The Etiology and Public Health Significance of Mycobacteriosis of Cattle in Kenya

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Abstract

Background: Zoonotic tuberculosis, commonly referred to as bovine, tuberculosis is mainly caused by Mycobacterium bovis subsp. bovis, a member of the mycobacterium tuberculosis complex (MTBC). Infection in humans resembles that caused by Mycobacterium tuberculosis. Mycobacteria Other Than Tuberculcous (MOTTs) are also increasingly being associated with tuberculosis humans but reports of infections in animals are scarce, their zoonotic potential being largely ignored. The study aimed to determine the aetiology and public health importance of mycobacteriosis in slaughter cattle in Kenya. Methods: Routine postmortem meat inspection was performed on a subpopulation of 1000 meat carcasses selected randomly from among 7,564 in a municipal abattoir, between January and July, 2015. Carcasses were examined for tuberculous lesions which were then examined for acid-fast bacilli, (AFB), cultured for isolation of mycobacteria and the isolates characterized by DNA analysis. Results: Of the carcasses examined, 218 (21.8%) had lesions in various parts of the carcasses. Acid-fast bacilli were observed in 63/218 of the lesions and suspected mycobacteria isolated from 35 of them. The isolates were identified as M. fortuitum (12), M. bovis subsp. bovis (3), M. shimoidei (2) M. asciticatum, M. interjectucatum, M. szulgal, M. celatum and M. kansaasi at one (1) each. Thirteen (13) of the isolates could not be speciated. Conclusion: The MOTTs identified in the study have been liked to various types of mycobacteriosis in humans. The study therefore highlights the pathogenic and zoonotic potential of MOTTs, indicating that zoonotic tuberculosis should not be restricted to Mycobacterium bovis subsp. bovis.

Keywords: Mycobacteriosis, slaughter cattle, public health

INTRODUCTION

Mycobacteriosis affects warm- and cold-blooded animals and is caused by obligatory, potentially pathogenic, or saprophytic Mycobacteria genus,¹ which currently comprises 163 species and 13 subspecies.² In cattle and other ruminants, tuberculosis (TB) is caused mainly by the obligate pathogen Mycobacterium bovis subsp. Bovis, but infections by M. bovis subsp. caprae, Mycobacterium tuberculosis, and Mycobacterium africanum have also been reported.³ The disease caused by M. bovis subsp. bovis is commonly referred to as bovine or zoonotic TB and is transmitted from animals to human through consumption of unpasteurized contaminated milk, ingestion of raw or undercooked meat, inhalation of cough aerosols from infected animals, and transcutaneously through handling of infected carcasses.⁴ In Africa, zoonotic TB has widespread occurrence due to unsanitary cultural practices and low hygiene standards.⁵ The disease in humans is clinically and pathologically indistinguishable from that caused by M. tuberculosis.⁶ However, given that ingestion is the main route of infection, extrapulmonary lesions such as submandibular and cervical lymphadenitis are common manifestations of M. bovis infections.⁷ Zoonotic TB has therefore an important public health concern worldwide. It is estimated to contribute to 5%–10% of human TB cases, but this varies between countries. Immunocompromised persons, especially by human immunodeficiency virus (HIV)/AIDS, are particularly at risk.¹⁷ Various studies have also shown the role of a wide range of Mycobacteria other...

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than tuberculous (MOTT) in etiology of TB in humans. MOTTs have been found to infect humans, causing lesions and symptoms similar to those caused by obligatory pathogenic Mycobacteria, implying their potential role as pathogens.\textsuperscript{[18,19]} However, reports of animal infections caused by MOTTs are few. Studies in animals have concentrated mainly on zoonotic TB, neglecting the potential pathogenic and zoonotic role of other types of Mycobacteria. This investigation aimed to establish the etiology and public health importance of TB in cattle in Kenya. The information generated will strengthen surveillance and control of bovine TB through improved diagnostic strategies to safeguard human health.

