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 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. canetti, 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 Km² 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, Del-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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction of primer sequence</th>
<th>Amplicon size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>(5’CCCCGCTGATGCAAGTGCC3’)</td>
<td>500</td>
</tr>
<tr>
<td>Reverse</td>
<td>(5’CCCCCACATCCCCAACC3’)</td>
<td></td>
</tr>
</tbody>
</table>

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 lyophilised mixture of Taq polymerase, reaction buffer, dNTPs( dATP,dGTP, dCTP, dTTP) , magnesium chloride (MgCl₂) and 13µl Denaturation 94°C for 1min
Annealing 70°C for 30 sec

Extension 72°C for 30 sec
Final Extension 72 °C for 15min

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 MgCl₂ 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 Culture of Mycobacteria on Lowenstein-media Lowenstein-Jensen medium with glycerol and with (4%) private instead of glycerol (to 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:

- 40 cycles
- 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% agarose 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).
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). 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 (Guerrero 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 cabinet. Condensation fluid (100ul) was added to the sample well. The inoculated cassette were kept undisturbed 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 15 minutes, 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 258 bp which is consistent with *M. bovis* (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 lacks 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 agarose 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).

<table>
<thead>
<tr>
<th>Test Method</th>
<th>Number of Positive</th>
<th>Positive detection Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZN Stain</td>
<td>10</td>
<td>6.8</td>
</tr>
<tr>
<td>Culture on LJ media</td>
<td>10</td>
<td>6.8</td>
</tr>
<tr>
<td>PCR</td>
<td>2</td>
<td>1.3</td>
</tr>
</tbody>
</table>

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

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

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

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

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®.

<table>
<thead>
<tr>
<th>Satellite town</th>
<th>Milk samples collected</th>
<th>Number of isolates</th>
<th>MPT64 Rapid test confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MTBC</td>
</tr>
<tr>
<td>Bwari</td>
<td>45</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dei-Dei</td>
<td>30</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Kubuwa</td>
<td>30</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Ushafa</td>
<td>40</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>145</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>

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

**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.

**REFERENCES**


