Development of a molecular tool for the differentiation of *Mycobacterium bovis* and molecular characterization of *Mycobacterium bovis* isolates in Malawi

(ウシ型結核菌遺伝子診断法の開発とマラウイにおけるウシ型結核菌分離株の遺伝学的解析)

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Table of Contents

List of figures

List of tables

Abbreviations

BCG	Bacillus Calmette–Guérin
BTB	Bovine tuberculosis
CFI	Colorimetric fluorescence indicator
CFP-10	Culture filtrate protein 10
CRISPR	Clustered regularly interspaced short palindromic repeat
DDW	Double distilled water
DNA	Deoxyribonucleic acid
DR	Direct repeat
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	Early secreted antigenic target 6
FPA	Fluorescence polarization assays
HDGI	Hunter-Gaston discriminatory index
IFN-γ	Interferon-gamma
IGRA	Interferon-gamma release assay
LAMP	Loop-mediated isothermal amplification
LJ	Löwenstein-Jensen
MEGA	Molecular evolutionary genetic analysis
MGIT	Mycobacterial growth indicator tube
MIRU	Mycobacterial interspersed repetitive units
MST	Minimum spanning tree
MTBC	*Mycobacterium tuberculosis* complex
NAAT	Nucleic acid amplification test
NTM	Non-tuberculous mycobacterium
NTRL	National tuberculosis reference laboratory
OIE	Office International des Epizooties
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PPD	Purified protein derivatives
PZA	Pyrazinamide
RD	Region of difference
REA	Restriction enzyme analysis
SICCT	Single intradermal comparative cervical tuberculin
SLV	Single locus variation
TB	Tuberculosis
UPGMA	Unweighted pair group method with arithmetic mean

UTH	University teaching hospital
VNTR	Variable number of tandem repeats
WHO	World Health Organisation

Notes

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Preface

Mycobacterium bovis is a species belonging to the *Mycobacterium tuberculosis* complex (MTBC), a group of bacteria that cause tuberculosis (TB) in a range of mammals, including humans [1]. Other MTBC species include *M. tuberculosis, M. africanum, M. caprae, M. microti,* and *M. pennipedii* [1]. The MTBC belongs to the genus *Mycobacterium*, order *Actinomycetales*, and family *Mycobacteriaceae* [2]. The genus *Mycobacteria* is divided into two groups: fast-growing and slow-growing. Phenotypically, the genus *Mycobacterium* is characterized as facultative intracellular, non-sporulated, non-motile, and non-capsular, measuring about 0.2-0.3 μm in width and 1.0-4.0 μm in length [3]. The cell wall comprises complex lipids, mycolic acid, and a thick waxy layer (Fig. 1, adapted from Gary Kaiser [4]). These provide special abilities to resist acids, hydrophobicity, physical injuries, antibiotics and contribute to the slow-growing characteristics. The mycolic acids are responsible for the acid-fast characteristics exhibited during staining procedures [4,5].

Comparative genomic studies have established that the MTBC shares more than 99.95% similarities at the nucleotide level [6–8]. These studies have shown that MTBC species underwent an evolutionary bottleneck from a common ancestor through deletion and insertion phenomenon estimated to have occurred between 15,000 and 35,000 years ago (Fig. 2, adapted from Rodriguez-Campos et al.,[8]). Despite the homogeneity shared within the MTBC, the species differ widely in host-related tropisms, phenotypes, and pathogenicity [6,9]. Notably, *M. tuberculosis* is the principal pathogen of humans, while *M. bovis* is the cattle's primary pathogen. Besides, *M. bovis* potentially infects a spectrum of other mammalian hosts, including domestic animals, wildlife, and humans [10].

The cultural, biochemical, and metabolic characteristics of *M. bovis* play an integral part in identifying the pathogen [11]. *M. bovis* exhibit dysgonic colony shaped characteristics on Löwenstein-Jensen (LJ) medium. Furthermore, *M. bovis* grows well in pyruvate supplemented medium, while *M. tuberculosis* does not require pyruvate supplementation. *M. bovis* shows negativity while *M. tuberculosis* is positive for niacin accumulation and nitrate reduction. On the Lebek medium, *M. bovis* displays microaerophilic growth and pyrazinamide (PZA) resistance characteristics. In contrast, *M. tuberculosis* shows aerophilic growth on Lebek medium and susceptible to PZA [12].

In cattle, bovine TB (bTB) is characterized by a chronic debilitating state with granulomatous nodular lesions. Generally, bTB affects the respiratory system (pulmonary

form). However, extra-pulmonary cases are frequently reported in generalized conditions [13]. In extra-pulmonary cases, the main symptoms include intermittent diarrhoea or constipation and weight loss [14,15]. These unspecific clinical signs challenge the discriminatory of bTB from other diseases that exhibit similar clinical signs. During the animal post-mortem examination, tuberculous granuloma and enlarged lymph nodes mainly from the head, thorax, visceral organs, and peritoneum are observed.

In humans, *M. bovis* causes the disease known as zoonotic TB. It is typically extra-pulmonary; however, pulmonary cases have been reported [15,16]. Zoonotic TB exhibits similar clinical signs as those caused by *M. tuberculosis* TB, potentially leading to misdiagnoses [5,17]. The manifestations of zoonotic TB are different in young children; abdominal infections are common signs. However, in older patients, the common signs are swollen and ulcerated lymph glands in the neck region. Usually, *M. bovis* infection in humans has a prolonged course with symptoms appearing after months or years. In some cases, *M. bovis* remains dormant without inducing the disease, and humans may be reservoirs.

The transmission of *M. bovis* can occur between animals (cattle to cattle, cattle to other domesticated animals, or cattle to wildlife or vice versa), from animals to humans, and vice versa, but seldom between humans [18,19]. Aerosol transmission is the most common among animals; however, transmission through other routes such as cutaneous, congenital, and genital have also been reported [15]. Intensive animal production increases the rate of transmission of bTB between animals through close contact and contamination of feed [13,20]. Long survivability of *M. bovis* in the environment is a huge risk of infection [13]. *M. bovis* survives for extended periods in favourable conditions. For instance, up to 11 months in cold, dark, and moist conditions (survivability rate is high in frozen tissues) and up to 2 years in artificially stored samples [21,22]. Suckling calves can get the infection through infected milk. There have been reports of bTB transmission through semen via artificial insemination [21]. The primary route through which humans get the infection is by consuming unpasteurized infected milk and ingestion of undercooked milk and meat products [15]. Additionally, humans get infected through contact with contaminated materials. Farmworkers, zookeepers, milkers, animal dealers, veterinarians, abattoir workers, meat inspectors, autopsy personnel, and laboratory personnel are mostly risk occupations [23].

Cattle vaccination has been proposed for the control of bTB, especially in industrialized countries such as the UK [24,25]. In 1921, Calmette and Guerin developed

the vaccine, Bacillus Calmette–Guérin (BCG) through serial passaging an *M. bovis* strain on glycerol-soaked potato slices [37]. The vaccine has been in use against TB in humans for over decades now. However, the challenge related to BCG use in cattle is its sensitization to single intradermal comparative cervical tuberculin (SICCT), making the differentiation of infected and vaccinated animals impossible [59]. Early studies have shown that BCG could protect against TB in cattle, although the protection wanes off after 1-2 years and vaccination may result in positive reactivity in the tuberculin test, suggesting confusion [26–29]. The findings of these studies concluded that "test and slaughter" control programs are better eradication strategies than relying on BCG vaccination. However, it was suggested that BCG vaccination could play a role in disease control in resource-limited areas where "test and slaughter" programs cannot be implemented for economic or social reasons. Currently, studies are underway in developing alternative vaccines that can be used for protecting cattle against *M. bovis* [30–32].

In cattle, the diagnosis of bTB is achieved by single intradermal comparative cervical tuberculin (SICCT). However, in absentia of SICCT, other methods such as history, clinical findings, and necropsy are used. The SICCT is based on the delayed-type hypersensitivity reaction elicited by bovine and avian purified protein derivatives (PPD) [33,34]. Elsewhere, suboptimal specificity has been reported linked to infections or exposure with other environmental non-tuberculous mycobacteria (NTM) such as *M. avium, M. interacellularae, M. scrofulaceum, M. paratuberculosis, M. kansasii,* and *M. fortuitum,* questioning the efficiency of SICCT [33,35,36]. Culture is also used for *M. bovis* identification. *M. bovis* generally grows within 3 – 6 weeks of incubation, depending on the media used. In liquid culture, growth is faster as compared to solid culture. LJ medium supplemented with pyruvate is the commonly used solid media. The media is examined for macroscopic growth at intervals during the incubation period. Following dysgonic colony shapes characteristic, smears are prepared and stained by the Ziehl Neelsen technique [12,13]. Liquid culture systems such as mycobacterium growth indicator tubes (MGIT) are used routinely in referral hospitals or veterinary laboratories; growth is checked automatically [37]. However, culture and microscopic characteristics do not definitively identify *M. bovis* because of the confusion due to the exhibition of similar characteristics by other MTBC species. As such other follow-up tests are required to make a conclusive diagnosis.

Several immunological and serological diagnostic tests are used as ancillary tests to SICCT in the diagnosis of bTB. The interferon-gamma release assay (IGRA) and the

lymphocyte proliferation assay measure cellular immunity, while the enzyme-linked immunosorbent assay (ELISA) measures humoral immunity [38]. The IGRA tests are based on the release of interferon-gamma (IFN-γ) from stimulated lymphocytes with specific antigens, e.g., early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) [39]. IGRAs have been used in many developed countries to speed up the eradication of infected animals in bTB outbreaks [35,38]. Other ancillary bTB diagnostic methods such as the antibody ELISA tests, lymphocyte proliferation assays, and fluorescence polarization assays (FPA) have been developed [40,41]. However, their application, mainly in developing countries, is still a topic of debate because they require standard laboratories and expertise.

Several polymerase chain reaction (PCR) based molecular techniques have been developed for differentiating *M. bovis* from the MBTC. PCR's principle hinges on the use of DNA polymerase which facilitates the replication of DNA sequences using specific target primers. For the detection of *M. bovis*, several PCR target loci or nucleotide polymorphisms have been reported. For example, multiplex PCR techniques targeting the region of difference 4 (RD4) differentiates *M. bovis* from *M. tuberculosis* [42]. Others include multiplex PCR targeting the genetic regions *cfp32*, RD9, and RD12 loci [43], *hup* B gene [44], and nucleotide polymorphism in the *pnc* A gene [45] have been suggested for MTBC species identification. Unfortunately, besides being able to differentiate MTBC species, these methods are not easily adapted for use in developing countries because they require the use of expensive equipment, complicated procedures and need for technical know-how.

Genotyping of MTBC strains has been achieved by several methods. The restriction enzyme analysis (REA) of mycobacterial DNA involves digestion of the target DNA using various restriction enzymes and separation, and visualization of the resulting fragments by gel electrophoresis. The obtained pattern of DNA fragments (genetic fingerprint) is characteristic for each strain. However, the challenges of this method include low sensitivity as a result of similar fragments from various strains. Furthermore, when more restriction enzymes are used, the high number of DNA fragments makes a reliable analysis impossible [46].

Pulsed-field gel electrophoresis (PFGE), is another molecular typing method that enables discrimination of large DNA fragments. PFGE is based on the use of an electric field that switches its orientation across a gel matrix achieved by varying electrical pulse and shifting the direction of the current frequently duration. PFGE involves the digestion

of bacterial DNA with restriction enzymes, followed by agarose gel electrophoresis and before analysis of the bands [46]. An important step is the preparation of mycobacterial genomic DNA that involves embedding the mycobacterial suspension in agarose plugs, lysing of the cells, and digestion of DNA with restriction enzymes. After the electrophoresis, the resulting banding patterns are compared, using a predefined set of criteria for strain relatedness [52]. The PFGE typing is seldom used in MTBC due to requirement of technical expertise, time-consuming, cost and low discriminatory power between the strains [47–49].

Insertion sequence 6110 (IS6110) based typing involves the digestion of MTBC genomic DNA with a restriction enzyme that cuts the IS6110 sequence into two fragments. These DNA fragments are separated through gel electrophoresis, then transferred onto a membrane, and hybridized [50]. Advantageously, IS6110 typing profiles are stable over time, allowing distinguishing epidemiologically related from unrelated isolates [51]. IS6110 typing has several disadvantages for instance the need for large amounts of high-quality DNA. Furthermore, requires technical know-how, sophisticated and expensive computer software. Additionally, the low discriminatory power of IS6110-RFLP typing is observed in strains with a low copy number of IS6110 [52–55]. In other mycobacteria such as NTMs have multiple IS6110 homologs and hence may hybridize with the IS6110 probe leading to false results [56].

Spacer oligonucleotide typing (Spoligotyping), a PCR-based molecular typing method, distinguishes the presence or absence of spacer sequences interspersed within the repeat sequences in the direct repeat (DR) locus of MTBC (Fig. 4 adapted from Comas et al.,[57]). The DR locus is part of the clustered regularly interspaced short palindromic repeats (CRISPRs) family that plays a significant role in replication partitioning [58]. The entire DR locus is amplified by PCR, using a set of two primers. The spoligotype membrane with 43 covalently bound synthetic oligonucleotides is hybridized by the different PCR products [59]. Individual strains are differentiated by the number of the spacers that are missing from the complete 43-spacer set [62]. For instance, *M. bovis* lacks spacers 3, 16, and 39 to 43. The lack of spacers results from various genetic deletions mechanisms, such as homologous recombination or transposition since IS6110 integration occur frequently in the DR region [60]. The incorporation of PCR procedures in spoligotyping makes it a relatively simple, cost-effective, and high-throughput method, whose results are accurate and reproducible. The reliability of the results is linked to the high stability of the DR locus. Additionally, spoligotyping can use small amounts of DNA e.g. 10 fg equivalent to two *M. bovis* genomic copies [61]. Spoligotyping has been used

for genotyping on isolates from various preparation such as paraffin-embedded tissues [62]. Nevertheless, spoligotyping discriminatory power is low, largely because it targets a single locus (the DR locus) that covers less than 0.1% of the MTBC genome.

MIRU-VNTR typing determines multiple independent loci encompassing variable numbers of tandem repeats (VNTR) of genetic elements collectively called Mycobacterial interspersed repetitive units (MIRU) (Fig. 4 adapted from Comas et al.,[57]) [63]. The principle of MIRU-VNTR typing is based on the amplification of each VNTR locus with specific PCR primers complementary to the flanking regions, and the resulting PCR products are visualized by standard gel electrophoresis. The results are expressed as a numerical code with each digit representing the copies number at a particular locus [64]. A 12 tandem repeat loci is the most common version of MIRU-VNTR. However, 15 and 24 MIRU-VNTR loci were standardized to increase the discriminatory power [63]. The discriminatory power of MIRU-VNTR analysis is proportional to the number of loci evaluated. MIRU-VNTR analyses have been used to understand the phylogenetic relationships of clinical isolates, trace *M. bovis* transmission, and elucidate the circulating strains' population [65–72].

The burden of bTB is high in developing countries as compared to developed countries. Previous reports have shown that approximately 168 million poor livestock keepers in the world live in sub-Saharan Africa and are severely affected by the consequences of bTB [73]. In 2016, the World Health Organisation estimated that 147,000 cases and 12,500 deaths in humans were due to *M. bovis*. Notably, 49.5% of the cases were recorded in the African region [74]. In developing countries, zoonotic TB's high burden is exacerbated by the presence of multiple risk factors such as increased HIV/AIDs prevalence, living in the same microenvironment with infected animals, and unhygienic practices such as consumption of unpasteurized milk [75]. In cattle, bTB is an economically significant disease resulting in high production costs, low revenue collection due to the condemnation of diseased organs and carcasses at slaughter, national and/or international trade restrictions [76,77]. The spillover to wildlife has unpredictable predicament; bTB in wildlife poses significant challenges to the diagnosis, eradication strategies and constitutes a threat to endangered species [76,78]. Wildlife is thus regarded as maintenance hosts of bTB because the disease circulates within them for a long time, and wildlife will seldom show clinical signs. The information about the importance of bTB in wildlife in the African region remains limited. However, few studies within the region have demonstrated that bTB in wildlife has multiple reservoirs. For instance, the lechwe antelopes (*Kobus leche Kafuensis*) have been described as reservoirs of bTB in

Zambia [66]. The Kafue lechwe (*Kobus leche*) and the buffalo (*Syncerus caffer*) have been documented as bTB reservoirs in Kruger National Park in South Africa [79]. Nevertheless, further studies are needed to understand the burden and importance of bTB in wildlife.

BTB is globally distributed; however, it has been eradicated mainly in industrialized countries except in a few countries where wildlife hinders the elimination efforts [80]. The disease is most prevalent in developing countries (Fig. 3 adapted from OIE [81]). There is a shortage of information about disease`s prevalence in developing countries, mainly due to insufficient surveillance systems, laboratory capacity, and veterinary expertise. This has contributed to the general underestimation of the disease prevalence in these regions [82]. Similarly, zoonotic TB`s distribution follows the same pattern. In a report by Muller and others [83], *M. bovis* caused 2.8% (range 0%–37.7%) of all TB cases in humans in Africa. However, there was a variation of bTB distribution; Tanzania had the highest proportion, 26.1% (range 10.8%–37.7%), while Malawi and Uganda respectively reported 3.3% (0.1–17.20%) and 0% (0.0 – 5.1%). However, these studies' significant limitation was the low sample size, resulting in a high statistical error, necessitating the significance of large-scale population-based studies. The zoonotic potential transmission of bTB to humans is of public health concern [84]. Therefore, there is a need to elucidate the burden of zoonotic in humans in Africa and other high burdened areas with studies covering large sample sizes.

The eradication of bovine TB in industrialized countries has been achieved mainly by the test and slaughter campaigns. During these campaigns, cattle are tested for bTB using other techniques such as tuberculin skin tests, and if positive, the animals are slaughtered. The government provides compensation to the farmers with regards to the number of animals removed. However, such methods may not be easily implemented in resource-limited areas due to financial constraints and social beliefs. In such a scenario, alternative bTB strategies are essential to control the disease. The OIE proposes creating disease-free zones by removing all the infected animals and repopulating disease-free animals. The implementation of such strategies may be challenging in extensive livestock production systems commonly practiced in many developing countries. In such cases, stringent cattle movement control measures seem appropriate.

In Malawi, like many developing countries, livestock plays a crucial role in the lives of people. More importantly, cattle are a source of dietary protein through meat and milk, a source of draught power for agricultural activities and transportation. Cattle also

serve as a repository of a family's wealth. The ownership of cattle is associated with high social status. Additionally, cattle play a significant role during ceremonies such as religious rites, marriages, where it can be paid for bride price, etc. Therefore, the interaction between people and cattle is unavoidable, providing leeway for easy transmission of diseases such as bTB.

It is worth mentioning that Malawi shares unmanned border segments with Zambia, Mozambique, and Tanzania. In these countries, bTB status has been documented as "enzootic." In Zambia, a study by Hang'ombe and others conducted in the Kafue basin, southern and central areas of the country reported that out of 315 specimens from cattle and 75 from lechwe antelopes, 37 and 15 *M. bovis* isolates were identified as *M. bovis* [66]. Another study conducted in Namwala district, Malama, and others identified *M. bovis* 25 isolates from 67 cattle samples [85]. Munyeme and others identified 31 out of the 98 suspected tissues as *M. bovis* [86]. A cross-sectional study from August 2003 to February 2004 in the Kafue basin reported the prevalence of bTB at 49.8% (95% CI: 37.9, 61.7%) using the SICCT [72].

In Mozambique, a prevalence study in cattle in Govuro District estimated bTB at 39.6% (95% CI 36.8–42.5) from a total of 1136 cattle using SICCT [87]. In Mozambique, the overall prevalence of bTB in cattle is estimated at 13.6% [68]. Previously information indicates that bTB is endemic in Tanzania's cattle, with regional prevalence ranging from 0.2% to 13.2% [88]. A high prevalence of 13.2% was reported in Tanzania's southern highlands, which shares borders with northern Malawi [89].

In Malawi, more than 85% of all the cattle are kept under the traditional farming characterized by communal grazing and this allows contacts of animals from different areas. People in rural areas share the same micro-environments with cattle, consume undercooked animal products, and seldom pasteurize milk. Granulomatous TB - like lesions are a common finding and primary reason for organ condemnation during meat inspection in slaughterhouses (Fig. 5). Unfortunately, in most cases, the heavily affected organs are simply trimmed, and the rest of the meat passed into the human food chain. There are no compensation programs to farmers whose animals are diagnosed with bTB, as such besides veterinary or public health officials declaring of the meat being unsafe for human consumption, in most cases, farmers do not comply; posing a serious public health threat. Malawi records one of the highest HIV prevalence in Sub-Saharan Africa region; in 2018, over 1 million people were living with HIV [90]. Besides these potential risk factors that could ease the transmission and circulation of *M. bovis* to and within Malawi,

the status of bTB remains unclear. A country-wide cross-sectional study performed in 1986 determined the prevalence of bTB at 4% from a random sample of 3481 cattle using SICCT [91]. Since then, there have been no studies to elucidate the prevalence of bTB at the national level [92]. The unavailability of active bTB surveillance systems has contributed to the failure to understand the disease's prevalence and underestimating its burden, resulting in less veterinary and public health attention.

Considering these challenges, I felt compelled to pursue a PhD in order to contribute to the body of knowledge the understanding of bTB in Malawi. The initial plan was to develop a simplified method for the rapid detection of *M. bovis* that can be easily used for bTB surveillance in Malawi. Furthermore, I planned to evaluate this tool on samples from cattle and humans indirectly elucidating the bTB apparent prevalence based on cattle slaughtered at major regional abattoirs in Malawi. Finally, I planned to genotype on recovered *M. bovis* isolates to provide substantial scientific evidence of bTB disease dynamics and encourage local stakeholders and policy formulators to strengthen control measures or implement new strategies.

Thus, this thesis is divided into three main chapters. Chapter one's purpose was to develop a simplified, rapid, and low-cost tool for the detection of *M. bovis*. In this chapter, a loop-mediated isothermal amplification assay (LAMP) for the specific detection of *M. bovis* was developed. LAMP method has been utilized to detect several pathogens because of its simplicity, rapidity, and use of inexpensive materials compared to PCR and other MTBC diagnostic methods [93–100]. The LAMP assay developed in this study targeted the region of difference 4 (RD4), a 12.7kb locus present in *M. tuberculosis* strains but deleted in all *M. bovis* strains.

In chapter two, utilizing the wet LAMP assay developed in the previous study, a dry LAMP version was developed by drying up and optimizing the reaction reagents. Dry LAMP is a promising tool for use in resource-limited areas because it does not require cold chain maintenance of reaction reagents. Furthermore, dry LAMP kits can be kept for more extended periods and easily transported to remote areas [100,101]. I prepared the dry LAMP kits at Hokkaido University, Japan, and transported them to Malawi for field evaluation. Samples were collected from cattle during the routine animal post-mortem examination at the regional abattoirs. Additionally, clinical samples from the National TB reference laboratory in Lilongwe were also subjected to dry LAMP assay.

