

Prevalence of Antibiotic Resistance among *Mycobacterium Tuberculosis* Complex Species from Camel Milk in Isiolo County, Kenya

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ABSTRACT---- *The rising levels of antibiotic resistance among bacterial pathogens is a major global concern of human and animal health. This has been attributed to unauthorized use of therapeutic drugs in livestock, a situation which is worse in pastoral system, which may result in residual antibiotic residues and antibiotic resistance in animal based-food such as milk, meat, eggs and fish. In Kenya, however, there is no formal surveillance system for drug resistance among livestock and human bacterial isolates. Therefore, there is limited data on prevalence of multi-drug resistant zoonotic diseases due to consumption of animal based foods. The aim of this study was to determine presence of Mycobacterium tuberculosis complex (MTBC) species and multi-antibiotic resistant MTBC species in camel milk in Isiolo County, Kenya. Raw camel milk was aseptically collected from 308 positive, negative and inconclusive bovine reactor*

, and positive avian reactor camels randomly selected from 15 camel herds in 3 major camel producing milk clusters in Isiolo County, Kenya. DNA was extracted directly from raw camel milk samples. A first single step Polymerase Chain Reaction (PCR) using primer set MTB-F 5'-CGGGTATGCTGTTAGGCGACG-3' and MTB-R 5'CCACCACAAGACATGCATG-3' was done to determine presence of MTBC species in camel milk samples. A second single step PCR using primer set RF1 5'-GGTCGCCGCGATCAAGGAGT-3' and RF2 5'TGCACGTCGCGGACCTCCA-3', targeting the rpoB gene, a marker of multi-drug resistant TB, was done to determine presence of multi-drug resistant MTBC species. The prevalence of MTBC species and multi-drug resistant MTBC species in raw camel milk was 3.1% and 1.55%, respectively. The presence of MTBC species and multi-drug resistant MTBC species in raw camel milk should be major public health concern to veterinary and human health medical services; especially among pastoralists who are traditionally accustomed to consuming raw camel milk.

Keywords---- Antibiotic resistance, Mycobacterium tuberculosis complex, camel, multi-drug resistance.

1. INTRODUCTION

The major concern globally has been the rising prevalence of foodborne diseases and antimicrobial resistance (AMR) of foodborne microorganisms (1), with serious implication on public health and social-economical aspects. It has been reported that Kenya is experiencing worsening high levels of antibiotic resistance (2). In Kenya, there is no formal system for surveillance of drug resistance in livestock bacterial isolates. In Kenya, like other African countries, the department of Veterinary Services monitors antibiotic residue in livestock products, these residues usually exceeded the WHO maximum residue levels in many cases. Antimicrobial resistance increases mortality, morbidity and health expenditures in human and animals. In 2016 a high level meeting of United Nations General Assembly with the participation of member states, non-governmental organizations, civil society, the private sector and academic institutions committed all stakeholders to take a broad, coordinated approach to address the root causes of AMR across multiple sectors, especially human health, animal health, environmental health and agriculture (3). Therefore, due to the

public health threat posed by antimicrobial resistance in animals and human, there is need for an integrated effective antimicrobial resistance surveillance programmes, especially in developing countries (4). In pastoral livestock production system, environmental issues are a major concern due to contamination of soil and water resources with both antibiotic residues and antibiotic resistant bacteria (5).

The unauthorized use of antibiotics for therapeutic purposes in livestock is causing serious problems associated with residual antibiotic residues in animal-based food such as milk, meat, eggs and fish (6, 7, 8). In pastoral areas of Sub-Saharan Africa, self-medication and misuse of antibiotics by pastoralists has been reported (8, 9, 10). The self-medication and misuse, either under- or over-dosing of livestock, may contribute to the selection of resistant pathogens that pose a danger to human and animal health. There is growing evidence that antibiotic use in livestock production systems be linked to antibiotic resistant human infections. This evidence has been supported by genomic sequencing of isolates from livestock and human (11).

Tuberculosis (TB), a worldwide zoonosis caused by *Mycobacterium tuberculosis* complex (MTBC) species in both human and livestock, has significant impact on the public health and socio-economical aspects of communities in developing countries. Kenya is ranked fifteenth among the twenty two high burden TB countries in the world (12). Bovine tuberculosis (BTB) transmission to human occurs mainly due to contact with infected animals, infected animal's materials and carcasses or through the consumption of contaminated animal-based foods. Therefore, pastoralists who are in most instances in closely in contact with livestock are at higher risk of contracting BTB infection. Despite this interaction, tuberculosis cases in human and animals are commonly treated as separate problems (13, 14). However, tuberculosis has serious consequences for both human and a broad range of animals, thus strongly advocated as a One Health issue (15, 16, 17). Currently, there is limited understanding of the relative importance of human, livestock and the environment in the emergence of antibiotic resistant genes (5).

The majority of reported studies on antimicrobial resistance have been based on human hospital cases rather than animal cases or animal-human interaction cases (18). Since MTBC species are intracellular pathogens, the treatment of tuberculosis must be of long duration and with agents that efficiently penetrate macrophages (19). In Kenya, several researchers have reported few cases of multi-drug resistant tuberculosis (MDR-TB) in patients at different hospitals (20, 21). This has been attributed to low level of detection, as the diagnostic methods are still based on sputum slide smear microscopy (22, 23). However, very few researchers have used molecular techniques in determining the antibiotic resistance among MBTC (21). Several studies from other East African countries have shown varied results of 3.9%, 6.2% and 3.9% in Rwanda (24); 2.7%, 9.9% and 2.7% in Northern Tanzania (25) and 1.6%, 3.2% and 4.8% in Uganda (26) of MDR, and isoniazid and rifampicin resistance, respectively.

In the pastoral system, human and livestock live in close proximity, therefore pathogen biodiversity among human and livestock is high and environmental conditions favour growth and survival pathogens. However, to date there are limited data on prevalence of MDR due to consumption of animal based foods, yet evidence indicate presence of zoonotic TB among human and livestock. Due to self-medication and misuse of veterinary drugs, and lack of information about antibiotics resistance in pastoral livestock system, this study was undertaken to detect the presence of antibiotic resistance among MTBC species in the camel milk produced by pastoralists in Isiolo County, Kenya.

2. MATERIALS AND METHODS

2.1 Sample Collection and Preparation

Raw camel milk was aseptically collected from 308 positive, negative and inconclusive bovine reactor, and positive avian reactor camels randomly selected from 15 camel herds along Mlango-Ngarendare-Burat, Kambi Garba-Ngaremara-Chumvi-Gambela and Boji-Kulamawe-Baranbate camel milk clusters in Isiolo County, Kenya (Figure 1). The camels were first screened for bovine tuberculosis with tuberculin skin test (results presented in a different Manuscript). The harvested milk samples were immediately kept in ice box containing ice packs and transported to the Isiolo County Veterinary Office laboratory where they were deep frozen at -20°C until transported to the Molecular Laboratory, University of Nairobi, where they were kept in a deep freezer maintained at -50°C until DNA extraction was done.

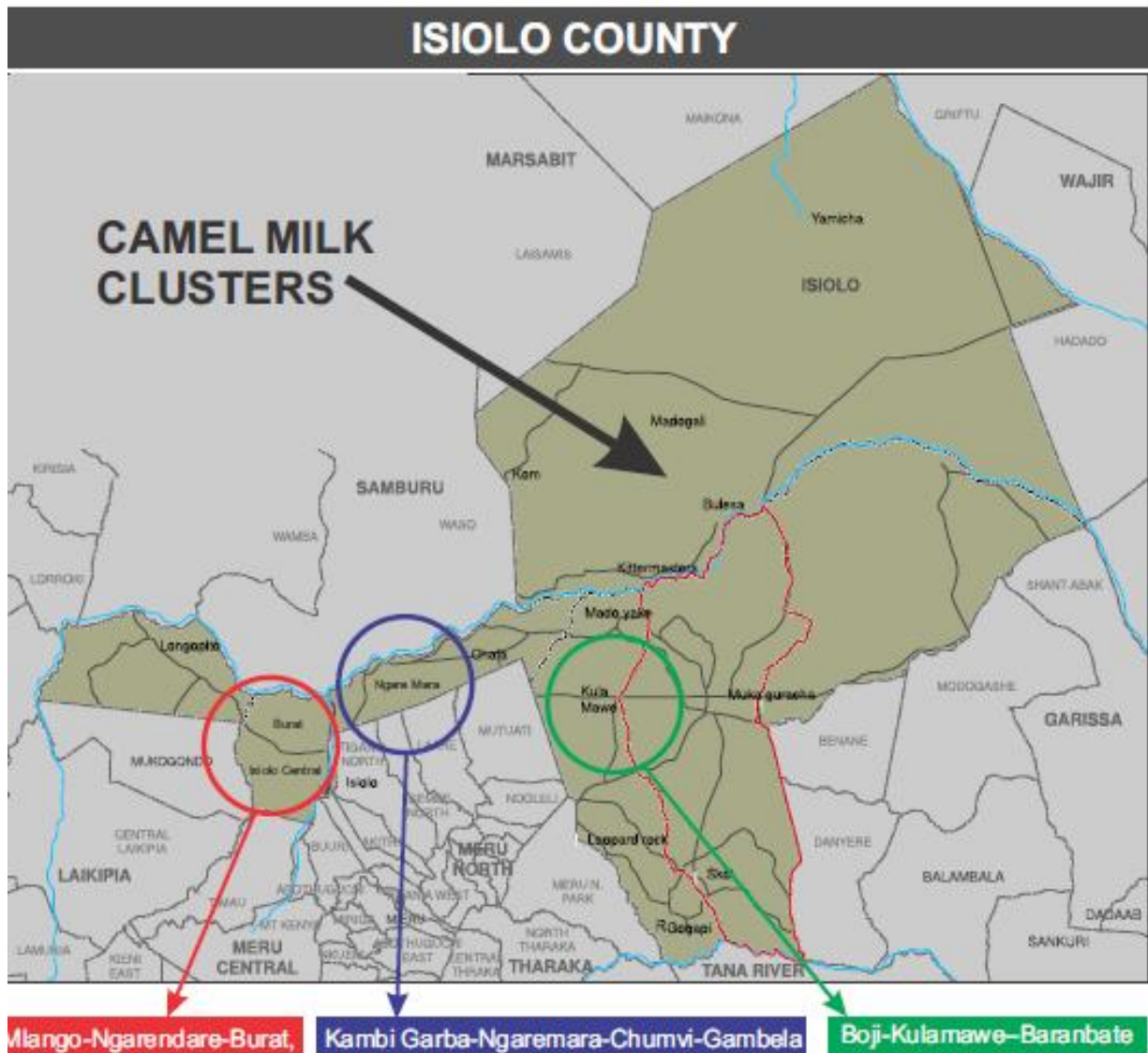


Figure 1. Map of Isiolo County showing camel milk clusters and study sites

2.2 DNA Extraction

DNA was extracted directly from raw camel milk samples according to the method of Leal-Klevezas et al. (28), as described by Ilhan et al., (29). Raw camel milk samples were thawed overnight at room temperature. Ten (10) millilitres of thawed raw camel milk samples were centrifuged at 13,000rpm for 15 minutes (Micro STAR 17, VWR, Germany). The top fat layer was removed using a sterile cotton swab and the supernatant was discarded. The pellet were re-suspended in 400 ml of lysis solution containing 100 mM Tris-HCl (pH 8), 100 mM Sodium chloride, 1% Sodium Dodecyl Sulfate (SDS) and 2% Triton-x 100 and 10 µl of proteinase K (10 mg/ml) (EMD Millipore Corporation, Merck, Germany) and incubated at 50°C for 30 minutes. Four hundred (400) µl of saturated phenol (liquid phenol containing 0.1% 8-hydroxyquinoline, saturated and stabilized with 10 mM Tris-HCl (pH 8) and 0.2 % of 2-mercaptoethanol) was added, mixed thoroughly and centrifuged at 8,000 x g for 5 minutes. The aqueous layer was transferred to a fresh tube and an equal volume of a mixture of chloroform-Iso amyl alcohol (24:1) added, mixed thoroughly and centrifuged at 8,000 x g for 5 minutes. The upper layer was transferred to a fresh tube and an equal volume of 7.5 M Ammonium acetate added and mixed thoroughly. The content was kept on ice for 10 minutes, centrifuged at 8,000 x g for 5 minutes and aqueous layer transferred to a fresh tube. Two (2) volumes of 95% ethanol were added to the aqueous layer, mixed thoroughly and the tubes stored at -20°C for 12 hours. The aqueous solution was centrifuged at 8,000 x g for 5 minutes to recovered DNA. The DNA pellets were rinsed with 1 ml of 70% ethanol, dried and re-suspended in 30 µl TE buffer. The extracted DNA was stored at -20°C until amplified.

2.3 Determination of presence of Genomic DNA