**Methods**

**Study sites**

The study comprised slaughterhouse meat inspection and laboratory analysis. Meat inspection was conducted at the municipal abattoir in Nanyuki, Laikipia County. The County is located in Central Kenya near the slopes of Mount Kenya and is predominantly a pasture land with a large population of cattle. Various breeds of cattle are raised under nomadic pastoralism, small-scale paddocks, zero-grazing, and commercial ranches. This abattoir was selected because it is the only main slaughterhouse and receives cattle for slaughter from the whole County. Laboratory analysis was carried out at the Department of Veterinary Pathology and Microbiology, University of Nairobi, the Central Tuberculosis Reference Laboratory, Ministry of Health, and the Central Veterinary Laboratory, Ministry of Agriculture and Irrigation.

**Sampling procedure**

Sampling was conducted between January and July 2015. There was no ethical consideration in the study as condemnation of diseased organs and carcasses is part of the routine meat inspection procedure. Postmortem meat inspection was carried out on 1000 meat carcasses selected randomly from among 7564 as described previously.\textsuperscript{[18]} Particular attention was given to the head and thoracic lymph nodes, lungs and associated lymph nodes, pleura, peritoneum, abdominal organs, and mesenterium. Superficial inguinal and supramammary lymph nodes in males and females, respectively, were also examined. In each case, the organ/tissue was palpated, incised, and examined. Lesions suggestive of mycobacteriosis (granulomatous, yellowish, purulent, caseous, or calcified) were excised, placed individually into 50 ml sterile Falcon\textsuperscript{®} tubes, labeled, placed into a cooler-box, transported to the laboratory, and preserved at $-20^\circ$C till processing.

**Laboratory analysis**

Lesion samples were examined for acid-fast bacilli (AFB) and cultured for bacterial isolation. The isolates were examined for AFB and then speciated by DNA analysis. All manipulations were carried out in Biosafety Cabinet (BSC) Level II. Before processing, tissue samples were allowed to thaw at 4°C for a day. The samples were then processed as described previously.\textsuperscript{[18]} Briefly, each sample was transferred into a sterile Griffith tube, 5 ml of sterile distilled water added, and the sample homogenized. Approximately 5 ml aliquots of the homogenates were then transferred into 50 ml Falcon\textsuperscript{®} tubes and equal volume of 4% NaOH was added to decontaminate the samples of other bacteria. The tubes were then tightly closed, vortexed for 5 min, and left to stand for 10 min to allow aerosols to settle. Phosphate buffer solution (PBS), pH 6.8 was added to the 45 ml mark of the tube to stop decontamination. The suspension was then centrifuged at 800 $\times g$ for 30 min at 4°C. The supernatant was discarded into a 5% phenol solution and the pellet completely resuspended in 3 ml PBS containing benzylpenicillin, 50 IU per ml, by vortexing. Smears were prepared with one drop of the suspension and Ziehl–Neelsen (ZN) staining carried out following the standard procedure. Stained slides were examined under oil immersion ($\times 1000$) for AFB. Two hundred microliters of the suspension was inoculated into three tubes of Lowenstein–Jensen (L-J) slant media containing either 0.4% pyruvate, 0.75% glycerol, or $p$-nitrobenzoic acid (PNB) at 0.5 mg/ml. The tubes were incubated at 37°C for up to 12 with weekly examination for growth.

Suspect colonies were then examined for AFB and isolation of DNA from positive cultures was carried out following the HAIN Lifescience manual (HAIN Lifescience, Nehren, Germany). Briefly, about three colonies of bacteria were collected from each AFB-positive culture and suspended in 300 µl of molecular grade water in a 1.5 ml sterile microcentrifuge tube. The tubes were tightly closed and incubated for 20 min in boiling water (95°C), followed by incubation in an ultrasonic bath for 15 min at room temperature. The tubes were then centrifuged at 11,000 $\times g$ for 5 min at room temperature and the supernatant, containing the DNA, transferred into fresh tubes, and stored at 4°C until use.

