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ARTICLE

The use of Deletion Analysis in the Detection of *Mycobacterium bovis, Mycobacteium tuberculosis and Mycobacterium africanum* among Slaughtered Cattle in Plateau State, North Central Nigeria.

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Summary

Bovine and human tuberculosis is endemic in Nigeria and apart from meat inspection at the abattoir, which is not very effective, no control measures are currently practiced against the disease in Nigerian livestock. A total of 3381 slaughtered cattle were examined for tuberculous lesions in the study area. Of these, 150 (4.4%) animals showed lesions typical of tuberculosis and 124 (3.7%) were acid fast positive. The major organs showing tuberculous lesions were lungs and lymph nodes. Out of 150 animals, 114 lungs (76%) and 27lymph nodes (18%) respectively were positive. 96 (64%) of the lungs were acid fast positive. Tuberculous organs were observed mostly in female animals. Following deletion analysis of 142 tissue samples using RD1, RD4, RD9, RD12, $RD1^{mic}$ and $RD2^{seal}$, 107 were identified as *M. bovis,* six as *M. tuberculosis* and two as *M. africanum.* The public health implication and control measures were discussed.

KEY WORDS: Tuberculosis, bovine, abattoir, deletion analysis, Nigeria, Butchers

INTRODUCTION

Mycobacterium tuberculosis and *Mycobacterium bovis* are very closely related and are referred to as members of the *Mycobacterium tuberculosis* complex (MTC) of organisms which also includes Bacillus Calmette–Guerin (BCG), *Mycobacterium africanum*, *Mycobacterium microti, Mycobacterium canetii* and some non-tuberculous *Mycobacteria* (Fifis *et al.*, 1991; Perez *et al.,* 2008). Bovine tuberculosis, caused by *Mycobacterium bovis*, is a highly significant zoonotic disease that can be transmitted through aerosols and by ingestion of unpasteurized milk (OIE, 2005). The disease is common in less developed countries including Nigeria and heavy economic losses can occur in cattle and African buffaloes from death, low productivity and due to trade restrictions (Alhaji, 1976; Alonge, 1988; Aliyu *et al*., 2001). Bovine tuberculosis in Nigeria appears to be mostly of the pulmonary form with gross lesions seen in the lungs and associated lymph nodes (Igbokwe *et al*., 2001). Bovine tuberculosis is a great threat to both animal and human health in many countries and human tuberculosis of animal origin particularly that caused by *Mycobacterium bovis*, is becoming increasingly important especially in developing countries (Ameni *et al*., 2007). In Africa, bovine tuberculosis has been given little attention despite being a public health problem and in this region many countries do not routinely undertake national tuberculin testing of cattle (Cook *et al*., 1996). There is a substantial lack of knowledge of the distribution, epidemiological patterns and zoonotic implications of bovine tuberculosis in developing countries especially in Africa (WHO, 1994). Farmers, abattoir workers, butchers and veterinarians are especially at risk of acquiring the disease from animals (Milian-Suazo *et al*., 2002). The high prevalence of human tuberculosis may be due to the high prevalence of tuberculosis in slaughtered cattle and the subsequent consumption of their products by humans (Asiak *et al*., 2007). Frequent cattle movement across borders favours the dissemination of the aetiologic agent (Njanpop-Lafourcade *et al*., 2001). The incidence of human tuberculosis due to species other than *Mycobacterium tuberculosis* has increased worldwide (Martinez *et al*., 2007). In a 9-year study in Taiwan, Chi-Cheng *et al.* (2010) reported non-tuberculous mycobacteria as accounting for 39.2% of positive mycobacterial cultures and this increased

significantly. Historically, tuberculosis caused by *Mycobacterium bovis* in humans was associated with consumption of unpasteurized milk and this is still the most important route of exposure in developing countries (Wilkins *et al*., 2008). Although bovine tuberculosis is known to be common in Africa, control policies have not been enforced in many countries due to cost implications, lack of capacity, and infrastructure limitations (Muller *et al*., 2009). Rapid and reliable diagnostic techniques for the identification of *Mycobacterium bovis* and for its differentiation from other members of the *Mycobacterium tuberculosis* complex are desirable for accurate diagnosis (Cobos Marin *et al*., 2003). Microscopy and culture are still the major backbone for laboratory diagnosis of tuberculosis even though new methods including molecular diagnostic tests have evolved over the last two decades (Sang-Nae and Patrick, 2007). Different molecular techniques that can differentiate between different Mycobacterial species have been developed (Oloya *et al*., 2007). The Restriction Fragment Length Polymorphism (RFLP) analysis using IS6110 is the gold standard and the most widely applied typing method for molecular epidemiology of *Mycobacterium tuberculosis* complex (Hilty *et al*., 2005). The development of PCR- based genotyping techniques offers promise for real time molecular epidemiological studies of tuberculosis and the techniques are faster and show more possibilities for automation (Scott *et al*., 2005; Hilty *et al*., 2005). A multiplex PCR technique based on genomic regions of difference has also been developed for accurate and faster way of differentiating members of the *Mycobacterium tuberculosis* complex (Waren *et al*., 2006). Molecular technologies are currently rarely used in developing countries, and where available, priority is usually given to human cases (Ayele *et al*., 2004).

This study was conducted to determine the *Mycobacterium* species causing bovine tuberculosis and its zoonotic implications among high risk groups in Plateau State with a view to suggesting measures that will improve the existing control programmes against the disease in both man and animals.

MATERIALS AND METHODS

Tissue Sample Collection

Tissue samples from tuberculous organs (lung, liver, spleen, lymph node, kidney, mammary gland and muscle) were collected from 150 slaughtered cattle showing lesions typical of tuberculosis. The samples were collected at Jos and Bukuru abattoirs (Plateau State) and this was done following thorough post mortem examination of the animals. Samples were collected using sterile scissors and forceps into clean, sterile, properly labeled plastic specimen containers with top screw caps. The samples were transported on ice to the laboratory for analysis. Samples that could not be processed immediately were stored in the refrigerator overnight. Abattoir records were also consulted in order to determine the number of cattle slaughtered during the study period.

Ziehl-Neelsen Test

Acid Fast Test (Ziehl-Neelsen Test) was carried out according to the method described (Baker and Silverton, 1985). Smears were prepared from a small portion of each of the samples using clean frosted microscope slides and these were fixed by heating with a gas flame. Following fixation, slides were flooded with carbol fuchsin and this was followed by heating until the stain began to steam but was not allowed to boil. Slides were washed with distilled water and decolourized with 3% acid alcohol before methylene blue was applied. This was followed by washing again for about 2 minutes; slides were air-dried and observed using a light microscope with $\times 100$ objective using oil immersion for the presence of acid- fast bacilli and the results recorded accordingly.

DNA EXTRACTION FROM TUBERCULOUS ORGANS

Tissue Homogenization

Using sterile forceps and blades, 2gm each of all the tissues collected were transferred into sterile pestle and mortar containing sterile glass beads and ground together. 1.5 ml of phosphate buffer saline (PBS) was added to the ground tissue and mixed well to form a suspension which was then transferred without sieving into 1.5 ml clean microcentrifuge tubes that were properly labeled. These were stored at -20°C until needed for DNA extraction.

DNA Extraction from Tissues

DNA extraction was carried out using kit extraction (Zymo Research®) according to manufacturer`s instructions. Samples stored at -20°C were removed and allowed to thaw at room temperature. Tissue homogenates were centrifuged at 10,000rmp for 5 minutes to obtain clear supernatants. 95μl of the supernatant, 95μl of 2X digestion buffer and 10μl of proteinase K (20mg/ml) were transferred into well labeled clean 1.5ml microcentrifuge tubes and the mixture was vortexed and incubated for 3 hours at 55°C. 700μl of genomic lysis buffer was then added, mixed thoroughly by vortexing and was followed by centrifugation at 10,000 rpm for one minute. The supernatant was transferred to a spin column in a collection tube and centrifuged at 10,000 rpm for one minute. 200μl of DNA pre-wash buffer was added to the spin column in a new collection tube and centrifuged at 10,000 rpm for one minute. This was followed by the addition of 400μl of g-DNA wash buffer to the spin column and centrifugation at 10,000 rpm for one minute. The spin column was then transferred to a clean microcentrifuge tube and 70μl of DNA elution buffer was added. The tubes were incubated at room temperature for 5 minutes and this was followed by centrifugation at 14,000 rpm for 30 seconds. The eluted DNA (70μl) was stored at -20°C until needed for molecular applications.

Deletion Analysis.

Deletion analysis was carried out as described (Warren *et al*., 2006), for the purpose of speciation using six primers namely Rd1, RD4, RD9, RD12, $RD1^{mic}$ and $RD2^{seal}$

Table II: Primer sequences used for deletion analysis

Primer sequence	RD	M. tb	M. bovis	M. africanum
AAGCGGTTGCCGCCGACCGACC	1	present	present	present
CTGGCTATATTCCTGGGCCCGG	1	(146bp)	(146bp)	(146bp)
GAGGCGATCTGGCGGTTTGGGG	1			
ATGTGCGAGCTGAGCGATG	4	present	absent	present
TGTACTATGCTGACCATGCG	4	(172bp)	(268bp)	(172bp)
AAAGGAGCACCATCGTCCAC	4			
CAAGTTGCCGTTTCGAGCC	9	present	absent	absent
CAATGTTTGTTGCGCTGC	9	(235bp)	(108bp)	(108bp)
GCTACCCTCGACCAAGTGTT	9			
GGGAGCCCAGCATTTACCTC	12	present	absent	present
GTGTTGCGGGAATTACTCGG	12	(369bp)	(306bp)	(369bp)
AGCAGGAGCGGTTGGATATTC	12			
CGGTTCGTCGCTGTTCAAAC	$RD1^{mic}$			present
CGCGTATCGGAGACGTATTTG	$RD1^{mic}$			(195bp)
CAATCAGCCAAGACGAGGTTG	$RD1^{mic}$			
TCAGCGGTCTCATAGCATTGC	$RD2^{seal}$			absent
CGGGTTGGGAATGTCAGAAAC	$RD2^{seal}$			(168bp)
GCGGCAAGGTACGTCAGAAC	$Rd2^{seal}$			

The PCR reaction contained 2.5μl green Gotag® flexir buffer (Promega, U.S.A), 2.5μl 25mM $MgCl_2$, 0.5μl 10mM dNTPs, 0.5μl (10pmol/ml) each of primer (forward, reverse, and internal), 0.125μl of Hotstar tag DNA polymerase, 17.875μl of Nuclease- free water, and 1μl of template DNA, making a final volume of 25μl. Amplification was initiated by initial denaturation at 96°C for 15minutes and this was followed by 40 cycles of 96°C for 1minute, 62°C for 1minute, and 72°C for 1minute. This was followed by incubation for a final extension at 72°C for 10 minutes. The PCR was carried out in a Thermal cycler (Applied Biosystems- Gene Amp PCR system 2700). PCR products were fractionated electrophoretically in 2% agarose gel in 1x TBE buffer, pH 8.3 for 1hour 30 minutes, and visualized under UV light after staining with ethidium bromide. The staining was done by immersing the gel in a solution of ethidium bromide in a shaker for 15 minutes. The size of the amplicons was determined by comparison with a 100bp DNA ladder.

RESULTS

Based on abattoir records, a total of 3381 cattle were slaughtered in Jos and Bukuru abattoirs during the study period between the months of , November, 2008, to March, 2009. Out of the 3381 slaughtered cattle, 2259 were in Jos abattoir while 822 were in Bukuru. 150 (4.4%) animals out of the total number (3381) had lesions typical of tuberculosis (plate 2) out of which 124 (3.7%) were acid- fast positive following Ziehl-Neelsen test (Plate 1). From the 2259 slaughtered in Jos abattoir, 120 (4.7%) had tuberculous lesions and 96 (3.8%) were acid fast positive. The total number slaughtered in Bukuru abattoir during the study period was 822 out of which 30 (3.6%) had tuberculous lesions and 29 (3.5%) were acid- fast positive (Fig.1). Of the 150 tuberculous organs detected during examination after slaughter, 114 (76%) were lungs, 27 (18%) lymph nodes, and 4 (2.7%) livers. 96 (64%) of the lungs were acid- fast positive while 22 (14.6%) of the lymph nodes, and 2 livers (1.3%) came out positive for acid-fast bacilli (Fig.1). Following Ziehl-Neelsen test, Acid fast bacilli appeared pinkish in a bluish background (Plate 1). Tuberculous lesions were observed more in female than male animals (Fig.2).

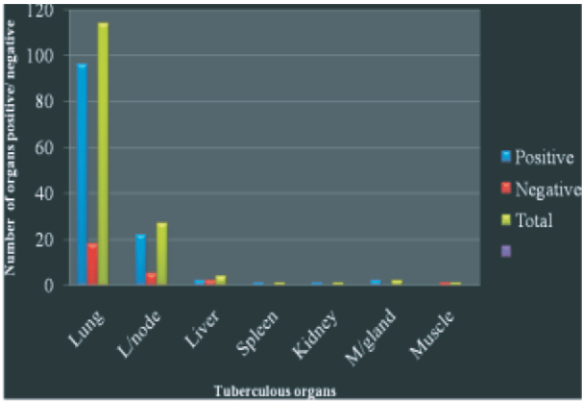

Fig 1: Detection of acid-fast bacilli in different organs of slaughtered cattle

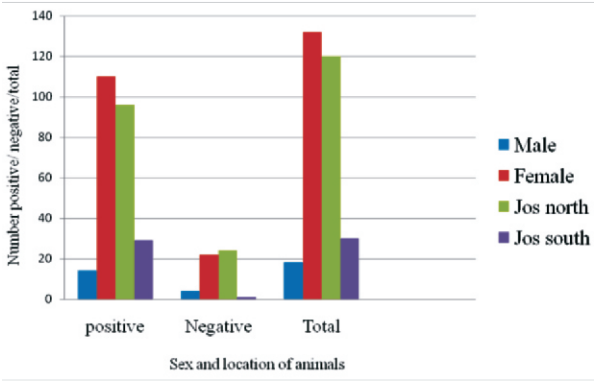

Fig 2: Tuberculosis in slaughtered cattle according to sex and location

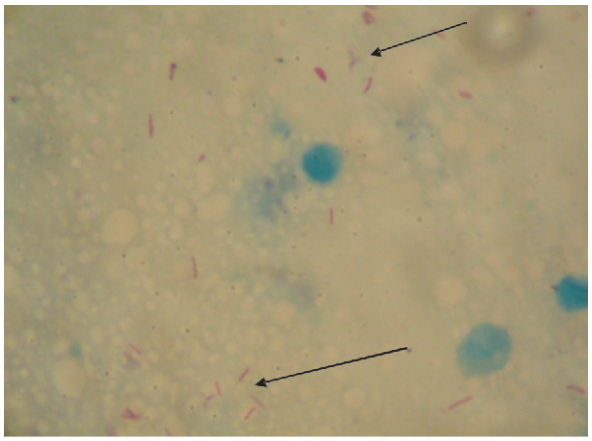

Plate 1 Pinkish Acid- fast bacilli (arrows) in a bluish background from a tuberculous lung

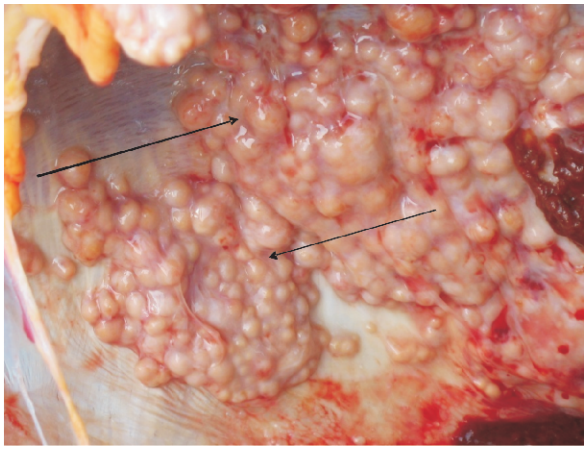

Plate 2 Image showing small discreet grayish nodules (arrows).

Deletion Analysis

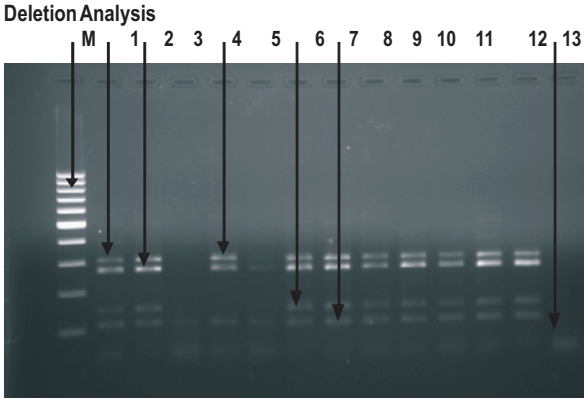

Fig 3: PCR products of regions of difference of Mycobacterium bovis using RD1, RD4, RD9, and RD12. Lane M- 100bp ladder, Lane 1- M. bovis positive control, Lane 2- RD4 absent (268bp), Lane 4- RD12 absent (306bp), Lane 6- RD1 present (146bp), Lane 7- RD9 absent (108bp), Lane 13- Negative control.

DISCUSSION

Based on abattoir records, a total of 3381 cattle were slaughtered in Jos and Bukuru abattoirs between the months of November, 2008, and March, 2009. Almost all the cattle slaughtered in both abattoirs came from nomadic herds within

and outside Plateau State. There are two major cattle markets located in Jos and Bukuru, and butchers in the two abattoirs usually buy their animals from these markets. Sometimes cattle from neighbouring countries like Chad, Cameroun, and Niger were brought to these markets for sale. Butchers handle a large number of animals (infected and non-infected) on daily basis. Slaughtered animals were usually dressed and processed without any protective clothing. During the course of processing carcasses, food and drinks are usually handled and consumed with blood- stained hands, a potential source of infection to the butchers and their families. One of the first studies indicating *Mycobacterium bovis* zoonotic transmission between cattle and humans in Africa was conducted in Tanzania where the same *Mycobacterium bovis* spoligotype was isolated from man and cattle (Hilty, 2006). Aerosol transmission of bovine tuberculosis to humans continues to occur among meat industry and slaughter house workers in regions where infection is still prevalent in cattle (Thoen *et al*., 2006). From the study conducted, the smuggling of tuberculous organs out of the abattoir was observed and these were usually sold to the public. In some cases, tuberculous organs were kept for customers who could be restaurants or beer parlour operators. In most developing countries, the laboratory diagnosis of pulmonary tuberculosis usually relies on the acid- fast bacillus (AFB) smear examination due to limited laboratory facilities (Thoen *et al*., 2006). An overall prevalence of 4.4% was observed in this study which is higher than the 2.8% reported in Borno and Yobe States by Igbokwe *et al*. (2001), but similar to the 4.3% reported by Cadmus *et al*. (2008), in South Western Nigeria. The major organs involved with tuberculous lesions were the lungs, 114 (76%) and the lymph nodes, 27 (18%) and occasionally the liver, spleen, kidney and the mammary gland. This is in agreement with the findings of Igbokwe *et al.* (2001), who reported that bovine tuberculosis in Nigeria is mostly of the pulmonary form involving mainly the lungs and associated lymph nodes. Hilty, (2006), reported bovine tuberculosis to be characterized by progressive development of granulomatous lesions in the lungs, lymph nodes, and other organs.

More female animals were slaughtered during the study period and were the most affected by tuberculous lesions. This may be due to the fact that cattle owners in the study area prefer to keep more female than male animals in their herds. Such animals are usually disposed and taken to the abattoir for slaughter when they can no longer reproduce due to old age or are observed to be sick by the owners. Belkhiri *et al*. (2009), reported bovine tuberculosis to be more often observed among oldest females because of the chronic nature of the disease and the possibility of exhibiting the infection increases with age. One of the essential methods of eradicating bovine tuberculosis is the epidemiologic surveillance of slaughtered animals in abattoirs through meat inspection and examination of samples taken from lesions typical of tuberculosis and subsequent confirmation of the disease through culture and molecular detection (Parra *et al*., 2007).

Deletion analysis confirmed the presence of *Mycobacterium bovis* and *Mycobacterium tuberculosis*. Out of a total of 142 samples analysed, 107 were *Mycobacterium bovis* 6 were *Mycobacterium tuberculosis*, 2 were *Mycobacterium africanum,* while 27 were untypeable. Deletion analysis is a much faster method of identifying members of the *Mycobacterium tuberculosis* complex compared to conventional methods like cultural isolation and biochemical tests which are time consuming and in most cases the facilities for such tests are not easily available in developing countries(Waren *et al*., 2006) including Nigeria. The presence of *Mycobacterium bovis* in slaughtered cattle signals a potential risk not only to cattle owners but also the general public that depend on these animals as source of animal protein. However, the herd owners who are mostly nomadic pastoralists may be more at risk due to their close association with the animals in addition to the reported practice of consumption of raw milk and other animal products. With RD4 and RD9 *Mycobacterium bovis* and *Mycobacterium tuberculosis* were differentiated. For *Mycobacterium bovis* both RD4 and RD9 are absent as these regions have been deleted in *Mycobacterium bovis* while in *Mycobacterium tuberculosis* the two regions are present. In *Mycobacterium africanum* RD4 and $RD1^{mic}$ are present while RD9 and $RD2^{seal}$ are

absent due to deletion of the regions in the genome of *Mycobacterium africanum*. The presence of *Mycobacterium tuberculosis* in bovine samples also indicates the possibility of transmission from humans to cattle.

CONCLUSION

From this study bovine tuberculosis can be said to be endemic in Plateau State, North Central Nigeria. Nationwide surveillance for bovine tuberculosis should be conducted to establish the actual situation of the disease among cattle population in the country. This will go a long way in helping to assess the risk posed by the disease to both livestock and humans particularly the high risk groups (herdsmen, butchers, abattoir workers, and cattle traders). The surveillance should be conducted through collaboration among appropriate Institutions and stakeholders in the country.

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