In chapter three, I described the molecular typing of sixty-three *M. bovis* isolates collected from cattle slaughtered at Lilongwe cold storage abattoir. The study's purpose was to elucidate the molecular epidemiology of *M. bovis* in the central parts of Malawi to gain insights into the transmission dynamics, sources, and circulating population of *M. bovis* strains. I utilized molecular typing tools, *M. bovis* clonal complex deletion analysis, spoligotyping, and MIRU-VNTR to characterize the isolates.

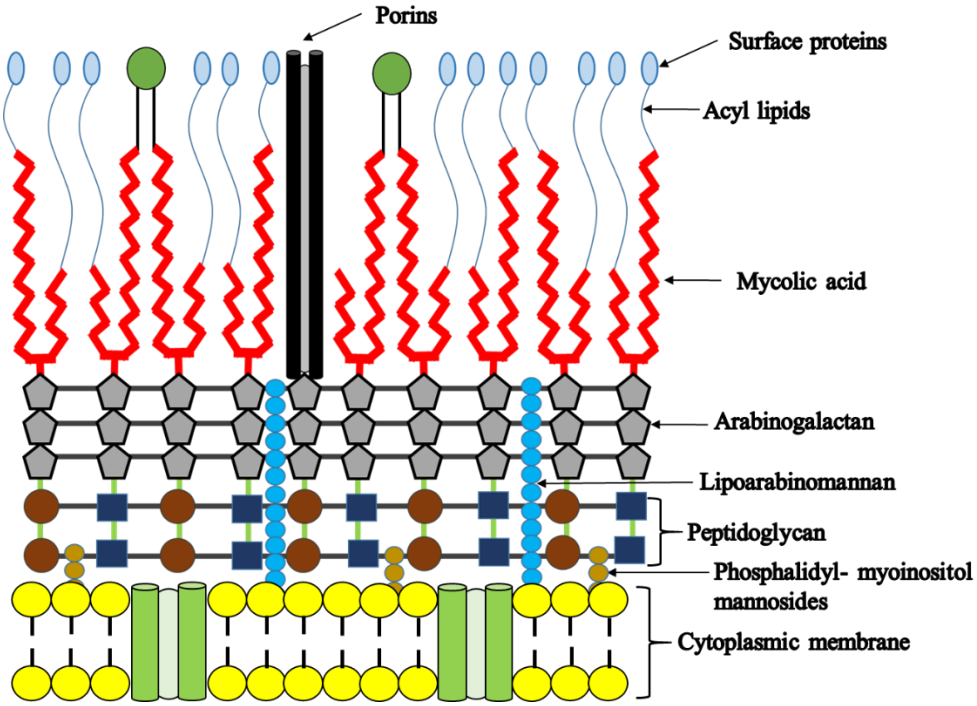


Fig. 1: Structure of *Mycobacterium* cell wall (adapted from Gary Kaiser [4]). The presence of different lipid layers confers resistance characteristics to external forces.

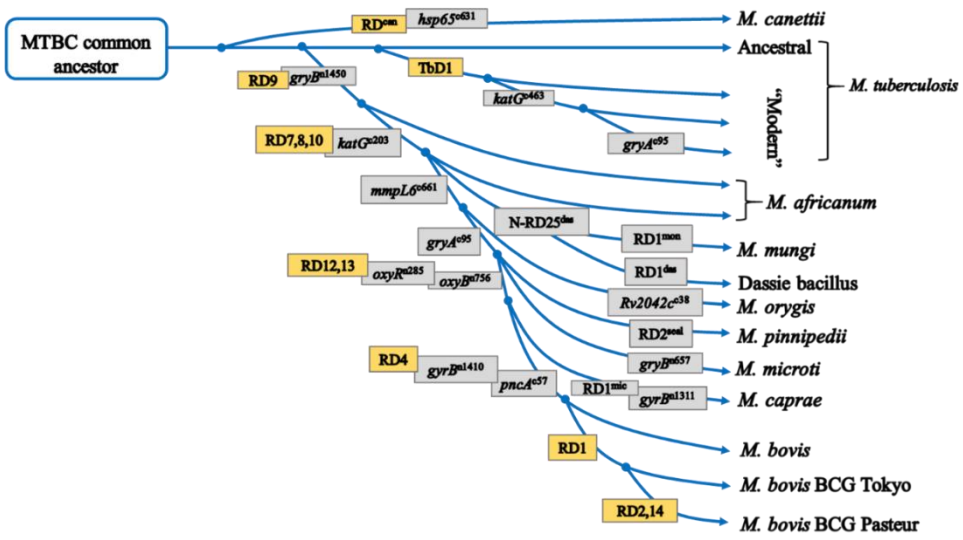


Fig. 2: MTBC evolutionary scenario. The region of differences (RD) and single nucleotide polymorphisms are indicated (adapted from Rodriguez-Campos et al., [8]). The genetic relatedness of the MTBC species infers originating from a common ancestor.

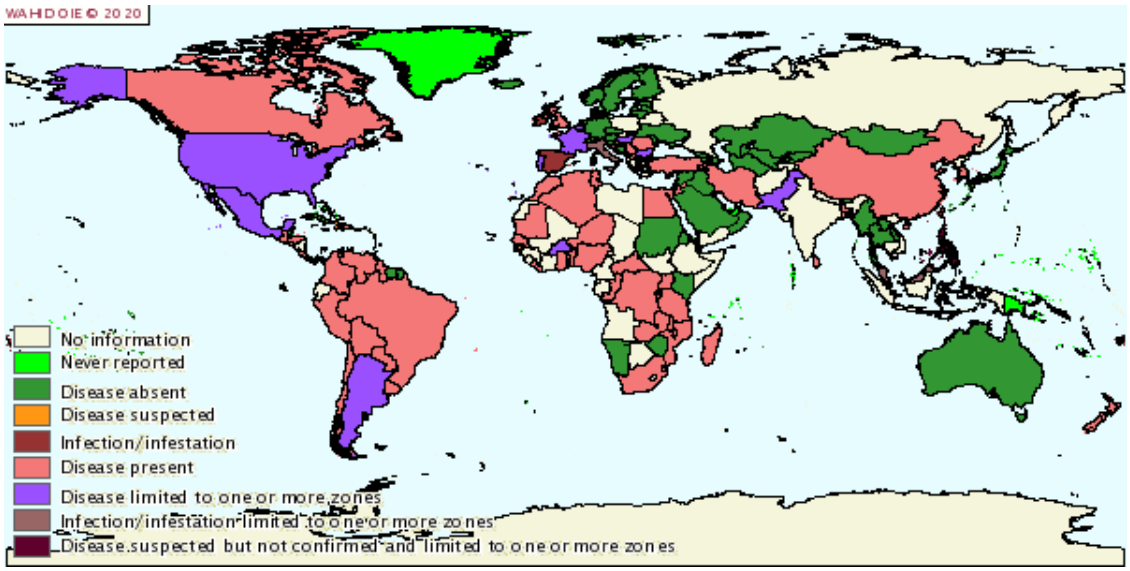


Fig. 3: Global distribution of bovine tuberculosis in domestic animals and wildlife (adapted from OIE, [81]). The burden is high in developing countries as compared to developed countries.

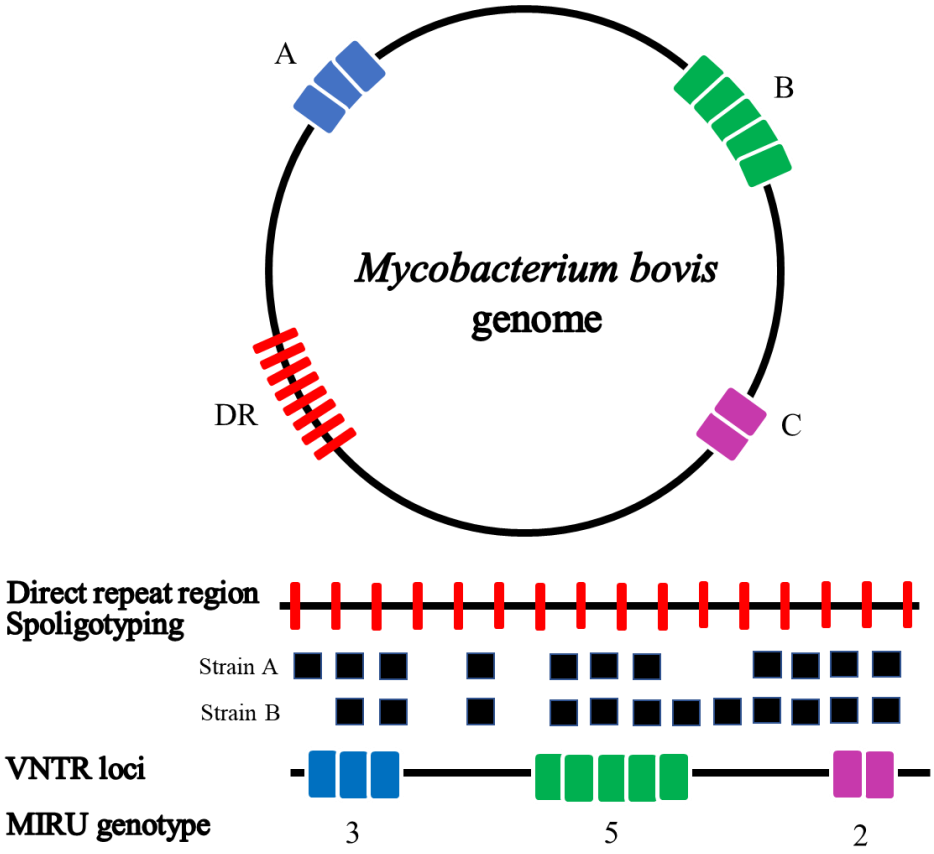


Fig. 4: Principles of spoligotyping and MIRU-VNTR in MTBC. Spoligotyping is based on detecting the 43 unique spacers located between direct repeats present in the DR locus. The deletion of some of these 43 spacers allows for differentiation between strains. MIRU-VNTR analysis relies on identifying the different numbers of repeats at several loci scattered around the bacterial genome (marked by A, B, and C in the figure). The number of repeats at each locus is combined to generate a unique numerical code used to establish phylogenetic and epidemiological links between strains (adapted from Comas et al., [57])

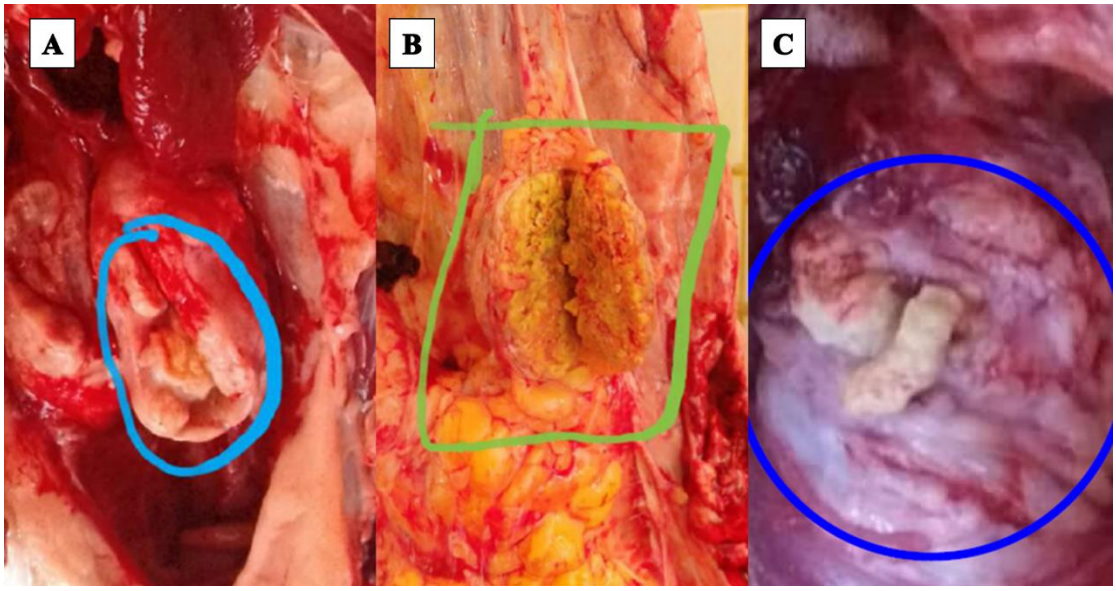


Fig. 5: Granulomatous TB - like lesions observed during a routine post-mortem at Lilongwe cold storage abattoir. (A) Retropharyngeal lymph node, (B) Lung tissue, and (C) Mesenteric lymph node (Pictures were taken during routine postmortem examination at Lilongwe cold storage abattoir).

Chapter I

Development of loop-mediated isothermal amplification (LAMP) method for specific detection of *Mycobacterium bovis*

Introduction

The burden of bTB in developing countries, mainly those from the African region, is indisputable. Unfortunately, routine diagnosis of TB in humans is still obtained by clinical symptoms, chest radiography, and sputum smear microscopy. Besides, MGIT culture or GeneXpert systems are also used mainly in referral hospitals or laboratories. Nevertheless, these methods do not distinguish MTBC to species level [102]. Modernized diagnostic methods capable of identifying *M. bovis* have various challenges prohibiting easy integration in resource-restricted areas. For example, biochemical tests such as nitrate reduction and niacin accumulation rely on bacterial growth and, hence, time-consuming, besides that, not all clinics could afford to buy the culture media and have the equipment and standardized laboratories to culture and maintain it [103]. Nucleic acid amplification tests (NAAT) such as PCR targeting nucleotide polymorphisms in the *pncA* gene and *oxyR* [11] involve complex processes and require the use of expensive equipment and a standardized laboratory infrastructure restricting usage in many developing countries [104]. In cattle, the lack of routine surveillance tools has contributed to the underestimation of bTB's burden. Therefore, there is a need for a rapid, simplified and low-cost method that could easily be integrated for use in resource-limited areas.

MBTC's high homogeneity level has significantly contributed to challenges in developing simplified tools to differentiate the group. However, comparative genomic studies proposed region of differences (RD) as reliable molecular markers to distinguish MTBC species [105,106]. The RDs are chromosomal regions that are present in one species but deleted in the other species. An example is RD4, a 12.7 kb locus that encompasses 11 genes, from Rv1506c - Rv1516c in *M. tuberculosis*, but deleted in *M. bovis* (Fig. 2) [106]. Other studies have developed a PCR targeting RD4 for specific differentiation of *M. tuberculosis* and *M. bovis* [42,43,107–110]. However, the use of these methods in resource-limited areas is still subjective due to the challenges explained in earlier sections.

Thus, utilizing RD4, I have developed a loop-mediated isothermal amplification (LAMP) assay for the specific detection of *M. bovis.* LAMP assay is a single tube NAAT technique first reported by Notomi et al. in 2000 [111]. LAMP`s advantages include the

use of inexpensive equipment such as a water bath for maintaining isothermal conditions. LAMP reactions are quicker as compared to PCR such that within 60 minutes, results are known. Additionally, results are visible to the naked eye, with no need for aided electrophoresis. Furthermore, the LAMP assay has high specificity because it uses more target primers (six or eight) than two primers by PCR. Due to these facts, LAMP is a potential candidate that could easily be integrated into resource-limited areas [112].

This study developed a LAMP assay for the specific detection of *M. bovis* by targeting the RD4 deletions.

Materials and Methods

Samples and DNA extraction

One hundred and thirty-nine mycobacteria and non-mycobacteria were used for specificity analysis. These included seven reference MTBC strains, twenty-two reference non-tuberculous mycobacteria (NTM) strains, five reference non-mycobacteria, and 105 clinical or field MTBC isolates or TB lesion specimens were used to determine the specificity of our LAMP assay (Tables 1 and 4).

Sixty-five isolates of *M. bovis* and forty of *M. tuberculosis* were used. Fifty-one isolates grown on Ogawa medium (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) and identified as *M. bovis* by an MTBC-discrimination multiplex PCR [43] and spoligotyping [59] were collected from cattle and wild lechwe antelope from 2004 to 2009 in Zambia [66]. Twenty clinical samples grown in MGIT (Becton, Dickinson and Company, NJ, USA) were obtained at the university teaching hospital (UTH) in Lusaka, Zambia from 2011 to 2016 [113]. Four were confirmed as *M. bovis,* and 16 were *M. tuberculosis* by Capilia TB (Tauns Laboratories Inc., Shizuoka, Japan) and spoligotyping. Ten samples collected from cattle suspected of TB during routine post-mortem at Lilongwe cold storage abattoir in Lilongwe, Malawi in November 2019 were used. Tissues were homogenized, decontaminated in 4% NaOH for 15 minutes, followed by neutralization in sterile phosphate buffer saline (PBS) and centrifuged at 3200 g for 20 minutes at 6ºC. The pellets were re-suspended in PBS, and DNA was isolated directly by heating at 95ºC for 15 minutes and immediately cooling at -20ºC for 30 minutes. Confirmation as *M. bovis* was done at Hokkaido University, Japan using *Mycobacterium* specific PCR targeting the IS6110 [114], MTC-discrimination multiplex PCR, and spoligotyping. The other 24 *M. tuberculosis* isolates were collected in Osaka, Japan, between 2000 and 2009 [115] and grown on Ogawa medium. Bacterial DNA from colonies on solid media was extracted as previously described [114]. For liquid medium

cultures, 500 µl of the MGIT contents were taken to a cryotube, and DNA was extracted by boiling at 95ºC for 15 minutes. All extracted DNAs were stored at -30ºC.

Primer design and screening

Molecular Evolutionary Genetics Analysis (MEGA) version 7 software (https://www.megasoftware.net/ Pennsylvania State University, USA) was used to align sequences of *M. bovis* AF2122/97 genome sequence (GenBank accession No.: LT708304.1) against *M. tuberculosis* H37Rv genome sequence (GenBank accession No.: AL123456.3). The target sequence was selected from upstream through to downstream, flanking the RD4 deletion of *M. bovis.* LAMP primers were designed by Primer Explorer V5 software (http://primerexplorer.jp/lampv5e/index.html, Eiken Chemical, Tokyo, Japan). A set of primers comprised four primers: outer primers (Forward outer primer-F3 and Backward outer primer-B3) and inner primers (Forward inner primer-FIP and Backward inner primer-BIP). Loop primers (Forward loop primer-FLP and Backward loop primer-BLP) were designed and added for the best performing primer set that was selected and optimized. In order to determine a set of primers specific for detection of *M. bovis*, primer design properties and position of RD4 deletions were manually adjusted with reference to the "Advanced primer design" manual [116]. Designed LAMP primers were screened by observing the specificity, cross-reactivity, and reaction speed to select the best performing primer set. All primers were synthesized by Life Technologies Japan Ltd. (Tokyo, Japan).

Optimization of LAMP reaction

Initial LAMP reactions were performed according to the procedures described previously [22]. Briefly, reactions were conducted at 64ºC for 120 minutes in a Loopamp real-time turbidimeter (LA-200: Teramecs Co. Ltd., Kyoto, Japan). *M. bovis* BCG Tokyo 172 genomic DNA was used as positive control while *M. tuberculosis* H37Rv DNA and double-distilled water (DDW) were both negative controls. Positive results were indicated by rising curve(s) of turbidity greater than 0.1 thresholds in the real-time turbidimeter (LA-200) and visual inspection of the colour change by colori-fluorometric indicator (CFI) [24]. Three key points were focused on to determine optimal conditions: temperature, primer concentration, and primer mixture final volume. LAMP reactions were performed at 1ºC interval from 60ºC to 67ºC. Subsequently, the concentration of loop primers was evaluated at 5.0 µM, 4.2 µM, 3.6 µM, and 2.8 µM. Inner primers (FIP/BIP) and outer primers (F3/B3) were kept fixed at 4.8 µM and 0.6 µM, respectively. The primer mixture's final volume per reaction was adjusted in 0.25 µl intervals from 2.0 to 2.75 µl.

Specificity and sensitivity analysis

Specificity was evaluated using bacteria listed in Tables 1 and 4. LAMP results were compared to the initial identification and confirmation of the bacteria. The cut-off point to determine positivity and negativity was set to 40 minutes. LAMP assay sensitivity was evaluated by diluted *M. bovis* BCG Tokyo 172 genomic DNA at 5 pg, 500 fg, 50 fg, 40 fg and 30 fg per reaction. LAMP reactions were monitored by the LA-200 (Teramecs Co. Ltd.) for 120 minutes.

Multiplex PCR reaction

LAMP sensitivity was compared to a multiplex PCR targeting RD4 for differentiating *M. bovis* and *M. tuberculosis* [42]. We slightly modified the protocol as follows; 4 µl 5× Go Taq buffer (Promega Co., WI, USA); 0.6 µl of 25 mM $MgCl_2$; 0.5 µl of 25 mM dNTP mix; 0.5 µl of 10 µM each primer; common forward primer - CBS1 (5'-TTCCGAATCCCTTGTGA-3'); *M. bovis* specific reverse primer - CBS2 (5'-GGAGAGCGCCGTTGTA-3') and *M. tuberculosis*-specific reverse primer - CBS3 (5'-AGTCGCCGTGGCTTCTCTTTTA-3'); 0.2 µl of 5 U/µl GoTaq DNA polymerase (Promega Co.); 1 µl of template DNA; and finally double-distilled water (DDW) to make up to a final volume of 20 µl. The cycling parameters consisted of an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C (1 minute), annealing at 52°C (1.5 minutes), and extension at 72°C (1 minute), with a final elongation step at 72°C for 5 minutes. The amplification products were analyzed by gel electrophoresis at 100V for 20 minutes. The predicted PCR products were 168 bp (*M. bovis)* and 337 bp (*M. tuberculosis*).

Confirmation of LAMP products

We first performed in-silico LAMP fragment analysis to predict the expected band sizes using the software found on http://creisle.github.io/creisle.lamprflp/. After that, enzymatic digestion of the LAMP products was performed in a reaction mixture comprising 1 µl of LAMP product, 1 µl of *Eco*RI, 2 µl of 10x enzyme buffer (New England Biolabs), and 16 µl of DDW. The mixture was incubated at 37ºC for 60 minutes, and the resultant products were analyzed by 2% agarose gel electrophoresis stained with Gel-Red (Biotium, Inc., CA, USA).

Results

LAMP primers screening and selection

Primer design properties and position of the RD4 deletion were manually configured while screening for the best performing set of primers for specific detection of *M. bovis,* and more than 40 sets of primers were designed and evaluated. A primer set where the F1 primer 3-prime section was located across the RD4 deletion-junction showed high specificity and repeatability and was selected for further optimization (Table 2). The target sequence and primer binding positions are shown in Fig. 6.

