovis*. M is molecular marker (1000bp DNA ladder, Bioneer Labs®) Line 4 is Positive control (BCG pasture strain).The remaining numbers are milk samples.

Study Limitations

The limitations of this study were screening of limited number of milk samples and the non inclusion of Mycobacteria isolated from patients diagnosed with tuberculosis from hospitals in Bwari Area Council, with a view to establishing link with either animals or consumption of milk or milk products as done by Byarugba *et al.*(2009) in Uganda.

CONCLUSION

Acid fast bacilli (AFB) were demonstrated in raw milk samples collected in Bwari council using ZN-stain technique. Also, *M. bovis* were isolated on LJ media and as well detected using PCR techniques. Consumption of raw milk or milk products from Fulani herds in Bwari area council poses a great danger to consumers. Therefore it becomes imperative to carry out surveillance program to forestall zoonotic spread.

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