Mycobacterium africanum and nontuberculous mycobacteria from fresh milk of pastoral cattle and soft cheese in Oyo State – implications for public health

CA Agada[1], HK Adesokan[2], D Igwe[3] and SIB Cadmus[2]
Department of Veterinary Public Health and Preventive Medicine[1], University of Agriculture, Makurdi, Department of Veterinary Public Health and Preventive Medicine[2], University of Ibadan and Virology and Molecular Diagnostic Laboratory[3], International Institute of Tropical Agriculture, Ibadan, Nigeria

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Abstract

Background: Milk and milk products from cattle in tuberculosis endemic countries like Nigeria where pasteurization is not enforced could be a source of health concerns to the consumers.

Methods: We assessed randomly selected fresh milk from pastoral cattle, cheese samples and sour milk (*nono*) for the presence of *Mycobacterium* species through cultural isolation, Multiplex Polymerase Chain Reaction (PCR) and PCR-restriction enzyme analysis (PRA)-*hsp*65.

Results: Out of 269 fresh milk, 295 cheese and 150 sour milk samples screened; 8.30%, 0.34% and 0.00%, respectively were positive for *Mycobacterium* species. Molecular characterization revealed three *M. africanum* (fresh milk = 2; cheese = 1) and a high prevalence of Non-tuberculous *Mycobacterium* (NTM; 89.29%) from fresh milk comprising *M. gordonae* (n=12), *M. fortuitum* (n=4), *M. senegalense* (n=7) and *M. avium* (n=1).

Conclusion: The isolation of *M. africanum* and NTM species from this study is a matter of public health concern considering the practice of pooling milk from different animals and the consumption of unpasteurized milk which characterize most pastoral communities in Nigeria. Given the predominance of NTM in this study, their potential to cause disease in humans should not be ignored. Urgent measures should also be taken to integrate molecular techniques that will differentiate NTM from members of the *M. tuberculosis* complex in the epidemiology of tuberculosis in order to avoid misdiagnosis in humans and thereby protect public health.

Keywords: *Tuberculosis, Mycobacterium africanum, nontuberculous mycobacteria, Southwestern Nigeria*

Correspondence: Dr. S.I.B. Cadmus, Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Ibadan, Nigeria. E-mail: simeonc5@gmail.com

Résumé

Contexte: Le lait et les produits laitiers provenant de bovins dans les pays d'endémie de tuberculose comme le Nigeria où la pasteurisation est pas appliquée pourrait être une source de préoccupations pour la santé des consommateurs.

Méthodes: Nous avons évalué choisi au hasard lait frais provenant de bovins pastorales, des échantillons de fromage et de lait caillé (nono) pour la présence d'espèces de Mycobacterium à travers l'isolement culturel, Multiplex Polymerase Chain Reaction (PCR) et l'analyse de l'enzyme PCR-restriction (PRA) -hsp65.

Résultats: Sur 269 du lait frais, 295 du fromage et 150 échantillons de lait aigre blindés; 8,30%, 0,34% et 0,00%, étaient respectivement positifs pour les espèces de Mycobacterium. La caractérisation moléculaire a révélé trois M. africanum (lait frais = 2; fromage = 1) et une prévalence élevée de non-tuberculousMycobacterium (NTM; 89,29%) à partir de lait frais comprenant M. gordonae (n = 12), M. fortuitum (n = 4), M. senegalense (n = 7) et M. avium (n = 1).

Conclusion: L'isolement de M. africanum et espèces de NTM de cette étude est un sujet de préoccupation de santé publique compte tenu de la pratique de la mise en commun du lait de différents animaux et la consommation de lait non pasteurisé qui caractérisent la plupart des communautés pastorales au Nigeria. Compte tenu de la prédominance de NTM dans cette étude, leur potentiel de provoquer une maladie chez l'homme ne doit pas être ignoré. Des mesures urgentes doivent être prises pour intégrer les techniques moléculaires qui permettront de différencier NTM des membres du complexe M. tuberculosis dans l'épidémiologie de la tuberculose afin d'éviter les erreurs de diagnostic chez l'homme et de protéger ainsi la santé publique.

