

Molecular detection of *Mycobacterium bovis* in cattle milk in Enugu State, Nigeria.

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ABSTRACT

The study was carried out to detect *Mycobacterium bovis* in cattle milk in Enugu. Fifty Milk samples were collected from cattle at Fulani settlements which comprised of 13 from Gariki, 16 from 9th Mile and 21 from Monarch in Enugu. DNA was extracted from milk samples using Relia prep DNA spin column method and screened for Tuberculosis using Nested Polymerase Chain Reaction (PCR) with specific Tuberculosis primer; Insertion sequence 6110 (IS6110) while Restriction Fragment Length Polymorphism (IS6110- RFLP) method was used to differentiate between *Mycobacterium bovis* and *Mycobacterium tuberculosis* using Nar 1 digestion enzyme. Statistical tools used to analyze the data were: Chi-square, fishers' exact test and non parametric t test. 9 (18%) samples out of the 50 milk samples were positive for tuberculosis with the PCR method. 1 (2%) out of the 9 positive milk samples was found to be *Mycobacterium tuberculosis* while the remaining 8 (16%) were detected to be *Mycobacterium bovis* after using the digestion enzyme. 1 (6.3%) of the 16 milk samples collected from the Fulani settlement in 9th Mile was positive for *M.bovis* while a total of 2 (15.4%) out of the 13 milk samples from Gariki were positive for *M.bovis* and a total positive of 6 (28.6%) were detected out of the 21 milk samples analyzed from Monarch, 5 (23.8%) of which were found to be *M.bovis* while *M. tuberculosis* was detected in 1 (4.8%). The differences however, were not statistically significant ($P>0.05$).

Key words: Tuberculosis, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, DNA

1.0 Introduction

Tuberculosis is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* which was first identified by Koch in 1882 and characterized by the formation of nodular lesions (tubercles) in the tissue (Oxford Medical Dictionary, 2003). Human and animal tuberculosis are widespread in Africa and are caused by organisms with very close genetic and antigenic similarities: *M. tuberculosis* and *M. bovis*, respectively both cause identical and clinically indistinguishable disease in humans (Denis *et al.*, 2007). The unusually extensive host range of *M. bovis*, represents a very significant economic problem in numerous countries in both developed and the developing world (Wonderwosen *et al.*, 2010).

Tuberculosis due to *Mycobacterium bovis* is a zoonosis that affects humans around the world. Although it has wide range of hosts, *M.bovis* primarily infects cattle which can transmit the agent to humans through the consumption of unpasteurized, contaminated dairy products (Michele *et al.*, 2008). Bovine tuberculosis (BTB) is a chronic infectious disease of animals characterised by the formation of granulomas in tissues and organs, more significantly in the lungs, lymph nodes, intestine and kidney including others. BTB is caused by slowly growing non-photochromogenic bacilli members of the *Mycobacterium tuberculosis* complex: *M. bovis* and *M. caprae* species. However, *M. bovis* is the most universal pathogen among *Mycobacteria* and affects many vertebrate animals of all age groups including human. BTB, apart from being the most important disease of intensification with a serious effect on animal production, also has a significant public health importance (Shitaye *et al.*, 2007). The actual impact of animal BTB on human health is generally considered low in developed and developing countries, which may be based on the rare identification of *M. bovis* isolates from human patients (Amanfu, 2006). In addition, the occurrence of BTB due to *M. bovis* in humans is difficult to determine accurately because of technical problems in isolating the micro-organism (Shitaye *et al.*, 2007). Currently, the BTB in humans is becoming increasingly important in developing countries, as humans and animals are sharing the same micro-environment and dwelling premises, especially in rural areas. At present, due to the association of *Mycobacteria* with the HIV/AIDS pandemic and in view of the high prevalence of HIV/AIDS in the developing world and susceptibility of AIDS patients to tuberculosis in general, the situation changing is most likely (Amanfu, 2006). Prevalence data on BTB infection in Africa is scarce. There is, however, sufficient evidence to indicate that it is widely distributed in almost all African countries and even is found at high prevalence in some animal populations (WHO, 1994; Ayele *et al.*, 2004; Zinsstag *et al.*, 2006).

Human to human airborne transmission of *M. bovis* does occur and it may be important where human immunodeficiency virus (HIV) infection in humans is prevalent. *M. bovis* infection in cattle is enzootic and pasteurization of dairy products is not routinely practiced. Eradication of *M. bovis* in cattle and pasteurization of dairy products are the cornerstones of prevention of human disease (Charles *et al.*, 2009). In developed countries, like the United States, the pasteurization of milk

and the testing and culling of infected cattle have resulted in steep decreases in incidence of *M. bovis* tuberculosis. *M. bovis* caused as much as 25% of case of human TB in developed countries in the late 19th and early 20th centuries (Michele *et al.*, 2008).

Nigeria has the fourth highest burden of human tuberculosis (TB) in the world, with an incidence in 2002 of 304 cases per 100,000 and a mortality rate of 89/100,000. These statistics are compounded by co-infection rate of tuberculosis patients with human immunodeficiency virus which stood at 27% in 2002 (Simon *et al.*, 2006). The Nigerian human population also carries the largest tuberculosis burden in Africa with an estimated 390,000 active infections and 107,000 deaths per year (WHO, 2002).

Determining which TB cases are caused by *M. bovis* is an essential first step to elucidating the epidemiology of *M. bovis* tuberculosis, which in turn would support the development and implementation of appropriate prevention strategies. Tuberculosis caused by *M. bovis* and tuberculosis caused by *M. tuberculosis* cannot be distinguished chemically, radiographically, or pathologically in individual patients, thus the identification to these causative agents requires laboratory testing. Epidemiological analysis of *M. bovis* infection has been hampered in the past by the difficulty associated with distinguishing between isolates originating from different sources and species. In recent years, molecular biological techniques have been employed to examine *M. bovis* bacilli at the DNA level and to identify genotypically distinct strain types that may be presumed to be epidemiologically unrelated. Such data may provide information pertinent to routine field investigations undertaken following herd breakdown and provide a greater understanding of disease transmission within the rational cattle herd and between cattle and non-bovine transmission vectors (Rory *et al.*, 2000; Tiruvilumala and Reichman, 2002).

Several strategies for typing *M. bovis* isolates on the basis of DNA polymorphisms have arisen in recent years. Techniques commonly used internationally include restriction fragment length polymorphism (RFLP) analysis, spoligotyping, Pulse-field gel electrophoresis and PCR-based techniques.

RFLP analysis has been demonstrated to be a robust and highly discriminatory typing procedure due to the availability of multiple DNA probes for the detection of polymorphic loci within the *M. bovis* genome and has been the method of choice (Rory *et al.*, 2000).

Tuberculosis is an important disease in humans and animals worldwide. It is estimated that 1.5 to 2 million people die from tuberculosis each year. A total 95% of cases occur in people in developing countries including Nigeria. Ofukwu (2008) carried out a study to determine the presence of *M. bovis* in freshly drawn cow milk and “nono” in market in Markurdi town, Nigeria and found 4 (18.22%) of 22 acid fast bacilli positive fresh milk and 2 (16.7%) of the 12 “nono” samples were positive for *M. bovis*. A cross sectional study was conducted from September, 2008 to March, 2009 by Ibrahim (2002) to identify risk factors for BTB in cattle and humans in Jigawa State, Nigeria and he found that 22 (5%) respondent amongst the families sampled had TB or clinical signs suggestive of TB, while 9 (2%) had reactor cattle in their herds and he reported that the habit of milk and meat consumption was found to be affected by occupation and location of the household residence. A study to generate epidemiological data on tuberculosis in cattle and humans in Enugu State, through a retrospective survey of abattoir and hospital records for a five-year period (2004 to 2008) was undertaken and overall mean prevalence of 1.4% and 12.9% was recorded for cattle and humans, respectively (Nwata *et al.*, 2011).