Optimization of the LAMP assay

During temperature optimization, an increase in temperature gave an improved detection speed and specificity. However, at 67°C the LAMP assay sensitivity decreased; thus, the optimal temperature was determined as 66°C. The effect of loop primers was tested at varying concentrations (Table 3). Upon adding 5.0 µM of loop primers to the primer mixture, the assay's detection speed improved with *M. bovis;* however, false positives from *M. tuberculosis* were observed. Reducing the concentration of loop primers (4.2 µM) improved the specificity. We observed that by using the lowest concentration of loop primers (2.8 µM) LAMP specificity improved, but sensitivity was reduced. Therefore, the optimal concentration of loop primers was determined as 3.6 µM. Primer mixture volume was optimized in the same way, and a 2.25 µl/reaction volume was chosen.

Considering these adjustments, LAMP reaction conditions were adjusted as follows: reaction temperature of 66°C; 2.25 µl of final primer mixture comprising of 0.6 µM of each outer primer, 4.8 µM of each inner primer, 3.6 µM of each loop primer; 20 mM Tris-HCl (pH 8.8, BIORAD), 10 mM KCl, 10mM (NH4)2SO4 and 0.1% Tween20; 6 mM MgSO4; 0.8 M betaine; 1.25 mM dNTP; 8U Bst DNA polymerase (Nippon Gene Co., Japan); 1 µl of CFI [24]; 2 µl of extracted DNA as a template and DDW up to a final volume of 25 µl.

The specificity of the established LAMP assay

The assay detected all sixty-five *M. bovis* isolates as positive, while all other bacteria tested negative. All isolates detected as *M. bovis* by our newly developed LAMP system were in agreement with their initial identification of the samples as *M. bovis* by other conventional methods (Table 4).

The sensitivity of LAMP assay and multiplex PCR

Sensitivity analysis was achieved by dilution of *M. bovis* BCG Tokyo 172

genomic DNA from 5 pg~30 fg/reaction. The established LAMP detection limit was 50 fg/reaction, equivalent to 10 copies of the *M. bovis* genome, in 40 minutes. These results were observed both on the LAMP turbidimeter (Fig. 7A) as well as visually by the naked eye under natural light (Fig. 7B) and under LED light (Fig. 7C). Comparatively, multiplex PCR [16] performed on the same samples detected up to 5 pg/reaction (Fig. 7D).

Confirmation of LAMP products

LAMP products were digested by restriction enzyme *Eco*RI to confirm successful amplification of the target sequence. Figures 8 and 9 show the three fragments 124, 169, and 239 bp observed.

Discussion

bTB represents a potential zoonotic threat to humans in developing countries, but its control has long been neglected. There is substantial evidence suggesting that developing countries bear the highest burden of zoonotic TB due to the existence of multiple risk factors maintaining the circulation of the disease in livestock [75]. In Africa, a range of 0% - 37.7% of all TB cases in humans are estimated to be caused by *M. bovis* [75]. However, detection of *M. bovis* is seldom performed, largely because routine TB diagnostic methods cannot differentiate *M. bovis* from other MBTC. Methods capable of delineating MBTC species, such as PCR or culture-based methods, are time-consuming, complicated, and prohibitively expensive and hence not easily adopted for widespread use. Consequently, all TB suspected cases adopt the standard *M. tuberculosis* treatment regimen that includes pyrazinamide, even though this approach has significant shortcomings in cases of *M. bovis* infection [117].

In this study, we describe the development of a LAMP-based method for specific identification of *M. bovis* by targeting the RD4 deletion. Thus, our newly established LAMP assay is important not only for guiding the appropriate choice of antimicrobials against zoonotic TB but also for surveillance purposes and disease control. The developed LAMP method accurately identified all *M. bovis* isolates, while no false-positive amplifications were observed with other MBTC species (Tables 1 and 4). One cattle sample on Ogawa medium was heavily contaminated with other bacteria, and *Pseudomonas* sp. sequence was detected by 16S rRNA gene sequencing (C-43, Table 4); however, the LAMP reaction became positive in less than 40 min in both duplicated test tubes. These results show that our established LAMP is highly specific for detecting *M. bovis* even in contaminated samples. Furthermore, the results show evidence for 'proof of concept' in using the RD4 deletion as a molecular signature for specific identification of

M. bovis amongst other MTBC species. Elsewhere, LAMP-based methods have been reported for differentiating *M. bovis* from MTBC targeting *mbp70* [118] and *mtp40* genes [119]. However, the specificity to detect *M. bovis* only is open to challenge because *mbp70* is also present in other MTC species [120], while *mtp40* is not present in all *M. tuberculosis* strains [121]. Given this, our LAMP is reliable for the identification and differentiation of *M. bovis* from MBTC because it targets RD4 which is the best target for this purpose as all *M. bovis* strains lack RD4 [6,106].

We used a variety of *M. bovis* and *M. tuberculosis* samples in our validation, encompassing *M. bovis* derived from different animal species, clinical samples from different countries, and *M. tuberculosis* strains from different lineages and a variety of spoligotypes (Table 4). All samples gave consistent LAMP results. Moreover, our method can be performed using DNA extracted directly from cattle specimens and a variety of growth media, liquid (MGIT) and solid culture (Ogawa medium). Regardless of the sample source, the performance of our LAMP assay was the same in all cases. The ability to use simply heat-killed MGIT culture is very advantageous in this regard since the medium is now widely used in developing countries, with MGIT positive culture growth indicated within 7 to 14 days. Hence this LAMP could be incorporated with standard MGIT to confirm and speciate positive growth results, allowing diagnosis at the earliest possible time.

We also performed the established LAMP system using larger amounts of mycobacterial DNA (50pg/reaction, an equivalent to smear-positive sputum+++) [122] to evaluate the applicability on samples with an unknown number of bacterial cells/DNA concentration. We observed no false amplification of *M. tuberculosis* until a reaction time of 92 minutes. Thus, the results show the ability of our established LAMP to specifically identify *M. bovis*. Despite the false amplification of *M. tuberculosis* when larger amounts of DNA were used, our LAMP reaction time could be shortened to 40 minutes to ensure specific identification of *M. bovis*. The established LAMP is highly sensitive, with the assay detecting as few as to 10 copies of *M. bovis* genomic DNA. In comparison to previous reports, a LAMP system targeting RD1 deletions for identification of *M. bovis* BCG required more than 200 copies of the targets for detection using a turbidimeter, and 2000 copies for detection with a visible colour change [123]. In other reports, a LAMP system targeting the *rim*-encoding 16S rRNA-processing protein for detection of *M. tuberculosis* and *M. bovis* detected as low as 200 copies [124]. Our results, therefore, indicate that the newly established LAMP assay has a superior limit of detection compared to previously described systems. Besides, the LAMP can be used together with other conventional MTBC identification methods, such as MGIT culture, to enhance

detection of *M. bovis* infections. Furthermore, as well as its application in human disease, our LAMP could be employed to detect *M. bovis* from cattle samples after postmortem assisting in tracing back sources of infection and the implementation of appropriate control measures.

Our established LAMP can easily be integrated for use in resource-limited settings due to its properties. It employs simple procedures that involve mixing the reaction reagents in a single tube before incubating at a constant temperature (66^{o}C) for 40 minutes. Furthermore, the incubation of LAMP reagents doesn't require the use of expensive equipment or a standardized laboratory; a heat block or water bath is sufficient, and results can be visualized with the naked eye, eliminating the need for procedures such as gel electrophoresis and photographic imaging as is the case with PCR-based methods.

One major challenge to enzymatic-based methods is cold chain maintenance and storage of reagents in resource-limited areas. To overcome this, Hayashida and others [24,44] used dried LAMP reagents, facilitating reagents' storage for an extended period without the need to maintain a cold chain. We plan to explore this option for our next study and further optimize the dry LAMP method for direct use against sputum samples and cattle post-mortem samples in a field setting. A further consideration in zoonotic TB is the emerging appreciation of the role of *Mycobacterium orygis* in human and bovine disease, particularly in South Asia [125–127] Hence, further development of our LAMP system could allow for differential identification of *M. orygis* vs *M. bovis*, for example, by targeting the distinct RD12 deletion locus in *M. orygis* [128].

In conclusion, we have established a LAMP system for specific detection of *M. bovis* by targeting the RD4 deletion. The established LAMP showed high specificity and sensitivity, with ease and use coupled with rapid detection time. This new RD4 LAMP assay employs simple procedures and does not require expensive equipment, such as thermocyclers. Additionally, one reaction cycle of the RD4 LAMP would cost ~US$1-2, hence it is a cheaper approach as compared to other NAATs. Our future development work will focus on establishing a dry LAMP kit to facilitate transport and storage, and evaluate it on field samples.

Summary

Loop-mediated isothermal amplification (LAMP) assay was developed for specific identification of *M. bovis* by targeting RD4, a 12.7 kb genomic region deleted in all *M. bovis* strains but present in other MTBC species. The established LAMP assay`s specificity was evaluated using 139 isolates comprising 65 *M. bovis* isolates, 40 *M. tuberculosis* isolates, seven *M. tuberculosis* complex reference strains, 22 non-tuberculous mycobacteria, and five other bacteria. LAMP detected only *M. bovis* isolates as positive, and all other mycobacteria and non-mycobacteria tested negative. The established LAMP assay detected as low as ten copies of *M. bovis* genomic DNA. This LAMP assay is rapid, with results observed in 40 minutes. The procedures are simple and involve mixing of the reaction reagents in one tube and incubate at 66^{o}C. Results are observed with the naked eye by a colour change, and there is no need to use expensive equipment. These properties make this LAMP a good fit for use in resource-limited areas.

Table 1. Bacteria used in this study to determine specificity.

	Bacterial species	Sample ID
MTC reference strains	*M. bovis BCG*	172-Tokyo
	M. tuberculosis	H37Rv
	M. tuberculosis	H37Ra
	M. africanum	KK 13-02
	M. caprae	EPDC01 [a]
	M. orygis	NepR1 [b]
	M. microti	ATCC 19422
MTC clinical isolates	*M. bovis*	65 isolates [c]
	M. tuberculosis	40 isolates [c]
NTM reference strains	*M. abscessus*	JATA 63-01
	M. asiaticum	KK 24-01
	M. avium	JATA 51-01
	M. chelonae	JATA 62-01
	M. fortuitum	JATA 61-01
	M. gastri	KK 44-01
	M. gordonae	JATA 33-01
	M. intermedium	JATA 9H-01
	M. intracellulare	JATA 52-01
	M. kansaii	KK 21-01
	M. lentiflavum	JATA 9N-01
	M. malmoense	JATA 47-01
	M. marinum	JATA 22-01
	M. mucogenicum	JATA 9P-01
	M. nonchromogenicum	JATA 45-01
	M. peregrinum	JATA 61-01
	M. scrofulaceum	JATA 31-01
	M. shimodei	JATA 54-01
	M. simiae	KK 23-01
	M. smegmatis	JATA 64-01
	M. szulgai	JATA 32–01
	M. terrae	JATA 46–01
Other bacteria	*Streptococcus pneumoniae*	NBRC 102642
	Klebsiella pneumoniae	NBRC 3318
	Pseudomonas aeruginosa	NBRC 12689
	Staphylococcus aureus	NBRC 100910
	Mycoplasma pneumoniae	NBRC 14401

[a] reference [91], [b] reference [92]

[c] More information in Table 4.

JATA, Japan Anti-Tuberculosis Association; JBCG, Japan BCG Laboratory; NBRC, NITE Biological Resource Center; ATCC, American Type Culture Collection.

Table 2 Nucleotide sequences and sizes of established LAMP primers

Primer	Length	Sequence (5'-3')
F3	20	GCCGCTCCCAAAAATTACCA
B3	18	GACGCTACTACGGCACGG
FIP	41	AGGCCACTCCAAGAGTGTTGCG-TGACGCCTTCCTAACCAGA
BIP	35	GCGCGGGCGTACCGGATAT-GCGCCCCGTAGCGTTA
FLP	19	CTTCTGCACGACTACGGCT
BLP	24	AGCCATTTTTCAGCAATTTCTCAG

FIP primer consisted of F1c and F2; BIP primer consisted of B1 and B2c.

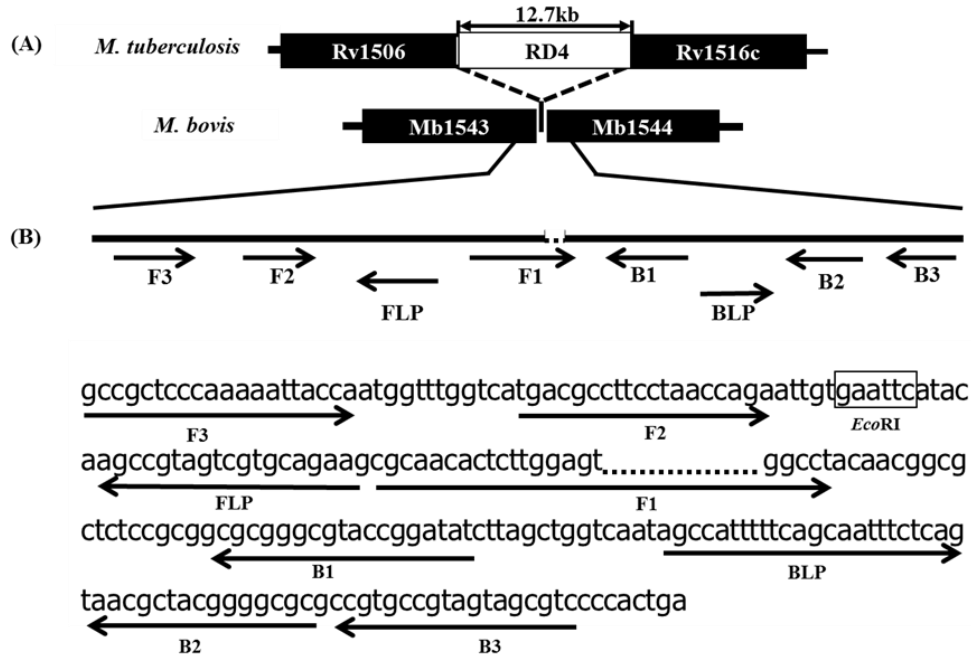


Fig. 6. LAMP primers for specific detection of *M. bovis* targeting the RD4 flanking region. (A) Position of the RD4 deletion on *M. bovis* genome, shown with reference to *M. tuberculosis.* The RD4 locus is present in *M. tuberculosis* but deleted from *M. bovis*. (B) The target sequence, individual primers, annealing positions, and directions of the primers with RD4 deletion at the 3-prime region of the F1 primer are shown. Restriction enzyme *Eco*RI target sequence is indicated in a box between F2 and FLP.

Table 3: Optimization of primer concentrations and ratios in LAMP reaction

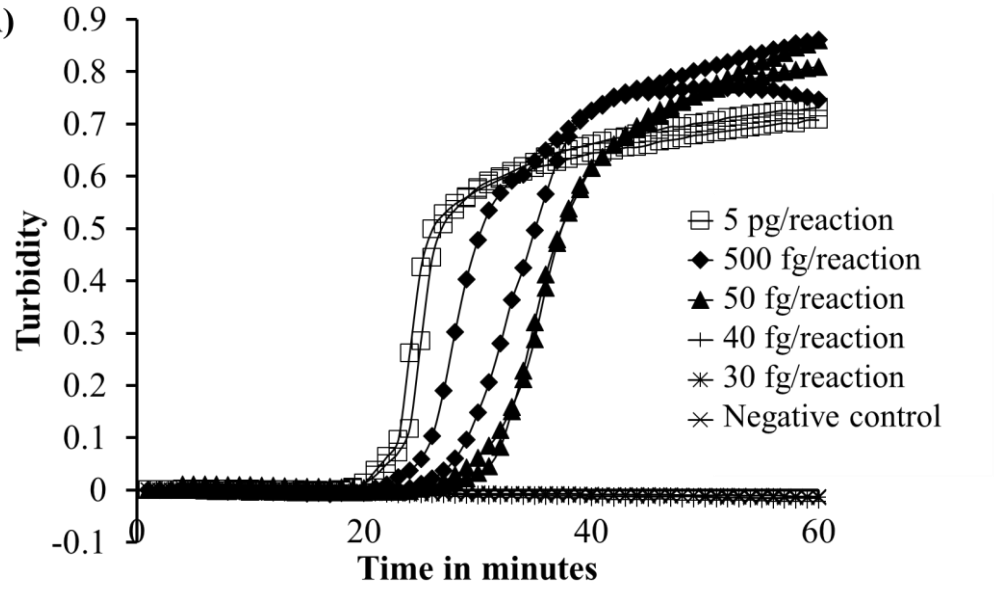
	Loop Primer concentration							
	5.0 µM		4.2 µM		3.6 µM		2.8 µM	
DNA concentration	BCG	H37Rv	BCG	H37Rv	BCG	H37Rv	BCG	H37Rv
5 pg	21.0±1.3	82.1±5.9	23.8±0.6	95.3±2.6[a]	24.8±0.6	NA	35.2±3	NA
500 fg	24±1.7	NT	24.9±1.2	NT	31.6±2.1	NT	39.9±10.1	NT
50 fg	27.1±0.9	NT	30.0±3.1	NT	36.1±4.0[b]	NT	NA	NT
NC	NA		NA		NA		NA	

	Primer volume quantity					
	2.0 µl		2.25 µl		2.5 µl	
DNA concentration	BCG	H37Rv	BCG	H37Rv	BCG	H37Rv
5 pg	37.6±1.6	117.3[c]	32.8±4	NA	23.8±2.1	101.3±10.1[d]
500 fg	41.9±6.2	NT	35.3±2.1	NT	31.9±7.0	
50 fg	70±15.7[e]	NT	45.1±9.0[f]	NT	34.4±1.9	
NC	NA		NA		NA	

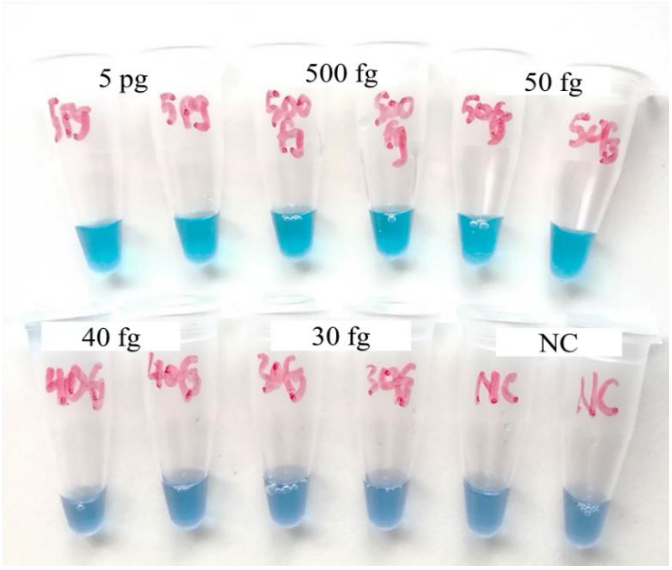
LAMP reactions were performed six times (duplicate x 3). Results are shown in time (min.) to be positive presented as mean ± standard deviation. BCG: *M. bovis* BCG 172 Tokyo; H37Rv: *M. tuberculosis* H37Rv; NA: No amplification observed within 120 min of reaction; NT: Not tested; NC: Negative control (DDW); NA: No Amplification

[a]2 of 6 reactions were positive; [b]5 of 6 reactions were positive; [c]1 of 6 reactions were positive; [d]4 of 6 reactions were positive; [e]3 of 6 reactions were positive; [f]4 of 6 reactions were positive

A)


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Turbidity
0
20
40
60
Time in minutes
5 pg/reaction
500 fg/reaction
50 fg/reaction
40 fg/reaction
30 fg/reaction
Negative control

B)


5 pg
500 fg
50 fg
40 fg
30 fg
NC

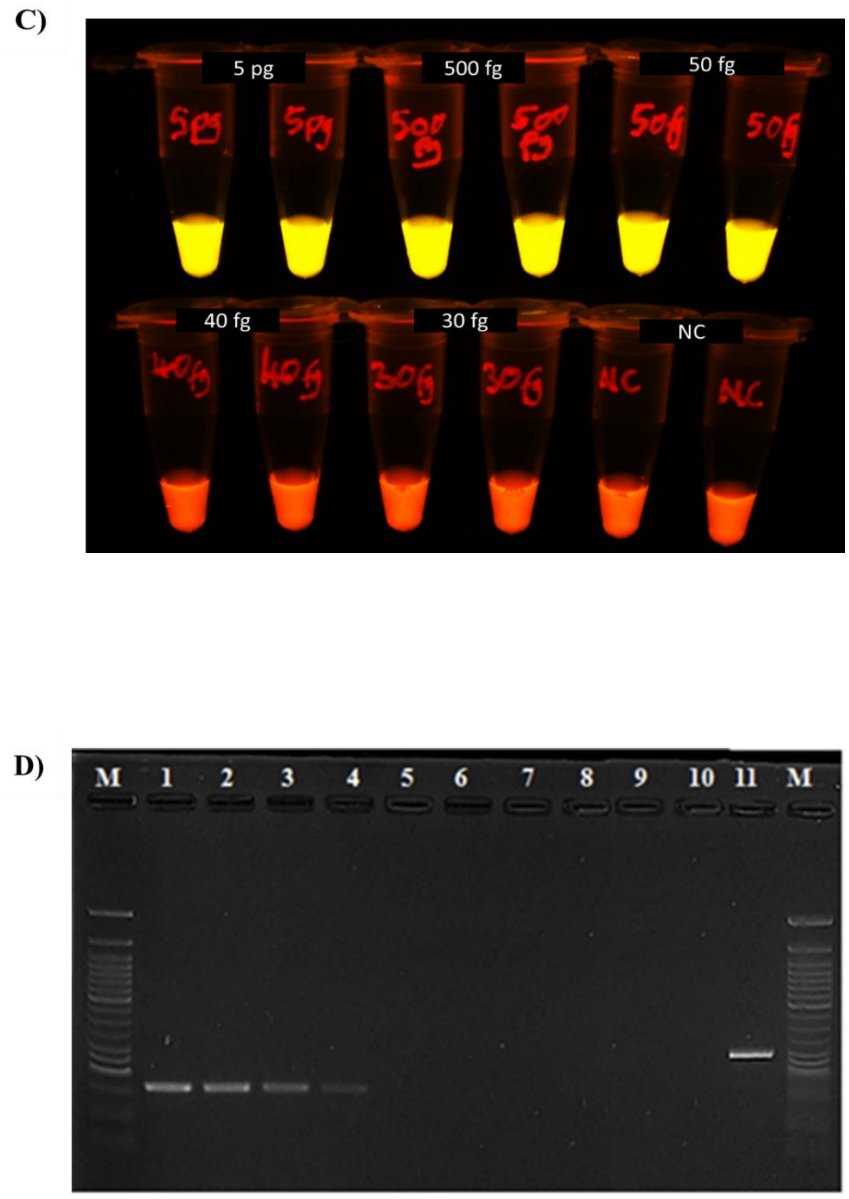


Fig. 7: Sensitivity of established LAMP assay and multiplex PCR. A) LAMP results observed by rising curves of turbidimeter. B) LAMP results observed by the naked eye under natural light; the colour shifts from violet to sky blue for positive samples. C) Under LED light, the colour change is from orange to light yellow for positive samples. D) The gel electrophoresis result of the multiplex PCR [16]. Lane M, 50 bp DNA marker (New England Biolabs); lanes 1 – 9, *M. bovis* BCG Tokyo 172 genomic DNA 500 pg, 50 pg, 20 pg, 5 pg, 500 fg, 50 fg, 40 fg, 30 fg, 20 fg/reaction; lane 10, Negative Control (NC); lane 11, *M. tuberculosis* H37Rv.