Mots-clés: *tuberculose, Mycobacterium africanum, mycobactéries non tuberculeuses, sud ouest du Nigeria*

Introduction

The increasing demand for protein in the developing countries as a result of malnutrition and among the immune-compromised individuals requires that animal

protein especially from cattle such as milk and meat should be safe and wholesome for human consumption. Zoonotic diseases such as bovine tuberculosis caused by *Mycobacterium bovis* are still being transmitted from cattle to man through the ingestion of infected animal products such as milk [1-3] in the developing countries mainly because pasteurization of milk is rarely observed and/or not enforced.

In most African countries as well as in Nigeria, consumption of unpasteurized milk is a regular practice in urban, peri-urban and rural areas where milk form part of the daily meals for most individuals [4]. Even when the milk is soured, it can still contain infective levels of mycobacteria [5-6]. An infected cow could produce milk containing mycobacteria, or cough infected droplets in the direction of the milker [6]. Since animals in traditional African farming systems are seldom culled, there is a greater chance for chronic tuberculosis in old cows, particularly those subjected to stress [7]. The risk is therefore high for humans to contract the disease through the consumption of contaminated milk or its products.

A recent report showed that Oyo State in south-western Nigeria has 3rd highest TB prevalence in the country, with about 6000 TB cases per annum and that the TB prevalence in the state increased by 46.5% from 2008 to 2010 with reported seven MDR-TB cases on treatment as at 2010 [8]. In addition, there are sub-ethnic groups with distinct dialect peculiarities besides livestock workers which are reported to cherish the consumption of "*wara*" soft cheese made from cow milk [9]. Despite the foregoing, there is paucity of information regarding the safety of milk and milk products consumed in the area with respect to mycobacterial organisms. This study therefore aimed to determine and characterize *Mycobacterium* spp contamination in pastoral cattle milk and its products in Oyo State, Nigeria.

Materials and methods

Study design and area

This cross-sectional study was conducted between January to December 2011 in Oyo State, south-western Nigeria. The State is bounded by Ogun, Kwara, Osun States and the Republic of Benin. It has two grazing reserves in Wasimi and Igangan where cattle graze throughout the year including a number of Government farm settlements in Ipapo, Ilora, Sepeteri, Eruwa, Ogbomosho, Iresaadu, Ijaiye, Akufo and Lalupon [10]. The state has a homogeneous population of about 4.5million [11] and predominantly occupied by Yoruba people.

Sample collection

Milk samples were randomly collected from pastoral cattle in the following areas in Oyo State: Wasimi, Igangan, Iganna and Ijaiye which are characterized by high population of livestock, where most of the cheese (wara) consumed by the residents of Ibadan, the state capital is produced. The choice of herds from which milk samples were collected was based on the cooperation of the animal owners. The samples were collected into 20ml sterile universal sample bottles and placed in cool boxes. Sour milk (*nono*) samples were obtained from the 150 vendors from Akinyele International Cattle Market and Bodija Market at various occasions. These markets have high population of pastoral females selling the products and buyers often come from various parts of Ibadan to buy and re-sell to the public. The *nono* samples were put directly into 20ml sterile universal sample bottles and placed in cool boxes. A number of cheese samples were also obtained at random from processors in the following rural areas: Igbo-ora, Wasimi Ijaiye, Igangan, Oyo and Iganna while another set of cheese samples were collected from eighteen market locations within Ibadan metropolis where an average of eight cheese samples purchased from the vendors. The cheese samples were placed in sterile polythene sample bags and placed in cool boxes. All the samples were transported to the Tuberculosis and Brucellosis Research Laboratories of the Department of Veterinary Public Health and Preventive Medicine, University of Ibadan where they were stored at 4°C until processing.

Sample processing and Isolation of mycobacteria

The samples were processed based on the Becton Dickinson digestion and decontamination procedure [12]. Using a sterile, 20ml centrifuge tube with a screw cap, 15ml of the fresh milk/nono sample was placed and centrifuged for 15mins. While about 5g of the cheese sample was homogenized with sterile distilled water using mortar and pestle and the homogenate placed into sterile 20ml centrifuge tube with a screw cap and centrifuged for 15mins. The supernatant of each sample was discarded and the residue re-suspended with equal amounts of activated NALC (N-acetyl-L-cysteine)-NaOH and shaken thoroughly to mix. The mixture was allowed to stand at room temperature for 15 min with occasional gentle shaking. Prepared