The presence genomic DNA in the unamplified DNA extract were analyzed in an electrophoresis system (MSCHOICETRIO, United Kingdom) in a 1.5% agarose gel in 1xTBE pH 8.3 (89 mM Tris-HCL, 89 mM borate, 2 mM EDTA), staining with ethidium bromide and visualized in UV Transilluminator Imaging System (BIODOC-IT Imaging System, Analytik Jena company, USA). The camel milk samples that showed presence of genomic DNA were subjected to single step polymerase chain reaction (PCR) to determine presence of MTBC. An extended 100bp DNA molecular marker was used.

2.4 Amplification of DNA

A first single step PCR was done to determine presence of MTBC species in camel milk samples. The primer set used were MTB-F 5'-CGGGTATGCTGTTAGGCGACG-3' and MTB-R 5'-CCACCACAAGACATGCATG-3' coding for the last five codons of *murA* gene, the promoter region of the *rRNA* operon and the 5' end of the 16S rRNA gene, respectively. The primers were obtained Inqaba Biotec, South Africa and were reconstituted according to manufacturer's instructions. The target for primer MTB-F was the sequence complementary to the last 15 nucleotides of the *murA* gene and the first 6 nucleotides of the promoter region of the *rRNA* operon (30). The target for primer MTB-R was position 192–210 of the V2 region located within the 16S rRNA gene (31).

The amplification was done in a thermocycler (T100 thermocycler, BIO-RAD, Singapore). The reaction mixture of 50 µl consisted of: - 1.7 mM Magnesium chloride, 200 µl dNTPs, 10% Dimethyl sulfoxide (DMSO), 0.125 µl Taq DNA polymerase (Peqlab, Germany) and 0.4 µl of each primer. The amplification consisted of: - 36 cycles at 94°C for 1 minute, 58°C for 1 minute 72°C for 2 minutes and a final extension step at 72°C for 5 minutes. The amplification products were analyzed in an electrophoresis system (MSCHOICETRIO, United Kingdom) containing 1.5% agarose gel in 1xTBE pH 8.3 (89 mM Tris-HCL, 89 mM borate, 2 mM EDTA and stained with ethidium bromide dye and visualized in a UV Transilluminator Imaging System (BIODOC-IT Imaging System, Analytik Jena company, USA). An extended 100bp DNA molecular marker was used.

The DNA extract of camel milk samples that showed presence of MTBC were amplified again in a thermocycler (T100 thermocycler, BIO-RAD, Singapore) together with 2 control samples that were negative for MTBC species. The reaction mixture of 50 µl consisted of: - 1.7 mM Magnesium chloride, 200 µl dNTPs, 10% Dimethyl sulfoxide (DMSO), 0.125 µl Taq DNA polymerase (Peqlab, Germany) and 0.4 µl of each primer. The amplification consisted of: - 36 cycles at 94°C for 1 minute, 58°C for 1 minute 72°C for 2 minutes and a final extension step at 72°C for 5 minutes. The amplification products were analyzed in an electrophoresis system (MSCHOICETRIO, United Kingdom) containing 1.5% agarose gel in 1xTBE pH 8.3 (89 mM Tris-HCL, 89 mM borate, 2 mM EDTA and stained with ethidium bromide dye and visualized in a UV Transilluminator Imaging System (BIODOC-IT Imaging System, Analytik Jena company, USA). An extended 100bp DNA molecular marker was used. The camel milk samples that showed presence of MTBC species were subjected to second single step PCR to determine antibiotic resistant MTBC species. An extended 100bp DNA molecular marker was used.

A second single step PCR was done to determine presence of multi-drug resistant MTBC species. Rifampin (RIF) resistance was used as marker of multi-drug resistant tuberculosis (MDR-TB) (32, 33, 34). The 157 bp fragment of the *rpoB* gene, a marker of multi-drug resistant TB, was amplified using PCR technique as described by Hermans et al. (35) and modified by Yam et al (36). The primer sets used were: - RF1 5'-GGTCGCCGCGATCAAGGAGT-3' and RF2 5'-TGCACGTCGCGGACCTCCA-3'; and 245 bp fragment of the insertion element sequence IS6110, as TB control. The primer INS1 5'- CGTGAGGGCATCGAGGTGGC-3' targeting sequence position 631-650 (5'-3') of the 16S rRNA coding region and INS2 5'-GCGTAGGCGTCGGTGACAAA-3' target sequence position 856 – 875 of the 16S rRNA coding region as described by Hermans et al. (35) were used. The reverse primers were biotin labelled.

The amplification was done in a thermocycler (T100 thermocycler, BIO-RAD, Singapore). The reaction mixture of 50 µL consisted: - 10 mM Tris-HCl (pH 8.3) at 25°C, 50 mM sodium chloride, 1.5 mM Magnesium chloride, 10% Dimethyl sulfoxide (DMSO), 0.01% (wt/vol) gelatin, 0.2 mM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 0.4 µl (each) primers INS1 and INS2 (i.e. 132ng/50µl), and 1 unit of Taq polymerase. The amplification was carried out as follows: 3 minutes at 95°C, 40 cycles of 1 minutes at 95°C, 1 minute at 60°C, 1.5 min at 72°C, and 4 min at 72°C. The PCR products were analyzed by electrophoresis in a 1.5% agarose gel. An extended 100bp DNA molecular marker was used.

3. RESULTS

Out of 308 raw camel milk samples examined, 129 camel milk samples showed presence of genomic DNA. Four (4) raw camel milk samples (sample number IG 137, IA 239, SI 94FL and AA 48FL) out of the 129 raw camel milk samples showed presence of MTBC species (Figure 1), indicating a prevalence of 3.1% (4/129) of MTBC species in raw camel milk.