There is an estimated population of 14 million cattle in Nigeria (Wosu, 2002) and 140,431,790 persons (National Population Commission, 2006), with significant continuously changing demographic factors such as population growth and structure. As a result, there is a considerable increase in the demand for animal products, in terms of both quantity and quality. TB remains a major public health problem worldwide. The disease is widespread and affecting livestock and human health in Africa (Corbett *et al.*, 2006). It is amongst the fastest killer diseases in Nigeria today (Anosike, 2011) and in developing countries as a whole (Seyed *et al.*, 2008).

1.1 Aim and Objective

To detect the presence of *Mycobacterium bovis* in raw cattle milk using molecular technique.

2.0 Sample collection

Milk sample were collected from 50 cows at Fulani settlements in Gariki, 9th mile and Monarch in Enugu. Udders of the cows were swabbed with 70% alcohol and milk was extracted aseptically from each cow into clean sterile 10mls universal bottles. Samples were transported to the laboratory in a cooler containing ice pack and stored at -20°C.

3.0 Extraction of Genomic DNA (gDNA) using Relia Prep DNA Spin Column

- Milk samples were allowed to thaw and then mixed thoroughly for 10 minutes at room temperature.
- 20µl of protein K (PK) solution was dispensed into 1.5 ml microcentrifuge tubes.
- 20µl of the milk sample was added to each of the tubes containing protein K solution and mixed briefly.

- 20µl of Cell Lyses Buffer (CLD) was added to each tube; the tubes were capped, mixed by vortex for 20 seconds and incubated at 56°C for 10 minutes.
- ReliaPrep binding column were placed in empty collection tubes and labelled according to the number of samples. Incubated tubes were removed from the heating block, then 250µl of Binding Buffer was added to each tube; the tubes were capped and vortex for 10 seconds.
- The contents of the tubes were added to the ReliaPrep Binding Column, capped and centrifuged for 1minute at 14000 rpm.
- Collection tubes that contain flow through were removed and discarded as hazardous waste.
- Binding columns were placed into fresh collection tubes and labelled accordingly; 500µl of column wash solution (CWD) was added to each column and centrifuged for 3 minutes at 14000 rpm. The flow through were discarded. This step was repeated twice for a total of 3 washes.
- The columns were placed in clean 1.5 ml microcentrifuge tube; 100µl of elution buffer (Nuclease free water) was added to each column and centrifuged for 1minute at 14000 rpm. ReliaPrep Binding Columns were discarded.
- DNA elute were labelled properly and stored at 4°C.

3.1 Polymerase Chain Reaction (PCR)

The primer sequence for the Mycobacterium PCR:

IS6110-Outer-F	CGGGACCACCCGCGGCAAAGCCCGCAGGAC
IS6110-Outer-R	CATCGTGGAAGCGACCCGCCAGCCAGGAT
IS6110-Inner-F	CCTGCGAGCGTAGGCGTCGG
IS6110-Inner-R	CTCGTCCAGCGCCGCTTCGG

HPLC grade. Manufactured by Integrated DNA Technology, Belgium.

3.2 Method: Nested PCR

The basic technique of PCR includes repeated cycles of amplifying selected nucleic acid sequences. Each cycle consists of three steps: (a) a DNA denaturation step, in which the double strands of the target DNA are separated; (b) a primer annealing step, performed at a lower temperature, in which primers anneal to their complementary target sequences; and (c) an extension reaction step, in which DNA polymerase extends the sequences between the primers. At the end of each cycle (each consisting of the above three steps), the quantities of PCR products were theoretically doubled. The whole procedure was carried out in a programmable thermal cycler and 35 thermal cycles result in an exponential increase in the total number of DNA copies synthesized. PCR was set in 2 rounds.

3.3 Agarose Electrophoresis

2% of agarose gel was prepared by dissolving 2g of agarose in 100ml of 1x TAE buffer; 10µl of Ethidium Bromide was added. It was sterilized in microwave oven for 3minutes at medium temperature. The gel was poured and allowed to solidify.

The gel was set in electrophoretic tank that contained 1x TAE buffer. 10µl of 2nd round PCR products were mixed with 2µl of 6x loading buffer and loaded into the gel with 100bp DNA ladder on the first lane and controls were set on the last two lanes.

Electrophoresis was run for 30minutes, then UV transilluminator was used to visualize the products and pictures were taken.

3.4 Restriction Fragment Length Polymorphism Analysis

Only positive samples for IS6110 were run for restriction enzyme digestion. 15µl each of the PCR products were transferred into 1.5ml tube, 5µl of restriction enzyme was added to each tube and mixed by repeated pipetting. The tubes were sealed with PCR caps, spun briefly and incubated at 37°C for 12hours.

The products were run on 3.0% agarose gel and pictures were taken.

All analysis was carried out at Safety Molecular Pathology Laboratory, Faculty of Health Sciences and Technology, University of Nigeria, Enugu Campus.

4.0 RESULTS

9(18%) samples out of 50 milk samples collected from different settlements were positive for tuberculosis with Polymerase Chain Reaction (PCR) method (Table 1), which showed band at 123base pair (123bp) on agarose gel electrophoresis.

In figure 1; Lane 1 is *Mycobacterium tuberculosis* positive sample, lane 2, 3, 4 are *Mycobacterium bovis* positive samples, lane 5 is *M. bovis* positive control while lane 6 is 100 base pair ladder and lane 7 is *M. tuberculosis* positive control Likewise in figure 2; lane 1 is 100base pair ladder, lane 2 is *M. bovis* positive control while lane 3, 4, 5, 6, 7 are *M. bovis* positive samples. As it is shown in Table 1 below, 1(2%) out of 9 positive milk samples was found to be *Mycobacterium tuberculosis* while the remaining 8 (16%) were detected to be *Mycobacterium bovis* after using digestion enzyme. Restriction endonucleases recognize specific nucleotide sequences in DNA and produce double-stranded cleavages that break the DNA into small fragments. These fragments were then separated by size with use of agarose gel electrophoresis.

Table 1: PCR result on milk samples obtained from different settlements in Enugu

Settlements	No of Milk Samples	Total +ve	Total -ve	<i>M. bovis</i>	<i>M.tuberculosis</i>
9 th Mile	16	1(6.3%)	15(93.7%)	1(6.3%)	0
Mornach	21	6(28.6%)	15(71.4%)	5(23.8%)	1(4.8%)
Gariki	13	2(15.4%)	11(84.6%)	2(15.4%)	0
TOTAL	50	9(18%)	41(82%)	8(16%)	1(2%)

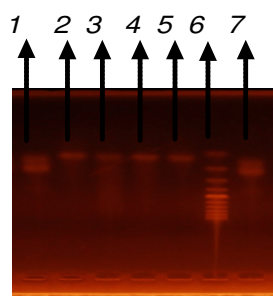


Fig 1: PCR products on agarose gel showing difference between *M. bovis* and *M. Tuberculosis*, using Nar 1 digestion enzyme. Lane 1 -*M.tuberculosis*, Lane 2, 3, 4 - *M. bovis*, Lane 5- *M. bovis* positive control, Lane 6 - 100bp Ladder, Lane 7 - *M. tuberculosis* positive control

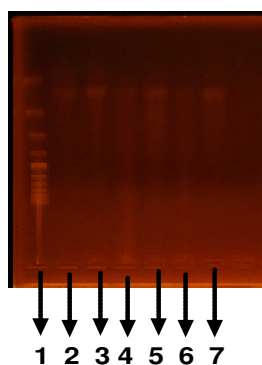


Fig 2: PCR product on agarose gel showing *M. bovis* after using Nar 1 digestion enzyme. Lane 1-100bp ladder, lane 2-*M. bovis* positive control, Lane 3,4,5,6,7-- *M.bovis*.