DNA analysis was done at three levels, using GenoType\textsuperscript{®} Mycobacterium DNA strip assay kits (Hain Diagnostica, Nehren, Germany). Samples were first analyzed using GenoType\textsuperscript{®} Mycobacterium Common Mycobacteria (CM) followed by GenoType\textsuperscript{®} Mycobacterium Additional Species (AS) Kits. M. tuberculosis complex (MTBC) species identified by the two kits were then speciated using GenoType\textsuperscript{®} Mycobacterium MTBC kit.

Master Mix preparation was done in a DNA cabinet. Thirty-five microliters of primer nucleotide mix (AM-B) and 10 µl of AM-A mixture were measured into a sterile 1.5 µl Eppendorf tube to make aliquots of the master mix. Aliquots (45 µl) were then transferred into labeled polymerase chain reaction (PCR) tubes which were then transferred to a BSC Class II and DNA template added. For CM and AS analysis, 100 µl of each DNA sample was first mixed with 2 µl of internal control DNA in a different labeled Eppendorf tube and 5 µl of the mixture transferred into the labeled PCR tubes. For MTBC analysis, 5 µl neat DNA template samples were transferred directly into the PCR tubes. The tubes were then loaded into a thermocycler (Techne TC-5000, Bibby
Scientific Ltd., China). Amplification protocol for CM and AS analysis consisted of one denaturation cycle of 15 min at 95°C, followed by 10 cycles comprising 30 s at 95°C and 2 min at 65°C, an additional 20 cycles comprising 25 s at 95°C, 40 s at 50°C, and 40 s at 70°C, and a final extension of 8 min at 70°C. The protocol for MTBC analysis consisted of one denaturation cycle of 15 min at 95°C, followed by 10 cycles comprising 30 s at 95°C and 2 min at 58°C, an additional 20 cycles comprising 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C, and a final extension of 8 min at 70°C. Hybridization and detection procedure were uniform for all three assays and were carried out using a semi-automated washing and shaking device (TwinCubator®, Hain Lifescience, Nehren, Germany). The hybridization buffer (HYB), stringent wash solution (STR), and TwinCubator were prewarmed to 45°C. Aliquots (20 μl) of denaturation solution were dispensed into one corner of the wells in a plastic reaction tray. Amplified product 20 μl was then added, gently mixed, and incubated at room temperature for 5 min. One milliliter of HYB was added into each well, allowed to mix, and labeled detection strips loaded. The loaded reaction tray was incubated for 30 min at 45°C followed by two washing steps using STR. The wells were then rinsed with 1 ml of rinsing solution (RIN). Diluted conjugate, 1 μl was added into each well and incubated at 45°C for 30 min followed by two successive rinsing using RIN and two using distilled water. Hybridized products were detected by addition of streptavidin conjugated with alkaline phosphatase, subsequent addition of the substrate, and incubation in the dark for 20 min. After a final rinse with distilled water, strips were dried on blotting paper and fixed on the data evaluation sheet.

Statistical analysis
All statistical analysis was undertaken in STATA (STATA/IC 11.0 for Windows, Stata Corp, College Station, TX, USA). The prevalence was determined as a proportion, at 95% confidence interval (CI), of the positive cases out of total carcasses examined.

RESULTS

Tuberculous lesions in meat carcasses
Two hundred and eighteen (21.8%) (95% CI: 19.35–24.46) of the 1000 carcasses examined had lesions located in one or more organs/tissues. In the affected organs or tissues, the lesions were either single or multiple. Lesions were observed in the lymph nodes of the head region, specifically, retropharyngeal, parotid, and submandibular 13 (6%) of the 1000 carcasses examined had lesions located in one or more organs/tissues. In the affected organs or tissues, the lesions were either single or multiple. Lesions were observed in the lymph nodes of the head region, specifically, retropharyngeal, parotid, and submandibular 13 (6%), bronchial lymph nodes 13 (6%), mediastinal lymph nodes 16 (7.3%), lungs 97 (44.5%), liver and portal lymph nodes 49 (22.5%), and mesenteric lymph nodes 2 (0.9%). Other organs affected were spleen and aortic serosa 27 (12.4%). The lesion sizes ranged from small (0–5 mm), medium (5–10 mm) to large (>10 mm), and the consistency was caseous (17.9%), fibrocasseous (27.1%), fibrocalcified (8.3%), or calcified (46.8%).

Laboratory analysis
On examination of direct smears stained by the ZN method, AFB were observed in 63 (6.3%) (95% CI: 4.95–7.98) of the 218 smears. Isolates were recovered from 35/1000 (3.5%) of the lesion samples. Of these, three were recovered in pyruvate and glycerol media, while 32 were recovered in all three media. DNA analysis identified 22 of the isolates as Mycobacterium fortuitum (12/35, 34%), M. bovis subsp. bovis (3/35, 8.5%), Mycobacterium shimoidei (2/35, 6%), and Mycobacterium asciticum, Mycobacterium interjectucct, Mycobacterium szulgi, Mycobacterium Celatum, and Mycobacterium kanssii at one (1/35, 2.8%) each. Thirteen of the isolates (37%) could not be speciated [Table 1]. GenoType® Mycobacterium CM kit identified M.fortuitum and Mycobacterium interjecturct; AS kit identified M. shimoidei, M. celatum M. szulgi, M. asciticum, M. kanssii, and Mycobacterium species; and MTBC kit identified M. bovis. The overall prevalence of mycobacteriosis in this study was 3.5% (35/1000) (CI: 2.53–4.83). Zoonotic and MOTTs mycobacteriosis prevalence was 0.3% (3/1000) (CI: 0.1–0.88) and 3.2% (32/1000) (CI: 2.28–4.48), respectively.

DISCUSSION
This study involved collection of tuberculous lesion samples from cattle meat carcasses and laboratory sample analysis to determine the etiology and public health importance of the lesions. In the study, tuberculous lesions were observed in 21.8% of the meat carcasses. Majority of the lesions were found in the lungs and associated lymph nodes (57.8%), which may indicate aerosol infection. Animals examined in this study were raised under nomadic or common grazing production system. This increases contact between animals raising chances of infection.[20] Similar findings have been reported elsewhere.[8,21-26]

In this investigation, majority of the lesions (46.8%) were calcified. TB is characterized by the formation of granulomas which can in the course of time regress, undergo necrosis, calcify, or liquefy.[27] The type of granuloma may therefore indicates the level of animal immunity and the duration of infection.[28]

Direct ZN staining confirmed AFB in 28.9% of the smears analyzed. Individual studies have reported a great disparity in the sensitivity of microscopic detection of AFB depending on the technician’s skill.[29] Mycobacteria are not evenly distributed in the tissue sample and may often be found in low numbers in smears due to sample taking technique, resulting in negative staining results.[30] In completely calcified lesions, ZN may stain negative due to low numbers of organisms or a genuine absence of mycobacteria in the necrotic area of the lesion.[31] Further, other bacterial pathogens such as Actinomyces bovis, Trueperella pyogenes, Corynebacterium, Gordonia, Nocardia, Rhodococcus, Tsukamurella, and Dietzia may macroscopically produce tuberculous-like lesions.[32,33] In this study, mycobacteria were isolated from 16% (35/218) of the lesions. DNA analysis identified the isolates as M. bovis subsp. bovis and seven species of MOTTs. The latter were M.fortuitum, M. shimoidei, M. asciticum, M. interjectuct,
M. szulgai, M. celatum, and M. kansasii. Some species (13/35) could not be speciated possibly because the kits used are optimized for detecting only 37 mycobacteria that are commonly encountered and the 13 MOTTs may not therefore be among them.

_M. bovis_ subsp. _bovis_ was recovered in L-J media containing either glycerol or pyruvate but not PNB. Growth of MTBC is selectively inhibited by PNB, but MOTTs are not and the criteria have been used for identification of the former.³⁴,³⁵ Growth of _M. bovis_ subsp. _bovis_ in this study was, however, not inhibited by glycerol as reported elsewhere.³⁶

The prevalence of _M. bovis_ subsp. _bovis_ in this study was 0.3% while that of MOTTs was 3.2%. Reports of MOTTs isolation in animals are scarce. A few studies in African region have reported isolation from granulomatous lesions in meat carcasses.⁸,³⁷,³⁸ In these studies, however, the MOTTs species were not determined.

Zoonotic TB is recognized worldwide as a public health concern and is transmitted from animals to humans through consumption of raw or undercooked animal products as well as through inhalation of infective aerosols.⁹,¹²,¹³,³⁹ MOTTs are widespread in the environment, especially water and soil, and have a high survival rate.¹⁰ They are potentially pathogenic and have been implicated in cases of mycobacteriosis in humans and animals.⁴¹ In humans, they can cause pulmonary disease, disseminated disease, or localized lesions in both immunocompetent and immunocompromised individuals.¹⁹ Seven species of MOTTs were identified in this study. _M. fortuitum_, a member of the _Mycobacterium avium_ complex (MAC) was identified in 12/50 (24%) of the specimens. Infection from this complex has been linked to severe cases of human and fish mycobacteriosis. Specifically, _M. fortuitum_ has been associated with cutaneous and deeper infections after trauma, pulmonary disease, and corneal infections. Other syndromes in human include osteomyelitis, joint infections, lymphadenitis, and endocarditis, especially in the immunosuppressed and especially by HIV/AIDS.⁴² _M. celatum_ has been recognized as a causative agent of pulmonary and disseminated infections in immunosuppressed humans,⁴³ especially those with preexisting lung infection. _M. szhimoidei_, also an opportunistic pathogen, has been previously isolated only among severely immunocompromised subjects and those with preexisting lung diseases such as emphysema, previous TB, silicosis, and lung carcinoma.⁴⁴ _M. interjectum_ has been well documented as a source of infection in children and immunocompromised adults where it is associated with necrotizing lymphadenitis.⁴⁵ _M. szulgai_ is a relatively uncommon MOTT, accounting for <1% of all MOTTs isolations. Diseases caused include pulmonary, cervical lymphadenopathy, cutaneous infections, osteomyelitis, and less common disseminated diseases.⁴⁶ _M. kansasii_ is the most common cause of nontuberculous mycobacterial lung disease in the western world⁴⁷ and the second most frequently isolated mycobacteria, after _M. avium_, in HIV-infected patients.⁴⁸ It causes lung disease that is clinically identical
to TB caused by *M. tuberculosis*, but asymptomatic infection occurs in some patients. *M. asciaticum* has been recovered from cases of pleuropulmonary and local disseminated cutaneous infections in organ-transplant recipients.[49]

**Conclusion**

This study demonstrated *M. bovis* subsp. *bovis*, seven species of MOTTs and 13 unidentified MOTTs as causative agents of mycobacteriosis in cattle in Kenya. The identified MOTTs have been found to cause various types of TB in humans. Previous studies on zoonotic TB in cattle have concentrated mainly on *M. bovis* subsp. *Bovis*, neglecting the presence and significance of MOTTs. This study demonstrated the pathogenic potential of MOTTs in cattle and also implied their zoonotic potential, concluding that to safeguard human health, diagnostic and surveillance strategies for zoonotic TB should not be restricted to *M. bovis* subsp. *bovis*.

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**Conflicts of interest**

There are no conflicts of interest.

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