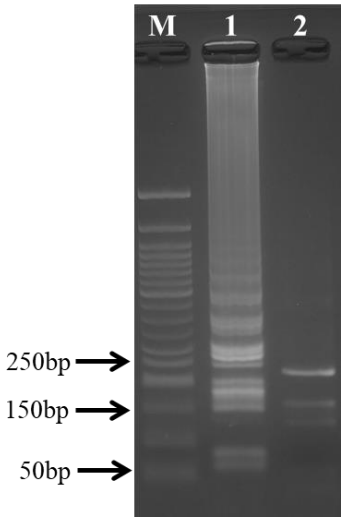


Fig. 8. Enzymatic digestion of target sequence by restriction enzyme *Eco*RI. Lane M, 50 bp DNA marker. Lane 1, LAMP products without the restriction enzyme. Lane 2, LAMP products digested by *Eco*RI. The predicted product sizes were 124 bp, 169 bp and 239 bp.

Table 4. List of bacterial isolates used for specificity analysis

	Bacteria species	Sample ID	Animal species	Country	Institute	Year	Culture media	RD4	16S	Used methods	Spoligotyping	MTB lineage
1	*M. bovis*	C2	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
2	*M. bovis*	C3	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0948, SCG-3	bovis
3	*M. bovis*	C-4	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
4	*M. bovis*	C5	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120, SCG-3	bovis
5	*M. bovis*	C-6	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
6	*M. bovis*	C-8	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
7	*M. bovis*	C11	cattle	Zambia	UNZA	2008	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
8	*M. bovis*	C-20	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
9	*M. bovis*	C-25A	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
10	*M. bovis*	C25J	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
11	*M. bovis*	C-26	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR	ND	bovis
12	*M. bovis*	C-28	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
13	*M. bovis*	C-34	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR	ND	bovis
14	*M. bovis*	C39	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120, SCG-3	bovis
15	*M. bovis*	C42	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
16	*M. bovis*	C-43	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, 16S	ND, contamination, Ps	bovis
17	*M. bovis*	C46	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
18	*M. bovis*	C-51	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
19	*M. bovis*	C1-1	cattle	Zambia	UNZA	2007	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131, SCG-2	bovis
20	*M. bovis*	C1-2	cattle	Zambia	UNZA	2007	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
21	*M. bovis*	C1-3	cattle	Zambia	UNZA	2007	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
22	*M. bovis*	C1-4	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
23	*M. bovis*	C1-5	cattle	Zambia	UNZA	2007	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
24	*M. bovis*	C1-7	cattle	Zambia	UNZA	2007	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
25	*M. bovis*	C1-15	cattle	Zambia	UNZA	2007	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
26	*M. bovis*	C1-16	cattle	Zambia	UNZA	2007	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
27	*M. bovis*	C1-17	cattle	Zambia	UNZA	2007	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
28	*M. bovis*	C1-18	cattle	Zambia	UNZA	2007	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
29	*M. bovis*	Cattle 1-44	cattle	Zambia	UNZA	2007	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
30	*M. bovis*	Lusaka 003	cattle	Zambia	UNZA	2009	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
31	*M. bovis*	Kabwe 1-10	cattle	Zambia	UNZA	2008	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
32	*M. bovis*	Kabwe 2-10	cattle	Zambia	UNZA	2008	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
33	*M. bovis*	Kabwe 3-10	cattle	Zambia	UNZA	2008	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
34	*M. bovis*	Kabwe 4-10	cattle	Zambia	UNZA	2008	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
35	*M. bovis*	6 Chongwe	cattle	Zambia	UNZA	2004	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131, SCG-2	bovis
36	*M. bovis*	Zam-3	cattle	Zambia	UNZA	2008	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0948, SCG-3	bovis
37	*M. bovis*	Zam-4	cattle	Zambia	UNZA	2008	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131, SCG-2	bovis
38	*M. bovis*	Zam-5	cattle	Zambia	UNZA	2008	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131, SCG-2	bovis
39	*M. bovis*	Zam-6	cattle	Zambia	UNZA	2008	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis
40	*M. bovis*	Zam-8	cattle	Zambia	UNZA	2008	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0131	bovis

41	*M. bovis*	BL-10	lechwe	Zambia	UNZA	2006	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
42	*M. bovis*	BL-14	lechwe	Zambia	UNZA	2006	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
43	*M. bovis*	BL-20	lechwe	Zambia	UNZA	2006	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
44	*M. bovis*	BL-21	lechwe	Zambia	UNZA	2006	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
45	*M. bovis*	LL-1	lechwe	Zambia	UNZA	2006	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
46	*M. bovis*	LL-2	lechwe	Zambia	UNZA	2006	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
47	*M. bovis*	LL-8	lechwe	Zambia	UNZA	2006	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120, SCG-3	bovis
48	*M. bovis*	LL-11	lechwe	Zambia	UNZA	2006	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
49	*M. bovis*	LL-12	lechwe	Zambia	UNZA	2006	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
50	*M. bovis*	LL-18	lechwe	Zambia	UNZA	2006	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
51	*M. bovis*	LL-20	lechwe	Zambia	UNZA	2006	solid, Ogawa	P	P	M-PCR, spoligotyping	SB0120	bovis
52	*M. bovis*	L090-1	human	Zambia	UTH	2011	liquid, MGIT	P	P	Capilia TB, spoligotyping	656573777777600	bovis
53	*M. bovis*	1199	human	Zambia	UTH	2013	liquid, MGIT	P	P	Capilia TB, spoligotyping	676773777777600	bovis
54	*M. bovis*	24189 (55)	human	Zambia	UTH	2016	liquid, MGIT	P	P	Capilia TB, spoligotyping	676773777777600	bovis
55	*M. bovis*	24208 (56)	human	Zambia	UTH	2016	liquid, MGIT	P	P	Capilia TB, spoligotyping	656573777761600	bovis
1	*M. bovis*	P2398/CS-L2	cattle	Malawi	CS abattoir	2019	Not cultured	P	P	M-PCR, spoligotyping	SB0131	bovis
2	*M. bovis*	P2398/CS-L3	cattle	Malawi	CS abattoir	2019	Not cultured	P	P	M-PCR, spoligotyping	SB0273	bovis
3	*M. bovis*	P2398/CS-L9	cattle	Malawi	CS abattoir	2019	Not cultured	P	P	M-PCR, spoligotyping	SB0425	bovis
4	*M. bovis*	P2398/CS-L12	cattle	Malawi	CS abattoir	2019	Not cultured	P	P	M-PCR, spoligotyping	SB0131	bovis
5	*M. bovis*	P2398/CS-L17	cattle	Malawi	CS abattoir	2019	Not cultured	P	P	M-PCR, spoligotyping	SB0425	bovis
6	*M. bovis*	P2398/CS-L20	cattle	Malawi	CS abattoir	2019	Not cultured	P	P	M-PCR, spoligotyping	SB0425	bovis
7	*M. bovis*	P2398/CS-L21	cattle	Malawi	CS abattoir	2019	Not cultured	P	P	M-PCR, spoligotyping	SB0131	bovis
8	*M. bovis*	P2398/CS-L23	cattle	Malawi	CS abattoir	2019	Not cultured	P	P	M-PCR, spoligotyping	SB0131	bovis
9	*M. bovis*	P2398/CS-L76	cattle	Malawi	CS abattoir	2019	Not cultured	P	P	M-PCR, spoligotyping	SB0273	bovis
10	*M. bovis*	P2398/CS-L79	cattle	Malawi	CS abattoir	2019	Not cultured	P	P	M-PCR, spoligotyping	SB0131	bovis
1	*M. tuberculosis*	3235	human	Zambia	UTH	2014	liquid, MGIT	N	P	Capilia TB, spoligotyping	577777606060771	Lineage 4
2	*M. tuberculosis*	4936	human	Zambia	UTH	2014	liquid, MGIT	N	P	Capilia TB, spoligotyping	577776777760601	Lineage 4
3	*M. tuberculosis*	5182	human	Zambia	UTH	2014	liquid, MGIT	N	P	Capilia TB, spoligotyping	677777607760771	Lineage 4
4	*M. tuberculosis*	5525	human	Zambia	UTH	2014	liquid, MGIT	N	P	Capilia TB, spoligotyping	703777740003171	Lineage 3
5	*M. tuberculosis*	5556	human	Zambia	UTH	2014	liquid, MGIT	N	P	Capilia TB, spoligotyping	777777606060771	Lineage 4
6	*M. tuberculosis*	6607	human	Zambia	UTH	2014	liquid, MGIT	N	P	Capilia TB, spoligotyping	777777606060731	Lineage 4
7	*M. tuberculosis*	7441	human	Zambia	UTH	2014	liquid, MGIT	N	P	Capilia TB, spoligotyping	777777777760771	Lineage 4
8	*M. tuberculosis*	7818	human	Zambia	UTH	2014	liquid, MGIT	N	P	Capilia TB, spoligotyping	743777777760700	Lineage 4
9	*M. tuberculosis*	7849	human	Zambia	UTH	2014	liquid, MGIT	N	P	Capilia TB, spoligotyping	777777604060731	Lineage 4
10	*M. tuberculosis*	8529	human	Zambia	UTH	2014	liquid, MGIT	N	P	spoligotyping	777777607760771	Lineage 4
11	*M. tuberculosis*	8816	human	Zambia	UTH	2014	liquid, MGIT	N	P	Capilia TB, spoligotyping	777417606060731	Lineage 4
12	*M. tuberculosis*	9114	human	Zambia	UTH	2014	liquid, MGIT	N	P	Capilia TB, spoligotyping	77777606060671	Lineage 4
13	*M. tuberculosis*	9711	human	Zambia	UTH	2015	liquid, MGIT	N	P	ND	ND	ND
14	*M. tuberculosis*	9783	human	Zambia	UTH	2015	liquid, MGIT	N	P	Capilia TB, pncA	ND	ND
15	*M. tuberculosis*	20011	human	Zambia	UTH	2015	liquid, MGIT	N	P	Capilia TB, spoligotyping	777177606060771	Lineage 4

16	*M. tuberculosis*	20049	human	Zambia	UTH	2015	liquid, MGIT	N	P	Capilia TB, spoligotyping	703377400001771	Lineage 3
17	*M. tuberculosis*	OM-1	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	Beijing	Lineage 2
18	*M. tuberculosis*	OM-3	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	Beijing	Lineage 2
19	*M. tuberculosis*	OM-5	human	Japan	OPIPH	2004	solid, Ogawa	N	P	spoligotyping	Beijing	Lineage 2
20	*M. tuberculosis*	OM-7	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	SIT42, LAM9	Lineage 4
21	*M. tuberculosis*	OM-15	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	SIT52, T2	Lineage 4
22	*M. tuberculosis*	OM-25	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	SIT523, pre-Beijing	Lineage 2
23	*M. tuberculosis*	OM-35	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	SIT53, T1	Lineage 4
24	*M. tuberculosis*	OM-49	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	Beijing	Lineage 2
25	*M. tuberculosis*	OM-76	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	SIT20, LAM1	Lineage 4
26	*M. tuberculosis*	OM-87	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	LAM, undesignated	Lineage 4
27	*M. tuberculosis*	OM-09-32	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	Beijing	Lineage 2
28	*M. tuberculosis*	OM-09-56	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	T3-OSA	Lineage 4
29	*M. tuberculosis*	OM-09-63	human	Japan	OPIPH	2008	solid, Ogawa	N	P	spoligotyping	Beijing	Lineage 2
30	*M. tuberculosis*	OM-09-67	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	Beijing	Lineage 2
31	*M. tuberculosis*	OM-09-88	human	Japan	OPIPH	2000~9	solid, Ogawa	N	P	spoligotyping	Beijing	Lineage 2
32	*M. tuberculosis*	06-13	human	Japan	OPIPH	2006	solid, Ogawa	N	P	rpoB, gyrA	PGG3	Lineage 4
33	*M. tuberculosis*	06-82	human	Japan	OPIPH	2006	solid, Ogawa	N	P	rpoB, gyrA	ND	ND
34	*M. tuberculosis*	07-21	human	Japan	OPIPH	2007	solid, Ogawa	N	P	rpoB, gyrA	ND	ND
35	*M. tuberculosis*	07-49	human	Japan	OPIPH	2007	solid, Ogawa	N	P	rpoB, gyrA	ND	ND
36	*M. tuberculosis*	07-61	human	Japan	OPIPH	2007	solid, Ogawa	N	P	rpoB, gyrA	PGG3	Lineage 4
37	*M. tuberculosis*	07-81	human	Japan	OPIPH	2007	solid, Ogawa	N	P	rpoB, gyrA	ND	ND
38	*M. tuberculosis*	08-10	human	Japan	OPIPH	2008	solid, Ogawa	N	P	rpoB, gyrA	ND	ND
39	*M. tuberculosis*	09-08	human	Japan	OPIPH	2009	solid, Ogawa	N	P	rpoB, gyrA	ND	ND
40	*M. tuberculosis*	09-09	human	Japan	OPIPH	2009	solid, Ogawa	N	P	rpoB, gyrA	ND	ND

UNZA: University of Zambia, Veterinary School; UTH: University Teacing Hostipal; OPIPH: Osaka Prefecture Institute of Public Health; M-PCR: MTC-discrimination multiplex PCR;

ND: Not determined

CS: Cold storage abattoir

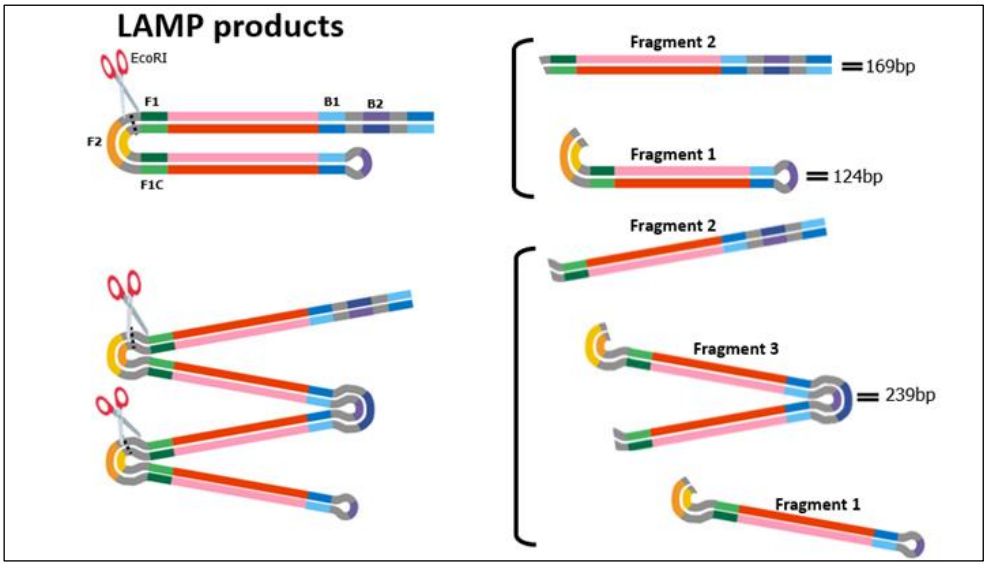


Fig. 9: *In silico* prediction of LAMP products digestion by *Eco*RI. Three different sizes of LAMP products were generated. Fragment 1 = 124 bp, fragment 2 = 169 bp and fragment 3 = 239 bp.

Chapter II

Field evaluation of dry loop-mediated isothermal amplification assay for specific detection of *Mycobacterium bovis*

Introduction

In the previous study, I developed a LAMP assay for specific detection of *M. bovis*, the causative agent of bTB and zoonotic TB in cattle and humans, respectively. One of the hiccups in implementing LAMP method in resource-limited areas has been the preservation of reagents, particularly enzymes that require storage at -20^{o}C. Hence the challenge is to develop a LAMP kit in a ready to use format with dried reagents useful for a quick and simple application in field conditions. Commercial LAMP kits for TB and Malaria diagnosis have been developed (https://www.human.de/products/molecular-dx/), and these have provided the hope of developing efficient dry LAMP systems.

Two methods for stabilizing LAMP reaction reagents in a dried kit format have been utilized, and these are, drying [100] and lyophilization [129]. The latter allows all LAMP reaction reagents to be initially included, while in the former, a single or two-step drying procedure is required using the direct flow of clean air. Trehalose is the major component in LAMP reagents drying based procedures. Trehalose resists extreme dehydration and temperature conditions. An advantage of trehalose is cheap availability because it is produced naturally by several microorganisms [130,131].

A dry LAMP system was developed in the present study by drying up liquid LAMP reagents developed in the previous study.

Materials and Methods

Preliminary preparation of dry LAMP reaction reagents

The dry LAMP reaction mixture was prepared in a final volume of 10.70 µl consisting of 2.0 µl of primer mix comprising of 100 µM mixed at 1:8:2 ratios of each outer primers (F3 and B3), inner primers (FIP and BIP) and loop primers (LF and LB); 1.4 µl of dNTPs (25 mM each); 2.5 µl of 2 M trehalose; 1 µl of 25×LAMP buffer (500 mM Tris-HCl [pH 8.8], 250 mM KCl); 1.8 µl of 100 mM MgSO4; 8 U Bst 2.0 warm start DNA polymerase (New England Biolabs) and 1 µl of colourimetric fluorescence indicator. The mixture was placed in the peripheral part of 0.2 ml microtube rid and dried inside a direct flow of clean air in a vacuum container with phosphorus oxide (P_2O_5) and silica gel. Upon confirmation of complete drying, the kits were stored at room temperature in

an opaque plastic with silica gel bags until use. Dry LAMP kits were transported from Japan to Malawi for field analysis.

Sampling, sample preparation and DNA extraction

Samples were collected during routine post-mortem examination from cattle slaughtered at three major regional abattoirs; Mzuzu in the Northern region, Lilongwe cold storage in the Central region, and Sidik and Abida (S and A) in the Southern region between August 2019 and February 2020 (Fig. 10). Fig. 11 summarizes sample collection, processing, and detection by the dry LAMP method. Cattle samples were collected during routine post-mortem examination as mandated by FAO [14]. The sample size was estimated based on a 50% prevalence assumption at a 95% confidence interval and 5% absolute precision since there were no previous studies on bTB prevalence in Malawi (Pearl, 2006). Cattle with suspected TB-like lesions had the tissue trimmed, packed in a zip-lock plastic bag, and transported (in a cooler box with ice packs) to the National TB Reference Laboratory, Lilongwe, for storage. The tissue was ground to a paste, decontaminated in 4% NaOH for 15 minutes followed by neutralization in PBS, and centrifuged at 3200 g for 20 minutes. The pellet was re-suspended in 800 µl PBS and divided into two folds; (i) 300 µl of contents for direct DNA isolation and, (ii) 500 µl was inoculated in BACTEC MGIT 960 tube (0.5 ml) following manufacturer's user manual. In both cases, DNA was isolated by boiling method at 95ºC for 15 minutes and immediately cooling at -20ºC; this was repeated twice.

Human samples

Eighty-six clinical isolates from National TB Reference Laboratory biobank previously cultured in MGIT and confirmed as MTBC by microscopy were used. The isolates were thawed, and DNA extracted by the boiling method as explained in earlier sections. The extracted DNA was used as a template for LAMP experiments.

Dry LAMP reactions

The dry LAMP reactions were performed by adding 23 µl of double distilled water and 2 µl of template DNA into the microtube with dried LAMP reagents. The tube was turned upside down for 2 minutes at room temperature to dissolve the dried reagents on the lid before mixing by inverting upside down. Isothermal incubation was carried out at 66ºC for 40 minutes in a battery-powered digital dry bath mini incubator (Mini BSH200-HL Benchmark Scientific, USA). Results were visualized by a hand-made LED illuminator powered by AA2 batteries using naked eyes. Colour shift from violet to blight yellow indicated positive for *M. bovis.* LAMP reactions were performed in duplicates for

each sample.

Confirmation of *M. bovis* isolates

A PCR targeting IS6110 was performed as described previously [114] to confirm if the isolates belonged to MTBC. A multiplex PCR targeting RD4 locus was performed as described previously [42] to confirm *M. bovis.*

Dry LAMP stability testing

For this purpose, *M. bovis* dry LAMP tubes were stored at 27ºC for five months. Additionally, dry LAMP tubes were stored in humid chambers (IG4-1; Yamato 167 Scientific, Tokyo, Japan) set to 40°C: 90% RH and 28ºC: 90% RH up to 30 days. Test tubes were stored in closed bags containing desiccants. Half of the tubes were left open, while the other half was closed entirely. After storage under each condition, the performance of dry LAMP was evaluated using serially diluted *M. bovis* BCG Tokyo 172 from 5 pg, 500 fg, 50 fg, 40 fg, and 30 fg/reaction to determine the sensitivity.

Ethical statement

The study was ethically approved protocol number 19/09/2398) by National Health Science Research Committee (NHSRC), Ministry of Health and Population, Lilongwe, Malawi.

Results

Demographics of cattle samples from the three regional abattoirs in Malawi

During the study period, a total of 1,547 cattle were inspected from the three abattoirs as follows; 313 from Sidik and Abida (S &A) abattoir Blantyre, 960 cattle from Lilongwe cold storage, 274 cattle from Mzuzu abattoir (Tables 5 and 7). A total of 146 bTB-like lesions, mainly from lymph nodes (Tables 7 and 8), were collected as follows; 24 from S and A abattoir, 104 from Lilongwe cold storage and 18 from Mzuzu abattoir. Analysis of all samples from the three abattoirs based on MGIT culture is summarized in Table 5. Out of 146 collected samples, ten samples from S & A abattoir and 11 from Lilongwe cold storage were not cultured due to logistical constraints, so the denominator for cultures is 125. Dry LAMP assay detected 82 (65.6%) as *M. bovis,* and multiplex PCR confirmed 78 (62.4%) as *M. bovis.*

Simple random selection criteria was used to select 43 samples from Lilongwe cold storage, and sample numbers were generated by Microsoft excel. The sample size was estimated based on a 50% prevalence assumption at a 90% confidence interval and

5% absolute precision since there were no previous reports about *M. bovis* (Pearl, 2006). DNA was extracted directly from processed homogenate without culturing (Fig. 11). Out of 43 samples, 28 (65.1% CI: 50.9 – 79.4) were detected as *M. bovis* by established dry LAMP, while PCR detected 25 out of 43 samples (58.1% CI: 43.3 – 72.9) (Tables 6 and 8). In the second analysis, the 11 samples were not cultured due to logistical constraints, so the denominator for MGIT cultures is 32. Out of these, 26 (81.3% 95 CI: 67.7 – 94.8) were detected as *M. bovis* by dry LAMP, while PCR detected 23 out of 32 samples (71.9% CI: 56.3 – 87.5) (Table 6).

Based on multiplex PCR as a reference method for the identification of *M. bovis*, the sensitivity of established dry LAMP was estimated at 97.4% (91.1 – 99.3%) on MGIT culture and 92.6% (76.6-97.8%) on directly extracted DNA samples. The specificity was estimated at 87.2% (74.8 – 94.0%) on MGIT culture and 81.3% (57.0 – 93.4% on direct extracted DNA samples. Overall, 4.91% (95% CI 3.83 – 5.99) of all cattle examined during this study were detected as *M. bovis*.

From 86 clinical isolates, one was detected as *M. bovis* positive. However, this study did not investigate further as it was not within the study's scope. These results demonstrate that the dry LAMP system described here could be efficiently applied for bovine TB diagnosis resource-limited areas.

Discussion

Fundamentally, eliminating bovine TB in humans cannot be fully achieved without tackling the disease in cattle as primary hosts. As such, surveying the disease in cattle plays an integral part in the disease's general prevention and control. In an effort to develop rapid, simplified, and low-cost methods that can easily be integrated into resource-limited areas, I have developed dry LAMP for specific detection of *M. bovis* by targeting a 12.7 kb locus RD4 deletion. The RD4 locus is deleted in all *M. bovis* isolates and inserted in all other MTBC species.

Initially, the LAMP assay was developed based on liquid reagents. Then, the reaction reagents were dried up to reconstitute the dry LAMP assay. The drying process described here is not cumbersome; it involves mixing the reagents into a master mix and aliquot to top of the lid of LAMP assay tubes. Then the reagents are let to dry under direct flow of air. The drying procedure is very simplified and easy to perform, unlike previous reports that indicated that drying procedures are laborious and require sophisticated

equipment [101].

This dry LAMP assay was evaluated using suspected TB-like samples collected from cattle during routine post-mortem examination at three major regional abattoirs (Fig. 10). Eighty-two isolates from MGIT culture were detected as *M. bovis* from a total of 125 suspected samples that were cultured (Table 5). *M. bovis* specific dry LAMP was also evaluated using DNA extracted directly from samples without culturing. Dry LAMP assay showed high sensitivity (92.6%) and specificity (81.3%) on these samples.

Dry LAMP established in this study has proved to be advantageous compared to PCR; first, this LAMP ably identified *M. bovis* within a short time, minimizing the time taken for PCR. The dry LAMP could take 60 minutes (from preparing reaction reagents to observing results) while the multiplex PCR for the differentiation of *M. bovis* and *M. tuberculosis* could take approximately 240 minutes. Second, LAMP assay procedures are simple and straightforward and do not require standardized laboratory equipment such as thermocyclers. In this study, a hand-made LED powered by AA2 batteries was used to visualize results. Third, the use of DNA isolated from fresh direct samples without culture reduces the time taken for *Mycobacterium*'s growth. In a standard setting, growth of *Mycobacterium* on MGIT is observed within 14 days. Forth, the drying up of reagents eliminates the need for cold chain maintenance of reagents as it is easy to transport and store the kits for more extended periods. As observed in this study, dry LAMP kits were prepared in Japan and transported to Malawi and used five months after preparation. This shows that this LAMP could be used for extended periods and still provide results are superior to PCR.

Dry LAMP developed in this study is an important tool for bovine TB surveillance in high burdened areas.

Summary

A dry LAMP assay for specific detection of *M. bovis* was developed. The kits were developed in Japan and transported to Malawi for field evaluation. Suspected TB samples were collected from cattle during routine post-mortem examination at three major regional abattoirs. Out of 1,547 animals examined, 146 samples were collected as follows; 24 from S and A valley abattoir (Southern region), 104 from Lilongwe cold storage abattoir (Central region), and 18 from Mzuzu abattoir (Northern region). Dry LAMP assay detected 82 samples as *M. bovis*. Comparatively, a multiplex PCR performed on the same samples detected 78. Forty-three tissues were sampled from Lilongwe cold storage and DNA extracted without culturing. Out of these 28, were detected and confirmed as *M. bovis*. Eighty-six clinical isolates were also analyzed, and only one was detected and confirmed as *M. bovis*.

Overall, 4.91% (95% CI 3.83 – 5.99) of all cattle examined during this study were detected as *M. bovis*. The dry LAMP developed and evaluated in this study provided results within 40 minutes, using low-cost tools to maintain the isothermal condition and detect results. Further, the drying of the reagents provided easy transportation and a long period of storage. Considering these facts, this LAMP can easily be integrated for use in resource-limited areas for the detection of *M. bovis*.

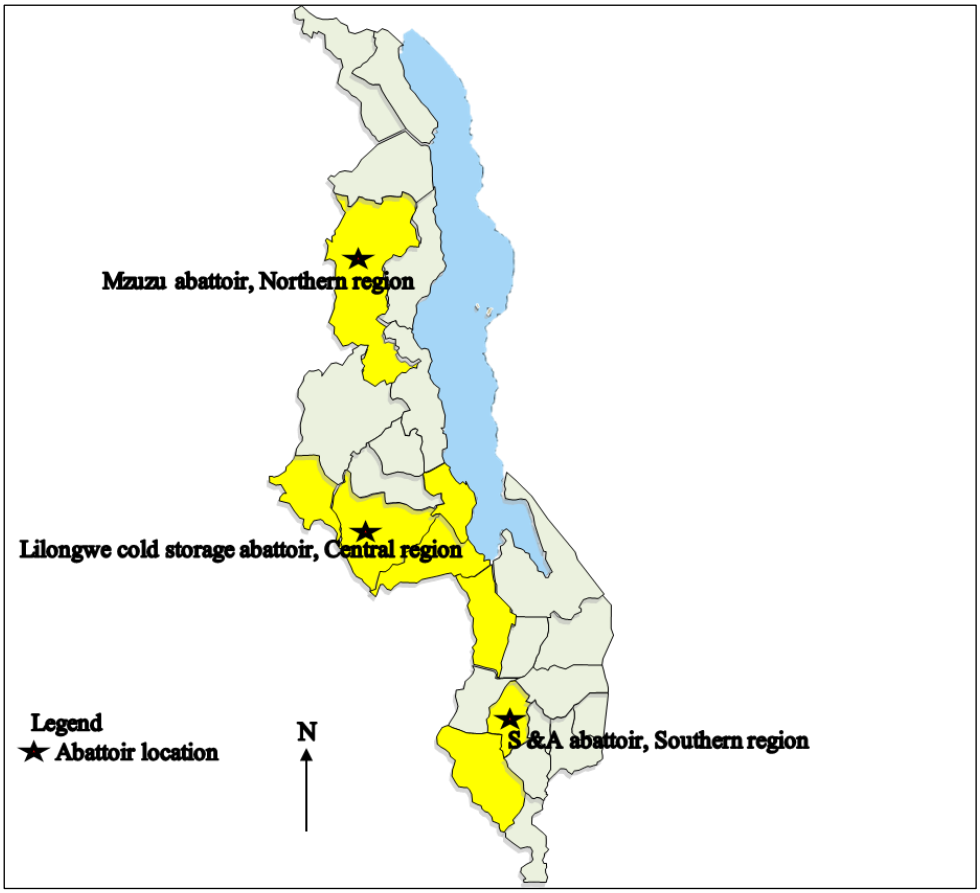


Fig. 10: Map of Malawi showing the 3 regional abattoirs where cattle samples were collected during routine post-mortem examination. S & A abattoir (Southern region), Lilongwe cold storage abattoir (Central region) and Mzuzu abattoir (Northern region). The yellow shaded areas are catchment areas supplying cattle to the abattoirs as shown on animal movement permits.

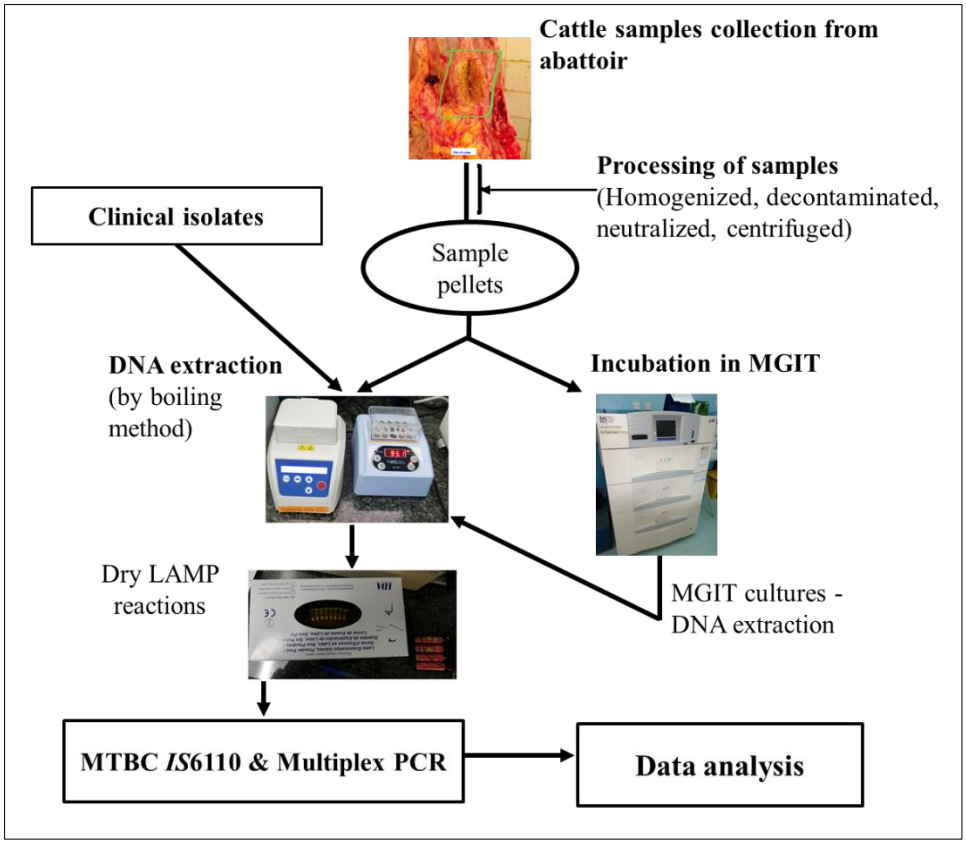


Fig. 11. Flow chart of the procedure of samples processing. Cattle samples were processed by homogenization, decontamination, neutralization and centrifugation. All the samples were cultured in MGIT. Additionally, 43 samples from Lilongwe cold storage had DNA directly extracted from the samples after initial processing.

Table 5: Summary of lesions collected from slaughtered cattle and detected as *Mycobacterium bovis* from MGIT culture

Abattoir (region)	No. of cattle inspected	No. Lesioned	Percentage of Lesioned (95% CI)	No. Cultured	No. positive on MGIT	No. *M. bovis* positive by dry LAMP	No. MTBC positive by IS6110 PCR	No. *M. bovis* positive by dry PCR	Overall percentage with *M. bovis*
S & A (Southern)	313	24	7.67 (4.72-10.62)	14*	9	6	6	5	
Lilongwe cold storage (Central)	960	104	10.83 (8.87-12.80)	93*	81	65	63	63	
Mzuzu (Northern)	274	18	6.57 (3.64-9.50)	18	13	11	10	10	
Total	1547	146		125*	103	82	79	78	4.91 (3.83 - 5.99)

*Ten samples from S &A and eleven samples from Lilongwe cold storage were not cultured due to financial constraints

Table 6: Summary of Dry LAMP performance on the 43 samples. DNA was extracted from sample tissues in the first place and later growing mycobacteria DNA was extracted from culture isolates

	DNA extracted directly from the tissue			**DNA extracted from culture isolates**			
	Dry LAMP	IS6110	PCR	MGIT	Dry LAMP	IS6110	PCR
Positive for *M. bovis*	28	25	25	30	26	23	23
Negative	15	18	18	2	6	9	9
Total	43	43	43	32[#]	32	32	32

[#] NB 11 collected tissues were not cultured due to logistical problems

Table 7: List of TB-suspected samples collected at three regional abattoirs

	Sample ID	District	Tissue	MGIT	Dry LAMP	IS6110	Multiplex PCR
1	P2398/CS-BT1	Blantyre	Lung tissue	P	N	N	N
2	P2398/CS-BT2	Blantyre	Bronchial-LN	P	N	N	N
3	P2398/CS-BT3	Blantyre	Bronchial-LN	P	P	P	N
4	P2398/CS-BT4	Blantyre	Liver tissue	N	N	N	Bovis
5	P2398/CS-BT5	Blantyre	Lung tissue	P	P	P	N
6	P2398/CS-BT6	Blantyre	Retro-pharyngeal	N	N	N	N
7	P2398/CS-BT7	Blantyre	Lung tissue	NC	NC	NC	NC
8	P2398/CS-BT8	Blantyre	Liver tissue	NC	NC	NC	NC
9	P2398/CS-BT9	Blantyre	Lung tissue	P	P	P	Bovis
10	P2398/CS-BT10	Blantyre	Parotid-LN	NC	NC	NC	NC
11	P2398/CS-BT11	Chikwawa	Bronchial -LN	NC	NC	NC	NC
12	P2398/CS-BT12	Chikwawa	Bronchial -LN	P	N	N	Bovis
13	P2398/CS-BT13	Chikwawa	Retro-pharyngeal	P	P	P	N
14	P2398/CS-BT14	Chikwawa	Mediastinal-LN	P	P	P	Bovis
15	P2398/CS-BT15	Chikwawa	Mediastinal-LN	NC	NC	NC	NC
16	P2398/CS-BT16	Chikwawa	Mediastinal-LN	N	N	N	N
17	P2398/CS-BT17	Chikwawa	Prescapular-LN	N	N	N	N
18	P2398/CS-BT18	Chikwawa	Prescapular-LN	NC	NC	NC	NC
19	P2398/CS-BT19	Chikwawa	Lung tissue	N	N	N	N
20	P2398/CS-BT20	Chikwawa	Lung tissue	P	P	P	Bovis
21	P2398/CS-BT21	Chikwawa	Lung tissue	NC	NC	NC	NC
22	P2398/CS-BT22	Chikwawa	Lung tissue	NC	NC	NC	NC
23	P2398/CS-BT23	Chikwawa	Lung tissue	NC	NC	NC	NC
24	P2398/CS-BT24	Chikwawa	Lung tissue	NC	NC	NC	NC
25	P2398/CS-MZ1	Mzuzu	Parotid -LN	P	P	P	Bovis
26	P2398/CS-MZ2	Mzuzu	Bronchial-LN	P	N	N	N
27	P2398/CS-MZ3	Mzuzu	Bronchial-LN	P	N	N	N
28	P2398/CS-MZ8	Mzuzu	Prescapular -LN	N	N	N	N
29	P2398/CS-MZ11	Mzuzu	Lung tissue	N	N	N	N
30	P2398/CS-MZ12	Mzuzu	Retro-pharyngeal	N	N	N	N
31	P2398/CS-MZ17	Mzuzu	Lung tissue	N	N	N	N
32	P2398/CS-MZ18	Mzuzu	Liver tissue	P	P	P	Bovis
33	P2398/CS-MZ19	Mzuzu	Liver tissue	P	P	P	Bovis
34	P2398/CS-MZ20	Mzuzu	Lung tissue	P	P	P	Bovis
35	P2398/CS-MZ21	Mzuzu	Bronchial -LN	N	N	N	N
36	P2398/CS-MZ23	Mzuzu	Bronchial -LN	P	P	P	Bovis
37	P2398/CS-MZ26	Mzuzu	Retro-pharyngeal	P	P	P	Bovis
38	P2398/CS-MZ27	Mzuzu	Mediastinal-LN	P	P	P	Bovis
39	P2398/CS-MZ28	Mzuzu	Mediastinal-LN	P	P	P	Bovis
40	P2398/CS-MZ29	Mzuzu	Mediastinal-LN	P	P	N	N
41	P2398/CS-MZ30	Mzuzu	Bronchial -LN	P	P	P	Bovis
42	P2398/CS-MZ31	Mzuzu	Lung tissue	P	P	P	Bovis
43	P2398/CS-L1	Ntcheu	Mediastinal-LN	P	N	N	N
44	P2398/CS-L4	Ntcheu	Bronchial -LN	N	N	N	N
45	P2398/CS-L5	Lilongwe	Lung tissue	P	P	P	Bovis
46	P2398/CS-L6	Mchinji	Bronchial -LN	P	P	P	Bovis

47	P2398/CS-L7	Mchinji	Bronchial -LN	P	P	P	Bovis
48	P2398/CS-L8	Ntcheu	Retro-pharyngeal	N	N	N	N
49	P2398/CS-L10	Ntcheu	Mediastinal-LN	P	N	N	N
50	P2398/CS-L11	Lilongwe	Liver tissue	N	N	N	N
51	P2398/CS-L13	Lilongwe	Parotid-LN	P	P	P	Bovis
52	P2398/CS-L14	Mchinji	Bronchial -LN	P	P	P	Bovis
53	P2398/CS-L15	Mchinji	Retro-pharyngeal	P	P	P	Bovis
54	P2398/CS-L16	Mchinji	Bronchial -LN	P	N	N	N
55	P2398/CS-L18	Mchinji	Bronchial -LN	P	N	N	N
56	P2398/CS-L24	Dedza	Mesenteric -LN	P	P	P	Bovis
57	P2398/CS-L25	Mchinji	Bronchial -LN	N	N	N	N
58	P2398/CS-L26	Mchinji	Mediastinal-LN	P	N	N	N
59	P2398/CS-L29	Ntcheu	Mesenteric -LN	P	P	P	Bovis
60	P2398/CS-L30	Ntcheu	Mesenteric -LN	P	P	P	Bovis
61	P2398/CS-L32	Lilongwe	Lung tissue	P	P	P	Bovis
62	P2398/CS-L33	Lilongwe	Mesenteric -LN	P	P	P	Bovis
63	P2398/CS-L35	Lilongwe	Right Brochial	P	P	P	Bovis
64	P2398/CS-L36	Lilongwe	Mediastinal-LN	P	P	N	N
65	P2398/CS-L39	Lilongwe	Right Bronchial	P	P	P	Bovis
66	P2398/CS-L43	Dedza	Lung tissue	P	P	P	Bovis
67	P2398/CS-L44	Dedza	Lung tissue	P	P	P	Bovis
68	P2398/CS-L45	Dedza	Bronchial -LN	P	P	P	Bovis
69	P2398/CS-L46	Lilongwe	Retro-pharyngeal	P	N	N	N
70	P2398/CS-L48	Lilongwe	Mediastinal-LN	P	P	P	Bovis
71	P2398/CS-L50	Mchinji	Parotid-LN	N	N	N	N
72	P2398/CS-L51	Mchinji	Bronchial -LN	N	N	N	N
73	P2398/CS-L53	Mchinji	Bronchial -LN	P	P	P	Bovis
74	P2398/CS-L54	Mchinji	Mediastinal-LN	P	P	P	Bovis
75	P2398/CS-L56	Mchinji	Mediastinal-LN	P	P	P	Bovis
76	P2398/CS-L57	Mchinji	Retro-pharyngeal	P	P	P	Bovis
77	P2398/CS-L60	Salima	Lung tissue	P	N	N	N
78	P2398/CS-L63	Lilongwe	Lung tissue	P	N	N	N
79	P2398/CS-L64	Mchinji	Lung tissue	N	N	N	N
80	P2398/CS-L65	Mchinji	Lung tissue	P	N	N	N
81	P2398/CS-L67	Lilongwe	Lung tissue	P	N	N	N
82	P2398/CS-L68	Lilongwe	Lung tissue	N	N	N	N
83	P2398/CS-L73	Lilongwe	Lung tissue	N	N	N	N
84	P2398/CS-L74	Salima	Lung tissue	P	P	P	Bovis
85	P2398/CS-L82	Lilongwe	Lung tissue	N	N	N	N
86	P2398/CS-L84	Lilongwe	Lung tissue	P	P	P	Bovis
87	P2398/CS-L85	Salima	Lung tissue	P	P	P	Bovis
88	P2398/CS-L86	Salima	Lung tissue	P	P	P	Bovis
89	P2398/CS-L87	Salima	Lung tissue	P	P	P	Bovis
90	P2398/CS-L89	Salima	Lung tissue	P	P	P	Bovis
91	P2398/CS-L90	Salima	Lung tissue	P	P	P	Bovis
92	P2398/CS-L91	Lilongwe	Lung tissue	P	P	P	Bovis
93	P2398/CS-L92	Lilongwe	Lung tissue	P	P	P	Bovis
94	P2398/CS-L93	Lilongwe	Lung tissue	P	P	P	Bovis

95	P2398/CS-L94	Lilongwe	Lung tissue	P	P	P	Bovis
96	P2398/CS-L95	Ntcheu	Lung tissue	P	N	N	N
97	P2398/CS-L96	Lilongwe	Lung tissue	P	P	P	Bovis
98	P2398/CS-L97	Lilongwe	Lung tissue	P	P	P	Bovis
99	P2398/CS-L98	Lilongwe	Lung tissue	P	P	P	Bovis
100	P2398/CS-L99	Lilongwe	Lung tissue	P	P	P	Bovis
101	P2398/CS-L100	Dedza	Lung tissue	P	P	P	Bovis
102	P2398/CS-L101	Dedza	Parotid-LN	P	P	P	Bovis
103	P2398/CS-L104	Dedza	Bronchial-LN	P	N	N	N

All samples were collected during routine cattle post-mortem examinamination

P: Positive, N: negative, NC: Not cultured, Bovis: *Mycobacterium bovis,* CS: Cattle sample, BT: Blantyre (S& A abattoir), LL: Lilongwe (Lilongwe cold storage abattoir), MZ: Mzuzu (Mzuzu abattoit)

Table 8: List of samples (DNA directly extracted) used for evaluation of dry LAMP