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phosphate buffer was then added to the 15 ml mark on the centrifuge tube and mixed, which was followed by centrifugation for 15 min at 3000 x g. The supernatant was carefully decanted, and 2 ml of phosphate buffer of pH 6.8 was added to re-suspend the sediment. The suspension was smeared on the slide for Zeihl Nelsen staining and microscopy while some were inoculated onto Lowenstein-Jensen (L-J) slopes with and without pyruvate and incubated at 37°C for at least 6 weeks with weekly observation for growth

Identification of positive culture

Smears of the colonies were stained by the Ziehl Neelsen (ZN) method [13] and examined for acid fast bacilli (AFB). The presence of AFB and cording of the bacilli were indicative of *Mycobacterium* species.

Molecular identification

All strains of the mycobacteria obtained were subjected to further characterization using two-step multiplex PCR technique based on genus typing and deletion typing as well as PCR-restriction enzyme pattern analysis (PRA-*hsp65*).

Genus typing

Genus typing was carried out according to the methods of Wilton and Cousins [14] and SOP CBU0247 [15]. The materials used for the PCR reaction include: Hotstar Taq DNA polymerase (10 μl) (Qiagen, Hidden, Germany) Mycgen-R 100μM (0.3 μl), Mycgen –F 100 μM (0.3 μl), Mycav –R 100μM (0.3 μl), Mycint –F 100μM (0.3 μl), TB1-F 100μM (0.3 μl), TB1-R 100 μM (0.3 μl), sterile water (6.2 μl) and DNA (isolate which has been heat-killed at 80^{0}C for 1 hour.) (2.0 μl). The reaction mixture was then heated in a Programme Thermal Controller (MyGene Series Peltier, Model MG 96$^+$) using the following amplification programs: 95°C for 15 min for enzyme activation, followed by 45 cycles at 94°C for 1 min for denaturation, 62°C for 1 min for annealing, and 72°C for 1 min for extension. After the last cycle, the samples were incubated at 72°C for 10 min and PCR amplification products were electrophoretically separated (fractionated) in a 1.5% agarose gel and 10×TAE running buffer at 10 V/cm for 2 h. Ethidium bromide at ratio of 1:5, 100 bp DNA ladder, and orange 6x loading dye were used in gel electrophoresis.

Deletion typing

This was carried out as described by Warren *et al.* [16]. The reagents used for the PCR reaction include Q-Buffer, 10xBuffer, 25mMgcl$_2$, 2.5mMdNTPs, and the primers which include RD1A, RD1B, RD1C, RD4A, RD4B, RD4C, RD9A, RD9B, RD9C, RD12A, RD12B and RD12C. The reaction mixture contains essentially the same component as that of genus typing.

PCR-restriction enzyme pattern analysis (PRA-*hsp65*)

This was carried out as described by Telenti *et al.*,[17] with slight modification.

Amplification

Five microliters of lysate was added to each reaction tube. The composition of the PCR mixture (25, μl) was 50mM (1.25μl) KCl, 5mM (2.5μl) Tris-HCl (pH 8.3), 0.75 mM (2.0 μl) MgCl$_2$, 200uM (0.2 μl) (each) deoxynucleoside triphosphate, 0.25uM (1.0 μl) (each) primer, and 1.25 U (4.0 μl) of Taq polymerase (Cetus or Boehringer Mannheim). The reaction was subjected to 45 cycles of amplification (1 min at 94°C, 1 min at 60°C, 1 min at 72°C); this was followed by 10 min of extension at 72°C. Primers Tbll (5'-ACCAACGATGGTGTGTCCAT) and Tb12 (5'-CTTGTCGAACCGCATACCCT) amplified a 439-bp fragment between positions 398 and 836 of the published gene sequence.

Restriction analysis

For BstEII digestion, 10μl RI of PCR product was added directly to a mixture containing 0.5 pl (=5 U) of enzyme, 2.5, ul of restriction buffer (5 x buffer B), and 12μl of water, and the mixture was incubated for 60 min at 60°C. Similarly, 10 μl of product was digested at 37°C in a solution containing HaeIII enzyme, the corresponding buffer (5 x buffer M), and water. Enzymes and buffers were purchased from Inqaba biotec South Africa.

Evaluation of restriction patterns

After digestion, 4.0μl of gel loading buffer (0.25% bromophenol blue, 40% sucrose in water) was added, and 10.0μl of the mixture was loaded onto a NuSieve 3:1 agarose gel (FMC Bioproducts). Fragments were visualized by ethidium bromide staining and UV light.

Ethical approval

Ethical approval was obtained from UI/UCH Ethics Committee with UI/UCH Ethics Committee assigned number: UI/EC/11/0238.

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Results

Culture

Out of the 269 fresh milk sampled (Wasimi: 89; Igangan: 48; Igana: 80 and Ijaiye: 52), 22 (8.2%) were positive by culture while none (0.0%) of the sour milk was positive. In addition, only one (0.3%) of the 295 cheese samples was positive by culture (Table 1).

Genus and deletion typing of the strains isolated from fresh milk and cheese

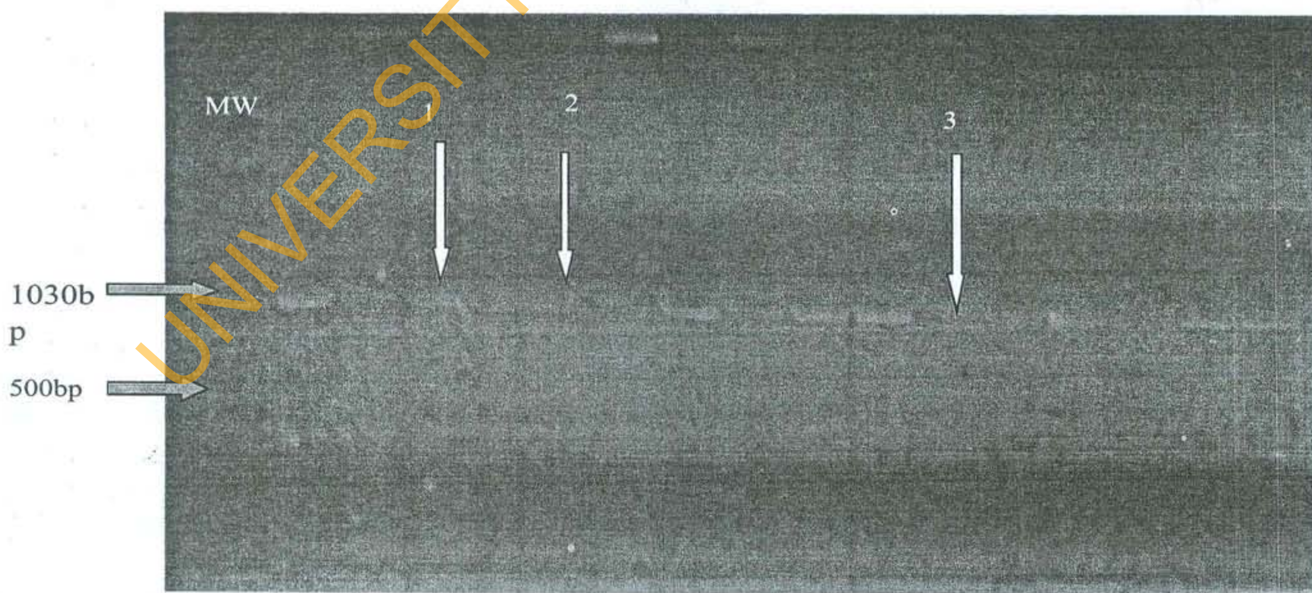
Out of the 22 fresh milk contaminated with the mycobacteria, seven (7) had isolates on both LJ with glycerol and pyruvate; as a result 29 isolates (seven samples with two isolates each = 14; 15 isolates from the remaining 15 milk samples) were obtained. The genus typing identified all the 29 isolates as

Table 1: Percentage distribution of cultured fresh milk, sour milk and cheese

Type of Sample	Total Number Sampled	Number (%) Positive	Number (%) Negative
Fresh Milk	269	22 (8.2)	247 (91.8)
Sour milk (Nono)	150	0 (0.0)	150 (100.0)
Cheese (Wara)	295	1 (0.3)	294 (99.7)

Table 2: Deletion typing showing regions of difference (RD) deleted

Region of Difference	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Sample	Fresh milk	Fresh milk	Fresh milk	Wara
RD 1	Present	Present	Present	Present
RD 4	Present	Present	Present	Present
RD 9	Absent	Absent	Absent	Absent
RD 12	Present	Present	Absent	Present
Mycobacterium Spp	*M. africanum*	*M. africanum*		*M. africanum*



MW 100bp molecular weight marker, 1, 2, *Mycobacterial* species, 3 not a *Mycobacterium*.

Fig 1: Gel electrophoresis separation of PCR products by multiplex PCR genus typing of mycobacteria isolated from fresh milk of pastoral cattle in parts of Oyo State.

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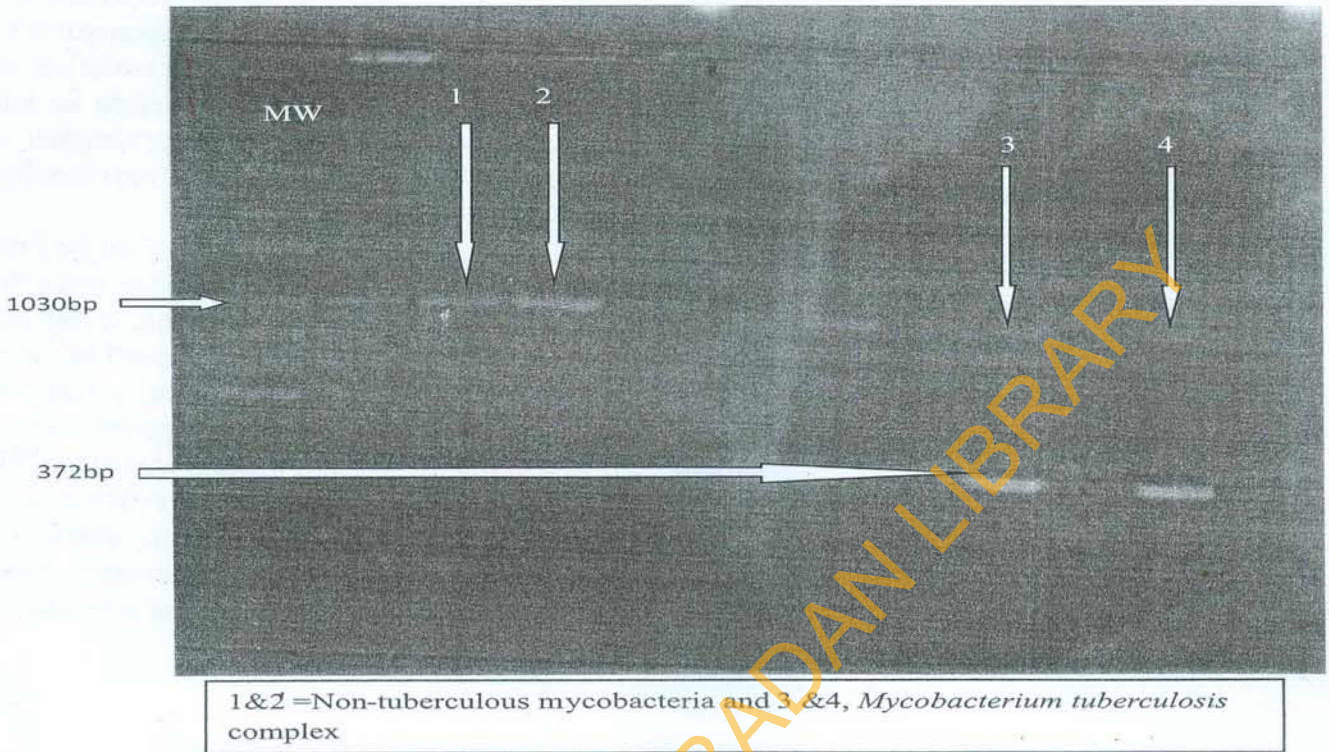

Fig 2: Gel electrophoresis separation of PCR products by multiplex PCR genus typing of mycobacteria isolated from fresh milk of pastoral cattle in parts of Oyo State.

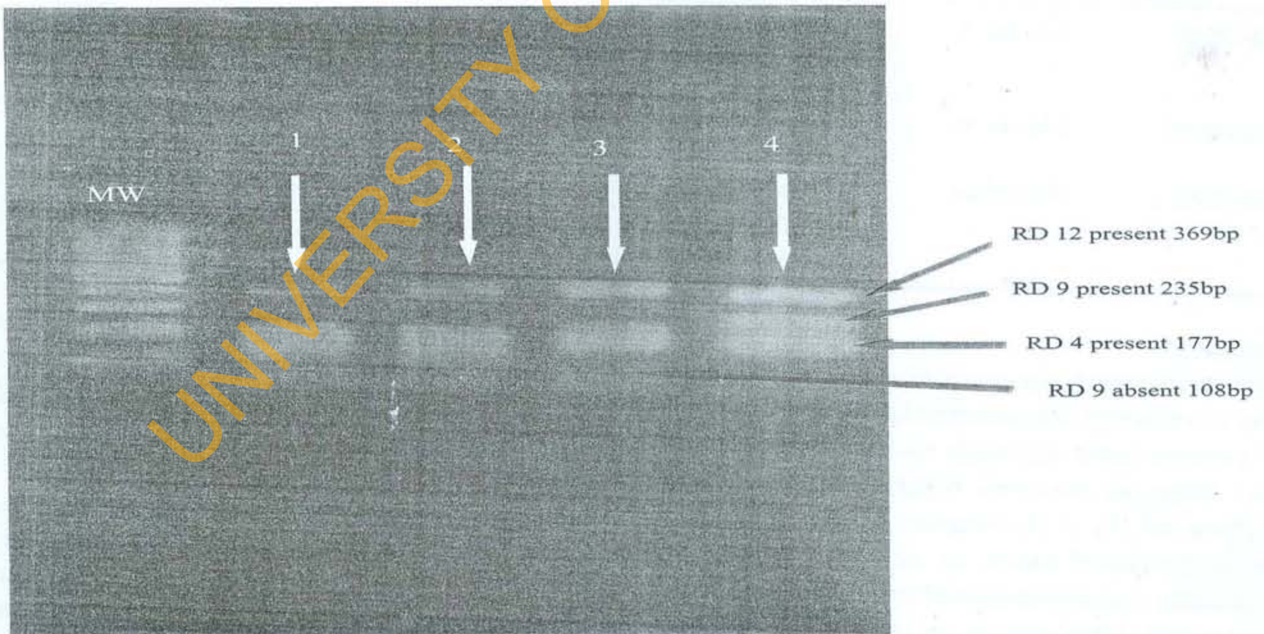

Fig 3: Gel electrophoresis separation of PCR products by multiplex PCR deletion typing of mycobacteria isolated from fresh milk of pastoral cattle in parts of Oyo State.

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Mycobacterium species of which 3 (10.3%) were identified as members of the *Mycobacterium tuberculosis* complex (MTC) group and the remaining 26 (89.7%) as nontuberculous mycobacteria (NTM) or mycobacteria other than *M. tuberculosis* (MOTT). Furthermore, the only strain isolated from the cheese was confirmed to be member of the MTC (Table 2; Figures 1 and 2).

Further characterization of the four MTC strains by deletion typing identified two of the strains from milk and the only strain from cheese as *Mycobacterium africanum*. This gives the prevalence of 0.7% and 0.3% of *M africanum* in fresh milk of pastoral cattle and cheese respectively (Figure 3).

PCR-restriction enzyme pattern analysis (PRA-*hsp65*)

The result of the PRA-*hsp*65 of the 26 NTM or MOTT indicates the following: 12 *Mycobacterium gordonae* (46.2%), seven *M. senegalense* (26.9%), four *M. fortuitum* (15.38%) and one *M. avium* (3.85%) and two (7.7%) unclassified isolate (Table 3).

pooling milk from various sources together and selling for public consumption either as sour milk or processed as cheese further exacerbates the situation which therefore enhances widespread transmission of infection to the consumers. Our finding is in agreement with previous reports which indicated isolation of *Mycobacterium spp* from cow milk brought for sale at the cattle market and cattle awaiting slaughter in Bodija Municipal Abattoir, Ibadan [22-23] and from cow milk in northern Nigeria [20,24,25].

The isolation of *M. africanum* from the fresh milk and cheese in the study area further re-iterates cattle as a reservoir of this infectious agent and consumption of milk and milk product such as 'wara' could be a source of the infection in humans. Meanwhile, *M. africanum* has been reported to be the cause of half of human tuberculosis in West Africa [26]. Previous studies in the study area reported the isolation of *M. africanum* from children with tuberculous lymphadenitis and in stool of children in an immunization clinic [27,28]. The role of consumption of unpasteurized

Table 3: Fragment sizes of mycobacterial 439bp *hsp*65 PCR products after digestion by Bst EII and HaeIII

BstEII Digestion	HaeIII Digestion	Mycobacterial isolates	PRAsite Database Result Score	Number of isolates
240/125/85	170/120/70	*Mycobacterium gordonae* type 1 *Mycobacterium gordonae* type 9	1722	12
230/140/85	200/140/90	*Mycobacterium senegalense* type 4	49	7
230/125/85	140/115/60	*Mycobacterium fortuitum* type 2	9	4
		Mycobacterium avium		1

Discussion

This study reports the prevalence as well as molecular characterization of mycobacterial species in fresh milk of pastoral cattle and ready-to- eat cheese in parts of Oyo State, southwestern Nigeria. The prevalence of 8.2% and 0.3% of *Mycobacterium* species in the fresh milk of pastoral cattle, as well as in cheese is an indication that mycobacterial infection is wide spread in the cattle population in the study area [18-21]. The presence of *Mycobacterium* species in milk in this study is a matter of public health concern considering the practice of consumption of unpasteurized milk especially among the pastoralists. Furthermore, the practice of

milk should therefore be considered in the epidemiology of *M. africanum* infection in humans in the study area.

Interestingly, a great number of NTM isolated (82.8%) from this study is similar to that reported by Kazwala *et al.*, [18] and Leite *et al.*, [19]. These NTM including *M. gordonae*, *M. senegalense*, *M. fortuitum* as well as *M. avium* are considered potentially pathogenic and cause variety of clinical manifestations in humans [19, 29] and they have been reportedly isolated from fresh milk samples in Tanzania and Brazil [18-19]. *M. avium* and *M. fortuitum* have also been isolated from humans with pulmonary infections in Lagos (south-western Nigeria) and Jos (northern Nigeria)

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[30,31] while a recent study also reported the isolation of these atypical mycobacteria from new and previously treated pulmonary tuberculosis patients in Ibadan, Nigeria [31]. *M. senegalense* has been associated with catheter-related hematological infection in a 49-year old woman with non-Hodgkin's lymphoma in Korea as well as tissue infection in a child after fish tank exposure in Canada [32-33]. In cattle, *M. senegalense* causes bovine farcy, a chronic suppurative granulomatous inflammation of the skin and lymphatics seen mostly in sub-Saharan Africa [34].

On the other hand, the prevalence of 1.1% MTC from fresh milk in this study is very low compared to 11.3% and 5.7% reported by Cadmus and Adesokan [22] and Cadmus *et al.* [23] respectively from cows' milk brought for sale at the cattle market and those awaiting slaughter in Bodija Municipal abattoir, Ibadan. The difference may be explained by the fact that the animals sampled by these workers were trade cattle which are often old animals which might have been infected due to longer exposure time. Also, the pastoralists hardly dispose or sell healthy animals except in extreme conditions, suggesting that the animals examined in the current study might be from healthier herds. However, the prevalence found in this study is similar to those reported in the Northern part of the country by Okaiyeto *et al.* [25] that reported 1.7% in Kaduna State, 1.4% in Makurdi [24] and 1.25% in Niger State [20].

The limitations of this study include the fact that the cattle handlers and other consumers of the milk and milk products were not screened for mycobacterial infection which would have further substantiated the transmission chain of the infection in the study area. Secondly, the herds selected for sampling were based on the co-operation of the livestock owners, therefore our findings may be under-reporting the magnitude of mycobacterial infection in milk and its products considering the fact that some of the unscreened herds may be major sources of these pathogens given the unrestricted interaction between infected and uninfected animals.

Despite these limitations, the isolation of *M. africanum* from fresh milk and cheese in this study requires urgent stakeholders' attention towards controlling the spread of bovine TB through enforcement of pasteurization of milk. In addition, routine screening of cattle for TB through enhanced surveillance should be on the front burner of the Nigerian government policy. Again, the predominance of NTM in this study signifies the importance of these species in the epidemiology of TB in Nigeria and other TB endemic countries. Finally, NTM should be considered to be dangerous to human health, particularly among the immuno-compromised.

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