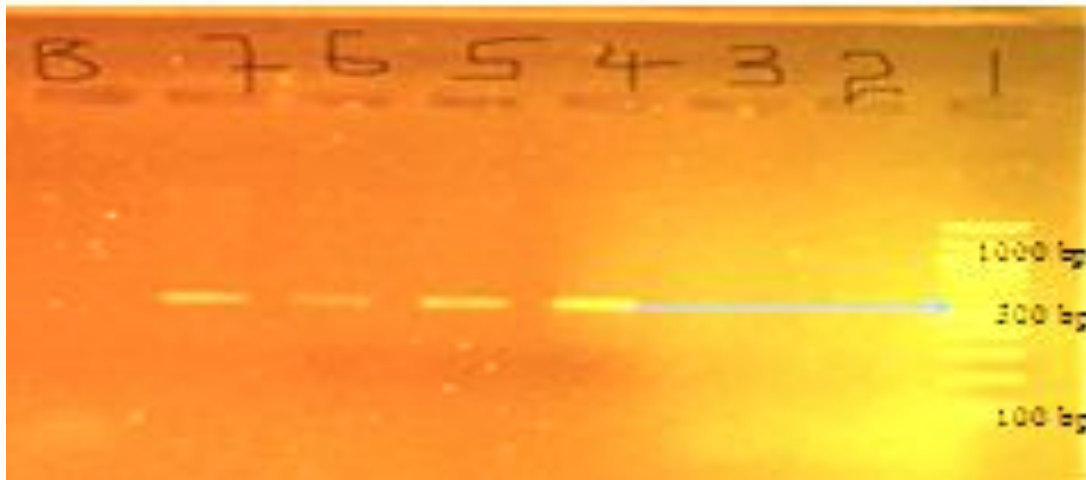


Figure 1. Electrophoresis products in 1% agarose during first stage single step PCR showing samples with presence of *Mycobacterium tuberculosis* complex (MTBC) species.

Two (2) raw camel milk samples (sample number IM75 and IM78) showed presence of multi-drug resistant MTBC species (Figure 2),

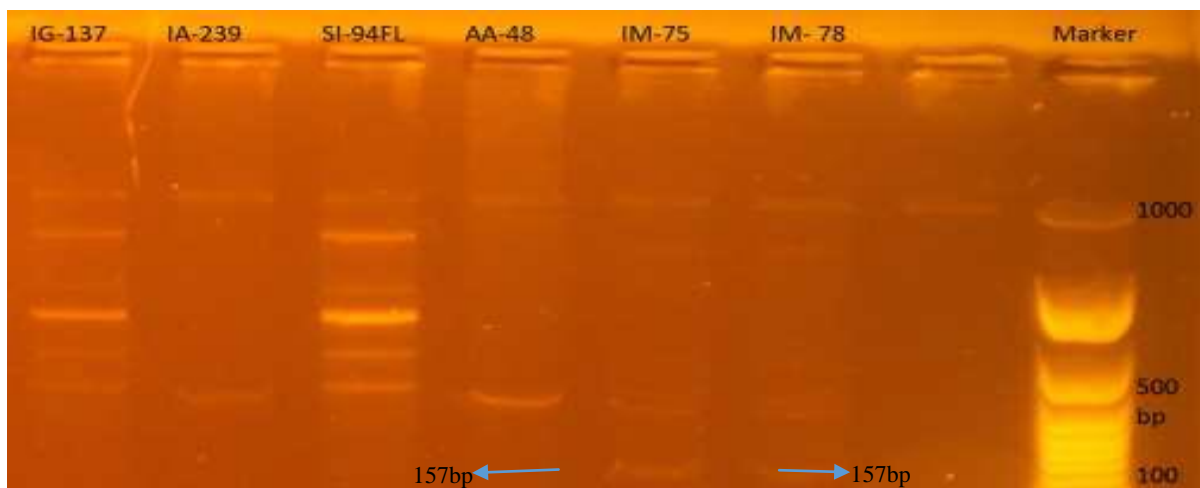


Figure 2. Electrophoresis products in 1% agarose during second stage single step PCR showing samples with presence of multi-drug resistant *Mycobacterium tuberculosis* complex (MTBC) species.

4. DISCUSSION

This study demonstrated prevalence of 3.8% and 3.1% of *Mycobacterium* genus and MTBC species, respectively in raw camel milk in Isiolo County, Kenya. This is a serious public health risk to consumers of raw camel milk, a practice common among pastoral communities in the arid and semi-arid lands (ASALs). The transmission of these MTBC species and other *Mycobacterium* species could be due to close contact of camel and other livestock during grazing, watering and in night shades “Bomas”. Camel milk production system in the study sites are characterized by dusty and unhygienic milk harvesting environment especially using non-food grade plastic containers and poor quality water. This results are in agreement with prevalence of 3.7% of our earlier study of bovine tuberculosis due to MTBC species in live camels screened using the Single Comparative Intradermal Tuberculin Skin (SCITS) test (Manuscript submitted).

The prevalence of 1.55% of multi-drug resistant MTBC species in this study could be attributed to untheorized use of antibiotics (misuse) and self-medication of camels by pastoralists/herders, a practice common in ASALs of Kenya. Most the therapeutic veterinary drugs label on the Kenyan market do not show the dosage for camels, therefore Veterinary Officers or pastoralists/herders usually extrapolate the camel dosage, a practice that may lead to either over- or under-dosing of camels (10). Antibiotic resistant strains of *Mycobacterium tuberculosis* can be selected by the over- or under-dosing or misuse of antimicrobial drugs. The misuse and over- and under-dosing can lead to acquired resistance

mediated through chromosomal mutation or and horizontal gene transfer arising due to selective pressure of antibiotic misuse. The *rpoB* used as a marker for MDR-TB, is a β -subunit of RNA polymerase and its mode of action is inhibition of transcription and its target is the drug. Exposure of bacterial isolates to sub-lethal or over-dose levels of bactericidal antibiotics may promote cellular mutagenesis resulting to increased mutations in drug resistance genes (37). Therefore the pastoralist's camel health management practices can favour development of antibiotic resistance among *Mycobacterium tuberculosis* species, which can be excreted in the camel milk.

The close contact of camel and other livestock during grazing, watering and in night shades “Bomas”, can significantly contribute to transmission of resistant *M. tuberculosis* and other Mycobacterial strains among the livestock. The practice of consumption of raw camel milk and harvesting, handling and transportation of camel milk under dusty and unhygienic conditions, in non-food grade and difficulty to clean plastic containers and at high temperatures can also contribute to development and transmission of antibiotic resistance, hence public health risk of pastoralists. Several authors have reported multi-drug resistant isolates from animal-based foods: - cow milk (38), animal environments (40), Japanese quails (43), pigs, chicken, eggs and cows (27), and chicken and swine (44). This was attributed to self-treatment of infected animals, availability of cheaper generic therapeutic drugs over the counter and without prescription, intensive housing conditions, poor hygienic conditions during animal product harvesting, handled and transportation, and handling animal-based foods in inappropriate containers and temperature abuse conditions. These bacterial isolates can be transmitted to human through consumption of contaminated animal-based foods or direct and indirect contact with the carrier animals, thus a high public health risk to the consumers. The close proximity of animals during grazing, watering and in night housing shades “bomas” provides high potential for the spread of multi-drug resistant MTBC species.

5. CONCLUSION AND RECOMMENDATIONS

This study demonstrates presence of MTBC species and multi-drug resistant MTBC species in raw camel milk, which can cause multi-drug resistant bovine tuberculosis among pastoralists who are traditionally accustomed to consuming raw camel milk. There is need for an interdisciplinary approach that that will advocate for reduce camel contribution to the global burden of antibiotic and multi-drug resistance. Therefore, from these study, any sustainable approach should be based on a sound understanding of the relative roles of human, animals and the environment in the emergence, spread and persistence of multi- drug resistance, hence a major concern to the stakeholders in veterinary and human medical services

6. ACKNOWLEDGEMENT

The authors sincerely acknowledge the Federal Ministry of Education and Research (BMBF) of Germany through the Reduction of Losses and Adding Value (RELOAD) Project, Kassel University for purchasing Polymerase Chain Reaction (PCR) equipment used in this study.

We highly acknowledge the technical assistance from Mr. Alfred Mainga of Department of Public Health, Pharmacology and Toxicology, University of Nairobi; Dr. Ruth Omani of Department of Veterinary Pathology, Parasitology and Microbiology, University of Nairobi; Miss Linnet Mwangi of Department of Dairy and Food Technology; Mr. Nicanor Odongo of Department of Food Science, Nutrition and Technology, University of Nairobi and Mr. Patrick Muthui of Isiolo County Veterinary Office. Our gratitude goes to Dr. Joseph Nduati Githinji, County Veterinary Officer, Isiolo County Veterinary Office for technical advice and allowing us to work in the county. We thank Mr. Abdi Ali of Kenya Camel Association and Isiolo County National government administration for their assistance with field logistics. We also wish to thank all herders and pastoralists for their willingness to participate in the study.

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