				Direct DNA extraction			Cultured			
No.	Sample ID	District	Organ	Dry LAMP	IS6110	mPCR	MGIT	Dry LAMP	IS6110	mPCR
1	P2398/CS-L2	Ntcheu	Lung tissue	P	N	N	NC	NC	NC	NC
2	P2398/CS-L3	Ntcheu	Retro-pharyngeal	P	P	Bovis	P	P	P	Bovis
3	P2398/CS-L9	Mchinji	Bronchial-LN	P	P	Bovis	P	P	P	Bovis
4	P2398/CS-L12	Mchinji	Liver tissue	P	P	Bovis	P	P	P	Bovis
5	P2398/CS-L17	Lilongwe	Mesenteric-LN	P	P	Bovis	P	P	P	Bovis
6	P2398/CS-L19	Mchinji	Mesenteric-LN	N	N	N	NC	NC	NC	NC
7	P2398/CS-L20	Lilongwe	Lung tissue	P	P	Bovis	P	P	P	Bovis
8	P2398/CS-L21	Lilongwe	Mediastinal-LN	P	P	Bovis	P	P	P	Bovis
9	P2398/CS-L22	Mchinji	Mesenteric-LN	N	N	N	NC	NC	NC	NC
10	P2398/CS-L23	Lilongwe	Mediastinal-LN	P	P	Bovis	P	P	P	Bovis
11	P2398/CS-L27	Mchinji	Bronchial-LN	P	P	Bovis	P	N	N	N
12	P2398/CS-L28	Ntcheu	Bronchial-LN	N	N	N	P	N	N	N
13	P2398/CS-L31	Ntcheu	Retro-pharyngeal	P	P	Bovis	P	P	N	N
14	P2398/CS-L34	Mchinji	Mediastinal-LN	N	N	N	NC	NC	NC	NC
15	P2398/CS-L37	Lilongwe	Prescapular-LN	N	N	N	N	N	N	N
16	P2398/CS-L38	Lilongwe	Mediastinal-LN	P	P	Bovis	NC	NC	NC	NC
17	P2398/CS-L40	Lilongwe	Lung tissue	P	P	Bovis	P	P	P	Bovis
18	P2398/CS-L41	Lilongwe	Mediastinal-LN	N	N	N	NC	NC	NC	NC
19	P2398/CS-L42	Lilongwe	Lung tissue	P	P	Bovis	P	P	P	Bovis
20	P2398/CS-L47	Mchinji	Mediastinal-LN	N	N	N	P	P	N	N
21	P2398/CS-L49	Lilongwe	Retro-pharyngeal	P	P	Bovis	NC	NC	NC	NC
22	P2398/CS-L52	Mchinji	Mediastinal-LN	P	P	Bovis	P	P	P	Bovis
23	P2398/CS-L55	Lilongwe	Mediastinal-LN	N	N	N	P	N	N	N
24	P2398/CS-L58	Lilongwe	Mediastinal-LN	P	P	Bovis	P	P	P	Bovis
25	P2398/CS-L59	Ntcheu	Partial-LN	P	N	N	N	N	N	N
26	P2398/CS-L61	Lilongwe	Brochial-LN	P	P	Bovis	P	P	P	Bovis
27	P2398/CS-L62	Lilongwe	Retro-pharyngeal	P	P	Bovis	P	P	P	Bovis
28	P2398/CS-L66	Lilongwe	Lung tissue	P	P	Bovis	P	P	P	Bovis
29	P2398/CS-L69	Mchinji	Lung tissue	P	P	Bovis	P	P	P	Bovis
30	P2398/CS-L70	Ntcheu	Retro-pharyngeal	P	P	Bovis	P	P	P	Bovis
31	P2398/CS-L71	Ntcheu	Lung tissue	N	N	N	NC	NC	NC	NC
32	P2398/CS-L72	Ntcheu	Liver tissue	P	P	Bovis	P	P	P	Bovis
33	P2398/CS-L75	Lilongwe	Lung tissue	N	N	N	P	P	N	N
34	P2398/CS-L76	Lilongwe	Lung tissue	P	P	Bovis	P	P	P	Bovis
35	P2398/CS-L77	Ntcheu	Lung tissue	N	N	N	P	N	N	N
36	P2398/CS-L78	Lilongwe	Lung tissue	N	N	N	NC	NC	NC	NC
37	P2398/CS-L79	Lilongwe	Lung tissue	P	P	Bovis	P	P	P	Bovis
38	P2398/CS-L80	Lilongwe	Lung tissue	P	P	Bovis	P	P	P	Bovis
39	P2398/CS-L81	Lilongwe	Liver tissue	N	N	N	P	P	P	Bovis
40	P2398/CS-L83	Lilongwe	Lung tissue	P	N	N	P	P	P	Bovis
41	P2398/CS-L88	Salima	Lung tissue	P	P	Bovis	NC	NC	NC	NC
42	P2398/CS-L102	Salima	Mediastinal-LN	N	N	N	P	P	P	Bovis
43	P2398/CS-L103	Dedza	Mesenteric-LN	N	N	N	NC	NC	NC	NC

P: positive, N: negative, NC: Not cultured, Bovis: *Mycobacterium bovis* , LN: Lymph node

Chapter III

Molecular characterization of *Mycobacterium bovis* isolates from the central parts of Malawi

Introduction

BTB remains a significant public health problem in Africa, mainly because of the existence of multiple risk factors, absence of surveillance and robust control strategies such as test and slaughter of reactive cattle. The HIV/AIDS epidemic's high prevalence has also significantly worsened the bTB situation [17,132,133]. In Southern Africa, especially Mozambique, South Africa, and Zambia, and the Eastern African Tanzania region, bTB molecular epidemiology has helped understand the disease's in-depth [66,68,85,86,134,135]. For instance, in South Africa, Mozambique and Zambia, the *M. bovis* European 1 (Eu1) clonal complex is dominantly circulating in cattle. Low prevalence of the BCG like spoligotypes has also been reported. The introduction of Eu1 in South Africa has been linked to colonial cattle trade from European countries [136]. In Tanzania, African 2 clonal complex has been described prevalent [137,138]. *M. bovis* has been linked to having originated from the east African region [138]. However, there is no information about the molecular characteristics of *M. bovis* strains in Malawi despite sharing open borders with Mozambique, Zambia, and Tanzania [139]. Past reports highlighted the presence of bTB in cattle in Malawi. However, this information is based on microscopy, tuberculin skin testing, and slaughterhouse post-mortem examination [27,91,140–142]. As seen by past investigations, the geographical variations of bTB in Malawi generally reflect the lack of routine surveillance systems [139].

In Malawi, cattle move within and inter-district, searching for grazing pasture mainly during the dry season. Elsewhere, such uncontrolled cattle movement has been linked to exacerbate bTB transmission and a significant drawback in implementing successful bTB control strategies [86]. Livestock trade and open borders between Malawi and neighbouring countries (Zambia, Mozambique, and Tanzania) could also promote the spread of bTB in the region. Since test and slaughter of reactive cattle can not be implemented in Malawi because of associated economic constraints, molecular studies provide a better option to understand the origin of infections, circulating pathogens populace, and transmission dynamics. Such information is indispensable in planning elective control strategies of the disease, such as zoning, livestock movement restrictions and so on. [143].

In the epidemiological studies of infectious diseases, defining the source of infection, routes of transmission and dissemination in the environment are considered key aspects. To achieve this, the ability to discriminate and tracing individual *Mycobacterium* strains is invaluable. Therefore, to understand the molecular epidemiology of *M. bovis*, in central parts of Malawi, I have employed molecular typing methods spoligotyping and MIRU-VNTR [67]. These techniques have contributed to the understanding of spatial dynamics and phylogeography of bTB and zoonotic TB [66,68,86,134,144–146]. Furthermore, spoligotyping and MIRU-VNTR have been utilized together with the region of difference (RD) deletion analysis to define the *M. bovis* global clonal complexes [137,147,148].

Materials and methods

Study design and sampling protocol

Sampling has been explained in chapter 2. Briefly, samples were collected during the routine abattoir cattle examination at Lilongwe cold storage abattoir, Malawi, and the procedures were carried out as described previously [149]. Suspected granulomatous bTB lesions were collected with accompanying demographical data such as the district of origin, type of organ or tissue, and sex of the animal. Samples were placed in sterile self-zipping plastic bags and placed in a cooler box with ice packs before transported to the National TB Reference Laboratory (NTRL). Samples were stored at -20^{o}C until use.

Mycobacterial culture and DNA extraction

Tissue was thawed and trimmed of fat using a pair of scissors and forceps. The sample was minced and grounded to paste in a sterilized glass homogenizer. One millilitre of PBS (pH 6.8) was added and decontaminated by adding 1 ml of 4% NaOH before incubating for 15 minutes at room temperature. Then, 10 ml of PBS was added and centrifuged at 3200 g for 20 minutes at 6^{o}C. The supernatant was discarded, and the pellet was suspended in 500 µl PBS and inoculated in BACTEC MGIT 960 tube following the manufacturer's user manual and monitored automatically. DNA was isolated by the boiling method by heating at 95^{o}C for 15 minutes and immediately cooling at -20^{o}C for 30 minutes; this was repeated twice.

Mycobacterial identification and confirmation

We screened all isolates using dry LAMP assay as described in chapter 2. Briefly, Twenty-three microliter of double distilled water and 2 µl of extracted DNA were added into the 0.2 ml LAMP tubes. The rid was tightly closed, turned upside down and, incubated at room temperature for 2 minutes. The contents were mixed by inverting

upside down five times. Isothermal incubations were carried out at 66^{o}C for 40 minutes in a battery-powered digital dry bath min incubator (Mini BSH200-HL Benchmark Scientific, USA). A hand-made LED illuminator was used to visualized results. DNA was transferred to Hokkaido University, Japan, for further analysis.

To confirm isolates as MBTC was achieved by a PCR targeting IS6110 as described previously [114]. A multiplex PCR targeting RD4 was used to confirm the isolates as *M. bovis* [42]. Gel electrophoresis was performed to visualize all PCR products.

Molecular typing of *Mycobacterium bovis* isolates

M. bovis clonal complex

We utilized a PCR targeting chromosomal deletions RDAf1, RDAf2, and RDEu1 for African 1, African 2, and European 1 clonal complex, respectively, as previously described [137,147,148]. Briefly, primers targeting RDAf1, FW-ACTGGACCGGCAACGACCTGG, Rev-CGGGTGACCGTGAACTGCGAC, and Int Rev-CGGATCGCGGTGATCGTCGA- were used to obtain a product of 350 bp (intact) or 531 bp (deleted) [148]. For RDAf2, FW-ACCGCCCTGTCCTATGTGAG, Rev-TGACGGTTGCCTTTCTTGAC, and Int Rev-CACTGTCTCCGCTCATCATG were used, and a PCR product size of 458 bp (intact) and 707 bp (deleted) was expected [137]. RDEu1 was amplified with primers FW-CCGATGAACTTGGCCCACAG, Rv-CGTGGTGGTGGGATGTCTTG, and a PCR product size of 1206 bp (intact) and a 401 bp (deleted) was expected [147].

Spoligotyping

Following the procedures previously described by Kamerbeek and others [118], spoligotyping was done with slight modification. Briefly, an initial PCR targeting the direct repeat region (DR) was performed in a total volume of 15 µl comprising 1 µl each of the 10 µM primer, 7.3 µl double distilled water, 3 µl of 5 x colourless Go Taq buffer (Promega™, Fitchburg, WI), 1.5 µl of PCR DIG Labeling Mix (Roche), 0.2 µl of Go Taq DNA Polymerase (5 units/µl; Promega) and 1 µl of DNA sample. The PCR reaction conditions were as follows; initial denaturation step (98°C for 1 minute), followed by 40 cycles of denaturation (98°C for 5 seconds), annealing (55°C for 10 seconds), and initial elongation (72°C for 30 seconds) and final elongation step (72°C for 1 minute). This was followed by hybridization, labelling and colouring of the spoligotyping membrane (Spoligo µ-PAS, Tohoku Bio Array). The presence or absence of spacers was recorded as ‘1’ or ‘0’ respectively. The decoded octal code number was entered into the database https://www.mbovis.org/database.php to generate the corresponding SB number.

MIRU-VNTR typing

We used 26 MIRU-VNTR loci described previously [66] in independent PCR reactions using previously reported primers [63] to genotype the isolates. Failed locus was repeated once, and the isolates that failed to amplify more than ten loci were removed from the analysis. Briefly, only MIRU26 was performed under GCII buffer (Takara) while the remaining 25 loci were performed under betaine 1.0 M. The standard PCR protocol for all loci except loci QUB11b, MIRU4, and QUB4156 was as follows; initial denaturation step at 95°C for 5 minutes and 32 cycles of denaturation at 95°C for 15 seconds, primer annealing 55°C for 20 seconds and extension at 72°C for 45 seconds with a final extension at 72°C for 1 minute. The primer annealing temperature was changed to 50^{o}C for loci QUB11b, MIRU4, and QUB4156. PCR products were visualized on 2% agarose gel prepared in 0.5 times TBE buffer stained with Ethidium bromide. The repeat numbers were deduced from PCR product bands using a reference chart.

Data management and statistical analysis

Spoligotyping and MIRU-VNTR typing data were imported as characters into Bionumerics software package version 8 (Applied Maths, Sint-Martens-Latem, 2020).

Phylogenetic analysis

Spoligotyping and MIRU-VNTR data were analyzed to determine *M. bovis* genotypes. An unweighted pair group average linkage algorithm (UPGMA) tree and Minimum spanning tree (MST) were used to present isolates' genetic relationships based on distance matrices with minimum phylogenetic distances set at a single locus variation (SLV). This study defined a cluster as two or more isolates sharing similar spoligotype or MIRU-VNTR pattern.

Allelic and genotypic diversity

Allelic diversity (h) for each of the 26-MIRU VNTR loci was estimated using the following formula.

$$h = 1 - \sum x_i^2 \left[\frac{1}{n(n-1)}\right]$$

where *n* is the number of isolates in the analysis and *xi* is the frequency of the ith allele at the locus [150]. MIRU-VNTR loci were classified as highly (h>0.6), moderately (0.3<h<0.6), or poorly discriminatory (h<03). The Homoplasy of MIRU-VNTR types was determined if more than one different spoligotype was shared. Spoligotyping and

MIRU-VNTR typing discriminatory power was estimated by the Hunter Gaston Discriminatory Index (HGDI) [151] using the following formula;

$$\mathrm{HGDI} = 1 - \frac{1}{\mathrm{N}(\mathrm{N}-1)} \sum_{\mathrm{J}=1}^{\mathrm{s}} \mathrm{nj}(\mathrm{nj}-1)$$

where N represents the total number of observed genotypes, s is the total number of unique genotypes, and nj is the number of genotypes belonging to the j^{th} genotype

Estimation of *M. bovis* clustering rate

The clustering rate was estimated using the formula (clustering rate = number of clustered isolates - number of clusters)/Total number of isolates [152]. We assumed that one sample corresponded to the index case at the origin of bTB infection from each district.

Genetic relationships of Malawian *M. bovis* with neighbouring countries

We combined *M. bovis* data from the current study (60 isolates) with Zambia (80 isolates), Mozambique (57 isolates) and, Tanzania (27 isolates) [66,68,85,137,148,151,153]. Among these studies, five divergent VNTR markers were shared, namely MIRU577, MIRU580 MIRU2165, MIRU2461, and MIRU3192. The VNTRs were used to construct the phylogenetic tree for their high resolution [154]. Sample selection was based on the availability of the 5 VNTRs.

Ethical statement

The study was ethically approved protocol number 19/09/2398) by National Health Science Research Committee (NHSRC), Ministry of Health and Population, Lilongwe, Malawi.

Results

Sample collection

All samples were collected during routine postmortem examination at Lilongwe cold storage abattoir in Lilongwe, Malawi (Fig. 12 (a)). A total of 960 cattle were examined during the study period (Table 9). Of these, 104 animals had granulomatous TB-like lesions, and samples were collected. Eleven samples were not cultured due to logistical constraints; hence the denominator is 93. Eighty-one samples were positive in MGIT, and from these, 63 (0.778: 95% Confidence Interval (CI) 0.687 – 0.868) were confirmed *as M. bovis* (Table 9).

Geographical distribution and diversity of *M. bovis* spoligotypes

Out of 63 isolates, 51 (81%) belonged to the European 1 clonal complex. Twelve isolates were classified as BCG-like derived for lacking additional spacers besides spacers 3, 16, and 39 - 43 markers of *M. bovis* strains. Spoligotyping revealed eight unique profiles (Fig. 13 and Table 14). SB0131 was the dominant spoligotype accounting for 56% (35/63) of the isolates (Table 10). SB0273 and SB0425, respectively, were revealed in 14% and 13% isolates. Spoligotypes SB0131 and SB0273 were recovered from all the districts (Fig. 12 (b)), while SB1056 and SB 0274 were exclusively identified from Lilongwe district. SB0425 was shared between Lilongwe and Mchinji districts (Fig. 12 (b)). SB0961was recovered from Mchinji, Dedza, and Lilongwe isolates and had spacer 11 present (Figures 12B). Spoligotyping discriminatory power was estimated at 0.6652 (95% CI: 0.693-0.637).

Mycobacterium bovis genotype diversity in Central parts of Malawi

Fig. 14 shows the minimum spanning tree depicting the isolates' phylogenetic relationships based on the 26 loci MIRU VNTR typing. The isolates were clustered into two main complexes 1 and 2. In both complexes, isolates were mostly recovered from Lilongwe and Mchinji with predominant spoligotype SB0131. Notably, SB0274 belonged to complex 1 only, while SB0961, SB1031, and SB1056 belonged to complex 2. SB0425 and SB0273 shared both complexes.

Results of the 26 loci allelic diversity are summarized in Table 11. Loci. QUB-3232 and MIRU-16 were highly discriminatory ($h > 0.6$) while seven loci, namely, MIRU-10, MIRU-26, ETR-A, ETR-B, ETR-C, QUB-11a, and QUB-11b were considered moderately discriminatory ($0.3 < h < 0.6$). Six loci, namely, ETR-F, MIRU-27, Mtub-30, QUB-26, Mtub-04, and MIRU-31, had low discriminatory power ($h<0.3$), while the remaining loci were monomorphic for all the isolates collected in this study. MIRU-VNTR's discriminatory power was 0.959 (95% CI: 0.963–0.955).

MIRU-VNTR typing discriminated the eight spoligotypes into 31 unique genotypes (Fig. 15). SB0131, the dominant spoligotype, was further differentiated into 19 genotypes (Table 12). SB0273 and SB0425 were discriminated into 6 and 5 genotypes, respectively. Lilongwe district had the highest genotypes diversity (0.995) (Fig 16), followed by Mchinji (0.969) (Fig. 17), Dedza (Fig. 18) and Ntcheu (Fig 19) with the same genotype diversity at 0.933, while Salima (Fig. 20) had the lowest genotypes at 0.717. Considerable homoplasy was observed with fifteen MIRU-VNTR types shared by more

than one spoligotype. MIRU-VNTR types V10 and V28 accounted for 46% of the observed homoplastic types and were mostly encountered in Lilongwe district (Table 13).

Estimating *M. bovis* clustering rate

We estimated the *M. bovis* clustering rate using the following formula; (clustering rate = number of clustered isolates -number of clusters)/Total number of isolates [152]. The overall clustering rate was estimated at 33%.

Genetic relationships of Malawian *M. bovis* with neighbouring countries

The minimum spanning tree of 221 isolates from Malawi (current study data), Zambia, Mozambique, and Tanzania is shown in Fig. 21. SB0131 strains from Malawi predominated group (A) alongside Zambian isolates with the same spoligotype. A few strains from Malawi, SB0961, and SB0131, clustered in group (B) dominated by Mozambican isolates with SB0961 spoligotype. Group C was shared by SB0273, SB0131and SB0425 isolates from Malawi and Mozambican SB0961. There was a distance relationship of 2-3 loci between isolates from Malawi and Tanzania sharing the same spoligotype SB0425.

Discussion

In the current study, the molecular epidemiology of *M. bovis* in cattle from the central parts of Malawi was carried out for the first time. *M. bovis* was isolated from 63/104 (0.615: 95% CI 0.522-0.709) cattle with granulomatous bTB-like lesions slaughtered at the Lilongwe cold storage abattoir (Fig 12 (a) and Table 9). The isolation rate of *M. bovis* was lower than the 73% observed previously in Tanzania [134] but higher than those observed in studies from Mozambique [68] and Zambia [66]. The variations in the isolation rate could result from differences in bTB prevalence in the specific study areas. For instance, a high prevalence of 13% was reported in Tanzania [89]. Additionally, the variation can result from different types of culture media used among the studies; MGIT culture has a higher recovery rate compared to solid media[155]. Finally, expertise in the identification of bTB-like lesions may differ across studies, leading to divergence in isolation rates from lesions.

Our investigation reveals that 81% of *M. bovis* isolates from the study sites in Malawi belonged to the Eu1 clonal complex. Evidence of high frequency of these strains has been documented in European countries; e.g., Great Britain (97%, of 490 isolates), Northern Ireland (100%, of 528 isolates), and the Republic of Ireland (100%, of 503 isolates) [156]. However, the Eu1 clonal complex has been rarely found in the African region except in Mozambique [68] and South Africa [136]. The distribution of Eu1 infers European countries as the possible source [147]. Previous reports suggested that the Eu1 clonal complex was introduced into Mozambique from South Africa and Malawi through cattle trade [68]. The spoligotype SB0131, reported in Zambia [66] is from the Eu1 clonal complex and indicates that this clonal complex is widespread in this region. In the current

study, we did not find strains belonging to Af1 and Af2 clonal complexes. This agrees with previous reports that the two *M. bovis* clonal complexes are mostly prevalent in the western and eastern African regions [137,148].

In this study, a total of eight unique spoligotypes were identified, and SB0131 was the predominant accounted for 56% of all isolates (Table 10). Evidence of *M. bovis* clonal expansion in the study setting is supported by the geographical localization and genetic association of spoligotypes (Figures 12 (a) and Fig. 15). For instance, SB0131 is widespread in the study areas; the high diversity in Lilongwe district suggests that the SB0131 was first introduced in the area and later spread to other districts. The data also show that SB0273 strains underwent a similar expansion to SB0131. Our study provides significant evidence supporting previous reports that indicated Lilongwe as the focal point of bTB outbreaks, linked to the high number of small dairy farms in the district [92]. SB0131 strains were previously reported circulating in Zambian and South African cattle but not in wildlife [66,70]. This scenario suggests that the introduction of SB0131 to Malawi was through cattle importation from other countries by the colonial settlers [132]. If this is the case, cattle importation and trade may be the driving forces responsible for expanding SB0131 in this region. SB0961, a BCG-like derived type, was isolated in Lilongwe and Dedza districts. This spoligotype was found predominantly (n = 109, 61.2%) in Mozambique [57]. The drivers for expansion in this region are linked to trade and unrestricted movement of cattle between Malawi and Mozambique. Of interest, the genetic characteristics of SB0425 strains. differed from those observed in Tanzania in the same spoligotype [137]. The genetic differences highlight the distinct provenance of these strains, as well as by their geographical distance, limited direct contact linkages, and absence of livestock trade between the study's setting (in Malawi) and Tanzania. In this study, the strains with BCG like pattern were less prevalent (Fig. 13). Our findings support previous findings that the BCG-like type has little discriminatory power [138].

