Prevalence and characterization of brucellosis and tuberculosis in cattle and its zoonotic risk associated factors in Rwanda

By

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DECLARATION

I declare that this thesis which I hereby submit for the fulfillment of the requirements of Doctor of Philosophy in Veterinary Science at the University of Pretoria is my original work and has not been previously submitted by me for a degree at any other higher learning institution.

Signed:

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This study derives from project V004-18 approved by the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria, South Africa, the research screening and ethical clearance committee of the College of Agriculture, Animal Sciences and Veterinary Medicine, University of Rwanda (Ref:026/DRIPGS/2017), and the Institutional review board of the College of Medicine and Health Sciences, University of Rwanda (N° 006/CMHS IRB/2017).

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LIST OF ABBREVIATIONS

BB: Bovine brucellosis

bTB: bovine tuberculosis

WHO: World Health Organization

EC: European commission

OIE: International Organization for Animal Health

IFAD: International Fund for Agricultural Development

LPS: lipopolysaccharide

Omp25: the outer membrane protein

T4SS: type 4 secretion system

BCV: *Brucella*-containing vacuole

MALT: mucosa-associated lymphatic tissue

EAC: East African community

SSA: Sub-Saharan Africa

RBT: Rose Bengal Test,

RSAT: rapid slide agglutination test

TAT: tube agglutination test

BPAT: buffered antigen plate agglutination test

FPA: fluorescence immunosorbent assays

CFT: complement fixation test

i-ELISA: indirect enzyme-linked immunosorbent assay

CITA: Centro de Investigación y Tecnología Agroalimentaria

VNTR: Multi locus variable number of tandem repeats

NGS: next generation sequencing

MRT: milk ring test

MTBC: *Mycobacterium tuberculosis* complex

NTM: non-tuberculous mycobacteria

MOTT: mycobacteria other than tuberculosis

RD: regions of differences

CR1 : complement receptors

BCG : Bacillus Calmette - Guerin

IFN-γ: gamma interferon test

PPD: purified protein derivative

CFT: caudal fold test

CCT: comparative cervical test

MIRU-VNTR: mycobacterial interspersed repetitive units - variable number tandem repeat typing

DR: direct repeat

MLVA: multilocus variable number of tandem repeats analysis

SNPs: single nucleotide polymorphisms

WGS: whole genome sequencing

NGOs: non-government organizations

SABAN : société des abattoirs de Nyabugogo

AIC: Alkaike Information Criteria

GLM: Generalized Linear Model

NRL: National Reference Laboratory

PPE: personal protective equipment

GPS: geographic positioning system

RAB: Rwanda Agriculture and Animal Resources Board

LJ: Lowenstein-Jensen

AFB: acid-fast bacilli

CI: confidence interval

REP: repetitive extragenic palindromic

BoHV-4: *Bovine herpesvirus 4*

Cff: *Campylobacter fetus* subsp. *fetus*

Cfv: *Campylobacter fetus* subsp. *venerealis*

DEDICATION

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THESIS SUMMARY

Prevalence and characterization of brucellosis and tuberculosis in cattle and its zoonotic risk associated factors in Rwanda

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Bovine brucellosis (BB) and bovine tuberculosis (bTB) are endemic in Rwanda; however, little is known about the diseases. Before this study, there were only three serological studies on BB, two serological studies on human brucellosis, and two studies on bTB. The aims of this study were to determine with the following objectives: to determine the prevalence and characterize Brucella in cattle from six districts which included the wildlife-livestock-human interface (5 districts) and peri-urban area together with the zoonotic associated risk factors; to characterize *Brucella* spp., and other abortigenic pathogens in aborted tissues from cattle from selected districts in Rwanda; to assess the awareness and occupational exposure to brucellosis, bTB, and other zoonotic diseases among abattoir workers at the six slaughterhouses in Rwanda and; to characterize BB and bTB from tissue samples collected from slaughtered animals in Rwanda using culturing and molecular characterization.

The BB prevalence was determined using Rose Bengal test (RBT) and indirect enzyme-linked immunosorbent assay (i-ELISA) in series and the animal-level seroprevalence was 7.4% (141/1907) in cattle from the six districts, 8.3% (141/1691) in cattle farmed at the wildlife-

livestock-human interface (5 districts), and 0.0% (0/216) in the peri-urban areas (1 district). The herd-level seroprevalence of BB was 28.9% (61/212) in herds from the six districts and 30.9% (61/198) in herds from the wildlife-livestock-human interface (5 districts). Multivariate analysis showed that old age (≥5 years), cattle from districts bordering national parks, history of abortions, and replacement animals were significantly associated with brucellosis ($p < 0.05$). Low awareness of zoonotic brucellosis transmission, assisting calving without biosafety protection, drinking raw milk, and manual milking were each observed in more than 21.7% of cattle keepers whose herds were seropositive. Whole blood (n=118), milk (41), and vaginal swabs (n=51) samples from brucellosis seropositive (n=183) and seronegative (n=27) cattle were cultured and *Brucella* cultures were identified using the 16S-23S ribosomal interspacer region (ITS) PCR assay. The culture prevalence determined by the gold standard (cultures and ITS-PCR) was 16.7% (35/210) and AMOS-PCR assay identified mixed *B. melitensis* and *B. abortus* (n=12) isolates, *B. melitensis* (n=3), *B. abortus* (n=19) while Bruce-ladder PCR assay identified *B. abortus* RB51 vaccine strain (n=2) amongst the *B. abortus* and *B. melitensis* cultures.

Aborting livestock samples (19 aborted tissues from isolated cases for cattle and 1 aborted tissue and 3 vaginal swabs from an abortion outbreak for goats) were investigated for brucellosis using culture and PCR assays. Two aborting cattle (2/19) were infected by *B. melitensis* (n=1), and *B. abortus* (n=1) while mixed *B. abortus* and *B. melitensis* were isolated from goats. The *Brucella* negative samples from cattle (n=17) where further characterize using a PCR abortion panel (*Anaplasma phagocytophilum*, *Bovine Herpes Virus Type 4*, *Campylobacter fetus*, *Chlamydophila* spp., *Coxiella burnetti*, *Leptospira* spp., *Listeria monocytogenes*, and *Salmonella* spp.). *Campylobacter fetus* (n=7), and *Leptospira* spp. (n=4) were identified including co-infections (n=2) of *C. fetus* and *Leptospira* spp.

BB seroprevalence using serological tests (RBT and i-ELISA) in parallel was 2.9% (8/300) from slaughtered cattle at six abattoirs in Rwanda. The culture prevalence determined by the gold standard method (culture confirmed by *Brucella* specific ITS-PCR) was 5.6% (11/300). AMOS-PCR assay identified mixed *B. abortus* and *B. melitensis* (n=3), *B. abortus* (n=3), and *B. melitensis* (n=5) isolated from lymph nodes while Bruce-ladder PCR assay identified *B. abortus*

and *B. melitensis*. The prevalence of bTB was 1.7% (5/300) and bTB was caused by *M. bovis* (n=4) and *M. tuberculosis* (n=1). Rifampicin-resistant (RR) *M. tuberculosis* (n=1) was recorded. The prevalence of non-tuberculous mycobacteria (NTM) was 12.0% (36/300).

The awareness of abattoir workers at these abattoirs was 82.2% (97/118) for zoonotic tuberculosis transmission, and 27.1% (32/118) for zoonotic brucellosis transmission, 8.5% (10/118) for Q-fever, 10.2% (12/118) for leptospirosis and 12.7% (15/118) for cysticercosis. Abattoir workers encountered diarrhea (5.9%), abortion (0.9%), orchitis (0.9%), fever (5.1%), fatigue (11.0%), flu (9.3%), headache (5.1%), and nephritis-related diseases (3.4%).

Results from this thesis surprisingly indicate the seroprevalence of BB at the interface and slaughtered cattle (8.3% and 2.9%) was less than the culture prevalence of 16.7% and 5.6%, respectively using the gold standard (culture and ITS PCR). The prevalence determined by the less sensitive culture method than the seroprevalence indicate that the i-ELISA needs to be validated in Rwanda. BB is endemic in Rwanda with higher rates at the wildlife-livestock-human interface and this highlights that control efforts should focus on the interface. Single and mixed infections by *B. abortus* and *B. melitensis* identified in cattle farmed at the interface, aborting cattle and goats, and slaughtered cattle pose a serious problem to public health. Thus, there is a need for strong brucellosis control involving systematic and coordinated vaccination combined with test-and-slaughter; and raising awareness among occupational groups would be of paramount importance. This first identification of abortigenic and zoonotic pathogens (*B. abortus*, *B. melitensis*, *C. fetus,* and *Leptospira* spp.) in aborting cattle in Rwanda indicates the enormous financial losses to cattle owners and a threat to public health. It is therefore essential to raise the awareness of caretakers, abattoir workers, and laboratory personnel. These identified pathogens should be included in the surveillance scheme of veterinary and human diseases. The zooanthroponotic transmission of RR *M. tuberculosis* indicates the risk of exposure of occupational groups. Therefore, we recommend the improvement in biosafety protection at the farm level and in the abattoirs. Educated farmers and educated abattoir workers had a high awareness of BB and zoonotic brucellosis, respectively, and this indicates the importance of education.

Chapter 1. Literature review

Bovine brucellosis (BB) and bovine tuberculosis (bTB) are contagious bacterial diseases of cattle and other domestic animals, wildlife, and humans (Corbel, 2006, Michel et al., 2010). BB and bTB are primarily caused by *Brucella abortus* and *Mycobacterium bovis,* respectively. Both diseases are of economic and public health importance worldwide but endemic in developing countries, including Rwanda, where little information is available on prevalence, bacterial species, and the risk factors driving the occurrence and persistence of these diseases. It was therefore essential to investigate the epidemiology of BB and bTB at the wildlife-livestock-human interface, and abattoirs to generate the baseline useful for the control of these two zoonotic diseases in Rwanda.

1.1. Introduction and rationale

BB and bTB are serious diseases of domestic animals, wildlife, and humans worldwide (Corbel, 2006, Michel et al., 2010). According to the World Health Organization (WHO), 832,633 humans acquire brucellosis annually with 4,145 deaths worldwide (Kirk et al., 2015) while 147,000 new cases of zoonotic tuberculosis with 12,500 deaths occurred in 2016 with the highest burden in developing countries (Cousins, 2018). Bovine brucellosis is usually a disease of which clinical form results in abortion, or placenta retention, and infertility (Corbel, 2006). The bTB is a debilitating chronic disease of cattle with a prolonged course of infection, however, in the late stage of infection or the case of stress, granulomatous tubercles develop in the lymph nodes of the head, thorax, and lungs (OIE, 2019). Brucellosis and bTB are notifiable diseases to the world organization for animal health, and both diseases cause economic losses related to trading restrictions, eradication costs, condemnations of carcasses, and compensations (Bamaiyi et al., 2012, Tschopp et al., 2013). The economic losses that were associated with brucellosis i.e., in Malaysia were USD 62,926,060 (Bamaiyi et al., 2012). The cost of national bTB eradication was estimated at USD 342 million between 2001 and 2009 in the USA (USDA, 2009), and almost £100 million in England (EC, 2013). The cost of bTB varied from USD 75.2 million in 2005 to USD 358 million in 2011 in Ethiopia (Tschopp et al., 2013). Brucellosis and bTB are worldwide distributed with high prevalence in low-income countries including Rwanda (De Garine-Wichatitsky et al.,

2013, McDermott et al., 2013). In Rwanda, 1683.5 kg of meat were condemned due to bTB-like lesions for eleven months (January – November 2009) of inspection and were estimated at USD 4,810 (Habarugira et al., 2014).

In Rwanda, the cattle population was estimated at 1,293,768 heads (Minagri, 2019) and cattle farming is one of the pillars for poverty alleviation with the dairy sector accounting for 10.5% of agricultural gross domestic product (IFAD, 2016). Bovine are the only species vaccinated against brucellosis using a live attenuated *B. abortus* RB 51 vaccine. Vaccination is voluntary and performed on demand by farmers who accept to pay approximately USD 0.6 per dose, thus, there is a need for systematic coordination. Brucellosis and bTB are prevalent in Rwanda but the associated risk factors remain poorly understood. However, the lack of valid and sufficient epidemiological data may account for this prevalence. Few studies have reported the individual seroprevalence of BB ranging from 2.3% to 34.9% (Akakpo and Bornarel, 1987, Chatikobo et al., 2008, Manishimwe et al., 2015, Ndazigaruye et al., 2018, Ntivuguruzwa et al., 2020). Furthermore, the seroprevalence rates of brucellosis in women with a history of abortions was 25.0% in Huye district (Rujeni and Mbanzamihigo, 2014) and 6.1% in Nyagatare district of the Eastern Province of Rwanda (Gafirita et al., 2017). The prevalence of bTB was 0.5% at abattoirs (Habarugira et al., 2014) and *M. africanum* was isolated from cattle in 1978 (David et al., 1978). About 40.0% of the national cattle population are found in the Eastern Province which harbors Akagera National Park and borders Tanzania in the east, Uganda in the north, and Burundi in the south. Cattle in the Eastern Province are on extensive farming and some herds share watering points. Similarly, cattle are farmed in Gishwati-Mukura National Park in the Western Province, but the park does not harbor hooved wildlife. In contrast, in the Northern Province, Virunga National Park is home of buffaloes, however, many cattle herds around the park, together with the remaining parts of the country, are mostly kept in a zero-grazing system. Some parts of the borders are porous and unauthorized movements of animals across borders and existence of wildlife may be potential risk factors for the transmission of brucellosis and bTB at the wildlife-livestock interface in Rwanda. In addition, there has been cohabitation between wildlife, livestock, and humans for many decades until the fencing of national parks in

2016. It is therefore essential to sample cattle at the wildlife-livestock-human interface and abattoirs to have the national baseline data of both diseases since abattoirs slaughter animals that come from different areas.

1.2. Objectives

This study aimed at investigating the seroprevalence and characterization of brucellosis and tuberculosis in cattle and its zoonotic risk associated factors in Rwanda. The objectives included:

1) To determine the seroprevalence, and associated risk factors of bovine brucellosis at the wildlife-livestock-human interface.
2) To characterize *Brucella* spp. from seropositive herds of cattle farmed at the wildlife-livestock-human interface.
3) To characterize *Brucella* spp., and other abortigenic pathogens in aborted tissues of cattle from selected districts.
4) To assess the awareness and occupational exposure to brucellosis, bovine tuberculosis, and other zoonotic diseases among abattoir workers in Rwanda.
5) To determine the seroprevalence of brucellosis and characterize *Brucella* spp. from slaughtered cattle in Rwanda.
6) To determine the prevalence of bovine tuberculosis and characterize *Mycobacterium* spp. in slaughtered cattle in Rwanda.

1.3. Brucellosis

1.3.1. The pathogen and affected species

Brucellosis is an infectious disease caused by bacteria of the genus *Brucella* spp. (Meyer and Shaw, 1920) that belongs to the group of Alphaproteobacteria (Moreno et al., 1990). *Brucella* spp. are gram-negative, coccobacilli, and facultative intracellular microorganisms (Moreno et al., 1990). The genus *Brucella* affects domestic, wildlife, and marine animals, as well as humans

(Corbel, 2006, Foster et al., 1996). The disease was first identified from a British soldier in 1887 in the Malta island and named "Malta fever" of which the causative agent was identified and named "*Micrococcus melitensis*" by David Bruce, a young British doctor and soldier (Bruce, 1887). The genus *Micrococcus* was later changed to *Brucella* by Louis Mayer and Wilbur Shaw in 1920 to acknowledge David Bruce (Meyer and Shaw, 1920). *Brucella melitensis* was transmitted to solders by drinking the milk of infected goats (Zammit, 1905) thus, pasteurization was later introduced as a preventive measure (Evans, 1918). With time, other classical *Brucella* species and biovars were identified and classified based on phenetic characteristics and their host preferences (Meyer and Shaw, 1920). The six classical species include *B. melitensis* for goats (Zammit, 1905), *B. abortus* for cattle (Bang, 1897), *B. ovis* for sheep (Buddle, 1956), *B. suis* for swine (Traum, 1914), *B. canis* for dogs (Kimberling et al., 1966), and *B. neotomae* for wood rats (Stoenner and Lackman, 1957). Three of these classical *Brucella* species are subdivided into biovars (bv.) namely, *B. abortus* bv. 1, 2, 3, 4, 5, 6, and 9, *B. melitensis* bv. 1, 2, and 3, and *B. suis* bv. 1, 2, 3, 4 and 5 (Alton et al., 1988, Jones, 1967). These classical species were found to be 96% genetically homologous and a monospecies nomenclature was proposed (Verger et al., 1985), but this was rejected by the international committee for prokaryotes nomenclature (Banai and Corbel, 2010). The classical species were further differentiated based on the polymorphisms found in the insertion sequence (IS) 711 (Halling et al., 1993) and other genetic markers (Cloeckaert et al., 1995, Ficht et al., 1990). Other *Brucella* spp. later isolated from marine animals are *B. pinnipedialis* for pinnipeds, *B. ceti* for crustaceans (Foster et al., 1996, Foster et al., 2007), *B. microti* for voles (Scholz et al., 2008), *B. innopinata* (Scholz et al., 2010), and *B. papionis* for baboons (Whatmore et al., 2014). The genus *Brucella* lacks most of bacterial virulence factors like exotoxins, endotoxic lipopolysaccharide, cytolysins, inducers of host cell apoptosis, capsule, fimbriae, flagella, plasmids, and lysogenic phages (Moreno, 1998), and therefore, the way *Brucella* spp. adapt within the host environment exclusively depends on mutation and internal genetic rearrangements (Moreno, 1998). *Brucella* spp. possess molecular markers essential for triggering the host immune system and cause disease in a wide range of domestic and wildlife animals, and humans (Moreno et al., 2002).

1.3.2. Virulence and pathogenesis

The ability of *Brucella* to penetrate, invade, survive, replicate, and infect the host cells depends on the virulence factors such as urease, bile salt hydrolase, two-component regulator system, cyclic β 1, 2-glucans, lipopolysaccharide (LPS; rough for *B. canis* and *B. ovis*, and smooth for other species), the outer membrane protein (Omp25), and the type 4 secretion system (T4SS) (Starr et al., 2008, Xavier et al., 2010). *Brucella* spp. penetrate the mucosal barriers of digestive, respiratory, and genitourinary tracts to reach the phagocytic cells (macrophages, dendritic cells), and non-phagocytic cells (epithelial cells and trophoblasts) (Anderson et al., 1986).

In the intestinal lumen, Brucellae produce bile salt hydrolase and urease to neutralize bile salts and gastric acid, respectively; then these Brucellae are actively transported by epithelial cells through M cells to the phagocytic cells which in turn transport the phagocytized Brucellae to the adjacent lymph nodes and then to systemic sites (Starr et al., 2008). The two-component regulatory system (BvrR/BvrS) and the outer membrane gene, Omp25, are responsible for adhesion, and invasion of macrophages (Edmonds et al., 2001, Lopez-Goni et al., 2002) by interacting with the superficial receptors leading to the internalization via complement receptors, and fibronectin receptors (Campbell et al., 1994). Most of Brucellae that enter via complement receptors are killed by oxygen radicals, nitric oxide, and enzymes but a few survive together with those entering by fibronectin receptors. The lipopolysaccharide and *Brucella* type 4 secretion system (BT4SS) are responsible for the survival within macrophages (O'Callaghan et al., 1999) while the cyclic β 1, 2-glucans protects the *Brucella*-containing vacuole (BCV) from digestion by lysosomes (Arellano-Reynoso et al., 2005). The BT4SS secretes effector proteins that are responsible for the maturation of BCV and its transport to rough endothelial reticulum for replication (Boschiroli et al., 2002) (Figure 1.1).

Trophoblasts are also target cells in which *Brucella* replicates rapidly and extensively due to high concentrations of steroid hormones (prostaglandin 2α, estrogen, and cortisol) which are increased during the third term of gestation (Samartino et al., 1994). At the early infection of trophoblasts, *B. abortus* inhibits the transcription of pro-inflammatory mediators (Carvalho Neta

et al., 2008) leading to an increased number of *Brucella* infecting the fetus followed by abortion (Xavier et al., 2010).

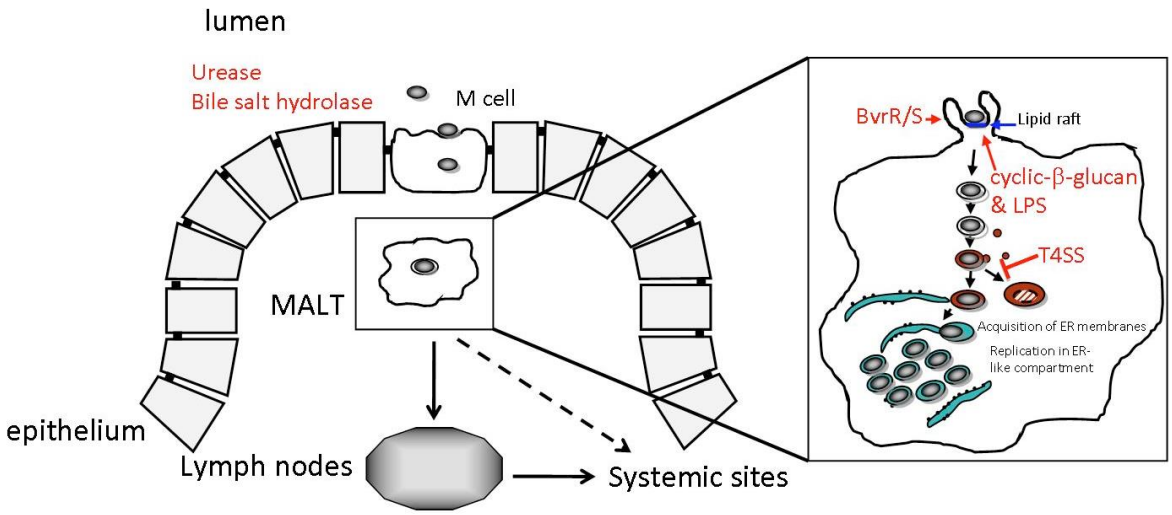

Figure 1.1. The pathogenesis of *Brucella* species via the digestive route (Starr et al., 2008). In the lumen, *Brucella* spp. survive the killing by hydrochloric acid and bile salts and adhere to the master cells (M cell) within the epithelium layer. The two-component regulatory system (BvrR/S) facilitates their internalization into the mucosa-associated lymphatic tissue (MALT) in which they use lipopolysaccharide (LPS) and *Brucella* type 4 secretion system (T4SS) to survive and multiplicate then disseminate within the entire body through lymphatic and blood circulations.

1.3.3. Epidemiology of brucellosis in livestock

Brucellosis is an infectious disease of livestock causing enormous economic losses in cattle, goats, sheep, and swine (McDermott et al., 2013).

1.3.3.1. Bovine brucellosis (BB)

1.3.3.1.1. The causative agents

Brucellosis in cattle is caused primarily by *B. abortus* (Corbel, 1997), occasionally by *B. melitensis* especially when cattle are mixed with small ruminants (Verger et al., 1985), and rarely by *B. suis* bv. 1 (Madsen, 1989).

1.3.3.1.2. Occurrence and temporospatial distribution

Brucellosis emerged at least 750 years BC (Roushan et al., 2014), but was first discovered in 1887 in Malta island, and later identified in all continents except in Antarctica (Bamaiyi, 2016). BB caused by *B. abortus* is endemic in central and south American countries and the prevalence varied between 4.0% and 8.0% with annual losses of USD 25 million (Moreno, 2002, Moreno, 2014, Pappas et al., 2006). BB caused by *B. abortus* and *B. melitensis* was hyperendemic in central and mediterranean European countries (Moreno, 2014, Pappas et al., 2006, Taleski et al., 2002). BB was also reported in Ireland (Abernethy et al., 2006, Reid, 2005). Brucellosis is hyperendemic in the Arabian Peninsula, (Moreno, 2014, Pappas et al., 2006), and India with 12.0% prevalence (Deka et al., 2018). The seroprevalence of brucellosis was 15.0% in Saudi Arabia, and *B. melitensis* accounted for 88.0 - 93.0% of cases (Memish, 2001). Brucellosis is hyperendemic in northern African countries namely Algeria, Tunisia, and Marocco (Moreno, 2014, Pappas et al., 2006), and endemic in sub-Saharan Africa with varying herd seroprevalences of 62.0% in Togo, 32.5% in Mali, 12.6% in Cameroon, 3.0% in Burkina Faso, 1.3% in Senegal, and 1.2% in Niger (Musallam et al., 2019). *Brucella abortus* bv. 3 strains that were significantly different from the European bv. 3 strains were commonly isolated in cattle in Senegal, Togo, Rwanda, Guinea Bissau, and Niger (Akakpo, 1987, Verger and Grayon, 1984). *Brucella melitensis* was isolated from cattle in African countries such as Kenya (Muendo et al., 2012), Uganda (Mugizi et al., 2015), and South Africa (Kolo et al., 2019). Furthermore, *B. suis* bv. 1 was isolated from cattle in Zimbabwe (Madsen, 1989).

Brucellosis is also endemic in countries of the East African community (EAC) including South Soudan; Kenya, Uganda, Tanzania, Burundi, and Rwanda; and this region has a high degree of under-reporting of cases and insufficient epidemiological data (McDermott et al., 2013). The herd seroprevalence of brucellosis in intensive dairy productions can reach up to

80.0% in eastern Soudan (Seifert, 1996), 4.7% in Uganda (Nizeyimana et al., 2013), 20.0% in Tanzania (Swai and Schoonman, 2010), 14.7% in Burundi (Musallam et al., 2019), and 30.0% in Rwanda (Chatikobo et al., 2008, Ntivuguruzwa et al., 2020). Except for *B. abortus* bv. 3 that has been isolated in Rwandan cattle in the 1980s (Verger and Grayon, 1984), there is no other study that identified *Brucella* species in Rwanda. Therefore, the identification of *Brucella* species is crucial for the control of the disease.

1.3.3.1.3. Transmission and predisposing factors

Healthy cattle acquire brucellosis by ingestion, inhalation, coitus, and cutaneous abrasion in contact with infected materials (Corbel, 2006, Kaufmann et al., 1980). Other modes of transmission are artificial insemination, and conjunctival routes (Alton, 1990). The sources of infection are the secretions and excretions from infected cattle from mucous membranes, aborted tissues, fluids, urine, milk, nasal discharges, and aerosols. These materials can contaminate the pasture and water and then become sources of infection for healthy animals (Bicknell and Bell, 1979, Corbel, 2006, Rhyan et al., 2013). *Brucella abortus* can persist for 81 days in foetal tissues, soil, and vegetation depending on exposure to sunlight (Aune et al., 2012). The sunny, dry, and hot weather reduces the viability of the pathogen (Esuruoso, 1974). Furthermore, the hot climate destroys *Brucella* while the infection increases in humid climate, and this was reported in Niger, Burkina Faso, Ivory Coast, Cameroon, and Mozambique (Akakpo, 1987).

The seroprevalence of brucellosis increases significantly in older dairy cattle (Ndazigaruye et al., 2018, Radostits et al., 2000). Animals that are kept for a longer period in the herds have more chance of exposure to *Brucella* spp. therefore brucellosis seropositivity increases with advanced age. It has also been reported that *Brucella* spp. have a tropism for reproductive organs of mature female animals, and the sex hormones and erythritol produced are responsible for survival and multiplication of *Brucella* spp. (Radostits et al., 2000). The BB seroprevalence also increases within herds of large size (Sagamiko et al., 2018), or the proximity to wildlife (Ndengu et al., 2017, Nthiwa et al., 2019, Ntivuguruzwa et al., 2020). Higher seroprevalences for brucellosis were recorded in Ankole breed compared to exotic and cross

breeds (Chatikobo et al., 2008, Kabi et al., 2015, Sagamiko et al., 2018). Indigenous cattle are more likely kept under free grazing, unlike the cross-bred and exotic animals that are mostly kept under zero grazing to intensify milk production, and therefore with lower likelihood of exposure to *Brucella* spp. Other predisposing factors include limited access to veterinary services, the introduction of newly infected animals into herds (Bugeza et al., 2019), and the low awareness and knowledge of brucellosis by farmers (Chaka et al., 2018). Moreover, extensive movement of cattle, nomadism, transhumance, and herds sharing pastures and watering points promote infection (Ducrotoy et al., 2017, McDermott and Arimi, 2002). However, only few developing countries report the brucellosis status to the OIE, and this may be due to the lack of accurate epidemiological data and confirmatory methods (Akakpo, Teko-Agbo & Kone 2010).

1.3.3.2. Caprine and ovine brucellosis

Caprine and ovine brucellosis caused by *B. melitensis* are geographically limited to the Mediterranean region, parts of Africa, Asia, and the Americas (Benkirane, 2006, Corbel, 2006, Lounes et al., 2014, Moreno, 2002). However, ovine brucellosis is also caused by *B. ovis* (OIE, 2018). The disease is endemic in south Asia, sporadic in south-east Asia, and hyperendemic in Mongolia (Benkirane, 2006). Caprine and ovine brucellosis are prevalent in Latin America (Benkirane, 2006). Ovine and caprine brucellosis due to *B. melitensis* is a problem of concern in southeastern Europe and Eurasia (Benkirane, 2006, Taleski et al., 2002). Caprine brucellosis due to *B. melitensis* bv. 3 is endemic in Maghreb countries (Algeria, Morocco, and Tunisia) in northern Africa (Benkirane, 2006, Lounes et al., 2014). Between 1986-1989, the flock seroprevalence was 43.5% and 42.0% in sheep and goats respectively in Algeria; 30.0% and 61.0% in 1991 in Tunisia, and 12.1% and 2.4% in 1996 in Morocco (Benkirane, 2006).

Epidemiological data on caprine and ovine brucellosis are few and limited to seroprevalence studies in Sub-Saharan Africa "SSA" (McDermott and Arimi, 2002). However, the disease was reported in Angola, Burkina Faso, Cape Verde, Djibouti, Eritrea, Ethiopia, and Kenya from 1993 to 2003 (Benkirane, 2006). *Brucella melitensis* bv. 1 and 3 were isolated in an outbreak of sheep and goats whose males were imported from Israel to Kenya (Philpott and

Auko, 1972). Furthermore, *B. melitensis* bv. 1 strain was isolated from goats in Zimbabwe (Madsen, 1989), and *B. ovis* was reported in sheep in Nigeria and South Africa (Ate et al., 2011, De Wet, 1984). The incidence of brucellosis in the tropics and eastern Soudan was 13.6% in sheep (Seifert, 1996). *Brucella abortus* has been reported from aborted ewes that were previously co-reared with cattle in other parts of the word (Allsup, 1969, Shaw, 1976). Caprine and ovine brucellosis have never been studied and reported in Rwanda.

1.3.3.3. Swine brucellosis

Brucellosis in pigs is a bacterial infection primarily caused by *B. suis* bv. 1, 2, and 3 with chronic inflammation in the reproductive system of females and males (Olsen, 2004). The disease caused by *B. suis* bv. 1 and 3 is similar in the geographic distribution and pathology and these strains are also pathogenic to humans, reindeer, caribou, hares, various marine species, and occasionally cattle and dogs (Olsen, 2004). *Brucella suis* bv. 2 is non-pathogenic for humans and affects hares (Olsen, 2004). In general, swine brucellosis is widespread with a low prevalence, except for south-east Asia, and South America (Olsen, 2004). Swine brucellosis due to *B. suis* bv. 2 is endemic in some regions of Yugoslavia (Taleski et al., 2002), and central American countries (Moreno, 2002). The prevalence of swine brucellosis is very low in Africa, and was 0.3% in Uganda (Erume et al., 2016), 0.6% in Nigeria (Onunkwo et al., 2011), and 0.0% in Zambia (Stafford et al., 1992). There is no report of the disease in Rwanda.

1.3.4. Epidemiology of brucellosis in wildlife

1.3.4.1. The causative agents and affected species

Brucellosis in wildlife is caused by *B. abortus*, *B. melitensis*, and *B. suis* (Godfroid, 2002). For instance, *B. abortus* has been isolated in buffaloes (*Syncerus caffer*), bison *(Bison bison),* waterbuck (*Kobus ellipsipymnus*), eland (*Taurotragus oryx*), and caribou (*Rangifer tarandus groenlandicus*) (Davis, 1990, Gradwell, 1977). *Brucella melitensis* has been reported in impalas (Schiemann and Staak, 1971), in chamois (*Rupicapra rupicapra*), and ibex (*Capra ibex*) (Ferroglio et

al., 2000). *Brucella suis* is usually isolated in wild pigs (*Sus scrofa*) (Olsen, 2004). *Brucella abortus* and *B. suis* are common in wildlife while *B. melitensis* is rarely isolated (Godfroid, 2002). Whether wildlife animals are the maintenance of infection or get infected from livestock remains debatable (Godfroid, 2002, Gorsich et al., 2015).

1.3.4.2. Occurrence and distribution

Brucellosis is prevalent in countries where domestic and wildlife animals are present and the demographic pressure has permitted the co-habitation of wildlife, livestock, and humans and this promotes the inter-species transmission of *Brucella* spp. (Bell et al., 1977). Brucellosis has been reported in bison in the Yellowstone National Park, the USA, with the first occurrence of seropositive and aborting bisons in 1917 (Williams et al., 1993), and with later isolation of *B. abortus* bv. 2 (Rhyan et al., 2001), and 1 (Rhyan et al., 1994). Brucellosis has been documented in wildlife in the southern and eastern Africa. In South Africa and Zimbabwe, brucellosis emerged into wildlife from livestock and then disseminated to other wildlife species without contact with cattle (Gradwell, 1977, Herr and Marshall, 1981, Madsen and Anderson, 1995). The presence of brucellosis in wildlife and livestock at the interface is of public health concern needing strong reinforcement to break the transmission of *Brucella* species. Studies in Africa showed the brucellosis seroprevalence in African buffaloes ranging from 7.9% to 20.7% and demonstrated the possibility of brucellosis transmission between cattle, African buffaloes, and humans (Assenga et al., 2015, Shirima et al., 2007, Motsi et al., 2013, Ndengu et al., 2017). Buffaloes carrying *Brucella* antibodies constitute a risk of transmission among other wildlife animals, farm animals, and humans residing in the proximity of their home-range (Assenga et al., 2015, Ndengu et al., 2017) and thus this interface needs attention in the establishment of strategic control programs (Ntivuguruzwa et al., 2020).

1.3.4.3. Modes of transmission

Infected animals shed the organism in milk, urine, vaginal discharges, semen, feces, and rarely saliva (Bicknell and Bell, 1979, McDiarmid and Sutherland, 1957, Serikawa and

Muraguchi, 1979). The mode of transmission depends on the type of wildlife host species. In buffaloes and other ungulates, the transmission is like that of cattle and includes ingestion of contaminated feeds and water, inhalation of aerosols, contact with infected herds, and by mating (Godfroid et al., 2013). In carnivores, infection is mainly by ingestion of infected ungulates, but cutaneous transmission through biting may also occur during aggression (Robertson, 1973).

1.3.5. Clinical signs of animal brucellosis

Animal brucellosis is mainly a chronic infection that affects mostly the reproductive system of sexually mature domestic animals (Corbel, 2006). In pregnant females, the infection causes placentitis leading to abortion, stillborn, placenta retention, vaginal secretions, low fertility rate, embryonic and neonatal death (Akakpo, 1987, Megid et al., 2010). The clinical manifestations in males include epididymitis, orchitis, testicular atrophy, sperm abnormalities, infertility (Megid et al., 2010), and hygroma in the carpal joint (Akakpo, 1987). The clinical signs are not conclusive of brucellosis and therefore infection by *Brucella* spp. should be confirmed by laboratory tests.

1.3.6. Diagnosis of animal brucellosis

The clinical diagnosis is not practical since reproduction disorders are not pathognomonic symptoms for brucellosis, however, the presence of hygromas in the carpal joint is a good suspicion of chronic infection in Africa (Akakpo, 1987, McDermott and Arimi, 2002). The diagnosis of brucellosis uses laboratory tests including modified acid-fast staining method, serological, bacteriological, biochemical, and molecular tests but, definitive diagnosis requires the use of different methods with culture being the gold standard method (OIE, 2018).

The Stamp's modified Ziehl–Neelsen's staining method consists of the observation of small red coccobacilli that may be arranged in pairs, or small groups under a microscope (Alton et al., 1988). Serological tests are commonly used and suitable for screening livestock. The

recommended tests by OIE for international trade include agglutination tests such as Rose Bengal Test (RBT), rapid slide agglutination test (RSAT), tube agglutination test (TAT), buffered antigen plate agglutination test (BPAT), fluorescence immunosorbent assays (FPA), the complement fixation test (CFT), indirect enzyme-linked immunosorbent assay (iELISA), and competitive (cELISA) (Nielsen, 2002, OIE, 2018). Other serological tests to screen milk include the milk ring test and milk ELISA (Nielsen, 2002, OIE, 2018). The diagnosis of brucellosis in animals requires a combination of more than one serological test of which RBT, BPAT, FPA are suitable screening and CFT, and ELISA for confirmation (Nielsen, 2002, OIE, 2018). However, the serological tests are not 100.0% specific, and bacteriological identification of *Brucella* spp. is the gold standard test (Alton et al., 1988, OIE, 2018).

Bacteriological isolation consists of culturing specimens on selective culture media such as Farrell's medium (Stack et al., 2002), modified Thayer-Martin is medium (Alton et al., 1988), Centro de Investigación y Tecnología Agroalimentaria (CITA) medium (De Miguel et al., 2011), and incubation at 37^{o} C with 10.0% CO_2 for at least one month depending on the type of sample and concentration of the organism (OIE, 2018). However, when the latter is sufficient *Brucella* grow within 4 days. The colonies should then be stained with the Stamp's modified Ziehl-Neelson's method and confirmed by biotyping which includes biochemical tests (OIE, 2018) such as the serum requirement, lysis by phages, oxidase, catalase and urease activity (Alton et al., 1988). However, the bacterial identification using these conventional phenotypic tests is time-consuming with an average of 10-14 days and requires trained personnel and appropriate biosafety, and therefore molecular methods may be a promising alternative (Bricker and Halling, 1994).

The main molecular methods are polymerase chain reaction (PCR) with different protocols such as one that is *Brucella* specific targeting the 16S-23S ribosomal DNA interspacer region (ITS) (Keid et al., 2007), a protocol that can differentiate four species: *B. abortus* bv. 1, 2, and 4, *B. melitensis* bv. 1, 2, and 3, *B. ovis*, and *B. suis* bv. 1 and hence named AMOS PCR (Bricker and Halling, 1994). Another PCR which can identify and distinguish all *Brucella* species from vaccine strains is known as Bruce-ladder PCR (Garcia-Yoldi et al., 2006, Lopez-Goni et al., 2008).

DNA-fingerprinting methods provide the maximum discrimination between strains and enable differentiation of the isolates that could not be obtained with multiplex PCR assays (Le Fleche et al., 2006). Multi locus variable number of tandem repeat (VNTR) assays (MLVA) can discriminate members of the *Brucella* spp., and identify species and biovars (Le Fleche et al., 2006). The nucleotide genome sequencing (NGS) of the pathogens allows the characterization of population genetics, trace back, epidemiological sources and explore the dynamics of each pathogen. The genome of *B. abortus, B. melitensis,* and *B. suis* is of 3.3 Mbp and comprise of two circular chromosomes (large with 2.1 Mbp and a small 1.5 Mbp). However, *B. suis* bv. 3 has only one chromosome of 3.1 Mbp (Halling et al., 2005). The comparison of the genomes of the three classical species showed some genetic variations such as the absence of large sequences in *B. abortus* that are shared by *B. suis* and *B. melitensis*, specific regions to *B. abortus*, genetic variations in the regions encoding outer membrane proteins, variable size of genes, and specific genes to *B. abortus* (Halling et al., 2005). These molecular assays have reduced the long procedure of conventional phenotypic identification of *Brucella* spp. (Bricker and Halling, 1994). However, serological methods are still prevailing in most developing countries, due to lack of appropriate knowledge, and biosafety facilities (Ducrotoy and Bardosh, 2017).

1.3.7. Prevention and control of animal brucellosis

World organization for animal health (OIE) recommends the following guidelines to prevent animal brucellosis: testing animals for replacement and quarantine them for one month, restriction of contacts and movement between healthy herds or flocks and those with unknown brucellosis status, testing of animals presenting abortions and hygromas, periodic surveillance of herds and flocks at least four times per year, burning or burial of abortion tissues and dead fetuses and disinfection of contaminated pastures and equipment, collaboration and informing human health professionals about animal cases especially those caused by *B. melitensis* to help them identify human cases, education campaigns to improve awareness of brucellosis and other zoonoses with emphasis on the mechanisms of transmission, test-and-slaughter in countries with a sustainable economy, hygiene, and vaccination (Corbel, 2006). The most successful

control program combines vaccination and test-and-slaughter. For instance, from 2002 to 2007, a control program in the Azores, Portugal, consisted of - vaccinating adult cows, heifers, and replacement calves aged 4-12 months using RB 51, screening animals using milk ring test (MRT), serological surveillance, and slaughtering of all reactors. This program reduced 69.3%, 39.3% and 75.4% of herd incidence, herd prevalence, and individual prevalence, respectively (Martins et al., 2009). Brucellosis remains endemic in northern Africa with poor reporting in the rest of Africa (Ducrotoy et al., 2017). Three vaccines that are presently available are *B. abortus* S19 and RB51 for cattle and *B. melitensis* Rev 1 for small ruminants (Ekron, 2008). Table 1.1 presents the properties of animal vaccines against brucellosis.

Table 1.1. Properties of vaccines against brucellosis in animals

Criteria	***B. abortus* S19**	***B. abortus* RB 51**	***B. melitensis* Rev 1**	**References**
Nature	Live smooth attenuated	Live rough attenuated	Live attenuated	(Dorneles et al., 2015, Schurig et al., 1991)
Host	Cattle	Cattle	Small ruminants	(OIE, 2018)
Protocol for quality control	Available	Unavailable	Available	(OIE, 2018)
Protection	Long-life immunity	Life-span immunity	Long-life useful immunity	(Nicoletti, 1990)
Interference with serological diagnosis	Yes	No	Yes	(Dorneles et al., 2015)
Abortifacient in pregnant females	Yes	More tolerant	Yes ++++	(Dorneles et al., 2015)
Infection in males	Yes	Yes	No	(Dorneles et al., 2015)
Virulence in humans	Yes	Yes	Yes++++	(Dorneles et al., 2015, McDiarmid and Sutherland, 1957)
Resistance to streptomycin	No	No	Yes	(Elberg and Meyer, 1958)
Resistance to rifampicin	No	Yes	No	(Schurig et al., 1991)

++++: strongly

These vaccines are used in specific hosts irrespective of the *Brucella* species and some studies have reported cross-infection with *B. melitensis* in cattle (Muendo et al., 2012, Mugizi et

al., 2015) and *B. abortus* in small ruminants (Bertu et al., 2015, Falade, 1981). There is no therapy for animal brucellosis, neither vaccination in humans, nor in wildlife (Dorneles et al., 2015). The vaccinal residues in milk predispose infection in humans and resistance to streptomycin and rifampicin should be considered when treating humans (McDiarmid and Sutherland, 1957). The *B. abortus* S19 vaccine causes chronic infections and abortions while and RB51 provides low levels of protection in different wildlife species of which some are considered maintenance hosts (Davis, 1990, Olsen, 2010), thus, the control of brucellosis should focus on the livestock to prevent the disease in wildlife and humans.

1.3.8. Epidemiology of brucellosis in humans or zoonotic brucellosis

1.3.8.1. The causative agents

Brucellosis in humans is caused by *B. melitensis* (Bruce, 1887), *B. abortus* (Spink and Thompson, 1953), *B. suis* (Forbes, 1991), *B. canis* (Lucero et al., 2010). *Brucella inopinata* was once isolated from human implants (Scholz et al., 2010). These *Brucella* species have their animal host preferences and since goats, sheep, and cattle are the most domesticated livestock, *B. melitensis* is more pathogenic and reported than *B. abortus* followed by *B. suis* (Bamaiyi, 2016, Moreno, 2014).

1.3.8.2. Occurrence and distribution

Brucellosis was first diagnosed in humans in Malta island in 1887 (Bang, 1897) and later reported in all continents with high incidence in Asian, North, South, and Latin America, Oceania, southeast of Europe, and Africa (Bamaiyi, 2016, Benkirane, 2006, Moreno, 2014). The incidence of zoonotic brucellosis in endemic zones ranged from 0.01 to 200 per 100,000 population in 1989 in Latin America (López-Merino, 1989). According to WHO estimates, the annual incidence increased from 500,000 cases in 2002 to 832,633 cases in 2015 (Kirk et al., 2015, Mangen, 2002). In the Maghreb, more than 600 and 400 human brucellosis cases were associated with the epizootic brucellosis in sheep and goats in Algeria, and Tunisia, respectively. Besides

85.0% of infected people consumed raw milk and milk products (Benkirane, 2006). Furthermore, *B. melitensis* bv. 3 strains were isolated from humans in the Maghreb (Benkirane, 2006, Lounes et al., 2014). In Rwanda, only two studies have reported the brucellosis seroprevalence of 25.0% and 6.1% in women with a history of abortion that occurred and reported in hospitals (Gafirita et al., 2017, Rujeni and Mbanzamihigo, 2014).

1.3.8.3. Modes of transmission

The modes of *Brucella* spp. transmission to humans are oral, inhalation, conjunctival, accidental self-inoculation with live vaccines or after blood sampling, and cutaneous contamination through cuts or skin abrasions (Ackermann et al., 1988, Anderson et al., 1986). Humans are mostly infected by the ingestion of untreated milk and contaminated animal products. The inhalation of dust and infected aerosols, and direct contact with infected animals especially during abortions, and parturition have also been reported (Young, 1983, Young, 1995). The disease is mostly found in occupational groups such as abattoir workers, butchers, cattle keepers, and handlers, laboratory workers, and health professionals (Baba et al., 2001, Bouza et al., 2005). Human-to-human transmission through tissue transplantation, breast feeding (Tuon et al., 2017), and sexual contact have also occasionally been reported (Mantur et al., 1996). Although, most consumers boil milk, consumption of untreated milk is still observed in sub-Saharan Africa (SSA) especially in pastoral groups, and this is usually associated with cultural events (McDermott and Arimi, 2002).

1.3.8.4. Clinical signs in humans

The disease in humans varies from an acute, sub-acute illness that may develop to a chronic condition with either localized infection or chronic fatigue syndrome (Young, 1995). The acute form is characterized by nonspecific clinical signs including undulant fever, joint pains, low back pain, weakness, nausea, vomiting, sweating, headache, loss of weight and pain, and edema in testes (Dean et al., 2012, Kose et al., 2014, Young, 1983). The occurring lesions are the swelling of the liver, spleen, and complications may occur with inflammation of the nervous

system in form of meningitis and meningoencephalitis (Ceran et al., 2011, Young, 1995), sacroiliitis, spondylodiscitis, endocarditis, and epidydimo-orchitis (Ceran et al., 2011, Kose et al., 2014, Young, 1995). The clinical signs resemble those of other diseases such as malaria, typhoid fever, salmonellosis, and therefore, the clinical diagnosis must be supported by the epidemiological history of the disease, and laboratory diagnosis (Crump et al., 2013).

1.3.8.5. Diagnosis in humans

Diagnosis of infection by *Brucella* spp. in humans is achieved by combining the clinical manifestation and history of the disease, supported by laboratory tests including RBT and SAT as screening tests and CFT and ELISA as confirmatory tests, however, the conclusive diagnosis should be supported by bacteriological tests (Díaz et al., 2011). RBT is more suitable for developing countries due to its sensitivity and low price (Díaz et al., 2011, Mantur et al., 2014). The most common specimen collected from humans for bacteriology is blood and this is initially inoculated into a basic medium such as serum dextrose broth, and solid and selective media are not necessary for human blood, and incubation is performed in 5% CO_2 (Corbel, 2006).

1.3.8.6. Treatment in humans

Brucella species are intracellular microorganisms requiring a long period of treatment and the antibiotics recommended by the WHO for severe brucellosis in adults are doxycycline 200 mg per os (PO) twice daily plus rifampicin 600 - 900 mg PO per day for a minimum of 6 weeks (WHO, 1986). However, a low proportion (1.1%) of resistance to doxycycline has been reported for *B. abortus* from bovine in Trinidad (Adesiyun et al., 2011).

1.3.8.7. Prevention and control in humans

The prevention of brucellosis in humans should primarily focus on the elimination of brucellosis in animals and the protection of people who are highly exposed to infected animals and animal products. The most exposed people are all farmworkers, abattoir workers, butchers,

veterinarians, artificial inseminators, and laboratory personnel (Corbel, 2006). According to the guidelines from the WHO, the following should always be observed: strict hygiene of premises and all workers whose occupation is at a high risk of exposure, safety measures in laboratories, education of health workers and the exposed community on zoonosis and food safety (Corbel, 2006). Other guidelines include serological surveillance of people at higher risk, wearing personal protection equipment (PPE) when handling and disposing abortion tissues, consumption of heat-treated food of animal origin including milk, milk products, and meat (Corbel, 2006). Brucellosis is one of the neglected and insidious zoonotic diseases that need attention to eradicate poverty and safeguard human health.

1.4. Bovine tuberculosis

1.4.1. The causative pathogen and affected species

Tuberculosis is a deadly disease that was devastating in animals and humans during the antiquity, but the first description of the pathogen (tubercle bacilli) was made in 1650 by Sylvius, while the infection was named "tuberculosis" in 1839 by Schonlein (Koch, 1882, Sakula, 1982). The causative agent, tubercle bacilli, was discovered on 14th March 1882 by a professor of bacteriology, Robert Koch (Koch, 1882, Sakula, 1982). The human and bovine tubercle bacilli were found to be different microorganisms in 1865 by Villemin and confirmed in 1898 by Professor Theobald Smith, however, it was in 1911 that the Royal Commission of tuberculosis recognized bTB as a hazard to humans and introduced hygiene and pasteurization of milk (Sakula, 1982). At this time, the bovine tubercle bacillus was considered as a variant and termed *M. tuberculosis var.* or *subsp. bovis* until it became *M. bovis* in 1970 (Karlson, 1970). *Mycobacterium bovis* belongs to the genus *Mycobacterium* in the unique family of *Mycobacteriaceae* of the order Actinomycetales; it is an aerobic, non-motile, fast acid-alcohol, non-sporulating, and slowly growing bacillus (Goodfellow and Wayne, 1982). Apart from *Mycobacterium laprae*, the genus comprises two groups, *Mycobacterium tuberculosis* complex (MTBC) and non-tuberculous mycobacteria (NTM) (Pfyffer et al., 1998). *Mycobacterium leprae* is an exceptional bacterium with a long generation time and no growth in artificial media; it affects the skin and peripheral

nerves of humans and some primates (Rastogi et al., 2001). *Mycobacterium tuberculosis* complex (MTBC) cause tuberculosis (TB) in their hosts and currently eleven species that are known to have reservoir hosts are *M. tuberculosis* for humans (Koch, 1882), *M. africanum* for humans (Castets et al., 1968), *M. canettii* for humans (Canetti, 1970), *M. microti* for voles (Wayne and Kubica, 1986, Wells, 1937). *M. pinnipedii* for rodents (Cousins et al., 2003), *M. caprae* for goats (Aranaz et al., 1999), *M. bovis* for cattle (Karlson, 1970), *M. bovis* BCG, a vaccine strain for humans (Calmette, 1927), *M. mungi* for banded mongoose (*Mungos mungo*) (Alexander et al., 2010), *M. suricattae* for meerkats (*Suricata suricatta*) (Parsons et al., 2013), *M. orygis* for oryxes (*Oryx* spp.) (van Ingen et al., 2012), and dassie bacillus for dassies (*Procavia capensis*) (Smith, 1960). Non-tuberculous mycobacteria (NTM) also known as atypical mycobacteria or mycobacteria other than tuberculosis (MOTT) (Pfyffer et al., 1998) are opportunistic and cause TB-like disease in immunocompromised hosts (Mfinanga et al., 2004).

The bTB is primarily caused by *M. bovis*, but other members of the MTBC have also been identified in diseased cattle and these are *M. africanum* (David et al., 1978), *M. caprae* (Pavlik et al., 2002), *M. tuberculosis* (Berg et al., 2009, Kazwala, 1996), and *M. orygis* (Dawson et al., 2012). Members of MTBC are 99.9% genetically identical and may have derived from a human-adapted *M. canetti*, from which successive DNA fragments deletions occurred chronologically to form regions of differences (RD) that led to the evolution of MTBC members (Brosch et al., 2002). In addition to cattle, *M. bovis* affects a wide range of other livestock species, wildlife, and humans (Edwards R, 2013, Michel, 2002, Michel et al., 2010). The host adaptation may have resulted from the interactions of the pathogen with the immune responses of the host species resulting in the genetic changes of the pathogen for survival and virulence (Gagneux et al., 2006). It is therefore essential to identify members of MTBC for further understanding their epidemiology and pathogenicity.

1.4.2. Virulence and pathogenesis

Pathogenic mycobacteria are intracellular microorganisms that activate the complement group C2a to produce an enzyme C3 convertase essential for their opsonization and adhesion to the complement receptors (CR1, and CR3) leading to their internalization into macrophages (Schlesinger and Horwitz, 1991, Schorey et al., 1997). Inside the macrophages, mycobacteria multiply and survive the killing by lysosomes by inhibiting their fusion with mycobacteria-containing macrophages (Armstrong and Hart, 1975). This property is conferred by the sulpholipids of the bacterial cell wall (Goren, 1970). Other virulence factors essential for the survival and control of the host immune responses are the structure and constituents of the cell envelop (Rastogi, 1990, Rastogi and Barrow, 1994). For instance, the mycolic acids are involved in the pulmonary inflammation (Vander Beken et al., 2011) and its biosynthesis plays considerable role in the physiology and intracellular survival of mycobacteria (Bhatt et al., 2007) while the lipoglycans control macrophage effector functions and cytokine secretion (Vercellone et al., 1998). Likewise, the superficial phenolic glycolipids and sulpholipids may also prevent intracellular killing by shifting reactive oxygen molecules (Rastogi and David, 1988).

1.4.3. Epidemiology of tuberculosis in cattle

1.4.3.1. The causative agents

As mentioned bTB in cattle is primarily caused by *M. bovis* (Karlson, 1970), and occasionally by *M. tuberculosis* (Berg et al., 2009) and *M. caprae* (Prodinger et al., 2005). We will mainly focus on bTB caused by *M. bovis*, the pathogen with the widest host range including domestic animals (cattle, goat, pig, sheep, horse, cat, dog, and camel), wildlife, primates, and humans (Good and Duignan, 2011, Michel et al., 2009, Michel et al., 2010). Thus, the disease negatively impacts the national, regional, and international economies (Tschopp et al., 2013).

1.4.3.2. Occurrence and temporospatial distribution

The bTB has been reported in all continents with varying prevalence (Pavlik et al., 2005, Pavlik et al., 2002). The animal level prevalence of bTB varied between 20.0 and 40.0% in Europe in 1882 (Francis, 1947), reduced to 16.3% in 1992, 3.4% in 1999 (Pavlik et al., 2002), and varied from 0.00 to 0.01% from 2000 to 2005 (Pavlik et al., 2005). Currently, the disease is completely eradicated in some countries but remains uncommon in some European countries (CFSPH, 2019). In the USA, in the 1890s, one-in-ten cattle were infected by bTB, but the USA was bTB free since 1940, a result of the massively and compulsory test-and-slaughter program that costed almost USD 1,100,000,000 (Olmstead and Rhode, 2004). However, some uncommon sporadic cases occur (CFSPH, 2019). In Australia, bTB was introduced in the 19th century (Seddon and Albiston, 1965) and except for some quarantined herds in Queensland state, Australia was bTB impending free in 1992 (Tweddle and Livingstone, 1994) and is presently completely bTB free due to strong eradication programs (CFSPH, 2019). Bovine TB is endemic in Latin America and the Caribbean with a constant prevalence above 1.0% (De Kantor and Ritacco, 1994, De Kantor and Ritacco, 2006). Bovine TB is endemic in Asia and 38.7% of MTBC were isolated from 1067 cattle positive to tuberculin skin test collected from different regions of China (Du et al., 2011).

Bovine TB is endemic in Africa and 80.0% (33/43) of African countries members of OIE reported the occurrence of the disease in the 1990s (Daborn and Grange, 1993). For instance, *M. bovis* was isolated in 18.3% of cattle in Burundi (Rigouts et al., 1996), in 26.0% of milk in Burkina Faso (Vekemans et al., 1999), in 7.8% of cattle in Uganda (Nalapa et al., 2017), 2.3% in slaughtered cattle in Kenya (Gathogo et al., 2012), and 13.2% and 17.0% of cattle were tuberculin reactors in Tanzania (Kazwala et al., 2001a) and Tchad (Schelling et al., 2000), respectively. Tuberculosis in cattle is endemic in Rwanda, although poorly documented. In 2002, Rwanda reported to the OIE 142 confirmed bTB cases, and 232 slaughtered cattle were suspected of bTB of which 38 were destroyed (OIE, 2002). Isolated studies reported the bTB prevalence of 0.5% in 2009 (Habarugira et al., 2014) and 13.4% between 2006 and 2010 (Nshimiyimana et al., 2013). However, there is no information on mycobacterial species circulating in cattle in Rwanda, therefore, the identification will contribute significantly to the understanding of their origin and transmission.

1.4.3.3. Transmission

Animals are commonly infected by inhalation, ingestion, and accidentally by direct contact with mucous membranes or skin abrasions (CFSPH, 2019). Transmission by inhalation requires a minimal dose compared to the oral route (O'Reilly and Daborn, 1995). The sources of infection in animals are contaminated water, milk, sputum, feces (Grange and Collins, 1987, Neill et al., 1989, Neill et al., 1988, Olmstead and Rhode, 2004). Animals with advanced bTB excrete and shed the pathogen in their urine, feces, and sputum in the pasture and the pathogen can persist for many weeks in the absence of ultraviolet radiation (Duffield and Young, 1985, Thoen et al., 2009). However, a study in South Africa demonstrated an absence of *M. bovis* in surface water and watering points of infected buffaloes (Michel et al., 2007). Factors facilitating transmission include confined spaces with little ventilation, old age, dairy herds, accidental contact at shared watering points, or livestock gatherings, introduction of an infected animal into a naïve herd, and importation of purebred herds (Grange and Collins, 1987, Olmstead and Rhode, 2004). The disease is insidiously widespread in healthy purebred cattle in the absence of early detection by the tuberculin test (Dormandy, 1999). The pathogen is also present in the milk implying the possibility of transmission via milk to neonates. The presence of tubercles in the genitourinary tract indicates the transmission through coitus however this requires a heavy infection (Thoen et al., 2009). The contact of livestock with infected buffaloes which are considered major maintenance hosts has also been incriminated in the transmission of *M. bovis* to livestock (Grange and Collins, 1987, Michel and Bengis, 2012).

1.4.3.4. Clinical manifestations

Diseased animals develop granulomatous tuberculous lesions in the lungs, lymph nodes of thorax, head, and other organs and tissues including bones (Grange and Collins, 1987, Olmstead and Rhode, 2004). Diseased animals lose weight and cows reduce between 10.0 - 25.0% of reproductive performances, dyspnea, and coughing followed by a premature death in the late stage of pulmonary infection (Grange and Collins, 1987, Olmstead and Rhode, 2004).

1.4.4. Epidemiology of bovine tuberculosis in wildlife

1.4.4.1. Affected species

Mycobacterium bovis is the widespread pathogen that has been isolated in a wide range of wildlife species such as fennec fox (*Vulpes zerda*), bison (*Bison bison*), deer (*Odocoileus virginianus*), possum (*Virginia possum*), badger (*Meles meles*), buffalo (*Bubalus bubalis*), ferret (*Mustela putorius furo*), hare (*Lepus timidus*), Lechwe antelope (*Kobus leche*), ilama (*Lama glama*), Arabian oryx (*Oryx leucoryx*), alpaca (*Vicugna pacos*), hyena (*Hyaena hyaena*), lion (*Panthera leo*), leopard (*Panthera pardus*), chaema baboon (*Papio ursinus*), kudu (*Tragelaphus strepsiceros*), black rhinoceros (*Diceros bicornis*), cheetah (*Acinonyx jubatus*), cayotes (*Canis latrans*), warthog (*Phacochoerus africanus*), lynx (*Lynx canadensis*), etc. (Arnot and Michel, 2020, Hlokwe et al., 2019, Michel et al., 2015, Michel and van Helden, 2019, Miller et al., 2019). Bovine TB has been reported in over 40 wildlife species (Michel et al., 2010) and in vervet monkeys (*Chlorocebus pygerythrus*) of the South African national zoological gardens, in chacma baboon (*Papio ursinus*), sable antelope (*Hippotragus niger*) (Michel et al., 2013), and in african elephant (*Loxodonta Africana*) (Miller et al., 2019). Other wildlife animals were susceptible to bTB and African buffalo (*Syncerus caffer*) is the well-known maintaince host (Michel et al., 2015). The clinical signs and lesions of bTB in wildlife resemble those in cattle with variations in the size, appearance, and distribution of lesions (Zanella et al., 2008).

1.4.4.2. Spatial distribution

Bovine TB remains maintained in some free-ranging hosts such as elk (*Cervus elaphus nelsoni*) and wood bison (*Bison bison athabascae*) in Canada, white-tailed deer (*Odocoileus virginianus*) in Michigan, wild boar (*Sus scrofa*) in southwestern Europe, brush-tailed possums (*Trichosurus vulpecura*) in New Zealand, badgers (*Meles meles*) in the UK and Ireland, African buffalo (*Syncerus caffer*), Kafue lechwe (*Kobus leche kafuensis*), and possibly greater kudu (*Tragelaphus strepsiceros*), and warthogs (*Phacochoerus aethiopicus*) in Southern Africa (CFSPH, 2019). A study conducted in South Africa reported that bTB in wildlife originated from

diseased cattle which transmitted the disease to buffaloes and from buffaloes to other ungulates, then carnivores, small mammals, and rodents (Michel et al., 2006). While bTB in wildlife has been reported in countries neighboring Rwanda such as Uganda (Kalema-Zikusoka et al., 2005, Woodford, 1982), and Tanzania (Cleaveland et al., 2005), similar studies are absent in Rwanda.

1.4.4.3. Transmission

The modes of transmission of bTB in wildlife depend mainly on the species' social and nutritional behaviors (Michel, 2002). For instance, the social behavior within herds and between herds of African buffaloes favors the transmission by inhalation (Cross et al., 2005a). The predisposing factor for the spatial distribution of the disease is the mixing of infected and naïve herds at watering points during drought seasons (Cross et al., 2005b). Diseased and old buffaloes are the sources of infection for predators such as lions, hyenas, and scavenging omnivores including baboons, honey badgers, and warthogs (Michel, 2002). In these species, the transmission is mainly by the oral route, inhalation but also by percutaneous route in case of aggression (Michel et al., 2015, Michel and van Helden, 2019). Infected animals may contaminate the environment by shedding the bovine bacilli through the feces, urine, and pus from infected bite wounds which constitute the sources of infection for herbivores (Grange and Collins, 1987).

1.4.5. Epidemiology of zoonotic tuberculosis

1.4.5.1. Causative agents

Zoonotic tuberculosis is mainly caused by *M. bovis* (Grange, 2001) and occasionally by *M. caprae* especially in Europe (Prodinger et al., 2014). Tuberculosis in humans was more prevalent before the discovery of the tubercle bacillus and the introduction of meat inspection and pasteurization of milk in 1911 significantly reduced the burden (Grange and Collins, 1987, Rastogi et al., 2001).

1.4.5.2. Distribution and transmission

In Australia, bTB was introduced in the 19th century and became an established zoonosis in children that were consuming milk (Seddon and Albiston, 1965). The prevalence of human TB due to *M. bovis* was estimated at 1.0% in the USA and Canada, and 7.0% in San Diego, CA, the USA owing to the ingestion of milk and cheese (De Kantor et al., 2010). *Mycobacterium bovis* was also isolated in Latin America and the Caribbean (De Kantor et al., 2010) with 13.8% of incidence in Mexico (Pérez-Guerrero et al., 2008). Zoonotic TB re-emerged and increased with the development of drug resistance, and human immunodeficiency virus especially in developing countries (Grange, 2001, Thoen Co, 1995). In 2013, almost 10.0% of humans TB cases were due to *M. bovis* in some African countries (OIE, 2013). In 2016, the global incidence of zoonotic tuberculosis was 147, 000 with 12, 500 human deaths of which the largest number was found in Africa and Asia (Cousins, 2018). Although, *M. bovis* was isolated from humans in countries neighboring Rwanda like Uganda (Oloya et al., 2008), Tanzania (Kazwala et al., 2001b, Mfinanga et al., 2004), and Democratic Republic of Congo (Mposhy et al., 1983), there is no single study on human tuberculosis due to *M. bovis* (Gafirita et al., 2012). Infection in humans is often by consumption of untreated milk and milk products (Grange and Collins, 1987, Thoen Co, 1995). However, the human-to-human transmission of *M. bovis* by inhalation has been reported in immunocompromised patients (Evans et al., 2007, O'Reilly and Daborn, 1995). Other sources of infection include eating undercooked meat, infection by skin abrasions and close contact with infected cattle can lead to aerosol transmission (Edwards et al. 2013). Oral ingestion of *M. bovis* leads to extra pulmonary TB and since 1840 *M. bovis* has been widely reported in human extrapulmonary infections with cervical lymphadenitis (Cicero et al., 2009, Hlavsa et al., 2008, Kazwala et al., 2001b). However, the mechanism driving this extrapulmonary infection is poorly understood and this compromises early treatment since human tuberculosis is mostly suspected in the lungs and *M. bovis* is not diagnosed in most laboratories (Kazwala et al., 2001b).

1.4.5.3. Treatment in humans

After Koch's discovery, the control of tuberculosis in humans consisted of surgical treatment and the use of X rays until the discovery of the vaccine, Bacillus Calmette - Guerin (BCG), that was first used in 1921 (Sakula, 1982). The first treatment started with the discovery of streptomycin in 1944 (Pfuetze et al., 1955), isoniazid (H) in 1954 (Hsu, 1974), and rifampin (R) in 1967 (Maggi et al., 1966). Except for pyrazinamide (Z), *M. bovis* is sensitive to other frontline antibiotics used to treat infection caused by *M. tuberculosis* and the therapy consists of isoniazid (H), rifampicin (R), ethambutol (E), and pyrazinamide (Z) for 2 months followed by 4 months of H and R (Daly et al., 2006, WHO, 2010, Romero et al., 2007).

1.4.6. Diagnosis of bovine tuberculosis

1.4.6.1. In humans

Most laboratories testing specimen collected from humans only detect MTBC without speciation (Kazwala et al., 2001).

1.4.6.2. In animals

The first location of clinical signs depends mostly on the route of transmission, and whether it is localized or generalized infection (Michel et al., 2010). The clinical signs can take several months and years to develop either in the intestines, skin, cervical lymph nodes, rarely the genitourinary tract, and other extrapulmonary sites (Cosivi et al., 1998, Grange and Collins, 1987, Wise and Marella, 2003).

The laboratory diagnosis consists of screening methods such as the intradermal tuberculation test which is usually complemented with the gamma interferon test "IFN-γ", (Buddle, Livingstone & De Lisle 2009, Michel et al. 2011), and confirmatory tests like bacteriology and biochemical tests, and molecular methods (Hlokwe et al., 2014, Michel et al., 2009). The intradermal tuberculation test consists of injecting the *M. bovis* antigen, purified protein derivative (PPD) into the skin at the base of the tail (caudal fold test "CFT"), or the neck

of animals (comparative cervical test "CCT") (Buddle et al., 2015). This test detects the delayed hypersensitivity response characterized by visible or palpable skin swelling within 72 hours of injection (Buddle et al., 2015). The principle of the IFN-γ test is that the T-cells are sensitized in whole blood cultures to produce and release IFN-γ when re-exposed to the *M. bovis* antigens in vitro (Buddle et al., 2015). Therefore, this test consists of short-time incubation (37°C, for 18-24 hours) of the whole blood in the presence of mycobacterial antigens followed by the measurement of IFN-γ from plasma using a sandwich ELISA. The blood must be processed within 8 hours after sampling (Buddle et al., 2015). The IFN-γ test allows re-testing with the CFT – false positive animals when used in series or the CFT – negative when used in parallel (Buddle et al. 2015). The culture consists of homogenization of affected tissue specimens followed by decontamination with 2% hydrochloric acid and with 4% NaOH and centrifugation at 3500 rpm for 10 min, then neutralization with sterile water and centrifugation at 3 500 rpm. The homogenate is then inoculated onto a Lowenstein-Jensen medium supplemented with pyruvate and then incubated at 37°C for 10 weeks (Alexander et al. 2002).

The identification of the members of the MTBC using culture and molecular techniques has significantly improved the bTB control. Molecular techniques include PCR, and DNA-fingerprinting techniques (OIE, 2019). The most used DNA-fingerprinting techniques include spoligotyping, and the mycobacterial interspersed repetitive units - variable number tandem repeat typing (MIRU-VNTR) (OIE, 2019). DNA-fingerprinting techniques distinguish strains for epidemiological purposes like the origin, transmission, and spread of *M. bovis* (Durr, Hewinson & Clifton-Hadley 2000). It is often recommended to combine techniques to gain maximum discrimination between strains (Jagielski et al., 2014, Michel et al., 2008). PCR can differentiate members of MTBC by detection of the presence or absence of RDs (Huard *et al.* 2003). This PCR assay targets the genes that are located within RD deletions loci and those genes include 16S rRNA which is common for *Mycobacterium* spp. (Springer et al., 1996). Rv0577 is the specific gene for MTBC members (Leclerc et al., 2000). The gene IS1561 is present in all members of MTBC except for *M. microti* (Gordon et al., 1999b). Rv1510 is the gene located to the RD4 deletion which is absent from all *M. bovis* and *M. bovis* BCG but present in other members of

MTBC (Gordon et al., 1999a). Rv1970 is the gene located within the RD7 deletion which is only present in *M. tuberculosis*, *M. canettii*, and *M. africanum* type II (Gordon et al., 1999a). The genes Rv3877 / Rv3878 that are located within the RD1 deletion locus are absent in *M. bovis* BCG but present in other MTBC members (Brosch et al., 2002). The gene Rv3120 that is located within the RD 12 and RD^{can} deletions is absent in *M. bovis*, *M. bovis* BCG, *M. caprae*, and *M. canettii* but present other members of MTBC (Brosch et al., 2002, Huard et al., 2003). **S**poligotyping exploits the DNA polymorphisms within the direct repeat (DR) locus to distinguish *M. tuberculosis* strains by detecting DRs and the presence or absence of spacer sequences (Groenen *et al.* 1993). Spoligotyping enabled the identification of clonal complexes of *M. bovis* including the African 2 (Af 2) which was isolated in cattle in Easter Africa (Berg *et al.* 2011) and African 1 (Af1) that was identified in the West Africa (Muller *et al.* 2009). *Mycobacterium bovis* strains belonging to the European 1 complex that was identified in South Africa, Tanzania, and Zambia may have originated from the UK through past livestock trade with these countries (Smith *et al.* 2011). The emergence of strains that are locally restricted to regions or countries suggests the local evolution of clonal strains due to the geographic and spatial adaptation to adverse unknown environmental or endogenous (host) conditions (Biffa et al., 2010, Michel et al., 2009).

Although spoligotyping, and the multilocus variable number of tandem repeats analysis (MLVA) help distinguish the genotypes of mycobacteria in epidemiological surveillance, they have a limited intrinsic discriminatory power because they only target polymorphic regions of less than 1% of the genome and cannot trace efficiently the origin of infection (Rodriguez-Campos et al., 2011). These limitations can be circumvented by single nucleotide polymorphisms (SNPs) analysis using whole genome sequencing (WGS) which provides a detailed genetic information including, all genomic targets, further evidence on genome evolution, virulence, and resistance determinants consequent to their high discriminatory power for closely related strains of *M. bovis* (Hauer et al., 2019).

1.4.7. Prevention and control of bovine tuberculosis

1.4.7.1. In animals

While the treatment is prohibited in domestic animals, non- human primates and wild animals in captivity can be treated using two or three drugs like isoniazid, rifampin, or ethambutol with variations in the dosage and duration depending on the wildlife species (Thoen et al., 2009). Whether the maintenance host is cattle or buffaloes, the effective eradication method that has been successful in some developed countries is the culling of all infected animals, but this method may not apply for developing countries (Thoen et al., 2009). Except for South Africa, other developing countries cannot afford the cost of test-and-slaughter due to compensations of farmers if the disease is maintained in cattle or cannot accept losing revenues from tourism if the disease is maintained in wildlife (Arnot and Michel, 2020, Michel, 2002, Thoen et al., 2009). In both scenarios, depopulation is not a good alternative, instead, immunization would be the best method to minimize the risk of transmission. However, there is no effective vaccine for animals (Michel, 2002). Vaccination with live Bacillus Calmette-Guerin (BCG) in animals (livestock or wildlife) requires elevated dose and does not provide complete protection. It interferes with diagnosis by tuberculin test and protection from environmental mycobacteria and sheds the bacillus in feces and urine (Buddle et al., 2002, Michel, 2002, Thoen et al., 2009). A vaccination trial demonstrated that the parenteral heat-killed vaccine provides humoral protection in calves, but this vaccine needs further investigations under field conditions for validation (Van Der Heijden et al., 2017). Separation of infected cattle from non-infected ones would reduce the degree of transmission (Michel, 2002, Thoen et al., 2009). The abattoir inspection of carcasses for organ lesions also contributes significantly to the bTB surveillance as symptoms take time to develop in chronic disease (Aylate et al. 2013). Monitoring, surveillance, and research studies would also provide essential epidemiological data of the disease dynamics between wildlife, livestock, and humans (de Lisle et al., 2001).

1.4.7.2. In humans

The control of zoonotic tuberculosis includes eradication of bTB in domestic animals, pasteurization of milk and milk products, meat inspection, hygiene in the abattoirs, treatment of diagnosed cases, and vaccination (Buddle et al., 2002, Grange and Collins, 1987, Michel, 2002,

Thoen et al., 2009). Live BCG vaccine provides strong immunity in humans but its use in immunodeficient people is prohibited and there is a need for the discovery of safe and efficient vaccines (Thoen et al., 2009).

1.5. The control scheme for brucellosis and bovine tuberculosis in Rwanda

The control of brucellosis, bTB, and other infectious diseases is governed by the animal health law which consists of regulations and procedures for reporting infectious diseases, restriction of animal movement, and the prohibition of illegal slaughtering (Minagri, 2009). The control for brucellosis consists of testing cattle and small ruminants before distribution to poor families by the government and other non-government organizations (NGOs), and annual surveillance using RBT (once per year) in areas with high dairy production. The control of bTB consists of surveillance using a tuberculin test once a year in critical zones, and abattoir surveillance by the main capital abattoir, société des abattoirs de Nyabugogo (SABAN). Furthermore, the national parks that harbor essential wildlife are fenced but small animals cross the electric fence to join livestock farms (Field observation). There is also a need for more effort in the implementation of the animal health law, checking the accuracy of abattoir records and make use of them, and testing animals at least three times a year with a representative sample size to build a stronger surveillance system.

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Chapter 2. Common Materials and Methods

2.1. Serological tests

2.1.1. Rose Bengal test (RBT)

Animal sera were screened for the presence of *Brucella* antibodies using Rose Bengal test (RBT, Onderstepoort Biological Products, South Africa) according to the protocol previously described by Alton et al. (1988). Briefly, equal volumes (30 µl) of serum and antigen were mixed for four minutes. A *Brucella* positive and one negative reference samples served as controls. An obvious, clear, and complete agglutination was recorded as a strong (+++) result, while a clear agglutination but not complete was recorded as a medium (++) result. An agglutination that was only visible at the margins was recorded as a weak (+) result.

2.1.2. Indirect ELISA

Indirect ELISA was used to confirm RBT positive results in series according to the manufacturer's instructions (IDvet Diagnostics, France). For each test microplate, samples were tested as singles while the positive and negative controls were tested in duplicates. The optical densities (ODs) of samples were determined at 450 nm using an ELISA reader (original multiscan Ex, Thermo Fisher Scientific, USA). The sera samples having 120% seropositivity and greater were confirmed positive. In this study, the sera samples showing seropositivity above 119.4% were rounded to 120% and considered positive.

2.2. Culturing

Tissue samples were processed and cultured in a biosafety level 3 at the National Reference Laboratory (NRL), Rwanda biomedical center, Kigali Rwanda. Tissues were sliced using sterile scissors and forceps into sterile mortars and grounded using a sterile pestle. An aliquot of pooled homogenate, milk, and vaginal swabs were spread into a modified Centro de Investigación y Tecnología Agroalimentaria (CITA) medium and incubated at 37°C with 5.0% CO_2 atmosphere while vaginal swabs and fluid were streaked out on the modified CITA

medium (Ledwaba et al., 2020). Plates were read for bacterial growth every day for three weeks. The morphology of *Brucella* organisms was tested using Stamp's modified Ziehl-Neelsen staining method (OIE, 2018). *Brucella* cultures from modified CITA were subcultured by streaking onto a modified CITA medium to obtain single purified colonies.

2.3. Molecular methods

2.3.1. DNA extraction from cultures

Genomic DNA was extracted from suspect cultures using ReliaPrep gDNA tissue Miniprep system following manufacture's guidelines (Promega, USA).

2.3.2. The 16S-23S ribosomal interspacer region (ITS) PCR assay

The identification of the genus *Brucella* was performed by amplification of the genomic DNA extracted from purified bacterial colonies using the gene-specific primers (Table 2.1) as previous described (Keid et al., 2007). *Brucella abortus* RB 51, *B. abortus* bv. 2 REF 544 strain served as positive controls. Sterile ultra-pure water served as a negative control. The 15 µl PCR reaction mixture contained 1x of MyTaq™ Red PCR Mix (Bioline, South Africa), primers at 0.2 µM and 2 µl of template DNA. The PCR cycling condition was initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 2 min and extension at 72°C for 2 min and a final extension step at 72°C for 5 min. The primers amplified a 214 bp fragment that was analyzed by electrophoresis using a 2% agarose gel stained with SYBR safe DNA staining gel (Invitrogen, ThermoFischer, South Africa) and visualized under UV light.

2.3.3. AMOS PCR assay

The *Brucella* spp. including *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis* were identified and differentiated using a multiplex AMOS PCR assay as previously described (Bricker and Halling, 1994). *Brucella abortus* bv.1 RB51, *B. abortus* bv.1 REF 544 strain, *B. melitensis* rev 1, and *B. melitensis* bv.1 16M strain served as positive controls while sterile ultra-pure water served as a negative control. A 25 µl reaction mixture contained 1x MyRaq Red PCR Mix (Bioline, South Africa), four species-specific forward primers and reverse primer IS711 (Table 2. 1) at final

concentration of 0.1 µM and 0.5 µM respectively, and 2 µl of template DNA. Thermocycling conditions included initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 2 min and initial extension at 72°C for 2 min and a final extension at 72°C for 5 min. PCR products were analysed by gel electrophoresis using 2% agarose stained with SYBR safe DNA staining gel (Invitrogen, ThermoFischer, South Africa) and visualised under UV light.

2.3.4. Bruce-ladder PCR assay

Vaccine strains and field isolates of *Brucella spp*. were identified and differentiated by a multiplex Bruce-ladder PCR assay developed as previously described (Garcia-Yoldi et al., 2006, Lopez-Goni et al., 2008). *Brucella abortus* bv.1 RB51, *B. abortus* S19, *B. abortus* bv.1 REF 544 strain, *B. melitensis* rev 1, *B. melitensis* bv.1 16M, *B. suis* bv.1 ZW45 served as positive controls. Sterile ultra-pure water served as a negative control. A 25 µl PCR reaction contained 1x MyTaq™ Red Mix (Bioline, South Africa), eight species-specific forward and revers primers at a final concentration of 6.25 µM (Table 2. 1) and 2 µl of template DNA. The PCR cycling conditions included an initial denaturation at 95°C for 3 min followed by 25 cycles of at 95°C for 30 s, at 64°C for 45 s, and at 72°C for 3 min and a final extension step at 72°C for 10 min. PCR products were analysed by gel electrophoresis using a 2% agarose stained with SYBR safe DNA staining gel (Invitrogen, ThermoFischer, South Africa) and viewed under UV light.

Table 2. 1. Sequences of oligonucleotide primers used for the distinction of *Brucella* spp. using ITS, AMOS, and Bruce-ladder PCR assays.

PCR name	Primer name	Sequence (5'-3')	Targets	Size (bp)	Conc. (µM)	References
ITS	ITS66 f	ACATAGATCGCAGGCCAGTCA	*16s-23s rRNA*	214	0.2	(Keid et al., 2007)
	ITS279r	ACATAGATCGCAGGCCAGTCA				
AMOS	*B. abortus*	GAC GAA CGG AAT TTT TCC AAT CCC	*IS711*	498	0.1	(Bricker and Halling, 1994)
	B. melitensis	AAA TCG CGT CCT TGC TGG TCT GA		731	0.1	
	B. ovis	CGG GTT CTG GCA CCA TCG TCG GG		976	0.1	
	B. suis	GCG CGG TTT TCT GAA GGT GGT TCA		285	0.1	
	IS 711	TGC CGA TCA CTT AAG GGC CTT CAT		-	0.2	
BRUCE- LADDER	BMEI0998f	ATC CTA TTG CCC CGA TAA GG	*wboA*	1682	6.25	(Garcia-Yoldi et al., 2005, Vemulapalli et al., 1999)
	BMEI0997r	GCT TCG CAT TTT CAC TGT AGC				
	BMEI0535f	GCG CAT TCT TCG GTT ATG AA	*bp26*	450	6.25	(Cloeckaert et al., 2000)
	BMEI0536r	CGC AGG CGA AAA CAG CTA TAA				
	BMEII0843f	TTT ACA CAG GCA ATC CAG CA	*omp31*	1071	6.25	(Vizcaino et al., 1997)
	BMEII0844r	GCG TCC AGT TGT TGT TGA TG				
	BMEI1436f	ACG CAG ACG ACC TTC GGT AT	*Deacetylase*	794	6.25	(Rajashekara et al., 2004)
	BMEI1435r	TTT ATC CAT CGC CCT GTC AC				
	BMEII0428f	GCC GCT ATT ATG TGG ACT GG	*eryC*	587	6.25	(Sangari et al., 1994)
	BMEII0428r	AAT GAC TTC ACG GTC GTTCG				
	BR0953f	GGA ACA CTA CGC CAC CTT GT	*ABC Transporter*	272	6.25	(Halling et al., 2005)
	BR0953r	GAT GGA GCA AAC GCT GAA G				
	BMEI0752f	CAG GCA AAC CCT CAG AAG C	*rpsL*	218	6.25	(Cloeckaert et al., 2002)
	BMEI0752r	GAT GTG GTA ACG CAC ACC AA				
	BMEII0987f	CGC AGA CAG TGA CCA TCA AA	*CRP Regulator*	152	6.25	(Rajashekara et al., 2004)
	BMEII0987r	GTA TTC AGC CCC CGT TAC CT				

2.4. References

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Chapter 3. Seroprevalence and Associated Risk Factors of Bovine Brucellosis at the Wildlife-Livestock-Human Interface in Rwanda

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3.1. Abstract

Bovine brucellosis is endemic in Rwanda; however, little information is available on seroprevalence and risk factors. Therefore, a cross-sectional study was conducted among cattle farmed at the wildlife-livestock-human interface (n = 1691) in five districts and one peri-urban district (n = 216). Cattle were screened using the Rose Bengal Test, then the results were confirmed by indirect enzyme-linked immunesorbent assay. Potential risk factors were determined with a questionnaire and analyzed for their association with seropositivity. In all districts, the animal and herd-level seroprevalence was 7.4% (141/1907) and 28.9% (61/212), respectively, 8.3% (141/1691) and 30.9% (61/198) at the interface, and 0.0% (0/216) in peri-urban areas. Among the potential risk factors, old age (≥ 5 years), cattle farmed close to wildlife, herds of cattle and small ruminants, history of abortions, and replacement animals were significantly associated with brucellosis (p < 0.05). Low awareness of zoonotic brucellosis, assisting calving without biosafety protection, drinking raw milk, and manual milking were each observed in more than 21.7% of cattle keepers whose herds were seropositive. This study confirmed brucellosis endemicity in cattle farmed close to wildlife in Rwanda, suggesting the

need to focus control efforts in these areas. Educated farmers with a high awareness of zoonotic brucellosis had low bovine brucellosis seropositivity, which emphasizes the importance of education.

Keywords: brucellosis; seroprevalence; potential risk factors; wildlife-livestock-human interface; Rwanda.

3.2. Introduction

Brucellosis is a neglected and contagious bacterial disease of veterinary and public health importance that remains endemic in developing countries including Rwanda (Ducrotoy et al., 2017). Brucellosis affects farm and marine animals, wildlife, and humans (Corbel, 2006, Galińska and Zagórski, 2013). The disease is caused by bacteria belonging to the genus *Brucella*. Each *Brucella* species has a preferential host, for instance, *Brucella abortus* has a preference for cattle (Bang, 1897), *B. melitensis* for goats and sheep (Zammit, 1905), *B. suis* for pigs (Traum, 1914), *B. ovis* for sheep (Buddle, 1956), and *B. canis* for dogs (Kimberling et al., 1966). Among these species, *B. melitensis*, *B. abortus*, *B. suis*, *B. canis* cause severe disease in humans (Galińska and Zagórski, 2013).

Brucellosis causes tremendous economic losses as a result of abortions, stillborn, placenta retention, decline in milk yield, and low fertility rate in both females and males (McDermott et al., 2013). The disease is usually chronic and asymptomatic animals shed infective discharges in the pasture or watering points, and these are important sources of infection for healthy animals (Corbel, 2006). Therefore, regular serological testing of herds would detect infected animals, and lead to control measures to limit the transmission of brucellosis in the herd. Serological testing in cattle involves a combination of more than one serological test (OIE, 2013). A combination of Rose Bengal Test (RBT) and Indirect Enzyme Linked Immuno-Sorbent Assay (i-ELISA) is among the tests of choice due to its high sensitivity and specificity (Chisi et al., 2017, Nielsen, 2002). Although, two serological tests are recommended for research and diagnosis of brucellosis, only RBT is widely used in most resource-poor countries.

In Rwanda, there are few published studies on bovine brucellosis, and the individual animal seroprevalence ranged from 2.2% in peri-urban areas of Kigali city to 18.9% in the Nyagatare district (Chatikobo et al., 2008, Manishimwe et al., 2015, Ndazigaruye et al., 2018, Rujeni et al., 2008). The seroprevalence of brucellosis in women with history of abortion was 6.1% at the Nyagatare district hospital (Gafirita et al., 2017) and 25.0% at Huye teaching hospital (Rujeni and Mbanzamihigo, 2014). Despite the efforts to control brucellosis in Rwanda, the factors responsible for its persistence remain poorly understood. However, the absence of sufficient epidemiological data on the seroprevalence of brucellosis and associated risk factors may impede the design of informed control strategies against brucellosis. The risk factors that have been found to be significantly associated with bovine brucellosis in Rwanda included herd size, breed, and animal age although that particular study was only conducted in Nyagatare district only and the impact of proximity of livestock to wildlife habitat was not assessed (Ndazigaruye et al., 2018).

The herd seroprevalence of brucellosis in cattle farmed at the wildlife – livestock - human interface in Easter African countries were reported to be 46.7% in Tanzania (Assenga et al., 2015, Shirima and Kunda, 2016), 26.7% (Enstrom et al., 2017) and 68.7% (Nthiwa et al., 2019) in Kenya. An increase of 42.0% from 2017 (Enstrom et al., 2017) to 2019 (Nthiwa et al., 2019) in Maasai Mara National Reserve, Kenya, may have resulted from the increased interactions of wildlife and livestock animals due to demographic pressures. Studies in Africa have documented brucellosis seroprevalence in African buffaloes ranging from 7.9% to 20.7% (Assenga et al., 2015, Motsi et al., 2013, Ndengu et al., 2017, Shirima and Kunda, 2016). Strategic control programs are needed for livestock farmed at the interface since the control of the disease in wildlife remains impossible. Although, brucellosis seroprevalence in cattle farmed at the wildlife-livestock-human interface has been documented in neighboring Uganda (Nina et al., 2018) and Tanzania (Shirima and Kunda, 2016), there is not a single similar study in Rwanda.

The aim of this study was therefore to investigate the seroprevalence of bovine brucellosis and associated risk factors at the wildlife–livestock–human interface. This will contribute to building a database about the occurrence of brucellosis and associated epidemiological factors,

and this is essential for providing informed advice to policymakers to improve the control strategies against brucellosis in Rwanda.

3.3. Materials and methods

3.3.1. Description of the study area

This study was conducted in six out of 30 districts of Rwanda; five of which have many dairy cattle at the proximity of national parks, and one district in Kigali city which has cattle farms in peri-urban areas. The five districts included - Nyagatare, Gatsibo, Kayonza which border Akagera National Park in the Eastern Province, Musanze district which borders Virunga National Park in the Northern Province, and Nyabihu district, which borders Gishwati-Mukura National Park in the Western Province. The sixth district, Gasabo, located in Kigali city was included to evaluate the brucellosis seropositive status of cattle in peri-urban areas, without a history of proximity with wildlife. Most cattle residing in Nyagatare, Gatsibo, Kayonza, and Nyabihu districts are crossbreeds, kept in a free grazing system, and most of the farms are fenced with *Euphorbia tirucalli*. On the other hand, the cattle from Gasabo and Musanze districts are kept under a zero-grazing system. The vaccination is not systematic at the national level and several herds located in districts of Eastern Province are in remote areas where access to veterinary services is limited. The climate in the Eastern Province is warmer and drier, characterized by annual average rainfalls ranging between 700 – 950 mm, and annual average temperatures ranging between 20°C and 21°C, while the vegetation is grassland with lowly inclined hills and an average altitude of 1513.5 m. In Northern and Western Provinces, the climate is the coolest and wettest characterized by annual rainfalls ranging from 1400 – 1600 mm and annual average temperatures ranging from 15 - 17°C, while the topology is mountainous with volcanoes, and the average altitude ranging between 2000 and 3000 m (USAID, 2019). Akagera and Virunga National Parks are home of buffaloes (*Syncerus caffer*) (Eugene and Martin, 2006). In this study, the wildlife-livestock-human interface was comprised of cattle farms in five districts that border the national parks. Further information on the study area is shown in Figure 3.1

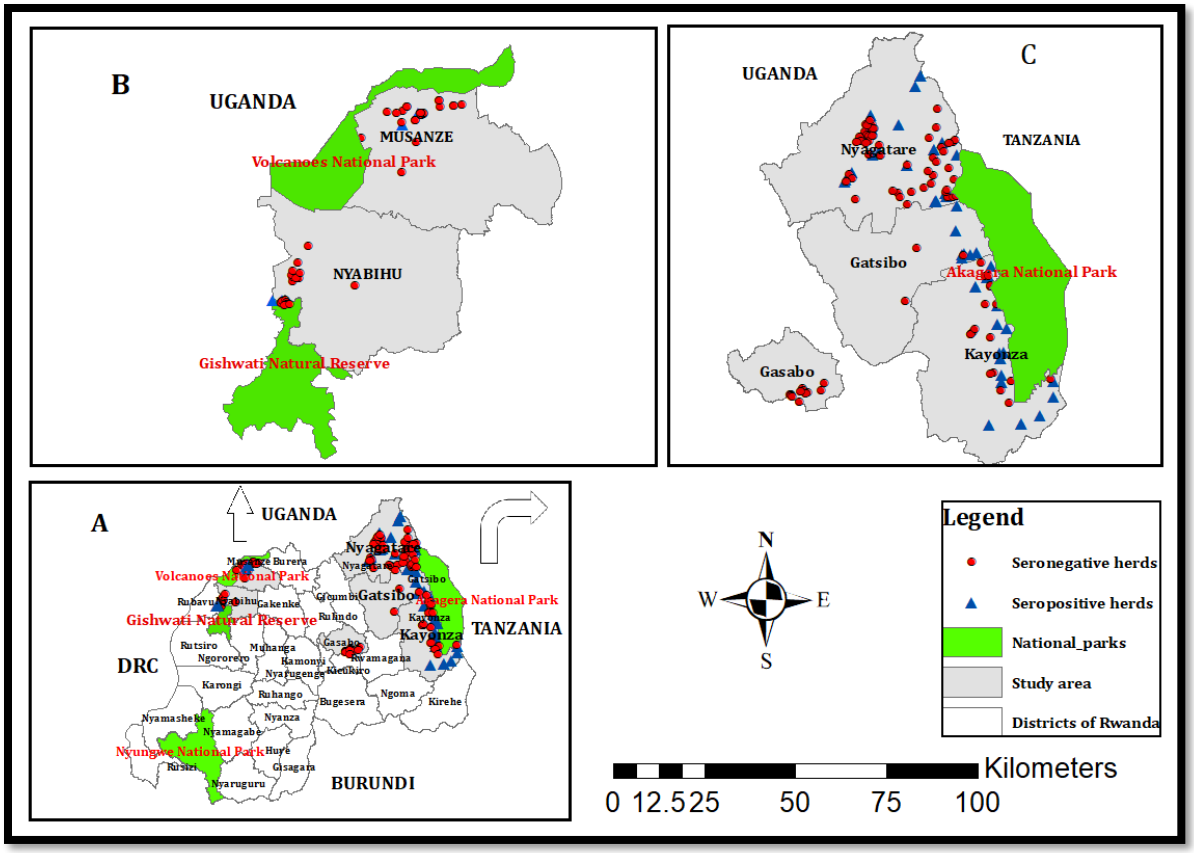

Figure 3. 1. Maps of (A) Rwanda with different districts, (B) Musanze and Nyabihu districts bordering Virunga and Gishwati national parks respectively, and (C) Nyagatare, Gatsibo, and Kayonza districts bordering Akagera National Park, and Gasabo, an urban district with peri-urban areas. Red circles and blue triangles indicate seronegative and seropositive herds obtained in this study.

3.3.2. Study design and sample size

The study was a cross-sectional design, conducted between May 2018 and September 2019 applying a multistage cluster sampling strategy, to select herds in the selected districts and individual animals within herds. A herd was classified as the sampling unit and this was stratified by districts. The target population was all dairy herds present in the vicinity of national parks or peri-urban areas of Kigali city. Cattle of 1-year-old age and above were selected for this study, and these were categorized as young (1 to 2 years old), adult of medium age (3 to 4 years old), and adult of old age (5 to 13 years old). The dental formula was used to

determine the age of animals as previously described (Pope, 1934). During sampling, a household that had dairy cattle on the same landsite, regardless of the size, was defined as a farm, whereas a farm owned by one or several people, regardless of the size, was considered as one herd. Within each district, households from all areas bordering a national park were randomly selected from sampling frames provided by the district and sector veterinary officers. The study involved blood sampling and herd data collection. The sample size was determined using the formula previously described (Dohoo et al., 2009):

$$\boldsymbol{Sample\ size\ (n) = \frac{Z^2\ \ P(1-P)}{d^2}}$$

Where Z^2 = 1.96 at 95% confidence level; P is the expected prevalence estimated to be 10% based on a previous study (Chatikobo et al., 2008) and d is a margin error of 5%. The total sample size per each district was adjusted for clustering using the following equation: $N = n\ (1 + \rho\ (m - 1)$; where N represents the new sample size, n stands for the original sample size, ϱ (= 0.2) for the intra-cluster correlation coefficient, and m (= 4) represents the number of cattle sampled per herd (Dohoo et al., 2009). The new sample size was 220 cattle per each district. To increase the precision and taking into consideration a large number of cattle in Nyagatare and Kayonza districts, the sample size was increased by 3 for Nyagatare and 1.5-fold for Kayonza, and this led to 654 and 375 cattle sampled from the two districts, respectively. The overall sample size was 1907, and these were selected from 212 herds. However, some households consented to participate in the study with a condition of testing all their animals. Therefore, a maximum of four, nine, 15, and more than 15 cattle were selected from 81, 68, 24, and 40 herds, respectively. The sample size at the wildlife-livestock-human interface was 1691 cattle that were selected from 198 herds, while that of peri-urban areas was 216 cattle that were selected from 14 herds.

3.3.3. Questionnaire design and data collection on individual cattle and the herds

Individual data, including the name of the owner, sample identification, age, sex, breed, and location were recorded in a separate list for all selected cattle in the 212 herds. A structured

questionnaire comprising of open-ended and closed-ended questions was then administered in a face-to-face interview with one respondent, a cattle keeper/owner in each of the 212 herds to obtain information about potential herd-level risk factors that could be associated with exposure to *Brucella* infection in both cattle and humans. The interviews were conducted in the herd owner's language (Kinyarwanda), by the primary investigator or a research assistant who was provided with prior training on all aspects of questionnaire administration in rural and peri-urban areas. The questionnaire was pre-tested in two herds that were not included in the final data set and subsequently adjusted to ensure precision and good flow of the questions and responses. The questionnaire data comprised potential herd-level risk factors, including herd size, herd composition (presence of small ruminants and/ or dogs), proximity or history of contact with wildlife, type of grazing system, access to veterinary services, disinfection of pastures and pens, and the knowledge of the disease by farmers, among others. The questionnaire also included questions related to public health to assess the knowledge of cattle keepers about the zoonotic aspect of bovine brucellosis and predisposing practices in cattle husbandry. The geographical coordinates of each location were recorded using the Global Positioning System (GPS) device (Etrex, USA) and were then used to generate a map of the study area using ArcGIS (ESRI ArcGIS, version 10.6).

3.3.4. Blood collection

Samples were taken without causing damage to the animals, respecting their welfare. Blood samples were collected aseptically in a 4-ml plain vacutainer tube from the jugular or tail vein of each selected animal. The vacutainer tubes labelled with each animal identification were transported to the nearest campus of the University of Rwanda where they were stored overnight at room temperature to allow clotting. The following day, sera were collected in a sterile microcentrifuge tube and kept at -20^{o}C waiting for serological testing at Rwanda Agriculture Board, Department of Veterinary Services, option Serology.

3.3.5. Serological tests: Rose Bengal test (RBT) and indirect ELISA

The serological tests were performed as described in chapter 2.

3.3.6. Data analysis

Individual or herd-level seroprevalence for each district and the entire study were calculated by dividing the total number of animals or herds simultaneously positive to RBT and i-ELISA by the total number of animals or herds sampled and multiplied by 100. A herd was considered positive if at least one animal tested positive. Data were recorded and analyzed in Microsoft Excel spreadsheets. Each potential risk factor from the individual- and herd-level data was assessed for significant statistical association with the serological status (considered as a binary outcome: positive or negative), using the Chi-square or Fisher's Exact tests of association. Variables that were significantly associated with brucellosis seropositivity ($p < 0.05$) at univariate analysis were selected and tested for collinearity using the chi-square test. If a pair of variables was found to be collinear, then only one variable considered to be more biologically associated with brucellosis was considered for multivariable analysis. The screened-in variables were then included in initial multivariable logistic regression models, separately for an individual- and herd-level data. The regression was performed by a Generalized Linear Model (GLM) function, considering a binomial distribution. Subsequently, a stepwise elimination procedure was conducted to arrive at the most adequate model that minimized the Alkaike Information Criteria (AIC). Univariate and multivariate analyses were performed using R Console version 3.5 (R Core Team, 2017) at a 5% level of significance. The selected model was then subjected to the goodness-of-fit test, by the Hosmer-Lemeshow (χ^2) test, followed by the determination of odds ratios (OR) for each variable in the final model (RCore-Team, 2020).

3.4. Results

3.4.1. Animal and herd-level seroprevalence of brucellosis in cattle in Rwanda

The total number of cattle samples analyzed using RBT was 1907, of which 13.6% (260/1907) tested positive. Among these, 260 RBT-positive samples, that is, 45.4% (118/260) were strong positive, 12.3% (32/260) were medium, and 42.3% (110/260) were weak positive. The 260 RBT-

positive sera were subsequently analyzed using i-ELISA to confirm the presence of anti-*Brucella* spp. antibodies. Of the 260 RBT-positive samples, 54.2% (141/260) tested positive for brucellosis. The overall true animal-level seroprevalence was 7.4% (141/1907, 95% CI: 6.1, 8.5) using both RBT and i-ELISA, and bovine brucellosis was detected in 83.3% (5/6) of the sampled districts (Table 3. 1). The true animal-level seroprevalence was 8.3% (141/1691, 95% CI: 7.0, 9.7) at the interface, and 0.0% (0/216) in peri-urban district.

Table 3. 1. Results of descriptive and univariate analysis between potential individual animal risk factors and the serological status of brucellosis in cattle sampled at the wildlife-livestock-human interface in Rwanda.

Variables	Categories	Positive to RBT			Positive to i-ELISA			Positive RBT &i-ELISA		
		NT	Total n+ (%)	*P value*	NT	Positive n^+ (%)	*P value*	NT	Positive n^+ (%)	*P value*
Districts	Gasabo	216	20 (9.3)	< 0.001	20	0 (0.0)	< 0.001	216	0 (0.0)	< 0.001
	Gatsibo	226	41 (18.1)		41	40 (97.6)		226	40 (17.7)	
	Kayonza	375	83 (22.1)		83	38 (45.8)		375	38 (10.1)	
	Musanze	215	13 (6.1)		13	7 (53.9)		215	7 (3.3)	
	Nyabihu	220	11 (5.0)		11	2 (18.2)		220	2 (0.9)	
	Nyagatare	655	92 (14.1)		92	54 (58.7)		655	54 (8.2)	
Age	Young (≥2years	273	26 (9.5)	0.013	26	9 (34.6)	0.098	273	9 (3.3)	0.002
	Medium (≤3≤4 years)	853	108 (12.7)		108	58 (53.7)		853	58 (6.8)	
	Older (≥5 years)	781	126 (16.1)		126	74 (58.7)		781	74 (9.5)	
Sex	Female	1803	249 (13.8)	0.43	249	136 (54.6)	0.56	1803	136 (7.5)	0.4
	Male	104	11 (10.6)		11	5 (45.5)		104	5 (4.8)	
Breeds	Exotic breeds*	43	6 (14.0)	<0.001	6	3 (50.0)	1	43	3 (7.0)	0.004
	Cross	1497	155 (10.4)		155	72 (46.5)		1534	99 (4.8)	
	Ankole	367	99 (27.0)		99	66 (66.7)		330	39 (18.0)	

RBT, Rose Bengal Test; i-ELISA, indirect Enzyme-linked immunosorbent assay; NT: number of cattle tested; n^+: number of positive animals; Exotic breeds* included Friesian and Jersey. The total number of samples analysed using RBT was 1907, of which 260 tested positive. The 260 RBT-positive samples were subsequently analysed using i-ELISA for confirmation of the brucellosis status.

The total number of herds analyzed using RBT was 212, of which 49.9% (89/212) tested positive. All the 89 RBT-positive herds were analyzed using i-ELISA to confirm the serological status of brucellosis, and 68.5% (61/89) tested positive for *Brucella* spp. infection. The overall true herd-level seroprevalence was 28.9% (61/212, 95% CI: 22.7, 34.9). Except for Gasabo district in Kigali city, positive herds were recorded in all the other sampled districts (5/6, 83.3%) (Figure 3. 1). The true herd-level seroprevalence recorded at the interface was 30.9% (61/198: 95% CI: 24.4, 34.2), and 0.0% (0/14) in the peri-urban district.

3.4.2. Univariate and multivariate analyses of individual risk factors

Univariate analysis of the individual animal risk factors showed that district, animal age, and breed were significantly associated with animal-level seroprevalence ($p < 0.05$). Cattle from the Gatsibo, Nyagatare, and Kayonza districts, which border the Akagera National Park in the Eastern Province showed higher seropositivity than other districts ($p < 0.05$) (Table 3. 1). Among these three districts, Gatsibo and Nyagatare showed significantly higher seropositivity than Kayonza ($p < 0.05$). Older cattle (≥ 5 years) showed the highest seropositivity (9.5%, 74/781) while young animals were least seropositive (3.3%, 9/273). The indigenous breed, "Ankole", was more exposed (18.0%, 66/367) to *Brucella* spp. compared to the cross-bred (4.8%, 72/1497) and exotic breeds (7.0%, 3/43). Although sex was not significantly associated with brucellosis seropositivity, female cattle were more seropositive (7.5%, 136/1803) than males (4.81%, 5/104) (Table 3. 1).

All of the three variables that were significantly ($p < 0.05$) associated with brucellosis seropositivity in the univariate analysis were included in the final multivariable logistic regression model. Cattle from Gatsibo (OR = 22.2), Nyagatare (OR = 9.7), Kayonza (OR = 7.8), Musanze (OR = 4.2), and Gasabo (OR = 10.0×10^{-7}) were associated with higher odds of brucellosis seropositivity compared with the Nyabihu district, although, the odds were not statistically significant ($p > 0.05$) for Musanze and Gasabo districts. Cattle of medium age (3 to 4 years old) (OR = 2.4, $p = 0.03$) or older (≥ 5 years) (OR = 3.0, $p = 0.005$) were associated with significantly higher odds of brucellosis seropositivity ($p < 0.05$) than young cattle (1 to 2 years).

The indigenous cattle breed, "Ankole", was associated with a higher likelihood of seropositivity (OR = 1.8) than the crossbreeds. "Exotic' breeds were not included in the final logistic regression due to the relatively small number of cattle (43) available for sampling. The Hosmer and Lemeshow goodness of fit test was not statistically significant ($\chi^2 = 3.04$, $p = 0.93$), showing that the model fitted the data well, with the observed data matching the values expected in theory (Table 3. 2).

Table 3. 2. Results of multivariable logistic regression between animal-level risk factors and serological status of brucellosis in cattle sampled at the wildlife-livestock-human interface in Rwanda.

Variables	**Category**	**Odds Ratios**	**95% CI**	***p*-Value**
Districts	Nyabihu [a]			
	Gasabo	10.0×10^{-7}	0.00–inf.	0.975
	Gatsibo	22.2	5.3–93.3	<0.001 [b]
	Kayonza	7.8	1.7–35.7	0.008 [b]
	Musanze	4.2	0.9–20.6	0.075
	Nyagatare	9.7	2.3–40.1	0.002 [b]
Age	Young [a]			
	Medium	2.4	1.1–5.1	0.025 [b]
	Older	3.0	1.4–6.3	0.005 [b]
Breeds	Crossbreed [a]			
	Ankole	1.8	1.0–3.3	0.067

[a] Reference categories for comparing serological status amongst cattle. [b]$p < 0.05$: significant difference in serological status as compared to the reference level for each variable. Hosmer and Lemeshow $\chi^2 = 3.5$, df = 8, p-value = 0.9.

3.4.3. Univariate and Multivariate Analyses of Potential Herd Risk Factors

Of the 20 variables considered in the univariate analysis, only 10 showed a significant association ($p < 0.05$) with herd-level seropositivity, and these included herd composition, grazing system, presence of endemic diseases, sharing watering points, history of abortion, good knowledge of bovine brucellosis, access to veterinary services, introduction of new cattle into the herds, and feeding abortion tissues to dogs (Appendix 1). Although other herd factors were not significantly associated with brucellosis ($p > 0.05$), high proportions of seropositive animals were observed between levels of variables and these data are available in the Supplementary Materials (Appendix 1).

Among the 10 variables that were significantly ($p < 0.05$) associated with brucellosis in the univariate analysis, only six comprised the final multivariate logistic model analysis (Table 3. 3). Herd owners without any level of education (OR = 7.2, p <0.05) and those with primary education (OR = 6.7, $p < 0.05$) were more likely to have seropositive herds than those with tertiary and secondary education, and the odds were statistically significant. Another important significant predictor for herd-level seropositivity included herd composition with herds that had both cattle and small ruminants being more significantly associated with brucellosis seropositivity (OR = 2.8, $p < 0.05$) compared to herds with cattle only. Good knowledge of animal brucellosis among herd owners was more likely to be associated with brucellosis seropositivity (OR = 5.5; $p < 0.05$). The history of abortions and the introduction of new animals into the herd were also significant predictors ($p < 0.05$) of brucellosis. Cattle reared under free-grazing were associated with higher odds of seropositivity (OR = 1.9) than those under zero-grazing, although, the odds were not statistically significant ($p > 0.05$). The Hosmer and Lemeshow goodness of fit test was not statistically significant ($\chi^2 = 3.9$, $p = 0.87$) showing that the model fitted the data well, with the observed data matching the values expected in theory (Table 3. 3).

Table 3. 3. Results of multivariable logistic regression between potential herd risk factors and the serological status of brucellosis in cattle farmed at the wildlife-livestock-human interface in Rwanda.

Variables	Category	Odds Ratio	95% CI	*p*-value
Education category	Tertiary [b]			
	Primary	6.7	1.9–23.3	0.003 [a]
	None	7.2	2.1–24.4	0.001 [a]
Herd composition	Cattle only [b]			
	Cattle and SR	2.8	1.1–6.7	0.024 [a]
	Cattle and dogs	1.4	0.6–3.4	0.458
Grazing system	Zero-grazing [b]			
	Free grazing	1.9	0.8–4.5	0.144
Brucellosis knowledge	No [b]			
	Yes	5.5	1.7–18.1	0.005 [a]
History of abortions	No [b]			
	Yes	2.5	1.2–5.1	0.014 [a]
New introduction	No [b]			
	Yes	2.7	1.3–5.9	0.011 [a]

[b] Reference categories for comparing serological status amongst cattle. [a] $p < 0.05$: Significant difference in serological status as compared to the reference level for each variable. Hosmer and Lemeshow $\chi^2 = 3.9$, df = 8, p-value = 0.9.

3.4.4. Potential Risk Factors Associated with Cattle Keepers Holding Seropositive Herds

Table 3. 4 shows the univariate associations between six risk factors and cattle keepers having seropositive herds. Brucellosis seropositive herds were significantly associated with cattle keepers with insufficient education ($p < 0.05$). Low awareness of zoonotic brucellosis was common in most cattle keepers, 85.9% (182/212) and among them, 26.9% had seropositive animals. Although calving was not significantly associated with herd seroprevalence, most

respondents, 76.9% (163/212) assist cattle during parturition without personal protective equipment (PPE), and 31.9% of them had seropositive herds. The number of cattle keepers who drink raw milk was 39.2% (83/212) and of them, 21.7% had seropositive herds. Manual milking was commonly observed in 98.6% of the herds and of them, 28.6% had seropositive herds (Table 3. 4).

Table 3. 4. Univariate associations between public health risk factors and herd brucellosis seropositivity among cattle keepers residing at the wildlife-livestock-human interface in Rwanda.

Variables	Categories	Sample Size	No. of responses (%)	Odds Ratio	95% CI	*p*-Value
Education level	Tert. & sec.	44	4 (9.1)	-	-	0.002 [a]
	Primary	83	25 (30.1)			
	None	85	32 (37.7)			
Zoonotic brucellosis	Yes	30	12 (40.0)	0.6	0.3–1.3	0.21
	No	182	49 (26.9)			
Boiling milk	Yes	129	43 (33.3)	0.6	0.3–1.0	0.094
	No	83	18 (21.7)			
Assisting calving	Yes	163	51 (31.3)	1.7	0.8–3.8	0.194
	No	49	10 (20.4)			
Using PPE	Yes	0	0 (0.0)	-	-	1
	No	163	51 (30.1)			
Milking method	Manual	210	60 (28.6)	2.5	0.1–98.1	0.493
	Machine	2	1 (50.0)			

No.: number; tert. & sec.: tertiary and secondary; [a] $p < 0.05$: significant difference in the frequency of responses; CI: confidence interval.

There was a significant correlation between awareness of zoonotic brucellosis and boiling milk and between education level and boiling milk ($p < 0.05$). Most of the cattle keepers, 80.0%

(24/30) that were aware of brucellosis being zoonotic also boiled their home milk before consumption. Of 182 cattle keepers that were not aware of brucellosis as a zoonotic disease, 42.3% (77/182) drank un-boiled milk. Educated cattle keepers, 79.5% (35/44) were more likely to boil their home milk before consumption compared to uneducated, 58.8% (50/85), and those with primary education, 53.0% (44/83) (Figure 3. 2).

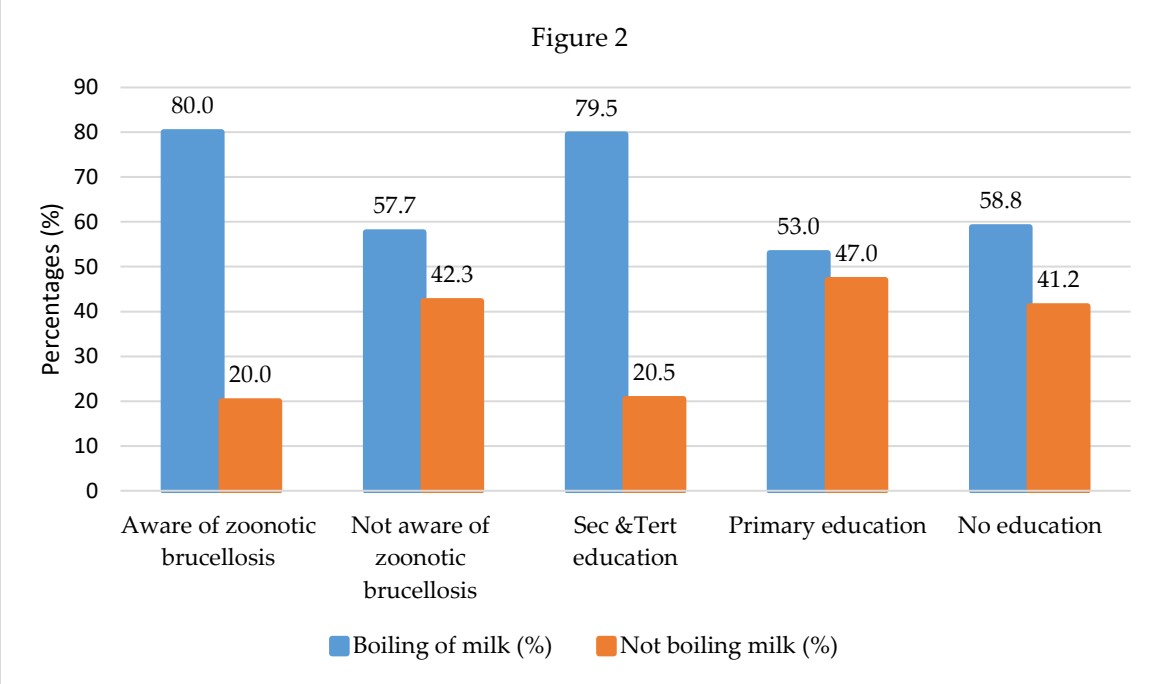

Figure 3. 2. Pairwise correlation between awareness of zoonotic brucellosis, education level, and drinking raw milk in cattle keepers in Rwanda.

3.5. Discussion

Bovine brucellosis is a contagious bacterial disease of veterinary and public health importance, and the disease is endemic in sub-Sahara African countries including Rwanda. This study, which was carried out in six districts, is the first to report on the seroprevalence of brucellosis and associated risk factors in cattle farmed at wildlife-livestock-human interfaces in Rwanda. The findings of the present study confirmed that brucellosis determined with serological tests (RBT and i-ELISA) is endemic in cattle farmed close to the national parks, especially those harboring several buffalos, and the occurrence therein was significantly higher

than that in peri-urban areas in the Gasabo district, Kigali city. The overall adjusted animal and herd seroprevalence rates (7.4% and 28.9%) obtained in cattle from six districts in this study as well as the previous rates (9.9–30.2%) obtained in the Nyagatare district of Rwanda using RBT alone (Chatikobo et al., 2008), the 7.4% rate reported in the Huye district of Rwanda using RBT alone (Rujeni et al., 2008), and the rate of 18.9% reported in the Nyagatare district using only RBT (Ndazigaruye et al., 2018), confirm that brucellosis is endemic in Rwanda.

Of the 260 (13.6%) sera that were detected as positive for brucellosis using RBT, 118 (45.4%) were strong positive, 32 (12.3%) were medium while 110 (42.3%) were weak positive. Of the 110 RBT-weak positives, 3 (2.7%) were confirmed seropositive using i-ELISA. Most veterinary laboratories in developing countries diagnose brucellosis by detecting only RBT strong positives (complete and clear agglutination) and medium positives (clear agglutination) due to the lack of expertise in detecting weak positives as RBT is a subjective test. Additionally, the confirmation test is not always performed due to either the lack of confirmatory test reagents or the limited number of personnel. Therefore, if the weak positive animals are undetected and then approved for trade, this could contribute to the spread of brucellosis to the naïve herds at the destination.

The animal-level seroprevalence (8.3%) observed in cattle at the wildlife-livestock-human interface is in line with the respective results (8.3% and 9.6%) reported in cattle at the wildlife-livestock-human interface in Zimbabwe using RBT and c-ELISA (Caron et al., 2013, Gomo et al., 2012), and (9.7%) in Ethiopia using RBT, and i-ELISA (Chaka et al., 2018). The herd-level seroprevalence of 30.9% observed at the interface in this study is comparable to that obtained in cattle at the wildlife-livestock-human interface in Kenya (26.7%) using i-ELISA (Enstrom et al., 2017), and in Ethiopia (32%) using RBT and i-ELISA (Chaka et al., 2018). On the other hand, our finding was lower compared to the results obtained in cattle farmed at the wildlife-livestock-human interface: in Kenya (68.7%) using i-ELISA (Nthiwa et al., 2019), and in Zambia (58.1%) using RBT and c-ELISA (Muma et al., 2007). This difference was explained by the absence of vaccination programs in the study area in Kenya (Nthiwa et al., 2019) while in Zambia, the high seroprevalence was associated with abortions and cattle shared grazing pastures and watering points with wildlife (Muma et al., 2007). Moreover, the seroprevalence of brucellosis was

reported in cattle, buffaloes, and humans at the interface in Tanzania (Assenga et al., 2015, Shirima et al., 2007) and in Zimbabwe (Motsi et al., 2013, Ndengu et al., 2017), and this suggests the spillover of brucellosis between these species. The current study together with the above studies confirmed that bovine brucellosis is prevalent in cattle farmed at the wildlife-livestock-human interface, and higher incidences of brucellosis occur in herds with increased interactions between livestock and wildlife.

The animal and herd-level seroprevalence rates observed in this study differed significantly among districts ($p < 0.05$). Cattle from districts that border national parks had higher animal and herd seroprevalences compared to those from peri-urban areas of Gasabo district where no animal was found positive. This difference can be ascribed to the relatively large size of herds, and the free grazing system observed in the Eastern and Western Provinces as compared to the zero-grazing system among cattle farms in Gasabo district, and in which animal health is managed better by the easily accessible veterinary services and readily available animal scientists. Zero-grazing system minimizes contacts between animals and thus reduces the risk of disease transmission. Districts bordering the Akagera National Park in the Eastern Province were more likely to have seropositive cattle ($p < 0.05$) compared to the Musanze and Nyabihu districts that border the Virunga and Gishwati-Mukura National Parks, respectively. In addition, the animal-level seroprevalence recorded in Musanze was high compared to that recorded in Nyahihu. This difference may be attributable to the presence and number of buffaloes within the various national parks. For instance, the Gishwati-Mukura National Park contains only monkeys, chimpanzees, and birds while the Akagera National Park contains many ruminants including buffaloes and the Virunga National Park alongside Musanze has buffaloes. Before the fencing of the Akagera and Virunga National Parks in 2014, cattle grazed and shared watering points with wild herbivores. Although these parks are now fenced, spotted hyenas cross the electric fence from the Akagera National Park to cattle farms (Field observation, 2019). We observed calves wounded around the anus and tail, and these wounds were caused by wild carnivores. These carnivores can move aborted tissue at the wildlife-livestock interface and a recent study isolated *Brucella abortus* and *Brucella suis* from lions and

hyenas in the Serengeti National Park, Tanzania (Sambu et al., 2020). *Brucella abortus* was isolated from 14 dogs in 10 brucellosis positive cattle farms (Forbes, 1990) and *Brucella* spp. were isolated from saliva, nasal discharges, and urine of dogs feeding on aborted tissue (Bicknell and Bell, 1979, Moore, 1969) and urine has been incriminated in the transmission of canine brucellosis (Serikawa and Muraguchi, 1979). In addition, occasional transmission of brucellosis through bites has been reported (Robertson, 1973). Thus, the movement of these carnivores feeding on aborted tissue and live calves and goats in both the park and cattle farms may play a role in the transmission of bovine brucellosis and other zoonotic diseases between the wildlife and livestock and vice-versa. Elsewhere in Africa, significantly higher brucellosis seroprevalences were reported in cattle in areas close to wildlife habitat compared to areas far from the home range of wildlife, i.e., in Uganda (Nina et al., 2018) and Tanzania (Assenga et al., 2016, Shirima et al., 2007). The significantly higher occurrence of brucellosis observed in districts that border national parks can be attributed to previous interactions between wildlife and livestock, and indirect interactions by carnivores and rodents. It is therefore worth further investigating the occurrence of brucellosis in buffaloes and other wild animals in Rwanda.

This study showed that the age of the cattle was a significant predictor of brucellosis seropositivity, with the medium adult age category (3 to 4 years) and the old cattle (≥ 5 years) being more affected (OR = 5, $p = 0.005$) than young animals. This finding is in agreement with other studies carried out in Rwanda (Chatikobo et al., 2008, Ndazigaruye et al., 2018), and in Uganda (Kabi et al., 2015). Animals that are kept for a longer period in the herds have more chances of exposure and acquiring brucellosis, and this translates into increased brucellosis seropositivity with increasing age. It has also been reported that *Brucella* spp. have a tropism for reproductive organs of mature female animals, and the sex hormones and erythritol produced are responsible for the survival and multiplication of *Brucella* species (Radostits et al., 2000); this contributes to the overall higher seropositivity in sexually mature females.

In this study, herds in which cattle grazed together with small ruminants were significantly more likely to be seropositive than cattle-only herds, which is consistent with similar studies that found that mixing cattle and small ruminants was a significant predictor of brucellosis

(Mugizi et al., 2015, Sagamiko et al., 2018). This suggests that small ruminants may play a role in the maintenance and persistence of brucellosis in cattle in Rwanda since the former are not vaccinated. This also indicates that there may be co-infection with *B. melitensis* and *B. abortus* in the same herd which is consistent with a recent study in South Africa that isolated both species in slaughtered cattle (Kolo et al., 2019).

A history of abortions was a significant predictor for herd-level seroprevalence, and this is in agreement with previous reports from Uganda (Kabi et al., 2015, Nina et al., 2018), and Tanzania (Asakura et al., 2018). Furthermore, this study also revealed that 98.6% of respondents did not dispose of abortuses properly and birth sites were not disinfected, which is consistent with a previous report in Nyagatare district (Ndazigaruye et al., 2018). Therefore, it is likely that there will be a continuous circulation of *Brucella* pathogens within and between herds. Various reproductive disorders that are associated with brucellosis have been reported in the cattle industry in Rwanda, including higher incidences of abortions, retained placenta, infertility of unknown origin, and longer calving intervals (Chatikobo et al., 2009). Such abortions can cause tremendous financial losses and wherever they occur in the herd, massive screening of the herd against brucellosis is very important and positive animals should be immediately slaughtered to stop the spread.

Our findings revealed that uneducated and less educated cattle keepers were significantly associated with higher herd-level seropositivity than herds whose owners had attained secondary and/or tertiary education. These findings are in agreement with those of Assenga *et al.* (2016), who reported lower *Brucella* infection exposure in the herds of educated livestock farmers (Assenga et al., 2016). Illiterate or less educated farmers are likely to be less informed or to adopt slowly to innovations, and this may be matched with poor management practices such as the hygiene of cattle and their environment, and weaker implementation of recommended control measures such as the restriction of animal movements and vaccination. Indeed, we found that among the 26 cattle herders who vaccinated their animals, 88.5% (23/26) were educated. Nevertheless, education and learning are processes, and owners with less or no education can be helped, for example, through regular consultation with professionals in

animal science. Furthermore, 78.8% (167/212) of cattle keepers had good knowledge of bovine brucellosis, which is known as "Amakore" in the Kinyarwanda language. The farmers knew that the disease is characterized by abortions and hygromas in the patellofemoral joint. However, herds belonging to such farmers had higher odds (5.5 times) of brucellosis seropositivity than herds with no awareness, which indicates that despite the knowledge, there is negligence in implementing recommended control measures such as restriction of movement and removal of seropositive animals from the herd. In contrast, several other studies reported poor knowledge of brucellosis among several cattle keepers (Ndazigaruye et al., 2018; Chaka et al., 2018), and this was associated with an increase of *Brucella* infection in herds.

The majority of cattle keepers (85.9%) did not know that brucellosis affects humans. It is therefore not surprising that 60.9% of cattle keepers mentioned that they drank boiled milk not to avoid brucellosis, but to prevent diarrhea or tuberculosis. Also, boiling milk was significantly associated with awareness of zoonotic brucellosis, and with education level. The low awareness of zoonotic brucellosis is further reflected in the observation that a high proportion (76.9%) of cattle keepers that assisted calving without wearing protective equipment or clothing—and given that manual milking was observed in almost all (98.8%) of the herds—this constitutes a high risk for cattle keepers. In congruence with our findings, low awareness of zoonotic brucellosis was also reported in more than 92.0% of cattle keepers in Ethiopia (Chaka et al., 2018), of which, most farmers did not regard exposure to abortion tissues, drinking and eating raw animal products as risk factors. In this study, boiling milk was significantly associated with awareness of zoonotic brucellosis, and with education level ($p < 0.05$). Within cattle keepers, the increase in awareness of zoonotic brucellosis or in their education level influences an increase in the number of cattle keepers boiling milk before home consumption. This finding indicates that continuing education of cattle keepers and other exposed groups on the epidemiology of zoonotic brucellosis and other zoonotic diseases should contribute significantly to preventing zoonotic diseases in humans.

3.6. Conclusions

This study confirmed that brucellosis is endemic in cattle farmed at the wildlife-livestock-human interface and found that the history of abortions and introduction of new animals into herds are the major predictors of brucellosis. Therefore, aborting cattle, and cattle for replacement should be quarantined, tested, and the positives slaughtered. The interface should be more targeted by control programs such as vaccinations, testing and slaughter, and the requirement of an annual brucellosis-free certificate for national and international trade. Most cattle keepers had low awareness of zoonotic brucellosis, and this was exemplified by them assisting calving without PPE and improper disposal of abortion tissues. This awareness should be raised among all stakeholders through education campaigns on zoonotic brucellosis. The One Health concept of involving veterinarians, environmentalists, and physicians could efficiently minimize zoonotic brucellosis, and the control of animal brucellosis would prevent the disease in humans since there is no vaccine for the latter. Further studies on brucellosis seroprevalence in wildlife, carnivores, and humans living at the interface are worthy of investigation in Rwanda.

3.7. Acknowledgments

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Chapter 4. Molecular characterization of *Brucella* spp. from seropositive herds of cattle farmed at the wildlife-livestock-human interface in Rwanda

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4.1. Abstract

Seroprevalence studies showed that brucellosis is prevalent in Rwanda, however, with no recent study on the characterization of *Brucella* spp. Therefore, the objective of this study was to characterize *Brucella* spp. in seropositive herds of cattle farmed at the wildlife-livestock-human interface. Whole blood samples (n=118), milk (n=41), and vaginal swabs (n=51) were collected from 64 seropositive herds. Samples (n=210) were inoculated onto modified Centro de Investigacion y Tecnologıa Agroalimentaria (CITA) selective medium. Cultures were analysed to detect *Brucella* using 16S-23S ribosomal DNA interspacer region (ITS), and the *Brucella* cultures were speciated using AMOS and Bruce-ladder PCR assays. *Brucella* spp. were detected in 16.7% (35/210) of the cultures established from the samples using ITS-PCR. The AMOS PCR assay identified mixed *B. abortus* and *B. melitensis* (n=6), *B. abortus* (n=7), and *B. melitensis* (n=1) from cultures from blood samples; mixed *B. abortus* and *B. melitensis* (n=1), and *B. abortus* (n=4) from cultures from milk samples; mixed *B. abortus* and *B. melitensis* (n=6), *B. abortus* (n=8), and *B. melitensis* (n=1) from cultures from vaginal swabs. Bruce-ladder PCR assay confirmed *B. abortus* and *B. melitensis* cultures. The isolation of *Brucella* spp. was significantly associated with districts with Nyagatare district having more isolates than other districts (p=0.01). This study identified for the first time in Rwanda single or mixed *B. abortus and B. melitensis* infections in cattle samples which emphasizes the need to improve brucellosis control and raise the awareness of cattle keepers, abattoir workers, laboratory personnel, and consumers of cattle products.

4.2. Introduction

Brucellosis is a widespread contagious bacterial disease of livestock, wildlife, marine animals, and humans (OIE, 2018). Brucellosis is caused by bacteria of the genus *Brucella* belonging to the family of alpha-2- *Proteobacteriaceae* (Moreno et al., 1990, Meyer and Shaw, 1920, Meyer, 1966). The genus was initially subdivided into six classical species based on their intracellular colonization and host species preference (Meyer, 1966). The six classical species include *B. melitensis* which affects primarily goats, *B. abortus* which affects cattle, *B. ovis* affecting sheep, *B. suis* affecting swine and rats, *B. canis* affecting dogs, and *B. neotomae* affecting wood rats (Alton et al., 1975, Meyer, 1966). Three classical *Brucella* species which are subdivided into biovars (bv.) include *B. abortus* with bv. 1, 2, 3, 4, 5, 6, and 9; *B. melitensis* with bv. 1, 2, and 3; and *B. suis* with bv. 1, 2, 3, 4, and 5 (Alton et al., 1988, Jones, 1967, OIE, 2018).

Brucella spp. are 96% genetically homologous (Verger et al., 1985), but can be distinguished based on their genetic polymorphisms (Cloeckaert et al., 2000, Cloeckaert et al., 1995, Halling et al., 1993, Halling and Zehr, 1990, Mercier et al., 1996). Two molecular markers (*omp2a* and *omp2b*) discovered within the outer membrane protein (*omp25*) were used in combination with restriction enzymes to differentiate *Brucella* spp. and some of their biovars (Cloeckaert et al., 1995, Ficht et al., 1990). Other *Brucella* spp. specific DNA sequences include repetitive extragenic palindromic (REP) (Mercier et al., 1996), two repeated palindromic DNA sequences (BRU: RS1, Bru: RS2) (Halling and Bricker, 1994), and the insertion sequence (IS) 711 (Halling et al., 1993). Insertion sequences are mobile genetic elements that code for proteins responsible for their transposition (Chandler, 1998). The IS711 that was first discovered in 1993 from *B. ovis* (Mayfield et al., 1988) has 35 copies of the element and is different from that of *B. abortus* which has 7 copies (Halling and Zehr, 1990). The IS711 is a unique sequence of *Brucella* spp. with multiple copies of which some occur at species and biovars-specific sites within the chromosomal locus, and this element is the basis of differentiation of the AMOS-PCR assay amongst *B. abortus* (bv. 1, 2, and 4), *B. melitensis* (bv. 1, 2, and 3), *B. ovis*, and *B. suis* bv.1 (AMOS PCR) (Bricker and Halling, 1994). Furthermore, IS711 is the basis of discrimination between

terrestrial *Brucella* spp. and biovars and vaccine strains using Bruce-ladder PCR assay (Garcia-Yoldi et al., 2006, Lopez-Goni et al., 2008). These molecular PCR assays have reduced the long procedure of conventional phenotypic characterization of *Brucella* spp. in developed countries. However, serological methods are still prevailing in most developing countries with a lack of appropriate knowledge, and biosafety facilities (Ducrotoy et al., 2017, Ducrotoy and Bardosh, 2017).

In Rwanda, the control of brucellosis falls under the animal health law which consists of regulations and procedures for reporting infectious diseases, guidelines for animal movement, and the prohibition of illegal slaughtering (Minagri, 2009). Apart from this animal health law, there is no other documentation about the brucellosis control scheme. However, routine serological testing of cattle and small ruminants is performed before important national trade for distribution to poor families by the government and non-government organizations, and during annual surveillance (once per year) in areas with high dairy production. Vaccination against brucellosis consists of administering *B. abortus* RB 51 to calves on demand by herders upon payment of RWF 500 ($ 0.5) per dose. Thus, vaccination is not systematic and coordinated at the national level. The individual seroprevalence of brucellosis in cattle varies from 2.3% to 18.4% in Rwanda (Manishimwe et al., 2015, Ndazigaruye et al., 2018, Ntivuguruzwa et al., 2020), and ranged from 6.1% to 25.0% in women with a history of abortions (Gafirita et al., 2017, Rujeni and Mbanzamihigo, 2014). However, apart from the study that characterized *B. abortus* bv. 3 from cattle in 1984 (Verger and Grayon, 1984), the current *Brucella* spp. circulating in Rwandan cattle remain unknown. The objective of this study was therefore to characterize the *Brucella* spp. that are circulating in cattle farmed at the wildlife-livestock-human interface to document the updated control scheme for brucellosis in Rwanda.

4.3. Materials and methods

4.3.1. Description of the study area

The study was conducted at the wildlife-livestock-human interface in Rwanda and described in Chapter 3 section 3.3.1. This study was conducted in six out of 30 districts of Rwanda; five of which have many dairy cattle at the proximity of national parks, and one district in Kigali city which has cattle farms in peri-urban areas. The five districts included - Nyagatare, Gatsibo, Kayonza which border Akagera National Park in the Eastern Province, Musanze district which borders Virunga National Park in the Northern Province, and Nyabihu district, which borders Gishwati-Mukura National Park in the Western Province. The sixth district, Gasabo, located in Kigali city was included to evaluate the brucellosis seropositive status of cattle in peri-urban areas, without a history of proximity with wildlife (Figure 3.1).

4.3.2. Study design and sample size

The target population (n=210) consisted of samples from all brucellosis seropositive to RBT and i-ELSIA cattle or seronegative cattle from brucellosis seropositive herds (Figure 3.1) determined in Chapter 3. The cross-sectional brucellosis seroprevalence study conducted between May 2018 to September 2019 was described in Chapter 3 section 3.3.2. The target population was all dairy herds present in the vicinity of national parks or peri-urban areas of Kigali city (Figure 3.1). During the household visit, for each selected cow, we collected clot blood (seroprevalence) and milk from lactating cows or vaginal swab from non-lactating cows. For logistic challenges encountered during sampling, no milk or vaginal swab were collected in Kayonza district instead whole blood was collected in cattle from Kayonza district and few cattle in the Gatsibo district (Table 4. 1). The samples subjected to bacteriological culture consisted of 41, 51 and 118 for milk, vaginal swabs and whole blood, respectively from seropositive herds which consisted of seropositive cattle (n=183) and seronegative cattle (n=27) (Table 4.1). Comparison between the type of samples was not analysed in this study.

4.3.3. Collection of whole blood, milk, and vaginal swabs

Animals were treated in humane care respecting their welfare. Whole blood was collected aseptically into a 4 ml vacutainer EDTA tube from the jugular or tail veins of each seropositive cattle. At least 20 ml of milk (5 ml from each teat) were collected into a 50 ml falcon tube. Each sample was labelled with the corresponding animal identification and transported chilled to the nearest campus of the University of Rwanda and kept at -20°C. Culturing of samples and DNA extraction were done in the biosafety level 3 at National Reference Laboratory, Kigali Rwanda. DNA was analyzed by PCR assays at Rwanda Agriculture Board, Department of Veterinary Services.

Table 4. 1. Bacteriology samples (n=210) from brucellosis seropositive herds in districts reported in chapter 3.

Districts	Animals	Samples selected for culturing			Sample types collected from animals in each district					
					Milk		Vaginal swabs		Whole blood	
	N	Seropositive	serone gative	Total	N	n for culturing	N	n for culturing	N	n for culturing
Gasabo	216	0	0	0	208	0	8	0	0	0
Gatsibo	226	46	4	50	118	12	92	5	33	33
Kayonza	375	84	0	84	0	0	0	0	375	84
Musanze	215	8	5	13	161	6	114	7	0	0
Nyabihu	220	5	7	12	150	6	75	6	0	0
Nyagatare	655	40	11	51	322	17	345	33	1	1
Total	1907	183	27	210	959	41	634	51	409	118

N: sample size, n: samples selected from seropositive herds of cattle for culturing from seropositive and seronegative animals in these herds.

4.3.4. Culturing

Culturing was performed as described in chapter 2.

4.3.5. Molecular methods

DNA was exacted from the bacterial cultures. *Brucella* DNA was detected using the 16-23S ribosomal RNA interspacer region (ITS) PCR specific for *Brucella.* The *Brucella* cultures were speciated using the AMOS- and Bruce-ladder PCR assays. The molecular methods were performed as described in Chapter 2.

4.3.6. Data analysis

Descriptive data were recorded and analysed in excel spreadsheets. The district of origin of samples was tested for significant associations with the prevalence of molecular detection of Brucella spp. using EpiInfo software version 7.2.4.0 at significance level of 95% and p value of 0.05.

4.4. Results

4.4.1. Culture and 16S-23S ribosomal interspacer region (ITS) PCR assay.

Of the 118 blood samples that were cultured, 14 amplified a 214 bp specific amplicon of the genus *Brucella* spp. Of the 41 milk samples that were cultured, 4 from seropositive cows and 2 from seronegative cows amplified a 214 bp sequence of the genus *Brucella* spp., respectively (Table 4. 2, Figure 4. 1). In total, 6 milk samples were positive to 16S-23S ribosomal DNA interspace region (ITS) PCR assay. Of the 51 vaginal swabs, 13 from seropositive cows and 2 from seronegative cows amplified a 214 bp sequence of the genus *Brucella* spp., respectively. The *Brucella* DNA was recovered in 15 of cultures of vaginal swabs (Table 4. 2, Figure 4. 1). Of the 183 samples from seropositive cows, 31 were identified as *Brucella* spp. Of the 27 samples from seronegative cows, 4 were identified as *Brucella* spp. In total, of the 210 samples that were inoculated onto modified CITA medium, 35 were ITS-PCR positive (Table 4. 3, Figure 4. 2). *Brucella* spp. were detected in 11.9% (14/118), 9.8% (6/41), and 29.4% (15/51) of the samples of whole blood, milk, and vaginal swabs, respectively. Altogether, *Brucella* spp. were detected in 16.7% (35/210) of seropositive herds of cattle farmed at the wildlife-livestock-human interface.

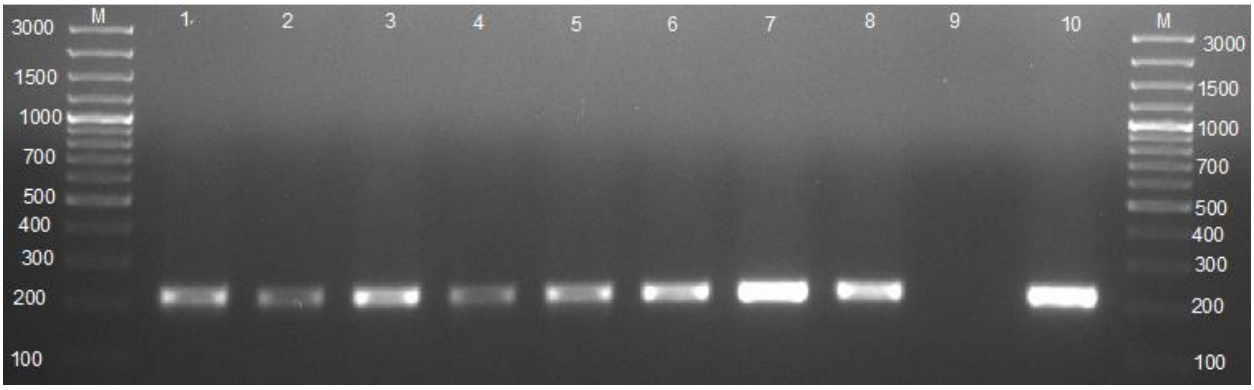

Figure 4. 1. Agarose gel electrophoresis of the 16S-23S ribosomal DNA interspace region (ITS) PCR product amplified from cultures of isolates from cattle samples from the wildlife-livestock-human interface. Lane M: GeneRuler 100 bp (Invitrogen, ThermoFischer, South Africa); lanes 1-3: *Brucella* spp. amplicon (214 bp) from whole blood; lanes 4-5: *Brucella* spp. amplicon (214 bp) from milk; lanes 6-8: *Brucella* spp. amplicon (214 bp) from vaginal swabs; lane 9: negative control containing sterile ultrapure water; lane 10: *B. abortus* bv. 2 REF 544 strain.

4.4.2. Speciation of *Brucella* spp. using AMOS PCR assay.

For whole blood, AMOS PCR assay identified *Brucella* spp. (n=14) including mixed *B. abortus* and *B. melitensis* (n=6, simultaneous amplification of 731 bp and 496 bp), *B. abortus* (n=7, amplification of 496 bp), and *B. melitensis* (n=1, amplification of 731 bp) (Table 4. 2, Figure 4. 2). For individual milk samples, AMOS PCR assay identified *Brucella* spp. (n=5) including a mixed *B. melitensis* and *B. abortus* (n=1), and *B. abortus* (n=4) (Table 4. 2, Figure 4. 2). For vaginal swabs, AMOS PCR assay identified *Brucella* spp. (n=15) including mixed *B. melitensis* and *B. abortus* (n=6), *B. abortus* (n=8), and *B. melitensis* (n=1) (Table 4. 2, Figure 4. 2).

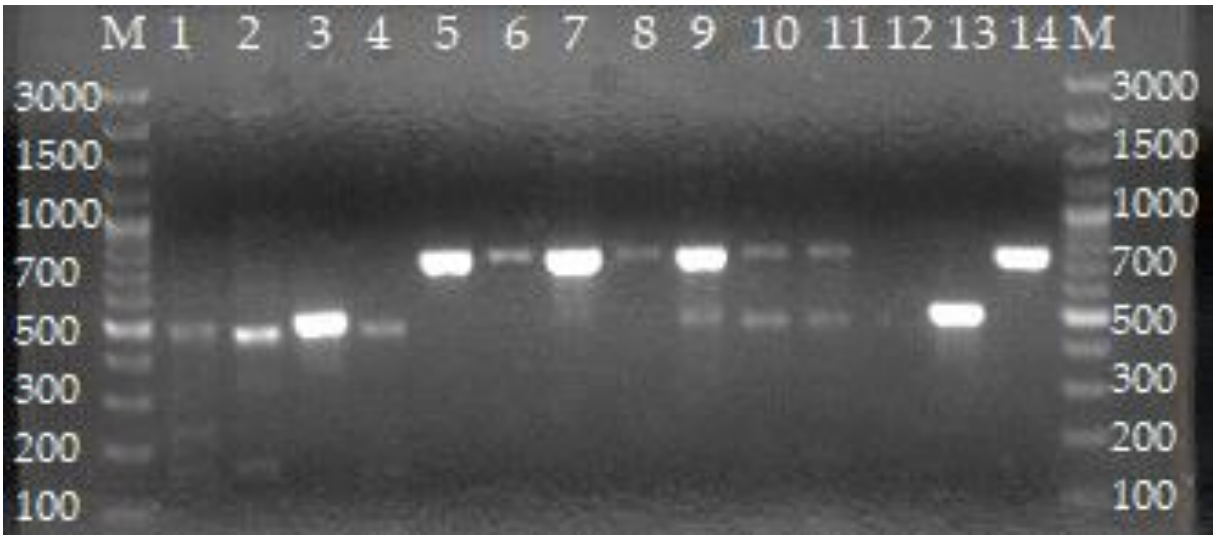

Figure 4. 2. Agarose gel electrophoresis of AMOS PCR from cultures of isolates from cattle farmed at the wildlife-livestock-human interface. Lanes M: GeneRuler 100bp (Invitrogen, ThermoFischer, South Africa). Lanes 1-4: *B. abortus*; lanes 5-8: *B. melitensis*; lanes 9-11: mixed *B. melitensis* and *B. abortus*; lane 12: negative control containing sterile water; lane 13: *B. abortus* bv. 2 REF 544 strain; lane 14: *B. melitensis* bv. 1 16M strain.

4.4.3. Distinction of terrestrial *Brucella* and vaccine strains using Bruce-ladder PCR assay.

For whole blood, Bruce-ladder PCR assay identified *B. abortus* (n=10), and *B. melitensis* (n=4) (Table 4. 2, Figure 4. 3). For individual milk samples, Bruce-ladder identified *B. abortus* (n=5) (Table 4. 2, Figure 4. 3). For vaginal swabs, Bruce-ladder identified *B. abortus* (n=10), *B. abortus* RB51 (n=2), *B. melitensis* (n=3) (Table 4. 2, Figure 4. 3).

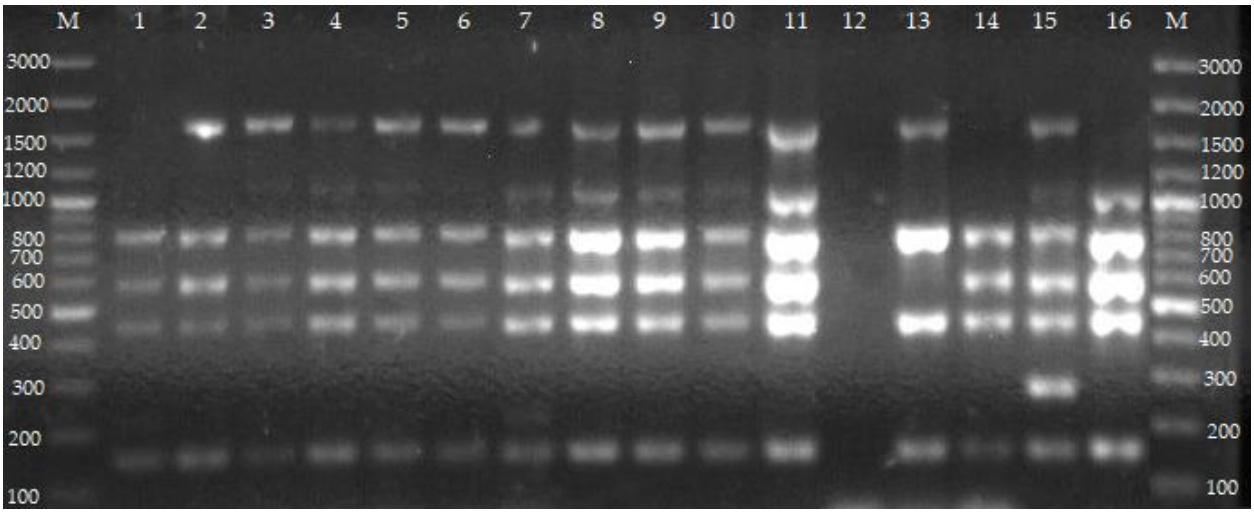

Figure 4. 3. Agarose gel electrophoresis of Bruce ladder PCR from cultures of isolates from cattle farmed at the wildlife-livestock-human interface. Lanes M: GeneRuler 100bp (Invitrogen, ThermoFischer, South Africa); lane 1: *B. abortus* RB51 from vaginal swabs; lane 2: *B. abortus* from whole blood; lanes 3-5: *B. melitensis* from whole blood; lane 6: *B. abortus* from milk; lanes 7-11: *B. melitensis* from vaginal swabs; lane 12: negative control containing sterile water, lane 13: *B. abortus* bv.1 S19; lane 14: *B. abortus* bv. 1 RB 51; lane 15: *B. suis* bv. 1 ZW 45; lane 16: *B. melitensis* bv. 1 16M strain.

Table 4. 2. Bacteriological, 16S-23S ribosomal DNA interspace region (ITS), AMOS, and Bruce-ladder PCR results of *Brucella* spp. isolated from seropositive herds of cattle farmed at the wildlife-livestock-human interface in Rwanda.

Type of samples	Seropositive cattle		Seronegative cattle		AMOS PCR assay		Bruce-ladder PCR	
	Cultured	ITS	Cultured	ITS	Pathogen	Number	Pathogen	Number
Whole blood	118	14	0	0	B.a	7	B.a	10
					B.a & B.m	3		
					B.m	1	B.m	4
					B.a & B.m	3		
Milk	28	4	13	2	B.a	4	B.a	5
					B.a & B.m	1		
Vaginal swabs	37	13	14	2	B.a	6	B.a	10
					B.a & B.m	4		
					B.a	2	RB51	2

				B.m 1 B.a & B.m 2	B.m 3	
Total	183	31	27	4		

B.a: *Brucella abortus*, B.m: *Brucella melitensis*.

The isolation of *Brucella* spp. was significantly associated with district with Nyagatare having more isolates [36.6%, (15/41)] than Nyabihu [36.4%, (4/11)], Kayonza [24.5%, (13/53)], Gatsibo [7.4%, (3/27)], and Musanze [0.0%, (0/9)] (p=0.01).

4.5. Discussion

Brucella spp. fall under category A pathogens and cause serious disease in a wide range of animals and humans (OIE, 2015, OIE, 2018). Bovine brucellosis negatively affects national economies and public health worldwide (Corbel, 2006, OIE, 2018, Young, 1995). Seroprevalence studies showed that bovine brucellosis is prevalent in Rwanda (Ndazigaruye et al., 2018, Ntivuguruzwa et al., 2020). However, serology does not provide a complete diagnosis and has drawbacks related to sensitivity and specificity (Corbel, 2006, Nielsen, 2002). Furthermore, *Brucella* spp. that are involved in *Brucella* infections remain unknown in Rwanda. This study isolated for the first time *Brucella abortus* and *B. melitensis* from blood, milk, and vaginal swabs of dairy cattle in Rwanda. some of the B. abortus isolated from vaginal swabs were identified as *B. abortus* RB51 using Bruce-ladder PCR assay. The identified *Brucella* included single and mixed *B. melitensis* and *B. abortus.*

The frequency of molecular detection of *Brucella* spp. (11.9%) from cultures of whole blood of cattle was higher than the finding (5.8%) obtained in South Africa (Caine et al., 2017), and other previous studies which did not detect *Brucella* spp. from cultures of blood (O'Leary et al., 2006, Yagupsky, 1999). This finding indicates that whole blood may be a good sample for isolation of *Brucella* spp. if processed immediately after collection (Caine et al., 2017, Gotuzzo et al., 1986). The frequency of isolation of *Brucella* spp. in 9.8% of milk samples in this study is higher than the 6.5% recovered from raw milk informally marketed on streets in Uganda

(Hoffman et al., 2016). The presence of *Brucella* spp. in milk is worrying since 21.7% of cattle keepers owning these seropositive cows reported drinking raw milk (Ntivuguruzwa et al., 2020) which might be reflected in the human brucellosis detected in Rwanda (Gafirita et al., 2017, Rujeni and Mbanzamihigo, 2014).

The isolation of *Brucella* spp. from seropositive and seronegative cows is consistent with earlier studies that also recovered *Brucella* spp. from seropositive and seronegative cows in Bangladesh (Islam et al., 2018), and China (Ning et al., 2012). The detection of *Brucella* spp. in seronegative animals may be due to the decrease of *Brucella* antibodies in seronegative and chronically diseased cows while the organism remains intracellular in different tissues (Islam et al., 2018). The detection of *Brucella* spp. in seronegative cattle indicates that serological tests such as i-ELISA with cut-off points determined in Europe where the prevalence is low or null, must be validated for Rwandan cattle. The isolation of *Brucella* spp. from the milk of seronegative cows is a problem of concern since serology is the only diagnostic method of brucellosis in Rwanda. False brucellosis seronegative dairy cows continue shedding the pathogen in milk which could be consumed unpasteurized and/or sold at the informal market (Kamana et al., 2017, Ntivuguruzwa et al., 2020). Furthermore, the traditional homemade cream milk known as "Ikivuguto" in the local language, is frequently made of raw milk by several Rwandan families (Karenzi et al., 2013). Therefore, there is a need to investigate the presence of *Brucella* spp. in the homemade cream milk "Ikivuguto" and to create an awareness of this risk in Rwanda.

The detection of *Brucella* spp. in 29.4% of vaginal swabs was higher than the 12.6% previously reported in Pakistan (Ali et al., 2014), 1.5% in Mongolia (Bayasgalan et al., 2018), and 1.1% in Nigeria (Ocholi et al., 2005). This difference may be associated with the origin of samples and in this study, samples were collected from seropositive cows farmed in high-risk zones (Ntivuguruzwa et al., 2020). In addition, the amount of *Brucella* isolation may also depend on the storage conditions and culture medium used (Falenski et al., 2011, Ledwaba et al., 2020). The isolation of *Brucella* spp. from vaginal swabs confirms that *Brucella* organisms have the tropism for the reproductive organs of mature animals and massively multiply in the presence

of reproductive hormones and erythritol (Keppie et al., 1965, Young, 1995). Therefore, this finding supports that vaginal swabs may be good specimen for rapid molecular detection of brucellosis in animals (Keid et al., 2007). It was not surprising to detect *B. abortus* vaccine strain RB51 which is the vaccine used in the vaccination of cattle in high-risk zones in Rwanda. The identification of vaccine strains RB51 from cattle farmed at the interface in Rwanda indicates that RB51 is not safe for cattle (Dougherty et al., 2013, Yazdi et al., 2009) and that vaccinating pregnant animals should be done with caution.

It is of diagnostic importance that the genus specific ITS-PCR detected *Brucella* DNA from 31 seropositive cows and 4 seronegative cows with an occurrence of 16.7% (35/210). The ITS-PCR was able to detect as little as 3.8 fg of *Brucella canis* DNA mixed with 54 ng of template canine DNA extracted from vaginal swabs of non-infected birches (Keid et al., 2007). The finding of this study confirms that the ITS-PCR can be used to detect *Brucella* spp. from vaginal swabs of animals that are seronegative, negative to blood culture, or blood PCR (Keid et al., 2007). However, there is a need to determine and validate the specificity and sensitivity for the ITS-PCR in Rwanda since closely related *Brucella* pathogens that were not analyzed by Keid et al., 2007 might be locally present and could generate false positives.

The recovery of *B. abortus* in the present study is consistent with earlier studies in the region (Hoffman et al., 2016, Mugizi et al., 2015). This finding confirms that *B. abortus* is the main causal agent of brucellosis in dairy cattle. Although, *B. melitensis* commonly cause the disease in goats, it was isolated in dairy cattle in the present study supporting the mixed farming of cattle and small ruminants that was earlier reported (Ntivuguruzwa et al., 2020). There is therefore, a need to strengthen the brucellosis control in cattle and goats as interspecies farming occurs in Rwanda. AMOS-PCR detected a mixed infection of *B. abortus* and *B. melitensis* in blood, milk, and vaginal swabs of cattle. Mixed infections of *B. abortus* and *B. melitensis* have been previously reported in herds where cattle graze together with small ruminants in South Africa, and Kenya (Kolo et al., 2019, Muendo et al., 2012). The purification of these cultures is recommended for future studies. *Brucella abortus* and *B. melitensis* cause brucellosis in cattle and humans (Corbel, 1997, Young, 1995). This is a problem of concern because diseased animals

reduce productions and *Brucella* spp. are present in the blood, milk, and vaginal secretions. This represents a great risk of contamination to handlers of live animals, carcasses, consumers of raw milk, and milk products.

4.6. Conclusions

This study identified mixed and single infections caused by *B. abortus* and *B. melitensis* from whole blood, vaginal swabs, and milk indicating the great risk of transmission to handlers of live cattle, and carcasses, and consumers of unpasteurized milk and milk products. The study also isolated the *B. abortus* RB 51 vaccine strain with Bruce-ladder PCR assay from seropositive cattle suggesting the occurrence of abortion cases in seropositive herds. Education about the epidemiology of brucellosis and other zoonotic diseases is of paramount importance to all stakeholders in the animal sector and consumers of animal products.

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Chapter 5. Characterization of *Brucella* spp., and other pathogens from aborted tissues of cattle and goats in Rwanda

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5.1. Abstract

This study aimed to characterize *Brucella* spp. from aborted tissues of cattle from five selected districts of Rwanda. For cattle, aborted tissues (n=19) were collected, cultured, and *Brucella* spp. were detected using genus specific 16S-23S ribosomal DNA interspacer region (ITS) and further speciated using AMOS and Bruce-ladder PCR assays. *Brucella* negative samples were screened using 8 abortion pathogens panel (*Anaplasma phagocytophilum*, *Bovine Herpes Virus Type 4*, *Campylobacter fetus*, *Chlamydophila* spp., *Coxiella burnetti*, *Leptospira* spp., *Listeria monocytogenes*, and *Salmonella* spp.). An abortion outbreak that occurred within a goat tribe in the Nyagatare district was included in this investigation. Sera of females (n=8), and males (n=2) were analysed using the Rose Bengal test (RBT), and indirect enzyme-linked immunosorbent assay (i-ELISA), while vaginal swabs (n=3), and aborted tissues (n=1) were cultured and molecular characterized. The ITS-PCR detected *Brucella* DNA in cultures from two aborted tissues of cattle two *Brucella* cultures [10.5%, (2/19)] which were identified as *B. melitensis* (n=1), and *B. abortus* (n=1) using AMOS and Bruce-ladder PCR assays. *Campylobacter fetus* (n=7) and *Leptospira* spp. (n=4) including co-infections (n=2) of *C. fetus* and *Leptospira* spp. were identified with the PCR panel from the *Brucella* negative samples of cattle. Goats (100.0%, 10/10) were brucellosis seropositive to RBT and i-ELISA. Mixed infection caused by *B. melitensis* and *B. abortus* were isolates from the vaginal swabs (n=3) and aborted tissues (n=1). The isolation of both *B. abortus* and *B. melitensis* indicated cross-infections and mixed livestock farming in Rwanda. This is the first identification of abortion associated pathogens (*B. abortus*, *B. melitensis*, *C. fetus,* and *Leptospira* spp.) in aborting cattle samples in Rwanda indicating the

enormous financial losses to cattle owners and a threat to public health. It is therefore essential to include these identified pathogens in the surveillance scheme of veterinary and human services, and raise the awareness of caretakers, abattoir workers, and laboratory personnel.

5.2. Introduction

Abortion is the premature expulsion of a dead foetus due to abnormalities of the reproductive tissues (Samartino and Enright, 1993). Abortions cause tremendous economic losses in food-producing animals and lead to food insecurity (Singh et al., 2015). Abortion is a clinical sign with multiple etiologies including nutritional deficiencies and infectious pathogens (da Silva et al., 2009). Infectious pathogens account for 90.0% of ruminants' abortions and these pathogens include bacteria, viruses, protozoans, and fungi (da Silva et al., 2009). Among these pathogens, the genus *Brucella* is among the major causes of infectious abortions in ruminants (da Silva et al., 2009). Other abortigenic pathogens include *Anaplasma phagocytophilum*, Bovine Herpes Virus Type 4, *Campylobacter fetus*, *Chlamydophila* spp., *Coxiella burnetti*, *Leptospira* spp., *Listeria monocytogenes*, and *Salmonella* spp. etc. (da Silva et al., 2009).

Brucella spp. are contagious pathogens causing abortions in the last term of gestation (Samartino and Enright, 1993) of domestic and wildlife animals, and humans (Corbel, 2006). Members of the genus *Brucella* and the most important species that were primarily isolated from aborting hosts. For instance, *B. melitensis* affects sheep and goats (Zammit, 1905), *B. abortus* affects cattle (Bang, 1897), *B. suis* affects pigs (Traum, 1914), and *B. canis* affects dogs (Kimberling et al., 1966). These species grow massively in the presence of erythritol, a normal constituent of amniotic fluid, leading to abortions in cows, ewes, does, and sow (Keppie et al., 1965, Pearce et al., 1962). During an abortion caused by *B. abortus*, aborted tissues contain more than 10^{14} bacteria, which is 10^5 times the estimated infectious dose of heifers vaccinated by *B. abortus* S19 (Corner et al., 1983).

Anaplasma phagocytophilum is a tick-borne alpha-proteobacterium that causes tick-borne fever characterized by fever and abortions in pregnant ruminants (Dugat et al., 2017). Bovine

herpesvirus 4 (BoHV-4) is a ubiquitous abortifacient pathogen belonging to the genus *Rhadinovirus* and causing abortion, postpartum metritis, and mastitis in cattle (Deim et al., 2007). The species *Campylobacter fetus* is a zoonotic pathogen of veterinary importance. It is divided into *C. fetus* subsp. *fetus* (*Cff*) and *C. fetus* subsp. *venerealis* (*Cfv*) (Véron and Chatelain, 1973). *Cfv* is a cattle restricted pathogen, which causes genital campylobacteriosis characterized by infertility, low conception rate, and abortions worldwide (Ishtifaq et al., 2020). *Cff* is a pathogen that cannot survive in the bovine intestine and causes reproductive disorders in sheep and cattle (Blaser et al., 2008). It is an opportunistic pathogen infecting mainly immune-compromised patients (Tremblay et al., 2003). *Chlamydia* ssp. are zoonotic, intracellular, obligate, gram-negative bacteria of the genus *Chlamydophila* causing abortions and endometritis in cattle (Godin et al., 2008, Hireche et al., 2016). *Coxiella burnetii* is a zoonotic gram-negative bacterium causing Q-fever characterized by fever, and abortion in ruminants worldwide (Frangoulidis and Fischer, 2015, Ghaoui et al., 2018). *Leptospira* spp. are zoonotic pathogenic spirochaetes of the genus *Leptospira* that cause abortion, stillbirth fetuses, decreased milk production, and low fertility (Momtaz and Moshkelani, 2012). *Listeria monocytogenes* is a zoonotic bacterial pathogen of the genus *Listeria* causing abortion and other reproductive disorders in cattle worldwide (Whitman et al., 2020). Certain members of *Salmonella enterica* subsp. *enterica* are bacterial zoonotic pathogens that cause salmonellosis in animals and humans. For instance, *Salmonella* Dublin, a cattle-adapted serotype, causes salmonellosis characterized by diarrhea, sepsis, mortality, and abortions (Hezil et al., 2021). Although these pathogens have never been reported in livestock in Rwanda, they are of considerable financial losses due to reproductive disorders.

In Rwanda, various reproductive disorders that have been reported in the cattle industry include higher incidences of abortions, retained placenta, infertility, and longer calving intervals (Chatikobo et al., 2009). Although a history of abortion was a significant predictor of brucellosis (Ndazigaruye et al., 2018, Ntivuguruzwa et al., 2020), there are several unreported cases of abortions in *Brucella* seronegative cattle. Furthermore, *Brucella* spp. or other abortigenic pathogens have never been detected from aborted tissues of cattle in Rwanda. This study

investigated therefore the presence of *Brucella* spp. and other eight pathogens from aborted tissues of cattle in Rwanda from January 2018 to October 2019.

5.3. Materials and methods

5.3.1. Description of the study area

This study was carried out in five districts of Rwanda. Three districts in the Southern Province: Kamonyi, Nyanza, and Ruhango; one district in the Northern Province: Musanze; and another district in the Eastern Province Nyagatare. The Southern Province comprises seven districts and borders the Republic of Burundi in the south. It covers an area of 5701 sq. km with 2,589,975 inhabitants. The Southern Province lies at an altitude of 1500 m to 2800 m with the temperature varying between 15°C and 28°C. Nyanza is a district of this Southern Province with a high production of milk. Cattle are mostly kept in a zero-grazing system. The Northern Province borders the Democratic Republic of Congo in the north and covers an area of 3293.3 Km^2. The province harbors mountains and volcanoes with good weather and comprises five districts and among them, Gicumbi is the basin of milk production. The Eastern Province is the largest and borders Uganda in the north and Tanzania in the east. The weather is warmer and drier, with yearly average temperatures varying between 20°C and 21°C, and characterized by yearly average precipitations fluctuating between 700–950 mm, while the flora is savanna with lowly inclined hills and an average altitude of 1513.5 m. The province is home to 40.0% of the national cattle population which was estimated to be 426,508 cattle in 2017. Cattle are mostly kept in the free-grazing system (Figure 5. 1). Bovine and caprine populations in Rwanda were estimated at 1,293,768 and 2,731,795 in 2018, respectively (Minagri, 2019).

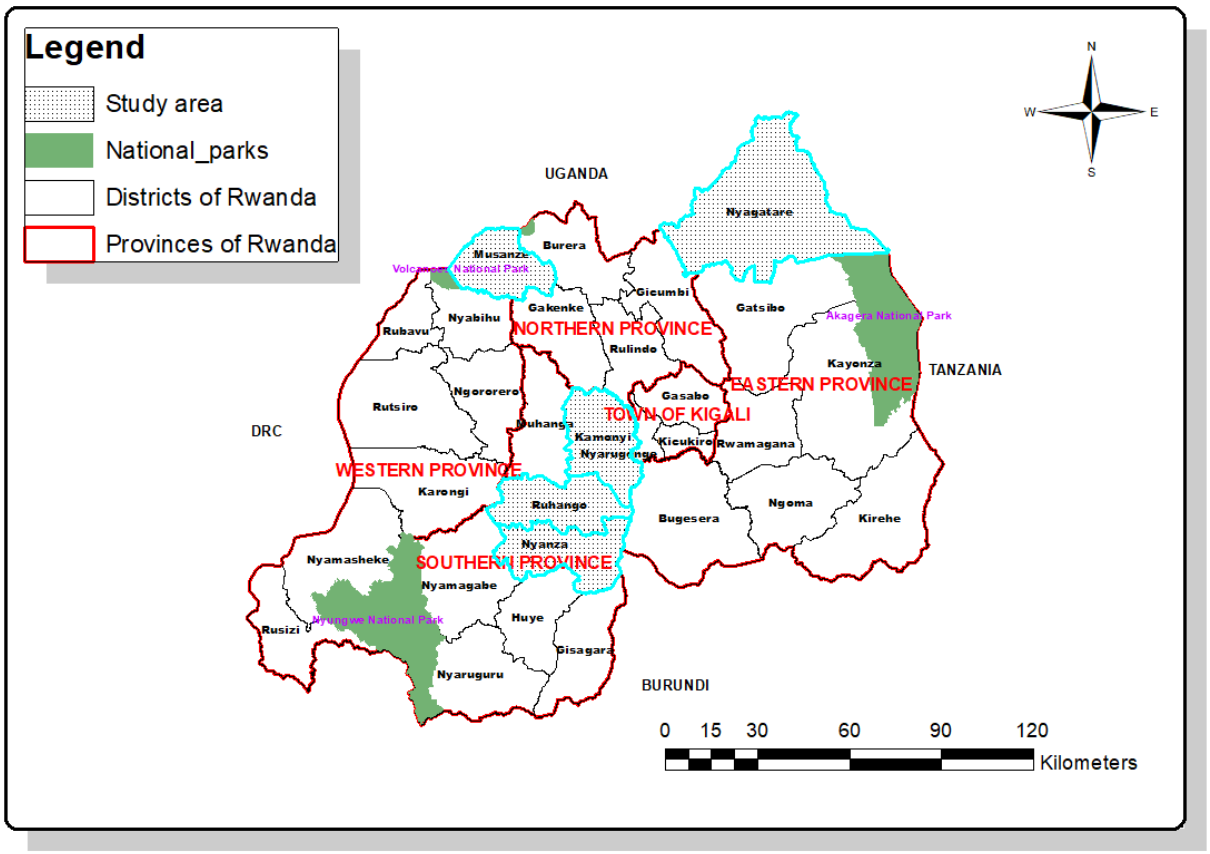

Figure 5. 1. Map of Rwanda with provinces and districts with the blue outlined districts indicating the origin of the samples from aborted animals.

5.3.2. Study design and sample size

A cross-sectional study was carried out from January 2018 through October 2019. The purpose of the study was explained to veterinarians of districts and sectors that were trained on biosafety and sample collection of aborted tissues comprising of cotyledons, amniotic fluid, and foetal lungs when available. The study population was cattle that aborted in the district areas (Figure 5.1). For cattle, aborted tissues (n=19) comprising of cotyledons and amniotic fluid were collected in the five districts. During the investigation, an abortion storm outbreak occurred in a flock of goats in the Nyagatare district and samples were included in this study. Samples of blood (n=10), cotyledons, and amniotic fluid (n=1), and vaginal swabs (n=3) were collected from the goats.

5.3.3. Blood and tissue collection

Aborted tissues comprised of cotyledons and amniotic fluids were collected from the aborting cattle into the sterile 50 ml tubes and double sealed in recyclable plastic bags by trained veterinarians wearing gloves, masks, and overall. Samples were kept in a cool box containing ice bags and quickly transported to the nearest laboratory of the University of Rwanda for storage at -20°C until further processing. Animals were treated in humane care respecting their welfare. Blood (4 ml) was aseptically collected from the jugular vein of goats into the plain vacutainer tubes by trained veterinarians. The tubes were transported to the nearest laboratory of the University of Rwanda and incubated overnight at room temperature to allow the serum to separate. The serum was collected into 2 ml vials and stored at -20°C until further testing.

5.3.4. Serological tests

The goat serum (n=10) was tested using the Rose Bengal test (RBT) and the iELISA as described in Chapter 2.

5.3.5. Culturing

Aborted tissue and vaginal swab samples were processed and cultured in a biosafety level 3 at the National Reference Laboratory (NRL), Rwanda biomedical center, Kigali Rwanda as described in Chapter 2.

5.3.6. Molecular methods

DNA was exacted from the bacterial cultures. *Brucella* DNA was detected using the 16-23S ribosomal DNA interspacer region (ITS) PCR specific for *Brucella.* The *Brucella* cultures were speciated using the AMOS- and Bruce-ladder PCR assays. The molecular methods were performed as described in Chapter 2.

5.3.6.1. Abortion PCR panel

Brucella negative DNA were screened for *Anaplasma phagocytophilum*, Bovine Herpes Virus Type 4, *Campylobacter fetus*, *Chlamydophila* spp., *Coxiella burnetti*, *Leptospira* spp., *Listeria monocytogenes*, and *Salmonella* spp. at the Molecular Diagnostics Services (Pty) Ltd (MDS), Westville, South Africa, using a PCR panel.

5.3.6.2. Data analysis

The proportions of positivity were calculated by dividing the total number of positive animals by the total number of sampled animals. Data were recorded in Microsoft Excel spreadsheets. Epi-Info 7 version 10 was used to calculate proportions. Individual risk factors and positive results were determined for significant levels at 95% confidence intervals and statistical significance at $p < 0.05$ using the chi-square test.

5.4. Results

5.4.1. Brucellosis from aborted tissues and culture of cattle

Out of 19 aborted tissues of cattle, 10.5% (2/19) were from Kamonyi district, [5.3% (1/19)] were from Musanze district, [26.3%, (5/19)] were from Nyagatare district, [47.4%, (9/19)] were from Nyanza district, and [10.5%, (2/19)] were from Ruhango district. These aborted tissues were cultured onto modified CITA medium and *Brucella* specific ITS PCR detected 10.5% (2/19) *Brucella* DNA (amplification of a 214 bp sequence, Figure 5. 2). *Brucella melitensis* (n=1), and *B. abortus* (n=1) were detected by AMOS and Bruce-ladder PCR assays (Figures 5.3; 5.4). In total [10.5%, (2/19)] DNA from aborted tissues of cattle were identified as *Brucella* spp.

5.4.2. Identification of pathogens from *Brucella* negative aborted tissues of cattle using a PCR panel

Campylobacter fetus (n=7) and *Leptospira* spp. (n=4) with two cases of co-infection caused by *C. fetus* and *Leptospira* spp. were identified from the non-*Brucella* abortion samples (n=17) using the PCR panel.

5.4.3. Brucellosis of goat's tribe with storm abortion

The abortion storm outbreak occurred in a tribe of 40 dams and 3 males in Rwimiyaga sector, Nyagatare district in June 2019. At the time of the visit, abortion had occurred in 16 dams while the other seven were monitored of which one aborted in our presence. The incidence of abortion was 60.0% (24/40) of the pregnant dams. Samples from 8 female and 2 male goats were RBT and i-ELISA positive (100.0%, 10/10).

The ITS-PCR amplified 214 bp *Brucella* DNA for cultures established from the aborted goat tissue (n=1) and vaginal swabs (n=3) (Figure 5. 2). The AMOS PCR detected *B. melitensis* and *B. abortus* with 731 bp and 498 bp amplification bands, respectively, from cultures DNA isolated from aborted tissue (n=1) and vaginal swabs (n=3) of goats (Figure 5. 3, lanes 4-5).

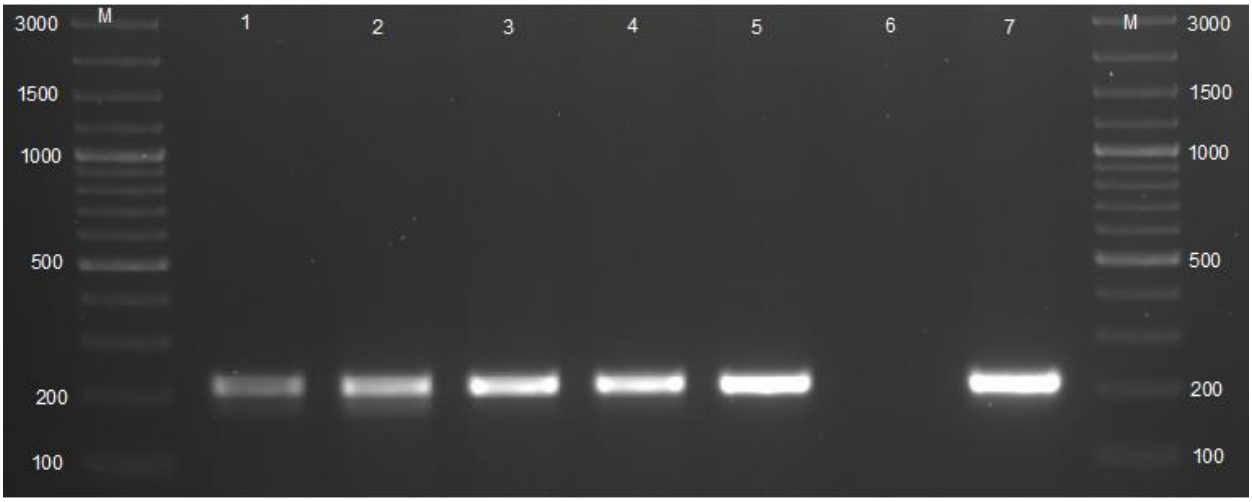

Figure 5.2. Agarose gel electrophoresis of the 16S-23S ribosomal DNA interspacer region ITS PCR products amplified from isolates from aborted tissues of cattle and goat. Lanes M: DNA GeneRuler 100 bp (Invitrogen, ThermoFischer, South Africa); lanes 1-2: isolates from aborted tissues from cattle, lanes 3-5: isolates from aborted tissues from goats (amplification of a 214 bp specific *Brucella* DNA region), lane 6: negative control, sterile water; lane 7: positive control, *B. abortus* RB51.

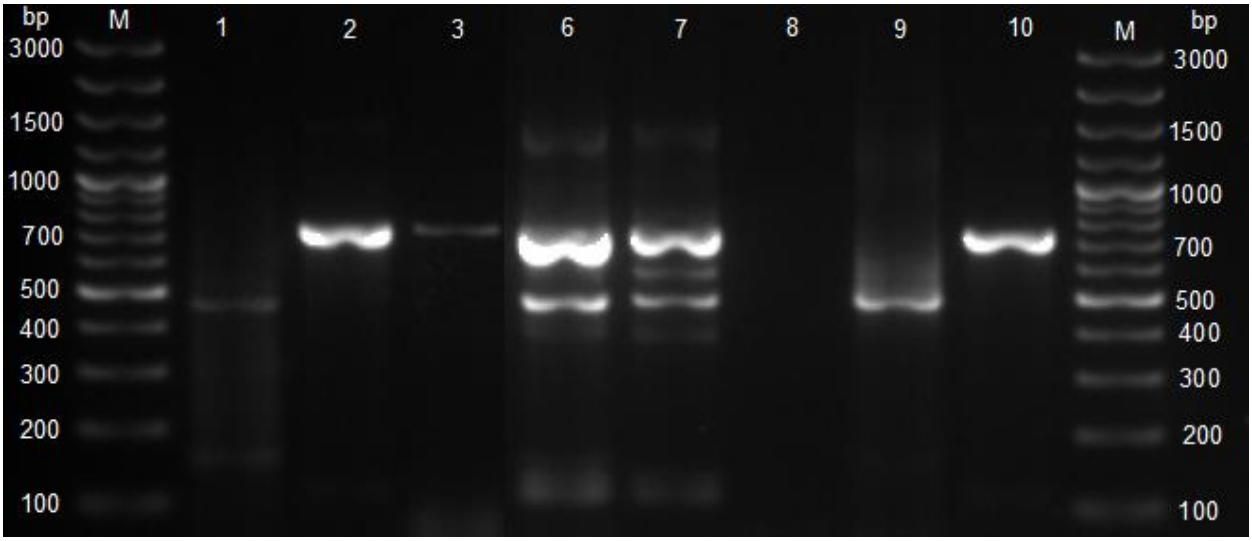

Figure 5.3. Agarose gel electrophoresis of AMOS PCR products amplified from *Brucella* cultures isolated from tissues of cattle and goats. Lane M: GeneRuler 100 bp (Invitrogen, Pretoria, South Africa). Lanes 1: *Brucella abortus* 498 bp amplicon from cattle, lane 2: *B. melitensis* 731 bp from

cattle, lane 3: *B. melitensis* 731 bp from goats, lanes 6-7 with mixed infection of *B. melitensis* 731 bp and *B. abortus* 498 bp from goat samples, lane 8: negative control sterile water, lane 9: *B. abortus* RB51 strain, lane 12: *B. melitensis* rev 1 strain.

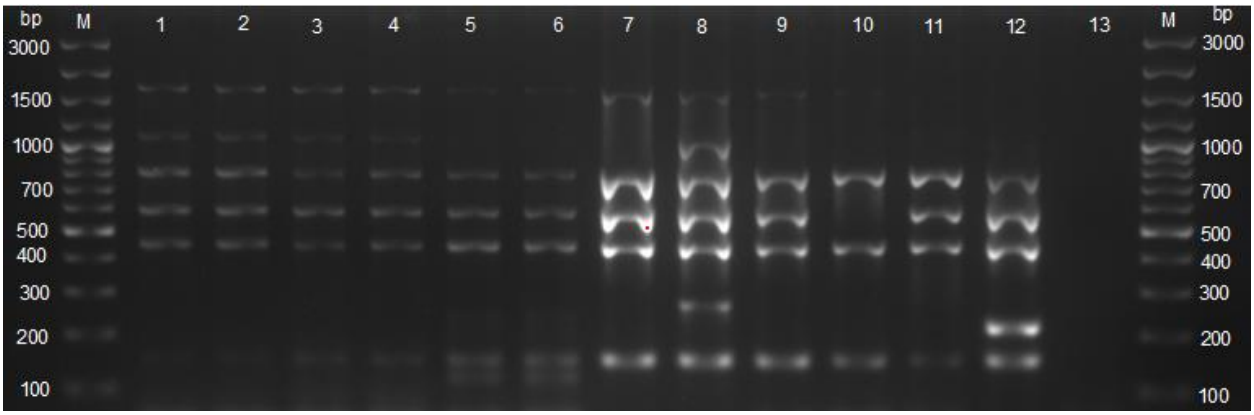

Figure 5.4. Agarose gel electrophoresis of Bruce-ladder PCR products amplified from cultures and tissues cattle and goats. Lane M: GeneRuler 100 bp (Invitrogen, ThermoFischer, Pretoria, South Africa), Lanes 1-4: *Brucella melitensis*, lanes 5-7: *B. abortus*, lane 8: *B. suis* ZW45 strain, lane 9: *B. abortus* bv. 2 REF544 strain, lane 10: *B. abortus* S19 strain, lane 11: *B. abortus* RB51 strain, lane 12: *B. melitensis* rev 1 strain, lane 13: Negative control, sterile water.

5.5. Discussion

This study is the first report of *B. abortus*, and *B. melitensis* confirmed with PCR assays from aborted tissues of cattle in Rwanda. This is also the first identification of *C. fetus*, and *Leptospira* spp. from aborted tissues of *Brucella* negative samples of cattle using multiplex PCR assays. This study also reports for the first time in Rwanda a mixed infection of *B. abortus* and *B. melitensis* isolated from aborted tissues and vaginal swabs collected during an abortion outbreak of goats in the Nyagatare district in June 2019. The identified abortigenic pathogens caused considerable financial losses to the animal owners and threaten their public health.

The causes of abortions include stress (Garcia-Ispierto and López-Gatius, 2019, Roth, 2020), nutritional disorders (Akar and Yildiz, 2005), and infectious pathogens such as fungi,

viruses, protozoans, and bacteria (Barkallah et al., 2014, Leaver and Hart, 1960). Abortions due to brucellosis led to the decline of milk production in 1952 with USD 400 million of losses in the USA (Acha and Szyfres, 2003) and negatively affect the livelihood of small farmers in Sub-Saharan Africa (McDermott et al., 2013). A comparison study of brucellosis seropositive and seronegative pregnant cows in southern Sudan showed that seropositive cows had about 10.0% fewer calves than seronegative cows and abortion occurred in 22.0% of seropositive versus 11.0% of seronegative cows (McDermott et al., 1987). Infectious pathogens contribute to 90% of ruminant abortions and the genus *Brucella* is among the major bacteria that cause abortions in livestock (da Silva et al., 2009). This study isolated *Brucella* spp. in 10.5% of aborted tissues of cattle.

The absence of *Brucella* spp. in many aborted tissues of cattle led to the screening of *Brucella* negative samples using eight abortion pathogens panel which identified *C. fetus*, and *Leptospira* spp. were identified. This is the first evidence of *C. fetus* and *Leptospira* spp. in aborted tissues of cattle in Rwanda. Infections caused *Campylobacter* spp. and *Leptospira* spp. have been reported in animals and humans in neighbouring Tanzania (Allan et al., 2020, Gahamanyi et al., 2020), and Uganda (Alinaitwe et al., 2019). This finding calls for active surveillance of genital campylobacteriosis and leptospirosis in aborting cattle, and occupational groups including animal caretakers and abattoir workers in Rwanda.

The combination of AMOS and Bruce-ladder PCR assays provides cohesive findings because AMOS does not identify all *Brucella* species and biovars but identifies mixed infections whereas Bruce-ladder will identify all *Brucella* species and biovars but does not detect mixed infections due to the multiple banding patterns of Bruce–ladder PCR assay. With the Bruce-ladder PCR assay mixed infections of *B. abortus* and *B. melitensis* will not be detected as *B. melitensis i*s identified by 152, 450, 587, 794, 1071 and 1682 bp bands while *B. abortus* is identified by 152, 450, 587, 794 and 1286 bp bands and thus the absence of 1071 bp band (Garcia-Yoldi et al., 2006). The present study identified mixed infections caused by *B. melitensis* and *B. abortus* in goats in Rwanda (Figure 5.3). *Brucella abortus* has been previously reported in goats in Mexico, and Egypt (Leal-Klevezas et al., 2000, Wareth et al., 2015) as well *B. melitensis* in aborting goats

in neighboring Uganda (Bruce et al., 1910, Philpott and Auko, 1972), Tanzania (Philpott and Auko, 1972), and Kenya (Muendo et al., 2012, Philpott and Auko, 1972). The mixed infections by *B. melitensis* and *B. abortus* in all the four abortion samples of goats indicates the cross-infections and herding different animal species on the same farm (Ocholi et al., 2005). Goats of the present study shared the same grazing pasture with cattle. In the present study, males of the flock were also seropositive to brucellosis, and this contributed to the propagation of disease in the whole tribe.

The introduction of caprine brucellosis in Rwanda may be associated with uncontrolled repatriation of Rwandans and their livestock from Uganda, and Tanzania in 1994, or importation of chronically diseased local goats, and Galla goats from East African countries and other countries for the distribution to poor families in Rwanda. Although, livestock are screened for brucellosis before importation, animals in early incubation, or chronically diseased may be seronegative due to the decline of antibody titres but remaining bacteriologically positive (Morgan and McDiarmid, 1960, Nicoletti and Muraschi, 1966, Zowghi et al., 1990). Therefore, screening before importation followed by quarantine and second serological screening would guarantee the brucellosis-free status.

Caprine brucellosis constitutes a public health concern as *B. melitensis* causes severe disease in humans (Bruce, 1887, Wallach et al., 1997). A study in Uganda has demonstrated a significant association between caprine brucellosis and brucellosis in owners of goats (Miller et al., 2016). It was thought for a long time that there is no brucellosis in goats in Rwanda, and the vaccination program against brucellosis targets exclusively cattle (Minagri, 2019). The discovery of *B. melitensis* and *B. abortus* in goats has public health implications since there exits few households still sleeping in the same house with their goats to prevent stealing and this may favor inhalation of *Brucella spp.* aerosols if ventilation is not sufficient in the houses (Kaufmann et al., 1980). In addition, there was a significant association between caprine and bovine brucellosis in Uganda; and goats play an important role in the transmission of brucellosis to cattle in Uganda (Miller et al., 2016).

This study isolated *B. melitensis*, and *B. abortus* in aborted tissues of cattle. A similar study in India isolated *B. abortus* and *B. melitensis* from different reproductive tissues of buffaloes, cows, does, and ewes (Verma et al., 2000). The proportion of isolation of *Brucella spp.* from aborted tissues of cattle (10.5%) obtained in the present study is comparable with serosurvey studies that reported abortions in 16.2% of seropositive cattle in Zambia (Muma et al., 2007), and 13.8% found in Ethiopia (Megersa et al., 2011).

The abortion cases reported in the present study caused tremendous financial losses in the livestock industry in Rwanda. The vaccination against brucellosis that is currently only focused on cattle should be expanded to include goats and sheep preferably in systematic and coordinated manner. The control program against brucellosis should focus on the hygiene of the animal environment, provision of separate maternity, early weaning, and before introduction into the herd or flock, animals should be screened using both buffered agglutination test like RBT and a confirmation test either ELISA or complement fixation test to distinguish early and latent infections. Any abortion case should be reported to the competent authority and the herd or flock should be massively screened against brucellosis, and the positive animals should be immediately slaughtered to stop spreading. *Brucella* negative animals should be screened for other abortigenic pathogens such as *C. fetus*, *Leptospira* spp. that were detected in this study.

5.6. Conclusions

This study identified for the first-time *B. melitensis*, *B. abortus*, *C. fetus*, and *Leptospira* spp. from aborted tissues of cattle. Mixed infections caused by *C. fetus* and *Leptospira* spp. were recorded in cattle indicating the severity of abortion in the herd. Co-infections of *B. melitensis* and *B. abortus* in aborted tissues of goats indicated cross-infection in cattle and goats. These abortions caused tremendous financial losses in the livestock industry in Rwanda. Any abortion case should be reported to the competent authority and the herd or flock should be massively screened against brucellosis, and the positive animals should be immediately slaughtered to

stop spreading. It is also important to screen all *Brucella* seronegative animals for other abortigenic pathogens to control ruminant abortions in Rwanda.

5.7. Acknowledgments

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Chapter 6. Assessment of the awareness and occupational exposure to brucellosis and other zoonotic diseases among abattoir workers in Rwanda

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6.1. Abstract

Brucellosis and bovine tuberculosis (bTB) are endemic in Rwanda, but little is known about the diseases among abattoir workers. A cross-sectional study was conducted to investigate the awareness, practices, and history of the diseases among 100 abattoir workers from four high throughput and 18 workers from two low throughput abattoirs. Data were collected by face-to-face interviews using a questionnaire, and exposure and outcome variables were analyzed for univariate and correspondence associations. It was found that 82.2%, 27.1%, 8.5%, 10.2%, and 12.7% of workers were familiar with tuberculosis, brucellosis, Q-fever, leptospirosis, and cysticercosis, respectively. The awareness for brucellosis was significantly higher in workers from low than high throughput abattoirs. There were poor practices of not wearing gloves (9.3%), overhead covers (1.7%), facemasks (0.0%), safety goggles (0.0%), eating at work (28.0%), and working with bare and injured hands (39.8%) which constitute a risk of exposure to zoonotic diseases. Transporters of carcasses, butchers, and workers with 3 years' experience and above were more likely to get sick than other groups. Abattoir workers encountered diarrhea (5.9%). Abattoir workers also reported abortion (0.9%), orchitis (0.9%), fever (5.1%), fatigue (11.0%), flu (9.3%), headache (5.1%), and nephritis (3.4%) which are common symptoms of brucellosis, leptospirosis, and Q-fever. The awareness of zoonotic brucellosis and tuberculosis was highest in educated workers indicating the need for education. Abattoirs are places for detection of zoonotic infectious diseases; thus, routine sampling and

testing of slaughtered animals and abattoir workers is needed for surveillance and control of zoonotic diseases.

6.2. Introduction

Brucellosis and bovine tuberculosis (bTB) are widespread bacterial diseases of a wide range of mammals, including humans especially those coming in close contact with animals or animal by-products (Corbel, 2006, Michel et al., 2010). The human brucellosis cases were estimated to be 830,000 worldwide in 2010 and 47.0% of these were foodborne cases (Kirk et al., 2015), while the World Health Organization reported 147, 000 cases of human tuberculosis (TB) due to *M. bovis* with 12,500 deaths in humans (WHO, 2017). Both diseases are neglected and endemic in sub-Saharan African countries including Rwanda where they cause disease burden in humans (Gafirita et al., 2017, Marcotty et al., 2009, Rujeni and Mbanzamihigo, 2014) and significant financial losses in animals (Habarugira et al., 2014, McDermott et al., 2013).

Zoonotic brucellosis is mainly caused by *Brucella melitensis* (Bruce, 1887), *B. abortus* (Spink and Thompson, 1953), *B. suis* (Forbes, 1991), and *B. canis* (Lucero et al., 2010). Zoonotic tuberculosis is mainly caused by *M. bovis* (Cosivi et al., 1998), and occasionally by *M. caprae* (Cvetnic et al., 2007, Prodinger et al., 2014), which belong to the Mycobacterium tuberculosis complex (MTBC) (Brosch et al., 2002). About 10.0-15.0% of human TB cases due to *M. bovis* occur in developing countries, where the test-and-slaughter control method is expensive and therefore not practiced (Cosivi et al., 1998, de la Rua-Domenech, 2006). Furthermore, in developing countries, zoonotic tuberculosis may be underestimated due to the lack of distinction between *M. bovis* and *M. tuberculosis* during diagnosis (Cosivi et al., 1995, Wedlock et al., 2002).

Animals are the reservoirs of brucellosis in humans, who contract the disease either directly by inoculation of the pathogen from infective animal tissues through cuts or skin abrasions, by ingestion of contaminated animal products (raw milk, and milk products), and inhalation of contaminated aerosols (Osoro et al., 2015, Sadler, 1960). The clinical signs of

human brucellosis, mimic acute febrile illnesses such as those of malaria, typhoid, and rheumatic fever and thus, lead to misdiagnosed and underdiagnosed cases of brucellosis (Crump et al., 2013). Brucellosis is an occupational disease affecting owners and keepers of animals, laboratory technicians, butchers, veterinarians, and abattoir workers (Aworh et al., 2013, Mirambo et al., 2018). The bTB is transmitted to humans through inhalation of infective particles from diseased cattle or through the ingestion of contaminated animal products such as unpasteurized milk and undercooked meat and is also an occupational disease affecting similar occupations as brucellosis (Cosivi et al., 1998, Thoen et al., 2014). *Mycobacterium bovis* - infected humans can transmit to other humans by the aerogenous route (Acha, 1987). However, human-to-human transmission is rare and limited to some circumstances.

For this study, other zoonotic diseases refer to leptospirosis, Q-fever, and cysticercosis. Leptospirosis and Q-fever are acute febrile diseases of both humans and animals and are caused by *Leptospira* spp. and *Coxiella burnetii*, respectively (Angelakis and Raoult, 2010, De Vries et al., 2014). The diseases are mainly transmitted to humans through contact with infectious tissues and the ingestion of contaminated food and water (Angelakis and Raoult, 2010, Bharti et al., 2003). However, these diseases have not yet been reported in Rwanda. Cysticercosis is a parasitic disease caused by the cysticerci of *Taenia saginata*, which is a human cestode (Karshima et al., 2013). Humans get infections by eating raw or undercooked beef (Cheruiyot and Onyango-Abuje, 1984).

In Rwanda, the seroprevalence of brucellosis ranges from 7.4% to 18.7% in cattle (Ndazigaruye et al., 2018, Ntivuguruzwa et al., 2020) and 6.1 to 25.0% in humans (Gafirita et al., 2017, Rujeni and Mbanzamihigo, 2014). The prevalence of bTB was 0.5% in slaughtered cattle (Habarugira et al., 2014), while the prevalence of *T. saginata* cysticerci, was 3.0% in slaughtered cattle (Nzeyimana et al., 2015) and 21.8% in people with epilepsy in Rwanda (Rottbeck et al., 2013). However, no information is available on abattoir workers, who are usually at high risk of exposure to zoonotic diseases (Awah Ndukum et al., 2010, Mirambo et al., 2018). In this study, abattoir-related-diseases are defined as diseases of animals that are transmissible to humans and these included febrile-like illnesses (brucellosis, leptospirosis, and Q-fever), tuberculosis,

and cysticercosis. The objective of this study was therefore, to investigate the awareness of zoonotic brucellosis transmission, zoonotic tuberculosis transmission, having heard of leptospirosis, Q-fever, and cysticercosis and assess occupational hazards among abattoir workers in Rwanda. The data from this study can be used by the competent authorities to raise the awareness level and attend to the preventive needs of abattoir workers.

6.3. Materials and methods

6.3.1. Study area

This study was conducted with 100 workers in four high throughput and 18 workers in two low throughput abattoirs. The six abattoirs are located in six of the 30 districts of Rwanda. In all the selected premises, cattle were the main-slaughtered animals. The cattle population in Rwanda was estimated to be 1,293,768 in 2018 (Minagri, 2019) and the current human population is 12,952,218 (Worldmeter, 2020). There are 12 modern abattoirs and other traditional slaughterhouses (unknown number) in each of the 30 districts of Rwanda. Of the modern abattoirs, 11 slaughter domestic ruminants (goats and cattle), while one slaughters only pigs. The selected abattoirs are shown in Figure 6. 1.

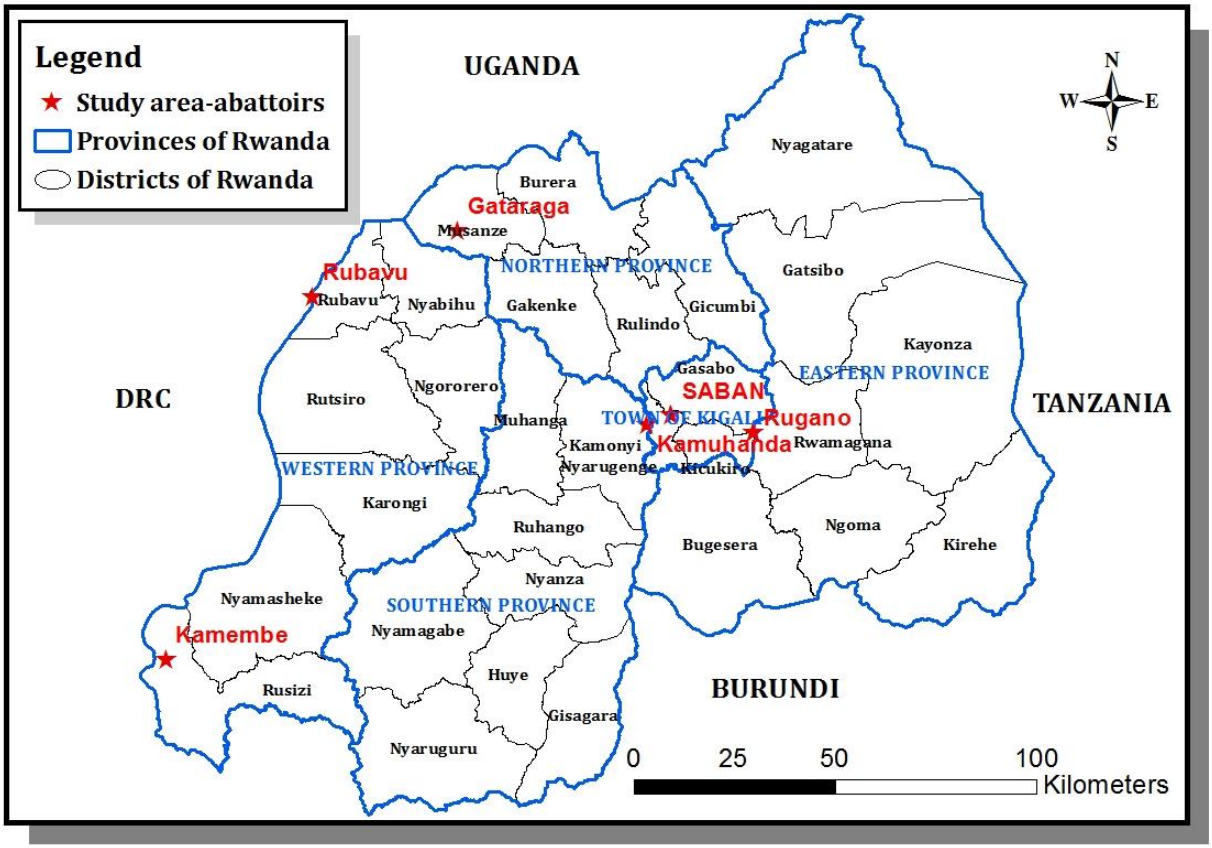

Figure 6. 1. Map of Rwanda showing the districts of Rwanda and the abattoirs and that were visited in this study

6.3.2. Study design and sample size determination

The study was a cross-sectional design, carried out from August 2018 through September 2019, with the aim of assessing the awareness of brucellosis, tuberculosis, and other zoonotic diseases among abattoir workers, and determining the knowledge, attitudes, and practices that pose risks of acquiring zoonotic diseases among workers. Abattoirs that consented to participate in this study were purposively selected. The selected abattoirs were classified as high throughput because they slaughtered more than 50 animals per day and low throughput for those that slaughtered 10 to 30 animals per day. Three of the six abattoirs slaughtered cattle and goats in the same building (n=2) and separate buildings (n=1), while the other three slaughtered only cattle. The sample size of the interviewed abattoir workers was determined using the formula previously described (Dohoo et al., 2009):

$$Sample\ size = \frac{Z^2\ \ P(1-P)}{d^2}$$

Where p is an expected prevalence of 10% based on a previous study in cattle in Rwanda (Chatikobo et al., 2008). A 95% confidence level (z) and a precision of 5% (d) were considered. The total sample size was estimated to be 138 abattoir workers but only 118 consented to participate in the study. In this study, people who were not abattoir employees but doing permanent jobs at abattoirs were also included in the study. These included sellers of viscera and cattle keepers. Each participant who consented to participate in the study was enrolled.

6.3.3. Participant's enrolment and data collection

All adult abattoir workers (from 18 years and above) of both genders, and working in the abattoir for at least one year, were listed in the sampling frame. The purpose of the study and implications of participating were explained to the abattoir workers. The participants were allowed to ask questions and to voluntarily agree to participate in the study by signing the consent form. Workers who needed further assistance to understand the consent form were given chance to get appropriate interpretation by their trusted colleagues. The questionnaire was pre-tested with five abattoir workers who were not included in the final interviews, and the questionnaire was adjusted accordingly. Data were collected in face-to-face interviews using a questionnaire. The interviews were administered in the participants' language "Kinyarwanda" by the primary investigator, and each interview took about 10 minutes. The collected data included demographic information of respondents (age, sex, marital status, education level, occupation, and duration at work). Other potential risk factors that were assessed included the awareness of brucellosis, tuberculosis, and other zoonotic diseases, and attitudes and daily practices at the abattoirs. The major risk factors assessed were wearing personal protective equipment (PPE), hand cuts, washing hands, eye splashes, and eating when working. As mentioned earlier, abattoir-related-diseases are defined as diseases of animals that are transmissible to humans and these included febrile-like illnesses (brucellosis, leptospirosis, and

Q-fever), tuberculosis, and cysticercosis. Thus, the awareness of other zoonotic diseases such as leptospirosis, Q-fever, and cysticercosis were also assessed.

6.3.4. Data analysis

The data were managed in Microsoft Excel spreadsheets and grouped into two categories. The first category comprised potential exposure variables, including kind of job, education level, duration at work, and cutting hands. The second category comprised likely outcome variables, including illness in the last five years, contracting diseases from abattoirs, becoming seronegative to malaria while presenting fever, awareness of zoonotic tuberculosis, and zoonotic brucellosis. Descriptive statistics were conducted to determine the proportions of abattoir workers by demographic information and each potential risk factor, as well as likely outcome from the survey. Univariate analyses were performed to assess pairwise associations between the potential exposure variables and likely outcome variables using the Chi-square test. Subsequently, significant variables from univariate analysis ($p<0.05$) were included in a correspondence analysis to explore the proximal associations between variables, namely education level, awareness about brucellosis or TB, years of work, occupation, and history of personal illness and hand-cut, and these relationships were visualized on a two-dimensional axis. Eigenvalues were estimated to determine the amount of variances expressed by each of the correspondence axis and the number of dimensions to retain. The asymmetric plot was generated to determine the distance between the variables. Data analysis was done using EpiInfo software version 7.2.3.1 and the R software version 4.0 (RCoreTeam, 2020) at a 5% level of significance. The coordinates of each slaughtering premises were recorded using a geographic positioning system (GPS) device (Garmin etrex 10, Lexena, KS, USA) and analyzed to produce a map using ArcGIS software version 10.2.

6.4. Results

6.4.1. Demographic information

Out of 180 abattoir workers (65.6%, 118/180) were interviewed, and of these, 84.7% (100/118) were from high throughput, while 15.3% (18/118) were from low throughput abattoirs (n=18). Among the respondents, 89.0% (105/118) were males, while 11.0% (13/118) were females. Most respondents 56.8% (67/118) were of the age category 18 - 30 years, followed by 40.7% (48/118) of the age category 31-60 years, and the old group (above 60 years) accounted for 2.5% (3/118) (Table 6. 1). The proportion of respondents according to their level of education is as follows: primary education 67.8% (80/118), secondary education 18.6% (22/118), and no formal education 13.6% (16/118). Respondents that were married accounted for 61.9% (73/118), followed by singles 36.4% (43/118), and divorced 1.7% (2/118). Of the respondents, butchers 52.6% (61/118), followed by the group of "others" (comprising of cleaners, permanent sellers of viscera, and animal keepers) 18.6% (22/118), transporters 15.5% (18/118), and inspectors 14.7% (17/118). The respondents with three years and longer of work experience were 67.8% (80/118), followed by those with one year-experience 24.6% (29/118), and the least were those with two years 7.6% (9/118). Respondents that worked on cattle were 79.7% (94/118), while 13.6% (16/118) worked on goats, and 6.8% (8/118) worked on both cattle and goats (Table 6. 1).

Table 6. 1. Sociodemographic information of abattoir workers participating in the study in Rwanda

Variables	Interviewed	Frequency n (%)	95% CI
Gender			
Male	118	105 (89.0)	[81.9 - 94.0]
Female		13 (11.2)	[6.0 - 18.1]
Age category			
Young (18-30)	118	67 (56.8)	[47.3 - 65.9]
Medium age (31-60)		48 (40.7)	[31.7 - 50.1]
Old ≥ 61		3 (2.5)	[0.5 - 7.3]
Level of your education			
No education	118	16 (13.6)	[8.0 - 21.1]
Primary		80 (67.8)	[58.6 - 76.1]
Secondary		22 (18.6)	[12.1 - 26.9]
Marital status			

Married	118	73 (61.9)	[52.5 - 70.7]
Single		43 (36.4)	[27.8 - 45.8]
Divorced		2 (1.7)	[0.2 - 6.0]
Occupation at work			
Butcher	118	61 (52.6)	[43.1 - 61.9]
Inspector		17 (14.7)	[8.8 - 22.4]
Transporter of carcasses		18 (15.5)	[9.5 - 23.4]
Others		22 (18.6)	[12.1 - 26.9]
Duration at working (abattoirs)			
1 year	118	29 (24.6)	[17.1 - 33.4]
2 years		9 (7.6)	[3.6 - 14.0]
≥ 3 years		80 (67.8)	[58.6 - 76.1]
Working on animal species			
Cattle	118	94 (79.7)	[71.3 - 86.5]
Goats		16 (13.6)	[8.0 - 21.1]
Cattle & goats		8 (6.8)	[3.0 - 12.9]

CI, confidence interval; n, number. Abattoir workers in six abattoirs interviewed about potential risk factors for brucellosis and human tuberculosis, using a semi-structured questionnaire

6.4.2. Awareness of the transmission of zoonotic brucellosis, and other zoonotic diseases, and history of diseases among abattoir workers

This study found a high frequency of awareness of zoonotic tuberculosis transmission (82.2%), low awareness of zoonotic brucellosis transmission (27.1%), and a low frequency of awareness for other zoonotic diseases, namely cysticercosis (12.7%), leptospirosis (10.2%), and Q-fever (8.5%). Of the 118 respondents, 80 (67.8%) confirmed that they had been sick and among them, 48.3% (57/118) suffered from malaria, while symptoms of flu and headache were each recorded in 5.1% (6/118) of the respondents. The history of symptoms such as abortion (0.9%) (1/118) and orchitis (0.9%) (1/118) were also recorded. Of the 118 respondents, 18 (15.3%) reported that they had been seronegative to malaria but presented symptoms of fever. The malaria seronegative patients presented also fatigue 11.0% (13/118), and flu 4.2% (5/118).

Respondents that contracted potentially abattoir-related diseases 7.6% (9/118) suffered from typhoid 1.7% (2/118) and presented symptoms of diarrhoea 5.9% (7/118) (Table 6. 2).

Table 6. 2. Awareness of zoonotic brucellosis, zoonotic tuberculosis, and other zoonotic diseases, and history of diseases among abattoir workers in Rwanda

Variables	Interviewed	Frequency n (%)	95% CI
Can you get infected with brucellosis from animals?			
No	118	86 (72.9)	[63.9 – 80.7]
Yes		32 (27.1)	[19.35 – 36.1]
Can you get infected with tuberculosis from animals?			
No	118	21 (17.8)	[11.4 - 25.9]
Yes		97 (82.2)	[74.1 - 88.6]
Have you ever heard of cysticercosis in animals or humans?			
No	118	103 (87.3)	[81.3 – 93.3]
Yes		15 (12.7)	[6.7 – 18.7]
Have you ever heard of leptospirosis in animals or humans?			
No	118	106 (89.8)	[84.4 – 95.3]
Yes		12 (10.2)	[4.7 – 15.6]
Have you ever heard of Q-fever in animals or humans?			
No	118	108 (91.5)	[86.5 – 96.6]
Yes		10 (8.5)	[3.5 – 13.5]
Have you ever been sick in the last 3 years of work at the abattoir?			
No	118	38 (32.2)	[23.9 - 41.4]
Yes		80 (67.8)	[58.57 - 76.1]
If yes, what was the diagnosis?			
Fatigue	118	1 (0.9)	[0.02 - 4.63]
Finger inflammation		1 (0.9)	[0.02 - 4.63]
Flu		6 (5.1)	[1.9 - 10.7]
High blood pressure & nephritis		1 (0.9)	[0.02 - 4.63]
Headache		6 (5.1)	[1.9 - 10.7]
Nephritis		1(0.9)	[0.02 - 4.63]
Malaria		57 (48.3)	[39.0 - 57.7]
Malaria & nephritis		2 (1.7)	[0.2 - 6.0]
Malaria & chest pain		1 (0.9)	[0.02 - 4.63]

Malaria & diarrhea		1 (0.9)	[0.02 - 4.63]
Malaria & flu		1 (0.9)	[0.02 - 4.63]
Mouth swelling		1 (0.9)	[0.02 - 4.63]
Wound		1 (0.9)	[0.02 - 4.63]
Not applicable[a]		38 (32.2)	[23.9 - 41.4]
Have you ever had a case of abortion/infertility/orchitis?			
No	118	116 (98.3)	[94.0 -99.8]
Yes		2 (1.7)	[0.21 - 6.0]
Have you ever been seronegative to malaria while presenting fever?			
No	118	100 (84.8)	[77.0 - 90.7]
Yes		18 (15.3)	[9.3 - 23.0]
If yes, what was the diagnosis in the case of seronegative malaria?			
Fatigue	118	13 (11.9)	[6 - 18.1]
Flu		5 (4.2)	[1.4 - 9.6]
Not applicable[a]		100 (84.8)	[77.0 - 90.7]
Did you ever get a sickness from abattoir?			
No	118	109 (92.4)	[86.0 - 96.5]
Yes		9 (7.6)	[3.6 - 14.0]
If yes, what was the diagnosis?			
Diarrhoea	118	7 (5.9)	[2.4 - 11.8]
Typhoid		2 (1.7)	[0.2 - 6.0]
Not applicable[a]		109 (92.4)	[86.0 - 96.5]

CI, confidence interval; n, number; [a]corresponds to the number of "No" responses from the previous question. Abattoir workers in six abattoirs interviewed about potential risk factors for brucellosis and human tuberculosis, using a semi-structured questionnaire.

The univariate analysis showed that the awareness of zoonotic brucellosis transmission was significantly higher in workers from low throughput abattoirs (50.0%, 9/18) (p=0.04) compared to those from high throughput abattoirs (23.0%, 23/100). However, the awareness of zoonotic tuberculosis transmission (85.0%, 85/100), leptospirosis (12.0%, 12/100), cysticercosis (13.0%, 13/100), and Q-fever (9.0%, 9/100) was higher in workers from high throughput abattoirs compared to those from low throughput abattoirs but this was not significant (Table 6. 3).

Table 6. 3. Awareness of transmission of zoonotic brucellosis, zoonotic tuberculosis, and other zoonotic diseases stratified by the capacity of abattoirs in Rwanda.

Variables	Categories	High throughput	Low throughput	Odds Ratios	P-value
Awareness of zoonotic	No	15.0% (15/100)	33.3% (6/18)		
transmission for TB	Yes	85.0% (85/100)	66.7% (12/18)	[0.12 - 1.17]	0.12
Awareness of zoonotic	No	77.0% (77/100)	50.0% (9/18)		
transmission for B	Yes	23.0% (23/100)	50.0% (9/18)	[1.19 -9.42]	0.04
Awareness of	No	88.0% (88/100)	100.0% (18/18)		
Leptospirosis	Yes	12.0% (12/100)	0.0% (0/18)	[0.00 -1.96]	0.2
Awareness of	No	87.0% (87/100)	88.9% (16/18)		
Cysticercosis	Yes	13.0% (13/100)	11.1% (2/18)	[0.08 - 4.29]	1
	No	91.0% (91/100)	94.4% (17/18)		
Awareness of Q-Fever	Yes	9.0% (9/100)	5.6% (1/18)	[0.01 - 4.8]	1

TB: tuberculosis; B: brucellosis

6.4.3. Behaviour and practices predisposing abattoir workers to zoonotic diseases

Consuming at least once raw meat and uninspected meat was each recorded in 0.9% (1/118) of the respondents, while none of the respondents consumed condemned meat. Most of the respondents 70.3% (83/118) had cut their hands at some point while working at the abattoir and among them, 39.8% (47/118) have been working with hand injuries, while only 7.6% (9/118) worked after treatment. Having splashes (animal fluid/blood) on the face was recorded in 74.6% (88/118) of respondents. Most of the respondents 95.8% (113/118) washed their hands and of these, 70.8% (80/118) washed their hands every time after touching potential contaminants, while the remaining 25.0% washed their hands once or twice daily. None of the 118 respondents wore a face mask or goggles, and only 9.3% (11/118) wore gloves, and of these, only 6.8% (8/118) used them regularly. Wearing overhead covers was recorded in 1.7% (2/118) of respondents. Most of the respondents 94.1% (111/118) had at least on overall clothing, but only 43.2% (48/118) always wore their overalls. Four respondents (3.4%) did not wear gumboots and among the

96.6% who wore gumboots, 11.4% (13/118) wore them occasionally. Twenty-eight percent [28.0%, (33/118)] of the respondents had their breakfast while wearing their overall clothing (Table 6. 4).

Table 6. 4. Behaviour and practices predisposing abattoir workers to occupational diseases in Rwanda

Variables	Interviewed	Frequency n (%)	95% CI
Have you ever eaten raw meat?			
No	118	117 (99.2)	[95.4 – 100.0]
Yes		1 (0.9)	[0.02 - 4.63]
Have you ever eaten uninspected meat?			
No	118	117 (99.2)	[95.4 – 100.0]
Yes		1 (0.9)	[0.02 - 4.63]
Have you ever eaten condemned meat?			
No	118	118 (100)	[96.9 - 100.0]
Have you ever cut your hand while working?			
No	118	35 (29.7)	[21.6 - 38.8]
Yes		83 (70.3)	[61.2 - 78.4]
Have you ever worked while injured?			
No	118	37 (31.4)	[23.1 - 40.5]
Yes		47 (39.8)	[30.9 - 49.2]
After treatment		9 (7.6)	[3.6 - 14.0]
Not applicable[a]		25 (21.2)	[14.2 - 29.7]
Have you ever had splashes on your face or eye?			
No	118	30 (25.4)	[17.9 - 34.3]
Yes		88 (74.6)	[65.7 - 82.1]
Do you wash your hands while working?			
No	118	5 (4.2)	[1.4 - 9.6]
Yes		113 (95.8)	[90.4 - 98.6]
How often do you wash your hands?			
Once	113	13 (11.5)	[6.3 - 18.9]
Twice		20 (17.7)	[11.2 - 26.0]
Every time		80 (70.8)	[61.5 - 79.0]
Do you wear a facemask?			
No	118	118 (100)	[96.9 - 100.0]
Do you wear gloves during work?			
No	118	107 (90.7)	[83.9 - 95.3]
Yes		11 (9.3)	[4.8 - 16.1]
How often do use gloves?			
Sometimes	11	3 (27.3)	[1.0 – 53.4]

Always		8 (72.7)	[46.4 – 99.0]
Do you wear an overhead cover?			
No	118	116 (98.3)	[96.0 - 100.0]
Yes		2 (1.7)	[0.0 - 4.0]
Do you wear overall clothing while working?			
No	118	7 (5.9)	[2.4 - 11.8]
Yes		111 (94.1)	[88.2 - 97.4]
How often do you wear the overall?			
Sometimes	111	63 (56.8)	[47.0 - 66.2]
Always		48 (43.2)	[33.9 - 53.0]
Do you wear gumboots while working?			
No	118	4 (3.4)	[0.9 - 8.5]
Yes		114 (96.6)	[91.6 - 99.1]
How often do you wear gumboots?			
Sometimes	114	13 (11.4)	[6.21 - 18.7]
Always		101 (88.6)	[81.3 - 93.8]
Do you wear glasses while working?			
No	118	118 (100)	[96.9 - 100.0]
Have you ever had your breakfaster with overall clothing?			
No	118	85 (72.0)	[63.0 - 80.0]
Yes		33 (28.0)	[20.1 - 37.0]

CI, confidence interval; n, number; [a]corresponds to the number of responses "No" of the previous question. Abattoir workers in six abattoirs interviewed about potential risk factors for brucellosis and human tuberculosis, using a semi-structured questionnaire.

6.4.4. Pairwise associations between exposure and outcome variables

The occupation was significantly associated with illness, awareness of zoonotic tuberculosis transmission, and awareness of zoonotic brucellosis transmission. Splitters & transporters of carcasses (77.8%, 14/18) were more likely to have been sick three years before the study ($p = 0.003$) and 22.2% (4/18) of them became seronegative to malaria while manifesting fever and headache ($p = 0.72$). Butchers (11.5%, 7/118) were more likely to contract potentially 'abattoir-related' diseases compared to other workers, but this was not significant ($p = 0.52$). All inspectors (100%, 17/17) were more aware of the zoonotic tuberculosis transmission ($p = 0.05$), and a large proportion of these (76.5%, 13/17) were aware of zoonotic brucellosis transmission ($p < 0.001$) compared to other workers.

The levels of education were significantly associated with illness, and workers with primary education (75.0%, 60/80) were more prone to sickness ($p < 0.003$). Although not significant, workers with primary education (10.0%, 8/80) have contracted 'abattoir-related' diseases, while (17.5%, 14/80) had been seronegative to malaria yet they presented fever as a symptom. Workers with secondary education were more aware of zoonotic tuberculosis transmission ($p < 0.001$), and zoonotic brucellosis transmission ($p < 0.001$).

Workers with three years' work experience and above (10%, 8/80) were significantly associated with being sick (77.5%, 62/80) compared to the one or two years-work experience categories ($p = 0.0006$). Workers with three years' experience and above contracted 'abattoir-related' diseases (10.0%, 8/80) more than other workers ($p = 0.61$). More workers with two years-work experience were seronegative to malaria (22.2%, 2/9) ($p < 0.01$) and were more aware of the zoonotic brucellosis transmission (6/9, 66.7%) ($p = 0.004$), than those of 1 year or ≥ 3 years-working experience. Most respondents (82.0%) irrespective of their duration at work were aware of zoonotic tuberculosis transmission, while the frequency of awareness of zoonotic brucellosis transmission was generally lower (21.1%, 32/118). A high proportion of abattoir workers with accidental cuts on their hands experienced sickness (71.1%, 59/83) ($p = 0.12$), contracted 'abattoir-related' diseases (10.9%, 9/83) ($p = 0.05$), were seronegative to malaria (20.5%, 17/83) ($p = 0.013$), were aware of zoonotic tuberculosis transmission (85.5%, 71/83) ($p = 0.23$), and zoonotic brucellosis transmission (30.1%, 25/83) ($p = 0.36$) (Table 6. 5).

Table 6. 5. Pairwise associations between exposure and outcome variables among abattoir workers in Rwanda

Exposure variables	Categories	Being sick	Diseases from abattoir	Seronegative to malaria	Awareness zoonotic tuberculosis transmission	Awareness zoonotic brucellosis transmission
Occupation	Butchers	46/61 (75.4%)	7/61 (11.5%)	10/61 (16.4%)	51/61 (83.6%)	11/61 (18.0%)
	Inspectors	5/17 (29.4%)	1/17 (5.9%)	2/17 (11.8%)	17/17 (100.0%)	13/17 (76.5%)
	Transporters	14/18 (77.8%)	0/18 (0.0%)	4/18 (22.2%)	14/18 (77.8%)	5/18 (27.8%)
	Others	13/22 (59.1%)	1/22 (4.5%)	2/22 (9.1%)	15/22 (68.2%)	3/22 (13.6%)
	P value	0.003	0.52	0.72	0.05	0.0000
Education	None	10/16 (62.5%)	1/16 (6.3%)	2/16 (12.5%)	6/16 (37.5%)	1/16 (6.3%)
	Primary	60/80 (75.0%)	8/80 (10.0%)	14/80 (17.5%)	71/80 (88.8%)	9/80 (11.3%)
	Secondary	8/22 (36.4%)	0/22 (0.0%)	2/22 (9.1%)	20/22 (90.9%)	22/22 (100.0%)
	P value	0.003	0.37	0.74	0.0000	0.0000
Duration at work	1 year	12/29 (41.4%)	1/29 (3.5%)	0/29 (0.0%)	26/29 (89.7%)	11/19(37.9%)
	2 years	4/9 (44.4%)	0/9 (0.0%)	2/9 (22.2%)	8/9 (88.9%)	6/9 (66.7%)
	≥ 3 years	62/80 (77.5%)	8/80 (10%)	16/80 (20.0%)	63/80 (82.2%)	15/80(18.8%)
	P value	0.0006	0.61	0.01	0.49	0.004
Cutting hand	No	19/35 (54.3%)	0/35 (0.0%)	1/35 (2.9%)	26/35 (74.3%)	7/35 (20.0%)
	Yes	59/83 (71.1)	9/83 (10.9%)	17/83 (20.5%)	71/83 (85.5%)	25/83 (30.1%)
	P value	0.12	0.05	0.013	0.23	0.36

6.4.5. Multivariate analysis

Significant variables from the pairwise associations were assessed further using correspondence analysis. The two correspondence dimensions, 1 and 2, explain a high percentage (96.4%) of the variance, implying that the variable mapping represents most of the information from the collected data (Figure 6. 2). The different levels of education showed varying degrees of association with the awareness of human brucellosis, with the highest association recorded for secondary education, and a negative association amongst workers with primary education or the illiterate. Inspectors were highly associated with awareness about human brucellosis, but at the same time more likely to cut their hands, compared to transporters, butchers, and other worker categories (Figure 6. 2). Respondents with personal illness most likely had three or more years-experience (compared to one year or two years), and those with primary education (compared to secondary education or illiterates). Workers with secondary education were highly associated with awareness about zoonotic tuberculosis, while negative associations were observed for primary or no education.

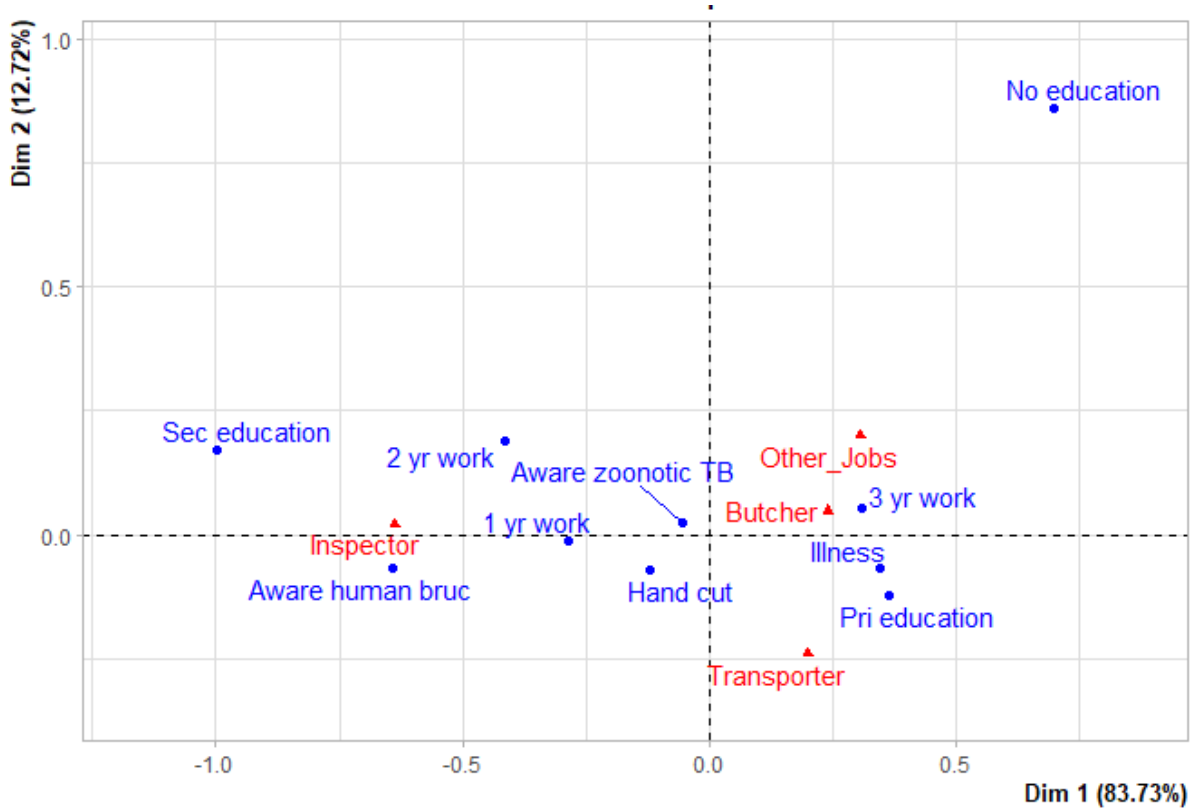

Figure 6. 2. A symmetric biplot of the first two axes of the correspondence analysis illustrating the association of potential risk factors related to human brucellosis and zoonotic tuberculosis among workers in six abattoirs in Rwanda.

6.5. Discussion

Brucellosis and bTB are zoonotic diseases that are endemic in developing countries including Rwanda. This study reports on the awareness of zoonotic brucellosis and tuberculosis transmission and assesses the occupational hazards among abattoir workers; hence, it is the first of its kind in Rwanda. The awareness for zoonotic brucellosis transmission was low and most of respondents have never heard of other zoonotic diseases (leptospirosis, Q-fever, and cysticercosis). Furthermore, there was a lack of epidemiological knowledge related to symptoms and transmission of the abovementioned zoonotic diseases among abattoir workers, but this was an observational finding that was not quantified. In contrast, a good awareness for zoonotic tuberculosis transmission was recorded among abattoir workers. Furthermore, this study

identified poor practices and gaps in the implementation of biosafety measures at abattoirs, predisposing abattoir workers to the high risks of contracting zoonotic diseases.

The awareness for zoonotic brucellosis transmission was significantly higher in workers from low throughput than that of those from high throughput abattoirs. This may be attributable to the limited sample size from low throughput abattoirs and the high education level of interviewed workers. The level of awareness of zoonotic brucellosis (27.1%) obtained in this study is lower than that (44.2%) reported in Ethiopia (Tsegay et al., 2017) and Tanzania (76%) (Luwumba et al., 2019) among abattoir workers. These differences may be due to the lower level of education of abattoir employees on zoonoses in Rwanda. The low awareness may also be attributable to a lack of routine diagnosis and treatment of human brucellosis in hospitals and health centers in Rwanda. Thus, in Rwanda, human brucellosis may be misdiagnosed for other acute febrile diseases namely typhoid fever and malaria (Crump et al., 2013). Furthermore, a recent study reported the lack of brucellosis knowledge in 88.4% of brucellosis seropositive women presenting abortions at Nyagatare district hospital, Rwanda (Gafirita et al., 2017). Another study at Huye teaching hospitals, Rwanda, reported that 25.0% of brucellosis seropositive women presenting abortions consumed unpasteurized milk or had been in contact with cattle or goats (Rujeni and Mbanzamihigo, 2014). Therefore, the low awareness observed in the present abattoir study may simply imply that the prevalence may be much higher in occupational groups who frequently come in contact with animals. There is thus a need for interdisciplinary collaboration, raising the awareness and knowledge of human health professionals and including human brucellosis among the routinely diagnosed and treated diseases in Rwanda.

The awareness for zoonotic tuberculosis transmission was higher in workers from high throughput than low throughput abattoirs, but this was not significant. High throughput abattoirs are modern with trained workers of sufficient work experience. The high level of awareness for zoonotic tuberculosis transmission (82.2%) in this study was consistent with a study in central Ethiopia that reported high awareness of bTB (95.0%) among abattoirs workers and 93.0% of them were aware that bTB can spread from animals to humans (Fekadu et al.,

2018). The high awareness of zoonotic tuberculosis transmission obtained in this study may be attributable to the sporadic occurrence of tuberculosis cases in slaughtered cattle resulting in the condemnation of the whole carcass (one case occurred during this study), and abattoir workers being aware of colleagues with tuberculosis infections, as mentioned by respondents during this study. Moreover, abattoir workers are required to present a medical check-up certificate every year which could have revealed pulmonary tuberculosis in some workers. For instance, one cleaner had recovered from tuberculosis in the past and during this study, two abattoir workers were diagnosed with TB and placed into isolation, but we could not trace the origin of the infection for all these cases. However, the estimated prevalence of human TB infection rate was 119.3 per 100,000 adult population in 2012 in Rwanda (Migambi et al., 2020). Besides, extra-pulmonary cases due to *M. bovis* (Gervois et al., 1972, Mfinanga et al., 2004), may be missed out because lymph nodes are rarely collected for the diagnosis of human TB. Instead, the sputum is the common specimen tested (Mfinanga et al., 2004), and there is inability of laboratory tests to distinguish MTBC species (de la Rua-Domenech, 2006, Wedlock et al., 2002).

The lever of awareness for leptospirosis (10.2%) recorded in this study was higher than the 4.2% recorded earlier in Nigeria and this low awareness in Nigeria was supported by the high (89.7%) number of workers testing positive for leptospirosis (Abiayi et al., 2015). A study in Kenya recorded awareness of 89.0% and 33.0% for cysticercosis and leptospirosis, respectively (Nyokabi et al., 2018), which is significantly higher than the 12.7% for cysticercosis and 10.2% for leptospirosis observed in this study. However, the awareness obtained in the current study (8.5%) for Q-fever is higher compared to the study in Kenya in which none of the respondents was aware of Q-fever (Nyokabi et al., 2018). This difference may be explained by variations in the level of education, and the acquired training on zoonosis among abattoir workers in the different countries. A 3.0% prevalence of bovine cysticercosis was reported at Nyagatare district slaughterhouse, Rwanda (Nzeyimana et al., 2015), and a high prevalence of 21.8% of cysticercosis was reported in people with epilepsy in the Southern Province of Rwanda (Rottbeck et al., 2013). These findings show that despite the scarcity of studies on leptospirosis, Q-fever, and cysticercosis in cattle and humans in Rwanda, these diseases may be endemic in

the country and may thus constitute a risk of infection for workers in abattoirs with poor hygienic and biosafety measures.

Seven respondents presented with symptom of diarrhoea which may have been caused by enteric pathogens such as zoonotic *Salmonella* spp., *Escherichia coli,* or *Campylobacter* spp. These pathogens may have been acquired by the workers by eating while working, as zoonotic salmonellosis and infections by *E. coli* and *Campylobacter* spp. are commonly reported in slaughtered cattle and abattoir workers (Akkaya et al., 2008, Milnes et al., 2008, Molla et al., 2003, Vaira et al., 1988). Fever (15.3%), fatigue (11.0%), flu (9.3%), headache (5.1%), and nephritis-related symptoms (3.4%) of unknown origin were reported in this study but these symptoms are commonly observed in humans with diseases like brucellosis (Dean et al., 2012), leptospirosis (Adler and de la Peña Moctezuma, 2010), and Q-fever (Derrick, 1937) as earlier reported in abattoir workers in other studies (Esmaeili et al., 2016, Mirambo et al., 2018). The nephritis-related symptoms may also be caused by *M. bovis* which has been isolated in the urogenital tract of humans (Grange and Collins, 1987). Abattoir workers being regularly exposed to carcasses, blood, urine, and hides from infectious animals are at high risk of contracting these diseases.

Given the history of abortion and orchitis each reported in one respondent in this study, education on brucellosis and other zoonotic diseases is essential since the seroprevalence of brucellosis ranges from 6.1% to 25.0% in women presenting abortions in Rwanda (Gafirita et al., 2017, Rujeni and Mbanzamihigo, 2014). Additionally, a 10.0% seropositivity to *Brucella* species has been reported among abattoir workers in neighboring Uganda (Nabukenya et al., 2013), and 19.5% in Tanzania (Mirambo et al., 2018), and the odds of contracting brucellosis were higher among abattoir workers compared to other occupational groups in Uganda (Swai and Schoonman, 2009). There is therefore a need to investigate the various diseases that may be acquired by abattoir workers during their duties. It is also necessary that the workers are facilitated with appropriate personal protective equipment (PPE) and other hygienic practices should be implemented.

Gloves, overhead covers, face mask, and goggles were only used by 9.3%, 1.7%, 0.0%, and 0.0% of inspectors and these findings are lower than those reported in Ethiopia where 18.6% and 13.4% of abattoir workers used gloves and face masks, respectively (Tsegay et al., 2017). Related to this, 70.3% of workers had cut injuries on their hands in the present study, and 74.6% reported the splashes of fluid or blood onto their faces (eyes, nostrils, and mouth) while on duty; this is of concern since a study in Tanzania demonstrated that abattoir workers that did not adhere to the use of protective gears were three times more at risk of contracting brucellosis (Nabukenya et al., 2013). The lack of PPE constitutes a high risk of exposure to zoonoses for abattoirs workers and indicated the failure of not implementing biosafety policy and to educate on the epidemiology of zoonotic diseases since *Brucella* spp., *M. bovis*, and other diseases can be transmitted through open wounds, ingestion, and inhalation (Corbel, 2006, Cosivi et al., 1998). Slaughtering with bare hands and injuries was observed in 39.8% of abattoir workers consistent with a study in Nigeria where 47.0% of abattoirs workers had hand-cut injuries while working (Abiayi et al., 2015). Furthermore, working with bare hands and injuries had been associated with the risk of contracting brucellosis in Nigeria (Aworh et al., 2013). In our study, 29.0% of respondents had their breakfast while wearing overall clothing and this proportion of workers was higher than 14.4% reported in Nigeria (Abiayi et al., 2015). The practices of working with bare hands, eating while working and the occurrence of injuries, as recorded in this study confirm the poor epidemiological knowledge of pathogens that cause zoonotic diseases. There is therefore a need for the concerned veterinary authorities to strengthen the implementation of rules and regulations regarding the biosafety in abattoirs and raise awareness of the abattoir workers and other stakeholders, through educational campaigns.

Univariate and correspondence analyses showed that the awareness of zoonotic brucellosis transmission was significantly associated with occupation and was consistent with studies in Tanzania (Luwumba et al., 2019), and in Ethiopia (Tsegay et al., 2017). In the present study, inspectors were more likely to be aware of zoonotic brucellosis transmission than other occupational categories. Awareness of zoonotic brucellosis transmission was also significantly associated with the level of education; the frequency was higher among workers with

secondary education than those with primary education and the illiterate respondents. This was in agreement with studies in Tanzania (Luwumba et al., 2019), Ethiopia (Tsegay et al., 2017), and Nigeria (Aworh et al., 2013). Our findings showed that 54.5% of workers with secondary level education were inspectors, and most had a veterinary education background and gained knowledge and skills through work experience which can explain their higher level of awareness. Our analysis demonstrated a significant association between occupation and awareness of zoonotic tuberculosis transmission, with inspectors more likely to know about zoonotic tuberculosis transmission than other workers and this was consistent with a study in Nigeria in which 100.0% of veterinarians and animal scientists were more aware of the bTB than other groups (Ismaila et al., 2015). Awareness of zoonotic brucellosis transmission was further significantly associated with the duration at work; the frequency was higher among workers with two years of experience which is in agreement with studies in Tanzania (Luwumba et al., 2019), Ethiopia (Tsegay et al., 2017), and Nigeria (Aworh et al., 2013).

Both univariate and correspondence analyses showed that illness during the past three years before the study was significantly associated with occupation, where transporters of carcasses (77.8%) and butchers (75.4%) were more likely to become sick. Furthermore, being sick during the past three years before the study was significantly associated with the level of education, and workers with primary education reported a higher frequency of illness, in agreement with the findings in Nigeria in which illiterate and workers with primary education were more likely to become seropositive to brucellosis (Aworh et al., 2013, Tsegay et al., 2017). Generally, workers with lower levels of education are assigned to cleaning and washing of viscera, jobs in which workers lack the understanding of the high burden of harmful microorganisms they are exposed to. Being sick during the past three years before the study was also significantly associated with the duration at work, and workers with experience of three years and above were more likely to get sick than other groups (one year, and two years) probably due to the longer and repeated exposure. This finding was consistent with studies in Nigeria in which abattoir workers with more than five years at work were more likely to be seropositive to *Brucella* spp. (Aworh et al., 2013), while those with more than 40 years at work

were more likely to be positive to *M. bovis* (Ismaila et al., 2015). This affirms the association between age and tuberculosis, in that, workers with advanced age and weakened immune systems may have been exposed to the pathogens for a long period, leading to a greater risk of contracting infections. From our observations, the slaughtering level of one of the abattoirs we visited was higher compared to its capacity and this led to poor hygiene of the premises and non-compliance with the recommended unidirectional slaughtering system, and thus lack of separation of dirty and clean operations. We noticed that - inspection results are not daily recorded and are not matched with the origin of animals. Most abattoirs were private and employed inspectors with only secondary veterinary education (98.0% of inspectors) and only two main abattoirs report monthly to the Department of Veterinary Services. Slaughtered cattle are the sentinels of zoonotic and other animal diseases. Thus, if the suspect inspection findings can be confirmed by laboratory evidence, this will play a significant role in the surveillance and control of infectious diseases.

6.6. Conclusions

This study demonstrated a low awareness of transmission of zoonotic brucellosis, leptospirosis, Q-fever, and cysticercosis among abattoir workers. Noncompliance with biosafety protection was associated with the lack of epidemiological knowledge on the transmission routes of zoonotic diseases. It is necessary to raise awareness through education campaigns and facilitate the workers with appropriate personal protective equipment (PPE). Our study has therefore, generated information for the concerned authorities to enforce appropriate practices. The coordination and collaboration between abattoirs and public health authorities are important for the surveillance and control of zoonotic diseases. There is therefore a need to further monitor the zoonotic diseases among abattoir workers.

6.7. Acknowledgments

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Chapter 7. Seroprevalence and molecular characterization of *Brucella* spp. from slaughtered cattle in Rwanda

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7.1. Abstract

Bovine brucellosis is endemic in Rwanda, although, there is no information about the disease in slaughtered cattle. A cross-sectional study was conducted in slaughtered cattle (n=300) to determine the seroprevalence of anti-*Brucella* antibodies using the Rose Bengal Test (RBT), and indirect enzyme-linked immunosorbent assay (i-ELISA). Corresponding tissues were cultured onto CITA medium and analyzed for *Brucella* spp. using the 16S-23S ribosomal interspacer region (ITS), AMOS, and Bruce-ladder PCR assays. The RBT seroprevalence was 20.7%, (62/300), and 2.9%, (8/300) with i-ELISA and 2.9%, (8/300) using both tests in series. *Brucella* specific 16S-23S ribosomal DNA interspace region (ITS) PCR detected *Brucella* DNA in 5.6%, (17/300; *Brucella* culture prevalence). AMOS-PCR assay identified mixed *B. abortus* and *B. melitensis* (n=3), *B. abortus* (n=3), and *B. melitensis* (n=5) while Bruce-ladder PCR also identified *B. abortus* (n=5), and *B. melitensis* (n=6). The gold standard culture method identified 5.6% *Brucella* cultures combined with PCR confirmation which is higher than the more sensitive seroprevalence of 2.9%. This emphasizes the need to validate the serological tests in Rwanda. The mixed infection caused by *B. abortus* and *B. melitensis* in slaughtered cattle indicates the cross-infection and poses a risk to abattoir workers. It is essential to urgently strengthen bovine brucellosis control through vaccination as well as test-and-slaughter.

7.2. Introduction

Brucellosis is a contagious widespread disease that causes not only substantial economic losses related to abortions, long conception intervals, and sterility in animals but also morbidity and reduced working capacity in humans (McDermott et al., 2013, Singh et al., 2015). The disease is caused by bacteria of the genus *Brucella* which belongs to the class alphaproteobacteria (Moreno et al., 1990). *Brucella* species are gram-negative microaerophilic coccobacilli, acid-fast intracellular, and hosts-specific microorganisms affecting a wide variety of terrestrial and marine mammals (Alton et al., 1975, Corbel, 1997). *Brucella* species are 96% genetically identical (Verger et al., 1985) with few polymorphisms that are essential for species and biovars differentiation (Bricker and Halling, 1994, Ficht et al., 1990). Classical species with their biovars (bv.) have specific hosts, for instance, *B. abortus* (7 biovars) infects primarily cattle, *B. melitensis* (3 biovars) infects goats and sheep, *B. ovis* infects sheep, *B. suis* (bv. 1, 3, 4, and 5) infects swine while *B. suis* bv. 2 infects rats, *B. canis* infects dogs, and *B. neotomae* infects wood rats (Alton et al., 1975, OIE, 2018).

The transmission of brucellosis in animals is through inhalation of *Brucella* aerosols (Kaufmann et al., 1980), direct contact with infective fetal membranes, vaginal discharges, placenta content, and ingestion of contaminated feeds (Corbel, 2006). There are no pathognomonic clinical signs for brucellosis, but cases of abortion or hygroma are suspicious signs that require laboratory diagnosis for confirmation (Akakpo and Bornarel, 1987, OIE, 2018).

As brucellosis is a herd disease, the most suitable tests are serological tests to determine the seroprevalence of the animal and or herd using the Rose Bengal test (RBT) followed by a confirmatory test like enzyme-linked immunosorbent assays (ELISAs) or complement fixation test (CFT) (OIE, 2018). However, serological tests do not provide a complete diagnosis, thus, the isolation of *Brucella* spp. remains the gold standard (OIE, 2018). The culturing and biotyping of *Brucella* cultures are expensive, time-consuming, and require trained personnel. PCR assays which differentiate *B. abortus* bv.1, 2, 4, *B. melitensis* bv.1, 2, 3, *B. ovis*, and *B. suis* bv.1 (AMOS PCR) in 24 hours from cultures (Bricker and Halling, 1994) and Bruce-ladder PCR assay can

differentiate all *Brucella* species and vaccine strains (Garcia-Yoldi et al., 2006, Lopez-Goni et al., 2008). Unfortunately, culture, phenotypic and genotypic isolation of *Brucella* spp. are not common in veterinary services in most developing countries owing to inadequate facilities and trained personnel; therefore, serology is in common practice with little knowledge on the causal *Brucella* spp. (Ducrotoy and Bardosh, 2017).

Brucellosis is an endemic disease in Rwanda with 7.4% to 18.7% seroprevalence in cattle reported (Ndazigaruye et al., 2018, Ntivuguruzwa et al., 2020) as well as seroprevalence in women with a history of abortions of 6.6% and 25.0% reported by Gafirita et al. (2017), Rujeni and Mbanzamihigo (2014). Although, cattle from various districts of the country are slaughtered at abattoirs, there is no single study on the seroprevalence of brucellosis and characterization of *Brucella* spp. in slaughtered cattle in Rwanda. Furthermore, apart from a single study that isolated *B. abortus* bv. 3 from Rwandan cattle in the 1980s (Verger and Grayon, 1984), *Brucella* spp. that are circulating in the animal population are not known. The objective of this study was, therefore, to determine the seroprevalence of brucellosis and characterize *Brucella* spp. from slaughtered cattle in Rwanda.

7.3. Materials and methods

7.3.1. Study area

This study was conducted in six abattoirs in Rwanda. Rwanda is a landlocked country of the East African community covering an area of 26,338 Km^2 in the southern hemisphere near the equator (West: 28.86; East: 30.89; North: - 1.04; South: - 2.83). The bovine population in Rwanda was estimated at 1,293,768 in 2018 (Minagri, 2019). The six abattoirs (société des abattoirs de Nyabugogo "SABAN", Rugano abattoir, Kamembe, Rubavu, Kamuhanda, Gataraga) consented to participate (Figure 6. 1). These abattoirs were selected based on their slaughtering capacity and their location to sample cattle from all the thirty districts of Rwanda. In this study, cattle collected at SABAN abattoir were from 19 districts including Rulindo, Ngoma, Muhanga, Nyagatare, Gasabo, Bugesera, Ngororero, Gakenke, Burera, Rutsiro,

Gicumbi, Nyarugenge, Kirehe, Ruhango, Kayonza, Karongi, Nyanza, Kamonyi, and Gatsibo. Cattle collected from Rugano abattoir were from 3 districts including Gasabo, Rwamagana, and Nyarugenge. Cattle collected at Kamembe abattoir were from 8 districts including Gisagara, Huye, Nyamagabe, Nyamasheke, Nyanza, Nyaruguru, Ruhango, and Rusizi. Cattle collected at Rubavu abattoir were from two districts including Nyabihu, and Rubavu. Cattle collected at Kamuhanda abattoir were from the Kamonyi district. Cattle collected at Gataraga abattoir were from the Musanze district. These abattoirs were classified into high throughput abattoirs (n=4) slaughtering more than 50 cattle daily and low throughput abattoirs (n=2) slaughtering 50 or less every day.

7.3.2. Study design and sample size

A cross-sectional study was carried out from August 2018 through October 2019 to determine the seroprevalence of brucellosis and characterize *Brucella spp.* from cattle tissue collected during slaughtering at abattoirs. The sample size was calculated using the previously described formula (Dohoo et al., 2009) which is common for cross-sectional studies.

$$N = \frac{Z^2 \ \ P(1-P)}{d^2}$$

Where N is the sample size, $Z^2 = 1.96$ the statistical constant at 95% confidence interval; P is the expected prevalence and was estimated at 0.5%, and the absolute precision, d = (P/2). According to the formula, the total sample size was 291 but it was rounded to 300 cattle to sample 10 animals per each of the 30 districts of Rwanda.

7.3.3. Sampling procedure

Our target was to sample five animals coming from the same district every day. The origin of animals was recorded on arrival using the movement permit. The age was determined using teeth erosion as previously described (Pope, 1934). Except for abattoirs that received mostly males, females of 1 year and above were selected using systematic random sampling. Animals were aligned in a crush and every fourth animal was selected for sampling. The

vaccination status and farm of origin of slaughter animals could not be traced because most of the animals were bought from the animal markets in different districts.

7.3.4. Collection of blood and tissues samples

After the selection and recording of individual demographic information (district of origin, age, breed, and sex), the animal was restrained, marked on the head, and released for resting waiting for the collection of blood after bleeding. Blood was collected into sterile 50 ml tubes after slaughter, aliquoted into 5 ml tubes, and then was immediately transported to the laboratory of the University of Rwanda (UR) and left overnight at room temperature to allow clotting. The following day, serum was collected into a sterile 2 ml micro-centrifuge tube and stored at -20°C until serological testing at Rwanda Agriculture and Animal Resources Board (RAB), Department of Veterinary Services, in the serology section. The head of the marked animal from which blood was collected was followed at head inspection station and the corresponding left and right retropharyngeal lymph nodes were collected into a sterile 50 ml tube.

7.3.5. Serological tests

Animal sera were screened for the presence of *Brucella* antibodies using Rose Bengal test and i-ELISA as described in Chapter 2.

7.3.6. Culturing

Culturing was performed as described in Chapter 2 using tissue from slaughtered cattle.

7.3.7. Molecular methods

DNA was exacted from the bacterial cultures. *Brucella* DNA was detected from bacterial cultures using the 16-23S ribosomal DNA interspacer region (ITS) PCR specific for *Brucella.* The *Brucella* cultures were speciated using the AMOS- and Bruce-ladder PCR assays. The molecular methods were performed as described in Chapter 2.

7.3.8. Data analysis

The overall seroprevalence was obtained by dividing the total number of animals simultaneously positive to RBT and i-ELISA by the total number of animals sampled. Data were recorded in Microsoft Excel spreadsheets. Epi-Info 7 version 10 was used to calculate proportions. Significant levels between individual risk factors and seroprevalence and molecular results were determined using the chi-square test. The odds-rations were determined for associated risk factors along 95% confidence intervals and statistical significance set at $p < 0.05$.

7.4. Results

7.4.1. Brucellosis seroprevalence among slaughtered cattle in Rwanda

Of the 300 cattle sera 95.7% (287/300) were from females while 4.3% (13/300) were from males. Most animals, 89.7% (269/300) were adults while young animals represented 10.3% (31/300). Twenty-seven percent [27.7%, (81/300)] of cattle sampled were local breed "Ankole", 67.0% (201/300) were crossbreeds and 5.3% (16/300) were Friesians. Samples were mainly collected from high throughput abattoirs (n=280) compared to low throughput abattoirs (n=20). The seroprevalence of brucellosis in parallel was 20.7% (62/300) and 2.9% (8/300) using RBT, and i-ELISA, respectively. The seroprevalence was 2.9%, (8/300) using both tests in parallel. Twenty-one percent [21.1%, (59/280)] of the RBT positive cattle were sampled from high throughput abattoirs, while 15.0% (3/20) were from the low throughput abattoirs. Eight samples that were simultaneously positive to RBT and i-ELISA were collected from high throughput abattoirs (Table 7. 1). Considering the RBT results alone, the highest RBT brucellosis rate of 30.0%, (21/70) was observed in the Eastern Province while the lowest 13.3%, (4/30) was recorded in the Kigali city (Table 7. 2). Twenty-one percent [21.6%, (58/269)] of RBT seropositive animals were adult animals while 12.9%, (4/31) of RBT seropositive animals were young. The eight animals that were seropositive to both tests were adults. Twenty percent [20.6%, (59/287)] of

RBT seropositive animals were female while 23.1%, (3/13) were young animals. The eight animals that were positive to both tests were all-female animals. The RBT seropositivity recorded according to the breeds of animals was as follows: 15.7%, (13/83) for Ankole, 22.0%, (44/201) for crossbreds, and 31.3%, (5/16) for Friesians with no significant statistical difference ($p = 0.28$). There was a significant association ($p = 0.04$) between seropositivity (RBT and i-ELISA) and breeds with Friesian being more seropositive 12.5%, (2/16) than crossbreds 2.5%, (5/201), and Ankole 1.2%, (1/83) (Table 7. 1).

Table 7. 1. Univariate associations between animal characteristics, seropositivity, and isolation of *Brucella* spp. using 16-23S ribosomal DNA interspacer region (ITS) PCR assay on the DNA extracted from bacterial cultures of tissues of slaughtered cattle in Rwanda.

Variables	Categories	Tested	RBT			i-ELISA			ITS PCR assay on culture isolates		
			n⁺ (%)	OR	*p*-value	n⁺ (%)	OR	*p*-value	n⁺/N (%)	OR	*p*-value
Abattoirs	High thr.	280	59 (21.1)	0.8 – 1.1	0.78	8 (2.9)	0.1-0.2	1	16/78 (20.5)	0.69-1.16	0.68
	Low thr.	20	3 (15.0)			0 (0.0)			1/9 (11.1)		
Provinces	Eastern	70	21 (30.0)	-	0.17	3 (4.3)	-	0.37	2/14 (14.3)	-	0.95
	Kigali city	30	4 (13.3)			3 (6.0)			3/11 (27.3)		
	Northern	50	7 (14.0)			1 (1.3)			4/18 (22.2)		
	Southern	80	14 (17.5)			1 (1.4)			3/17 (17.6)		
	Western	70	16 (22.7)			1 (1.4)			5/27 (18.5)		
Age	Adults	269	58 (21.6)	0.8 – 1.0	0.35	8 (3.0)	0.95-0.99	1	17/77 (22.1)	0.69-0.88	0.19
	Young	31	4 (12.9)			0 (0.0)			0/10 (0.0)		
Sex	Females	287	59 (20.6)	0.8– 1.4	0.74	8 (2.8)	0.95-0.99	1	17/80 (21.3)	0.70-0.88	0.34
	Males	13	3 (23.1)			0 (0.0)			0/7 (0.0)		
Breed	Ankole	83	13 (15.7)	-	0.28	1 (1.2)	-	0.04	1/21 (4.8)	-	0.02
	Cross	201	44 (22.0)			5 (2.5)			13/61 (21.3)		
	Friesian	16	5 (31.3)			2 (12.5)			3/5 (60.0)		

RBT: Rose Bengal Test, i-ELISA: indirect enzyme-linked immunosorbent assay, Thr. = throughput; OR = odds ratio; CI = confidence interval; n+: number of positives; n+/N: number of positive animals over the total number of tested animals; %: percentage.

7.4.2. Bacteriology and the 16S-23S interspacer region (ITS) PCR assay

Of the tissues (n=300) that were cultured onto the modified CITA medium, ITS-PCR confirmed 5.6% (17/300) (Figure 7. 1). The *Brucella* culture prevalence obtained by bacteriology and confirmed by ITS PCR was 5.6% (17/300).

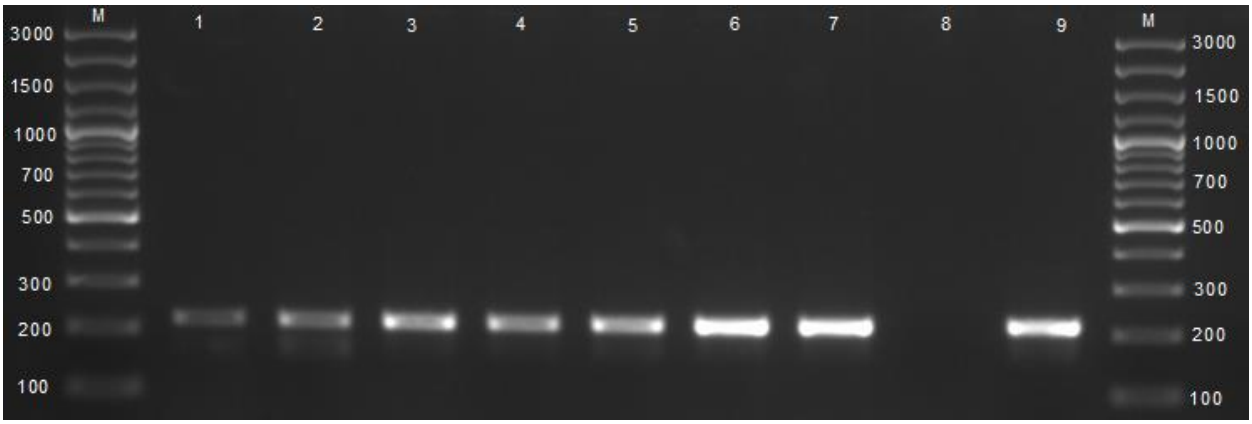

Figure 7. 1. Agarose gel electrophoresis of the 16-23S interspacer region (ITS) PCR products amplified from cultures of tissues from slaughtered cattle. Lanes M: DNA GeneRuler 100bp plus (Invitrogen, Pretoria, South Africa), lanes 1 – 7: amplification of a 214 bp sequence of the genus *Brucella* spp., lane 8: negative control containing sterile water, lane 9: positive control with *B. abortus* REF 544.

7.4.3. Differentiation of *Brucella* spp. by AMOS and Bruce-ladder PCR assays

The AMOS PCR identified *B. melitensis* and *B. abortus* (n=3) mixed cultures, *B. abortus* (n=3), and *B. melitensis* (n=5) (Figure 7. 2) from the 17 *Brucella* culture (impure culture). The Bruce-ladder PCR assay identified *B. abortus* (n=5), *B. melitensis* (n=6) (Figure 7. 3).

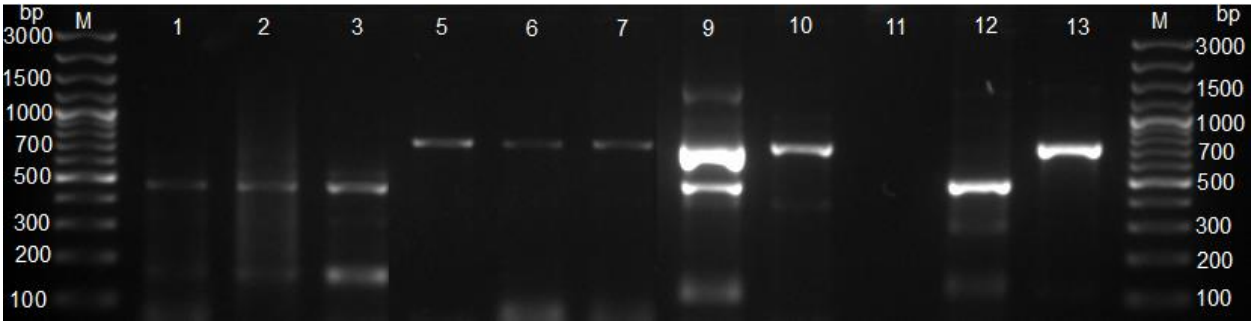

Figure 7. 2. Agarose gel electrophoresis for AMOS PCR products amplified from cultures of tissues from slaughtered cattle. Lanes M: GeneRuler 100 pb plus (Invitrogen, ThermoFischer, South Africa), lanes 1-4: *Brucella abortus* (496 bp), lanes 5-7: *B. melitensis* (731 bp), Lanes 9-10: mixed *B. melitensis* and *B. abortus*, lane 11: negative control containing sterile water, lane 12: positive control, *B. abortus* RF544, lane 13: positive control, *B. melitensis* rev 1.

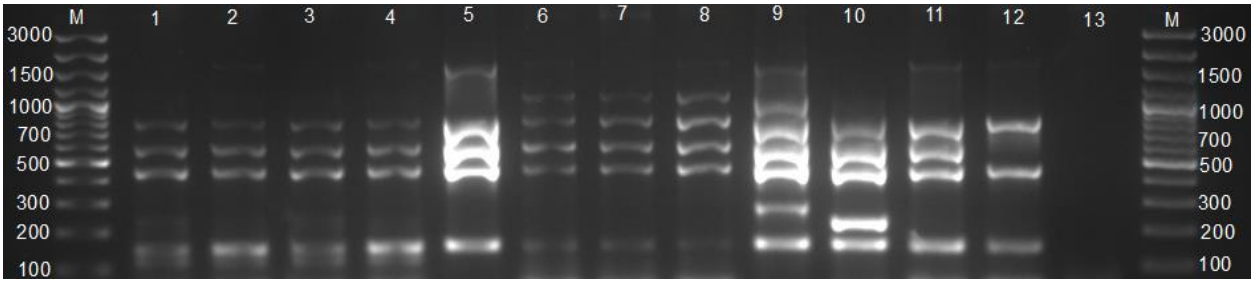

Figure 7. 3. Agarose gel electrophoresis for Bruce–ladder PCR products amplified from cultures of tissues from slaughtered cattle. Lanes M: GeneRuler 100 bp (Invitrogen, ThermoFischer, South Africa); lanes 1-5: *B. abortus*; lanes 6-8: *B. melitensis*; lane 9: positive control, *B. suis* ZW45, lane 10: positive control, *B. melitensis* rev 1, lane 11: *B. abortus* (REF 544), lane 12: positive control, *B. abortus* S 19, lane 13: negative control with sterile water.

7.4.4. Culture prevalence amongst slaughtered cattle in Rwanda

The *Brucella* culture prevalence obtained by culture and confirmed by ITS PCR was 5.6% (17/300). Of the *Brucella* spp. obtained by culture, 20.5% (16/78) were collected from high throughput abattoirs, while 11.1% (1/9) were from low throughput abattoirs (OR= 0.69 – 1.16, p=0.68). The *Brucella* spp. obtained by culture were collected from provinces (p=0.95) as follows: Eastern (14.3%, 2/14), Kigali city (27.3%, 3/11), Northern (22.2%, 4/18), Southern (17.6%, 3/17),

Western (18.5%, 5/27). All the *Brucella* obtained by culture (21.3%, 17/80) were adult (OR=0.69-0.88, p=0.20) and females (OR=0.70-0.88, p=0.34). There was a significant association between the culture prevalence and breed (p=0.02) with Friesians having more isolates (60.0%, 3/5), followed by crossbreds (21.3%, 13/61), and Ankole (4.8%, 1/21) (Table 7.1).

The *Brucella* DNA detected by ITS, AMOS, and Bruce-ladder PCR assays (100.0%, 11/11) were from cattle that were either seropositive to RBT or i-ELISA. Of these 11 *Brucella* isolates, 10 were isolated from slaughtered cattle collected at high throughput abattoir. The 11 *Brucella* isolates were identified in provinces are as follows: Eastern (n=1), Kigali city (n=2), Southern (n=3), Western (n=2), Northern (n=3). The *Brucella* isolates (n=11) were isolated from female and adult cattle. Of the *Brucella* isolates (n=11) were identified from Ankole (n=1), crossbreds (n=8), and Frisians (n=2). There was no significant difference between the category of abattoirs, provinces, age, sex of animals, and the detection by ITS, AMOS, and Bruce-ladder PCR assays.

7.5. Discussion

This is the first report of *B. abortus* (either bv. 1, 2, or 4) and *B. meletensis* cultures isolated from cattle tissues collected from abattoirs. The overall seroprevalence obtained in this study among slaughtered cattle selected from all the thirty districts of Rwanda (2.9% for RBT and i-ELISA) was lower than the culture prevalence 5.6% (17/300). This suggests that the seroprevalence rates observed in abattoirs are usually lower compared to the seroprevalence recorded at the farm level which usually focuses on endemic zones while slaughtered cattle come from various locations (endemic and non-endemic zones). The fact that the lower sensitivity culture method is higher than the seroprevalence is a clear indication that the confirmatory i-ELISA test must be validated for bovine in Rwanda as the cut-off values were determined in developed countries with low brucellosis prevalence and thus clearly underestimate the prevalence due to high cut-off values.

Frisians were more likely to be seropositive in this study consistent with earlier studies in Pakistan where Holstein and Frisian cattle were more seropositive than indigenous breeds (Mangi et al., 2015), and in Ethiopia (Omer et al., 2000). This supports that exotic pure breeds like Frisians are more susceptible to brucellosis than crossbreeds and indigenous breeds (Akhtar et al., 2019) or were introduced in the herd with seronegative status but being chronically infected (Akakpo and Bornarel, 1987, Roux, 1979)(Akakpo and Bornarel, 1987, Roux, 1979). When the acute brucellosis has passed, the infection stabilizes with the acquisition of herd immunity leading to less infectious discharges and non-visible symptoms (Ducrotoy et al., 2017). The overall seroprevalence of 2.9% using RBT and i-ELISA is different from the rate (7.4%) reported in cattle from six districts of Rwanda using both RBT and i-ELISA in series (Ntivuguruzwa et al., 2020). The seroprevalence obtained in this study was comparable with 3.4% reported at Gaoundere municipal abattoir in Cameroun using RBT and i-ELISA (Awah-Ndukum et al., 2018), and the 3.9% reported among slaughtered cattle in Nigeria (Akinseye et al., 2016), and the 5.5% reported among slaughtered cattle in Gauteng province, South Africa (Kolo et al., 2019).

The mixed infection caused by *B. abortus* and *B. melitensis* and the isolation of *B. melitensis* from slaughtered cattle indicate the cross-infection between both *Brucella* spp. and mixed farming of cattle and goats or sheep. The mixed infection and mixed farming were reported in our study that identified both pathogens in aborting goats in Rwanda (unpublished data). The co-infection of *B. abortus* and *B. melitensis* has also been reported in slaughtered cattle in South Africa (Kolo et al., 2019). The isolation of *B. melitensis* in slaughtered cattle poses a risk to abattoir workers and consumers of contaminated milk and milk products as *B. melitensis* and *B. abortus* cause severe brucellosis in humans (Sadler, 1960, Sayour et al., 2020). There is a need for improvement in the brucellosis control using vaccination, test-and-slaughter as well as raising awareness of all occupational groups as education was associated with a high awareness of brucellosis in Rwanda (Ntivuguruzwa et al., 2020).

AMOS and Bruce-ladder PCR assays identified *B. abortus* and *B. melitensis* with the *B. abortus* being either biovars 1, 2, or 4 (identified by AMOS PCR) which will be identified in the

future after purification of cultures using biotyping. A previous study *B. abortus* bv. 3 was identified in humans and animals in 1987 in Rwanda (Verger and Grayon, 1984). *B. abortus* bv. 3 and *B. melitensis* bv. 1 were reported in neighboring Uganda (Mugizi et al., 2015), Tanzania (Mathew et al., 2015), and Kenya (Muendo et al., 2012). Biotyping of *B. abortus* biovars is complex as characteristic typical for *B. abortus* bv .1, except CO_2 requirement for growth by Alton *et al.* (1988). However, the *B. abortus* bv. 3 ref strain Tulya isolated from human patient in Uganda grows in the absence of CO_2 and has been observed to occur within some biovars and changes with OIE biotyping profile (Mathew et al., 2015, OIE, 2009, OIE, 2016). Hence classifying *B. abortus* bv. 3 strains should be carefully considered. The *B. abortus* and *B. melitensis* isolated in this study could originate from neighboring countries due to repatriation of Rwandans and their livestock from Uganda, and Tanzania as well as importation of improved cattle breeds from various countries cannot be eliminated despites testing procedures (Akakpo and Bornarel, 1987). Purifying and biotyping these cultures will be able to identify the biovar(s) and molecular characterization of the strains will allow trace back studies.

Brucella spp. were mostly isolated from adult females, and this is not surprising as cattle industry focuses mostly on dairy production while commercial beef production is emerging in Rwanda. Almost half (47.0%) of the milk produced in 2008 was destined for sale at the informal market, with 16.0% for home consumption, while, 35.0% represented spoiled milk (Techoserve, 2008). Therefore, the occurrence of brucellosis in slaughtered cattle is not only a risk to abattoir workers but also consumers of milk and milk products. Several cattle and tons of beef are sold to Bukavu and Goma, the towns of the Democratic Republic of Congo neighboring Rwanda where the brochettes of the udder are expensive and frequently consumed. The udder is among the predilection sites of *Brucella* spp. (Caine et al., 2017, Fero et al., 2020) and meat inspection should focus on the udder. Meat inspection provides safe meat and contributes to the monitoring and surveillance for animal infectious diseases and zoonoses (Vågsholm, 2014). Furthermore, these brochettes should be consumed well done. It is also important to raise the awareness of involved stakeholders through education campaigns or media.

7.6. Conclusions

The present study found the seroprevalence lower than the gold standard rate indicating that cut-off points of i-ELISA determined in Europe with brucellosis free status or low prevalence, should be optimized for Rwanda as also reported by Mathew *et al.* (2015). This study identified *B. abortus* and *B. melitensis* as well as mixed infection in slaughtered cattle which is a result of the mixed livestock farming in Rwanda. These infections pose a risk of contamination to handlers of cattle, carcasses, and consumers of unpasteurized milk and milk products. Thus, vaccination and test-and-slaughter would significantly contribute to mitigate the disease. Furthermore, the introduction of an annual brucellosis-free certificate for large herds would contribute to mitigating brucellosis.

7.7. Acknowledgments

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Chapter 8. Prevalence of bovine tuberculosis and characterization of mycobacterial species from slaughtered dairy cattle in Rwanda

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8.1. Abstract

Bovine tuberculosis (bTB) is an endemic disease in Rwanda, but little is known about its prevalence and responsible mycobacterial species. bTB causes tremendous losses in livestock and wildlife and remains a significant threat to public health. A cross-sectional study employing a systematic random sampling of cattle (n=300) with collection of retropharyngeal lymph nodes and tonsils (n=300) with or without pathological lesions was carried out at six abattoirs to investigate the prevalence and identify mycobacterial species using culture, acid fast bacteria (AFB) staining, polymerase chain reaction (PCR), and GeneXpert assay. Individual risk factors and origin of samples were analysed for association with the prevalence. Our findings demonstrated the presence of MTBC in 1.7% of sampled slaughtered cattle. *M. bovis* was isolated from 1.3% (4/300) animals while 1 case was caused by *M. tuberculosis*. This study is the first in Rwanda to confirm both *M. bovis* and *M. tuberculosis* infection in cattle. Non-tuberculous mycobacteria (NTM) were identified in 12.0% (36/300) of the sampled cattle. There were no significant associations between the prevalence and abattoir category, age, sex, and breeds of slaughtered cattle. The study also identified rifampicin-resistance (RR) in the *M. tuberculosis* isolate which implies multidrug resistance (MDR). MDR is associated with a delayed

appropriate treatment which leads to human suffering and deaths. The isolation of RR *M. tuberculosis* from cattle indicates possible zooanthroponotic transmission of *M. tuberculosis* through exposure to cattle. It is essential to raise the awareness of zoonotic diseases among cattle owners, abattoir workers, and other stakeholders as well as to reinforce biosafety at farm level and in the abattoirs.

8.2. Introduction

Apart from *Mycobacterium leprae*, the genus *Mycobacterium* comprises two groups, Mycobacterium tuberculosis complex (MTBC) and non-tuberculous mycobacteria (NTM) also known as atypical mycobacteria or mycobacteria other than tuberculosis (MOTT) (Pfyffer et al., 1998). The bTB is a mycobacterial disease of cattle, other domestic and wild animals, as well as humans (Michel et al., 2010, Thoen et al., 2009). The disease is characterized by granulomatous lesions in affected tissues (Michel et al., 2010, Thoen et al., 2009). The disease is primarily caused by *M. bovis* (Karlson, 1970), and occasionally by *M. caprae* (Pavlik et al., 2002). *Mycobacterium tuberculosis* infection in cattle has been identified more recently and is of concern (Ameni et al., 2011, Berg et al., 2009, Malama et al., 2014). These species belong to the MTBC whose members share 99.9% of their genome (Brosch et al., 2002). MTBC members evolved from a human-adapted species, *M. canetti* (Canetti, 1970). The latter has successively lost DNA fragments to form regions of differences (RDs) which led to the evolution of *M. tuberculosis* affecting humans (Koch, 1882), *M. africanum* for humans (Castets et al., 1968), *M. microti* for voles (Wells, 1937), *M. pinipedii* in wild rodents (Cousins et al., 2003), *M. caprae* for goats (Aranaz et al., 1999), and *M. bovis* BCG, an attenuated vaccine strain (Calmette, 1927). The RDs form the basis of molecular differentiation of MTBC members (Huard et al., 2003). Likewise, *M. bovis* causes tuberculosis in humans and the disease resembles that caused by *M. tuberculosis* in terms of virulence, pathogenesis, and lesions (Cosivi et al., 1998, Moda et al., 1996). Zoonotic TB is a neglected disease due to the lack of discrimination between MBTC species in human TB laboratories (Cosivi et al., 1995, Wedlock et al., 2002), and therefore, the precise prevalence of zoonotic TB remains unknown. The prevalence is associated with immunosuppression and occupational groups that get infected by ingestion of untreated food of animal origin and inhalation, as well

as through transcutaneous route by handlers of animal carcasses (Kaneene, 2006, Thoen et al., 2009).

Cattle get infected mainly by erogenous route and ingestion of highly infected feeds (O'Reilly and Daborn, 1995). The disease is insidious with a progressive development of tubercles in the lungs, lymph nodes of the head, thorax, mesentery, liver, spleen, and other organs. The subclinical form involves weakness, anorexia, emaciation, dyspnea, and cough in the late stage of infection (Grange and Collins, 1987, OIE, 2019). These clinical signs are not specific and take several years to develop thus the diagnosis consists of routine post-mortem inspection at the abattoirs (Griffin and Buchan, 1994). The bTB herd prevalence in cattle varies from 0.2% to 13.2% in Tanzania (Katale et al., 2019) while the animal and herd bTB prevalence reported in cattle in Karamoja region and Mbarara districts of Uganda were 1.4% and 6.0%, and 51.4% and 74.1%, respectively (Oloya et al., 2006). Little information is available on bTB in Rwanda, and only two studies reported the bTB prevalence in Rwanda. A low prevalence (0.5%) was recorded in slaughtered cattle at société des abattoirs de Nyabugogo (SABAN Nyabugogo) (Habarugira et al., 2014). A retrospective study on TB-like macroscopic lesions at the same abattoir from 2006 to 2010 reported prevalence ranging from 1.4% in Kigali city to 11.8% in Eastern province (Nshimiyimana et al., 2013). In Rwanda, there is not any veterinary service unit that is capable of isolating airborne pathogens owing to the lack of appropriate facilities and adequately trained personnel. The control program for bTB relies mostly on monthly reports of gross TB-like lesions from the main private abattoir, SABAN Nyabugogo.

The cattle population in Rwanda was estimated at 1,293,768 in 2018 (Minagri, 2019). Although, informal slaughtering of goats, sheep, chicken, and rabbits for family or small bar consumption do occur in Rwanda, it is estimated that 95.0% of slaughtered cattle are processed by abattoirs. Determining the bTB prevalence and identification of MTBC members are essential to understand the transmission dynamics at the animal-human interface and to design adequate control programs. The objective of this study was therefore to determine the prevalence of bTB and characterize mycobacterial species in slaughtered cattle in six abattoirs in Rwanda. The

findings of this study will contribute to building the bTB database essential for policymakers to establish informed control policies and strategies to mitigate bovine tuberculosis in Rwanda.

8.3. Materials and methods

8.3.1. Study area

The present study was carried out in six abattoirs in Rwanda as described in chapter 6 (Figure 6.1). Rwanda is a member of the East African Community (EAC) located in the southern hemisphere, near the equator. Six abattoirs that consented to participate in this study included high throughput abattoirs (n=4) slaughtering more than 50 cattle daily, and low throughput abattoirs (n=2), slaughtering 50 or less every day. Three of these slaughtering premises slaughtered cattle and goats and three others were mono species (cattle). The location of the six abattoirs is shown on the Figure 6. 1.

8.3.2. Study design and sample size

A cross-sectional study was carried out from August 2018 through October 2019 to determine the prevalence of bTB and characterization of *Mycobacterium spp.* in cattle slaughtered at abattoirs. The abattoirs that accepted to participate in the study were purposively selected based on their strategic locations and the slaughtering capacity. The strategy was to gain insight into the bTB situation in cattle from each of the thirty districts of Rwanda (Figure 6.1). The sample size was calculated as previously described (Dohoo et al., 2009).

$$N = \frac{Z^2 \ P(1-P)}{d^2}$$

Where N is the sample size, a 95% confidence level (z) equivalent to 1.96; P is an expected of 0.5% based on a previous study in cattle in Rwanda (Habarugira et al., 2014), and the absolute precision (d = P/2) of 0.25%. According to the formula, the total sample size was supposed to be 291 but it was rounded to 300 cattle to respect the systematic sampling of 10 cattle per each of the 30 districts of Rwanda.

8.3.3. Sampling procedure and tissue collection

A systematic random sampling procedure among dairy cattle was adopted, and the strategy was to sample five animals from the same district per day. The movement permit was collected on arrival to record the origin of animals. The age was determined using dental erosion as previously described (Pope, 1934). Animals of two years and above were selected with a target on dairy except for some low throughput abattoirs that slaughtered mostly young male cattle. Animals were aligned in a crush and every fourth animal was selected, individual demographic information (district of origin, age, breed, and sex) recorded, restrained, marked on the head with original paint, and released for resting waiting for slaughter. Upon decapitation, the marked head was inspected, and left and right retropharyngeal lymph nodes and lingual and palatine tonsils with or without visible lesions were aseptically collected into a sterile 50 ml tube and stored at - 20^{o}C until processing.

8.3.4. Culture and detection of acid-fast bacilli (AFB)

Retropharyngeal lymph nodes and their respective tonsils (n=300) were decontaminated as previously described (Alexander et al., 2002). Briefly, tissues were thawed, sliced, grounded in a sterile mortar with sea sand (Glentham Life Sciences, UK). decontaminated as previously described (Alexander et al., 2002). An aliquot of each tissue sample was kept in -20^{o}C. Another aliquot was divided into two 50 ml falcon tubes. The content of one tube was decontaminated with an equal volume of 2% hydrochloric acid and the other one with an equal volume of 4% sodium hydroxide for 10 min at room temperature, then centrifuged at 3500 rpm for 10 min. The pellet was neutralized with 25 ml of sterile water centrifuged at 3500 rpm for 10 min. The pellet that was decontaminated by hydrochloric acid and sodium hydroxide were each inoculated onto duplicate slopes of Lowenstein-Jensen (LJ) with glycerol and duplicate slopes of LJ with sodium pyruvate (Labkem, Spain), and then incubated at 37^{o}C for 10 weeks with weekly readings. Cultures were scored positive, negative, or contaminated. When contamination occurred, the original sample was reprocessed and reinoculated. Any suspected growth was tested for morphology using auramine O staining and fluorescence microscope as previously

described (Clancey et al., 1976). All manipulations of samples including processing, inoculation and DNA extraction were performed in the biosafety level three at National Reference Laboratory (NRL), Kigali, Rwanda.

8.3.5. Molecular Assays

8.3.5.1. DNA extraction

Lysate DNA was extracted from each AFB culture isolate as previously described (Hlokwe et al., 2013). Briefly, two loopful bacterial cells were suspended in 300 µl of distilled sterile water, then boiled at 95°C for 25 min, quickly cooled and stored at - 20°C until required. Genomic DNA was extracted from inactivated grown cultures using a DNA extraction kit according to the manufacturer's instructions (Promega, USA).

8.3.5.2.Conventional PCR

Isolates that were confirmed AFB were screened for the presence of 16S rRNA sequence specific for the genus *Mycobacterium* and a sequence encoding the MPB 70 antigen which is specific for members of MTBC using specific primers (Mycgen–F and Mycgen-R, TB1-F and TB2-F, respectively) as previously described (Wilton and Cousins, 1992). PCR assay based on genomic deletions differentiated members of MTBC (refer to as MTBC differential PCR assay) using primers targeting the regions of difference, RD1, RD4, RD9, and RD12 as previously described (Table 8. 1) (Warren et al., 2006). *Mycobacterium tuberculosis* 25177 was used as a reference. For all multiplex PCRs, the 15 µl PCR reaction mixture contained 1x MyTaq™ Red PCR Mix (Bioline, South Africa), primers at 0.2 µM, and 2 µl of template DNA. The PCR cycling condition was as follows: initial denaturation at 94°C for 5 min followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 1 min, and extension at 72°C for 1 min and a final extension step at 72°C for 8 min. Primers amplified a 1030 bp, 372 bp, 108 bp, and 268 bp fragments for the genus, MTBC, and *M. bovis*, respectively and were analyzed by electrophoresis using a 2% agarose gel stained with red gel nucleic acid stain and visualized under UV light. The PCR experiments were performed at Rwanda Agriculture and Animal

Resources Board, Department of Veterinary services in the virology and molecular biology sections.

8.3.5.3. GeneXpert MTB/RIF assay

MTBC isolates characterized by conventional PCR were also tested by GeneXpert MTB/RIF molecular diagnostic assay following the manufacturer's instructions (Cepheid, Sunnyvale, USA). GeneXpert MTB/RIF is a real-time PCR for the detection of MTBC and rifampin resistance. Briefly, 0.5 ml of the cell suspension was transferred into a conical-screwed tube and 1 ml of sample reagent was added. The mixture was vortexed for 10 sec and incubated for 15 min with vortexing for 10 sec after 8 min of incubation. The liquefied sample was then dispensed into the sample chamber of the cartridge containing five probes (A-E), integrated reagents tubes, a sample processing control (SPC), and a probe check control (PCC). Cartridges were then installed into the GeneXpert^{R}Dx system version 4.8 (Cepheid, Sunnyvale, USA) and the amplification was run for two hours by activating the software installed in the computer. This assay was performed at NRL, Kigali, Rwanda.

Table 8. 1. Oligonucleotides used to identify mycobacterial species isolated from slaughtered cattle in Rwanda

PCRs	**Primer name**	**Nucleotide sequence (5'----------3')**	**Target**	**Size (bp)**	**Tm (^{o}C)**	**References**
Multi plex 1	MYCGEN-F	AGA GTT TGA TCC TGG CTC AG	16s rRNA	1030	62	(Wilton and Cousins, 1992)
	MYCGEN-R	TGC ACA CAG GCC ACA AGG GA				
	TB1-F	GAA CAA TCC GGA GTT GAC AA	MPB 70	372		
	TB2-R	AGC ACG CTG TCA ATC ATG TA				
Multi plex	RD1-1	AAGCGGTTGCCGCCGACCGACC		146	62	(Parsons et al., 2002)
	RD1-2	CTGGCTATATTCCTGGGCCCGG				

2*	RD1-3	GAGGCGATCTGGCGGTTTGGGG				
	RD4-1	ATG TGC GAG CTG AGC GAT G	Rv 1510	268 RD is absent	62	(Warren et al., 2006)
	RD4-2	TGT ACT ATG CTG ACC CAT GCG				
	RD4-3	AAA GGA GCA CCA TCG TCC AC				
	RD9-1	CAA GTT GCC GTT TCG AGC C	Rv 2073	108 RD is absent	62	
	RD9-2	CAA TGT TTG TTG CGC TGC				(Parsons et al., 2002)
	RD9-3	GCT ACC CTC GAC CAA GTG TT				
	RD12-1	GGGAGCCCAGCATTTACCTC		306		(Warren et al., 2006)
	RD12-2	GTGTTGCGGGAATTACTCGG				
	RD12-3	AGCAGGAGCGGTTGGATATTC				

*MTBC (*Mycobacterium tuberculosis* complex) differential PCR assay, MPB 70 stands for protein from *M. bovis* with 0.70 mobility by native polyacrylamide gel electrophoresis at pH 9.4 gel but it an antigen common to all MTBC, Rv: refers to rough morphology and virulent MTBC strain.

8.3.6. Data analysis

Data were recorded in the Microsoft Excel spreadsheet and descriptive and inferential statistics were performed using EpiInfo software version 7.2. The significance level of 95% and p-value less or equal to 5% were considered for all analyses. The prevalence of isolation of *Mycobacterium* spp. and MTBC was tested for association with individual animal characteristics such as age, sex, breed, and sampled abattoir in the univariate logistic analysis using Chi-square or Fischer exact.

8.4. Results

Out of the 300 samples collected from 300 cattle, 94.0% (282/300) were collected from the high throughput abattoirs while 6.0% (18/300) were from the low throughput abattoirs. Of the 300 samples, 95.3% (286, 95% CI: 92.3 – 97.4) were collected from female cattle while 4.7% (14, 95%CI: 2.6 - 7.7) were from male cattle. The majority 90.3% (271/300, 95% CI: 86.4 – 93.4) were from adult cattle while 9.7% (29/300, 95% CI: 6.6 - 13.6) were collected from young animals. Most samples, 67.9% (203/300, 95% CI: 62.3 – 73.2) were collected from crossbreeds, 25.8% (77/300, 95%CI: 20.9 – 31.1) were collected from local breed "Ankole", while 6.7% (20/300, 95%CI: 3.8 – 9.5) were collected from a pure breed "Friesian".

Of the 300 samples that were inoculated, 55.0% (165, 95%CI: 49.2 – 60.7) had bacterial growth of which 30.9% (51/165, 95% CI: 23.9 – 40.0) were AFB as indicated by auramine fluorescence staining method. Of the 51 AFB, the PCR identified 80.4% (41/51, 95% CI: 69.5 – 91.3) as *Mycobacterium* spp. (amplification of 1030 bp fragment, Table 8. 1). Of the 41 *Mycobacterium* spp., 87.8% (36/41, 95%CI: 77.8-97.8) were NTM, while the remaining 12.2% (5/41, 95% CI: 2.2 – 22.2) were MTBC (amplification of a 372 bp fragment, Table 8. 1). MTBC differential PCR assay identified 80.0% (4/5, 95% CI: 44.9 – 100.0) as *M. bovis* (amplification of 108 bp, 146 bp, and 268 bp) and 20.0% (1/5, 95% CI: 0.0 – 55.1) as *M. tuberculosis* (amplification of 146 bp, 172 bp, 235 bp, 369 bp) (Figure 8. 1). Overall, NTM were identified in 12.0% (36/300, 95%CI: 8.3-15.7) of the sampled cattle, MTBC were isolated in 1.7% (5/300) of the sampled cattle and among these, four were *M. bovis*, while one was *M. tuberculosis*.

Among the six samples that were collected with visible pathological lesions (four with generalized lesions and two localized to popliteal lymph node), only one was confirmed as *M. bovis*, another one was NTM, while the remaining four were not identified as *Mycobacterium* spp.

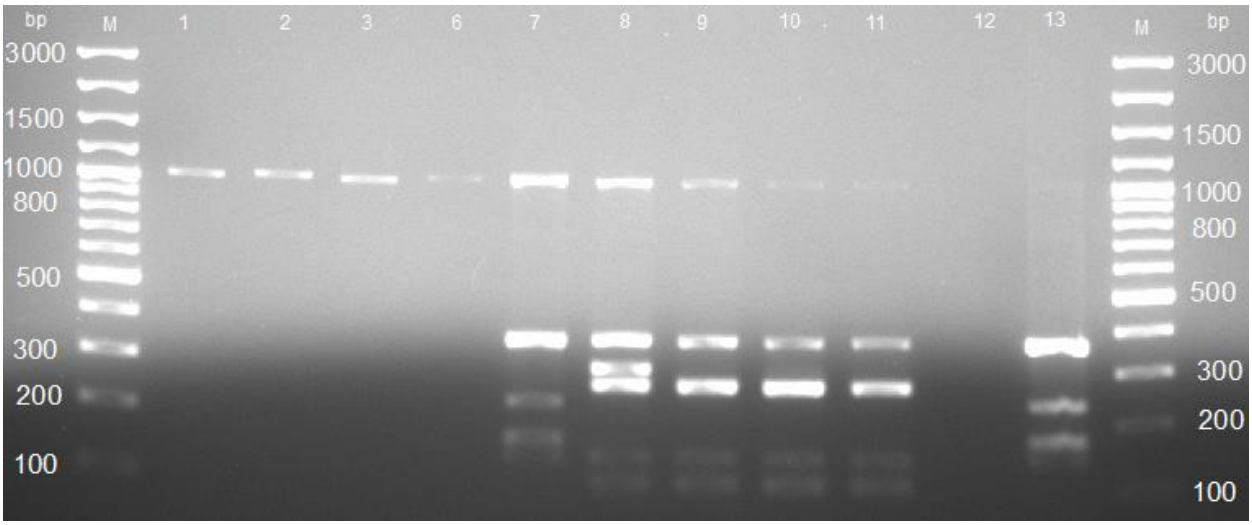

Figure 8. 1. Agarose gel electrophoresis of the Mycobacterium tuberculosis complex differential PCR assay. Lane M: GeneRuler 100 bp (Invitrogen, ThermoFischer Scientific, South Africa), lanes 2-4: Non-tuberculosis mycobacteria (NTM) which amplified 1030 bp; lane 7: *M. tuberculosis* which amplified 1030 bp, 372 bp, 235 bp, 172 bp, and 146 bp; lanes 8 – 10: *M. bovis* which amplified 1030 bp, 372 bp, 268 bp, 146 bp, and 108 bp; lane 11: negative control; lane 12: *M. tuberculosis* reference strain 2517.

Mycobacterium spp. isolates were found in 83.3% (5/6) of the abattoirs; 80.9% (38/47) in high throughput abattoirs and 75.0 (3/4) in low throughput abattoirs, although the difference was not significant (Table 8. 2). All the MTBC isolates were found in the high throughput abattoirs. MTBC were isolated in cattle from Nyarugenge district (n=1) of Kigali city, Karongi (n=1), Nyabihu (n=1), and Rubavu (n=2) districts of western province. All five MTBC isolates were identified from adult and crossbred cattle (Table 8. 2). The GeneXpert MTBC/RIF assay confirmed MTBC isolates. Resistance to rifampicin was detected in one isolate.

Table 8. 2. Mycobacterial culture results for 300 slaughtered cattle and PCR results of AFB isolates stratified by the abattoir, age, breed, and sex of slaughtered cattle in Rwanda.

Variables	Categories	Mycobacterial culture and AFB results		PCR results of AFB isolates for detection of *Mycobacterium* spp.				PCR results of AFB isolates for detection of Mycobacterium tuberculosis complex (MTBC)			
		Growth % (n)	AFB positive	Positive % (n)	95% CI	Chi ²	*p*-value	Positive n (%)	95% CI	Chi ²	*p*-value
Abattoirs	High throughput	53.9 (152/282)	16.7 (47/282)	80.9 (38/47)	0.3 - 4.8	0.8	1	10.6 (5/47)	0.0 - 16.3	0	1
	Low throughput	72.2 (13/18)	22.2 (4/18)	75.0 (3/4)				0.0 (0/4)			
	Total	**55.0 (165/300)**	**17.0 (51/300)**	**80.4 (41/51)**				**9.8 (5/51)**			
Provinces	East	55.7 (39/70)	17.1 (12/70)	83.3 (10/12)	-	1.6	0.9	0.0 (0/12)	-	10.2	0.04
	West	62.7 (44/70)	25.7 (18/70)	72.2 (13/18)				22.2 (4/18)			
	North	56.0 (28/50)	16.0 (8/50)	87.5 (7/8)				0.0 (0/8)			
	South	45.0 (36/80)	13.8 (11/80)	81.8 (9/11)				0.0 (0/11)			
	Kigali city	60.0 (18/30)	6.7 (2/30)	100.0 (2/2)				50.0 (1/2)			
	Total	**55.0 (165/300)**	**17.0 (51/300)**	**80.4 (41/51)**				**9.8 (5/51)**			
Age	Young	65.5 (19/29)	20.7 (6/29)	50.0 (3/6)	0.2-1.7	2.1	0.1	0.0 (0/6)	0.0–9.3	0.02	1
	Adult	53.8 (146/271)	16.6 (45/271)	84.4 (38/45)				11.1 (5/45)			
	Total	**55.0 (165/300)**	**17.0 (51/300)**	**80.4 (41/51)**				**9.8 (5/51)**			
Sex	Female	55.6 (159/286)	16.8 (48/286)	79.2 (38/48)	-	0.01	1	8.3 (4/48)	0.4 - 74.8	0.2	0.3
	Male	42.9 (6/14)	21.4 (3/14)	100.0 (3/3)				33.3 (1/3)			
	Total	**55.0 (165/300)**	**17.0 (51/300)**	**80.4 (41/51)**				**9.8 (5/51)**			
Breeds	Ankole	54.5 (42/47)	13.0 (10/77)	80.0 (8/10)	-	0.8	1	0.0 (0/10)	-	1.9	0.7
	Crossbred	56.2 (114/208)	18.7 (38/203)	79.0 (30/38)				13.2 (5/38)			
	Friesians	42.1 (8/19)	15.0 (3/20)	100.0 (3/3)				0.0 (0/3)			
	Total	**55.0 (165/300)**	**17.0 (51/300)**	**80.4 (41/51)**				**9.8 (5/51)**			

8.5. Discussion

Bovine tuberculosis (bTB) causes financial losses in livestock and remains a significant threat to public health worldwide. This study on the prevalence of bTB and identification of mycobacterial species in slaughtered cattle using bacterial culture and polymerase chain reaction (PCR), is the first report in Rwanda to identify *M. bovis* (n=4) and *M. tuberculosis* (n=1), and non-tuberculous mycobacteria (n=41) in a total of 300 cattle from 30 districts and animals were slaughtered in six abattoirs in Rwanda. Although the prevalence of bTB (1.7%, 5/300) was low, the identification of *M. bovis* and *M. tuberculosis* with rifampicin resistance in cattle indicates the cross-transmission from humans to cattle but also highlights the great risk of exposure of handlers of live infected cattle and animal carcasses.

Considering the relatively large sample size (n=300) and the random selection of animals found without visible lesions (96.7%), the prevalence observed for MTBC in this study (1.7%) is higher than 0.5% reported at SABAN Nyabugogo abattoir, Rwanda (Habarugira et al., 2014) given the small sample size (n=36) of previously selected gross bTB-like lesions. However, considering that samples were collected at a single point in time and the small sample size (n=10) per district, the prevalence of this study is lower than the real prevalence in slaughtered cattle in Rwanda. The prevalence (1.7%) obtained in this study is consistent with 2.1% obtained in Kenya using bovine TB-like lesions, AFB staining, bacterial culture, and PCR (Gathogo et al., 2012) but lower compared to 7.6% obtained in Uganda using bovine TB-like lesions, bacterial culture, AFB staining, and capillia TB-neo assay for detection of MPT 64 antigens of the MTBC (Nalapa et al., 2017). The prevalence obtained in this study also falls in the range of bTB herd prevalence (0.2 to 13.2%) reported in cattle in Tanzania (Katale et al., 2019). Therefore, bTB may be circulating in cattle of the Easter African region through animal trading and animal movement across borders. Furthermore, Rwanda supplies daily many tones of live animals, carcases, red offal's and viscera in neighbouring Goma and Bukavu in the Democratic Republic of Congo hence there is a need for testing bTB before animal trading. The prevalence obtained in this study was lower than 5.0% obtained in Cameroun using bovine TB-like lesions, AFB

staining, bacterial culture, SD bioline TB Ag MPT 64 rapid test, and PCR (Ibrahim et al., 2018). The low prevalence observed in this study may be associated with the low concentration of the pathogen in the sample due to the low average age of cattle at slaughter (5 years). It is known that the age of cattle is often associated with the low concentration since the bTB prevalence increases with advanced age from 5 and greater (Ameni et al., 2007, Awah-Ndukum et al., 2012). Furthermore, a certain concentration of the bacterial load may be lost during the decontamination process (Mtafya et al., 2019, Peres et al., 2009) leading to the absence of bacterial growth in the LJ medium. The low prevalence may also be a result of restriction of cattle movement and zero-grazing practised in Rwanda to mitigate the propagation of infectious diseases.

This study demonstrated that bTB is prevalent in Rwandan cattle but at low prevalence since samples were collected from all the 30 districts of Rwanda. Four high throughput abattoirs received cattle from several districts (close and districts from far in more than 90 km). For instance, cattle that were sampled at SABAN abattoir located in Kigali city, were from 19 districts including Nyarugenge, Gasabo (Kigali city), Ngoma, Kirehe, Nyagatare, Gatsibo, Bugesera, Kayonza (Eastern Province), Gakenke, Burera, Rulindo, Gicumbi (Northern Province), Rutsiro, Karongi, Ngororero (Western Province), Ruhango, Nyanza, Kamonyi, Muhanga (Southern Province). Cattle that were sampled at Rugano abattoir located in Kigali city, were from 4 districts including Gasabo, Kicukiro, Nyarugenge (Kigali city), and Rwamagana of Eastern Province. Cattle that were sampled at Kamembe abattoir located in the Western Province, were from eight districts including Gisagara, Huye, Nyaruguru, Ruhango, Nyanza, Nyamagabe (Southern Province), Nyamasheke, and Rusizi (Western Province). Cattle that were sampled at Rubavu abattoir located in the Western Province, were from two districts including Nyabihu, and Rubavu (Western Province). However, three animals that were positive to MTBC were detected in cattle from Rubavu (n=2) and Nyabihu (n=1) districts which supply cattle to the nearby abattoirs (less than 20 km) in the Western Province. Of the other two cattle that were positive to MTBC, one was from Ngororero district of the Western Province and

another one from Nyarugenge district of Kigali city and both were found at SABAN abattoir located in Kigali city.

This study identified for the first time *M. bovis* (1.3%, 4/300) and *M. tuberculosis* (0.3%, 1/300) in slaughtered cattle in Rwanda. The isolation of *M. tuberculosis* in retropharyngeal lymph nodes of cattle indicates that close contact of tuberculous humans with animals pose a risk of transmission of human TB to animals. Similar studies in Africa isolated *M. bovis* (2.0%, 19/929) and *M. tuberculosis* (0.2%, 2/929) in slaughtered cattle in Kenya (Gathogo et al., 2012), and *M. bovis* (4.0%, 12/300), and *M. tuberculosis* (0.7%, 2/300) in slaughtered cattle in Cameroun (Ibrahim et al., 2018). However, the prevalence of *M. tuberculosis* in cattle is commonly below 1.0% (Gathogo et al., 2012, Ibrahim et al., 2018, Ocepek et al., 2005), apart from some areas (27.0%) in Ethiopia where cattle owners had the habit of discharging the chewed tobacco into the mouth of their cattle (Ameni et al., 2011). The prevalence of *M. tuberculosis* is also high in areas or countries with a high prevalence of TB in humans owning cattle (Regassa et al., 2008). For example, a study in the Eastern Cape province in South Africa identified more *M. tuberculosis* (41.8%, 157/376) than *M. bovis* (1.3%, 5/376) from slaughtered cattle (Bhembe et al., 2017). Cattle may acquire *M. tuberculosis* through the respiratory route due to close contact of tuberculous humans with cattle or via ingestion of materials contaminated by humans presenting urogenital tuberculosis and urinating in the pasture (Grange and Collins, 1987). The transmission of *M. tuberculosis* from humans to cattle is not surprising since 92.0% of Rwandans owning cattle are small dairy farmers practicing a zero-grazing system (IFAD, 2016). The latter promotes a close contact with animals leading to the risk of cross-infection by respiratory route from cattle to humans and vice-versa. This cross-infection has negative financial and public health implications on the households and other occupational groups. Despite the little attention given to the zoonotic TB caused by *M. bovis* (Marcotty et al., 2009), several studies have isolated *M. bovis* in extrapulmonary lymph nodes of humans in neighbouring Uganda (Oloya et al., 2008), and Tanzania (Mfinanga et al., 2004). It is hence essential to raise the awareness among veterinary and human health professionals about the anthropozoonotic transmission of TB in Rwanda. Further studies on the identification of *M. bovis* in humans are

worthy investigating to provide epidemiological data that are indispensable for the eradication of tuberculosis by 2035.

This study identified NTM in 12.0% of slaughtered cattle, and this prevalence is consistent with 8.4% obtained in Uganda (Nalapa et al., 2017), but higher than 3.9% in Tanzania (Kazwala, 1996). This study considered the presence of NTM in the environment, hence, tissues were aseptically (changing gloves and sterilization of the knife into hot water) collected, stored, and processed, thus, it can be assumed that the identified NTM were recovered from the tissues of animals, but it does not prove any pathological effect, it is just colonization. The fact that no speciation was conducted, it would be important to determine the potential significance for the health of the cattle. NTM have been isolated in cattle and sometimes cause localized lymphadenitis, skin infections, TB-like pulmonary infections, and systemic diseases in immunodeficient cattle (Primm et al., 2004). The presence of NTM in cattle may interfere with immune-diagnostic methods such as comparative tuberculin test and may negatively impact vaccination (Gcebe and Hlokwe, 2017).

In this study, among the samples with pathological lesions (n=6) submitted by inspectors for confirmation of bTB, only one popliteal lymph node was associated with NTM species, and one lung was associated with *M. bovis*. A retrospective study reported the prevalence of 11.8% based on TB-like lesions recorded during routine meat inspection from 2006 to 2010 at SABAN Nyabugogo abattoir, Rwanda (Nshimiyimana et al., 2013). TB-like lesions might therefore be a poor reflection of bTB in the absence of a confirmatory laboratory test. TB-like lesions from routine meat inspection should, therefore, be confirmed by laboratory tests to obtain accurate results essential for surveillance of bTB, but also improve the knowledge of inspectors.

Mycobacterium spp. isolates (76.7%) were more frequently isolated in adult than young cattle (40.0%) and all MTBC were isolated from adults consistent with a study in Ethiopia (Ameni et al., 2007). The isolation of *Mycobacterium* spp. depends a lot on the dose and

frequency of exposure to *M. bovis*. Therefore, higher infection rates in adult cattle results from a cumulative risk of infection in adult cattle. In other words, the older an animal the more opportunities it had to contract *M. bovis*. Furthermore, the literature states that young cattle are less susceptible to mycobacteria owing to the high concentration of T cells in the blood circulation and T cells play a role in the immunity against mycobacteria (Mackay and Hein, 1989).

This study isolated for the first-time rifampicin-resistant (RR) *M. tuberculosis* (n=1) from slaughtered cattle in Rwanda. This is consistent with a study in Italy which reported RR *M. bovis* isolates from cattle (Sechi et al., 2001). Rifampicin is a cornerstone antibiotic of the first-line regimen (WHO, 2010), and resistance to rifampicin is considered as multidrug resistance tuberculosis (MDR-TB) and indicates a mutation in the *ropB* gene (Jain and Mondal, 2008). RR *M. tuberculosis* isolated in cattle is most likely of human origin and rifampicin resistance tuberculosis (RR-TB) is commonly reported in Rwandans with TB. For instance, 876 cases of RR-TB were recorded from 2005 to 2016 and these were associated with deaths due to delayed diagnosis and inadequate treatment (Ngabonziza et al., 2020). Interestingly, the RR-TB mortality reduced from 30.6% in 2006 to 6.9% in 2016 following the introduction of rapid diagnosis with GeneXpert MTB/RIF assay and access to the second-generation regimen (Ngabonziza et al., 2020). However, this effort deals with treatment of RR-TB while RR MTBC strains remain prevalent. It is essential to raise awareness of the Rwandan community especially the owners of cattle about anthropozoonotic transmission of TB that was demonstrated in this study. Another thought is that cattle can be considered sentinels for *M. tuberculosis* in settings where human TB is not effectively controlled in humans. It is therefore also an alert for improved TB control in humans in rural settings.

8.6. Conclusions

This study demonstrated that bTB is prevalent in Rwanda at low prevalence. The present study reports for the first time MTBC in cattle in Rwanda and the presence of RR *M.*

tuberculosis indicating possible cross-infection between humans and cattle. There is therefore a need for raising awareness among veterinary and human health professionals about the zooanthroponotic transmission and cross infection of TB in Rwanda. Further studies on the identification of *M. bovis* in humans are worth investigating to provide epidemiological data that are indispensable for the eradication of tuberculosis by 2035, a global movement led by the World Health Organization.

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8.8. References

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Chapter 9. General discussion and conclusions

9.1. General discussion

Bovine brucellosis (BB) and bovine tuberculosis (bTB) are widespread bacterial diseases that affect several animals and humans (Arnot and Michel, 2020, Godfroid et al., 2010). These diseases cause considerable financial losses and threaten public health worldwide (McDermott et al., 2013, Zinsstag et al., 2006). Although BB and bTB are endemic in Rwanda (Habarugira et al., 2014, Ntivuguruzwa et al., 2020), little is known about their epidemiology and there is a lack of sufficient information, research, and official documentation about BB and bTB. It is known that BB is mainly caused by different biovars of *B. abortus*, *B. melitensis*, and occasionally *B. suis* (OIE, 2018) while the bTB is mostly caused by *M. bovis*, *M. tuberculosis*, and *M. caprae* (Berg et al., 2009, Karlson, 1970, Prodinger et al., 2005). However, there is not recent documentation on the *Brucella* spp. and *Mycobacterium* spp. that are circulating in Rwanda. It was, therefore, important to investigate the prevalence of BB at the wildlife-livestock-human interface and slaughtered cattle, identify *Brucella* spp. that are circulating in Rwanda, identify *Mycobacterium* spp. and determine the prevalence of bTB at the abattoirs, as well as assess the potential risk factors that are associated with BB in animals, farmworkers, and abattoir workers, and bTB, leptospirosis, Q-fever, and cysticercosis in abattoir workers.

The control scheme of brucellosis in developing countries including Rwanda mainly focuses on high-risk zones (Zhang et al., 2018). It is in this context that this study investigated the brucellosis status at the wildlife-livestock-human interface. This was the first study of BB within five districts (bordering national parks) and one peri-urban district of Rwanda using RBT and i-ELISA. The overall individual seroprevalence rate of brucellosis (8.3%) at the interface falls in the range (0.2% – 43.8%) reported for cattle in East African countries (Djangwani et al., 2020). This study found that brucellosis is endemic in districts of Eastern Province with Gatsibo (17.7%), Kayonza (10.1%), and Nyagatare (8.2%) having higher rates than

other districts ($p < 0.001$). This was consistent with previous studies which also reported brucellosis in the Nyagatare district of Eastern Province (Chatikobo et al., 2008, Ndazigaruye et al., 2018). The identification of single and mixed *B. abortus* and *B. melitensis* from cultures of whole blood, milk, and vaginal swabs sampled from seropositive herds of cattle is a problem of concern as low awareness of zoonotic brucellosis transmission was observed among cattle keepers and more than 27.0% of cattle keepers having seropositive cattle reported to assist calving without biosafety protection, drinking raw milk, and manual milking (Ntivuguruzwa et al., 2020). *Brucella abortus* and *B. melitensis* that were identified in cattle also cause brucellosis in humans (Bamaiyi, 2016, OIE, 2018). In Rwanda, the seroprevalence of brucellosis varied from 6.7% to 25.0% in women with a history of abortion (Gafirita et al., 2017, Rujeni and Mbanzamihigo, 2014). This suggests that the disease may be prevalent among farmworkers and other occupational groups who are daily working without wearing PPE while exposed to aborted tissues, and contaminated carcasses. This study also found a history of abortion, old age of animals, cohabitation of cattle and goats, and introduction of replacement animals of unknown brucellosis status into the herd were significant predictors of brucellosis ($p < 0.05$) (Ntivuguruzwa et al., 2020). In this study, the association of brucellosis with cohabitation of cattle and goats was confirmed by the isolation of mixed infections caused by *B. abortus* and *B. melitensis* in both cattle, and goats.

This study found that the history of abortion was significantly associated with brucellosis in cattle farmed at the wildlife-livestock-human interface (Ntivuguruzwa et al., 2020), consistent with previous reports in Uganda (Kabi et al., 2015, Makita et al., 2011, Nina et al., 2018), and Tanzania (Asakura et al., 2018). This finding was confirmed by the isolation of single and mixed infections caused by *B. abortus* and *B. melitensis* from blood, milk, and vaginal swabs of seropositive herds of cattle farmed at the wildlife-livestock-human interface. It is believed that *Brucella* spp. cause most of the abortion cases (da Silva et al., 2009), although in Rwanda some cases are sometimes reported in cattle that are seronegative to brucellosis, and in these cases, further diagnostic options are limited. This study isolated for the first time *B. abortus*, *B. melitensis*, *Leptospira* spp., and *C. fetus* from aborted tissues of cattle. The isolation of

C. fetus and *Leptospira* spp. from aborted tissues of cattle suggests that a multiplex PCR should be a good alternative to diagnose other abortigenic pathogens from *Brucella* negative samples. In Rwanda, various reproductive disorders that have been reported in the cattle industry (Chatikobo et al., 2009) can be associated with infections caused by *Brucella* spp., *C. fetus*, or *Leptospira* spp. These disorders included higher incidences of abortions, retained placenta, infertility of unknown origin, and longer calving intervals (Chatikobo et al., 2009). Such abortions cause tremendous financial losses and wherever they occur in the herd, massive screening of the herd against brucellosis is very important and positive animals should be immediately slaughtered to stop spreading.

This study demonstrated a low awareness of cysticercoses as an animal and human disease among abattoir workers while the disease was reported in 3.0% of slaughtered cattle at Nyagatare slaughterhouse, Rwanda (Nzeyimana et al., 2015), in 21.8% of patients presenting epilepsies at Kabutare hospital, Rwanda (Rottbeck et al., 2013), and 13.3% (76/572) of children in Gakenke district, Rwanda (Soto et al., 2021). We, therefore, advise health professionals both in veterinary and human medicine to share information and educate the population about cysticercosis in Rwanda.

This study also isolated *Leptospira* spp. from aborted tissues of cattle while the awareness of leptospirosis either in animals or humans was low (10.2%) among abattoir workers. This is the first report of the disease in cattle and there is no evidence of the disease in humans in Rwanda however the disease was reported in abattoir workers in Nigeria (Abiayi et al., 2015). Further investigations on leptospirosis are worthwhile among exposed groups in Rwanda.

To have an idea of the brucellosis status in the whole country, we investigated the brucellosis at the abattoirs by sampling blood and corresponding lymph nodes for 10 slaughtered cattle coming from each of the thirty districts of Rwanda. The prevalence (5.6%) obtained using the gold standard (bacterial culture and ITS PCR) was higher compared to the seroprevalence (2.7%) indicating that the cut-off points of i-ELISA determined in Europe where

brucellosis has been eradicated need to be validated for Rwandan conditions where brucellosis is endemic. This was also supported by the identification of *Brucella* spp. in seronegative cattle in this study. The seroprevalence of brucellosis obtained at the interface of 8.3% is comparable to the prevalence obtained at the abattoirs (5.6%) using the gold standard. These findings indicate that BB is endemic in Rwanda with high rates in the high-risk areas such as the wildlife-livestock-human interface, and areas with high milk production. These high-risk areas need special attention. Single and mixed infections caused by *B. abortus* and *B. melitensis* that were observed in cattle at the interface and in slaughtered cattle pose a problem to public health and thus, there is a need for improved control of brucellosis in animals and raising brucellosis awareness among abattoir workers.

This study also found poor practices that can be associated with brucellosis among abattoir workers. The identified poor practices included working without wearing gloves, cutting hands, eating while working and these habits commonly predispose abattoir workers to zoonotic diseases (Luwumba et al., 2019, Mirambo et al., 2018). Brucellosis in humans is an insidious disease with clinical signs like those of other febrile illnesses such as malaria, typhoid, etc. leading to misdiagnosis (Crump et al., 2013). The awareness of zoonotic bTB transmission was high because tuberculosis cases are easily diagnosed at post-mortem inspection and TB cases among abattoir workers were reported in this study. This study found that education contributes to a reduction of infectious diseases in animals and humans, and this is in agreement with the literature (Assenga et al., 2016).

Brucellosis and bovine tuberculosis were both detected in slaughtered cattle for the first time in Rwanda indicating the high risk of exposure of abattoir workers to the pathogens. One sample (NRG04) with bTB lesions in the lungs and lymph nodes had both *B. abortus* and *M. bovis* representing a high risk of contamination to farm and abattoir workers. Furthermore, this study identified RR *M. tuberculosis* indicating the transmission of rifampicin-resistant (RR) *M. tuberculosis* from humans to cattle either via erogenous contamination facilitated by close contact or ingestion of materials contaminated by urine or sputum of an infected patient

(Grange and Collins, 1987). The tubercle bacilli are excreted via milk promoting the spreading of the pathogen through a repeated cycle of exposure among farmworkers, and abattoir workers. Therefore, we recommend the improvement of biosafety protection at the farm level and in the abattoirs.

9.2. General conclusions

The seroprevalence of brucellosis obtained at the interface of 8.3% is comparable to the prevalence obtained at the abattoirs (5.6%) using the gold standard indicating that BB is endemic in Rwanda with high rates in the high-risk areas such as the wildlife-livestock-human interface, and areas with high milk production. These high-risk areas need special attention in the control of brucellosis. Single and mixed infections caused by *B. abortus* and *B. melitensis* were observed in cattle farmed at the wildlife-livestock-human interface, in slaughtered cattle, and aborted tissues of cattle. However, *C. fetus* and *Leptospira* spp. were the most recovered abortigenic pathogens from aborted tissues of cattle. Furthermore, *M. bovis* and RR *M. tuberculosis* were identified in slaughtered cattle. Additionally, low awareness of the transmission of zoonotic brucellosis and other zoonotic diseases (leptospirosis, Q-fever, and cysticercosis) was recorded among farm and abattoir workers. However, educated farm and abattoir workers had a higher awareness of the transmission of zoonotic diseases compared to uneducated. Therefore, the education of farm workers, abattoir workers, and other stakeholders would significantly contribute to the control of brucellosis, bovine tuberculosis, leptospirosis, and cysticercosis in animals and humans in Rwanda.

9.3. Recommendations

Based on the findings of this study we recommend:

- ✓ For each large herd to possess an annual brucellosis-free certificate issued by competent authorities after serological testing (RBT and i-ELISA, or milk ring test),
- ✓ Brucellosis testing of domestic animals (cattle, sheep, goats, and pigs) before animal trade,

- ✓ Systematic and coordinated vaccination of all calves using already used *B. abortus* RB51 vaccine strain in Rwanda,
- ✓ Biannual surveillance of brucellosis in high-risk zones and at the abattoirs,
- ✓ Validation of i-ELISA for Rwanda, and screening of animal tissues using the 16S-23S interspacer region (ITS) PCR assay,
- ✓ Respect of test-and-slaughter and compensation of animal owners,
- ✓ To avoid mixed farming and introduction of replacement animals with unknown brucellosis status,
- ✓ Reporting each abortion occurring in food-producing animals followed by a massive brucellosis screening of the herd, cooling of positive animals to stop spreading, screening *Brucella* negative samples for other abortigenic pathogens using a multiplex PCR assay, and incorporating this method in the routine tests done at the national veterinary laboratory,
- ✓ Inclusion of brucellosis, and leptospirosis in the list of diseases for diagnosis in malaria negative patients,
- ✓ Education of the exposed occupational groups, stakeholders, and the public on zoonotic diseases, good practices for farm and abattoir workers, hygiene of food-producing animals and food such as milk and milk product especially the homemade cream milk (Ikivuguto) using different types of communication such as TV, Radio, teaching materials, etc.
- ✓ Supporting and implementation of the One Health Approach to facilitate collaboration and information exchange between veterinarians, medical doctors, and environmentalists about zoonotic cysticercosis, leptospirosis, genital campylobacteriosis, and extrapulmonary cases of TB due to *M. bovis*.

This control program should be coordinated, continual with biannual monitoring and evaluation reports.

9.4. Limitations of the study

We could not test all the 1907 sera against brucellosis using i-ELISA and this may have led to missing chronic infections because IgM detected by RBT are earlier induced and

disappear quickly while IgG detected by i-ELISA are induced later and last for longer (Godfroid et al., 2013). Due to the large sample size, in different six districts which are dispersed in four of the five provinces of Rwanda, and due to financial constraints, we could not systematically sample for all the 1907 cattle sera, the corresponding whole blood, vaginal swabs, and milk, therefore, a comparison of results from different type of samples was not possible. The sample size for the abattoir study that consisted of 10 samples per district was not representative of the whole district and consequently the whole country. We, therefore, recommend a longitudinal abattoir study involving a large sample size per district for different seasons combining sampling slaughtered cattle and abattoir workers.

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APPENDIXES

Appendix 1: Univariate associations of potential risk factors with herd-level seropositivity of *Brucella* spp. in cattle sampled at the wildlife - livestock - human interface in Rwanda.

Variables	Category	Interviewed	RBT & i-ELISA		*p*-value
			No. (%)	[95% CI]	
Level of education	Tertiary-secondary	44	4 (9.09)	[0.60 – 17.58]	0.002[a]
	Primary	83	25 (30.12)	[20.25 - 39.99]	
	No education	85	32 (37.65)	[27.35 - 47.95]	
Herd size	Small ≤ 10	72	20 (27.78)	[17.43 - 38.12]	0.33
	Medium ≤ 11 ≤ 30	116	31 (26.72)	[18.67 - 34.78]	
	Large ≥ 31	24	10 (41.67)	[21.94 - 61.39]	
Herd composition	Cattle only	108	22 (20.37)	[12.78 - 27.97]	0.007[a]
	Cattle-SR*	44	20 (45.45)	[30.74 - 60.17]	
	Cattle-dog	60	19 (31.67)	[19.9 – 43.44]	
Grazing system	Zero grazing	73	10 (13.7)	[5.81 – 21.59]	< 0.001[a]
	Free grazing	139	51 (36.69)	[28.68 – 44.7]	
Proximity to wildlife	Yes	65	24 (36.92)	[25.19 - 48.65]	0.072
	No	147	37 (25.17)	[18.15 - 32.19]	
Endemic diseases in the region	Brucellosis	44	20 (45.45)	[30.74 - 60.17]	0.022[a]
	HP diseases*	62	17 (27.42)	[16.32 - 38.52]	
	Viral diseases*	81	21 (25.93)	[16.38 - 35.47]	
	Mastitis	25	3 (12)	[0.0 – 24.74]	
Sharing watering points	Yes	150	50 (33.33)	[25.79 - 40.88]	0.034[a]
	No	62	11 (17.74)	[8.23 – 27.25]	
Fenced farms	Yes	114	30 (26.32)	[18.23 – 34.4]	0.48
	No	98	31 (31.63)	[22.48 - 40.84]	
History of infertility	Yes	115	36 (31.30)	[22.83 - 39.78]	0.46
	No	97	25 (25.77)	[17.07 - 34.48]	
History of abortions	Yes	88	37 (42.05)	[31.73 - 52.36]	< 0.001[a]
	No	124	23 (19.35)	[11.71 - 25.39]	
Knowledge of brucellosis	Yes	167	57 (34.13)	[26.94 - 41.32]	< 0.001[a]
	No	45	4 (8.89)	[0.57 – 17.2]	
Vaccination	Yes	26	6 (23.08)	[6.88 – 39.27]	0.64

last two years	No	186	55 (29.57)	[23.01 - 36.13]	
Breeding system	Natural	192	57 (29.69)	[52.04 - 71.88]	0.44
	AI	20	4 (20)	[2.47 – 37.53]	
Having own bull	Yes	78	22 (28.21)	[18.22 - 38.19]	0.832
	No	114	35 (20.70)	[22.24 - 39.17]	
Access to veterinary services	Yes	105	23 (21.90)	[24.32 - 42.35]	0.042[a]
	No	107	38 (35.51)	[26.45 - 44.58]	
Regular testing	Yes	39	8 (20.51)	[7.84 – 33.18]	0.29
	No	173	53 (30.64)	[23.77 – 37.5]	
Disinfection of abortion site & pastures	Yes	3	0 (0)	[0.0 – 0.0]	0.56
	No	209	61 (29.19)	[23.02 - 35.35]	
Introduction of new cattle	Yes	115	41 (35.65)	[26.9 – 44.41]	0.024[a]
	No	97	20 (20.62)	[12.57 - 28.67]	
Screening before introduction	Yes	2	0 (0)	[0.0 – 0.0]	0.54
	No	113	41 (36.28)	[27.42 - 45.15]	
Feeding abortive tissues to dogs	Yes	101	42 (41.58)	[31.97 – 51.2]	< 0.001[a]
	No	111	19 (17.12)	[10.11 - 24.12]	

No.: number of responses from owners of seropositive cattle

AI: artificial insemination

Cattle-SR: cattle and small ruminants

HP diseases: Hemoparasitic diseases (Theileriosis, Trypanosomiasis)

VD: Viral diseases (Foot and mouth disease)

[a] $p < 0.05$: proportions are significantly different.

Appendix 2. Questionnaire for cattle owners, farmworkers, and veterinarians

QUESTIONNAIRE FOR FARMERS, FARM WORKERS AND VETERINARIANS

This questionnaire (interview) is designed to collect information about the prevalence and risk factors of brucellosis and abortions in cattle and goats. These data will be used to inform policy makers and service providers to improve monitoring and surveillance of infectious diseases. This interview will take 5 min. The owner's names, farm's name, and names of farm workers will be kept confidential.

This questionnaire contains 4 sections: information about the interview, respondent address and information, herd management, and knowledge of the diseases by respondents.

I. Information about the interview

1.1. Interview language: ----------
1.2. Interviewer name: ----------

	Day/month/year
Date of first interview attempt	
Date and time arranged for second interview attempt	
Date and time arranged for third interview attempt	
Date of interview	
Date form checked by supervisor	
Date entered in computer	

II. Respondent information

2.1. Sectors of origin

Rwimiyaga ◯ Karangazi ◯ Bigogwe ◯ Kinigi ◯ Ndera ◯

2.2. Cell and village of origin / GPS

Cell ……..Village…….GPS…….

2.3. Sex of respondents: Female ◯ Male ◯

2.4. Are you? Herd owner ◯ farm worker ◯ sector veterinarian ◯ district veterinarian ◯

2.5. How long have you been in this job < 1 year ◯ 2 - 3 years ◯ 4-5 years ◯ > 5 years ◯

2.6. Education level: Tertiary education ◯ Secondary education ◯ Primary ◯ None ◯

III. HERD MANAGEMENT

3.1. How many cattle do you have on this farm/sector/district? Less than 10 ◯ Between 11-20 ◯

Between 21-30 ◯ between 31 – 40 ◯ between 41 – 50 ◯ Above 50 ◯ ……

3.2. What are the species present on this farm? Cattle ◯ Goats ◯ Sheep ◯ Dog ◯

3.1. What is the purpose of cattle production?

Meat ◯ Milk ◯ Mixed ◯ Prestige ◯

3.2. What is the market of milk produced?

Own consumption ◯ Inyange industries ◯ cooperatives ◯ others ◯

If others which ones?

3.5. Milking procedures: manual ◯ milking machine ◯

3.6. Do you boil your milk before selling or before home consumption? Yes ◯ No ◯

3.7. Do you make cream milk at home? Yes ◯ No ◯

3.8. Do you make butter, cow oil from milk at home? Yes ◯ No ◯

3.9. If your answer is yes, do you boil milk before? Yes ◯ No ◯

3.10. Do you use cow oil at home? Yes ◯ No ◯

3.11. If yes do you mix with food or fly the food? Mix ◯ fly ◯

3.12. Is there any cohabitation between wildlife and domestic animals? Yes ◯ No ◯

If yes go to question 3.13 if no go to question 3.14

3.13. Which kind of wildlife animals do you always see in contact with domestic animals?

Buffalos ◯ zebra ◯ gazelles ◯ elephants ◯ others ◯

If others please mention them

3.14. How often do you see wildlife animals?

Regularly ◯ sometimes ◯ rarely ◯

3.15. In which season or month do you see wildlife animals living the park?

Heavy sunny (June – September) ◯ light rainy (October – December) ◯

light sunny (January – February) ◯ heavy rainy season (March – may) ◯ regularly ◯

3.16. Do you share water sources with others? Shared ◯ personal ◯

3.17. What is the source of water? REG ◯ rain ◯ river ◯ other ◯

If others please mention ……..

I. KNOWLEDGE OF THE DISEASES

4.1. Are you aware of the transmission of contagious diseases from wildlife to domestic animals?

Yes ◯ No ◯

4.1. If yes which of the following diseases do you often find in your animals?

Tuberculosis ◯ Brucellosis ◯ Typanosomiasis ◯ Anaplasmosis ◯ Easter cost fever ◯

If others which ones: ……

4.2. Do know brucellosis? Yes ◯ no ◯

4.3. Did you ever face infertility cases in your herd? Yes ◯ no ◯

4.5. Can brucellosis affect humans? Yes ◯ no ◯

4.6. Did you ever get sick of brucellosis or disease similar to Malaria? Yes ◯ no ◯

4.7. How often does calving occur? Naturally ◯ assisted ◯ c – section ◯

4.8. If assisted do you wear protective clothes during assisted calving? Yes ◯ no ◯

4.9. Did you ever see some abortions in your herd? Yes ◯ no ◯

4.10. How do you dispose the abortive organs? Dogs ◯ deep burial ◯ open air ◯

4.11. Do you vaccinate against brucellosis? Yes ◯ No ◯

If yes when last did you vaccinate against brucellosis? Every year ◯ this year ◯ last year ◯

4.12. Do you consult veterinarians? Yes ◯ no ◯

4.13. Do veterinarians come regularly to collect samples? Yes ◯ no ◯

If yes which ones?

Blood ◯ faeces ◯ foetuses ◯ abortion tissues ◯

4.14. What type of reproduction do you use? Natural mating ◯ artificial insemination ◯

4.15. Do you have your own bull? Yes ◯ no ◯

4.16. Do you know other diseases that are characterized by abortions? ◯ Yes ◯ no

If yes which ones? ……

3.14. Do you clean & disinfect animals, stall, and pasture? Yes ◯ no ◯

3.15. If yes how many times a month? once ◯ twice ◯ more than twice ◯

3.16. What chemical do you usually use for cleaning & disinfection? ……

3.17. Where did animals come from? In country ◯ Uganda ◯ Tanzania ◯

3.18. Did you perform any test before their entry? Yes ◯ No ◯

If yes which one? Rose Bengal test ◯ if others please specify……

3.14. Do you sometimes see animals crossing borders? Yes ◯ No ◯

If yes by which means of transport? By vehicle ◯ by feet ◯

Note: Questions highlighted in Gray are related to public health

IKINYARWANDA

IBIBAZO BIZIFASHISHWA MU KIGANIRO N' ABOROZI, ABAKOZI, N'ABAVUZI Z' AMATUNGO

Ibi bibazo bizifashishwa mu gushakisha amakuru ajyanye n'indwara y'amakore n' ukuramburura mu nka n' ihene. Aya makuru azafasha abashinzwe ubuhinzi n'ubworozi gukaza ingamba zo gukumira indwara zandura zifata abantu, amatungo, n'inyamaswa. Iki kiganiro kizamara iminota itanu (5). Amazina yanyu cyangwa y' ifamu azagirwa ibanga.

Ibi bibazo bigabanyijemo ibice bine (4): gahunda y' ikiganiro, umwirondoro w'ubazwa, imicungire y'ubushyo, n' ubumenyi ku ndwara z'amatungo n'abantu.

1. Gahunda

1.1. Ururimi ruzakoreshwa: ----------
1.2. Izina ry' uzayobara ikiganiro:----------

	Umunsi / ukwezi/umwaka
Itariki ya mbere y'ikiganiro	
Itariki n'isaha by' ikiganiro ku inshuro ya 2	
Itariki n'isaha by' ikiganiro ku inshuro ya 3	
Itariki n'isaha by' ikiganiro nyirizina	
Itariki ibi bibazo byasuzumwe na mwarimu	
Itariki ibibazo n'ibisubizo byinjiriye muri mudasobwa	

2. Umwirondoro w'ubazwa

2.1. Umurenge ukomokamo

Rwimiyaga ◯ Karangazi ◯ Bigogwe ◯ Kinigi ◯ Ndera ◯

2.2. Icyo akora: Umworozi ◯ Umukozi ◯ Umuveterineri w'Umurenge ◯ w'Akarere ◯

2.3. Akagari, Umudugudu, aho aherereye (GPS)

Akagari ……..umudugudu..……GPS…….

1.1. Igistina : Gore ◯ Gabo ◯

1.2. Umaze igihe kingana iki muri aka kazi? umwaka 1 ◯ imyaka 2 - 3 ◯ imyaka 4 ◯

1.3. Wize ayahe mashuri? Kaminuza ◯ Ayisumbuye ◯ Abanza ◯ Nta mashuri ◯

2. **IMICUNGIRE Y' UBUSHYO**

2.1. Mufite inka zingahe muri ubu bworozi / Umurenge / Akarere? Munsi y'icumi 10 ◯ Hagati 11-20 ◯ Hagati 21-30 ◯ Hagati 31 – 40 ◯ hagati 41 – 50 ◯ Hejuru ya 50 ◯ ……

2.2. Ni ayahe matungo mufite hano? Inka ◯ ihene ◯ intama ◯ imbwa ◯

2.3. Intego yanyu ni ukorora inka zitanga amata cyangwa umukamo?

Inyama ◯ Amata ◯ byombi ◯ icyubahiro ◯

2.4. Niba ari umukamo, amata muyagurisha he?

Kwinywera mu rugo ◯ Inyange ◯ Koperative ◯ abandi ◯

Niba ari abandi ni bande?

3.5. Uburyo bwifashishwa mu gukama: amaboko ◯ imashini ikama ◯

3.6. Mubanza guteka amata mbere yo kuyagurisha cyangwa kuyanywa? Yego ◯ oya ◯

3.7. Mujya mukore ikivuguto mu rugo? Yego ◯ oya ◯

3.8. Mujya mukora amavuta y' inka mu rugo? Yego ◯ oya ◯

3.9. Niba mukora ikivuguto cyangwa amavuta mujya mubanza kubiza amata? Yego ◯ oya ◯

3.10. Mujya murya amavuta y'inka? Yego ◯ oya ◯

3.11. Niba ari yego murayarunga cyangwa murayakarangisha? kurunga ◯ gukaranga ◯ byombi ◯

3.12. Mujya mubona amatungo arisha ari kumwe n'inyamaswa? Yego ◯ Oya ◯

Niba ari yego komeza ku kibazo cya 8 niba ari oya komeza ku cya 9

3.13. Ni izihe nyamaswa mukunda kubona ziri hamwe n' amatungo?

Imbogo ◯ imparage ◯ gazelle ◯ inzovu ◯ izindi ◯

Niba hari izindi wazivuga

3.14. Izo nyamaswa uzibona kangahe?

Buri munsi ◯ rimwe na rimwe ◯ gake cyane ◯

3.12. Mu kihe gihe cyangwa ukwezi mukunze kubona inyamaswa ziva muri pariki?

impeshyi (Kamena – Nzeli) ◯ Umuhindo (Ukwakira – Ukuboza) ◯

Urugaryi (Mutarama – Gashyantare) ◯ Itumba (Werurwe – Gicurasi) ◯ buri gihe ◯

3.13. Amazi y'inka (ibumbiro) hari abo murifatanyije? Yego ◯ Oya ◯

3.14. Ayo mazi aturuka he? REG ◯ imvura ◯ umugezi ◯ ahandi ◯

1. UBUMENYI KU NDWARA Z' AMATUNGO

1.1. Muzi ko indwara z'inyamaswa zafata n' amatungo? Yego ◯ Oya ◯

1.2. Ni izihe ndwara mu kunze kubona mu bworozi?

Igituntu ◯ amakore ◯ indwara y'ibitotsi ◯ Gasheshe ◯ Ikibagarira ◯

niba hari izindi ni izihe? ……

1.3. Muzi amakore? Yego ◯ Oya ◯

1.4. Hari inka zanze kwima inshuro nyinshi? Yego ◯ Oya ◯

4.5. Ese abantu barwara amakore? Yego ◯ Oya ◯

4.6. Waba warigeze kurwara amakore cyangwa indi ndwara imeze nka Malariya? Yego ◯ Oya ◯

4.7. Ese hari inka zigeze kuramburura? Yego ◯ Oya ◯

4.8. Ibirambu bishyira he? Imbwa ◯ kubitaba ◯ turabyihorera ◯

4.9. Ese inka zikunze kubyara gute? Ku bwazo ◯ zifashijwe ◯ zibazwe ◯

4.10. Iyo muzifasha kubyara muba mwambaye akarindantoki cyangwa itaburiya? Yego ◯ oya ◯

4.11. Mujya mukingira amakore? Yego ◯ Oya ◯

Niba ari yego uheruka gukingira ryari? Buri mwaka ◯ uyu mwaka ◯ umwaka ushize ◯

4.12. Mujya mwitabaza abavuzi b' amatungo? Yego ◯ Oya ◯

4.13. Abavuzi b'amatungo bajya baza gufata ibizami? Yego ◯ Oya ◯

Niba ari yego bafata ibihe bizami?

Amaraso ◯ amase ◯ ibirambu ◯ uturemangingo tw'ibirambu ◯

4.14. Ni ubuhe buryo mukoresha mubangurira? Ikimasa ◯ Gutera intanga ◯

4.15. Ufite ikimasa cyawe? Yego ◯ Oya ◯

4.11. Hari izindi ndwara muzi zitera inka kuramburura? Yego Oya ◯

Niba ari yego ni izihe? ……

4.12. Mujya musukura ikiraro, ubwatsi n' amatungo mukoresheje umuti? Yego ◯ Oya ◯

Niba ari yego ni kangahe mu kwezi? Rimwe ◯ Kabiri ