The prevalence and risk factors of *Mycobacterium bovis* infections in domestic animals and man in Eritrea

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Prevalentie en risicofactoren van *Mycobacterium bovis*
infecties in gedomesticeerde dieren en mensen in Eritrea
(met een samenvatting in het Nederlands)

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Contents

Chapter 1

General Introduction

Mycobacterium bovis (M. bovis) is the etiologic agent of bovine tuberculosis (BTB). It is a slow-growing, aerobic, acid fast bacterium with a broad host range [Nugent, 2011]. The disease is characterized by the formation of granulomas in tissues and organs, mainly in the lungs, lymph nodes, intestines and kidneys [Amanfu, 2006]. *M. bovis* belongs to the genus Mycobacterium of which most of the members are nonpathogenic, saprophytic mycobacteria and of which the majority are found in the environment [Quinn et al., 2001]. Few are obligate pathogenic, above all, the *Mycobacterium tuberculosis* complex (MTBC) species [Kazda & Pavlik, 2009]. MTBC includes *M. tuberculosis* (human), *M. africanum* (human*), M. canettii* (human), *M. bovis BCG, M. microti* (vole), *M. caprae* (goats), *M. pinnipedii* (seal), *M. mungi* (mongoose), in addition to *M. bovis* [Khan et al., 2011; Gyles et al., 2010; Quinn et al., 2001; Alexander et al., 2010] and other mycobacteria that seem to have a wide host range within the wildlife, i.e., *M. orygis* (oryx, antelope, gazelles, waterbucks, deer), *M. surricatae* (suricate/meerkat) and *Dassei bacilli* (dassie/rock hyrax) [Van Ingen et al., 2012; Parsons et al., 2004].

M. bovis, though it mainly affecting cattle, it also affects humans and many other animals including wildlife [Michel et al., 2009]. *M. bovis* and *M. tuberculosis* share > 99.9% genetic identity and induce similar host immune responses and disease manifestations upon infection [Waters et al., 2011; Atkins, 2016] except in some wildlife, i.e., badgers, suricates, lions, mongoose, where draining skin wounds are manifest [Radostits et al., 2007; Clifton-Hadley et al., 2001].

In addition to a zoonotic risk, the disease causes major problems in animal health and welfare, and economic losses resulting from reduction in milk production, draught power, emaciation and death of animals, and condemnation of carcasses and organs in the slaughterhouses and at household level. At macro level, it tremendously affects the economy of a country because of trade bans.

M. bovis transmission

The principal route of transmission of *M. bovis* is via inhalation, as this route requires as few as one bacillus to induce infection [OIE, 2008; Radostits et al., 2007; Skuce et al., 2012]. Infection through ingestion of contaminated pasture (feed) and drinking of contaminated water, and milk (calves) are also known routes of *M. bovis* transmission, though they require more (> 10^7) bacilli as compared to the respiratory route [Radostits, et al., 2007].

Less common routes of *M. bovis* infection are congenital (via umbilical vessel to the foetus from infected uterus of the dam), via wound or broken skin, and seldom, via infected genitalia when either male or female is infected, and sometimes when the preputial orifice is contaminated by infected dust [Thoen et al., 2006; Neill et al., 1994; Humblet et al., 2009]. Intrauterine infection of *M. bovis* is also reported to have resulted from coital transfer [Andrews et al., 2004]. *M. bovis* transmission between animals depends on frequency of excretion, infective dose, route of infection, the period of communicability, and host susceptibility [Griffin & Dolan, 1995], including the number of infected animals present and their proximity to the susceptible animal(s).

Clinical signs of BTB

Bovine tuberculosis being a chronic disease can remain subclinical for a long period [De la Rua-Domenech et al., 2006] whereas animals may become infectious even before manifestation of clinical signs and lesions. Some cows with extensive miliary tubercular lesions may seem clinically healthy [Radostits et al., 2007]. Clinical signs are loss of appetite, fluctuating temperature (intermittent fever), rough hair coat, cough that might progress to dyspnea, progressive emaciation and enlargement of lymph nodes. Infected animals may become more docile and sluggish [Radostits et al., 2007; OIE, 2009].

Diagnosis of BTB

Currently, there are two categories of diagnostic tests to detect *M. bovis* infection in animals, i.e., indirect and direct tests. The former, indicate infection in live animals indirectly, using indicators of *M. bovis* specific immune responses. These indirect tests are in turn divided into those that detect cellular immune responses (tuberculin skin test and interferon gamma test), and those that detect antibodies (multi-antigen print immunoassay; MAPIA, and lateral flow based rapid test; RT) [OIE, 2009; De la Rua-Domenech et al., 2006]. Direct tests identify the organism directly at postmortem examination, bacterial culture and by molecular methods).

The tuberculin skin test is a standard method of BTB diagnosis in living animals that involves injection into the skin of a small amount (0.1 ml) of tuberculin, a purified protein derivative (PPD) of *M. tuberculosis*, *M. bovis*, or *M. avium.* Skin testing is used in cattle and a wide range of mammal species including humans (Mantoux). Tuberculin induces a type IV hypersensitivity reaction that results in thickening (swelling) of the skin in the site of injection within 72 hours, in most of the animals, in case of prior exposure to *M. bovis*/other mycobacteria. Skin testing can be either a single intradermal (SIT) or a single intradermal comparative tuberculin test (SICTT).

SIT uses only one tuberculin, which is the bovine PPD-B. In the SICTT both bovine and avian tuberculin (PPD-A) are used allowing the differentiation between animals infected by *M. bovis* and those infected by Mycobacteria other than *M. bovis*, usually represented by *M. avium* [Waters et al., 2011; OIE, 2009; De la Rua-Domenech et al., 2006]. Many countries have managed to eradicate BTB with the help of the skin test alone and slaughter of all test reactors.

The Gamma interferon (IFN-γ) release assay is an in vitro cellular test for the diagnosis of BTB. The principle of the test is detection of gamma interferon which is released after whole blood stimulation with TB antigens and control stimulants [De la Rua-Domenech et al., 2006; Wood et al., 1991; OIE, 2009]. The sensitivity of the IFN-γ assay for the detection of BTB was found to be higher than that of SIT [Wood et al., 1991; Clegg et al., 2017; De la Rua-Domenech et al., 2006] as this test can detect considerable proportion (about

28%) of infected cattle that were not detected by the skin test [Wood et al., 1991].

Antibody tests are useful ancillary tests, especially in detecting infection at the chronic stage of diseases that cannot be detected by the intradermal skin test or the gamma interferon test. Wernery et al. (2007) indicated the usefulness of the two antibody tests (MAPIA and RT) in having the potential to improve surveillance and ante-mortem diagnosis of TB in camels when compared to tuberculin skin test. As there is no perfect diagnostic test that can 100% detect the truly infected animals, combined tests are recommended to increase the sensitivity and specificity of the diagnosis [Buddle et al., 2009]. Combination of the IFN-γ assay with skin testing was postulated to improve the detection of *M. bovis* infected cattle. Such combined testing is expected to reduce the unnecessary slaughter of false-positive reactors [Buddle at al., 2009]. In a recent study, the combined use of three diagnostic tests (SICTT, IFN-γ assay, and RT) was shown to be more effective in detecting infected animals [Ameni et al., 2010].

In countries with low disease prevalence or disease-free status (OTF), meat inspection is used for diagnosis and surveillance. Definitive diagnosis of BTB is only possible by culture of mycobacteria as a gold standard [OIE, 2009; Wood et al, 1991], followed by confirmation of the identity of the organism by polymerase chain reaction (PCR) [Scherer et al., 2011]. Both direct methods are more specific than the indirect immune response. However, the limitation of both methods is that, they can only be performed on samples collected post mortem.

PCR can identify the MTBC members, which is vital in sorting out whether the pathogen in question is *M. bovis*, *M. tuberculosis* or any other member of the group. Genotyping using spoligotyping and/or VNTR profiling are classical typing tools for MTBC species [Hlokwe et al., 2014; Hlokwe et al., 2013; Mignard et al., 2006]. Recently, whole genome sequencing (WGS) with single nucleotide polymorphisms (SNP) analysis has been shown to have much higher resolution than the former more traditional approaches [Kao et al., 2014; Glaser et al., 2016]. WGS can more precisely determine genetic relationships via common ancestors and provides insight into local

transmissions between herds when combined with traditional epidemiologic investigation data.

BTB control and its challenges

The essence of BTB control in cattle and other species is the accurate detection and removal of animals infected with *M. bovis* from the herd [De la Rua-Domenech et al., 2006]. For this to be achieved, early detection of infection is crucial which is possible using ante-mortem cellular immuno-assays that are capable to detect cellular immune response before any antibody responses are elicited [Buddle et al., 2009]. Control and eradication programs for BTB were successful in developed countries through voluntary removal of positive reactors at the beginning of the BTB control campaign in the 19th century, with subsequent implementation of mandatory test and slaughter policies in the 1950s [Cosivi et al., 1998; Ayele et al., 2004; De la Rua-Domenech et al., 2006]. In developing countries, BTB remains a major animal health problem due to the financial constraints faced by these countries to implement the control program and compensate for slaughtered animals [Ayele et al., 2004]. Other major constraints may be poor awareness of BTB, low level of education and lack of effective disease diagnosis and surveillance.

Vaccination of animals against BTB is suggested to be a viable strategy in domesticated animals in developing countries, and in wildlife and feral reservoirs of disease in industrialized countries where test and slaughter programs have failed to achieve elimination of the disease [Cosivi et al., 1998]. Vaccines are being developed and evaluated for use in domestic bovine and wildlife species, but, except BCG in badgers, have so far not been registered. Since routine administration may compromise the use of the tuberculin skin test and IFN-y assay, efforts are being made to design [Vordermeier et al., 2011a; Vordermeier et al., 2011b] a test that can *Differentiate between Infected and Vaccinated Animals* (DIVA). Suggestions are however there to vaccinate wildlife and cattle [Buddle et al., 2013], that may also be applicable for livestock within the extensive system [Nugent et al., 2017], where BCG vaccination or vaccination of calves in general may be seen as alternative control strategy, since such vaccination has proven to have

70% protection against BTB in efficacy trial [Ameni et al., 2010; Vordermeier et al., 2012] and indicated to slowdown the disease progression and reduce the incidence of detectable lesions [Nugent et al., 2017].

Treatment

Treatment is not deemed feasible in animals due to high costs, lengthy time required, as well as the risk of the animals serving as reservoirs if treatment is not effective [Michel et al., 2006].

Bovine tuberculosis in cattle in Africa

In Africa, about 85% of the cattle population lives in areas where BTB is either not or only partly controlled [Cosivi et al., 1998]. Animal husbandry and management systems contribute to the development and dissemination of BTB. The disease is endemic in various livestock production systems [Regassa et al., 2010; Radostits et al., 2007; Munyeme et al., 2009; Shirima et al., 2003].

In Ethiopia, where similar livestock management exists as in Eritrea, several studies have reported a high BTB prevalence in intensive dairy farms [Shitaye et al., 2007; Regassa et al., 2010; Fetene et al., 2011; Fetene & Kebede, 2009; Firdessa et al., 2012]. Similarly, in Eritrea, the prevalence of BTB in intensive dairy farms in and around the capital Asmara was found to be 14.5% [Omer et al., 2001]. In such a production system, animals, mostly exotic dairy breeds are kept indoors with zero-grazing, aiming at high milk yield. Housing that allows close contact between animals, exotic breeds, poor ventilation, and fence-line contact between herds, may facilitate within and between herd transmission of BTB [Ameni et al., 2006; Menzies & Neill, 2000; Costello et al., 1998; Elias et al., 2008; Radostits et al., 2007; O'Reilly & Daborn 1995; Omer et al., 2001; Ameni et al., 2007; Inangolet et al., 2008; Vordermeier et al., 2012; Griffin et al. 1993].

In extensive livestock husbandry systems in Africa, *M. bovis* can easily spread between herds during the free movement of cattle, gathering at

communal resting sheds and at watering points [Radostits et al., 2007; Durnez et al., 2009]. Cattle-to-cattle contact at pasture is related to greater risk of *M. bovis* transmission [Dommergues et al., 2012]. In UK badger contaminated pastures were implicated in the transmission of BTB to cattle [APHA, 2017]. Apart from animal-to-animal transmission of *M. bovis*, though rarely, humans with open tuberculosis due to *M. tuberculosis* or *M. bovis* can also be sources of infection to animals. Such infections might occur through the respiratory route when handling the animals or through contamination of their environment by spitting or urinating on pasture (in the case of clinical genitourinary tuberculosis) [Radostits et al., 2007; Ayele et al., 2004; Grange et al., 1994; Ameni et al., 2011; Berg et al., 2009]. In addition, animals grazing on mineral deficient soils are prone to develop chronic tuberculosis unless supplemented with mineral licks, as mineral deficiency is directly associated with immune depression [Cadmus & Arinola, 2007; Griffin et al., 1993]. As an example, the copper metabolism affects T and B cells, neutrophils, and macrophages [McDowell, 1992]. Despite the higher potential risks for transmission of BTB in the extensive production system reported prevalences are low [Tschopp et al., 2011; Tschopp et al., 2010; Gumi et al., 2012]. This may be attributed to the lower contact rate in the extensive ranges, the fact that predominantly indigenous local cattle are kept, presence of co-infections with other bacteria, viruses and infestation with parasites that might lower the sensitivity of the SICTT, hence the chance of accurate detection of *M. bovis* in cattle [Monghan et al., 1994; Humblet et al., 2009]. Besides, the high temperature, prevailing solar radiation, arid and semi-arid climate are not ideal for the growth and multiplication of *M. bovis* [Radostits et al., 2007; Humblet et al., 2009].

BTB in other animal species in Africa

Caprine tuberculosis

Caprine tuberculosis affects the livelihoods of the farmers by reducing milk and meat production, culling of the affected animals, death of the animals, trade restrictions, and by its ability to infect other animals, including wildlife and humans [Seva et al., 2002; Daniel et al., 2009; Vordermeier et al., 2002;

Cvetnic et al., 2007; Pate et al., 2006; Kubica et al., 2003; Sharpe et al., 2010]. In general, goats are very susceptible to tuberculosis (Napp et al., 2013), and if kept with infected herd(s) of cattle the incidence may reach 70% [Bezos et al., 2012; Radostits et al., 2007], presumably, as cattle are the main sources of infection and goats are susceptible to *M. bovis* infection. On the other hand, if infected goats are kept with cattle or use the same grazing areas, they can be potential sources of infection to cattle [Bezos et al., 2012]. Mortality rates ranging from 20-50% per year were reported in different milking flocks in Spain [O'Reilly & Daborn, 1995]. The causative agents of tuberculosis in goats can be *M. bovis*, *M. caprae* and occasionally, *M. tuberculosis* [Muller et al., 2013; Aranaz et al., 2003; Crawshaw et al., 2008; Cadmus et al., 2009]. The presence of these pathogenic agents was confirmed by their isolation from affected tissues and milk samples [Hiko et al., 2011; Cadmus et al., 2009; Quintas et al., 2010; Shanahan et al., 2011; Daniel et al., 2009]. In Spain, *M. caprae* is shown to be the major cause of tuberculosis in goats, representing about 7.4% of all MTBC isolates from domestic and wild animals in the country [Rodríguez et al., 2011]. A recent study in Ethiopia indicated the prevalence of TB in goats to be higher under traditional husbandry practices, than in organized (research) farms [Tafess et al., 2011]. In the traditional husbandry system, farmers either share their dwellings with goats or keep them in separate houses with calves. In both cases there is a risk of TB transmission from animals to animals, animals to humans or vice versa [Radostits et al., 2007; Hutson, 1941; Deresa et al., 2013]. In countries where BTB eradication was successful, human *M. bovis* or *M. caprae* isolates are rare [Cosivi et al., 1998; De la Rua-Domenech et al., 2006].

Tuberculosis in camels

Like cattle and goats, camels are affected by tuberculosis. As cited by Mustafa [1987], tuberculosis in camels was first reported by Littlewood as early as 1888 in Egypt. When kept in contact with cattle, camels are found to be more prone to *M. bovis*, and have more tuberculosis lesions in the abattoirs than those coming from regions where only camels are herded [Manal et al., 2008; Mustafa, 1987]. Several recent studies in Ethiopia indicated tuberculosis in

camels with isolation of *M. bovis* and *M. tuberculosis* from tuberculous lesions collected at slaughterhouses [Mamo et al., 2009; Mamo et al., 2012; Beyi et al., 2014; Gumi et al., 2012].

TB in humans in Africa

Tuberculosis in humans is mainly caused by *Mycobacterium tuberculosis (M. tuberculosis)* and occasionally by *Mycobacterium bovis* (*M. bovis*), both belonging to the *Mycobacterium tuberculosis complex* (*MTBC*), > 90% genetically identical, and causing similar clinical diseases in both humans and animals [Warren et al., 2006; Alexander et al., 2010]. Humans may be infected by *M. bovis* due to consumption of unpasteurized dairy products, inhalation of infected aerosols because of close contact with animals having chronic cough due to tuberculosis [WHO, 2017; Michel et al., 2015; Harris et al., 2007; Milian-Suazo et al., 2010]. *M. bovis* was detected in goats' milk, as well as in cow's milk where *Mycobacterium africanum* (*M. africanum*) was also identified [Kazwala et al., 2001; Shanahan et al., 2011; Cadmus et al., 2010; Amanfu, 2006; Boukary et al., 2011; Nasr et al., 2014; Cezar et al., 2016].

In most developing countries where *M. bovis* infection is present in animals, little is known about the distribution, epidemiological patterns and zoonotic implications of the infection [Cosivi et al., 1998]. In addition, due to technical difficulties, only few laboratories in developing countries can differentiate *M. bovis* from *M. tuberculosis* [Moda et al., 1996].

In Africa, about 82 % of the human population lives in areas where BTB is either not or partly controlled [Cosivi et al., 1998]. In 2015, among the 30 high TB burden countries, 16 (53%) were in Africa [WHO, 2016]. In this continent, *M. bovis* is implicated for 0.4-10% of pulmonary, in addition to extra pulmonary TB in humans [Byarugaba et al., 2009; Cosivi et al., 1995; Muler et al., 2013]. In developing countries, mainly in rural areas, sharing of the same dwellings and close contact between animals and humans facilitates zoonotic BTB transmission [Amanfu, 2006]. Once infected, human-to-human *M. bovis* transmission is rare but possible [Sunder et al., 2009; Evans et al., 2007; Thoen et al., 2009], especially in immunocompromised people. This agent was identified from sputum and cervical lymphadenitis of TB patients

[Cosivi et al., 1998; Cadmus et al., 2006; Gumi et al., 2012; Boukary et al., 2011; Shitaye et al., 2007]. Occasionally, *M. bovis* can be introduced to the human body through the mucous membranes and broken skins [De la Rua-Domenech et al., 2006].

In general, TB in humans gets worst when HIV-TB co-infection exists. Such situation is common in Sub-Saharan Africa [O'Brien & Perriens, 1995; Narain et al., 1992]. In addition to domestic animals, wildlife is also implicated as a cause for *M. bovis* transmission to humans [Wilkins, 2008]. Zoonotic TB presents a treatment challenge as extra-pulmonary cases are resistant to pyrazinamide, one of the first-line anti-TB drugs. But if combined drugs (isoniazid, rifampin, pyrazinamide & ethambutol) are used, the treatment becomes effective against both *M. bovis* and *M. tuberculosis* [De Kantor et al., 2010]. However, some *M. bovis* isolates are indicated to have developed resistance to rifampin and isoniazid, in addition to pyrazinamide, resulting into multidrug resistant *M. bovis* [WHO, 2017]. Apparently, if the medication starts without identifying the agent, the patient may receive inadequate treatment that eventually might lead to development of resistance to other anti-TB drugs [WHO, 2017].

Nontuberculous mycobacteria (NTM)

Opportunistic mycobacteria species, that are not members of the MTBC, can cause TB-like pulmonary lesions, localized lymphadenitis, and skin lesions in humans. These mycobacteria are known as non-tuberculous mycobacteria (NTM), mycobacteria other than tuberculosis (MOTT) or environmental mycobacteria (EM) [Primm et al., 2004; Rice et al., 2005; Covert et al., 1999]. They are the major causes of opportunistic infection in immunocompromised human patients, and some also infect animals and cause diseases [Mirsaeidi et al., 2014; Gyles et al., 2010; Quinn et al., 2001]. NTM have been found in drinking water, soil, dairy products, tuberculosis-like lesions (TBL), and animal derived swap samples [Gcebe et al., 2013; Rice et al., 2005; Harris et al., 2007; Berg et al., 2009], and the environment is considered a likely source of infection [Covert et al., 1999]. The routes of transmission of NTM are postulated to be via inhalation of aerosols or ingestion of water and soil [Van Ingen et al., 2009]. NTM pose diagnostic challenges as they cannot be

differentiated from the pathogenic mycobacteria using the acid fast (Ziehl-Neelsen) staining. They require time consuming and more expensive techniques like bacterial culture and molecular diagnostic tools for their identification [Gcebe et al., 2013].

Livestock production systems in Eritrea

Eritrea is in the Horn of Africa and lies north of the equator between latitudes 12°22′ N and 18°02′ N, and longitudes 36°26′ E and 43°13′ E. It has an area of 124,400 square kilometers. The country is bordered to the east by the Red Sea, southeast by the Republic of Djibouti, south by Ethiopia and to the northwest by Sudan. Eritrea is divided into six (Maekel, Debub, Anseba, Gash Barka, Northern, and Southern Red Sea) regions, 57 sub-regions and 699 administrative areas. About 80% of the population lives in rural areas and agriculture is the main source of livelihoods. Livestock form an important component of the agricultural production system in Eritrea. According to the Department of Animal Resources of the Ministry of Agriculture [Anonymous, 1997] in Eritrea, there are 1.9 million cattle, 4.6 million goats, 2.1 million sheep and 0.3 million camels. These are distributed into three main livestock production (husbandry) systems: intensive urban and peri-urban dairy husbandry system, the extensive mixed crop-livestock system, and the extensive pastoral system.

The dairy farms are mainly located in and around the major towns of the regions (Maekel, Debub & Anseba). Dairy farming was initiated by the Italian settlers during the colonial period in the 19th century. During the establishment and till 1970s, adequate land and proper dairy cattle houses were available in the outskirts of the cities. However, the escalation of the war for independence (1962-1991) in Eritrea forced the dairy farmers to relocate their farms (near their houses and in their compounds) in the cities for safety reasons [Mogos, 2003].

In Maekel region, currently, the dairy farms are located in the capital Asmara and its environs. Dairy farmers in this region have shortage of land for housing, thus keep their dairy herds in confined areas, in small houses that lack windows for adequate ventilation.

In Debub, most of the dairy farms are located outside the major towns with few farms within the towns. The dairy farms outside the towns have adequate space for housing and grazing. Those dairy houses located in the towns have small sizes but with windows for ventilation. In this region dairy cattle are allowed to partially graze within the compounds of the farms.

In Anseba, most of the dairy farms are located outside the major towns and have adequate spaces for partial grazing and housing and the dairy houses have adequate spaces and bigger windows. During the day the dairy cattle spend most of their time under shades outside the buildings.

The current total dairy cattle population in Eritrea is estimated around 16,000 exotic breeds (Holstein Friesians, HF) and their crosses, 13,000 local breeds, and 1,500 Sudanese breeds [MOA, Kahsay Negash, personal communication, 2010]. In 2013, the total amount of milk produced by the exotic and local dairy cattle managed within the 'intensive' system was estimated 17 million and 3 million litres, respectively [Anonymous, 2015].

In the extensive, mixed crop-livestock system, livestock are sedentary, kept in villages where cereal and livestock are managed in an integrated system and where grazing is communal, and where mixing of livestock from different villages is rare. The mixed crop-livestock production system is one in which more than 50% of household gross revenue comes from farming, and 10-50% from pastoralism (livestock) Morton and Meadows, 2000, cited by, Dinucci and Fre (2003). In the mixed crop-livestock system, animals are sources of food and cash with oxen constituting the animal power for agricultural activities, which is the vital element of the production system. The manure is also used as nitrogen source of fertilizer. Mixed crop-livestock system is also practiced in some parts of Gash Barka region where rainfall is favorable.

In the pastoral system, large mobile herds are kept in the lowlands. 'Pastoral production systems are those in which 50% or more of household gross revenue (i.e. the total value of marketed production plus the estimated value of subsistence production consumed within the household) comes from livestock or livestock-related activities, or where more than 15% of household food energy consumption consists of milk or milk products produced by the household' Morton and Meadows, 2000, cited by, Dinucci and Fre, (2003).

Such a husbandry system is mainly practiced in the western and eastern lowlands of Eritrea, namely, in Gash Barka, Northern and Southern Red Sea regions, and partially in Anseba. The herds in this production system encounter other herds during their movement. The livestock in these regions extensively move from one village to others without restriction, and thus livestock from different villages usually mix and share common grazing areas and watering points. The largest proportion of the livestock (about 60 %) is kept in the lowlands under the pastoral system [Anonymous, 2015]. The livestock species in these regions are predominantly small ruminants, cattle and camels. The total amount of milk produced in 2013 by cattle within the extensive system was estimated around 300 million liters [Anonymous, 2015].

Goats are vital components of the agricultural system of Eritrea. Because of the unpredictable and low rainfall, cropping is often at risk in the arid and semi-arid areas of Eritrea. Hence, goat production under a pastoral system can compensate failing crop harvests in most parts of the country. Moreover, goat production could be a reliable potential source of livelihood provided that effective disease control measures are undertaken. It is estimated that 60-70 % of households in Eritrea own an average flock size of 10-20 small ruminants which are considered major source of household income (Dr. Ogubeab, Director Animal Health services, MOA, Eritrea, personal communication, 2012). The biggest population of goats in Eritrea is found at Gash Barka, followed by Northern Red Sea, Debub, and Anseba regions, respectively [Anonymous, 1997].

Camels are another very important animal species with a significant economic importance in Eritrea. In pastoral areas camels' milk is sold as a source of cash as well as shared among neighbors. Camels' milk is available throughout the year, including in times of drought and in dry season. During the drought period, 50-100% of the nutrient intake of some of the pastoralists depends on camels' milk [Gebrehiwet, 1998]. Camels' milk, beyond its nutritive value, it is considered to have medicinal value in Eritrea. In the urban areas camels' milk is consumed more for its medicinal than its food value and is consumed raw. Studies conducted in Israel and Dubai have demonstrated its medicinal importance [Zagorski et al., 1998; Mal et al., 2006; Shabo, 2005; Wernery et al., 2003].

Tuberculosis situation in Eritrea

Bovine tuberculosis in animals in Eritrea

The first report of BTB in Eritrea was in 1929 (Pirani, 1929), followed by isolation of acid fast bacilli from milk in 1944 by Sfroza, who subsequently isolated eight strains of what he named '*Mycobacterium tuberculosis* bovine type', 'seemingly, *M. bovis'*, in the same year, as cited by Omer et al. (2001). A more recent study [Omer et al, 2001] demonstrated high individual animal (14.1%) and herd prevalence (41.7%) of bovine tuberculosis in dairy cattle farms in and around Asmara, the capital. But this study [Omer et al., 2001] was limited to dairy cattle in and around Asmara, Maekel region. The present overall status of BTB in the dairy sector in Eritrea is not known.

In the rural areas, raw goats' milk consumption is common, goats and cattle are herded together, and people have close contact with their animals, as well as share the same dwelling, in some cases. Such type of husbandry and housing system can be an important risk factor for transmission of tuberculosis (TB). The transmission can be multi directional: animals to animals, animals to humans, humans to animals, and humans to humans [Boukary et al., 2011; Tschopp et al., 2011; Byarugaba et al., 2009; Amanfu, 2006]. Despite the apparent economic and potential zoonotic impact of BTB in goats, the BTB status in goats is not known in Eritrea.

As camels are susceptible to tuberculosis [Manal et al., 2008; Wernery et al., 2007] milk from infected camels may contain *M. bovis* [Mustafa, 1987]. Gebrehiwet (1998) has noted his observation of chronic cough and pneumonia in camels in Eritrea. From this observation it may not be possible to conclude the presence of BTB in camels, but might indicate occurrence of BTB in camels in Eritrea that warrants further study.

Overall, the main constraints to livestock production in Eritrea are the presence of endemic diseases and shortage of feed. Most of the classical animal diseases are prevalent [FAO, 1994] in Eritrea; 100 years ago the veterinary institute was established in the country to carry out research on animal diseases and to develop vaccines. One of the important diseases with both economic and zoonotic potential in Eritrea is bovine tuberculosis (BTB).

Tuberculosis in humans in Eritrea

In Eritrea, the TB trend in humans is in a steady decline with an average rate of 2% per year. However, notification of extra-pulmonary TB, relatively, seems to rise, though new smear positive cases are reported to decrease [WHO, 2012]. Recent reports have shown that Eritrea is a low TB burden country with incidence of 78/100,000, prevalence of 123/100,000, and mortality of 14/100,000, where multidrug resistant TB (MDR-TB) occurs in 1.7% of new and 17% of re-treatment cases [WHO, 2015]. The number of pulmonary and extrapulmonary TB in humans that were notified in Eritrea during 2014 and 2015 were 2,425 and 2,075, respectively. Of these, extra-pulmonary TB cases accounted for 32% and 34%, respectively [MOH, 2014 & 2015]. The current TB detection approaches in the country are mainly screening for clinical signs and symptoms, acid fast staining (AFS), and radiology that cannot distinguish *M. bovis* from *M. tuberculosis* or other mycobacteria. Recently, liquid mycobacterial culture (MGIT system; Becton Dickinson) and GeneXpert MTB/RIF (Real-Time PCR based technique that detects presence of *M. tuberculosis* and resistance to rifampin), were established and used mainly for human case of treatment failure, or detection of drug resistance, in the National Health Laboratory, in Asmara.

The role of NTM in human and animal tuberculosis is not known in Eritrea.

Aims and content of the thesis

Since Robert Koch isolated tubercle bacilli more than 100 years ago in 1882, tuberculosis has been the focus of intense research, followed by rigorous global BTB control and eradication programs, mainly in the 1950s. Though bovine tuberculosis was reported in Eritrea in 1929, very little has been done to determine its prevalence, risk factors and its potential link with human tuberculosis. This thesis explores the overall status of bovine tuberculosis in dairy cattle within the major milk producing regions of the country, and in cattle, goats and camels managed within the mixed crop-livestock and pastoral system in Eritrea. The Single Intradermal Comparative Tuberculin Test (SICTT), besides face-to-face interviews of the livestock owners using structured questionnaires were utilized to elicit the major risk factors

associated with the current tuberculosis status in animals. In addition, it explores the potential link of bovine tuberculosis with human tuberculosis. Thus, the overarching aim of the study was: 'To investigate the prevalence and risk factors of *M. bovis* infections in domestic animals and humans in Eritrea'. To determine the BTB prevalence in dairy cattle, the major milk producing regions of the country were included in a study covering more than 50% of the dairy cattle and the findings of this study are presented and discussed in **Chapter 2.** To elucidate and better understand the major risk factors for the presence of BTB in dairy herds in Eritrea, the potential risk factors were also explored by a case control study within randomly selected dairy farms in the major milk producing regions of the country, and the findings of the study are presented and discussed in **Chapter 3**. Livestock (cattle, goats and camels) managed under the traditional farming systems (mixed crop-livestock, and pastoral) in Eritrea are the mainstay for the livelihoods of the vast majority of the Eritrean rural community, where consumption of raw milk and close animal-human contact exists. Since prevalence of BTB in these farming systems was not known, control measures could not be implemented. BTB prevalence was studied in cattle, goats and camels and the findings of the study are presented and discussed in **Chapter 4**. Though, previously, prevalence of BTB was studied using SICTT, isolation of *M. bovis* was never attempted in the country and thus presence of *M. bovis* was not confirmed. Therefore, to confirm *M. bovis* presence and the species and strains circulating in the dairy cattle in Eritrea, bacterial culture and genetic profiling of *M. bovis* from tissues with TBL collected from the Asmara municipal slaughterhouse in Eritrea was conducted using the classical genotyping methods (Spoligotyping and VNTR typing) and whole-genome sequencing (WGS), and the findings are presented and discussed in **Chapter 5**. Although *M. bovis* is known to infect humans and cause similar clinical signs as *M. tuberculosis* and rarely nontuberculous mycobacteria (NTM) in humans, its precise involvement in TB in humans was never studied in Eritrea. Hence, a study with the aim to define the presence of *M. bovis* in sputum samples collected from multidrug resistant (MDR) TB suspected patients was conducted using molecular detection techniques and classical genotyping methods. The findings of this study are presented and discussed in a paragraph within the summarizing discussion of this thesis, **Chapter 6**.

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Chapter 2

Prevalence and risk factors of bovine tuberculosis in dairy cattle in Eritrea

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Abstract

Background: The prevalence of bovine tuberculosis (BTB) in dairy cattle in the three major milk producing regions of Eritrea was assessed by subjecting 15,354 dairy cattle, 50% of Eritrea's dairy cattle population, to the single intradermal comparative tuberculin test (SICTT). Skin test results were interpreted according to guidelines of the World Organization for Animal Health (OIE) with > 4 mm as cut-off in skin thickness increase. In addition, we studied the relation between 'physiological' variables related to pregnancy and lactation, and the variable 'region' on the probability to be skin test positive.

Results: The BTB prevalences at animal and herd levels were: 21.5% and 40.9% in Maekel, 7.3% and 10% in Debub, and 0.2% and 1.6% in the Anseba region, respectively. Overall, in the regions included, prevalence was 11.3% (confidence interval (CI) 95% CI, 11.29-11.31%) and 17.3% (95% CI, 17.27-17.33%), at animal and herd level, respectively. Considering positive herds only, the animal BTB prevalence was 36.8%, 30.1%, and 1.8%, in Maekel, Debub and Anseba, respectively, and the overall animal prevalence within these herds was 32%. In adult dairy cattle the probability of positive reactivity in the SICTT test was highest in pregnant animals as compared to the other categories.

Conclusion: This study reports persistent prevalence of BTB as defined by positive SICTT tests in the dairy sector of Eritrea, especially in the regions of Maekel and Debub that are located in the central highlands of the country. To our understanding this is the first report that has encompassed all the major dairy farms in Eritrea and it will be instrumental in advocating future BTB control programs in the dairy sector.

Keywords: bovine tuberculosis (BTB); prevalence; comparative tuberculin test; dairy; Eritrea.

Background

Bovine tuberculosis (BTB) is a chronic, infectious and contagious disease caused by *Mycobacterium bovis (M. bovis)* affecting cattle and other species. The disease in cattle appears both on intensive dairy farms and in extensive pastoral systems [1-5]. The standard diagnostic test used in our study and many others is the 'single intradermal comparative tuberculin test' (SICTT). This test has moderate to high sensitivity (68-95%) and high specificity (96-99%) [6-9]. *M. bovis* is zoonotic, and can be transmitted from animals to humans through consumption of raw milk, inhalation, and direct contact with saliva. Beside its zoonotic importance, BTB affects the livelihood of people in developing countries by compromising sustainable food supply, income and social status [10]. In Africa, for example, BTB is ubiquitous: about 85% of the cattle and 82% of human populations live in areas where BTB is either not or only partly controlled [11]. In 2004, BTB was reported by 26 of the 51 African countries who filed statistics with OIE [12].

BTB is present in Eritrea [13], where the integrated farming system includes a dairy cattle population of around 16,000 animals of exotic breed (Holstein Friesians [HF]) and crossbreds, 13,000 cattle of the indigenous Barka breed, and 1,500 animals of Sudanese breeds (MOA, Kahsay Negash, personal communication, 2010). BTB, first reported in Eritrea by Pirani (1929), was reported to be highly prevalent in the capital city Asmara (Maekel region) and its surroundings in 2001 [13]. The BTB status in other regions of the country was never addressed.

Thus, the current study focused on the major milk producing regions (Maekel, Debub, Anseba), and its samples included more than 50% of the total dairy cattle population of the country. It aimed to determine BTB prevalence and its association with various animal 'physiological statuses' related to pregnancy and lactation and 'region' in relation to positive reactivity in the SICTT throughout Eritrea's dairy sector.

Methods

Study Area

Eritrea is located in the Horn of Africa and lies north of the equator between latitudes 12°22′ N and 18°02′ N, and longitudes 36°26′ E and 43°13′ E. It has an area of 124,400 square kilometres and is divided into six regions (zones) administratively, namely: Maekel, Debub, Anseba, Gash Barka, Southern and Northern Red Sea (Figure 1), each of them comprising of several sub-regions. The country has diverse climatic zones. The mean annual temperature ranges from 16°C in the highlands (around the capital Asmara) to extremely high temperatures (31°C) in the lowlands. In the highlands, where the Maekel and Debub regions are located, the hottest months are usually May to June with the highest temperature reaching around 27°C to 30°C. Whereas in the western lowlands, where the Anseba region is located, the temperature ranges from 28°C to 46°C, except in December month when the temperature falls as low as 15°C. The altitude ranges from 120 meters below sea level to 2,400 meters above sea level in the central highlands. Dairy farms in the highlands of Eritrea are mainly concentrated in the Maekel and Debub regions [14], and in the lowlands the major dairy region is Anseba.

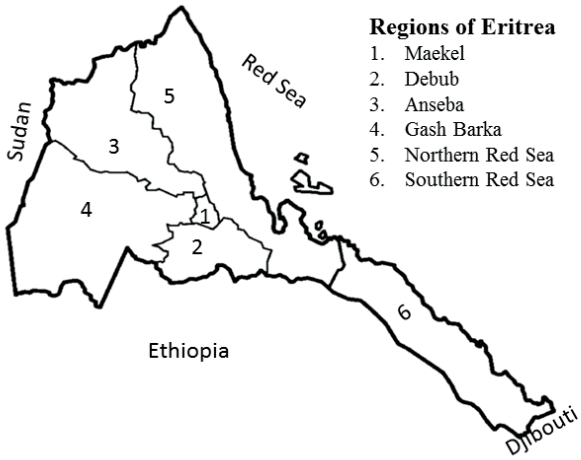

Figure 1 Map of Eritrea and the study areas. The map shows the six administrative regions of Eritrea and the three major milk producing regions of the country where the study was conducted: Maekel (1), Debub (2) and Anseba (3)

Adapted from https://commons.wikimedia.org/wiki/File:Eritrea_regions_blank.png (website re-visited: 06/05/2016)

The livestock production system in the country is divided into three parts: intensive urban and peri-urban dairy husbandry system, extensive mixed crop-livestock system, and extensive pastoral system. The dairy farms are mainly located in and around the major towns of the regions. In Maekel the dairy farms are located in the capital Asmara and its environs. Dairy farmers in this region have shortage of land for housing, thus keep their dairy herds in confined areas in small houses. Most of the houses lack windows for adequate ventilation. In Debub, most of the dairy farms are located outside the major towns with few farms within the towns. The dairy farms outside the towns have adequate space for housing and grazing. Those dairy houses located in the towns have small sizes but with windows for ventilation. In this region dairy cattle are allowed to partially graze within the compounds of the farms. In Anseba, most of the dairy farms are located outside the major towns and have adequate spaces for partial grazing and housing. The dairy houses have adequate spaces and bigger windows. During the day the dairy cattle spend most of their time under shades outside the buildings.

In the mixed crop-livestock system, livestock are sedentary, kept in villages where grazing is communal. This production system is practiced mainly in the highlands (Maekel & Debub) and partially in Anseba (in the highland areas). In the pastoral system, large mobile herds are kept in the lowlands. Such husbandry system is mainly practiced in the western and eastern lowlands of the country, namely, in Gash Barka, Northern and Southern Red Sea regions, and partially in Anseba (Figure 1). The herds in this production system come in contact with other herds during their movement. The largest proportion of the livestock (about 60%) is kept in the lowlands under traditional system. The cattle population and its distribution in the six regions is shown in Table 1. The livestock species in these regions are predominantly small ruminants, cattle and camels.

Study population and skin testing

In 2011 a cross-sectional survey was conducted to assess BTB prevalence in the three major milk producing regions, Maekel, Debub, and Anseba, in Eritrea (Figure 1). All herds and all individual animals above six weeks of age were included in the study within the selected regions. The inclusion criteria were

the same in all the regions. In total 15,354 dairy cattle in 3,149 herds were tested for BTB using the single intradermal comparative tuberculin test (SICTT). The survey was organized and coordinated by the Ministry of Agriculture, Eritrea.

Table 1 Official cattle population in 10^3 in the six regions of Eritrea (Anonymous, 1997)

Region	Cattle population
Anseba	219
Debub	490
Gash Barka	917
Maekel	40
Northern Red Sea	178
Southern Red Sea	82
Total	**1,927**

The SICTT was used based on the guidelines of the OIE [6]. Two sites on the left side of the mid-neck, 12-15 cm apart, were shaved. The skin thickness was measured with a 'Vernier caliper' and recorded. The upper site was injected with 0.1 ml containing 2,500 IU/ml avian PPD (Instituto Zooprofilatico Sperimentale dell' Umbria e delle Marche, Italia) using McLintock pre-set automatic syringes. Likewise, 0.1 ml of 2,500 UI/ml bovine PPD (Instituto Zooprofilattico Sperimentale deli Abruzzo e del Molise G. Caporale®, Italia) was injected into the lower site. Bovine PPD (PPD-B) and avian PPD (PPD-A) are defined as Purified Protein Derivatives of *M. bovis* and *M. avium*, respectively. All animals were ear tagged and their identities were confirmed at the time of reading. After 72 hours, the skinfold thickness at the injection sites was re-measured by the same operator and with the same calliper used before. A skin reaction was considered positive when the skin thickness increase at the bovine site of injection was more than 4 mm greater than the reaction at the avian injection site. The reaction was considered inconclusive when the increase at the bovine site was between 1 and 4 mm greater than the avian reaction. The reaction was considered negative when the increase in skin thickness at the bovine site

was less than or equal to the increase in the skin reaction at the avian site of injection in the absence of clinical signs at the bovine injection sites. Moreover, information relating to age, sex, physiological status (respectively, bull (castrated/entire), heifer empty, heifer pregnant, lactating empty, lactating pregnant, dry pregnant) and region, was recorded for all the tested cattle (Table 2).

Table 2 Groups of cattle in different 'Physiological statuses' and different age categories in the study (Eritrea, 2011)

Variables	Description
Calf	Young female/male animal < 2 years old
Bull	Male animal (entire/castrated) ≥ 2 years old
Heifer empty	Young female animal ≥ 2 years old that hasn't calved and is not pregnant
Heifer pregnant	Young female animal ≥ 2years old that is pregnant and hasn't calved before
Lactating empty	Mature ≥ 3 years old, lactating cow that is not pregnant
Lactating pregnant	Mature ≥ 3 years old, lactating and pregnant cow
Dry pregnant	Mature > 3 years old pregnant cow that is approximately 4 to 8 weeks from calving, and not lactating
Age (three categories)	< 3 years old 3 to ≤ 5 years old > 5 years old

Data entry and analysis

The data was first entered in an Excel spreadsheet and checked for correctness by computing frequencies (pivot tables) using SPSS IBM version 20 software. Analyses of the association of the animals 'physiological statuses' on the prevalence of BTB as assessed by SICTT were performed using the statistical package R version 3.1.0 [15]. A 'Generalized linear mixed model fit by maximum likelihood' [16] was used with BTB as binary outcome (BTB present: yes or no) and with herd as random effect to account for correlated observations within herd. Explanatory variables were 'region' (forced in the model), 'physiological statuses' and 'age category' (the latter variable was included in the model on data of adult animals as 'age' is highly correlated

with 'physiological status'). In both models (on all animals and adult animals respectively) the final model contains 'region' and 'physiological status'.

The AIC (Akaike's Information Criteria) selection criterion was used to select the 'best model'. The analysis was performed in positive herds (at least one reactor animal) with ≥ 5 animals only (Model 1 & 2) (Table 3 & 4). The variable 'physiological status' included calf, bull, heifer empty, heifer pregnant, lactating empty, dry pregnant, lactating pregnant. The variable 'age' included different age groups, namely; < 3 years old, 3 to ≤ 5 years old, and > 5 years old (Table 2).

Results

Descriptive analysis

Overall, 15,354 individual animals in 3,149 herds were tested in the three regions. The highest proportion of the cattle population under study was that of young stock < 3 years (60%) followed by mature animals 3 to ≤ 5 years (24%). The prevalence of SICTT reactors was 11.3% (95% CI, 11.29-11.31%) at individual animal level and 17.3% 95% CI, 0.1727-0.1733) at herd level. The individual animal prevalence would have increased by 0.4% had we used single intradermal tuberculin test instead of SICTT. The number of individual animals and herds tested in each of the regions were: Maekel (n = 5,667 and 927), Debub (n = 6,827 and 1,839), Anseba (n = 2,860 and 383), respectively. The region with the highest animal and herd BTB prevalence was Maekel (21.5% and 40.9%), followed by Debub (7.3% and 10%) and Anseba (0.2% and 1.6%). Considering only the positive herds (n = 545) in the three regions, the total number of animals tested was 5,272. These comprised 3,226 animals in Maekel, 1,647 in Debub and 399 in Anseba. The BTB animal prevalence in the positive herds was 36.8% (n = 1,188), 30.1% (n = 496), and 1.8% (n = 7) in Maekel, Debub, and Anseba, respectively. The reaction to bovine tuberculin test was strong with differences in skin thickness (PPD-B minus PPD-A) reaching 4.5 mm to 73 mm (Additional File 1). The additional file shows the bovine versus avian immune reactivity in the skin test in dairy cattle in Eritrea. This file will be useful as a baseline document for further studies in the area.

Table 3 Model 1 'Physiological status' and 'region' as potential risk factors for reactivity in the comparative tuberculin test on positive herds with ≥ 5 tested animals (4,776 observations within 344 positive herds) and animals tested, number and proportion of positive reactors in all positive farms (5,269 observation). Estimated variance for herd: 1.118 on the logit scale

Physiological status and region	OR	95% confidence interval		Number and % tested and % positive reactors in positive farms		
		Lower bound	Upper bound	Tested	% tested	% positive reactors
Calf (reference)	1.0			1613	30.6	14.6
Bull	5.2	3.3	8.3	147	2.8	30.6
Heifer empty	2.5	1.8	3.5	533	10.1	22.1
Heifer pregnant	5.8	4.2	8.1	460	8.7	34.3
Lactating empty	8.2	6.3	10.6	996	8.7	43.9
Lactating pregnant	10.8	8.4	13.9	1191	22.6	46.9
Dry pregnant	10.2	7.0	14.8	329	6.2	42.2
Anseba region (reference)	1.0			397	7.5	1.8
Debub region	8.5	1.9	37.1	1647	31.3	30.1
Maekel region	13.0	3.0	46.1	3225	61.2	36.8

Risk factors analysis

The highest prevalence of SICTT reactors was found in 'lactating-pregnant cows (Table 3). In Table 3 the Odds ratios (OR), with 95% confidence interval, of BTB status with animal group and region in positive farms with at least 5 tested animals are presented. The category 'calf' (animals of < 2 years age) was taken as a reference for the 'physiological status' groups. Compared to calves animals in all different 'physiological statuses' groups were significantly more at risk of being skin test positive, with pregnant lactating cows being most at risk (Table 3, Model 1). Animals in Maekel were most at risk to be SICTT positive reactors, followed by those in Debub when compared to the Anseba region.

The odds ratios of BTB statuses of 2,356 adult animals in 326 positive herds with 'physiological status' as potential risk factor in relation to positive skin test results, taking 'region' into account are presented in table 4 (Model 2). Compared to bulls, as reference, females tend to have a higher risk, with pregnant animals significantly so.

Table 4 Model 2 'Physiological status' as potential risk factor for reacting to tuberculin testing in adult dairy cattle in positive herds with ≥ 5 tested animals (2,356 observations in 326 herds) in Eritrea-2011, corrected for region. Estimated variance for herd: 1.245 on the logit scale

Physiological status	OR	95% confidence interval	
		Lower	Upper
Bull (reference)	1.00		
Lactating empty	1.55	0.99	2.43
Lactating pregnant	2.24	1.43	3.50
Dry pregnant	2.01	1.19	3.40

Discussion

The current study included more than half of the dairy cattle population of Eritrea and reports an overall prevalence of BTB at animal (11.3%) as well as at herd (17.3%) level. The individual animal BTB prevalence differed between the three regions included, ranging from 21.5% in Maekel to 7.5% in Debub and 0.2% in Anseba. The BTB prevalence in skin test positive herds was not uniform; it was highest (37%) in Maekel, followed by Debub (30%), and Anseba (2%).

The risk of having a BTB skin test positive animal was 13 times more likely in Maekel and ~9 times more likely in Debub than in Anseba (Table 2, Model 1). Differences in BTB prevalence may be due to risk factors varying across regions, as geographical location is in general an accepted risk factor. Herds and individual animal factors [17] may be attributed to the presence or absence of proper housing, adequate space for grazing and exercise, the type of breeds kept, and differences in climate.

Apart from differences in climatic circumstances between the regions the dairy housing system differs as well. In the highlands the farmers keep their animals indoors, in small houses with few or no windows [18], and as reported poor ventilation and housing may facilitate transmission of *M. bovis* [2, 19-21]. Close contact between animals is known to be a major risk factor as BTB is mainly transmitted via the respiratory route [22-24]. Similarly, close proximity of dairy farms and fence-line contact between animals, like in Maekel may be another important risk factor [25, 26]. These might have contributed over time to the persistence and high prevalence of BTB in the region, especially since no control program is in place in Eritrea. In contrast, in Anseba the dairy houses have adequate windows for ventilation and the animals spend more time outside in shaded areas and these factors might have contributed to the low number of SICTT reactors in the dairy farms of the region.

Asmara and its surroundings (Maekel region) was the first place in the country where exotic breeds were introduced for dairying. It was also in this area that BTB was reported for the first time in Eritrea, in 1929, and as already indicated in the previous study [13] as well as by the present study, the BTB prevalence is still on-going with an increasing trend. This might

indicate that BTB has established in this region as it is likely for herd breakdowns to occur repeatedly in the same areas [27]. It may also be reasonable to argue that the subsequent development of an intensive dairy production system contributed to the establishment of *M. bovis* in this production environment as previously stated by various investigators [11, 28]. There is a good reason therefore to suspect that Maekel region might be acting as a source for *M. bovis* spread in the country, as it is a pioneering region for commercial dairying and a source of stocking and restocking of the dairy farms of the different regions in the country.

A major limitation of the present study was that the 'breed' of the animals tested was not sufficiently recorded by the operators during the skin testing period and thus could not be included in the analysis. However, we assume that the different breeds kept in the different regions of the country may have contributed to the relatively higher BTB prevalence observed in Maekel and Debub regions where the presence of the HF breed predominates at the farms as opposed to Anseba. Several studies have shown indigenous zebu cattle to be relatively tolerant to *M. bovis* infection as compared to exotic dairy breeds [3, 13, 20, 28-30]. To address this important aspect a relevant study is currently underway.

The present study indicated that pregnancy and lactation were significantly linked with the reactivity in SICTT. Lactating -pregnant cows were most at risk (OR~11) to show positive reactions in the skin test compared to calves, and 2 times more compared to bulls. These results are in agreement with a similar study in Ethiopia [20] that reported that among 'physiological status' groups pregnant lactating animals had the highest prevalence of BTB. Kazwala et al., [31] also reported high reactivity in SICTT in lactating cows. Pregnant animals are reported to have two to threefold more severe inflammatory lesions with a rise to miliary tuberculosis, compared to non-pregnant young female adults [32]. It is difficult to explain the strongly increased odds ratio for SICTT positive responses in lactating-pregnant animals. Physiological alterations in immune responsiveness during pregnancy and lactation might influence skin test responsiveness and potentially lead to improved detectability of skin test positive females.

A case control study has shown that susceptibility of animals to BTB infection increases when they are fed deficient rations [33]. Currently, the available animal feeds in Eritrea are deficient quantitatively (inadequate in amount) as well as qualitatively (inadequate in their contents of carbohydrate, proteins, minerals, etc.) [34]. Feeding on such feeds might have contributed to the higher susceptibility to BTB in the pregnant cows as they are more demanding in their nutrient requirement.

Our risk factor analyses showed that 'physiological status' was strongly associated with SICTT positive reactivity (Table 4, Model 2). Since age and herd sizes have been indicated to be associated with the prevalence of BTB in several studies [13, 35-39], we included age and herd size as risk factors in the model. When the variable 'age' (3 levels) was added to the adult animal model on 'physiological status', age was not significant and did not influence the model estimates (results not shown). In the model with all age groups, calves clearly had the lowest risk of being SICTT positive as expected. Besides, including animals from all herd sizes, instead of only herds of five animals and above, did not have an important effect on the odds ratios of 'physiological status' vis-a-vis BTB prevalence (results not shown).

When compared to calves, bulls were at higher risk (OR~5) to react to the skin test (Table 2, Model 1). The increased risk of bulls to be skin test positive as shown may be due to frequent contact of these animals with other herds during breeding, as sharing of bulls is a common practice in Eritrea [40], which is also considered to be a risk factor for BTB transmission between herds of animals [33]. In addition, in view of age, the potential cumulative exposure time for bulls is higher than for calves.

The overall results of the current study resemble those of similar studies in Ethiopia [1, 41-43]. The prevalences in Maekel were, however, higher than those reported by a study conducted previously by Omer et al. in the same region in Eritrea [10], which reported lower (14.5%) animal, and comparable (41.7%) herd BTB prevalences. As expected the animal prevalence was higher than in the previous study since no BTB control measures were in place. The slightly higher herd prevalence in the previous study [10] might be due to the fact that they only considered animal herd sizes of > 9 whereas in our study

herd sizes of ≥ 1 were taken into account. Smaller herds have a lower prevalence [37, 39, 40].

In this study bacterial culture was not conducted due to the fact that skin test positive dairy cattle were not slaughtered after the disclosure of the results due to a lack of a compensation scheme.

In order to get more insight in the importance of BTB and its epidemiology in Eritrea, as a potential basis for a control program, further investigations in dairy cattle as well as in indigenous cattle, goats and camels kept under traditional livestock production systems are underway.

Conclusion

The current study has brought to light the persisting prevalence of BTB as well as some animal characteristics, related to pregnancy and lactation, linked with SICTT positive skin test in the dairy sector in Eritrea. The BTB risk tended to vary by region and adult lactating-pregnant animals were the most likely to be test positive.

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Supporting information

Additional File 1 Frequency of skin thickness (mm) after 72 h post inoculation in both the avian and bovine sites and the differences within the two (Bovine 72 h -Avian 72 h). The first two columns show the skin thicknesses on the avian site of injection after 72 h of the PPD-A inoculation and their frequencies (frq). The following four columns show the skin thicknesses on the bovine site of injection after 72 h of PPD-B inoculation and their frequencies. The following six columns show the skin thickness differences on the bovine sites versus avian sites of injection after 72 h of inoculations of both PPD-A and PPD-B (PPD-B minus PPD-A) and their subsequent frequencies.

Avian 72 hrs		Bovine 72 hr				Bovine - Avian					
Skin thickness	Frq	Skin thickness	Frq	Skin thickness	Frq cont..	Skin thickness	Frq	Skin thickness	Frq cont..	Skin thickness	Frq cont..
1	44	1	41	22.5	3	-16	1	15.5	5	48	1
2	1180	2	1085	23	52	-13	1	16	52	49.5	1
2.5	12	2.5	12	24	49	-12	2	16.5	2	50	2
3	2306	3	2036	24.5	1	-11	1	17	52	52.5	1
3.5	163	3.5	157	25	51	-9	5	17.5	2	53	1
4	2976	4	2613	26	36	-8	1	18	59	54	1
4.5	356	4.5	350	27	31	-7	9	19	49	56	3
5	2654	5	2425	28	20	-6	9	19.5	2	59	1
5.1	1	5.1	1	29	22	-5	13	20	34	61	2
5.5	476	5.5	463	30	24	-4	32	21	32	62	1
5.7	1	5.7	1	30.5	1	-3.5	1	21.5	3	73	1
6	2041	6	1942	31	26	-3	41	22	35		
6.1	1	6.5	381	31.5	1	-2	59	22.5	1		
6.5	392	7	919	32	27	-1.5	1	23	26		
7	995	7.1	1	33	15	-1	70	23.5	4		

7.1	2	7.5	205	33.5	2	-0.5	1	24	30
7.5	227	8	491	34	20	0	13294	24.5	1
8	544	8.5	99	35	23	0.5	85	25	12
8.5	107	9	237	36	7	1	50	26	20
9	261	9.1	1	37	6	1.5	8	26.5	2
9.5	47	9.5	49	38	9	2	21	27	12
10	207	10	219	38.5	1	2.5	5	27.5	1
10.5	18	10.5	19	39	5	3	12	28	19
11	132	11	149	40	10	3.5	2	29	6
11.5	6	11.5	6	40.5	2	4	64	29.5	1
12	94	12	131	41	6	4.5	13	30	17
12.5	8	12.5	13	42	9	5	102	31	9
13	42	13	77	43	2	5.5	8	32	6
13.5	5	13.5	8	44	5	6	109	33	7
14	28	14	98	45	7	6.5	8	33.5	1
15	33	14.3	1	46	6	7	102	34	10
15.5	1	14.5	2	47	5	7.5	9	35	3
16	20	15	97	48	1	8	112	36	3
16.5	1	15.1	1	49	2	8.5	4	37	2
17	10	15.5	12	50	5	9	95	38	6
18	15	16	113	51	4	9.5	5	38.5	1
19	9	16.5	7	52	3	10	108	39	2
20	9	17	70	53	1	10.3	1	39.5	1
21	4	17.5	5	55	3	10.5	10	40	3

22	6	18	72	57	1	11	87	40.5	2
23	4	18.5	2	60	6	11.5	8	41	3
25	2	19	69	61	2	12	92	42	7
27	1	19.5	4	65	3	12.5	2	43	3
28	2	20	91	69	1	13	85	43.5	1
31	2	20.5	1	70	1	13.5	4	44	1
		21	69	72	1	14	59	44.5	1
		21.5	4	76	1	14.5	4	46	3
		22	76	80	1	15	60	47	1

Chapter 3

Farm level risk factors associated with bovine tuberculosis in the dairy sector in Eritrea

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Summary

The aim of our study was to determine the association of selected potential risk factors with the presence of bovine tuberculosis (BTB) in dairy herds in Eritrea. A case control study was conducted in the three major milk producing regions of the country by stratified random sampling of 61 case and 65 control herds combined with completion of a standardized pretested questionnaire pertaining 36 relevant risk factors (variables). The variables were divided into two clusters, based on potential association with either 'introduction', or 'establishment' of BTB on the farms to elucidate association with incident or prevalent cases separately. Subsequent to univariable analysis of the 36 risk factors at herd level, 14 of these were offered to multivariable logistic regression models. Farms with higher numbers of cows, and those with concrete floors, were 3.6, and 7.5 times more at risk for presence of BTB, respectively, compared with their references. These findings will be useful as entry points for future informed decision making towards BTB control and eradication program in the country.

Keywords: bovine tuberculosis, case control study, risk factors, dairy, Eritrea

Introduction

Bovine tuberculosis (BTB) caused by *Mycobacterium bovis* (*M. bovis*) (OIE, 2009) is a debilitating, infectious and contagious disease that affects many animal species and occasionally humans. Animal husbandry and management systems contribute to the development and dissemination of BTB. The 'intensive husbandry system' has shown to be a predisposing factor to BTB in animals (Griffin et al., 1993). Since the principal route of transmission of *M. bovis* is via aerosols (OIE, 2009), close contact between animals enhances the transmission of the disease (Neill et al., 1991). Apart from animal-to-animal transmission as a cause of infection, humans with open tuberculosis due to *M. tuberculosis* or *M. bovis* may also be sources of infection to animals (Radostits et al., 2007).

In Eritrea BTB is one of the most important zoonotic diseases already reported by Pirani (1929), as cited by Omer et al. (2001). Recently, Ghebremariam et al.

(2016) reported 17.3% herd prevalence of bovine tuberculosis in the dairy cattle in the country. The goal of the current study was to investigate the association of potential farm level risk factors with this herd prevalence.

Material and Methods

Study area and selection criteria

To define farm level risk factors associated with positive results in the 'single intradermal comparative tuberculin test (SICTT reactors)', as indicator of *M. bovis* infection, we conducted a case control study with a 1:1 ratio. A herd was considered a 'case herd' when it included at least one SICTT reactor (positive) animal during the 2011 BTB prevalence survey in the dairy sector in Eritrea (Ghebremariam et al., 2016). In the previous prevalence study (Ghebremariam et al., 2016), all herds (3,149) and all individual animals (15,354) above six weeks of age, from the three selected regions (Maekel, Debub, & Anseba) were tested using the SICTT according to OIE. A herd was considered a 'control herd' when none of the animals reacted to the test. Herds with (n = 545) and without SICTT (n = 2,604) reactors were identified in each of the three major milk producing regions (Maekel, Debub & Anseba; Figure 1). Relative to the number of reactor herds in Maekel and Debub, the largest proportions of herds, n = 56 and, n = 65, respectively, were selected from these two regions and only few (n = 5) from Anseba. Prior to the selection, all the herds in the three regions were stratified according to their herd sizes, categories being 9-20, 21-30, 31-40, and > 40 animals. Only farms with a herd size of ≥ 9 head of cattle were selected. Numbers of herds, representative for the number of herds in each stratum, were randomly drawn using a computer generated random table (Epi Calc 2000 version 0.1), resulting in inclusion of 61 case and 65 control herds.

To obtain information on the potential risk factors associated with the reactivity to SICTT in a standardized fashion, a pre-tested questionnaire was used. It included 36 relevant variables that captured the farmers' and farms' characteristics. The variables were divided into two clusters, based on potential association with either 'introduction' (entry to the farms from an external source), or 'establishment' (persistence on the farm) of BTB. The selection process was based on

epidemiological importance of the factors as described by Griffin et al. (1996). All 36 factors selected for the study and their categories are shown in Table S1.

Data analysis

Data were first entered in Excel and then exported to SPSS IBM version 20 software and analyzed.

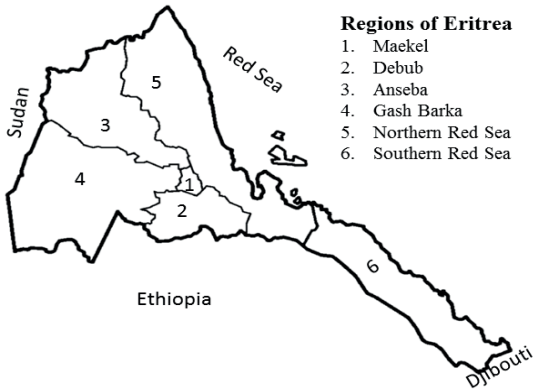

Figure 1 Study area map: Maekel (1), Debub (2) and Anseba (3), the three major milk producing regions of Eritrea. Adopted from Ghebremariam et al. (2016)

Associations between the farm level risk factors (variables) and herds with reactor(s) were tested in two stages: First, all the variables (n = 36; Table 1) were screened using the Fisher's Exact Test in univariable analyses and those variables with p-value < 0.25 (n = 14) (Table 2) were tested for collinearity. The variable 'reproduction method' could not provide any interpretable differences between case and control herds (Table 2). Due to the lack of AI-only reproduction methods in control herds, the Firth's correction was applied to allow for estimation of the odds ratio (OR) (Table 2), but the variable ('reproduction method') was excluded from further analysis as it did not give interpretable result in the multivariable analysis. Hence, 13 variables remained to be offered to either or both of the multiple logistic regression

models, for 'introduction' and 'establishment' of BTB (presence of SICTT reactors) on the farms (Table 3). During the multivariable logistic regression model building, using manual (Enter) and automated (Backward) procedures for the possible risk factors screened, respectively, potential confounders were identified by the changes in the coefficients (β) (i.e., if inclusion of a variable to the baseline model altered the coefficient of the model by > 10%, one of the variables, depending on its importance, was retained/ dropped), and p-values < 0.05, to decide for definite inclusion or exclusion of the variables in the models.

Two variables, 'age of respondents' and 'major activity', representing farmers' characteristics for 'establishment' of BTB in the farms were highly related to case control status. However, they were not used in the analysis as they overshadowed the potential influence of more biological factors. Besides, two variables ('number of bulls' and 'availability of bulls') that showed high correlation with 'number of cows' and 'herd size' were excluded from the models, since the latter two were considered more plausible risk factors for 'introduction' and 'establishment' of BTB, respectively. The herd size, number of cows, and number of heifers, were arbitrarily categorized as shown in Table 2. The model for introduction of BTB included four variables ('number of cows', 'species of animals sharing water point', 'sharing of water point', and 'same source of bull purchase') and that for establishment of BTB six variables ('herd size', 'number of heifers', 'housing type', 'housing system', 'building status', and 'feeding system'). Finally, two variables ('number of cows', and 'building status' from both 'Introduction' and 'Establishment') retained in the final model (Model 3; Table 3) as potential risk factors for the presence of BTB reactors in the farms.

Results

Univariable analysis

A summary of the results of the univariable analysis is shown in Table 2.

The variables 'age of respondent' and 'major activity' were selected to demonstrate the role of farmers' characteristics in relation to SICTT reactivity between the case and control farms. 'Age of respondents' coded in three

categories [25-45 years, 46-65 years, and > 65 years] was significantly associated (p-value = 0.036) with the presence of SICTT reactor animal(s) on the farms. Farms owned by the 46- to 65- year age group (Table 2) were the most affected by BTB (OR = 3.5), followed by farms owned by those aged > 65yrs (OR = 1.9) when compared with farms managed by younger farmers (25-45 yrs).

'Major activity' of the farmers grouped into two categories (full-time dairy and part-time dairy farming) was significantly associated (p-value = 0.004) with the presence of SICTT reactor animal(s). Farms owned by full-time dairy farmers were more at risk (OR = 3.3) as compared with those owned by part-time dairy farmers.

The remaining thirteen variables described farm characteristics as risk factors for the presence of one or more SICTT reactors on the farms.

One of the potential risk factors, 'number of cows' on the farms, screened in the univariable analysis, was significantly (p-value = 0.001) associated with the presence of SICTT reactors. Farms with larger (> 10) numbers of cows were more at risk (OR = 4.0) compared to those with smaller numbers of cows. The mean number of cows in the dairy herds included in the study was 10.2 (range: 0-120), whereas the mean numbers of cows in the cases and controls were 13.52 (range: 0-120) and 7.09 (range: 2-20), respectively.

'Herd size' was also significantly (p-value = 0.002) associated with the presence of SICTT reactors and was coded in two categories (Table 1). The risk increased with herd sizes > 26 animals (OR = 5.0). The mean herd size for cases was 27 (range: 9-180) as compared to 16 (range: 9-60) for controls.

The number of heifers was significantly associated (p-value = 0.018) with the presence of SICTT reactors in dairy farms and herds with larger (> 8) numbers of heifers had a higher risk (OR = 4.1) than herds with fewer heifers. The mean number of heifers in case farms was 6.8 (range: 0-40) as compared to 4.1 (range: 0-20) in controls.

Table 1 Names, descriptions and coding of the 36 variables and their categories included in the study as potential risk factors for 'introduction' (Intro), and 'establishment (Est) of BTB in the 61 case and 65 control farms in Eritrea. Frq, frequency

Variables	Description	Coding of the variables
Age respondent (Est)	Age of the farmer in years (yrs)	25-45 yrs, 46-65 yrs, and > 65 yrs
Educational level (Both)	Farmers' educational level	Low (can/not read and write), Medium (1-8 grade education), High (secondary/tertiary education)
Major activity (Est)	Major activity (occupation) of the farmer	Full-time dairy, part-time dairy
Breed (Est)	Breed of cattle available in the farm	Holstein Friesians (HF), others (HF crosses, local, Sudanese breeds
Herd size (Est)	Total number of cattle in the farm (cows, heifers, bulls, etc.)	Small herds (9-26), large herds (> 26)
Number of cows (Intro)	Number of matured ≥ 3 yrs old cattle that has calved	Small size (0-10), large size (> 10)
Number of heifers (Est)	Number of matured (≥ 3 yrs old) female cattle that have not calved	Small size (0-8), large size (> 8)
Availability of bulls (Both)	Availability of matured (≥ 2 yrs old) male animals at farm for natural breeding	Yes/No
Number of bulls (Intro)	Number of bulls available at farm	No bulls, one bull, ≥ 2 bulls
Stock density (Est)	Number of animals per given spaces	Number of cattle per square meter
Labor source (Est)	Source of work force used in the farm	Family, hired, both
Distance from other dairy farms	Distance of the dairy farms from other dairy farms in the vicinity	In meters (m)
Intro. of new animals (Intro)	Introduction of new animals in to the existing herd	Yes/No
Cattle purchase frq (Intro)	Number of times the farm purchases cattle	No purchase, every 1-2 yrs, every 3 and more yrs
Reproduction method (Intro)	Method of reproduction used for breeding of the cattle	Artificial insemination (AI), natural method

		using bulls, both
Source of bulls for mating (Intro)	The sources or origins of bull(s) used for breeding in the farm	Not applicable, home grown, not home grown
Bull purchase frequency (Intro)	The number of times a farm purchases bull(s)	No purchase, every 1-2 yrs, every 3 and more yrs
Same source of bull purchase? (Intro)	Is the farm purchasing bull(s) from the same source(s)?	Not applicable (no purchase), yes, no
Other animals present at the farm (Intro)	Other species of animals available in the farm in addition to cattle	Not available, sheep and goats, equids, dogs and chicken, multispecies
Cattle contact with (Intro)	With which species of animals do your animals get contact	No contact, one species multispecies
Wild animals present (Intro)	Wild animals noticed in the farm or around the farm premises	No wildlife, one species, multispecies
Source of water (Intro)	Source of drinking water for the animals in the farms	Water at farm, water outside farm, both
Sharing of water point (Intro)	Sharing of water points with animals from other farms	Yes/No
Species of animals sharing water point (Intro)	The species of animal from other farms sharing the same water point	Not sharing, one species, multispecies
Signs of respiratory diseases? (Est)	Any clinical signs observed in relation to respiratory diseases	Yes/No
Clinical signs observed (Est)	Signs of respiratory diseases manifested by any animal(s) in the farm that were observed by the farmer	Not applicable, difficult breathing and anorexia, anorexia, listlessness and emaciation
Housing type (Est)	Dairy house types in which either the animals are kept tied (stanchioned) in the house/shade or kept at a liberty to move freely	Stanchion(tied) system, other
Housing system (Est)	System of housing that either keeps the animals all the time inside, or allows the animals to stay both inside or outside, or outside all the time	Housed indoors all the time, not all the time housed indoors

Building status (Est)	The floor types in the dairy houses, whether the dairy farms have concrete floors or other types of floor (example: sand)	With concrete floors, without concrete floors
Ventilation status (Est)	The status of the houses in terms of air supply and removal by natural means through windows and doors	Good, bad
Frequency of cleaning (Est)	The number of times the dairy houses are cleaned	Once every day, twice a day, once a month
Feeding of green feeds (Est)	Provision of green feed to the animals either by grazing, by cut and carry or purchase	Yes/No
Source of green feed (Est)	Where does the green feed come from: own farm or purchase from other farms?	Not applicable, home grown, purchased, both
Feeding system (Intro)	The system of feeding that allows grazing of animals or that does not allow grazing but rather depends on cut and carry (bringing the green fodder to the barn)	Zero grazing, grazing
Concentrate feeding (Est)	The provision of the animals with concentrate (formulated) feed that fulfills the requirement of dairy cattle for milk production	Yes/No, not disclosed
Mineral supplement (Est)	Provision of mineral supplements to the animals in the form of mineral licks	Yes/No

Finally, the 'availability of bulls' (categories: yes/no), and 'number of bulls', were associated with the presence of SICTT reactors (p-values = 0.199, and 0.077, respectively). Farms with bull(s) were more at risk (OR = 1.2) compared with farms without bulls, and those with two or more bulls were most at risk (OR = 2.8), followed by farms with one bull (OR = 1.2). The mean number of bulls in case farms was 1.2 (range: 0-4) as compared with 0.8 (range: 0-3) in control farms.

In addition to the aforementioned risk factors, related to numbers of animals within a herd, management factors associated with indoor/outdoor keeping of animals were assessed. 'Species of animals sharing water point' was associated (p-value = 0.10) with the presence of reactor animals in the farms, and herds sharing with one species of animals (OR = 0.2) and those sharing with multispecies (OR = 0.8) were protective. Besides, 'sharing a water point' (categories: yes/no) was associated (p-value = 0.116) with having SICTT reactor animal(s) in the farms and was a protective (OR = 0.5) factor.

The use of stanchions on the farm was identified as a risk factor (OR = 1.8) and found to be associated (p-value = 0.135) with being a case herd.

The variable 'housing system' was grouped into two categories: farms that kept their animals indoor permanently, compared with those that did not do so. Farms that kept their cattle entirely indoor were at higher risk (OR = 1.8) of having SICTT reactor(s) as compared with farms that allowed animals outdoors as well.

The 'building status' was grouped into two categories: dairy farms with concrete floors, and without concrete floors. This variable was significantly (p-value = 0.033) associated with the presence of SICTT reactor animal(s) in the farms, and those that housed their animals on concrete floors were more (OR = 8.4) at risk.

The variable 'feeding system' was categorized into two groups (zero-grazing and grazing) and was significantly associated (p-value = 0.061) with the presence of SICTT reactor(s) on the farms. Farms using zero-grazing were at higher (OR = 4.1) risk than the others.

Finally, the univariable analysis has shown that reproduction method (artificial insemination (AI) versus AI and natural breeding) was significantly (p-value = 0.024) linked to the presence of SICTT reactor(s) in the farms. Farms using both 'AI and natural breeding' had (OR = 0.08) a protective effect (Table 2).

Multivariable analysis

From the three potential risk factors related to 'introduction' of BTB on the farm that were offered to the first multivariable model (Model 1, Table 3) to analyze the occurrence of SICTT reactivity in the dairy farms, the final model retained only the 'number of cows' in the farms. Farms with larger numbers of cows (> 10) were more at risk (OR = 4.0; 95% CI: 1.7-9.7) than farms with ten cows or less.

Likewise, from the six variables that were offered to the second model (Model 2, Table 3) as possible risk factors for 'establishment' of BTB in the farms, two variables, namely 'herd size', and 'building status' were retained in the model. Larger herd sizes (> 26) were associated with a higher risk (OR = 4.8; 95% CI: 1.6-14.4) compared to smaller herd sizes (9-26).

Herds kept on concrete floors were more at risk (OR = 8.6; 95% CI: 1.0-75.7) to have SICTT reactors when compared with those kept on other types of floors (Table 3). This variable ('building status') had a borderline significance (p-value = 0.052), but was retained in the model given its biological importance as a risk factor. In the final multivariable model (Model 3; Table 3), only two variables; 'number of cows' from the 'introduction model' and 'building status' from the 'establishment model' were retained.

Table 2 Variables (risk factors) screened by univariable analysis with p-values < 0.25, their distribution, and p-values of Fisher's Exact Test, odds ratios (OR) and 95% confidence intervals for 61 case and 65 control herds as determined by SICTT reactivity in the dairy sector of Eritrea. NI, not interpretable due to empty cells; Ref, reference

Variables	Categories	Case (n = 61) % (n)	Control (n = 65) % (n)	OR	95% CI	P-value
'BTB introduction'						
Number of cows	0-10	60.7 (37)	86.2 (56)	1.0	Ref	.001
	> 10	39.3 (24)	13.8 (9)	4.0	1.7-9.7	
Reproduction method	Natural and both	91.8 (56)	100 (65)	NI		.024
	AI	8.2 (5)	0.0 (0.0)	0.0	Ref	
Number of bulls	No bulls	31.1 (19)	43.1 (28)	1.0	Ref	.077
	One bull	34.4 (21)	40 (26)	1.2	0.5-2.7	
	≥ 2 bulls	34.4 (21)	16.9 (11)	2.8	1.1-7.2	
Species of animals sharing water point	No sharing	86.9 (53)	75.4 (49)	1.0	Ref	.10
	One species	3.3 (2)	13.8 (9)	0.2	0.04-1.0	
	Multispecies	9.8 (6)	10.8 (7)	0.8	0.2-2.5	
Sharing water point	Yes	13.1 (8)	24.6 (16)	0.5	0.2-1.2	.116
	No	86.9 (52)	75.4 (49)	1.0		
Same source of bull purchase	Not applicable	70.5 (43)	78.5 (51)	0.3	0.1-1.1	.167
	Yes	14.8 (9)	4.6 (3)	1.0	Ref	
	No	14.8 (9)	16.5 (14)	0.3	0.1-1.3	
Availability of bulls	No	31.1 (19)	43.1 (28)	1.0	Ref	.199
	Yes	68.9 (42)	56.9 (37)	1.7	0.8-3.5	

'BTB establishment'						
Age-respondent	25-45 yrs old	11.5 (7)	27.7 (18)	1.0	Ref	.036
	46-65 yrs old	68.9 (42)	47.7 (31)	3.5	1.3-9.4	
	> 65 yrs old	19.7 (12)	24.6 (16)	1.9	0.6-6.1	
Major activity	Full time	80.3 (49)	55.4 (36)	3.3	1.5-7.9	.004
	Part time	19.7 (12)	44.6 (29)	1.0	Ref	
Herd size	9-26	70.5 (43)	92.3 (60)	1.0	Ref	.002
	> 26	29.5 (18)	7.5 (5)	5.0	1.7-14.6	
Number of heifers	0-8	78.7 (48)	93.8 (61)	1.0	Ref	.018
	> 8	21.3 (13)	6.2 (4)	4.0	1.5-13.5	
Housing type	Stanchion system	72.1 (44)	58.5 (38)	1.8	0.9-3.9	.135
	Others	27.9 (17)	41.5 (27)	1.0	Ref	
Housing system	Inside the house all time	55.7 (34)	41.5 (27)	1.8	0.9-3.6	.153
	Not all the time inside the house	44.3 (27)	58.5 (38)	1.0	Ref	
Building status	With concrete floors	98.3 (58)	87.3 (55)	8.4	1.0-69.7	.033
	Without concrete floors	1.7 (1)	13.6 (8)	1.0	Ref	
Feeding system	Zero grazing	96.7 (59)	87.7 (57)	4.1	0.8-20.3	.061
	Grazing	3.3 (2)	12.3 (8)	1.0	Ref	
Availability of bulls	No	31.1 (19)	43.1 (28)	1.0	Ref	
	Yes	68.9 (42)	56.9 (37)	1.7	0.8-3.5	.199

Table 3 Final logistic regression models of the risk factors associated with herds having SICTT reactor(s) in the study areas within the dairy sector of Eritrea, as they are grouped into factors for 'Introduction' (Model 1) and 'Establishment' (Model 2) of BTB with the final model (Model 3) for the presence of SICTT reactor(s) on the farms

Variables	P-values	OR	95% CI	
			lower	upper
Results of multivariable logistic regression for model 1				
'Number of cows'				
0-10 (reference)		1.0		
> 10	.002	4.0	1.7	9.7
Results of multivariable logistic regression for model 2				
'Herd size'				
9-26 (reference)		1.0		
> 26	.006	4.8	1.6	14.4
'Building status'				
Without concrete floors (reference)		1.0		
With concrete floors	.052	8.6	1.0	75.7
Results of multivariable logistic regression for model 3, based on variables from model 1 + 2				
Number of cows				
0-10 (reference)		1.0		
> 10	.005	3.6	1.5	8.9
Building status				
Without concrete floors (reference)		1.0		
With concrete floors	.066	7.5	0.9	64.1

Discussion

To the best of our knowledge, this is the first study in Eritrea that has systematically identified farm level risk factors for the presence of SICTT reactor(s) in the three major milk producing regions. Both univariable (Table 2) and multivariable logistic regression (Table 3) analyses for potential risk factors related to presence of SICTT reactor(s) on the farms have been performed. Overall, the major risks seemed to be associated either with numbers of cattle on the farms ('number of cows', 'herd size', 'number of heifers', 'number of bulls' and 'availability of bulls'), or with whether or not the

animals were kept outdoors for parts of the day, e.g. for grazing and/or access to and sharing of water.

'Major activity' of the farmers, and 'age of respondents' were the two farmers' characteristics found significant risk factors for 'establishment' of BTB in the farms as identified by the univariable analysis. The majority of farmers, above 45 years old, were full-time dairy farmers. Both age of respondents', and 'major activity' were independently associated with the SICTT reactivity in the herds. The lower risk associated with farms owned by the younger age groups may be explained by younger age groups engaging more actively with and seeking advice from animal health experts and veterinary professionals if their dairy cattle showed reduced productivity or ill health. Surprisingly, farms owned by full-time dairy farmers were three times more at risk as compared to part-time dairy farmers.

'Number of cows' was identified as a risk factor for the 'introduction' of BTB to the case farms (Model 1, Table 3) and was retained in the final Model 3 (Table 3) as potential risk factor for the presence of BTB reactor(s). Farms with larger (> 10) numbers of cows being approximately four times more at risk than those with lower numbers of cows. This might be attributed to the inclination of farmers in Eritrea to purchase pregnant cows to cope with the high demand for milk in the market. The more pregnant cows are purchased the higher the risk of introducing a *M. bovis* infected cow. Purchase of adult pregnant cows also implies larger numbers of older animals on the farm. The longer an animal stays in a case herd, the higher will be the cumulative increase in the chances of being infected (Humblet et al., 2009, Cleaveland et al., 2007, Proano-Perez et al., 2009).

Availability and number of bulls were significant risk factors in the univariable analyses for 'introduction' of BTB in the case farms which might have been attributed to the discontinuation of artificial insemination and thus the use of bulls for reproduction by natural mating in the study area. Sharing of bulls is a common practice and those having more bulls are inclined to share them more frequently. The observed higher SICTT reactivity in those herds having bulls could be due to frequent and direct close contact between the bulls and animals in several herds (Skuce et al., 2011). Although farms not owning bulls also use bulls from other farms to breed their animals, they

experience a shorter contact time and hence a lower risk of transmission from potentially infected bulls.

'Sharing of water points' and 'species of animals sharing water point' were associated with a decreased risk of BTB 'introduction' in the farms. The water points used in our study areas were surface waters (rivers and dams), that were indicated not to be significant risk factors for BTB transmission in cattle (Griffin et al., 1993), and in buffaloes under free ranging condition (Michel et al., 2007). During the dry season, in our study area, farmers used their own mobile watering troughs to water their animals from water holes manually dug out from river beds. In addition, 'sharing of water points' entails walking the animals to water points that may result in minimizing, within herd, animal-to-animal contacts, due to lower density of cattle on pasture, besides providing them with an opportunity of, beneficial, physical exercise 'en route'.

'Herd size' was identified as one of the major risk factors for the 'establishment' of BTB in the case farms (Model 2). Farmers try to increase herd sizes for increased efficiency gains, but increase in herd size may lead to overcrowding causing enhanced cattle-to-cattle transmission of *M. bovis.* This finding is in agreement with those of similar studies that indicated the association of herd size with the prevalence of BTB (Wright et al., 2015; More & Good, 2015; Proano-Perez et al., 2009; Pavlik et al., 2005), including that of Omer et al. (2001) who identified herd size as one of the major risk factors for SICTT reactivity in the Maekel region, one of our current study areas in Eritrea. As shown by the univariable analyses, farms with larger numbers (> 8) of heifers were four times more at risk to have SICTT reactors than farms with fewer heifers (Menzies & Neill, 2000; Neill et al., 1994) in relation to 'establishment' of BTB in the farms. Increased animal-to-animal contact, normal behavior during estrous, might explain this finding (Hurnik et al., 1975).

The other major risk factor for the 'establishment' of BTB in our study was 'building status' (floor types) (Model 2), which was the second most important risk factor retained in the final Model 3 (Table 3). Those farms using concrete floors were about eight times more at risk when compared to those farms without concrete floors. Most of the dairy farms in the urban and peri-urban areas in Eritrea house their animals on concrete floors. Due to lack of

maintenance such floors are indicated as culprits for causing digital dermatitis which was shown as one of the major constraints in the dairy farms in and around the capital by Nsahlail and Mogos (2007). Eventually, due to the tendency of affected animals to lie down for prolonged times; their feed intake time may be compromised, causing emaciation and enhanced susceptibility to disease, including BTB (De Vries et al., 2015; Nordlund et al., 2004; Cook & Nordlund, 2009).

'Feeding system' was one of the risk factors for 'establishment' of BTB in the farms identified by the univariable analysis, where farms with a zero-grazing system were four times more at risk than farms that do not make use of this practice. Similarly, farms that kept their cattle permanently indoor encountered double the risk for 'establishment' of BTB in the farms compared with those letting animals go outdoors. The plausible reasons for this, as already indicated by other studies (Ameni et al., 2006; Skuce et al., 2012; Skuce et al., 2011; Griffin et al., 1996; Griffin et al., 1993; Ayele et al., 2004), may be that indoor housing enhances closer contact between cattle, and more likely transmission of respiratory disease (Skuce et al., 2011). For infection to occur in cattle via inhalation, it requires as little as one bacillus as compared to ingestion that requires quite a large number of *M. bovis* (10^7) (Humblet et al., 2009). On the other hand, being outdoors, the animals literally reduce the contact among themselves and thus with the pathogenic agent (*M. bovis*). Besides, allowing animals to graze is directly connected with the animals' health and welfare. Relatively intense solar radiation of animals (e.g., hair, skin) (Kazda, 2010) and their excrements might play a bactericidal role and thus reduce mycobacterial burden (Fine et al., 2011).

'Housing type' was also one of the significant risk factors for 'establishment' of BTB in the farms identified by the univariable analyses, where dairy farms with stanchion (tied-up) were two times more at risk as compared with farms having other types of housing. Such housing might facilitate transmission of *M. bovis* from affected animals to the susceptible ones as they are tied-up closely together in dairy barns. To make the situation worse, windows were very small or absent in most of the dairy barns, especially in the Maekel region.

In our attempt to assess a relatively large number of risk factors it appeared impossible to rule out all potential biases, unobserved confounders and lack of independence between some of the factors. For that reason we retained only those factors that were most strongly associated with the risk of having SICTT reactor(s) in a case farm. Although, currently, SICTT is the most widely applied screening test for BTB in live cattle (OIE, 2009; De la Rua-Domenech et al., 2006), it is imperfect but performs better than other ante mortem tests currently available for the detection of BTB.

Although the advantages of grazing on pasture are apparent in relation to hoof health, exercise and the access for more space that might minimize animal-to-animal contact, switching from indoor housing to pasture is not a realistic option for many farms in the urban areas because of unavailability of land for pasture. In such herds, a regular test and control system related to animal movements might help to control BTB in the future.

Dairy farms that allowed their animals outdoors, that kept smaller numbers of cattle (fewer cows and smaller total herd size), or that did not have concrete floors were less at risk to be BTB positive as indicated by the SICTT. Introduction of changes in management according to these findings may lead to an improved BTB status of the dairy farms. The use of sand floors or other alternative beddings (straw or saw dust) could improve the current prevailing problems related to floor type and thus reduce the number of farms with SICTT reactive animals. Purchasing replacement dairy cattle from farms with no SICTT reactors and testing for BTB before introduction of new cattle to farms might assist in controlling spread of BTB in the country.

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Supporting information

Table S1 Names of categorical variables (risk factors) in the study of 61 case (one or more SICTT reactors) and 65 control (no SICTT reactor) farms, their categories, distribution (% and number) and p-values of the Fisher's Exact Test, odds ratio (OR) and 95% Confidence Interval from univariable logistic regression analysis, with the 14 most significant variables ($p < 0.25$) screened for multivariable logistic regression in gray

Variables and their categories	Case (n = 61)	Control (n = 65)	OR	95%CI
Age respondent	**P-value = 0.036**			
25-45 yrs old(Reference)	11.5% (7)	27.7% (18)	1.0	
46-65 yrs old	68.9% (42)	47.7% (31)	3.5	1.3-9.4
> 65 yrs old	19.7% (12)	24.6% (16)	1.9	0.6-6.1
Educational level	**P-value = 0.48**			
Low	19.7% (12)	15.4% (10)	1.0	0.3-3.0
Medium	50.8% (31)	61.5% (40)	0.7	0.3-1.5
High (Reference)	29.5% (8)	23.1% (15)	1.0	
Major activity	**P-value = 0.004**			
Full-time dairy	80.3% (49)	55.4% (36)	3.3	1.5-7.3
Part-time (Reference)	19.7% (12)	44.6 (29)	1.0	
Breed	**P-value = 0.642**			
HF	80.3% (49)	76.9% (50)	1.2	0.5-2.9
Others (Reference)	19.7% (12)	23.1% (15)	1.0	
Herd size	**P-value = < 0.002**			
9-26 cattle (Reference)	70.5% (43)	92.3% (60)	1.0	
> 26	29.5% (18)	7.5% (5)	5.0	1.7-14.6
Number of cows	**P-value = 0.001**			
0-10 (Reference)	37.(60.7%)	56 (86.2%)		
> 10	24.(39.3%)	9 (13.8)	4.0	1.7-9.7
Number of heifers	**P-value = 0.018**			
0-8 (Reference)	48.(78.7%)	61 (93.8%)	1.0	
> 8	13.(21.3)	4 (6.2%)	4.1	1.3-13.5
Availability of bulls	**P-value = 0.199**			
No (Reference)	31.1% (19)	43.1% (28)	1.0	
Yes	68.9% (42)	56.9% (37)	1.7	0.8-3.5
Number of bulls	**P-value = 0.077**			
No bulls (Reference)	31.1% (19)	43.1% (28)	1.0	
One bull	34.4% (21)	40% (26)	1.2	0.5-2.7
≥ 2bulls	34.4% (21)	16.9% (11)	2.8	1.1-7.2

Stock density	**P-value = 0.595**			
≤ 0.06 cattle per square meter (Reference)	45.9% (28)	36.9% (24)	1.0	
0.07-0.48	50.8% (31)	58.5% (38)	0.7	0.4-1.4
≥ 0.49	3.3% (2)	4.6% (3)	0.6	0.1-3.7
Labour source	**P-value = 0.998**			
Family (Reference)	45.9% (28)	46.2% (30)	1.0	
Hired	21.3% (13)	21.5% (14)	1.0	0.4-2.5
Both	32.8% (20)	32.3% (21)	1.0	0.5-2.3
Distance from other dairy farm(s)	**P-vale = 0.285**			
0-30 meters	41% (25)	27.7% (18)	1.9	0.9-4.1
31-60	16.4% (10)	18.5% (12)	1.1	0.4-3.0
≥ 61 (Reference)	42.6% (16)	53.8% (35)	1.0	
Introduction of new animals	**P-value = 0.512**			
Yes	16.4% (10)	12.3% (8)	1.4	0.5-3.8
No (Reference)	83.6% (51)	87.7% (57)	1.0	
Cattle purchase frequency	**P-value = 0.817**			
No purchase (Reference)	82.0% (50)	86.2% (56)	1.0	
Every 1-2 years	6.6% (4)	6.2% (4)	1.1	0.3-4.7
Every 3 and more years	11.5% (7)	7.7% (5)	1.6	0.5-5.3
Reproduction method	**P-value = 0.024**			
Natural and both	91.1 (56.5)	100% (65.5)	0.08	0.0042-1.5
Artificial insemination (Reference)	8.9 (5.5)	0.0% (0.5)	1.0	
Source of bull for mating	**P-value = 0.433**			
Not applicable	8.2% (5)	3.1% (7)	3.3	0.6-18.8
Home grown (Reference)	31.2% (19)	38.5% (44)	1.0	
Not home grown	45.9% (28)	49.2% (60)	1.2	0.5-2.5
Both	14.8% (9)	9.2% (15)	2.0	0.6-6.5
Bull purchase frequency	**P-value = 0.329**			
No purchase (Reference)	70.5% (43)	81.5% (53)	1.0	
Every 1-2 yrs	4.9% (3)	3.1% (2)	1.9	0.3-7.5
Every 3 and more yrs	24.6% (15)	15.4 (10)	1.9	0.8-4.5
Same source of bull purchase?	**P-value = 0.153**			
Not applicable	65.6% (40)	73.8% (48)	0.3	0.1-1.1
Yes (Reference)	14.8% (9)	4.6% (3)	1.0	
No	19.7% (12)	21.5% (14)	0.3	0.1-1.3

Other animals present at the farm	**P-value = 0.672**			
Not available (Reference)	16.4% (10)	10.8% (7)	1.0	
Sheep and goats	1.6% (1)	3.1% (2)	0.4	0.03-4.7
Equids	44.3% (27)	52.3% (34)	0.6	0.2-1.7
Dogs and/chicken	11.5% (7)	6.2% (4)	1.2	0.3-5.9
Multispecies	26.2% (16)	27.7% (18)	0.4	0.2-2.0
Cattle contact with	**P-value = 0.660**			
No contact (Reference)	16.4% (10)	18.5% (12)	1.0	
One species	6.6% (4)	10.8 (7)	0.8	0.03-3.2.1
Multispecies	77.0% (47)	70.8% (46)	0.6	0.2-2.0
Wild animals present	**P-value = 0.590**			
No wildlife (Reference)	29.5% (18)	24.6% (16)	1.0	
One species	54.1% (33)	63.1% (41)	0.7	0.32-1.62
Multispecies	16.4% (10)	12.3% (8)	1.1	0.4-3.5
Source of water for animals	**P-value = 0.303**			
Water at the farm (Reference)	90.2% (55)	83.1% (54)	1.0	
Water outside the farm and both	9.8 % (6)	16.9% (11)	0.5	0.2-1.6
Sharing of water point	**P-value = 0.166**			
Yes	13.1% (8)	24.6% (16)	0.5	0.2-1.2
No (Reference)	86.9% (52)	75.4% (49)	1.0	
Species of animals sharing water point	**P-value = 0.10**			
No sharing (Reference)	86.9% (53)	75.4% (49)	1.0	
One species	3.3% (2)	13.8% (9)	0.2	0.04-1.0
Multispecies	9.8% (6)	10.8% (7)	0.8	0.3-2.5
Signs of respiratory disease	**P-value = 0.837**			
Yes	8.2% (5)	9.2% (6)	1.0	0.1-2.7
No (Reference)	91.8 (56)	90.8% (59)	1.0	
Clinical signs observed	**P-value=1.0**			
Not applicable (Reference)	91.8% (56)	92.3% (60)	1.0	
Difficult breathing and anorexia	6.6% (4)	7.7% (4)	1.1	0.3-4.5
Anorexia, listlessness and emaciation	1.6% (1)	1.5% (1)	1.1	0.1-17.5
Housing type	**P-value=0.133**			
Stanchion system	72.1% (44)	58.5% (38)	1.8	0.9-3.9
Others	27.9% (17)	41.5% (27)	1.00	
Housing system	**P-value=0.153**			
All the time housed indoors	55.7% (34)	41.5% (27)	1.8	0.9-3.6
Not all the time housed indoors (Ref)*	44.3% (27)	58.5% (38)	1.0	

Building Status	**P-value=0.033 (n=59)**	**(n=63)**		
With concrete floors	98.3 % (58)	87.3% (55)	8.4	1.0-69.7
Without concrete floors (Reference)	1.7% (1)	13.6% (8)	1.0	
Ventilation status of the house	**P-value=0.857**			
Good (Reference)	57.4% (35)	60% (39)	1.0	
Bad	42.6% (26)	40% (26)	1.1	0.6-2.3
Frequency of cleaning	**P-value=0.571**			
Once every day	91.8% (56)	95.4% (62)	0.7	0.2-3.2
Twice a day (Reference)	6.6% (4)	4.6% (3)	1.0	
Once a month	1.6% (1)	0.0% (0)	NI	
Feeding of green feed?	**P-value = 0.634**			
Yes (Reference)	93.4% (57)	95.4% (62)	1.0	
No	6.6% (4)	4.6% (3)	1.5	0.3-6.8
Source of green feed	**P-value = 0.877**			
Not applicable	6.6% (4)	4.6% (3)	1.5	0.3-7.5
Home grown (Reference)	42.6% (26)	46.2% (30)	1.0	
Purchased	23.0% (14)	18.5% (12)	1.4	0.5-3.4
Both	27.9% (17)	30.8% (20)	1.0	0.4-2.3
Feeding system?	P-value = 0.061			
Zero grazing	96.7% (59)	87.7% (57)	4.1	0.8-20.3
Grazing (Reference)	3.3% (2)	12.3% (8)	1.0	
Concentrate feeding?	**P-value = 0.484**			
Yes (Reference)	96.7% (59)	95.4% (62)	1.0	
No	1.6% (1)	4.6% (3)	0.4	0.4-1.6
Not disclosed	1.6% (1)	0.0% (0)	NI	
Mineral supplement?	**P-value = 0.593**			
Yes (Reference)	59.0% (36)	53.8% (35)	1.0	
No	41.0% (25)	46.2% (30)	0.8	0.4-1.6

Chapter 4

Prevalence of bovine tuberculosis in cattle, goats, and camels of traditional livestock raising communities in Eritrea

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Abstract

Background: The aim of the current study was to assess the prevalence of bovine tuberculosis (BTB) in cattle, goats, and camels, and its zoonotic potential within the traditional livestock raising communities in four regions of Eritrea. The Single Intradermal Comparative Tuberculin Test (SICTT) as indicator of *M. bovis* infection was conducted on 1077 cattle, 876 goats, and 195 camels. To elucidate possible risk factors for BTB transmission between animals and its potential zoonotic implication, questionnaire based face-to-face interviews were conducted in households of which 232 raised cattle, 128 goats, and 29 camels.

Results: The results of the SCITT were interpreted using the OIE standard (> 4 mm cut-off) for positive responses. In cattle, individual animal (n = 1077) and herd (n = 413) prevalences were 1.2% (n = 13) [Confidence Interval (CI) 95% CI, 1.0-1.3%] and 3.2% (n = 13) (95% CI, 3.0-3.4%), respectively. In goats (n = 876), none of the animals was positive. In camels, individual animal (n = 195) and herd (n = 70), BTB prevalences were 1.5% (n = 3) (95% CI, 1.4-1.6%) and 2.9 (n = 2) (95% CI, 0.9-4.6%), respectively. Overall, male animals were more at risk (OR = 2.6; 95% CI, 1.0-8.7) when compared to females. Sharing of water points, introduction of new animals into herds and migration of animals over large distances were common events that may contribute to intra and inter-species transmission of BTB. Consumption of raw milk, lack of BTB transmission awareness, and low levels of education were common in the farming communities.

Conclusion: The current study highlighted a low prevalence of *M. bovis* in cattle, goats and camels in extensive traditional livestock in Eritrea. Despite this, the spatial distribution of affected animals across most of the sampled regions and consumption of unpasteurized milk warrants surveillance, cautious and timely control measures for the disease.

Keywords: Bovine tuberculosis, Camels, Eritrea, Goats, Mixed crop-livestock system, Pastoral system, Single intradermal comparative tuberculin test (SICTT)

Background

Bovine tuberculosis (BTB) is a chronic bacterial disease caused by *Mycobacterium bovis* (*M. bovis*), a member of the group known as *Mycobacterium tuberculosis* complex (MTC), that has a wide host range. It predominantly affects cattle, but also other domesticated species, like goats [1-4], and camels [5, 6], as well as many wildlife species [7, 8]. In general, in traditional livestock raising systems, cattle and goats are often herded together and watering points are shared by many animal species. Such livestock husbandry and management systems can be an important risk factor for animal-to-animal, animal-to-human, human-to-animal, and human-to-human *M. bovis* transmission [9-13]. *M. bovis* infected animals, as indicated by SICTT, were present in the 'intensive' dairy husbandry system of the major milk producing regions in Eritrea [14, 15]. Besides, the presence of *M. bovis* was confirmed by bacterial culture and molecular diagnostic tools from bovine tissues collected at the Asmara slaughterhouse (Ghebremariam, unpublished data). However, the BTB status was never studied in the extensive traditional livestock (pastoral and mixed crop-livestock) system, which comprises the largest percentage of the livestock population (approx. > 99.9%) [14]. In neighbouring Ethiopia, with similar agricultural settings, BTB was reported to be prevalent in the 'intensive' dairy cattle in different studies (11.6% and 22.1%, respectively) [16, 17], as well as in cattle in the traditional extensive livestock husbandry system (8.2% and 11%, respectively) [16-18].

In developing countries, especially in rural settings, where dwelling areas may be shared between humans and animals, humans may become infected. This may occur through the inhalation of cough sprays released by chronic coughing animals [9, 12], or/and by drinking raw milk from infected animals [1, 19, 20]. The aim of the current study was to assess the prevalence of BTB in cattle, goats, and camels, and its zoonotic potential within the traditional livestock raising communities in the four regions (Debub, Anseba, Gash Barka, and Southern Red Sea) in Eritrea that share borders with at least one of the neighbouring countries (Figure 1).

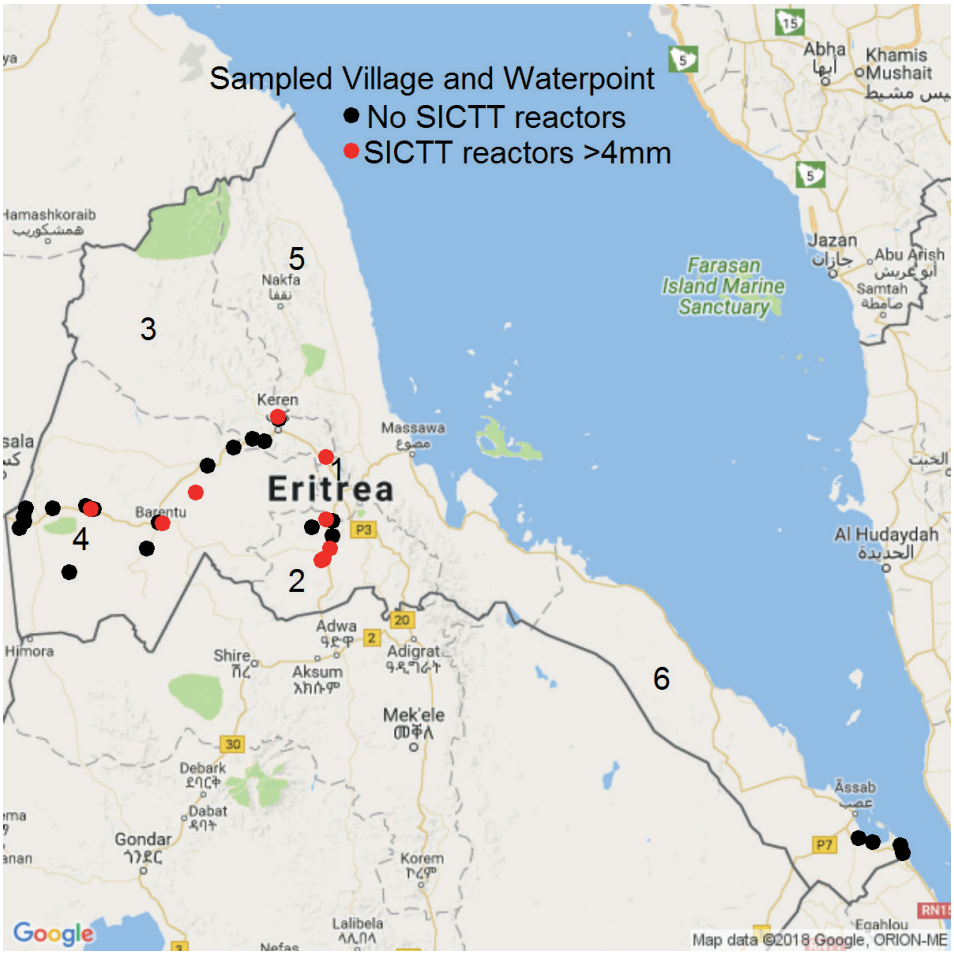

Figure 1 Map of Eritrea depicting the study areas (n = 31) and water points (n = 4, shared by animals from 11 study areas), having skin test reactors with > 4 mm cut-off (red dots) and those with no reactors using > 4 mm (black dots) in the selected study areas within the traditional livestock husbandry system in Eritrea. The numbers (1-6) indicated on the map show the six administrative regions of Eritrea (1 = Maekel; 2 = Debub; 3 = Anseba; 4 = Gash Barka; 5 = Northern Red Sea; 6 = Southern Red Sea)

(Adapted using: Markus Loecher and Karl Ropkins [54]
RgoogleMaps and loa: Unleashing R Graphics Power on Map Tiles. Journal of Statistical Software 63(4), 1-18. URL http://www.jstatsoft.org/v63/i04/)

Methods

Study population and sample size determination

We conducted a BTB prevalence study using multistage sampling in Debub, Anseba, Gash Barka, and Southern Red Sea regions of Eritrea (Figure 1), from October to November 2013, and September to December 2014. First, we selected the regions with the target species of animals (cattle, goats and camels or at least two of the species) and those sharing common borders with neighbouring countries (Ethiopia, Sudan, Djibouti) (Figure 1). For the second stage official lists of sub-regions, including villages that were connected by paved and dry weather roads were used to randomly select villages where two or three of the target species were present. Participation of livestock owners/herdsmen present at the testing sites was voluntary, and only animals of those who gave their consent to participate were recruited for the study. Convenience sampling was applied by selecting animals that could be caught and restrained for testing, and if possible, the questionnaire was concurrently completed by the owners/herdsmen. In the Debub region, as camel raising is not a common practice, hence camels were not included in the study there. Since the prevalence of BTB in the extensive livestock production system in Eritrea was unknown, required sample sizes were determined considering the livestock population size per species (data provided by the Ministry of Agriculture, not shown) of the selected sub-region and assuming 3% prevalence as found in a similar study in extensive traditional livestock farming in Ethiopia [21], and 95% level of significance, using the WIN EPISCOPE 2.0, veterinary epidemiological computer software.

Outcomes of calculations dictated a minimum of 99 animals from each species per study region to be tested to find at least one positive animal. The precision for the estimated prevalence assuming 3% prevalence and the calculated sample size is between 0.6 and 8.5%. But as animals are clustered within herds we aimed for a higher number of cattle and goats by selecting 3% of each herd per species for testing. All the target species of animals were sampled up to a herd size of three and at least up to a convenient higher number for larger farms.

That is, in the presence of three or less animals in a herd of a given species (cattle, goats or camels), the whole herd was subjected to testing. However, although the sample size (3% prevalence) requires 99 animals to be tested per region, it was not possible to reach that number at all times. Particularly, camels were less abundant and not represented in all herds.

The test sites were water points (n = 4, where animals from 11 study areas were tested), villages (n = 21), livestock gathering (resting) (n = 4 sites) and grazing areas (n = 6 sites) as they provide frequent opportunities for inter-and intra-species contact.

Geographic coordinates were registered at the test sites (areas) by the global positioning system (GPS) (Figure 1). Information relating to age, sex, region, sub-regions, and study areas was recorded for all the tested cattle, goats and camels (Additional file 1). In the testing areas animals were restrained by manually handling them using 3-4 men, and were ear tagged for identification purposes.

Questionnaire survey among livestock owners

In total, 232 cattle, 128 goats, and 29 camel owners, who presented their animals for tuberculin testing, were interviewed face-to-face using a standardized questionnaire consisting of both open and closed questions, based on their consent to voluntary participation (Additional files 2, 3, 4 & 5). The questionnaire included potential risk factors (variables) for *M. bovis* transmission amongst animals, and between animals and humans: housing of animals, source of water for the animals, sharing of water points, livestock migration, presence of wild animals, farmers' BTB transmission awareness, raw milk consumption habits, and farmers' level of education, etc.

The questionnaires were translated from English into two local languages (Tigre and Tigrigna) that are widely spoken in the study areas. Initially, the questionnaire was pre-tested by 10 randomly selected households in one of the villages to verify if the questions were clearly understood by the respondents. Subsequently the questionnaire was fine-tuned (ambiguous/misleading questions were corrected or omitted) and used. Administrators of the selected sub-regions, villages, including village elders,

were informed of the testing program through regional administration offices to make them aware of and sensitize them to it. The respondents were informed about the confidentiality of data collected through the questionnaire and the interviews were conducted upon the consent of the respondents. It was not possible to interview all the farmers that presented their animals for testing, since at some moments, sites were too crowded to allow timely completion of the questionnaires, and the farmers were leaving the sites.

Intradermal tuberculin skin testing

The skin testing was conducted according to the OIE [22] standard. Briefly, two sites on the left side of the mid-neck in cattle, right and left neck in goats, and upper and lower neck, closer to the shoulder, in camels, were shaved 12-15 cm apart. At the indicated sites skin thickness was measured with a 'Vernier calliper' and recorded, and the sites were injected with 0.1 ml containing 2,500 IU avian PPD (Prionics, Lelystad, The Netherlands) and 0.1 ml of 3,000 IU bovine PPD (Prionics, Lelystad, The Netherlands) using McLintock pre-set automatic syringes. Correct injection was confirmed by palpation of a small pea-like swelling at each site of injection.

Seventy-two hours after inoculation, the skinfold thicknesses at the injection sites were re-measured by the same operator and with the calliper used before. The readings were interpreted using the standard (> 4 mm cut-off; OIE) method [22]. An animal was considered positive if the differential increase in skin thickness between the bovine and avian injection was greater than 4 mm, inconclusive when the reaction difference was 1-4 mm, and negative when the bovine reaction was less or equal to the avian reaction in the absence of any clinical signs at the injection site. For the logistic regression analysis, we considered the inclusive results as negatives. As an incentive for participation by the owners, their animals were treated for internal and external parasites with ivermectin (anthelmintics) following the reading of the results. Though we have used standard interpretation of the results throughout the manuscript, we have included severe (> 2 mm cut-off) method for comparison with other studies (Additional files 6 & 7). According to the severe method, an animal was considered positive if the bovine minus the avian reaction was greater than 2 mm.

Data analysis

Data were entered in an Excel spreadsheet and then exported to SPSS IBM version 20 for analysis and all the analyses were conducted using this statistical package. Descriptive analysis was conducted at both individual animal and herd levels. Due to repeated samples within herds a mixed effect logistic regression model would be most appropriate to account for this dependency. A considerable number of farms have only one sample which causes problems in the estimation of the farm effects (farm level is the level of the only sampled animal). Secondly the outcome is binary (pos/neg per animal) which means that with three animals tested in a herd only a few levels for estimation maybe observed (0, 33, 66, 100% positive). The expected prevalence is 3%, so at farm level the prevalence will be mostly 0%. Therefore, we used an ordinary logistic regression model to investigate the relationship between the potential risk factors and outcomes. First, in univariable analysis, the measure of association between each of the potential risk factors and BTB (skin test positive score as a parameter), was examined for each factor individually and evaluated for statistical significance using a Fisher's Exact test for independence; secondly, multivariable logistic regression was applied to estimate the measures of associations between the potential risk factors (species, age, and sex of the tested animals) and the outcome were tested resulting in odds ratios (OR). The outcome of all statistical analyses were individual animal and herd level binary outcomes. A herd was considered positive if it had at least one skin test positive reactor using the > 4 mm cut-off.

Results

The Single Intradermal Comparative Tuberculin Test (SICTT) was conducted in 1077 cattle, median herd size 3.00 (range: 1-31), from 36 study areas and 11 sub-regions, 876 goats, median herd size 4.00 (range:1-15) from 27 study areas and nine sub-regions, and 195 camels, median herd size 6.0 (range: 1-11) from 16 study areas and seven sub-regions (Table 1). In total, 413 cattle, 243 goat, and 70 camel herds were tested (Table 2). Overall, females

accounted for 71% of animals tested (n = 764) in cattle, 95.5% (n = 838) in goats, and 75% (n = 146) in camels (Table 3).

Tuberculin reactors in cattle, goats and camels at individual and herd levels

Cattle

Results of the SICTT are shown in Table 2. The overall individual animal and herd prevalences, using the standard method were 1.2% (13/1077) [Confidence Interval (CI), 95% CI, 1.1-1.3%] and 3.2% (13/413), (95% CI, 3.0-3.4%), respectively. Within the cattle rearing villages, 22% (8/36) of the herds had positive reactors (Tables 1 & 2). Whereas, using the severe method (> 2 mm cut-off), the individual and herd prevalences were 5.5% (59/1077) and 13% (54/413), respectively, and more (58%; 21/36) villages had at least one reactor cattle when compared with the standard method (22%; 8/36) (Additional files 6 & 7).

Goats

No reactor animals were detected in goats in all the tested regions using the standard method (> 4 mm cut-off). Using the severe method (> 2 mm cut-off), 2.2% (19/876) of the individual goats and 4.9% (12/243) of the herds were reactors. With this method about 30% (7/27) of villages had at least one reactor goat (Additional files 6 & 7).

Camels

In camels, the animal and herd prevalences were 1.5% (3/195) (95% CI, 1.4-1.6%) and 2.9% (2/70), (95% CI, 0.9-4.9%), respectively (Table 2), with > 4 mm cut-off. Only one village had reactors (Table 1). In contrast, using the severe method (> 2 mm cut-off), the animal (11.8%; 23/195) and herd (26.8%; 19/70) prevalences were higher and about 56% (9/16) of the villages had at least one reactor camel (Additional files 6 & 7).

Statistical analysis

In the univariable analysis male camels and male cattle were at approximately 2-3 times at higher risk to be test positive when compared to females (Table 4). Similarly, in the multivariable analysis of the risk factors, only 'sex' of the animals remained in the final reduced model, though with borderline significance as shown for the univariable results (Table 4). Overall, male animals had around three times higher odds to be test positive than females. The variables 'species' and 'age', though, apparent potential risk factors, were not statistically significant.

Descriptive epidemiology of the animal and human risk factors on farm level

Risk factors for animal BTB exposure

Median numbers of cattle, goats and camels owned per farmer (household) interviewed were six (Range: 1-208), 11 (Range: 2-250), and three (Range: 1-47), respectively. Out of the 232 interviewed households (farmers) keeping cattle, 5.6% (n = 13) had positive animals, whereas this was the case in 6.9% (n = 2) of camel keeping households. None of the households with goats had SICTT positive animals. Out of the two households with SICTT reactor camels, one also owns dairy farm (MK Ghebremariam, personal experience). Among the interviewed households, 96.6% (n = 224) of cattle, 93.8% (n = 120) of goat, and 100% (n = 29) of camel owners allowed their animals to share watering points with other animals. Besides, 18.1% (n = 42) of the cattle, 3.9% (n = 5) of the goats, and 13.8% (n = 4) of the camel owners reported that they bought and introduced new animals to their existing herds in the last 2-3 years (Table 5). Likewise, 22.0% (n = 51) of the cattle, 10.2% (n = 13) of the goat, and 55.2% (n = 16) of the camel owners reported that their animals migrate to other regions during the dry seasons (Table 5).

Of the farmers interviewed 38.4% (n = 89) reported that their cattle spend the nights in the open including night grazing, 52.7% (n = 106) in separate animal houses, 13.8% (n = 14) in enclosures, and 2.3% (n = 5) share houses with humans at nights (Table 6). They also indicated that 74.2% (n = 95) farmers house their goats in separate animal houses, whereas 23.4% (n = 30), and 2.3% (n = 3), keep them in 'enclosures made of thorns' and 'free roaming in compounds' at night, respectively (Table 6).

Table 1 Number (and herd) of skin tested cattle, goats and camels, and number (herd) of reactors at region, sub-region and study areas levels using the standard (> 4 mm cut-off) method in the selected study areas within the traditional livestock husbandry system in Eritrea'0' = zero animals tested from zero herds. NA = not applicable

Regions	Sub-region	Study Areas	Tested cattle (herd)	Reactor cattle (herd) > 4 mm	Tested goats (herds)	Reactor goats (herd) > 4 mm	Tested camels (herds)	Reactor camels (herd) > 4 mm
Debub	**Dbarwa**	DRW1	61 (20)	0 (0)	60 (13)	0 (0)	NA	NA
		DRW2	44 (29)	3 (3)	15 (6)	0 (0)	NA	NA
		DRW3	38 (26)	0 (0)	17 (7)	0 (0)	NA	NA
		DRW4	48 (15)	0 (0)	25 (6)	0 (0)	NA	NA
	Total		**191 (90)**	**3 (3)**	**117 (32)**	**0 (0)**	**NA**	**NA**
	Mendefera	MFR1	104 (35)	4 (4)	38 (7)	0 (0)	NA	NA
		MFR2	52 (23)	1 (1)	16 (5)	0 (0)	NA	NA
		MFR3	38 (15)	1 (1)	0 (0)	-	NA	NA
	Total		**194 (73)**	**6 (6)**	**54 (12)**	**0 (0)**	**NA**	**NA**
Anseba	**Hagaz**	HAZ1	3 (2)	0 (0)	15 (2)	0 (0)	0	-
		HAZ2	12 (2)	0 (0)	10 (1)	0 (0)	1 (1)	0 (0)
		HAZ3	3 (1)	0 (0)	0	-	0	-
		HAZ4	8 (2)	0 (0)	0	-	0	-
		HAZ5	0	-	13 (2)	0 (0)	10 (1)	0 (0)
		HAZ6	0	-	3 (1)	0 (0)	0	-
		HAZ7	0	-	5 (1)	0 (0)	0	-
	Total		**26 (7)**	**0 (0)**	**46 (7)**	**0 (0)**	**11 (2)**	**0 (0)**
	Hamelmalo	HAM1	14 (6)	0 (0)	0	0 (0)	0	-
		HAM2	1 (1)	0 (0)	0	0 (0)	0	-
		HAM3	61 (27)	0 (0)	37 (7)	0 (0)	10 (3)	3 (2)
		HAM4	45 (11)	0 (0)	11 (2)	0 (0)	0	-
		HAM5	0 (0)	-	0 (0)	-	0	-
	Total		**121 (45)**	**0 (0)**	**48 (9)**	**0 (0)**	**10 (3)**	**3 (2)**
	Adi-Tekelezan	ATK1	72 (35)	1 (1)	39 (9)	0 (0)	0	-
	Total		**72 (35)**	**1 (1)**	**39 (9)**	**0 (0)**	**0**	**-**

Gash Barka	**Barentu**	BAR1	89 (32)	1 (1)	0	-	0	-
		BAR2	28 (6)	0 (0)	7 (2)	0 (0)	0	-
		BAR3	6 (4)	0 (0)	49 (13)	0 (0	13 (10)	0 (0)
		BAR4	22 (10)	0 (0)	72 (18)	0 (0)	2 (2)	0 (0)
	Total		**145 (52)**	**1 (1)**	**128 (33)**	**0 (0)**	**15 (12)**	**0 (0)**
	Tessenei	TES1	28 (12)	0 (0)	163 (70)	0 (0	1 (1)	0 (0)
		TES2	11 (4)	0 (0)	22 (9)	0 (0)	0	-
		TES3	0	-	0	-	71 (20)	0 (0)
		TES4	30 (10)	0 (0)	55 (21)	0 (0)	1 (1)	0 (0)
		TES5	15 (5)	0 (0)	10 (5)	0 (0)	0 (0)	-
	Total		**84 (31)**	**0 (0)**	**250 (105)**	**0 (0)**	**73 (22)**	**0 (0)**
	Hykota	HYK1	17 (5)	0 (0)	0	-	0	-
		HYK2	16 (8)	0 (0)	0	-	1 (1)	0 (0)
		HYK3	11 (11)	1 (1)	0	-	10 (2)	0 (0)
		HYK4	8 (1)	0 (0)	0	-	0	-
		HYK5	28 (8)	0 (0)	0	-	0	-
		HYK6	14 (14)	0 (0)	0	-	48 (6)	0 (0)
		HYK7	31 (1)	0 (0)	0	-	0	-
		HYK8	28 (1)	0 (0)	0	-	0	-
	Total		**153 (49)**	**1 (1)**	**0**	**-**	**59 (9)**	**0 (0)**
	Mogolo	MOG1	46 (9)	1 (1)	25 (2)	0 (0)	11 (8)	0 (0)
	Total		**46 (9)**	**1 (1)**	**25 (2)**	**0 (0)**	**11 (8)**	**0 (0)**
	Akurdet	AKU1	25 (15)	0 (0)	0	-	0	-
		AKU2	18 (5)	0 (0)	0	-	0	-
	Total		**43 (20)**	**0 (0)**	**0**	**-**	**0**	**-**
Southern Red Sea	**Debub Dankalia**	DANK1	0	-	17 (4)	0 (0)	3 (3)	0 (0)
		DANK2	0	-	10 (1)	0 (0)	2 (2)	0 (0)
		DANK3	0	-	54 (13)	0 (0)	0	-
		DANK4	2 (2)	0 (0)	35 (10)	0 (0)	0	-
		DANK5	0	-	53 (6)	0 (0)	11 (9)	0 (0)
	Total		**2 (2)**	**0 (0)**	**169 (34)**	**0 (0)**	**16 (14)**	**0 (0)**
	Grand Total		**1077 (413)**	**13 (13)**	**876 (243)**	**0 (0)**	**195 (70)**	**3 (2)**

Table 2 BTB prevalence in cattle, goats and camels at individual animal and herd levels within the traditional livestock husbandry system in the study regions using standard cut-off (> 4 mm). NA= not applicable

Number and herds of cattle, goats and camels tested	Anseba	Debub	Gash Barka	Southern Red Sea	Overall
Number (%) of cattle	219 (20.3)	385 (35.7)	471 (43.7)	2 (0.2)	1077 (100)
Number (%) of goats	133 (15.2)	171 (19.5)	403 (46)	169 (19.3)	876 (100)
Number (%) of camels	21 (10.8)	NA	158 (81.0)	16 (8.2)	195 (100)
Total number (%) tested/region	**373 (17.4)**	**556 (25.9)**	**1032 (48)**	**187 (8.7)**	**2148 (100)**
Individual animal Prevalence (%)					
Cattle	1 (0.5)	9 (2.3)	3 (0.6)	0 (0.0)	13 (1.2)
Goats	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Camels	3 (13.6)	NA	0 (0.0)	0 (0.0)	3 (1.5)
Herds of cattle, goats and camels tested					
Herds (%) of cattle	87 (21.1)	163 (39.5)	161 (38.9)	2 (0.5)	413 (100)
Herds (%) of goats	25 (10.3)	44 (18.1)	140 (57.6)	34 (14.0)	243 (100)
Herds (%) of camels	6 (8.3)	0 (0.0)	50 (72.2)	14 (19.4)	70 (100)
Herd prevalence (%)					
Cattle	1 (1.2)	9 (5.5)	3 (1.9)	0 (0.0)	13 (3.2)
Goats	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Camels	2 (33.3)	NA	0 (0.0)	0 (0.0)	2 (2.9)

Table 3 BTB prevalence as associated to sex in cattle and camels within the traditional livestock husbandry system using > 4 mm

Status	P-value	Female 764 (79.9)	Male 313 (29.1)
		Number (%)	Number (%)
Cattle: > 4 mm cut-off	**(0.089)**		
Inconclusive		146 (19.1)	67 (21.4)
Negative		612 (80.1)	239 (76.4)
Positive		6 (0.8)	7 (2.2)
Camels: > 4 mm cut-off	**(0.053)**	**146 (74.9)**	**49 (25.1)**
Inconclusive		22 (15.1)	15 (30.6)
Negative		122 (83.0)	33 (67.4)
Positive		2 (1.4)	1 (2.0)

Risk factors for human BTB exposure

Out of the farmers interviewed, 24.6% (n = 57) of cattle, 51.7% (n = 15) of camel, and 38% (n = 49) of goat owners indicated that raw milk and milk products are consumed in their households regularly (Table 7). Moreover, 4.7% (n = 11) of the cattle, and 2.3% (n = 33) of the goat owners reported the presence of respiratory diseases in their family members. Of these, one cattle herd was positive with > 4 mm cut-off and two with > 2 mm cut-off. None of goats' farms that reported the presence of tuberculosis in their families had positive animals. The questionnaires did not include this variable with reference to camels. Among the total number of farmers interviewed, 57.6% (n = 224) indicated that BTB awareness campaigns had never been held in their areas. Of the respondents, 42.9% (n = 167) had no education (cannot read and write), 37.5% (n = 146) had a low (literate; primary level education; grade 1-5), 17.5% (n = 68) medium (grade 6-8), and 2.1% (n = 8) higher levels of education (grade 9-12/college level education) (Table 7).

Table 4 Species of animals (cattle and camels) and sex as potential risk factors for the presence of BTB reactors at > 4 mm cut-off within the tested animals in the study regions within the extensive traditional livestock husbandry system analyzed by univariable and multivariable logistic regression

Species	P-value	OR	95% CI	
			Lower bound	Upper bound
Univariable analysis				
Camel versus cattle	0.703	1.3	0.4	4.5
Sex				
Male versus female cattle	0.058	2.9	1.0	8.7
Male versus female camel	0.743	1.5	0.13	16.9
Male versus female (overall)	0.06	2.6	1.0	8.7

Table 5 Risk factors for the presence of SICTT reactors as compared between cattle, goats and camels in the extensive livestock husbandry system within the study regions in Eritrea

Variables	Cattle herds (n = 232)	Goat herds (n = 128)	Camel herds (n = 29)	Overall herds (n = 389)
	Number (%)	Number (%)	Number (%)	Number (%)
Water point sharing				
Yes	224 (96.6)	120 (93.8)	29 (100)	373 (95.9)
No	8 (3.4)	8 (6.3)	0 (0.0)	16 (4.1)
Introduction of new animals				
Yes	42 (18.1)	5 (3.9)	4 (13.8)	51 (13.1)
No	190 (81.9)	123 (96.1)	25 (86.2)	338 (86.9)
Livestock migration				
Yes	51 (22.0)	13 (10.2)	16 (55.2)	80 (20.6)
No	181 (78.0)	115 (89.8)	13 (44.8)	309 (79.4)
Source of water				
Outside farms	128 (55.2)	46 (35.9)	11 (37.9)	185 (47.6)
Inside farms	49 (21.1)	60 (46.9)	17 (58.6)	126 (32.4)
Inside and outside farms	55 (23.7)	22 (17.2)	1 (3.4)	78 (20.0)

Discussion

This study presents the first efforts to assess the prevalence of BTB in cattle, goats and camels, and its zoonotic potential within the extensive traditional livestock husbandry system in Eritrea (Figure 1). Focusing on cattle, our study reports low (1.2%) individual animal and 3.2% herd prevalences of BTB. Similar findings for individual animal prevalence were reported in Ethiopia, Gumi et al. [21] 5.5%, Ameni et al. [23] 1.8% and 4.7%, Ameni et al. ([24, 25], reported) 7.9-11.6%, Tschopp et al. ([26, 27], 0.8%; 0.9%), and Mamo et al. [18] reported 11% and a herd prevalence of 44% in Ethiopia in the Afar region with similar conditions as the pastoral areas in our study. In Ghana 13.8% of individual animal prevalence was observed [28]. Although low, the prevalence in our study differed between regions, Debub showing the highest prevalence, 2.3%, where also a high (7.3%) BTB prevalence in dairy cattle was recorded in our previous study [14]. Contact between the dairy cattle and cattle within the extensive system may be postulated as a potential risk factor for the transmission of BTB. This region, located in the central highland of Eritrea, where mixed crop-livestock farming is conducted, is endowed with relative mild temperature and higher precipitation, hence environmental conditions more favourable for survival of *M. bovis* as compared with the arid and semi-arid regions of Gash Barka, Southern Red Sea and Anseba (partially) [14, 29]. In the latter areas, climatic conditions, lower cattle density, housing of animals in open areas (Table 6) may explain the low prevalence of BTB in the extensive cattle production system in general. In our current study, in Anseba region, BTB prevalence was very low (0.5%) in cattle within the extensive livestock production system (Tables 1 & 2). In contrast to Debub region, in Anseba, very low (0.2%) BTB prevalence was reported in dairy cattle [14], thus, in this case, transmission of BTB from the dairy cattle to the indigenous cattle within the extensive farming may be less likely when compared to Debub region.

Although the observed BTB prevalence was low, it is noteworthy that the presence of infection was indicated in many of the study areas (Table 1 & Figure 1). This may suggest that BTB was introduced to these areas sporadically from various sources but spread was limited. This can be explained by investigating the generally accepted drivers of BTB prevalence,

i.e. breed of cattle, farming system (intensive/extensive), housing and gathering of animals at grazing and watering points. Our current study was conducted exclusively in indigenous (zebu) cattle which are considered relatively resistant to BTB as compared to exotic breeds [13, 25, 30]. Likewise, the extensive livestock management practiced in our study areas is known to pose a far lower risk for BTB progression and transmission than the intensive dairy farming system. It can be argued that under these circumstances and in combination with the prevailing climatic conditions, the risk for BTB transmission is effectively reduced as evidenced by the current low prevalence.

Table 6 Housing of cattle and goats at night in Debub (in central highlands with high altitude and mild temperature), Anseba (partially in the central highlands and partially in the lowlands with hot and arid climate), Gash Barka (in the western low lands with hot and arid climate) and Southern Red Sea (in Eastern low land with hot and arid climate) regions within the extensive livestock husbandry system

Variables	Debub (n = 106)	Anseba (n = 61)	Gash Barka (n = 64)	Southern Red Sea (n = 1)	Overall (n = 232)
	Number (%)	Number (%)	Number (%)	Number (%)	Number (%)
Cattle housing					
Open area	9 (8.5)	32 (52.5)	48 (75.0)	0 (0.0)	89 (38.4)
Separate animal houses	95 (89.6)	8 (13.1)	3 (4.7)	0 (0.0)	106 (52.7)
Enclosures	0 (0.0)	18 (29.5)	13 (20.3)	1 (100.0)	32 (13.8)
Share houses with humans	2 (1.9)	3 (4.9)	0 (0.0)	0 (0.0)	5 (2.2)
Goat housing	**n = 39**	**n = 17**	**n = 39**	**n = 33**	**n = 128**
Open area	0 (0.0)	0 (0.0)	3 (7.7)	0 (0.0)	3 (2.3)
Separate animal houses	39 (100)	6 (35.3)	19 (48.7)	31 (93.9)	95 (74.2)
Enclosures	0 (0.0)	11 (64.7)	17 (43.6)	2 (6.1)	30 (23.4)

Table 7 Bovine tuberculosis awareness, education levels, and raw milk consumption habit among farmers keeping cattle, goats and camels, within the traditional livestock farming system in Eritrea

Variables	Cattle owners (n = 232)	Goats owners (n = 128)	Camel owners (n = 29)	Overall (n = 389)
	Number (%)	Number (%)	Number (%)	Number (%)
TB awareness				
Yes	109 (47.0)	46 (35.9)	10 (34.5)	165 (42.4)
No	123 (53)	82 (64.1)	19 (65.5)	224 (57.6)
BTB animal to humans				
Yes	151 (65.1)	91 (71.1)	13 (44.8)	255 (65.6)
No	24 (10.4)	9 (7.0)	10 (34.5)	43 (11.0)
I don't know	57 (24.7)	28 (21.9)	6 (20.7)	91 (23.4)
TB human to animals				
Yes	23 (9.9)	22 (17.2)	5 (17.2)	50 (12.9)
No	79 (34.1)	37 (28.9)	15 (51.7)	131 (33.7)
I don't know	130 (56.0)	69 (53.9)	9 (31.0)	208 (53.5)
Level of education				
No education (cannot read and write)	93 (40.1)	53 (41.4)	21 (72.4)	167 (42.9)
Low (literate; grade 1-5 of formal education)	87 (37.5)	56 (43.8)	3 (10.3)	146 (37.5)
Medium (grade 6-8 of formal education)	46 (19.8)	18 (14.1)	4 (13.8)	68 (17.5)
Higher (grade 9-12 formal/college level)	6 (2.6)	1 (0.8)	1 (3.4)	8 (2.1)
Raw milk consumption				
Yes	57 (24.6)	31 (24.2)	25 (86.2)	113 (29.0)
No	175 (75.4)	97 (75.8)	4 (13.8)	276 (71.0)

Nevertheless, the existing potential for spread of BTB due to inter-species herd mixing at water points and resting areas where livestock congregate and as well as due to migration and uncontrolled livestock movement must not be underestimated, nor ignored (Table 5). On the other hand, it must be kept in mind that several host related factors like malnutrition, recent infection with *M. bovis*, co-infection with non-tuberculous mycobacteria, infestation with

gastrointestinal parasites, and generalized tuberculosis [31-34] are able to decrease reactivity to the SICTT and cannot be ruled out to have influenced our study outcome.

In goats, no positive reactors were found in our current study, and this finding was in agreement with what was reported by Tschopp et al. (2011 & 2010b) [10, 27]. A similar study in Ethiopia reported 0.5% in small ruminants, [35]. The absence of BTB positive goats in our current study (mixed crop-livestock, and pastoral systems) might be attributed to the restriction of grazing of the flocks within their villages or housing of goats separately from cattle and camels at night. In addition, separate herding may have contributed to a low contact rate between goats and the other species of animals, unlike in the neigbouring Ethiopia where there is congregation and interspecies as well as wildlife mixing in grazing areas [18]. Infestation with liver flukes (*Fasciola hepatica*) and other helminths in the relatively wet highlands and arid low land areas are commonly encountered in slaughtered goats ([36], T Ghebremeriam, senior meat inspector, MOA, personal communication, and MK Ghebremariam, personal experience) perhaps causing reduced reactivity to the SICTT as reported by several studies [31-33]. In addition, the sensitivity of SICTT in goats, as in cattle, might be compromised by co-infections with viral diseases in chronic stages such as peste des petits ruminants (PPR), Contagious Caprine Pleuropneumonia (CCPP); or sheep and goat pox (MK Ghebremariam, personal experience). In general, the possibilities for goats to become infected are less, as they are browsers and rarely graze pastures that may have been contaminated. Studies also suggest that small ruminants are only spillover hosts that cannot maintain the disease in a herd [37], unless they are in close contact with cattle with high BTB prevalence [9, 38] or managed under intensive production system [39-41]. There is no information on the status of paratuberculosis (*M. avium* subspecies paratuberculosis) in Eritrea, that is known to interfere with the skin test when present [23, 42]. Though our current study was not able to show the presence of BTB in goats, several studies in Africa and Europe with different as well as similar agricultural settings as in Eritrea showed the presence of *M. bovis* and *M. caprae* in goats [1-4, 35, 39-45] thus we need to approach the current finding cautiously since only the OIE standard was used to interpret the

results. In indigenous (zebu) cattle as well as in goats, the severe (> 2 mm cut-off) method showed better sensitivity without affecting the specificity of the SICTT as compared to the standard method [21, 44-47]. The use of severe method in our study might have increased the sensitivity of the test. Results that compare the number of positive animals when both the standard (> 4 mm cut-off) and the severe (> 2 mm cut-off) methods are used are presented in additional files (Additional files 6 & 7). Might be good to emphasize the increase spatial spread in case of the severe interpretation/implying increase risk for infection of animals and humans.

Our study has shown that camels were more at risk of being SICTT reactors as compared with cattle (Table 4), though the association was not significant as only few were positive. BTB is prevalent in dairy cattle in Anseba region, as reported by Ghebremariam et al. [14]. The overall individual animal prevalence in camels was 1.5%. This is considered low as compared with similar studies in Ethiopia and Kenya that showed 6% (29/480) and 37% (15/41) prevalences, respectively, based on standard interpretation [48, 49]. Relatively, the prevalence was higher in the Anseba region (1.5%; Table 2) when compared to other regions of Eritrea where the study was conducted. In this region mixing of camels and cattle is relatively common, and in some cases camel owners also own dairy farms and use their camels to transport animal feed to the farms that may allow camel-cattle contact. Such interactions may have contributed to the presence of more SICTT reactor camels in Anseba as compared with the other study regions. Camels in close contact with cattle were found to be more prone to *M. bovis* infection and to have more tuberculosis lesions in the abattoir than those not having contact with cattle [5, 6, 50-52]. No reactor camel was found in Gash Barka and Southern Red Sea regions. In these regions camels are herded separately from cattle, but trekked long distances where they may come into contact with other animal species en route and at water points. The low prevalence of BTB in camels in the lowlands can be understood as they are browsers, in addition to being herded separately from cattle in a region with low prevalence of BTB in cattle. However, there is no information on the status of helminths, paratuberculosis (*M. avium* subspecies paratuberculosis), other non-tuberculous mycobacteria or viral infection in camels in Eritrea, that may

interfere with the skin test when present [31-34, 41, 42]. Comparatively, the overall BTB prevalence at animal level in all the tested species and the number of study areas with reactors was highest in Debub (Figure 1). Out of the nine positive reactor animals in this region, seven were males. As mixed-crop livestock production system is practiced in this region, male cattle are mostly used either as oxen or for mating purposes, and thus kept longer in the herd than females [29].

Gash Barka is the region where approximately 60% of the livestock population is located and it is the destination for all the animals migrating from different regions of the country, especially, during the dry season. Besides, this region shares borders with Sudan and Ethiopia where uncontrolled movement of animals is possible. The low BTB prevalence in this region might be due to the arid and hot climate which is not suitable for the survival of *M. bovis* as it is readily destroyed by direct sunlight under dry condition [13], in contrast to Debub region.

Focusing on the risk factors for human BTB exposure, overall, within the traditional extensive livestock husbandry system in the selected study areas, 29% of the households consume raw, untreated milk (Table 7). Such milk consumption habit might serve as a vehicle for BTB transmission from infected animals to humans as several studies have shown the presence of *M. bovis* in camels', goats' and in cows' milk [1-4, 53]. Moreover, among the interviewed cattle owners, 2.4% share their houses with their cattle (Table 6). Sharing of the same microenvironment and dwelling between humans and animals has been identified as one of the routes of animal-to-human BTB infection or vice versa, mainly in rural areas in developing countries [12]. The presence of tuberculosis within some of the cattle rearing families and their animals warrants suspicion of the presence of *M. bovis* within the animal human interface in this production system.

BTB in camels and goats was not considered of veterinary concern in Eritrea, thus, so far, no attempt has been made to conduct BTB testing or routine post mortem examinations in the slaughterhouses for the detection of TB-like lesions. The current study indicated the presence of BTB in cattle and camels, and its spread throughout the study regions within the extensive

livestock production system in Eritrea, though at a low prevalence. It warrants future, more in-depth, studies on BTB in these livestock species.

Our study has one major limitation, i.e., the number of the farmers that completed the questionnaires were fewer than the animal herds tested. This was mainly attributed to the overcrowding of the testing sites and the hot and arid climate that forced the farmers to leave the testing sites without filling the questionnaires (even after presenting their animals for testing). As the observed level of animal prevalence is low, a multilevel analysis of the data was not estimable although such a model would give more precise estimates if feasible. Such a model also needs sufficient observations within each cluster which was not the case in our study as many very small herds are present, most of the herds consist of 1-3 animals.

Conclusion and recommendation

The current study has shown that SICTT reactors are rare in cattle and camels and were not found in goats. However, though rare, the spatial distribution of the affected animals across most of the selected regions (Figure 1), where consumption of unpasteurized milk is common, warrants continuous surveys, cautious and timely control measures of the disease. We recommend the testing of the animals to be conducted during mild weather seasons so as to be able to conduct face-to-face interviews and complete the questionnaires that would match the number of herds tested.

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Supporting information

File 1 Regions, sub-regions and villages included in the study, and species, breed, sex and age of each animal tested using the SICTT during the study periods

https://static-content.springer.com/esm/art%3A10.1186%2Fs12917-018-1397-0/MediaObjects/12917_2018_1397_MOESM1_ESM.xlsx

File 2 Questionnaire for BTB risk factors study within the cattle raising communities in the extensive livestock husbandry system in Eritrea

https://static-content.springer.com/esm/art%3A10.1186%2Fs12917-018-1397-0/MediaObjects/12917_2018_1397_MOESM2_ESM.pdf

File 3 Questionnaire for BTB risk factors study within the goat raising communities in the extensive livestock husbandry system in Eritrea

https://static-content.springer.com/esm/art%3A10.1186%2Fs12917-018-1397-0/MediaObjects/12917_2018_1397_MOESM3_ESM.pdf

File 4 Questionnaire for BTB risk factors study within the camel raising communities in the extensive livestock husbandry system in Eritrea

https://static-content.springer.com/esm/art%3A10.1186%2Fs12917-018-1397-0/MediaObjects/12917_2018_1397_MOESM4_ESM.pdf

File 5 Consolidated criteria for reporting qualitative studies (COREQ): 32-item checklist and answers

https://static-content.springer.com/esm/art%3A10.1186%2Fs12917-018-1397-0/MediaObjects/12917_2018_1397_MOESM5_ESM.docx

File 6 Number (and herd) of skin tested cattle, goats and camels, and number (herd) of reactors at region, sub-region and study areas levels using the standard (> 4 mm cut-off) method in the selected study areas within the traditional livestock husbandry system in Eritrea presented for comparison. '0' = zero animals tested from zero herds. NA = not applicable

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5842630/bin/12917_2018_1397_MOESM6_ESM.docx

File 7 BTB prevalence in cattle, goats and camels at individual animal and herd levels within the traditional livestock husbandry system in Eritrea using the standard and severe cut-offs (> 4 mm and > 2 mm) presented for comparison. NA = not applicable

https://static-content.springer.com/esm/art%3A10.1186%2Fs12917-018-1397-0/MediaObjects/12917_2018_1397_MOESM7_ESM.do

Chapter 5

Genetic profiling of *Mycobacterium bovis* strains from slaughtered cattle in Eritrea

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Abstract

Mycobacterium bovis (*M. bovis*) is the main causative agent for bovine tuberculosis (BTB) and can also be the cause of zoonotic tuberculosis in humans. In view of its zoonotic nature, slaughterhouse surveillance, potentially resulting in total or partial condemnation of the carcasses and organs, is conducted routinely. Spoligotyping, VNTR profiling, and whole genome sequencing (WGS) of *M. bovis* isolated from tissues with tuberculosis-like lesions collected from 14 cattle at Eritrea's largest slaughterhouse in the capital Asmara, were conducted. The 14 *M. bovis* isolates were classified into three different spoligotype patterns (SB0120, SB0134 and SB0948) and six VNTR profiles. WGS results matched those of the conventional genotyping methods and further discriminated the six VNTR profiles into 14 strains. Furthermore, phylogenetic analysis of the *M. bovis* isolates suggests two independent introductions of BTB into Eritrea possibly evolving from a common ancestral strain in Europe. This molecular study revealed the most important strains of *M. bovis* in Eritrea and their (dis)similarities with the strains generally present in East Africa and Europe, as well as potential routes of introduction of *M. bovis*. Though the sample size is small, the current study provides important information as well as platform for future in-depth molecular studies on isolates from both the dairy and the traditional livestock sectors in Eritrea and the region.

This study provides information on the origin of some of the *M. bovis* strains in Eritrea, its genetic diversity, evolution and patterns of spread between dairy herds. Such information is essential in the development and implementation of future BTB control strategy for Eritrea.

Keywords: Bovine tuberculosis; Eritrea; Genotyping; *M. bovis*; Spoligotyping; VNTR; Whole genome sequencing

Author summary

The livestock sector plays a major role in poverty and hunger reduction in the vast majority of Africa, as a source of food, cash income, manure, draught power, transportation, savings, insurance and social status. However, for livestock to play this vital role, the impact of diseases of economic and zoonotic importance need to be reduced. Bovine tuberculosis, mainly caused by *Mycobacterium bovis*, is such an infectious disease. Slaughterhouse (gross pathology) surveillance, followed by bacterial culture and genotyping, are options to identify the disease-causing agents, their distribution, and enabling trace back of the sources of infections, in order to prevent their re-introduction and spread. Unfortunately, genotyping is by far not generally introduced in the continent. In the present study, tissues with tuberculosis-like lesions were collected from the Asmara municipal slaughterhouse, the largest slaughterhouse in Eritrea, and bacterial culture, classical *Mycobacterium tuberculosis* complex typing (Spoligotyping and VNTR profiling), as well as whole genome sequencing (WGS) were used to gain insight into the spatial and temporal distribution, genetic diversity and evolution of *M. bovis* strains circulating in Eritrean dairy cattle. The results revealed (dis)similarities of the Eritrean *M. bovis* strains with the strains generally present in Africa and Europe, potential routes of introduction to Eritrea and genetic diversity of the *M. bovis* strains. Future in-depth molecular studies including more samples from dairy cattle as well as cattle and goats from the traditional livestock sector are recommended.

Introduction

Mycobacterium bovis (*M. bovis*) is the causative agent of bovine tuberculosis (BTB), achronic, infectious and contagious disease that also affects other domestic animals as well as humans [1, 2]. Although BTB is prevalent in dairy cattle in Eritrea as shown by Omer et al. (2001) [3] and Ghebremariam et al. (2016) [4] by skin-test based survey, detection and isolation of the causative agent has never been done. Routine meat inspection at municipal slaughterhouses is performed for identifying tuberculosis-like lesions (TBL)

that usually results in either total or partial condemnation of carcasses depending on the level of TBL dissemination, however, confirmatory testing or trace back epidemiological investigations are not conducted in Eritrea. Genotyping is a vital tool for trace back in epidemiological investigations, and according to Biek et al. (2012) [5] results from WGS alone can provide insight into TB epidemiology even in the absence of detailed contact data. Despite the usefulness of genotyping, it is rarely used in developing countries, i.e., in Africa, Asia, and South America [6-9]. The routine use of such tool in these countries could be instrumental in complementing BTB control strategies.

Spoligotyping and variable number of tandem repeat (VNTR) profiling have been used extensively in many countries to document the molecular epidemiology of *Mycobacterium tuberculosis* complex (MTBC) species [7, 10-14]. For this reason, the digital MTBC molecular genotypes are predominantly stored in these two forms globally [15-18].

The recent technological advancements in molecular genetics imply that we can now more than ever understand the molecular epidemiology of MTBC at amore granular level. In the last few years, whole genome sequencing (WGS) for typing of pathogens has been explored and yielded important additional information on strain diversity in comparison to the classical DNA typing methods. Analysis of data from WGS also allows detection of minute differences in genetic diversity and this has contributed retrospectively to outbreak investigations [19-23]. Significantly, WGS allows for better genomic coverage with single nucleotide polymorphisms (SNP) profiling than the two classical typing methods [24, 25]. WGS has also led to a significant growth in quantitative methodology that allows for a robust estimation of phylogenetic and temporal relationships between samples [26]. All these aspects are essential in enhancing our understanding of local and distant, recent and historical dynamics of BTB [5, 24]. Although several reports predict that the use of WGS for genotyping will eclipse the classical MTBC typing tools [27], this will likely take longer to occur in Africa. It is therefore important to compare their utility in resource limited settings. Although such tools have never been used in Eritrea, their use would greatly enhance our understanding of: a) the genetic diversity of *M. bovis,* b) its evolution and c) the patterns of spread (spatial and temporal) between dairy herds, in the country and region.

Such data (information) would be critical for safeguarding and further development of the dairy industry of Eritrea. In the present study, the classical MTBC typing tools (Spoligotyping and MIRU-VNTR) as well as WGS were used to gain insight into the spatial and temporal dynamics, genetic diversity and evolution of *M. bovis* strains circulating in Eritrean dairy cattle. Furthermore, to infer local and international historical phylogenetic relationships.

Materials and methods

Data and sample collection

Pooled tissue samples (lungs and pleura, mediastinal, bronchial, deep inguinal and lung lymph nodes), were collected from 15 animals that showed TBL in gross pathology, at the Asmara municipal slaughterhouse from March 2014 to May 2015. These 15 animals were all those with TBL during the study period. The animals were slaughtered for meat purpose and processed as part of the normal work of the abattoir. Approximately 5-10 grams of pooled tissues from each sampled animal were collected in sterile specimen containers, and immediately transported on icepacks to the National Animal and Plant Health Laboratory (NAPHL), Asmara, and stored at -20^{0}C until processing for culture.

Data collected from individual animals (Table 1) included: source of the animal slaughtered, date of slaughter, species, breed, sex, age, pregnancy status (pregnant/ non-pregnant), ante mortem clinical signs, post mortem lesions, and type of the tissue samples collected. In addition, retrospective meat inspection data for the period 2010 to 2015 were retrieved from the logbook of the slaughterhouse.

Isolation and identification of *Mycobacterium bovis*

Samples were processed for *M. bovis* culture as follows: approximately 5 g of each pooled tissue sample with TBL per animal was cut into small pieces and covered with 100 ml of sterile distilled water in a biohazard cabinet (Esco Class II BSC; Labotec, SA). The samples were homogenized using anUltra-Turrax homogenizer at 17500 rpm (Separation Scientific, SA). Seven millilitres of the homogenate was poured into each of two separate 15 ml falcon tubes,

and the remaining homogenate was poured into individual 50 ml centrifuge tubes and stored at -20°C as reference samples. The samples were decontaminated with 7 ml of 2% HCL (final concentration of 1%) and 7 ml of 4 % NaOH (final concentration of 2%), respectively, and incubated at room temperature (18-25°C) for 10 min. After subsequent centrifugation (Heraeus Labofuge 400) of the samples at 3500 rpm for 10 min, supernatants were poured off and 7 ml of sterile distilled water was added. After washing, the centrifugation step was repeated and most of the supernatant was poured off. The pellets were re-suspended in a volume of approximately 1 ml using a sterile inoculation loop. Two loops of each of the pellets were spread evenly onto two Löwenstein-Jensen (L-J) media slants supplemented with pyruvate (National Health Laboratory Service, SA) and onto one L-J medium slant supplemented with glycerol (BD Diagnostics), and incubated at 37°C for up to ten weeks. The slants were monitored weekly for mycobacterial growth.

Ziehl-Neelsen staining was conducted and lysate (DNA) of acid fast bacteria was subjected to polymerase chain reaction (PCR) testing to identify bacteria as MTBCas previously described [28, 29]. Subsequently, deletion analysis was performed on the isolates using PCR primers targeting the RD4 (region of difference-4) as previously described for *M. bovis* identification [30].

Genotyping

Genotyping was conducted first using the standard, spoligotyping and VNTR profiling methods, followed by bioinformatics tools as described below to analyse the WGS data.

Spoligotyping

Spoligotyping was conducted according to previously used standard methodology [14] using a commercial kit (SPOLIGO TB, Mapmygenome, India), *M. bovis* BCG and distilled sterile water were used as positive and negative controls, respectively. Briefly, DNA samples from fresh isolates of the identified MTBC, confirmed through deletion typing, were used. The direct-repeat (DR) region was amplified with primers DRa (biotinylated) and DRb, and the amplified DNA was hybridized to inter-DR spacer oligonucleotides covalently bound to a membrane.

Table 1 Tissues with tuberculosis-like lesions (TBL) collected at the Asmara slaughterhouse, animals' characteristics, ante mortem signs (AM signs), post mortem signs (PM signs), and origin of the slaughtered animals

Date	TB number	Species	Breed	Sex	Age (yrs)	AM signs	PM lesions	Tissues collected	Origine of animals
28/03/14	TB8599	Bovine	HF*	F	5	Poor	TBL on lung and chest cavity	Lung and chest cavity tissues, bronchial and mediastinal. LN**	Asmara area
17/06/14	TB8600	Bovine	HF	F	7	Emaciated	TBL on chest cavity	Tissues with TBL from chest cavity	Asmara
09/04/14	TB8613	Bovine	HF	F	7	Normal	Traumatic pericarditis and TBL	Lung tissues and lung LN with TBL	Asmara area
10/11/14	TB8601	Bovine	Local	M	7	Normal	TBL on peritoneum	Peritoneum and inguinal LN	Embaderho (Maekel)
19/11/14	TB8602	Bovine	HF	F	6	Emaciated	TBL on the chest	TBL from lung tissues and lung LN	Not available
24/11/14	TB8603	Bovine	HF	F	7	Emaciated	TBL on the chest & lung	Tissues of lung and sternum with TBL	Dekemhare (Debub)
29/12/14	TB8604	Bovine	HF	F	6	Normal	TBL on chest and lung	TBL from lung and chest	Not available
16/01/15	TB8605	Bovine	Cross	M	7	Normal	Abscess on chest and abdominal cavities	TBL from chest and abdominal cavity	Not available
02/10/15	TB8606	Bovine	HF	F	6	Normal	TBL in abdominal and chest cavity	Indguinal LN	Asmara
28/02/15	TB8607	Bovine	HF	F	4	Bloating	Lesions on abdominal and chest cavity	TBL from chest and abdominal cavity	Asmara area
20/04/15	TB8608	Bovine	HF	F	5	Normal	TBL in body cavity	Inguinal and sternal LN	Asmara
18/05/15	TB8609	Bovine	HF	F	4	Normal	Miliary TBL	Pleural and deep inguinal LN	Asmara

19/05/15	TB8910	Bovine	HF	F	8	Emaciated	Few TBL on the chest	Lung and bronchial LN	Asmara Unaminassie
23/05/15	TB8611	Bovine	HF	F	7	Normal	TBL on chest cavity	TBL from pleura and mediastinal LN	Not available
30/05/15	TB8612	Bovine	Cross	F	6	Normal	Hyperemic lesions on pleura and its cavity	TBL from chest, inguinal and bronchial LN	Not available

*HF = Holstein-Friesian; **LN = lymph nodes

Variable Number of Tandem Repeat (VNTR) typing

PCR amplification of DNA for VNTR typing was performed using a set of 13tandem repeat loci recently identified as stable and polymorphic for South African *M. bovis* isolates [11]. These included the (4) ETRs loci, (4) QUB loci, (3) MIRU and (2) Mtub loci (i.e. ETR-A, -B, -C, and -E; Qub-11a, -11b, -18 and -26, MIRU 16, 23 and 26, as well as Mtub 12 and 21). The loci were amplified individually as previously described [31]. The band sizes were converted into number of tandem repeats at each locus based on the allele naming table provided [31].

Identification of *M. bovis* clonal complexes

The three features used to distinguish *M. bovis* clonal complexes were: a) they are a derivative of most recent clonal ancestors (MRCA) spoligotype b) region of difference deletion and c) geographic restriction (Example: African 1 is localized in West Africa)

a) Clonal complexes African 1 and 2

The status (presence or absence) of the regions of difference for African 1 and 2 (RDAF1 & RDAF2) in the isolates was assessed by multiplex PCR following procedures described earlier by Müller et al. [17], and Berg et al. [16] with minor modifications (2 µl of DNA template was used to make a final reaction volume of 21 µl each), respectively.

b) Clonal complexes European 1 and 2

The status of the European 1 region of difference (RDEu1) was determined by PCR using two primers targeting the flanking regions of the Eu1 deletion boundary as previously described by Smith and co-workers [18]. Whereas, the status of the European 2 region of difference (RDEu2) was determined by performing a PCR restriction endonuclease analysis to determine the presence of the SNP in *gua*A gene [15].

Whole genome sequencing (WGS)

To obtain the whole genome sequences, DNA of the 14 Eritrea *M. bovis* isolates was extracted (dx.doi.org/10.17504/protocols.io.nsgdebw) and sequenced on a MiSeq instrument (Illumina, San Diego, CA) using 2 x 250 paired-end chemistry and the Nextera XT library preparation kit (Illumina, San Diego, CA). FASTQ files from the instrument were put through the National Veterinary Services Laboratories (NVSL) in-house pipeline (see https://github.com/USDA-VS). Briefly, reads were aligned to the reference genome AF2122/97, NCBI accession number NC_0002945, using BWA and Samtools [32, 33]. A depth of coverage of 80X was targeted. BAM files were processed using Genome Analysis Toolkit (GATK)'s best practice workflow. SNPs were called using GATK's HaplotypeCaller outputting them to variant call files (VCF) [34-36]. Results were filtered using a minimum QUAL score of 150 and AC = 2. From the VCFs, SNPs gathered were outputted to three formats: an aligned FASTA file; tab-delimited files sorted by position location and by SNP groups; and a maximum likelihood phylogenetic tree created with RAxML [37]. The tree was built using a GTR-CAT model with input taken as an alignment file containing only informative and validated SNPs. SNPs were visually validated using Integrative Genomics Viewer (IGV) [38]. Because WGS isolates from this region of the globe are not readily available, databases from three laboratories (United States Department of Agriculture, Centre de Recercaen Sanitat Animal (CReSA) - Institute de Recerca i Technologia Agroalimentáries (IRTA), Spain, and Tuberculosis Research Laboratory, Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Nigeria) that are actively sequencing *M. bovis* isolates were queried and field isolates that were within 150 SNPs of the Eritrea isolates were included in our analysis. Also included for perspective were widely available reference strains, AN5, Ravenel, 95-1315, AF2122/97, BCG, and BZ-31150. FASTQ files from the isolates sequenced were uploaded into NCBI short read archive. Accession numbers Bioproject and sample numbers are listed in supplemental S1 Table.

Results

TBL detection at meat inspections

During the period 2010 to 2015, 78,820 cattle were slaughtered and 38 carcasses, originating from Maekel and Debub regions, were totally condemned due to generalized TBLs showing caseous necrosis identified in gross pathology in the lungs, livers, pleura (chest), peritoneum, and lymph nodes. Besides, fore quarters of three animals, plucks, shoulders, chests and heads of six cattle were partially condemned due to the presence of TBL (Table 2). All, except one (local breed), of the condemned carcasses were of the exotic HF breed or their crosses.

Table 2 Total number of cattle slaughtered, and number of totally and partially condemned carcasses and organs due to the presence of tuberculosis-like lesions (TBL) from 2010 to 2015 (inclusive) at Asmara municipal slaughterhouse in Eritrea

Year	Number of cattle slaughtered	Number of carcasses totally condemned	Body parts and organs partially condemned	Number of animals	Cattle breeds and sex
2010	14,919	6	Fore quarters, pluck (thoracic viscera and liver) and chest	1	Exotic (HF*), male
2011	11,976	8	Fore quarters, plucks, heads and shoulders	2	Exotic (HF), females
2012	12,307	5	Head, plucks and shoulders	3	Exotic (HF), 1 male and 2 females
2013	13,018	10	Heads, Plucks and shoulders	3	Exotic (HF), females
2014	13,359	1	Plucks and shoulders	1	Local, male
2015	13,244	8	Plucks and shoulders	2	Exotic (HF), females

*HF = Holstein-Friesian

Out of the 15 animals sampled from March 2014 to May 2015, nine originated from Maekel and one from Debub, while the origin of the other five slaughtered animals was unknown due to lack of records. Detailed gross

pathology information on the tissues collected is presented in Table 1. During this period 26,603 cattle were slaughtered and nine out of the 15 carcasses sampled, were totally condemned due to generalized TBL. In addition, the entire plucks and shoulders of three animals were partially condemned (Table 2), and from three other animals, tissues with TBL were collected and the carcasses passed for consumption.

Mycobacterium bovis culture and identification

Out of the 15 pooled tissue samples cultured on L-J media slants supplemented with pyruvate, 14 yielded smooth dysgonic growth, suggestive of *M. bovis* presence. All the 14 isolates were identified as MTBC. Subsequent examination by *M. bovis* specific PCR targeting the RD4, yielded banding patterns typical of *M. bovis* with a 268 bp product indicating RD4 deletion.

Mycobacterium bovis PCR based genotyping

Spoligotyping

The spoligotyping resulted in 3 distinct spoligotype profiles (Figure 1). The predominant spoligotype was SB0120 (9/14; 64%), characterized by the absence of spacers 3, 9, 16, and 39-43; followed by SB0134 (4/14; 29%), that showed absence of spacers 4 and 5 in addition, and lastly SB0948 (1/14; 7%); with the absence of spacers 1, 3, 9, 16, and 39-43. Designations for the spoligotypes corresponding to the spoligotype profiles in our isolates were obtained from http://www.*M.bovis.org* database.

VNTR typing

From the 14 *M. bovis* isolates*,* VNTR typing using a 13-loci VNTR panel revealed six VNTR profiles. Within the strains analyzed, only VNTR loci QUB26, ETR E, B, and MIRU 26 showed variations amongst the isolates, whereas the remaining loci (ETR A, C, Qub11a, 11b, 18, MIRU 16, 23, Mtub16 and 21) were monomorphic (Figure 1). One isolate exhibited two different VNTR alleles (3 & 4 tandem repeats) for locus ETR-E. For convenience reasons, the six VNTR profiles found were designated: VNTR profiles-ER-1 to -ER-6.

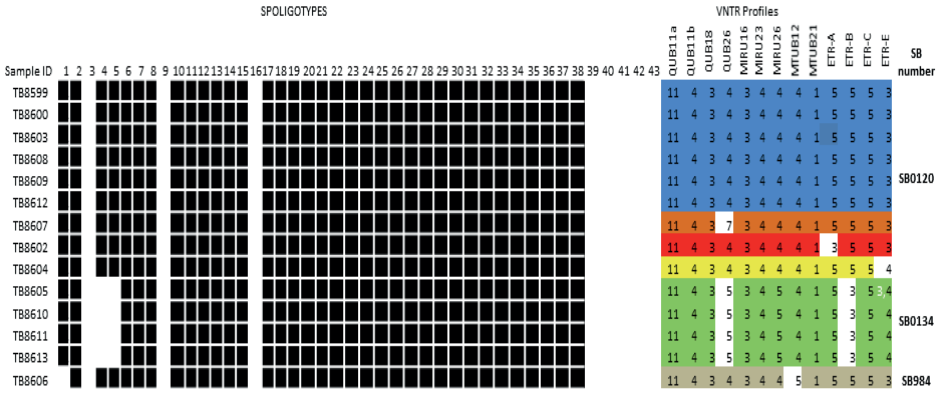

Figure 1 Spoligotype patterns with their SB-numbers retrieved from www.*Mbovis.org* of 14 *M. bovis* isolates (TB numbers) and VNTR profiles with their designations (ER-1 -ER-6) from tissues with TBLs collected at the Asmara municipal slaughterhouse. https://doi.org/10.1371/journal.pntd.0006406.g001

VNTR profile-ER-1 shared by six isolates (TB8599, TB8600, TB8603, TB8608, TB8609 & TB8612) was the most common one; VNTR profile-ER-5 was shared by four isolates (TB8605, TB8610, TB8611 & TB8613); VNTR profile-ER-2, -3 -4 and -6, were represented by one isolate each (TB8607, TB8602, TB8604 & TB8606, respectively). Four VNTR profiles (ER-1 to ER-4) corresponded to the SB0120 spoligotype, and two VNTR profiles, i.e., ER-5 and ER-6 corresponded to the SB0134 (TB8605, TB8610, TB8611, TB8613), and SB0948 (TB8606), respectively (Figure 1). The spoligotype SB0948 was clustered within the SB0120 group in its VNTR profile, with only one locus (Mtub21) difference from the rest of the group (Figure 1).

Clonal complex characterization

None of the 14 *M. bovis* isolates belonged to the RDAf1 (PCR product size of 350bp); RDAf 2 (PCR product size of 458bp), RDEu 1 (PCR product size of 1206bp), and RDEu 2. The spacers known to be deleted in the respective clonal complexes (i.e. spacer 30 in Af1, spacer 3 to 7 in Af2, spacer 11 in Eu1 & spacer 21 in Eu2) are intact in the Eritrean strains. The *M. bovis* positive control used (South African isolate; TB8569) was Eu1 clonal complex that demonstrated intact RDAf1 and 2, spacer 21, and the absence of spacer 11. Of

the 14 *M. bovis* isolates, two isolates (TB 8603 & 8613) were found to have the guaA mutated as indicated by the presence of the SNP leading to a single band of 179 bp following a PCR-restriction endonuclease analysis conducted to determine the presence of the SNP in guaA. The absence of a SNP in the guaA gene was demonstrated by two bands of 145 and 34 bp.

***Mycobacterium bovis* SNP based genotyping and phylogenetic relationships**

WGS and SNP analysis (Figure 2), shows that the Eritrean isolates clustered into two distantly related groups, containing an additional 135-159 SNPs since sharing a common ancestor (Labeled A in Figure 2) along with isolates from Spain and the USA, respectively. The Eritrean cluster consisting mostly of SB0120 isolates which were more diverse than those in the SB0134 cluster i.e. a SNP difference ranging from 8-30 from a common ancestor (Labeled B in Figure 2). There were also sub-clusters within this group; five isolates were only 5-6 SNPs from a common ancestor. Eritrean samples with the SB0134 spoligotype were only 10 SNPs from a shared common ancestor (Labeled C in Figure 2) with two isolates from Ethiopia. Interestingly the four Eritrean isolates were within a distance of 5-6 SNP from each other. Supplemental S2 Table contains the location and annotation of each SNP identified in the sequences from Figure 2. The overall phylogenetic structure of *M. bovis* isolates in the NVSL database are shown in Supplemental S2 Figure.

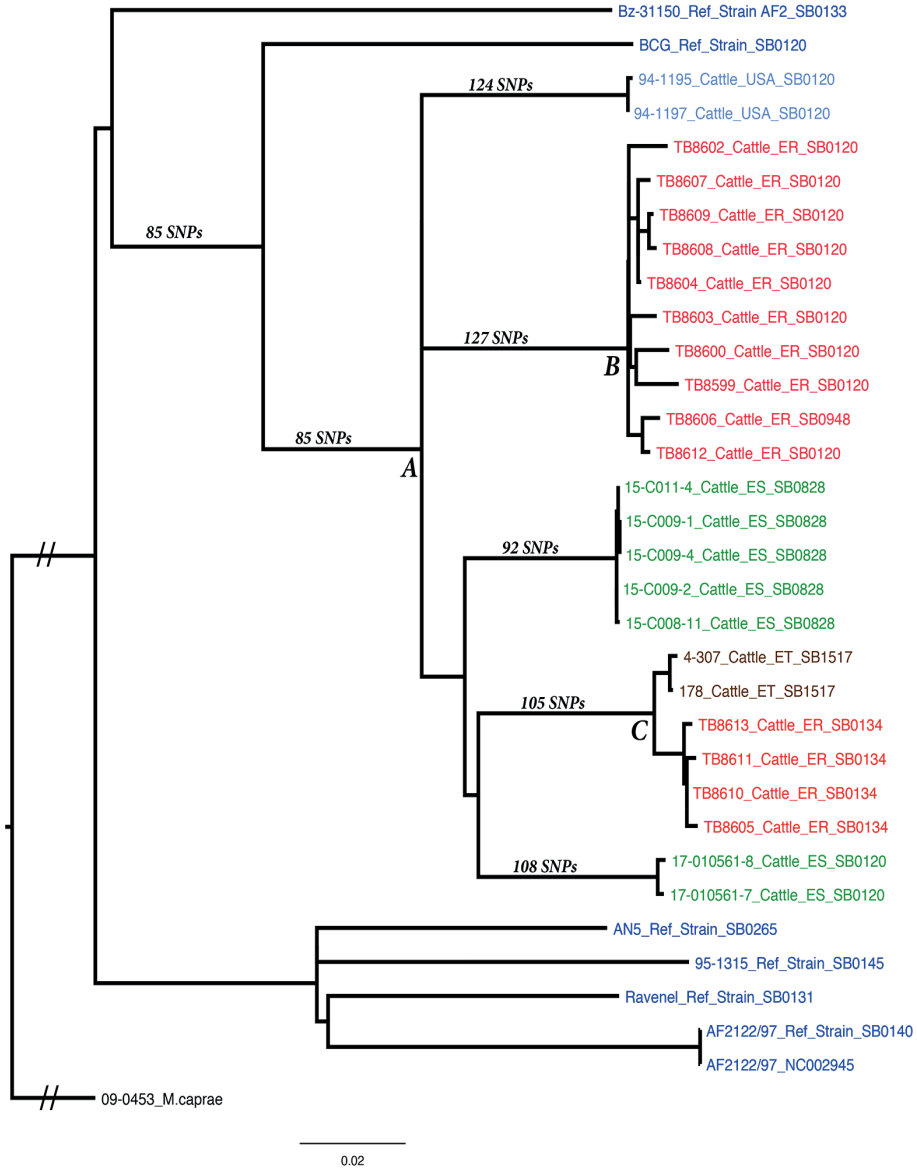

Figure 2 Whole genome sequence phylogenetic tree created using RAxML of 14 Eritrea dairy cattle abattoir samples and other field isolates that happen to share the same common ancestor with Eritrean isolates in the National Veterinary Services Laboratories' database. These include cattle isolates from Ethiopia, United States and Spain. Also included in the tree are well known type strains, including BCG, Bz-31150 – a recently sequenced AF2 strain, AN5 – used in PPD production, 95-1315 – Michigan deer strain, Ravenel and AF2122/97.

http://doi.org/10.1371/journal.pntd.0006406.g002

Discussion

Livestock production in general ('intensive dairy' and extensive traditional livestock rearing) is the main stay of the vast majority of people in Africa. In the Horn of Africa, where Eritrea is located, about 41 million people keep some livestock as a source of food, cash income, manure, draught power, transportation, savings, insurance and social status [39]. Hence, livestock plays a major role in poverty and hunger reduction. For livestock to play its crucial role in achieving food security and become economically viable in Eritrea, the impact of major transboundary diseases needs to be reduced. One of these diseases is BTB. Genotyping plays vital role in highlight of transmission networks of pathogens and enables trace back sources of infections, in order to prevent their re-introduction and spread. For this purpose, classical and state-of-the-art genotyping tools were used in the present study of *M. bovis* isolates circulating in dairy cattle in Eritrea.

Mycobacterium bovis PCR based genotyping

The dominant spoligotype identified in our study was SB0120, named BCG-like by Haddad et al. [13] and considered as parental strain for the *M. bovis* vaccine strain. It accounted for 64% of the isolates, whereas the other two spoligotypes SB0134 and SB0948 did so for 29% and 7%, respectively. The first two strains are widely distributed in a number of African countries, namely; Ethiopia, Algeria, Zambia, South Africa [6, 10, 16, 40-43] as well as in Italy, Spain, other European countries and Mexico [13, 44-49]. In addition to cattle, SB0120 affects wildlife and humans in Africa and Europe [50-53]. The third spoligotype (SB0948) has been reported in France, Italy, and in Zambia [13, 41, 44].

The relatively high frequency of the spoligotype SB0120 found in the present study may indicate its predominance in Eritrean dairy cattle, though difficult to conclude with such small sample size. The second predominant spoligotype (SB0134) appears to have evolved from spoligotype SB0120 by the loss of spacers 4 and 5 in addition to spacers 3, 9, 16, and 39-43 that classify spoligotype SB0120. This finding might not be surprising, in view of the past trade relations between Eritrea and Ethiopia, as both SB0120 and

SB0134 spoligotypes are also present in Ethiopia. Besides, these two countries share open borders that consequently allowed the uncontrolled movement of animals as obtained in most African countries. Therefore, it might be plausible to speculate that these strains of *M. bovis* are shared between Eritrea and Ethiopia. On the other hand, it might also be plausible to suggest Italy as a possible source of these strains, on the following grounds: a) long historical ties (1900 to 1970s) between Eritrea and Italy existed, b) Italian settlers initiated the establishment of dairying in Eritrea in the 19th century by importing exotic breeds (Holstein-Friesian), c) the *M. bovis* spoligotypes detected in our study are also wide spread in Italy. Although the reason for the apparent predominance of the two spoligotypes (SB0120 & SB0134) needs further study, as this may indicate an epidemiological link between different dairy farms/regions in Eritrea, as buying and selling of cows between dairy farms is common in the country [54] without following strict sanitary rules. Since not all the slaughtered cattle with TBL had records of their farm of origin, it may also be possible to suspect that some of the slaughtered animals might have originated from the same farm. It is noteworthy, however, that based on the WGS data there appears to be at least two introductions of *M. bovis* into Eritrean dairy cattle, an SB0120 strain and SB0134 strain. The SB1517 (Ethiopian strain; Figure 2) is an offspring of SB0134 suggesting that the common ancestor of the cluster was SB0134.

Spoligotype SB0948 was found in only one animal. It is a descendant of spoligotype SB0120 as it differs by the absence of spacer 1 only, and deviates only by the Mtub21 locus in its VNTR profile from the other members of the SB0120 group (Figure 1). Further, the WGS data confirmed that SB0948 is a recent descendant of a sub-cluster of SB0120 isolates. Though unclear what its relevance is in neighboring Ethiopia, this spoligotype was reported in several countries in Africa and Europe [13, 41, 44, 48, 55]. The African and global comparisons of spoligotype profiles (Figure 3 & S1 Figure) demonstrated the regional and global distribution of the spoligotype and VNTR profiles and their similarities with the Eritrean ones. These similarities could be attributed to the following two plausible reasons: a) inter-regional and global livestock trade, b) colonial livestock and livestock product trade within their then colonies and outside.

Variable number tandem repeat (VNTR) profiles are considered appropriate to complement spoligotyping due to their ability to discriminate between *M. bovis* strains as defined by spoligotyping [15, 55, 56]. The three spoligotypes were clustered into six VNTR profiles (Figure 1). The diversity seen in the VNTR profiles may suggest that *M. bovis* has been circulating in the dairy herds of the country for quite a long time with only minor mutations as the BCG-like spoligotype (SB0120) is the predominant one. Four of the VNTR profiles (ER-2, -3, -4 & 6) may have derived from the predominant VNTR profile ER-1, that corresponds to spoligotype SB0120. According to Smith et al. [49], strains bearing the same spoligotype pattern are assumed to be a set of individuals derived relatively recently by clonal replication from a single ancestral cell. On the basis of the VNTR profile, both strains, SB0948 and SB0134, are clustered within the SB0120 group with a loss of only one locus (Mtub12) in the former and two loci (ETR-B & ETR-E), in the latter strain, respectively. One of the VNTR profiles within the SB0134 strain exhibited two different VNTR alleles (3 & 4 tandem repeats) for locus ETR-E (Figure 1), suggesting either a mixed infection with two distinct strains or a microevolution in this strain. The VNTR profiles found in our study showed clonal variants differing at their loci as compared to what was reported in other parts of Africa (i.e., Zambia) (Figure 1 & Figure 3), though they were all *M. bovis* strains belonging to SB0120 spoligotype. This clonal difference (Figure 1 & Figure 3) seen in our study may have been attributed to the absence of active livestock (dairy cattle) trade between Eritrea and other parts of Africa (Zambia) or due to the different geographical locations and livestock management systems between the countries, that might have dictated the microevolutions (mutations) differently. The possible reason for having the same spoligotype (SB0120) in Eritrea and other African countries (S1 Figure), might be that the source of the cattle for Eritrea and the other countries was Europe, as Europe is the source for the high yielding dairy cows, like the Holstein Friesians, that are imported by most African countries with the aim of improving milk production in their countries in order to realize food security.

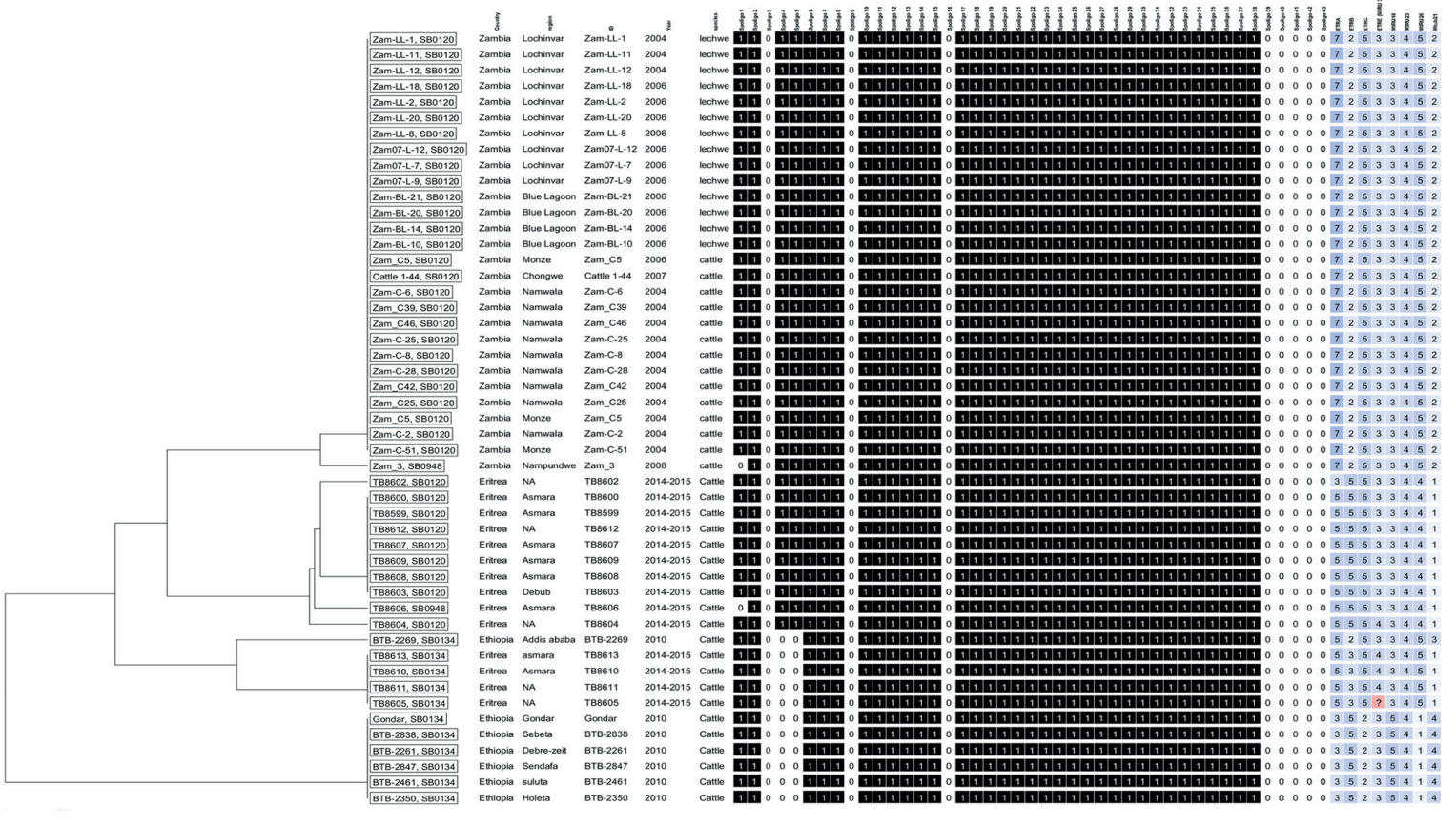

Figure 3 African comparison of spoligo patterns and VNTR profiles showing common traits with the Eritrean samples. https://doi.org/10.1371/journal.pntd.0006406.g003

Clonal complex characterization

The investigation of the 14 *M. bovis* isolates for clonal complex differentiation revealed that they belonged to none of the complexes identified so far i.e., African 1 (RDAf1), African 2 (RDAf2), European 1 (RDEu1) and European 2 (RDEu2) [15-18]. The absence of members that belong to clonal complex African 1 and 2 in our samples could suggest limited introduction of such strains from the neighboring Eastern and Western African livestock movement routes. It is noteworthy, that these two strains (SB0120 & SB0134) are present in Ethiopia [16], although most of the other strains in this country belong to Af2. In the current study, little strain diversity is recorded (Figure 1) as compared to studies conducted in other countries with similar agricultural setting like Eritrea [6, 42].

***Mycobacterium bovis* SNP based genotyping and phylogenetic relationships**

The WGS results matched the conventional laboratory methods with better resolution. These data support two separate introductions of *M. bovis* into Eritrea, with subsequent localized spread. The common ancestor of these two groups is shared widely with isolates in the USA and Spain, with greater diversity found in Spain suggesting an introduction from Europe.

The presence of a common ancestor in these distantly located countries may be due to the international livestock trade between these countries, geographical proximity and similar livestock production systems. Example: the origin of the high yielding dairy breed (Holstein Friesian) is Europe. As indicated in the spoligotyping section above, the spoligotype SB0120, predominant in our study, is also ubiquitous in Europe, especially in France [13], Italy [44], Portugal [45], and Spain [48], most likely as a result of geographical closeness and trade relations between these countries. Therefore, our finding may not be a surprise, given the historical establishment of 'intensive' dairy farming by the Italian settlers in Eritrea through the importation of high yielding dairy breeds (Holstein Friesians) to meet the high demand for milk and dairy products. The fact that the Eritrean strains are between (close to) Spain samples (Figure 2) may suggest two introductions or may be just one introduction; i.e., from Europe (Italy). Since

we do not have information that shows historical, political or trade ties between Spain and Eritrea, we can speculate that either the strains are circulating in Italy and Spain. Or that, the Italian settlers during the establishment of dairy farms may have imported the cattle from Spain or other European countries where the same strains of *M. bovis* might have been circulating. A classical analogy for this speculation may be rinderpest that was brought to Sub-Saharan Africa by Italian forces in 1889, with infected cattle they had imported from India, Aden, South Russia to feed their army that had then occupied Massawa (Eritrea) [57]. However, although phylogenetic comparison with Italian *M. bovis* isolates could not be done in our study, we cannot refute the possibility that these strains originate from Italy or via the above indicated routes from other countries.

The second probable route of introduction for one of the groups of the Eritrean strains, but not for the other, may be Ethiopia considering the long and close historical relationship and uncontrolled livestock movement between these two countries. The Ethiopian and Eritrean samples have accumulated 8-16 additional SNPs since sharing a common ancestor suggesting a recent common source and regional spread. But the four Eritrean samples (strains) are within 5-6 SNPs from sharing a common ancestor suggesting these isolates have established and spread within Eritrea, though it might be premature to reach into conclusion with such small sample size. Eritrea, on the other hand, might have introduced this strain to Ethiopia. This is plausible because both intensive dairy farming, established 100 years ago in Eritrea and the first report of BTB (Pirani, 1929), cited by Omer et al. [3], occurred earlier than in Ethiopia where 'intensive' dairy farming started in the 1950s (1947) by importing Friesians and Brown Swiss [58]. This was followed by the detection of acid fast bacilli in a cow's milk, in one study, and detection of what was called '*Mycobacteria tuberculosis* bovine type' seemingly, *M. bovis* from 18 cattle, in another study, in Eritrea, by Sfroza in 1944 [3].

The samples collected in this study are not considered representative of all strains possibly circulating in Eritrea. However, Asmara slaughterhouse, as the country's biggest facility mostly slaughters exotic cattle breeds from various regions in Eritrea in which previously a high BTB prevalence was reported [4]. Therefore, the panel of samples still provides a valuable insight

in the genetic strain composition from mostly dairy producing regions in Eritrea and a valuable basis for future investigations.

The current study characterized strains of *M. bovis* in Eritrea and revealed their (dis)similarities with the strains generally present in Africa and Europe, as well as potential routes of introduction of *M. bovis*. Though the sample size is small, our study provides important information as well as availability of technology for future in-depth molecular studies including more samples from dairy cattle as well as cattle and goats from the traditional livestock sector. This study provides information on the origin of the *M. bovis* strain in Eritrea, its genetic diversity, evolution and patterns of spread (spatial and temporal) between dairy herds. The information obtained will be instrumental in making informed decisions in future BTB control strategy for Eritrea.

Limitations

Our study has some limitations. The samples were collected from one slaughterhouse and were few due to the absence of tissues with TBL during the study period. The low prevalence of BTB in the traditional livestock raising system [59] where majority of slaughtered animals come from, has limited the possibility of detecting more *M. bovis* strains from different geographical regions of Eritrea.

Conclusion and recommendations

Genetic profiling of *M. bovis* strains is a highly useful approach which can aid in the study and control of the temporal and geographical disease spread in the country and the African continent where BTB is largely uncontrolled. We recommend future studies in Eritrea to include genetic profiling of Italian isolates so as to support or negate our hypothesis with certainty than just live with speculation that the origin of the Eritrean *M. bovis* strains was Italy.

In future studies in Eritrea, inclusion of more regional slaughterhouses including animal traceability will enable us gain greater insight into the

epidemiology of BTB in the country which will allow the *M. bovis* genotype to be linked to the population from which it was obtained.

We also recommend that simultaneous detection and strain differentiation of *M. bovis* isolates should become a reality in the routine of human tuberculosis reference laboratories, as well as in the routine meat inspection at municipal slaughterhouses. Therefore, using the One Health paradigm (i.e. interdependence between the medical and veterinary fields), greater integration between agriculture and health sectors could be an important strategy to control *M. bovis* in several places in the world where the agent is disseminated between animals and humans.

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Supporting information

S1 Table Accession numbers, Bioproject and sample numbers in 'FASTQ' files from the isolates sequenced and uploaded into NCBI short read archive. The Eritrean *M. bovis* isolates are compared with the widely available *M. bovis* reference strains, the field strains from the collections of United States Department of Agriculture, Centre de Recerca en Sanitat Animal (CReSA) - Institute de Recerca i Technologia Agroalimentáries (IRTA), Spain, and University of Ibadan, Nigeria, that shared the same common ancestor.

http://journals.plos.org/plosntds/article/asset?unique&id=info:doi/10.1371/journal.pntd.0006406.s001

S2 Table SNP table comparing the Eritrean strains with Ethiopian, Spanish and American (USA) strains with reference to Bz-31150_Ref_Strain AF2_SB0133 and BCG_Ref_Strain_SB0120

http://journals.plos.org/plosntds/article/asset?unique&id=info:doi/10.1371/journal.pntd.0006406.s002

http://journals.plos.org/plosntds/article/asset?unique&id=info:doi/10.1371/journal.pntd.0006406.s003

S1 Figure Global comparison of spoligotypes in relation to the spoligotypes of the Eritrean *M. bovis* isolates

http://journals.plos.org/plosntds/article/asset?unique&id=info:doi/10.1371/journal.pntd.0006406.s004

S2 Figure The overall phylogenetic structure of *M. bovis* isolates in the NVSL database with the Eritrean and related strains

Chapter 6

Summarizing Discussion

The studies presented in this thesis aimed to determine the prevalence as well as the potential risk factors for the transmission of *Mycobacterium bovis* (*M. bovis*) between domestic animals and to man in Eritrea. First, the status of bovine tuberculosis (BTB) within the dairy sector of Eritrea was investigated by subjecting about 50% of the country's dairy cattle to diagnosis of BTB by the single intradermal comparative tuberculin test (SICTT). This study demonstrated a considerable prevalence of BTB in the dairy sector of Eritrea, ranging from 21.5% (the highest) in Maekel, to moderate (7.3%) in Debub, and low (0.2%) in Anseba region. Furthermore, it aimed at elucidating the possible risk factors that may have contributed to the observed prevalence at individual animal level and to the differences within the regions tested. The potential risk factors considered were age and sex, lactation and pregnancy status, and the regions where the testing was conducted (**Chapter 2**). It was highlighted that pregnant-lactating animals were more at risk when compared to the other categories of animals (**Chapter 2**). This study, however, was not able to show the factors that might have contributed to the between herds (farms) BTB prevalence, as not all farms had SICTT reactor animal(s). Therefore, a second study was performed to elucidate the potential risk factors for BTB as represented by presence/absence of SICTT reactors in the tested dairy herds (farms) and the regions of the country. Farms with SICTT reactor animal(s) (case farms), and without reactor animal(s) (control farms), were identified, and stratified according to their herd sizes at 61 case and 65 control farms were randomly selected for the study. Face-to-face interviews using standardized pretested questionnaire addressing potential risk factors were conducted. This study showed that farms with higher numbers of cows, and those with concrete floors were more at risk to have SCITT reactors, compared with their references (**Chapter 3**). Though the dairy cattle in and around the cities are the major sources of milk for the urban dwellers, the majority of the Eritrean population depends on livestock kept within the traditional livestock system (mixed crop-livestock, and pastoral systems). To increase milk production and to meet the high demand for milk in the country, the farmers tend to cross breed their indigenous cattle with high yielding Holstein Friesian (HF) breeds. Given the importance of the extensive livestock system, it was vital to explore BTB prevalence and its potential risk factors within the livestock kept in this system. Therefore, a third study assessed whether BTB

was also present in the livestock managed within the traditional livestock farming system, moreover, to address its zoonotic implication. To answer these questions, cattle, goats, and camels in four selected regions within this farming system were tested for BTB using the SICTT, and questionnaire based face-to-face interviews were conducted in households raising cattle, goats and camels to associate potential risk factors with the presence of BTB in these animals. This study has shown that SICTT reactors, though rare, were present in cattle, goats and camels within the traditional livestock husbandry system in Eritrea and the spatial distribution of affected animals was vast, covering most of the study regions (**Chapter 4**). Since so far BTB was only indicated by SICTT reactivity, a fourth study done was to confirm the actual presence of *M. bovis* and the diversity of strains circulating in the livestock of Eritrea. For this purpose, tissues with tuberculosis-like lesions (TBL) were collected from the Asmara municipal slaughterhouse (Eritrea's largest slaughterhouse) and bacterial culture, spoligotyping, VNTR profiling, and whole genome sequencing (WGS) of *M. bovis* isolated from these tissues were conducted. The findings of this study revealed three different spoligotype patterns, six VNTR profiles using Spoligotyping and VNTR profiling techniques, respectively, and 14 strains of *M. bovis* using WGS (**Chapter 5**).

BTB in livestock in Eritrea

BTB in the intensive dairy husbandry system

Previous studies have reported the presence of BTB in dairy cattle in Eritrea with the highest prevalence being in Asmara area (the capital), in Maekel region [Omer et al., 2001], where the first BTB report was filed by Pirani (1929), as cited by Omer et al. (2001). Our current study, has also found high (21.5%) BTB prevalence in Maekel, with moderate (7.3%) to low (0.2%) prevalences in Debub and Anseba regions, respectively. The risk of having a BTB skin test positive animal in the dairy farms was 13 and ~9 times more in Maekel and Debub regions, respectively than in Anseba region [Ghebremariam et al., 2016]. These differences in BTB prevalence within the dairy sector may be attributed to the presence or absence of proper housing, adequate space for grazing and exercise, and differences in climate between the regions.

Maekel and Debub regions, showing the higher prevalences, are endowed with relatively mild and wet climate that might be conducive for the survival and multiplication of *M. bovis*, as compared to the arid and semi-arid climate of most part of Anseba region [Ghebremariam et al., 2017; Ghebremariam et al., 2016; Humblet et al., 2009; Skuce et al., 2012; Mogos, 2003]. In addition, keeping dairy cattle indoors, in small houses with few or no windows may have contributed to the high prevalence of BTB in the two regions (Maekel & Debub), in contrast to housing practices in the Anseba region, as such conditions lead into poor ventilation facilitating *M. bovis* transmission [Ghebremariam et al., 2017; Radostits et al., 2007; Costello et al., 1998; Elias et al., 2008; O'Reilly et al., 1995] via the respiratory route [Ameni et al., 2006; Menzies et al., 2000; Johnston et al., 2011], through which as few as one bacillus may cause infection [Ayele et al., 2004; Griffin et al., 1993; Griffin et al.,1996; Skuce et al., 2012; Skuce et al., 2011; Humblet et al., 2009]. In addition, Asmara and its surroundings being the pioneering city for the initiation of dairy farming, the importation, hence abundant presence of exotic dairy breeds, might be the reason behind the high BTB prevalence in Maekel region. The absence of control measures may also have contributed to the high BTB prevalence in this region. Our *M. bovis* genetic profiling study [Ghebremariam et al., 2018a] has reaffirmed this suggestion by revealing two independent introductions of *M. bovis* into Eritrea with the most probable source the importation of exotic dairy breeds from Europe. The plausible reasons for the low prevalence of BTB in Anseba region may be the fact, that in this region the dairy houses are well ventilated and the animals spend more time outside in shaded areas. Importantly, in addition, the dairy association in Anseba region took a timely measure to control BTB by compensating the farmers for slaughtered reactor animals through a mutual funding scheme. At individual animal level, the major risk factors for reacting in the skin test were pregnancy and lactation. Pregnant animals are reported to have two to threefold more severe inflammatory lesion with a rise to miliary tuberculosis, compared to non-pregnant young female adults [Weinberg, 1983]. It is difficult to explain the strongly increased odds ratio for SICTT positive responses in lactating-pregnant animals in our study [Ghebremariam et al., 2016]. Immunosuppression during periparturient and during the early phases of lactation might increase susceptibility of the animals to *M. bovis* infection

which is eventually detected by SICTT [Lloyd, 1983]. In addition, farms with higher numbers (> 10) of cows and those with concrete floors were more at risk to have positive BTB reactors than their references [Ghebremariam et al., 2017]. In contrast, smaller herds have a lower prevalence [Griffin et al., 1996; Wright et al., 2015; Humblet et al., 2009]. The role of concrete floors as a potential risk factor for farms to have positive BTB reactor animal(s) may be attributed to the lack of maintenance of such floors that might enhance the development of digital dermatitis [Nsahlail & Mogos, 2007]. Affected animals tend to lie down for prolonged times, that in turn may compromise their feed intake and time used for feeding, causing emaciation and enhanced susceptibility to disease, including BTB [Cook & Nordlund, 2009; De Vries et al., 2015; Nordlund et al., 2004]. The inclination of dairy farmers in Eritrea to purchase pregnant cows to cope with the high demand for milk in the market might have attributed to the higher risk of having BTB positive reactors due to the risk of introducing a *M. bovis*-infected cows. The longer an animal stays in a case herd, the higher will be the cumulative increase in the chance of being infected [Cleaveland et al., 2007; Humblet et al., 2009; Proano-Perez et al., 2009]. The overall results of the case control study [Ghebremariam et al., 2017] resembled those of similar studies in Ethiopia [Regassa et al., 2010; Ameni et al., 2003; Shitaye et al., 2006; Gumi et al., 2011]. Our studies were able to report the prevalence, interregional differences, and persistence of BTB within the dairy sector of Eritrea, and identified the major potential risk factors implicated for this prevalence.

BTB in extensive livestock husbandry system

The extensive livestock husbandry system in Eritrea is crucial for the livelihoods of the vast majority of the farming communities, and to the economy of the country. This husbandry system is primarily carried out within the mixed crop-livestock, and pastoral systems. In this system, 18% of the total (around 2 million) cattle population are lactating cows with milk output averages of 4 liters per day over 270 days lactation period. The current study [Ghebremariam et al., 2008b] was the first to assess the prevalence of BTB in cattle, goats and camels and its zoonotic implication within the extensive traditional livestock system. The findings showed low BTB prevalence in cattle

(1.2%), and camels (1.5%) with none in goats (0%), using a standard (> 4 mm) cut-off [Ghebremariam et al., 2018b]. Prevalence figures increased, when the severe (> 2 mm cut-off) interpretation was used, in all the tested species, i.e. in cattle 5.5%, in goats 2.2%, and in camels 11.8%. This interpretation was used in several studies in Africa and its usefulness was indicated in indigenous livestock [Ameni et al., 2008; Gumi et al., 2011; Gumi et al., 2012; Tafess et al., 2011; Ngandolo et al., 2009; Tschopp et al., 2011]. In addition, interregional differences were observed in the BTB prevalence, which was highest (2.3%) in cattle in Debub region, where also moderate (7.3%) prevalence was recorded in the dairy sector study [Ghebremariam et al., 2016]. The overall results in our extensive livestock system study concurred with findings of similar studies in Ethiopia [Mamo et al., 2013; Ameni et al., 2013; Ameni et al., 2003; Ameni et al., 2007; Tschopp et al., 2009]. The comparatively high prevalence in Debub may be attributed to the mild and wet climate in the region which is more favourable for survival of *M. bovis* as compared with the arid and semi-arid regions of Gash Barka, Southern Red Sea and Anseba (partially) [Ghebremariam et al., 2017; Humblet et al., 2009; Skuce et al., 2011]. In the latter areas, climatic conditions, lower cattle density, and keeping of animals in open areas may explain the low prevalence of BTB in the extensive cattle husbandry system in general. On the other hand, several animal conditions like malnutrition, recent infection, infestation with gastrointestinal parasites, breed of animals [Flynn et al., 2007; Claridge et al., 2012; Ameni & Medhin, 2000; Ameni et al., 2007; Voldermeier et al., 2012] may be behind the low skin test reactor rate as measured with the SICTT in such a husbandry system in general. Both the relative resistance of the indigenous breeds for *M. bovis* infection as well as the alteration of cell mediated responsiveness due to these animal conditions may be explanation for these findings. In our study within the extensive farming system [Ghebremariam et al., 2018b], indigenous zebu cattle that are considered more BTB resistant when compared to exotic breeds were predominantly used. Despite the overall current low BTB prevalence, the fact that the presence of infection was indicated in many of the study villages, holds the risk of spread and increase in the future due to inter-species herd mixing at water points and resting areas where livestock congregate, migration and uncontrolled livestock movement in the extensive livestock

raising system in Eritrea [Shanahan et al., 2011; Quintas et al., 2010; Daniel et al., 2009; Cadmus et al., 2009; Tschopp et al., 2011; Mamo et al., 2012; De la Rua-Domenech et al., 2006; Crawshaw et al., 2008; Tafess et al., 2011].

Molecular identification of *M. bovis*

In the present study [Ghebremariam et al., 2008a] the classical *Mycobacterium tuberculosi*s complex (MTBC) typing tools (Spoligotyping and variable number tandem repeat (VNTR) typing) as well as advanced methods such as whole genome sequencing (WGS) were used to better understand the spatial and temporal distribution, genetic diversity and evolution of *M. bovis* strains circulating in Eritrean dairy cattle. Accordingly, several *M. bovis* strains in Eritrea and their (dis)similarities with the strains generally present in East Africa and Europe, as well as potential routes of introduction were revealed. The WGS results matched the conventional genotyping methods (thus confirming their findings) with better resolution. The data support two separate introductions of *M. bovis* into Eritrea. The dominant spoligotype was SB0120, suggesting its predominance in Eritrean dairy cattle. The second predominant spoligotype (SB0134) appears to have evolved from SB0120. Both spoligotypes are widely distributed in East, West, South, and North Africa [Berg et al., 2011; Biffa et al., 2010; Tsegaye et al., 2010; Firdessa et al., 2012; Sahraoui et al., 2009; Hang'ombe et al., 2012; Hlokwe et al., 2014] as well as in Italy, Spain, and other European countries and Mexico [Boniotti et al., 2009; Haddad et al., 2001; Rodriguez et al., 2010; Duarte et al., 2008; Hauer et al., 2015; Milian-Suazo et al., 2016; Smith et al., 2006]. The common ancestor of these two groups (SB0120 & SB0134) is shared widely with isolates in the USA and Spain [Ghebremariam et al., 2018a], with greater diversity found in Spain suggesting an introduction from Europe. The presence of a common ancestor in these distantly located countries may be due to the international livestock trade, specifically HF, between these countries, geographical proximity and similar livestock production systems. Although the reason for the apparent predominance of the two spoligotypes (SB0120 & SB0134) in Eritrea needs further study. It cannot be ruled out that some of

the slaughtered animals have originated from the same farm, given the fact that not all the slaughtered cattle with TBL had records to trace back their farms of origin, and this may indicate an epidemiological link between different dairy farms/regions in Eritrea, as trade of cows between dairy farms is common in the country [Ghebremariam et al., 2017] without following strict sanitary rules, and thus higher risk of BTB infection [Palisson et al., 2016].

Role of *M. bovis* in human tuberculosis

Determining whether human TB cases are caused by *M. bovis* is a vital step to elucidate the epidemiology of *M. bovis* TB, which in turn would be additional support to the application of proper prevention strategy for human infection [Hlavsa et al., 2008]. Spoligotyping and VNTR typing are vital tools to distinguish *M. bovis* isolates of both human and bovine origins, with VNTR having a better discriminatory power and is considered the most suitable as a complimentary or an alternative method to spoligotyping [Kamberbeek et al., 1997; Hlokwe et al., 2013; Boniotti et al., 2009; Rodriguez-Campos et al., 2011].

Presence of MTBC and a high prevalence of MDR within the MTBC in dairy milk were confirmed in a study conducted in South Africa [Silaigwana et al., 2012] and MDR *M. bovis* in milk, and from clinical human samples in Brazil [Parreiras et al., 2004; Vazquez-Chacon et al., 2015]. The role of *M. bovis* in human tuberculosis was not known in Eritrea and in a preliminary study we aimed at defining its presence from 77 sputum samples collected from MDR-TB suspected patients of which 33 were preliminarily suspected for *M. tuberculosis* or potentially other MTBC members, and 44 for non-tuberculous mycobacteria (NTM) otherwise known as mycobacteria other than tuberculosis (MOTT) or environmental mycobacteria (EM) by liquid culture BACTEC 960 MGIT system, and GeneXpert (MTB/RIF assay that is based on Realtime PCR and detects the presence of *M. tuberculosis* and rifampin resistance, simultaneously). These samples were further analysed using classical molecular detection techniques (spoligo- and VNTR typing) and 16S rRNA and 16S rDNA gene sequencing. Overall, seven (of 14) samples were identified as *M. tuberculosis* and seven (of 14) as NTM [*M. sherrisii* (1 patient, 2 samples at

different sampling times). *M. yongonense* (2 patients), *M. intracellulare* (2 patients), and *M. avium* (1 patient).

Although one of the reasons for not detecting *M. bovis* from the samples may be: a) only sputum samples were analyzed and samples from other organs (example: lung aspirates) were not included that might have represented the extra-pulmonary cases, though *M. bovis* can also be isolated from sputum samples [Cosivi et al., 1998; Byarugaba et al., 2009; Milian-Suazo, et al., 2010]; b) the bacterial culture conducted in the National Health Laboratory, Eritrea, mainly targets *M. tuberculosis,* and the growth medium is thus not supplemented with pyruvate to enhance *M. bovis* growth; c) or there may be no *M. bovis* in human patient from whom the samples were collected. The National Health Laboratory was recommended to include L-J media with pyruvate during bacterial culture of samples collected from MDR-TB patients and include PCR and genotyping tools to be able to detect *M. bovis* and NTM in the future.

Messages and recommendations from this thesis

Future BTB control options in Eritrea

BTB control is not an easy task since it requires skilled manpower, resources, political goodwill of the governments and high level of public awareness. Though rigorous efforts were made, few success stories exist on BTB control and eradication on the globe [More et al., 2015]. Although it has been more than 100 years since in several countries the control and eradication programs started, BTB seems to linger in most, even in countries with adequate resources [Fischer et al., 2005; Good, 2006].

Knowledge gained through the present and other research can only become instrumental to make informed and evidence based policies for sustainable control and eradication of BTB when translated into action. With the aim of translating the knowledge gained from our current BTB studies in Eritrea, the findings and recommendations of our studies were communicated through seminar presentations that were attended by veterinarians, animal production experts, academia, farmers and policy makers.

Experts' workshop on future BTB control in Eritrea

A one-day experts' workshop with the theme 'Bovine tuberculosis status and control options in Eritrea', was organized to discuss and enrich the BTB control options recommended by our study for Eritrea. It is worthwhile noting that in 2012 the animal health professionals from the Ministry of Agriculture (MOA) and academia had a 10 days OIE experts' consultation workshop that aimed at evaluating the performance of veterinary services (PVS), where future BTB control options were touched upon. The current workshop was based on better information on the prevalence of BTB in both intensive and extensive livestock farming system, potential risk factors, the etiologic agent and its origin(s) as evidenced in this thesis. In the OIE expert consultation (2012) and in the present workshops the participants helped develop action steps towards the future BTB control and eradication. While the workshop was not intended to reach 100% consensus on each issue of BTB control, the input gathered and described in this thesis within this section, represents key animal health and production experts' opinions on the future of the National BTB control Program in Eritrea.

The following recommendations of our studies were presented and discussed: conduct rigorous awareness raising campaign to sensitize the communities on the danger of BTB and its zoonotic potential; voluntary test and slaughter of BTB reactors; compulsory test and slaughter; popularizing mutual compensation schemes by the farmers; strict livestock movement control. A discussion guide on the overall BTB situation in Eritrea (past, present and future) had been provided to workshop participants prior to the meeting to stimulate discussion.

The workshop participants reached into a consensus on the following control options to implement them step by step: 1) voluntary test and removal, and 2) compulsory test and removal. Both approaches may have their own advantages and disadvantages, nonetheless, in the absence of strictly enforced national and international livestock movement control, and lack of awareness on the risk of keeping of infected animals in the herd, neither method will provide for complete eradication [Good, 2006; Humblet et al., 2009; Palisson et al., 2016]. The presence of BTB in camels, in addition to cattle, within the extensive livestock system [Ghebremariam et al., 2018b] will

be an additional control challenge. These control options may be achievable if combined with effective awareness raising campaign for easily applied, compliance friendly, preventive measures. In both control options, positive reactors to SICTT should immediately be segregated from the other animals. In addition, inconclusive reactors need to be isolated from the other animals in the herd until their status has been clarified by a further test after 60 days. Goats can be potential sources of both *M. bovis* and *M. caprae* infection to cattle if they co-exist or share the same grazing areas [Bezos et al., 2012; Duarte et al., 2008; Crawshaw et al., 2008; Humblet et al., 2009; More et al., 2015]. Test and slaughter of reactor cattle in the dairy sector might not be successful as a control strategy without testing and culling of the co-existing, other susceptible animals, like goats [Humblet et al., 2009]. Therefore, skin testing of goats and other susceptible reservoir or spillover hosts co-existing with dairy cattle will be required for effective and sustainable control of BTB in the dairy sector in Eritrea. In developing countries, all species of animals co-existing with dairy herds are required to be monitored [Humblet et al., 2009]. The role of wildlife in maintaining *M. bovis* and the potential role of *M. bovis* in human tuberculosis in Eritrea is unknown. Therefore, control strategies should initially focus mainly on dairy cattle, with special attention to regional differences, followed by cattle, goats and camels in the extensive farming system, and studies should be initiated to elucidate the role of wildlife in maintaining *M. bovis* and the role of *M. bovis* in human tuberculosis. In the future, the SICTT needs to be complemented with the 'gamma interferon release assay' (IGRA) to increase the sensitivity of the cell mediated immunity (CMI) testing [De la Rua-Domenech et al., 2006] and positive reactors should immediately be removed from the herd. This might be challenging in remote areas where laboratory facilities are not available, thus requires strengthening of the regional veterinary laboratories in this regard. Even though, eradication may be an unlikely outcome at this stage, but this should not prevent the effort of achieving the public health benefit that will result from controlling the disease to reduce the level of risk from exposure.

'Test and voluntary removal'

This is much slower regarding the control and eradication of BTB when compared to the 'test and removal' method. Nonetheless, much easier to attain farmers' compliance. Those countries that have succeeded in controlling BTB have followed these steps, i.e., from test and voluntary removal to compulsory test and removal [Good, 2006]. This method will require rigorous awareness campaigns of the danger posed by bovine tuberculosis on animal health and production, and also the public health risk if test positive animals are kept in the farms. In the Eritrean perspective, this approach is most likely to succeed if implemented through dairy farmers' associations where peer pressure can be brought to bear on individual farmers who may otherwise resist compliance.

'Compulsory Test and slaughter'

This is a very well-known method through which successful control and eradication of bovine tuberculosis can be attained. The challenge of this method is that the farmers may not cooperate as they cannot see the direct impact of the disease due to its chronic and insidious nature. It is also the most expensive method since it requires payment of compensation for the slaughtered animals. Test and slaughter can only be successful provided that 100% compliance of dairy farmers is achieved which requires registration of all dairy farmers; identification of all dairy cattle; regular skin testing (twice a year/at least once a year), removal of all positive reactors from the herds, strict livestock movement control, and traceability of infected animals encountered at slaughterhouses. This applies true also for the livestock within the extensive livestock system. In the extensive livestock system control and eradication might be easier as the prevalence is low [Ghebremariam et al., 2018b] and only few animals are required to be slaughtered, though testing the entire livestock is difficult to achieve. But if back-tracing of the origin of the slaughtered animals is possible, targeted skin testing can be conducted and the positive reactors be removed.

In both approaches the farmers' associations will have a pivotal role in implementing the control and eradication program by making sure the proper disposal of the positive reactors within a given time frame, if they are

adequately sensitized about the program. The farmers' association should be informed of the test status of the animals of all member farmers and entrusted to oversee the proper disposal of reactor animals. The positive contribution of farmers' associations in controlling BTB was evidenced in Anseba region, where low prevalence was reported when compared to the other region of the country [Ghebremariam et al., 2016]. The dairy farmers' association in this region pioneered a 'mutual funding system' through which the association compensated for the slaughtered test positive reactors. This method is recommended to be replicated in the other regions of the country [Blanc & Denormandie, 2009]. Currently, Debub region has also initiated a similar program. Maekel region, where a high prevalence is recorded, needs to follow the good example of the former regions.

Only farms attested BTB free in three or more consecutive tests should achieve 'tuberculosis free' status with publicly awarded certificates, that should allow them to sell their animals to other dairy farms as replacement stocks, and should receive a premium payment for the milk they sell. Such benefits will encourage the farmers to strictly comply with the control and eradication scheme.

At any case to initially diminish the zoonotic risk, pasteurization of milk must be compulsory and the overall program must be organized at national level. In addition, to have a successful and sustainable control and eradication program for BTB in Eritrea, epidemiological zoning, livestock movement control, and breeding for resistance of BTB may play pivotal role.

Epidemiological zoning for BTB control and eradication

As it is very difficult to establish and maintain a BTB free status for the entire country, 'zoning' is recommended in view of defining BTB free sub-populations (herds) in the regions or sub-regions of the country for BTB control [OIE, 2007; Max et al., 2011]. This needs to be underpinned by regular testing of animals, on-going awareness raising programs to encourage reporting of all cases suggestive of tuberculosis, surveillance for the disease, and an official certification system for the livestock coming from BTB free herds or zone attesting that they are free from BTB [OIE, 2007]. For this purpose, animal

identification and traceability are very important requirements. Zoning may also be applied at farm level [OIE, 2007]. With the available information, we suggest dividing the regions into two epidemiological zones: 'control and eradication'. Maekel and Debub regions, with high and moderate BTB prevalence, respectively, will be within the control zone. Anseba and the rest of the regions, with low BTB prevalence, will be within the 'eradication zone'. The objective of the 'control zone' will be to reduce the BTB prevalence to less than 5%, and for the 'eradication zone' to eradicate BTB from all herds within the zone [Max et al., 2011].

Livestock movement control

Strict livestock movement control from the 'control zone' to the 'eradication zone', as well as from the dairy sector, high prevalence zone, [Ghebremariam et al., 2016] to the extensive livestock production system, low prevalence zone, [Ghebremariam et al., 2018b] must be enforced. The dairy sector mainly consists of exotic breeds, and the extensive livestock system of indigenous Zebu breeds. In the extensive livestock system, inter- and intra-herd contact at water points, pasture, en route during trekking and migration is common in Eritrea. Similar studies in Ethiopia have also shown comparable findings both in exotic dairy cattle kept within the intensive system and in local cattle kept within the traditional system [Tschopp et al., 2010; Ameni et al., 2006; 2007; Berg et al., 2011; Omer et al., 2001; Elias et al., 2008; Vordermeier et al., 2012; Ameni & Erkihun, 2007; Inangolet et al., 2008]. But the current situation may change due to increased demand for milk that necessitates importation of exotic breeds and intensification of the production system. Such activity will encourage the introduction of exotic breeds into the extensive livestock farming system with inevitable change in the management. Increased milk production is achieved through either purchase of exotic breeds or cross-breeding of the local ones with exotic breeds. The currently available exotic dairy cattle in the dairy sector are the candidates for cross-breeding. Such practice may have implications for reversing the current BTB status. Live animal movements are a major transmission route for the spread of infectious agents such as *M. bovis* [Pallison et al., 2016; Humblet et al., 2009]. Countries that were once free of BTB and that attained officially tuberculosis

free (OTF) status are experiencing resurgence of BTB [Palisson et al., 2016; Dommergues et al., 2010]. Spatial proximity to infected herds was found to be one of the major risk factors for BTB transmission [Palisson et al., 2016]. In France and Great Britain an increased risk of BTB infection was documented in herds located up to around 12 and 6 km distances from infected herds, respectively [Palisson et al., 2016; Green et al., 2008]. Therefore, strict livestock movement control and regular BTB surveillance should be in place with special attention to the hot spot areas to prevent further spread. Improving milk production of local indigenous breeds to limit importation of exotic breeds to the country (area) should be considered in the control program. Local breeds appear to be more resistant to BTB when compared to the exotic breeds [Humblet et al., 2009; Ameni et al., 2006; Vordermeier et al., 2012].

Eventhough eradication may be an unlikely outcome at this stage, this should not prevent the effort of achieving the public health benefit that will result from controlling the disease to reduce the level of risk from exposure.

This thesis provides baseline information on BTB prevalence, based on SITCC testing, in the dairy sector of Eritrea as well as in cattle, goats and camels within the extensive livestock system. It has indicated the presence of BTB throughout all study regions, though at a low prevalence, in cattle, camels and potentially in goats raised within the traditional extensive livestock system in Eritrea and regions. Major potential risk factors for the presence of BTB were identified and the actual presence of *M. bovis* was confirmed. This is an indicator for the need of immediate action to control BTB in dairy cattle and for more in-depth studies on BTB within the livestock kept in the extensive farming system.

Any future planning of importing high milk producing exotic breeds should make sure that the animals are pretested in situ and are free from the disease. Purchasing replacement dairy cattle from BTB free farms and testing for BTB before introduction of new cattle to farms will assist in controlling spread of BTB in the country [Humblet et al., 2009]. Improving local breeds for better milk yield may be a better option. Breeding programs could be considered. Furthermore, regional and local area variations in BTB prevalence, with some villages as hot spots, was revealed. Risk factors for such variations

should further be studied to develop locally adapted control strategies. Dairy farms that allowed their animals outdoors, kept smaller numbers of cattle (fewer cows and smaller total herd size) or that did not have concrete floors were less at risk to have SICTT reactor(s) when compared to their references [Ghebremariam et al., 2017]. Introduction of changes in management according to these findings may lead to an improved BTB status of the dairy farms. The use of sand floors or other alternative types of bedding (straw or saw dust) in dairy farms could improve the current prevailing problems related to floor type and thus reduce the number of farms with SICTT-reactive animals [De Vries et al., 2015].

Camels and goats were tested using the protocol developed for cattle to perform PPD skin testing which was not validated for these species. The validation of the SCITT in camels and goats should be under taken in the future studies in Eritrea.

BTB remains of public health importance in the intensive as well as in the extensive livestock husbandry systems in Eritrea and warrants the development and strict enforcement of control strategies. Particular attention should be given to TB treatment programs in areas where *M. bovis* is a potential etiologic agent in human pulmonary/extra pulmonary TB. Significance of this zoonotic disease in impairing animal welfare and the health of the community needs to be further studied in the future.

This thesis, has demonstrated the importance of classical and advanced molecular techniques in revealing the presence of *M. bovis* in tissues, and in characterization of the strains of *M. bovis* in Eritrea. It revealed (dis)similarities with the strains generally present in Africa and Europe, as well as potential routes of introduction of *M. bovis*. The whole genome sequencing (WGS) has allowed the detection of minute differences, local and distant, recent and historical dynamics, as well as, the strain diversity of *M. bovis* in Eritrea [Ghebremariam et al., 2018a]. Up until this state-of-the-art genotyping tool establishes itself in Africa, the classical methods will be as useful and we recommend their establishment in Eritrea. We also recommend future genetic profiling of *M. bovis* in Eritrea to include more samples with TBL from slaughterhouses of the country to have a better insight on the spatial and temporal distribution of *M. bovis* strains in Eritrea and their origins (sources).

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Chapter 7

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English Summary

The aim of this thesis was to explore the prevalence of *Mycobacterium bovis* (*M. bovis*) infection (bovine tuberculosis; BTB) and its potential risk factors for animals and humans in Eritrea. To that end, BTB prevalence in the intensive livestock production system (dairy cattle) in three major milk producing regions (Maekel, Debub & Anseba), and in the extensive livestock (mixed crop-livestock, and pastoral) system in cattle, goats, and camels in four regions (Debub, Anseba, Gash Barka, and Southern Red Sea) of Eritrea was investigated. The investigation in the intensive system was conducted by subjecting more than halve of Eritrea's dairy cattle population (around 15,000), to the single intradermal comparative tuberculin test (SICTT). Skin test results were interpreted according to guidelines of the World Organization for Animal Health (OIE) with > 4 mm as cut-off in skin thickness increase. In addition, we studied the relationship between 'physiological' variables related to pregnancy and lactation, and the variable 'region' on the probability to be skin test positive. The individual animal and herd BTB prevalence in the dairy sector were variable across the country with Maekel having the highest (21.5% & 40.9%), followed by Debub, 7.3% and 10%, and Anseba, 0.2% and 1.6%, respectively. The overall individual animal and herd prevalence within the dairy sector was 11% and 17.3%, respectively, and pregnant-lactating cattle were more at risk for being SICTT positive, as compared to the other categories. This study was followed by an investigation that attempted to associate the potential herd-level risk factors with the herd prevalence reported, by including case and control herds with and without reactor animal(s), respectively. A questionnaire provided relevant variables (risk factors). The result of this study showed that herds with higher numbers of cows, and those at farms with concrete floors, were more at risk for being SICTT positive, compared with their references in the dairy sector.

The BTB prevalence study in the extensive livestock production system examined cattle (n = 1077), goats (n = 876), and camels (n = 195) using SICTT, and studied its potential risk factors in animals (migration, multispecies mixing at water points and pasture, introduction of new animals into existing herds) and in humans (lack of awareness of the consequences of drinking raw

contaminated milk, close contact with their animals, sharing of the same dwelling with their animals), using questionnaire. The skin test results were interpreted using the standard (> 4 mm cut-off) and severe (> 2 mm cut-off) methods. The overall prevalence of BTB in the livestock within this system was low, but was spread throughout most of the sampled regions. In addition, consumption of unpasteurized milk was common.

To determine if *M. bovis* was circulating in the dairy cattle in Eritrea, and to gain insight into its spatial and temporal distribution, a molecular study was conducted on tissues with tuberculosis-like lesions (TBL) collected at the Asmara municipal slaughterhouse, the largest in Eritrea. The results revealed the most important strains of *M. bovis* in Eritrea and their (dis)similarities with the strains generally present in East Africa and Europe, as well as potential routes of introduction of *M. bovis* in the country.

To gain insight into the role of *M. bovis* in human tuberculosis in the country, sputum samples from multidrug resistant tuberculosis (MDR-TB) suspected patients were investigated using molecular detection techniques. This study was not able to identify *M. bovis* in the collected samples, but it did identify *M. tuberculosis* strains and four NTM species [*M. sherrisii, M. yongonense*, *M. intracellulare*, and *M. avium*].

In conclusion, the studies in this thesis highlighted high BTB prevalence in the intensive (dairy sector), and conversely, low prevalence in the extensive livestock systems in Eritrea. In addition, the studies have highlighted the presence of NTM in human patients in Eritrea and identified several risk factors for *M. bovis* infection in animals and humans.

The lesson learned are: strict livestock movement control must be in place and extra caution must be taken when moving animals from high BTB prevalence areas to the low prevalence areas; purchase of animals, especially for dairy purposes, should be tested for BTB; rigorous awareness raising campaign must be executed in regard to the danger of keeping of infected animals in the herds, and drinking of unpasteurized contaminated milk; the presence of NTM must be suspected in human patients with MDR-TB and the presence of *M. bovis* should be further investigated including the use of pyruvate enriched L-J medium to enhance the chance of *M. bovis* detection in humans.

The information obtained from this thesis will be instrumental in making informed decisions in the design of future BTB control and eradication strategies for Eritrea.

Nederlandse Samenvatting

Het onderzoek beschreven in dit proefschrift had tot doel de prevalentie van *Mycobacterium bovis* (*M. bovis*) infectie (bovine tuberculose; BTB) en de potentiële risicofactoren voor dieren en mensen in Eritrea in kaart te brengen. De prevalentie van BTB in runderen in intensieve melkveehouderij in de drie belangrijkste melk producerende regio's, Maekel, Debub en Anseba en daarnaast die in runderen, geiten en dromedarissen in extensieve veehouderij systemen (gemengde akkerbouw-veeteelt, zowel als pastorale) in de regio's Debub, Anseba, Gash Barka en Southern Red Sea van Eritrea, werden bepaald.

In het onderzoek in de intensieve melkveehouderij werd meer dan de helft van Eritrea 's melkvee (ca. 15.000 runderen) getuberculineerd. Resultaten van deze tuberculine huidtest werden geïnterpreteerd volgens de richtlijnen van de Wereldorganisatie voor diergezondheid (OIE), waarbij een huidzwelling van > 4 mm als grenswaarde voor een positief resultaat werd gehanteerd. Daarnaast werd de relatie tussen fysiologische variabelen zoals drachtstatus en melkgift en de variabele 'regio' met positieve huidtesten onderzocht. De individuele dier en kudde prevalenties van BTB in de melkvee sector varieerden binnen Eritrea. De regio Maekel had de hoogste prevalenties (21,5% resp. 40,9%), gevolgd door Debub, 7,3% resp. 10% en Anseba, 0,2% resp. 1,6%. De totale dier en kudde prevalenties in de melkvee sector waren 11% resp. 17,3%. De kans dat drachtige en melkgevende runderen positief waren in de tuberculine huidtest was groter dan die van dieren in de overige categorieën. Deze studie werd gevolgd door een case-control onderzoek gericht op de associatie tussen potentiële risicofactoren op kudde- en dierniveau met de gerapporteerde kudde prevalenties, in kuddes met respectievelijk zonder huidtest reactor dieren. In een vragenlijst werden de relevante variabelen (risocofactoren) aan de orde gesteld. Deze studie liet zien, dat voor melkvee kuddes met hogere aantallen runderen en die, gehuisvest op vloeren van cement, de kans hoger was positief te zijn in de tuberculine huidtest dan in overige kuddes.

De BTB prevalentie studie in de extensieve veehouderij systemen betrof runderen (n = 1077), geiten (n = 876) en dromedarissen (n = 195), die ook werden onderworpen aan de tuberculine huidtesten en schatte de sterkte van associatie van positieve testresultaten met potentiële BTB risico factoren voor dieren (migratie; multi-species interacties bij waterpunten en in het veld; introductie van dieren in bestaande kuddes) en voor mensen (gebrek aan kennis van de consequenties van het consumeren van rauwe geïnfecteerde melk; nauw contact met de dieren; gezamenlijke huisvesting) op basis van een vragenlijst. De huidtest resultaten werden geïnterpreteerd met standaard (> 4 mm) of aangepaste (> 2 mm) grenswaarden. De totale prevalentie in dieren in de extensieve veehouderij systemen was laag, maar gespreid over alle regio's. Consumptie van rauwe melk, een van de risicofactoren, was gemeengoed.

Ter bepaling van circulatie van *M. bovis* stammen in melk producerende runderen in Eritrea en om inzicht te krijgen in de verspreiding in ruimte en tijd, werden weefsels met tuberculose-achtige laesies, verzameld op het gemeentelijke slachthuis van Asmara (het grootste in Eritrea), onderzocht met behulp van moleculaire technieken. De belangrijkste *M. bovis* stammen in Eritrea werden geïdentificeerd en de mate van overeenkomst met de algemeen aanwezige stammen in Oost Afrika en Europa werd in kaart gebracht, en daarmee de potentiële introductie routes.

Om inzicht te krijgen in de mogelijke rol van *M. bovis* in humane tuberculose in Eritrea werden sputum monsters van, van multidrug resistentie verdachte, tuberculose (MDR-TB) patiënten verzameld en onderzocht met behulp van moleculaire technieken. In deze studie kon *M. bovis* niet worden geïdentificeerd, maar werden *M. tuberculosis* stammen en vier NTM species (*M. sherrisii, M. yongonense*, *M. intracellulare*, and *M. avium*) gevonden.

Concluderend, de studies beschreven in dit proefschrift lieten een hoge prevalentie van BTB in de intensieve melkveehouderij zien en, in tegenstelling daartoe, lage prevalentie in de extensieve veehouderij systemen in Eritrea. Bovendien werd de aanwezigheid van NTM in humane patiënten in Eritrea aangetoond en werden een aantal risicofactoren voor *M. bovis* infecties in dieren en mensen geïdentificeerd.

Geleerde lessen zijn: strikte vervoerscontrole van vee moet worden geïmplementeerd, met name bij beweging van dieren vanuit hoog BTB prevalente naar laag prevalente gebieden; dieren aangekocht met name voor melkproductie, moeten worden getest voor BTB; het gevaar van het houden van geïnfecteerde dieren in kuddes en het drinken van ongepasteuriseerde geïnfecteerde melk moet in bewustwordingscampagnes nadrukkelijk aan de orde worden gesteld; de aanwezigheid van NTM moet worden verwacht in humane patiënten met MDR-TB: de aanwezigheid van *M. bovis* moet verder worden onderzocht gebruikmakend van pyruvaat verrijkt L-J medium om de kans op aantonen in humane sputum monsters te vergroten.

De informatie verkregen in de studies beschreven in dit proefschrift zijn belangrijk bij het nemen van goed onderbouwde besluiten ten aanzien van toekomstige BTB controle en eradicatie strategieën voor Eritrea.

ጽሚቕ ጽሑፍ ብትግርኛ

ቀንዲ ዕላማ ናይዚ መጽናዕቲ ህላወ፣ ዝርጋሔን ጠንቂን ናይ ማይኮባክቴርዩም ቦቪስ (*M. bovis*) (ዓባይ ሳዓል ናይ ከብቲ) ኣብ እንስሳ ዘቤትን ሰብን ኣብ ኤርትራ ንምግምጋም እዮ። ማይኮባክቴርዩም ቦቪስ ዓሌት ባክቴርያ ኮይኑ ቀንዲ ጠንቂ ናይ ዓባይ ሳዓል ኣብ ከብቲ እዩ። ብተወሳኺ ንካልኦት እንስሳ ዘቤት (ኣጣል፣ ኣግማልን ኣባጊዕን) እንስሳ ዘገዳምን ከምኡ'ውን ንሰባት የጥቅዕ። እዚ ሕማም ኣብ ምሉእ ዓለም ተዘርጊሑ ዝርከብ ኮይኑ ብብዝሒ ኣብ ምምዕባል ዝርከባ ሃገራት ተስፋሕፊሑ ይርከብ። ቀንዲ ናብ ሰባት መተሓላለፊ መንገዲ ዘይመኽነ (ጻልጣ) ጸባ ብምስታይ እዩ። ኣብ ዝማዕበላ ሃገራት ሳላ ግዱድ ምምኽካን ጸባን ምእላይ (ምሕራድ) ቢቲ ሕማም ዝተጠቕዑ እንስሳታትን፣ ዓባይ ሳዓል ኣብትሕቲ ቁጽጽር ካብዝኣቱ ነዊሕ ጊዜ ኣቑጺሩ ይርከብ። ይኹን ደኣ'ምበር ብሰንኪ ህላወ ነዚ ባክቴርያ ክዕቅቡ ዝኽእሉ ኣንስሳ ዘገዳም ዓባይ ሰዓል ኣብ እንስሳ ዘቤት ዳግም ከቀላቕል ጀሚሩ ኣሎ። ዓባይ ሰዓል ኣብ ጥዕናውን ፋይናንሳውን መዳይ ናይ ሓንቲ ሃገር ዓቢ ዕንወት የስዕብ። ኣብ ዝተፈላለየ ዕድመ ዝርከቡ ጥሪት ኩሎም ንዓባይ ሰዓል ዝተቓልዑ እዮም። እዚ ሕማም ሕዱር ብምኽኑ በዚ ባክቴሪያ ዝተጠቕዑ እንስሳታት ንጹር ዝኾነ ምልክት ሕማም ኣየርእዩን እዮም። ምልክት ሕማም ከርኢ ዝጀመረ እንስሳ ግን ቀጻሊ ይስዕል፣ ኣካላቱ እናማህመነ ይኸይድ፣ ኣብ መወዳእታ'ውን ከመውት ይኽእል። ቀንዲ ኣታሓላለፍቲ ዓባይ ሳዓል ቢቲ ሕማም ዝተጠቕዑ እንስሳታት እዮም። እቲ ባክቴርያ ካብ ኣካላት ናይ'ቲ ሕሙም ብትንፋስ፣ ብንፋጥ፣ ብቐልቀል፣ ብሸንቲ፣ ብጸረርታ ርሕሚ፣ ብጸባ፣ ካብ ዝተቓልዑ ቁስሊ ጽኪታት ዝወጽእ ጸረርታ ኣቢሉ ንደገ ይወጽእ። እቶም ቀንዲ መተሓላለፍቲ መንገድታት ስርዓተ ምስትንፋስን ስርዓተ ምሕቓቕ መግብን እዮም። ብዓባይ ሳዓል ዝተለኽፈ እንስሳ ምሕካም (ምፍዋስ) ቁጠባዊ ረብሓ ስለዘይብሉን ኣዝዩ ነዊሕ ጊዜ ስለዝወስድን ዝተባባዕ ኣይኮነን። እቲ ዝሓሸ ኣገባብ ምክልኻል ዓባይ ሳዓል ብቐጻሊ መርመራ ምክያድን ኑቶም በዚ ሕማም ዝተጠቕዑ እንስሳታት ብኣግዑ ምእላይን እዩ።

ነቲ ኣብ መእተዊ ናይዚ ጽሑፍ ዝተጠቕሰ ቀንዲ ዕላማ ንምርግጋጽ ኣብተን ብዘመናዊ ኣገባብ ዝእለያ ናይ ጸባ ከብቲ፣ ብፍላይ ከኣ ኣብተን ሰለስተ ፍሉጣት ኣፍረይቲ ጸባ ዝኾና ዞባታት (ማእከል፣ደቡብ፣ዓንሰባ)፣ ከምኡ'ውን ኣብተን ብያታዊ ኣገባብ ዝእለያ ጥሪት (ኣጣል፣ ከብትን ኣግማልን) ኣብ ኣርባዕተ ዞባታት (ደቡብ፣ዓንሰባ፣ጋሽ ባርካ፣ ደቡባዊ ቀይሕ ባሕሪ) ኣብ ኤርትራ መጽናዕቲ ዓባይ ሳዓል ተኻይዱ። እቲ ኣብ ናይ ጸባ ከብቲ ዝተገብረ መጽናዕቲ ኣስታት ሓምሳ ሚእታዊት (ኣስታት 15,000) ኣብ ኤርትራ ዝርከባ ናይ ጸባ ከብቲ "ብሲንግል ኮምፓራቲቭ ቱበርኩሊን ቴስት (SICTT)" ተመርሚረን። ውጽኢት ናይ'ዚ መርመራ መለከዒ ዓለምለኻዊ ውድብ እንሰሳ (OIE) (>4 ሚሊ ሜትር፡ ሚሜ) ብዝእዝዞ ኣገባብ ተገምጊሙ። ብተወሳኺ ምልኣ፣ሕልቦ ከምኡ'ውን እቶን ጥሪት ዝርከባሉ ዞባ ኣብ ህላወን ዝርጋሐን ዓባይ ሳዓል ከህልዎም ዝኽእል ጽልዋ ተጸኒዑ። ኣብ ጸባ ከብቲ ህላወ ዓባይ ሳዓል ኣብ ውልቀ እንስሳን መጓሰን ኣብ ዝተፈላለዩ ዞባታት ውጽኢቱ ዝተፈላለየ ኔይሩ። ኣብ ዞማ ማእከል ዝላዓለ መጠን (ውልቀ እንስሳ=21.5%፡ መጓሰ=40.9%) ዝተለኽፉ እንስሳታት ከህልዉ እንከለዉ ብተዛማዲ ኣብ ዞባ ደቡብ፣ ማእከላይ (ውልቀ እንስሳ=7.3%፡መጓሰ=10%)፣ ከምኡ'ውን ኣብ ዞባ ዓንሰባ ዝዋሓደ ህላወ (ውልቀ እንስሳ=0.2%፡ መጓሰ=1.6%) ኔይሩዎም። ሓፈሻዊ ህላወ ዓባይ ሳዓል ኣብ ናይ ጸባ ከብቲ 11% ኣብ ውልቀ እንስሳ ከኽውን እንከሎ

ኣብ መጓሰ ድማ 17.3% ኔይሩ። መላኣት ኮይነን ዝሕለባ ዝነበራ ናይ ጸባ ከብቲ ብተዛማዲ ነዚ ሕማም'ዚ ናይ ምቅላዕ ዝላዓለ ተኽእሎ ኔይሩወን።

ህላወ ዓባይ ሳዓል ኣብ ብመጓሰ ዝመሓደራ ጥሪት (ኣጣል፣ከብትን ኣግማልን) እውን መጽናዕቲ ተኻይዱ። ኣብ'ዚ መጽናዕቲ'ዚ 1077 ከብቲ፣ 876 ኣጣልን 195 ኣግማልን ብሲንግል ኮምፓራቲቭ ቱበርኩሊን ቴስት (SICTT) ተመርሚረን። ብተወሳኺ ነዚ ሕማም ከስዕቡ ዝኽእሉ ጠንቅታት ንምልላዮም ብቃለ-መሕትት ዝተደገፈ መጽናዕቲ ተኻይዱ። ውጽኢት ናይዚ መጽናዕቲ ከም ዝሕብሮ ኣብ ምስጋም፣ ማይ መስተዪ ንቑጣታትን ቦታ መጋሃጫን ዝተፈላለዩ እንስሳታት ብብዝሒ ምእካብን ሓደሽቲ እንስሳታት ብዘይ መርመራ ምስ ነባራት ምሕዋስን ዝብሉ እዮም። ከምኡ'ውን ጠንቅታት ዓባይ ሳዓል ኣብ ሰባት ዘይመኽነ (ዘይፈልሐ) ጸባን ፣ጥቡቕን ቀጻሊን ምትንኽኻፍ ምስ ጥሪቶምን ከምኡ'ውን ምስእን ኣብ ትሕቲ ሓንቲ ጽላል (ገዛ) ብምንባሮምን እዩ። ውጽኢት መርመራ ኣብ መዓቀኒ ዓለምለኻዊ ውድብ ጥዕና (>4 ሚሜ) ከምኡ'ውን ኣብ ቀረባ እዋናት ዝተኣታተወ ጽንኩር ኣገባብ (severe method) መዓቀኒ (>2 ሚሜ) ዝተሞርኮሰ ኔይሩ። ሓፈሻዊ ህላዌ ዓባይ ሳዓል ኣብተን ብመጓሰ ዝዓሰያ ጥሪት በተዛማዲ ትሑት ክኸውን እንከሎ ዝርገሐኡ ግን ዳርጋ ኣብ ኩለን መጽናዕቲ ዝተገብረለን ቦታታት እዩ። በተወሳኺ ዘይመኽነ (ዘይፈልሐ) ጸባ ምስታይ ኣብ መብዛሕቲኤን መጽናዕቲ ዝተገብረለን ቦታታት ልሙድ ተርእዮ እዩ።

ብምቕጻል ህላወ ማይኮባክቴርዩም ቦቪስ ኣብ ናይ ጸባ ከብቲ ንምርግጋጽ ካብ ቤት ሕርዲ ስጋ ኣስመራ ምልክት ዓባይ ሳዓል መሰል ዘርኣዩ ውሽጣዊ ኣካላት ከም ሰናቡእ፣ ጸላም ከብዲን ጽኪታትን ተኣኪቦም ተመርሚሮም። እዞም ውሽጣዊ ኣካላት ብግቡእ መስርሕ ተዳልዮም ኣብ ምቹእ መራብሒ ተራቢሖምን መንነቶም'ውን ብፒሲአር (PCR) ተረጋጊጹ። ውጽኢት ናይዚ መጽናዕቲ መንነት ናይቶም ቀንዲ ኣብ ኤርትራ ዝርከቡ ዓሌታት ማይኮባክቴርዩም ቦቪስ ኣብ ናይ ጸባ ከብቲ ኣንፈት ሂቡ። ብተወሳኺ መቦቀል፣ ሓረጋዊ ቅርበትን ፍልልይን ናይዚ ማይኮባክቴርዩም ቦቪስ ምስቲ ኣብ ምዕራብ ኣፍሪቃን ኣውሮፓን ዝርከብ ተመሳሳሊ ማይኮባክቴርዩም ቦቪስ ከምኡ'ውን ተኽእሎ ኣመጻጽኣኡ ናብ ኤርትራ ተጸኒዑ።

ህላወ ማይኮባክቴርዩም ቦቪስ ኣብ ሰባት ኣብ ኤርትራ ኣፍልጦ ንኽህልወና ካብ ብዓባይ ሳዓል ዝተለኽፉን ከምኡ'ውን ናይ ጸረ ተህዋስያን ተጻዋርነት ዘማዕበሉን ሰባት ዓኽታ ተኣኪቡ ብፒሲአር ዝተደገፈ መጽናዕቲ ተኻይዱ። ኣብዝተኻየደ መጽናዕቲ ማይኮባክቴርዩም ቦቪስ ክረክብ ኣይተኻእለን። ይኹን እምበር ማይኮባክቴርይም ቱበርኩሎሲስ (ብፍላይ ንሰባት ዘጥቅዕ)፣ ከማኡ'ውን ኣርባዕተ ኣብ ኣከባቢና ዝርከቡ ዓሌት ተበለጽቲ ማይኮባክቴርያ [Environmental mycobacteria; ማይኮባክቴርዩም ሸሪሲ (*M. sherrisii)*፣ ማይኮባክቴርዩም ዮንጎንጎንሰ (*M. yongonense)*፣ ማይኮባክቴርዩም ኢንትራሴሉላረ (*M. intracellulare)*፣ ማይኮባክቴርዩም ኤቭዩም (*M. avium)]* ተለልዮም።

ኣብ መደምደምታ፡ እዚ መጽናዕቲ ኣብ ኤርትራ ኣብ ናይ ጸባ ከብቲ ዝለዓለ ዓቕን ህላወ ዓባይ ሳዓል (ማይኮባክቴርዩም ቦቪስ) ከምዘሎ ይሕብር። ብኣንጻሩ ኣብ ብመጓሰ ዝመሓደራ ጥሪት (ከብቲ፣ ኣጣልን ኣግማልን) ዝተሓተ መጠን ዓባይ ሳዓል ምህላዉ የረጋግጽ። ብተወሳኺ እዚ መጽናዕቲ ኣብ ኤርትራ ሰባት ብኣብ ኣካባቢና ዝርከቡ ተበለጽቲ ምይኮባክቴርያ (ኤንቫይሮመንታል ማይኮባክቴሪያ) ከምዝጥቅዑ ይሕብር።

ካብዚ መጽናዕቲ ዝተረኽቡ ኣስተምህሮታት እዞም ዝስዕቡ እዮም፡ ምንቅስቓስ እንስሳታት ካብ ልዑል ህላው ናይ ዓባይ ሳዓል ዝራኣየሉ ከባቢታት ናብ ትሑት ደረጃ ህላው ናይ ዓባይ ሳዓል ዝተመዝገበሎም ቦታታት ከማኣ ወይ ከኣ ብኣፍልጦ ከፍሊ ሕክምና እንስሳ ክኸውን ይግባእ፡ ናይ ጸባ ከብቲ ክዕደጋ/ክሸየጣ እንከለዋ ብቐዳምነት ኩነታት ጥዕናኡን (ዓባይ ሳዓል) ብመርመራ ክረጋገጽ ኣለዎ፡ ንሕማም ዓባይ ሳዓል ዝምልከት ከምኡ'ውን ብዓባይ ሳዓል ዝተለኽፉ እንስሳታት ኣብ ሕርሻ ምዕቃብ (ምሓዝ) ዘምጽኦ ሳዕቤን ኣመልኪቱ ኣስተምህሮ ብቐጻሊ ክወሃብ ኣለዎ፡ ምስታይ ዘይፈልሐ (ዘይመኽነ) ጸባ ኣብ ሰባት ዘምጽኦ ሳዕቤን፡ ከምኡ'ውን ህላው ንሰባት ከጥቅዑ ዝኽእሉ ኣብ ኣካባቢና ዝርከቡ ተበለጽቲ ማይኮባከቴርያ ኣብ መጻኢ ጽጹይ መጽናዕቲ ክግበረሎም ኣለዎ። ብፍላይ ብዓባይ ሳዓል ዝተጠቕዑ ሰባት ከምኡ'ውን ንጸረ ተህዋስያን ተጻዋርነት ኣሕዲሮም ተባሂሎም ዝተጠርጠሩ ሕሙማት ብተበለጽቲ ማይኮባከቴርያን ማይኮባከቴርዩም ቦቪስን ተለኺፎም ከይኮኑ ኣብ ግምት ምእታው የድሊ።

እዚ ካብዚ መጽናዕቲ ተረኺቡ ዘሎ ሓበሬታ ንመጻኢ ኣብ ኤርትራ ንዝካየድ ናይ ዓባይ ሳዓል ምቕጽጻርን ምጥፋእን ንጥፈታት ብኣፍልጦ ዝተደገፈ ስትራቴጂ ንምሕንጻጽ ዓቢ ኣበርክቶ ክህልዎ እዩ።

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Curriculum Vitae

Michael Kahsay Ghebremariam was born in Massawa (on the Red Sea coast), Eritrea, in 20 April 1969. In 1986 he joined Addis Ababa University, to study 'Animal Sciences and Technology' at the then Junior College of Agriculture in Awasa town. In the meantime, he developed a dominating passion for animal health and welfare, thus made a great effort to get the opportunity to study veterinary medicine. In 1991, he was awarded a scholarship to study veterinary medicine in the Faculty of Veterinary Medicine, National Agricultural University, Kiev, Ukraine, and graduated with a 'Doctor of Veterinary Medicine (DVM)' in July 1997. In 1998, he joined the College of Agriculture and Aquatic Sciences, Asmara University, Eritrea, as a lecturer until he was awarded a scholarship in 2003, to study 'Animal Pathology' in the Faculty of Veterinary Medicine, Department of Pathobiology, Pathology Division, Utrecht University, The Netherlands, at MSc level. In 2005, he successfully completed his MSc study under the supervision of Prof. Dr. Eric Gruys. During this MSc study he conducted research on Post Weaning Multisystemic Wasting Syndrome (PMWS) in pigs. Results of the MSc study were published in peer reviewed journals as well as in 'Annals of the World Association on Animal Pathology (AWAAP)'. In August 2005, Michael re-joined Asmara University and resumed teaching courses in veterinary sciences, that included Veterinary Pathology, Veterinary Public Health, and other related courses. In 2006, the College of Agriculture of Asmara University merged with a new agricultural college 'Hamelmalo Agricultural College' and as a result, Michael was transferred to this new college as an assistant professor and Head of the Department of Veterinary Sciences. In addition to teaching veterinary courses, Michael participated in curriculum development, teaching materials preparation, and consultancy. In 2012, Michael was awarded a PhD scholarship through the PhD-NFP, by NUFFIC. Since 2012, he did his PhD studies into '*The prevalence and risk Factors of Mycobacterium bovis infections in domestic animals and man in Eritrea*' under supervision of Prof. Dr. VPMG Rutten, Prof. Dr. M Nielen, and Prof. Dr. AL Michel at the division of Immunology, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. The studies conducted are described in this thesis

and are published in peer-reviewed scientific journals. During his PhD study period, Michael attended courses in immunology, infection biology, epidemiology, writing and presenting of scientific papers, poster presentation, grant proposal writing, data analysis, data management, among others. In addition, he attended international scientific conferences related to bovine tuberculosis, and presented his research. He also gave several presentations to veterinary professionals and college communities on bovine tuberculosis and control options. To translate the knowledge gained during the PhD study to action, he organized a consensus workshop on the challenges and opportunities of bovine tuberculosis control in Eritrea, where policy makers, veterinary professionals, and animal production experts participated.

Michael's research interest is mainly on the areas of veterinary public health (zoonoses), with emphasis on 'bovine tuberculosis, nontuberculous mycobacteria, brucellosis, and hydatidosis, at animal-human-environment interface.

List of Publications

In this thesis

Ghebremariam MK, Rutten VPMG, Vernooij JCM, Uqbazghi K, Tesfaalem T, Butsuamlak T, Idris AM, Nielen M, Michel AL. Prevalence and risk factors of bovine tuberculosis in dairy cattle in Eritrea. *BMC veterinary research*. 2016; 12(1): 80.

Ghebremariam MK, Michel AL, Nielen M, Vernooij JCM, Rutten VPMG. Farm-level risk factors associated with bovine tuberculosis in the dairy sector in Eritrea. *Transboundary and emerging diseases*. 2017; 65(1): 105-113.

Ghebremariam MK, Michel AL, Vernooij JCM, Nielen M, Rutten VPMG. Prevalence of bovine tuberculosis in cattle, goats, and camels of traditional livestock raising communities in Eritrea. *BMC veterinary research*. 2018; 14(1): 73.

Ghebremariam MK, Hlokwe T, Rutten VPMG, Allepuz A, Cadmus S, Muwonge A, Robbe-Austerman S, Michel AL. Genetic profiling of Mycobacterium bovis strains from slaughtered cattle in Eritrea. *PLoS neglected tropical diseases*. 2018, 12(4).

Additional publications

Samson Teweldebrhan, Yishak Ghebrekidan, **Michael Kahsay**. Prevalence of brucellosis in dairy farms in Berik sub-zone, Central Region. *EJSE*. 2017; 3(1): 61-88.

Ghebremariam MK, Gruys E. Postweaning multisystemic wasting syndrome (PMWS) in pigs with particular emphasis on the causative agent, the mode of transmission, the diagnostic tools and the control measures. A review. *Veterinary quarterly*. 2005; 27(3): 105-116.

Ghebremariam MK, Devarajan S, Ahmed B. Prevalence of Helminth Parasites in Indigenous Fowls of Zoba Anseba of Eritrea, North-East Africa. Veterinary World. 2011; 4(11): 492.

Ghebremariam MK, Debesai MG, Sanjay D, Basharat AP. Hydatidosis as a major cause of liver condemnation among parasitic diseases in goats and sheep in Keren slaughterhouse, Anseba zone, Eritrea. *Veterinary World*. 2014; 7(4).

Varghese R, Sjostrom PD, **Ghebremariam MK**. Surgical management of atresia ani et recti in a perosomus acaudatus Holstein calf. *Indian Journal of Veterinary Surgery (India)*. 2010; (31): 1.

Ghebremariam MK. Viral causes of respiratory diseases in piglets: emphasis on PCV2, PRSV and SIV. *Indian Journal of Veterinary Pathology*. 2010; 34(1): 29-31.

Ruiz León JR, **Kahsay M**, Milán López WA, Sánchez Bell Los W. Hallazgos Hematológicos en dromedarios (*Camelus dromedarius*) aparentemente sanos en la región de Keren en Eritrea - Hematological finding in dromedary (*Camelus dromedarius*) apparently healthy in the Keren region in Eritrea. *REDVET Rev Electrón vet*. http://www.veterinaria.org/ revistas/redvet. 2013; 14: 9. http://www.veterinaria.org/revistas/redvet/n090913.html. REDVET-Revista electrónica de Veterinaria ISSN 1695-7504.

Ghebremariam MK, Van Asten AJAM, Kroese MV, Toussaint H, Gruys E. Co-existence of PCV2 and PRRSV infection in postweaning multisystemic wasting syndrome (PMWS): by chance or by choice? A study in suspected cases by in situ hybridization (ISH). *Journal of the World Association on Animal Pathology (WAAP)*. 2006; 4: 4-14.

Kothalawala H, **Ghebremarium M**, Toussaint MJM, Van Asten AJAM, Van Ederen AM, Gruys E. Detection of porcine reproductive and respiratory syndrome virus (PRRSV) genotypes in tissue sections of piglets with respiratory lesions using cDNA oligonucleotide probes encoding the conserved region of ORF 5 genes of Lelystad and VR-2323 viruses. *Annals of the World Association on Animal Pathology (AWAAP)*. 2007; Volume 5.

Contribution to scientific conference

Poster presentation

Michael K, Ghebremariam, Rutten VPMG, Vernooij JCM, Ogbazghi K, Tesfaalem T, Butsuamlak T, Idris A, Nielen M, Michel AL. International *M. bovis* conference, Cardiff, Wales, UK, 2014.