MIRU-VNTR typing differentiated the unique eight spoligotypes into 31 genotypes (Fig. 15). Our report agrees with previous reports that MIRU-VNTR provides high discrimination, an attribute of individual loci's diversity [157,158]. MIRU-VNTR locus QUB3232 and QUB11a showed high discrimination of the isolates (Table 11). QUB11a was also reported as having high discriminatory power in Mozambique. MIRU40, QUB4156, MIRU02, MIRU20, and MIRU23 had no discrimination in the current study (Table 11); these loci have shown similar results in Mozambique [57]. Our study concurs with Malama et al., (2014) that a 9 loci MIRU-VNTR analysis is a suitable alternative in this region. MIRU-VNTR typing has demonstrated evidence of *M. bovis* evolution and expansion in the study area. For example, SB1031 clustered with SB0131 (V14), SB0273 with SB0131 (V28) with one locus loss, while SB0425 clustered with SB0131 (V1) with a loss of 2 loci (Fig. 15). Additionally, the relatively high genetic diversity of *M. bovis* in Lilongwe, Mchinji, Dedza, and Ntcheu districts (Figures 16 - 20) highlights the importance of uncontrolled cattle movement occurring within and between the districts, thus driving the dissemination of *M. bovis* strains. The within- and inter-districts livestock movements generally result from searching for pasture, especially during the dry season. Additionally, unregulated livestock markets and movements between Malawi, Mozambique, and Zambia allow cattle contacts, hence resulting in the transmission and dissemination of strains of *M. bovis*. The overall clustering rate was

estimated at 33%, suggesting earlier introduction and that *M. bovis* has been circulating for quite a long time, supporting the facts that bTB was introduced in Malawi well before the country's independence in 1964 [92] and that the east African region could be the ancestral origin of *M. bovis* [138].

We observed genetic similarities among isolates from Malawi, Mozambique, and Zambia (Fig. 21). SB0961 strain recovered from Malawi shared the same cluster with Mozambique isolates, while SB0131 from Malawi clustered with isolates from Zambia bearing the same spoligotype. In both cases, the strains shared the same number of MIRU-VNTR repeats. These similarities can be attributed to the pre - and post-colonial livestock trade between these countries. Livestock reports indicate that exotic dairy breeds were imported from Zambia and South Africa to improve Malawi's dairy industry. Although Malawi and Mozambique were colonized by the British and Portuguese, respectively, the open borders and unregulated livestock movement contributed to the spread of bTB across the two countries.

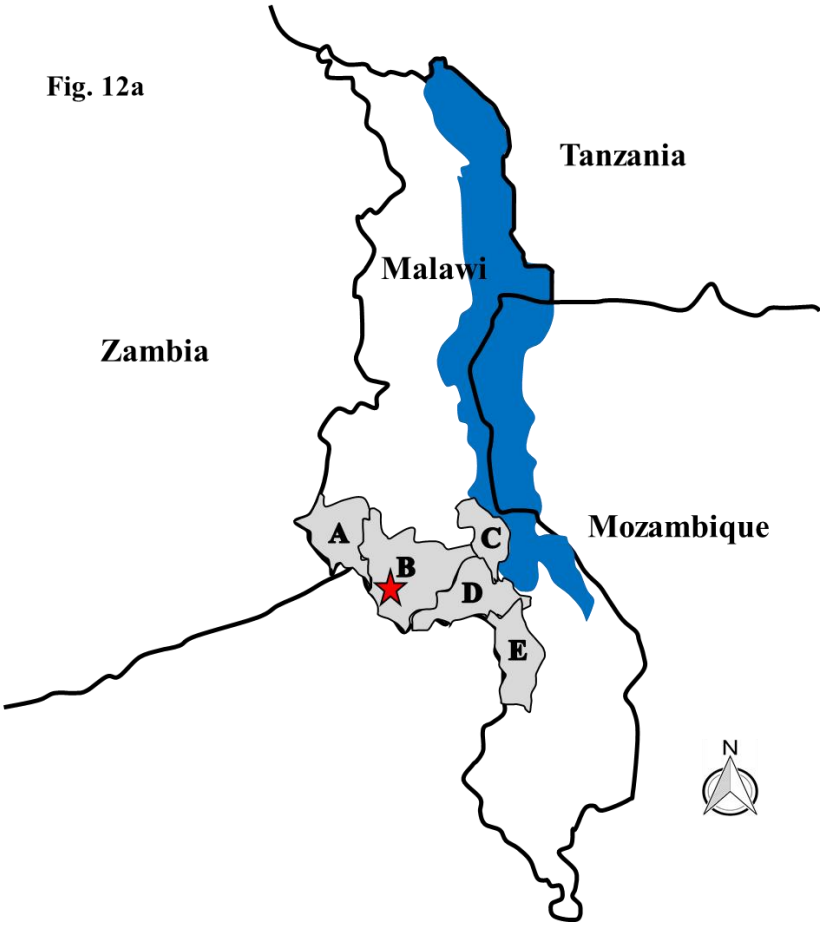
We understand that the results of this study cannot be generalized for the whole country. However, it provides an overview of what could be happening in Malawi in terms of bTB prevalence and transmission. In the future, we plan to conduct similar studies in other parts of the country to elucidate the molecular characteristics of *M. bovis* in Malawi in greater detail to provide a basis for the formulation of improved control policies.

In conclusion, this study has provided evidence of a high genetic diversity of *M. bovis* circulating in central parts of Malawi, with the isolated strains showing genetic relationships with *M. bovis* isolates previously reported in neighbouring countries. The information greatly increases our understanding of bTB in the study area and suggesting the need for the development of bTB control strategies to enhance the current control programs.

Summary

I have performed a molecular characterization on isolates from central parts of Malawi. Out of 104 samples collected during routine cattle post-mortem, 63 samples were identified and confirmed as *M. bovis.* Out of 63 samples, 51 belonged to Eu1 clonal complex, while 12 isolates were categorized as BCG-like derived for lacking additional spacers other than 6, 9, 16, and 39-43 that are molecular markers for *M. bovis*. Spoligotyping analysis reviewed 8 unique patterns that were further differentiated into 31 genotypes by MIRU-VNTR. The discriminatory power of MIRU-VNTR typing was 0.959 (95% CI: 0.963–0.955). The study found a high diversity of *M. bovis* strains circulating within Lilongwe and surrounding districts. I have highlighted the population of circulating *M. bovis* strains as predominated by SB0131 and likely imported a long time during colonial times through livestock trading systems. Further, I have linked the relatedness of isolates from Malawi to Mozambique due to regional cattle trade and unrestricted cattle movements because of open borders. The information provided by this study brings the understanding of bTB in central parts of Malawi and can aid in developing other bTB control strategies to enhance the current control programs.

Fig. 12a



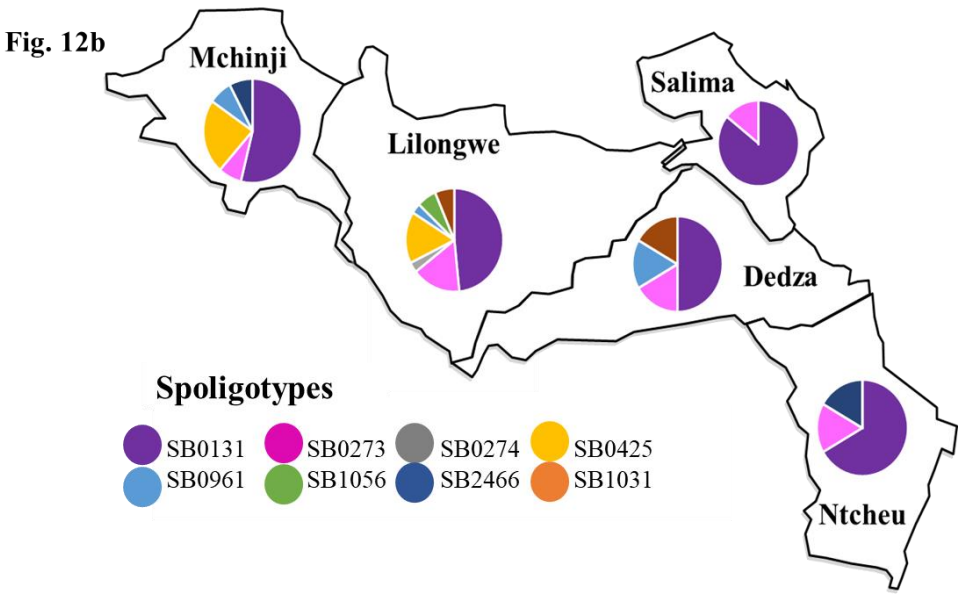


Fig. 12: Study area in Malawi and the distribution of spoligotypes in sampled districts. (a) Map of Malawi, showing administrative districts of the central region covered by this study. **A**: Mchinji, **B**: Lilongwe (Star: Lilongwe cold storage abattoir location), **C**: Salima, **D**: Dedza, and **E**: Ntcheu. (b): Spatial distribution of spoligotypes recovered from cattle from central parts of Malawi. SB0131 was recovered from recovered in all districts.

Table 9: Isolation of *Mycobacterium bovis* from cattle slaughtered at Lilongwe cold storage abattoir August – November 2019

District	No. of animals examined	No. of animals with lesions	Percentage Lesioned (95% CI)	No. confirmed as *M. bovis*	Percentage with *M. bovis* (95% CI)
Dedza	67	8	11.94 (4.18-19.70)	6	8.96 (2.12-15.79)
Salima	126	9	7.14 (2.65-11.64)	7	5.56 (1.56-9.56)
Lilongwe	415	46*	10.84 (7.85-13.83)	31	7.47 (4.94-10.00)
Ntcheu	109	16	14.68 (8.04-21.32)	6	5.50 (1.22-9.79)
Mchinji	243	25	10.29 (6.47-14.11)	13	5.35 (2.52-8.18)
Total	960	104*		63	

*Eleven samples were not cultured because of logistical constraints.

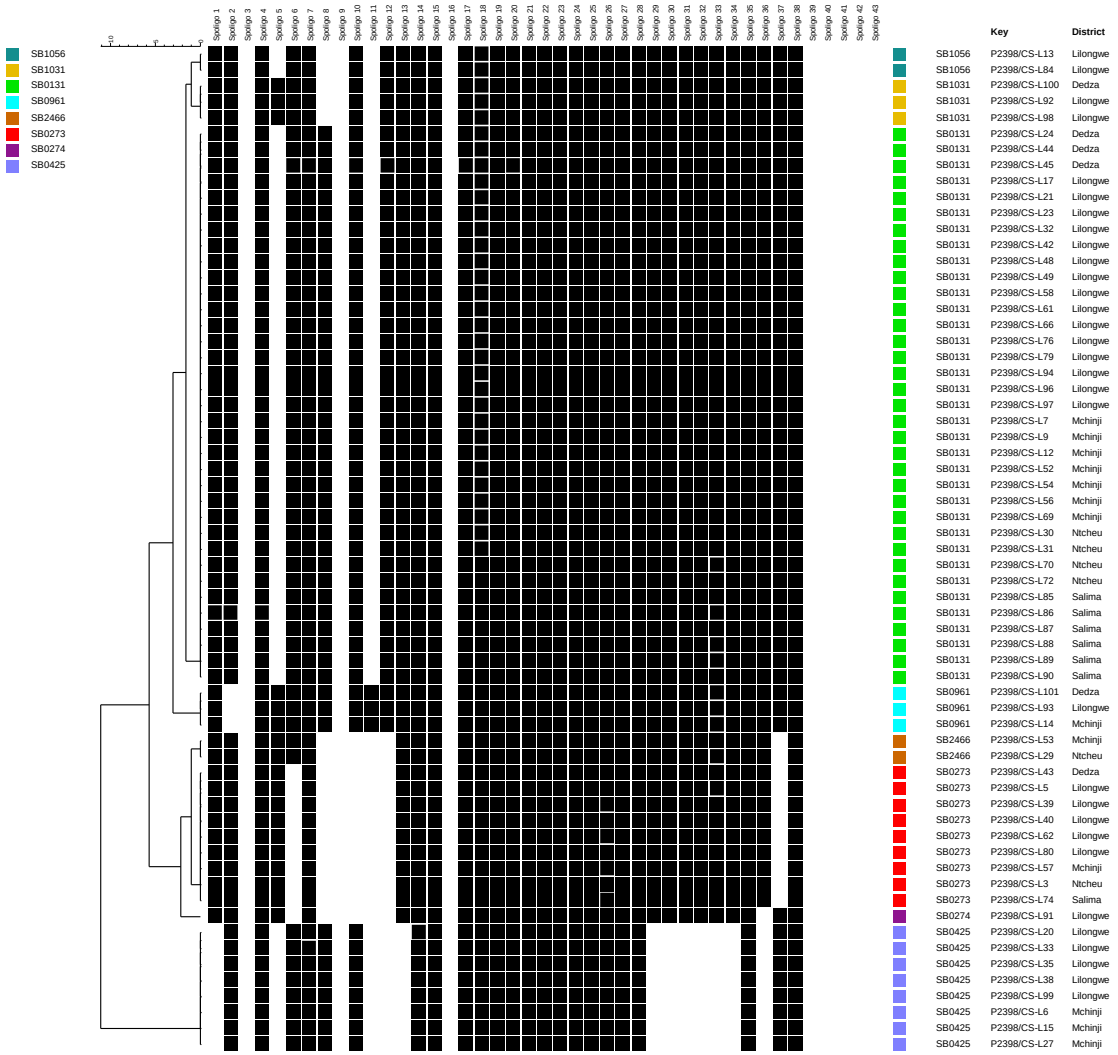


Fig. 13: A UPGMA phylogenetic tree showing the relationships of spoligotypes recovered from cattle slaughtered at Lilongwe cold storage abattoir, Lilongwe Malawi. The tree is based on the standard 43 spoligotype spacers. Eight unique profiles were recovered.

Table 10: Distribution of spoligotypes of *M. bovis* recovered from cattle slaughtered at Lilongwe cold storage abattoir between August and November 2019.

Spoligotype number	District of origin					Overall	Frequency (%)
	Lilongwe	Dedza	Mchinji	Salima	Ntcheu		
SB0131	15	3	7	6	4	35	56
SB0273	5	1	1	1	1	9	14
SB0274	1	0	0	0	0	1	2
SB0425	5	0	3	0	0	8	13
SB0961	1	1	1	0	0	3	5
SB1056	2	0	0	0	0	2	3
SB2466	0	0	1	0	1	2	3
SB1031	2	1	0	0	0	3	5
Total	**31**	**6**	**13**	**7**	**6**	**63**	**100**

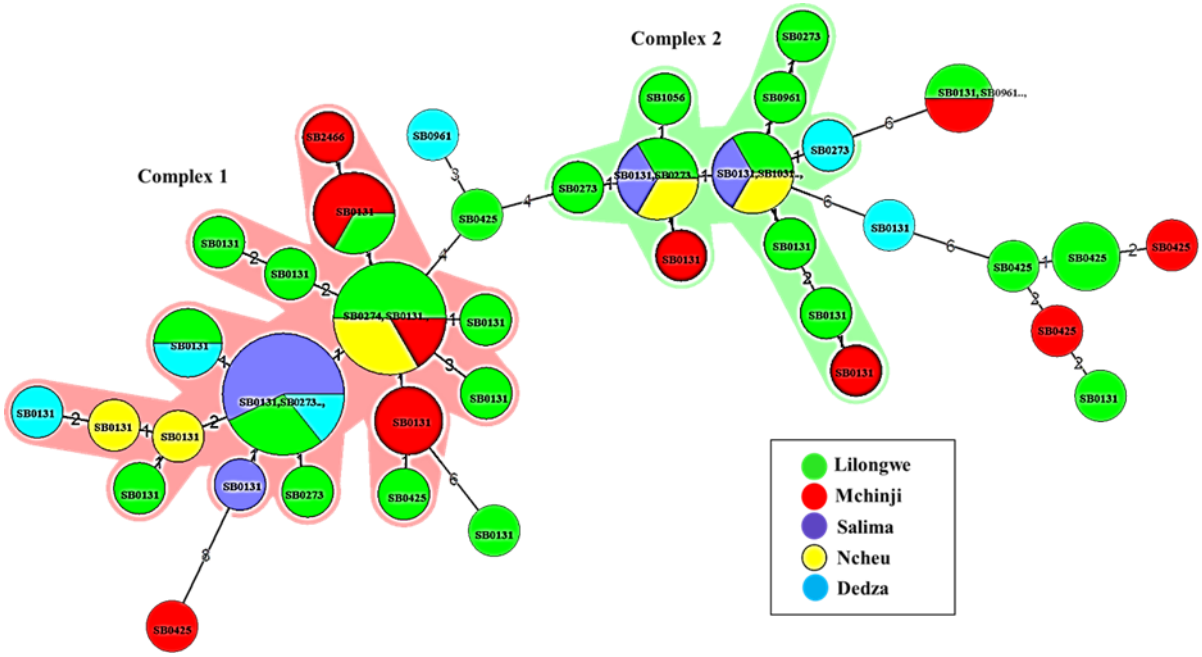


Fig. 14: The minimum spanning tree (MST) of 60 *M. bovis* "genotypes" based on 26 loci MIRU-VNTR typing of cultured isolates from cattle slaughtered at Lilongwe cold storage abattoir. MST nodes are highlighted by the district of origin (Green: Lilongwe, Red: Mchinji, Purple: Salima, Yellow: Ntcheu, and Umber: Dedza) and coded with spoligotype number. It should be noted that three isolates were removed from MIRU - VNTR analysis because they failed to amplify more than ten loci.

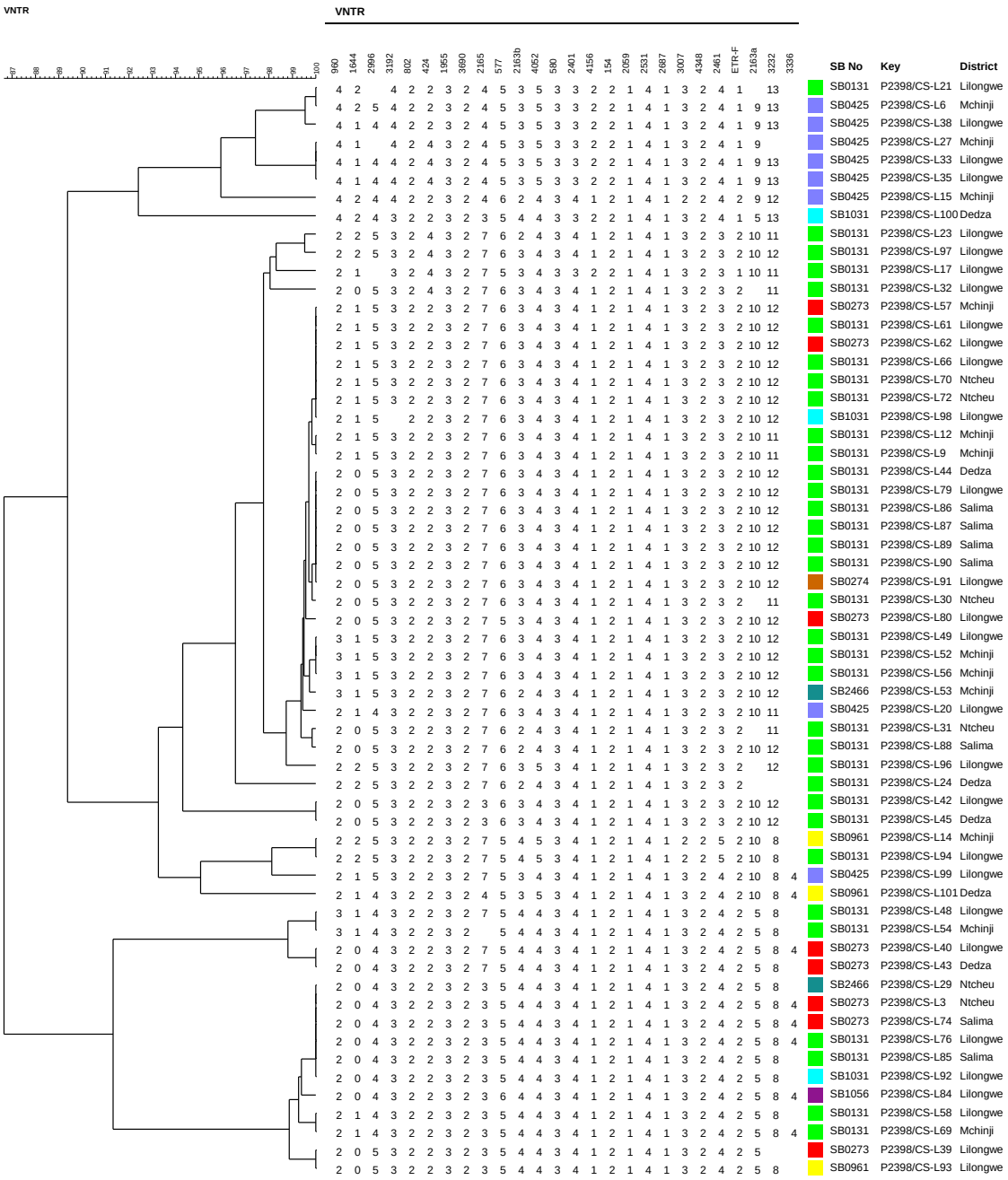


Fig. 15. A UPGMA phylogenetic tree showing the relationships of spoligotypes isolated from cattle slaughtered at Lilongwe cold storage abattoir. The tree was constructed based on 26 loci MIRU-VNTR, and 31 genotypes were revealed.

Table 11. Allele diversity of the MIRU-VNTR analysis performed on *M. bovis* isolates from bTB-like lesions in cattle slaughtered at Lilongwe cold storage abattoir

Locus name	**Number of isolates with indicated MIRU allele**															**AV**	**Allele diversity**
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**NA**		
QUB3232									18			8	25	6	3	4	0.689
MIRU16	25	25	10													3	0.636
QUB11a						16				6	32				6	3	0.559
QUB11b			6	36	18											3	0.549
ETR-A				14	8			37							1	3	0.541
ETR-B				33	25	2										3	0.532
ETR-C						27	33									2	0.503
MIRU26					20	37									3	2	0.464
MIRU10			46	6	8											3	0.391
QUB26					50	10										2	0.282
QUB4156		52	8													2	0.235
Mtub30				8	52											2	0.235
ETR-F		8	52													2	0.235
MIRU3				52	7										1	2	0.213
Mtu04			53		7											2	0.21
MIRU27			3	57												2	0.097
MIRU40			60													1	0
Mtub21				60												1	0
Mtub39			60													1	0
MIRU04				60												1	0
MIRU02			60													1	0
MIRU20		60														1	0
MIRU23					60											1	0
MIRU24		60														1	0
MIRU39			59												1	1	0
QUB3336					8										52	1	0

Allelic diversity was estimated by the Hunter Gaston Discriminatory Index (HGDI)

NA: Not amplified, AV: Allelic variants

Table 12: Discrimination and diversity of spoligotypes by MIRU-VNTR analysis

Spoligotype	**MIRU-VNTR Types**
SB0131	V1, V6, V7, V8, V9, V10, V11, V12, V13, V15, V18, V19, V20, V21, V22, V23, V26, V28, V30 (19)
SB0273	V10, V14, V27, V28, V31 (5)
SB0274	V12 (1)
SB0425	V1, V2, V3, V4, V17, V24 (6)
SB0961	V23, V25, V31 (3)
SB1056	V29 (1)
SB2466	V16, V28 (2)
SB1031	V5, V10, V28 (3)

Table 13: MIRU VNTR homoplasy among spoligotypes

MIRU-VNTR type	**Spoligotype**
V28	SB2466, SB0273, SB0131, SB1031
V10	SB0273, SB1031, SB0131
V31	SB0273; SB0961
V23	SB0961; SB0131
V12	SB0274, SB0131
V1	SB0131, SB0425

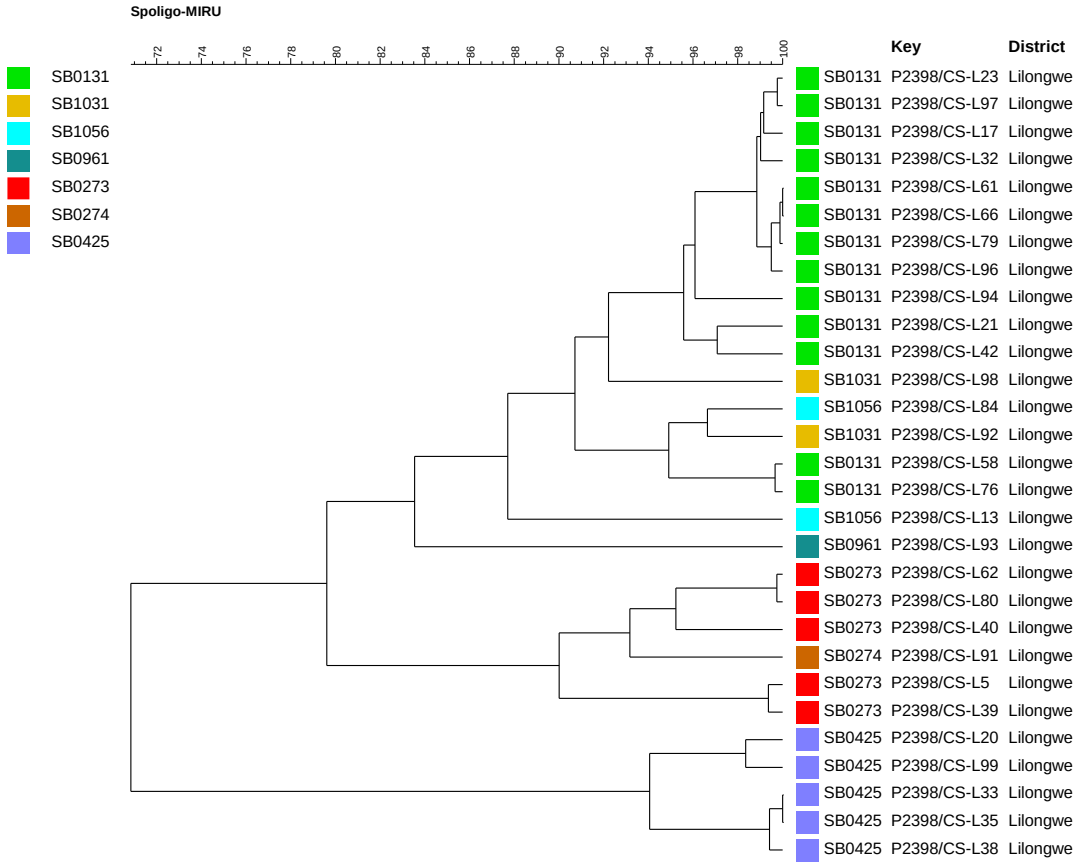


Fig. 16: Genetic diversity of isolates from Lilongwe district based on the combination of spoligotyping and MIRU-VNTR.

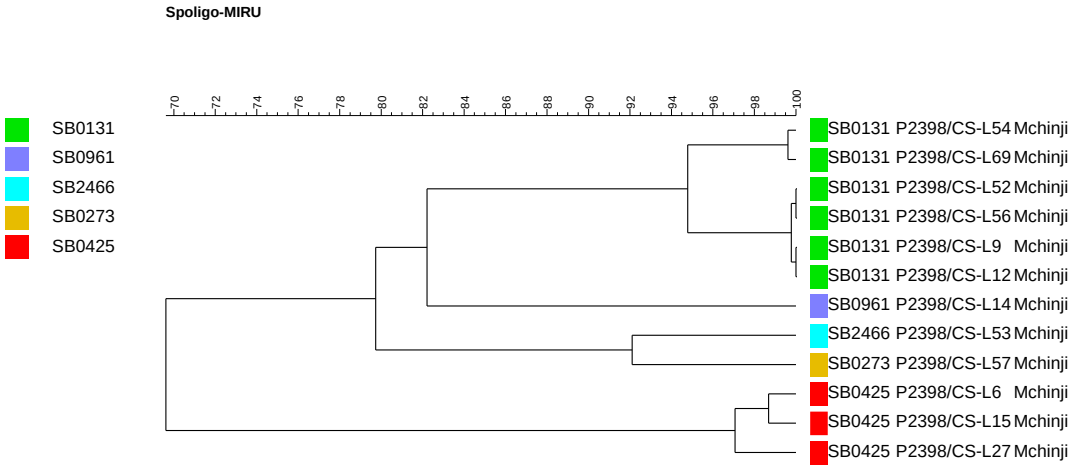


Fig. 17: Genetic diversity of isolates from Mchinji district based on the combination of spoligotyping and MIRU-VNTR.

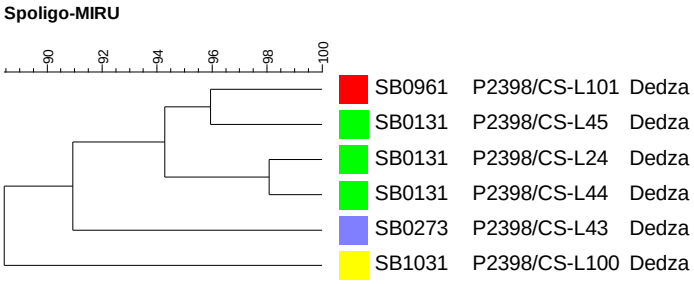


Fig. 18: Genetic diversity of isolates from Dedza district based on the combination of spoligotyping and MIRU-VNTR.

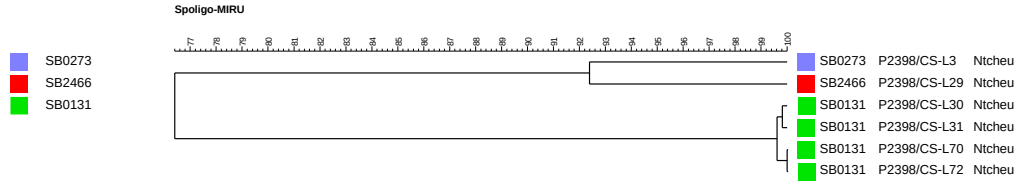


Fig. 19: Genetic diversity of isolates from Ntcheu district based on the combination of spoligotyping and MIRU-VNTR.

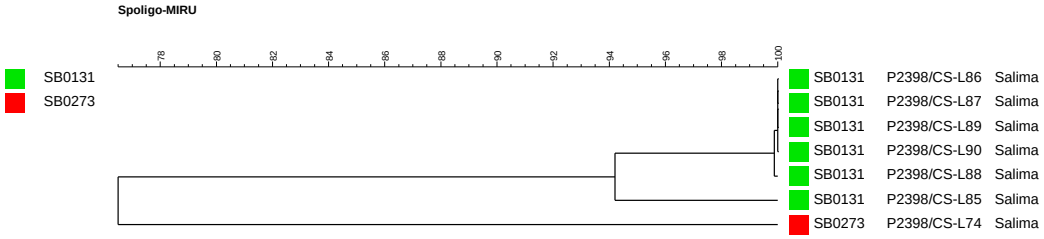


Fig. 20: Genetic diversity of isolates from Salima district based on the combination of spoligotyping and MIRU-VNTR.

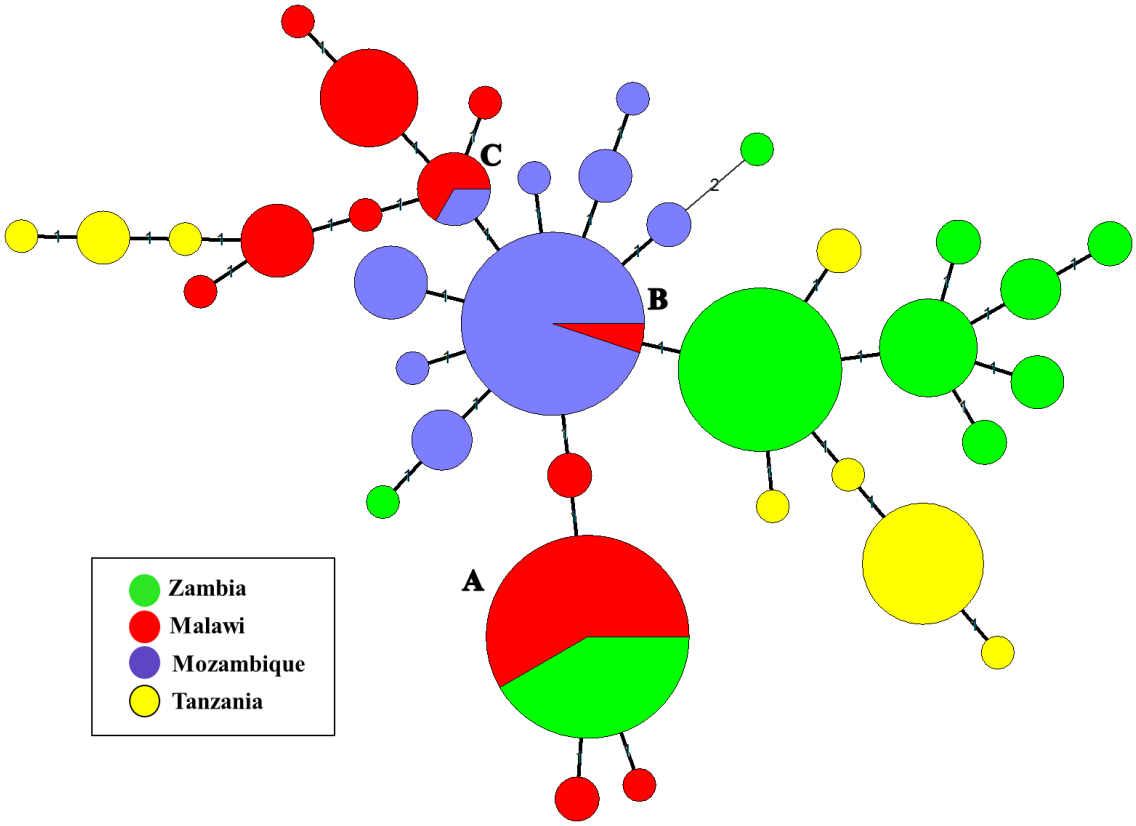


Fig. 21: Minimum spanning tree (MST) of isolates from Malawi and neighbouring countries. The MST is based on the five MIRU VNTR loci, MIRU577, MIRU580 MIRU2165, MIRU2461, and MIRU3192. Nodes are coloured by country of isolates collection Zambia (green), Malawi (red), Mozambique (purple), and Tanzania (yellow). Three groups were revealed (A, B and C) where isolates from Malawi clustered together with those from neighbouring countries.

Table 14: Analysis of the samples collected from Lilongwe cold storage abattoir.

			M. bovis clonal complex analysis				Spoligotyping	26 loci MIRU VNTR typing																								
Sample ID	District	Country	RDEu1	RDAf1	RDAf2	Clonal complex	SB Number	MIRU10	MIRU16	MIRU26	MIRU31	MIRU40	VN424	VN1955	VN3690	ETR-A	ETR-C	QUB11B	QUB26	MIRU04	VN2401	VN4156	MIRU02	MIRU20	MIRU23	MIRU24	MIRU27	MIRU39	ETR-B	ETR-F	QUB11A	QUB3232	QUB3336
P2398/CS-L3	Ntcheu	Malawi	D	I	I	Eu1	SB0273	2	0	4	3	2	2	3	2	3	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	4
P2398/CS-L5	Lilongwe	Malawi	D	N	I	Eu1	SB0273	2	1	5	NA	2	NA	NA	NA	3	NA	4	4	3	4	NA	NA	NA	4	1	NA	NA	4	NA	5	8	NA
P2398/CS-L6	Mchinji	Malawi	I	I	I	BCG-Like	SB0425	4	2	5	4	2	2	3	2	4	5	3	5	3	3	2	2	1	4	1	3	2	4	1	9	13	NA
P2398/CS-L7	Mchinji	Malawi	D	N	N	Eu1	SB0131	NA	2	4	NA	2	NA	NA	NA	4	NA	2	NA	3	NA	NA	NA	NA	4	1	NA	NA	3	NA	NA	NA	NA
P2398/CS-L9	Mchinji	Malawi	D	I	I	Eu1	SB0131	2	1	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	11	NA
P2398/CS-L12	Mchinji	Malawi	D	I	I	Eu1	SB0131	2	1	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	11	NA
P2398/CS-L13	Lilongwe	Malawi	N	N	N	BCG-Like	SB1056	NA	NA	4	NA	2	NA	NA	NA	7	NA	4	NA	0	NA	NA	NA	NA	4	1	NA	NA	5	NA	10	NA	NA
P2398/CS-L14	Mchinji	Malawi	I	I	I	BCG-Like	SB0961	2	2	5	3	2	2	3	2	7	5	4	5	3	4	1	2	1	4	1	2	2	5	2	10	8	NA
P2398/CS-L15	Mchinji	Malawi	I	I	I	BCG-Like	SB0425	4	2	4	4	2	2	3	2	4	6	2	4	3	4	1	2	1	4	1	2	2	4	2	9	12	NA
P2398/CS-L17	Lilongwe	Malawi	D	I	I	Eu1	SB0131	2	1	NA	3	2	4	3	2	7	5	3	4	3	3	2	2	1	4	1	3	2	3	1	10	11	NA
P2398/CS-L20	Lilongwe	Malawi	D	I	I	Eu1	SB0425	2	1	4	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	11	NA
P2398/CS-L21	Lilongwe	Malawi	I	I	I	BCG-Like	SB0131	4	2	NA	4	2	2	3	2	4	5	3	5	3	3	2	2	1	4	1	3	2	4	1	NA	13	NA
P2398/CS-L23	Lilongwe	Malawi	D	I	I	Eu1	SB0131	2	2	5	3	2	4	3	2	7	6	2	4	3	4	1	2	1	4	1	3	2	3	2	10	11	NA
P2398/CS-L24	Dedza	Malawi	D	I	I	Eu1	SB0131	2	2	5	3	2	2	3	2	7	6	2	4	3	4	1	2	1	4	1	3	2	3	2	NA	NA	NA
P2398/CS-L27	Mchinji	Malawi	I	I	I	BCG-Like	SB0425	4	1	NA	4	2	4	3	2	4	5	3	5	3	3	2	2	1	4	1	3	2	4	1	9	NA	NA
P2398/CS-L29	Ntcheu	Malawi	D	I	I	Eu1	SB2466	2	0	4	3	2	2	3	2	3	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	NA
P2398/CS-L30	Ntcheu	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	NA	11	NA
P2398/CS-L31	Ntcheu	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	2	3	2	7	6	2	4	3	4	1	2	1	4	1	3	2	3	2	NA	11	NA
P2398/CS-L32	Lilongwe	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	4	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	NA	11	NA
P2398/CS-L33	Lilongwe	Malawi	I	I	I	BCG-Like	SB0425	4	1	4	4	2	4	3	2	4	5	3	5	3	3	2	2	1	4	1	3	2	4	1	9	13	NA
P2398/CS-L35	Lilongwe	Malawi	I	I	I	BCG-Like	SB0425	4	1	4	4	2	4	3	2	4	5	3	5	3	3	2	2	1	4	1	3	2	4	1	9	13	NA
P2398/CS-L38	Lilongwe	Malawi	I	I	I	BCG-Like	SB0425	4	1	4	4	2	2	3	2	4	5	3	5	3	3	2	2	1	4	1	3	2	4	1	9	13	NA
P2398/CS-L39	Lilongwe	Malawi	D	I	I	Eu1	SB0273	2	0	5	3	2	2	3	2	3	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	NA	NA
P2398/CS-L40	Lilongwe	Malawi	D	I	I	Eu1	SB0273	2	0	4	3	2	2	3	2	7	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	4
P2398/CS-L42	Lilongwe	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	2	3	2	3	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L43	Dedza	Malawi	D	I	I	Eu1	SB0273	2	0	4	3	2	2	3	2	7	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	NA
P2398/CS-L44	Dedza	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L45	Dedza	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L48	Lilongwe	Malawi	D	I	I	Eu1	SB0131	3	1	4	3	2	2	3	2	7	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	NA
P2398/CS-L49	Lilongwe	Malawi	D	I	I	Eu1	SB0131	3	1	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L52	Mchinji	Malawi	D	I	I	Eu1	SB0131	3	1	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L53	Mchinji	Malawi	D	I	I	Eu1	SB2466	3	1	5	3	2	2	3	2	7	6	2	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L54	Mchinji	Malawi	D	I	I	Eu1	SB0131	3	1	4	3	2	2	3	2	NA	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	NA
P2398/CS-L56	Mchinji	Malawi	D	I	I	Eu1	SB0131	3	1	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L57	Mchinji	Malawi	D	I	I	Eu1	SB0273	2	1	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L58	Lilongwe	Malawi	D	I	I	Eu1	SB0131	2	1	4	3	2	2	3	2	3	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	NA
P2398/CS-L61	Lilongwe	Malawi	D	I	I	Eu1	SB0131	2	1	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L62	Lilongwe	Malawi	D	I	I	Eu1	SB0273	2	1	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L66	Lilongwe	Malawi	D	I	I	Eu1	SB0131	2	1	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L69	Mchinji	Malawi	D	I	I	Eu1	SB0131	2	1	4	3	2	2	3	2	3	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	4
P2398/CS-L70	Ntcheu	Malawi	D	I	I	Eu1	SB0131	2	1	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L72	Ntcheu	Malawi	D	I	I	Eu1	SB0131	2	1	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L74	Salima	Malawi	D	I	I	Eu1	SB0273	2	0	4	3	2	2	3	2	3	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	4
P2398/CS-L76	Lilongwe	Malawi	D	I	I	Eu1	SB0131	2	0	4	3	2	2	3	2	3	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	4
P2398/CS-L79	Lilongwe	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L80	Lilongwe	Malawi	D	I	I	Eu1	SB0273	2	0	5	3	2	2	3	2	7	5	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L84	Lilongwe	Malawi	D	I	I	Eu1	SB1056	2	0	4	3	2	2	3	2	3	6	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	4
P2398/CS-L85	Salima	Malawi	D	I	I	Eu1	SB0131	2	0	4	3	2	2	3	2	3	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	NA
P2398/CS-L86	Salima	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L87	Salima	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L88	Salima	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	2	3	2	7	6	2	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L89	Salima	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L90	Salima	Malawi	D	I	I	Eu1	SB0131	2	0	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L91	Lilongwe	Malawi	D	I	I	Eu1	SB0274	2	0	5	3	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L92	Lilongwe	Malawi	D	I	I	Eu1	SB1031	2	0	4	3	2	2	3	2	3	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	NA
P2398/CS-L93	Lilongwe	Malawi	D	I	I	Eu1	SB0961	2	0	5	3	2	2	3	2	3	5	4	4	3	4	1	2	1	4	1	3	2	4	2	5	8	NA
P2398/CS-L94	Lilongwe	Malawi	I	I	I	BCG-Like	SB0131	2	2	5	3	2	2	3	2	7	5	4	5	3	4	1	2	1	4	1	2	2	5	2	10	8	NA
P2398/CS-L96	Lilongwe	Malawi	D	I	I	Eu1	SB0131	2	2	5	3	2	2	3	2	7	6	3	5	3	4	1	2	1	4	1	3	2	3	2	NA	12	NA
P2398/CS-L97	Lilongwe	Malawi	D	I	N	Eu1	SB0131	2	2	5	3	2	4	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L98	Lilongwe	Malawi	D	I	I	Eu1	SB1031	2	1	5	NA	2	2	3	2	7	6	3	4	3	4	1	2	1	4	1	3	2	3	2	10	12	NA
P2398/CS-L99	Lilongwe	Malawi	I	I	I	BCG-Like	SB0425	2	1	5	3	2	2	3	2	7	5	3	4	3	4	1	2	1	4	1	3	2	4	2	10	8	4
P2398/CS-L100	Dedza	Malawi	I	I	I	BCG-Like	SB1031	4	2	4	3	2	2	3	2	3	5	4	4	3	3	2	2	1	4	1	3	2	4	1	5	13	NA
P2398/CS-L101	Dedza	Malawi	D	I	I	Eu1	SB0961	2	1	4	3	2	2	3	2	4	5	3	5	3	4	1	2	1	4	1	3	2	4	2	10	8	4

I: Intact, D: Deleted, NA: not amplified

Conclusion

TB caused by *M. bovis* is a significant global concern on three counts; (1) the economic impact on cattle due to high production costs, organ condemnation, and trade barrier. (2) public health threat due to potential zoonotic transmission to humans, and (3) the spill-over to wildlife risking endangered species. The eradication of the disease has been hampered mainly by (1) the unavailability of surveillance tools that are simple and low-cost for easy integration in high burdened areas, (2) lack of information about the disease, especially from endemic regions resulting in underestimation of the burden and failure of the application of appropriate control strategies. Therefore, in the present study, I have developed a LAMP assay for the specific detection of *M. bovis* and characterized isolates from the central parts of Malawi to gain insight into their molecular structure, disease dynamics, and relationships with isolates from neighbouring countries like Zambia, Tanzania, and Mozambique.

In chapter I, a LAMP method was developed by targeting the region of difference (RD4) deletion. RD4 is deleted in all *M. bovis* strains and inserted in all other MTBC species, including *M. tuberculosis*. This LAMP assay showed high specificity and sensitivity for the detection of *M. bovis*.

In chapter II, the wet LAMP developed in a previous study was converted to the dry form by drying the reaction reagents. The LAMP kits were developed in Japan and transported to Malawi for the field evaluation on cattle samples collected at three regional abattoirs. All the isolates detected as *M. bovis* by established dry LAMP were confirmed so by a multiplex PCR for detecting *M. bovis* and *M. tuberculosis*. This is a significant breakthrough in developing rapid, low-cost, and simplified methods that can be easily used in resource-limited areas for the surveillance of bTB.

In chapter III, molecular typing of *M. bovis* isolates has indicated that the majority of the circulating strains belong to the Eu1 clonal complex and that SB0131 is dominant in central parts of Malawi. The high diversity of *M. bovis* in Lilongwe points at the district as the epicentre of bTB in the study area. The genetic similarities with isolates from neighbouring countries, especially Zambia and Mozambique, suggest transmission links. Thus, these results highlight the need to institute stringent bTB control measures within this region to curb the transmission and spread. Further, this calls on the veterinary and public health sectors in Malawi to consider implementing other control measures to beef-up the current bTB control efforts.

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