5.0 Discussion

From the study conducted, 9(18%) of the 50 milk samples collected from cattle in three different location in Enugu (9th mile, Monarch and Gariki) were positive for tuberculosis, 1(2%) of which was found to be caused by *Mycobacterium tuberculosis* which is known to be causative agent of tuberculosis in human. This finding clearly shows that *M. tuberculosis* can also infect animal and vice versa. This supports the findings of some researchers like Danima *et al.* (2011), who used deletion analysis method to detect 6 *M. tuberculosis* out of 142 samples analyzed from slaughtered cattle in Plateau State, Nigeria. Ayele *et al.* (2004) reported that farm workers urinating in cowsheds may represent a source of infection for animals. This is common in rural Africa, where patients with genitourinary tuberculosis may urinate on Pasture; animals craving salt preferentially graze on this grass and may succumb to infection (Grange and Yates, 1994).

The remaining 8 (16%) of positive milk samples were found to be *M. bovis*, this is higher than findings of Cadmus *et al.* (2004) who detected 6 (11.3%) of *M. bovis* from 53 milk samples screened in Ibadan, Oyo state of Nigeria, but lower than findings of Ofukwu *et al.* (2008) who reported 4 (18.2%) of *Mycobacterium bovis* from 22 fresh cow milk in Makurdi, Nigeria. Compared to the findings in this study, Abubarka, (2007) also reported a lower prevalence rate of 14 (12.6%) of *M. bovis* from 111 fresh cow milk samples in Federal capital territory and Kaduna State of Nigeria.

1 (6.3%) of 16 milk samples collected from Fulani settlement in 9th mile was positive for *M.bovis* while total of 2 (15.4%) out of 13 milk samples from Gariki were positive for *M. bovis* and total positive of 6(28.6%) were detected out of 21 milk samples analyzed from Monarch which differences were not statistically significant ($P>0.05$). Total number of 41 (82%) negative samples was recorded in milk samples.

From personal discussion with Fulani herds' men during this study, it was found out that they move their cattle from one place to another from time to time in searching for green pasture; this may increase the cross infection from one farm to another because if infected animals urinate on grasses and drinks water from available rivers during their movements and other animal may feeds on this contaminated grasses and water and may be infected by *Mycobacterium*. This also serves as a potential danger to the public, most of our local foods are being processed by the river side; especially "AKpu" which the process involves soaking of cassava in water for some days. The villagers prefer to make use of the available rivers for this process and if contaminated river is used, infection by *Mycobacterium* may occur.

Also, considering what happened about one or two years ago in Ebonyi and Benue State where there was a clash between Fulani herds' men and farmers who claimed that cattle destroy their farms. If truly these cattle enter the farm, not only farm products will be destroyed, shedding of mycobacterium in the urine of infected cattle will definitely occur and this serve as source of infection to the farmers as invisible droplets containing tuberculosis bacteria may be inhaled.

Indeed, detection of *Mycobacterium bovis* from cattle milk is a serious threat to human life as the small quantity of produced milk may not be sold to dairy industry for pasteurization, but sold at retail and may be consumed raw or used for producing fermented dairy products. One of these fermented dairy products is called "nono" and Ofukwu *et al.* (2008) reported the presence of *Mycobacterium bovis* 2 (2.2%) of 90 "nono" samples analyzed in Makurdi, Benue State of Nigeria. Ben *et al.* (2011), in his work carried out in Tunisia detected 4.9% out of 102 of the cattle as shedders of *M.bovis* in the milk.

5.1 CONCLUSION

From this study, bovine tuberculosis showed a prevalence rate of 16% in milk, which is very high and this indicates the potential treat by tuberculosis infection in Nigeria as people are still fun of taken raw milk without pasteurization, also couple with alarming increase rate of HIV infection which depress immunity and make people more susceptible to infection.

5.2 RECOMMENDATION

For the effective control of Bovine tuberculosis in Nigeria, it is worthwhile to apply the following measures as fundamental practice:

- Pasteurisation of milk and milk products should be done as routine practice most notably in rural communities.
- Government should create a strong policy that will guide the movement of animal from one place to the other, especially from other country.
- In general, information about zoonotic disease and their potential impact on human health should be disseminated appropriately.

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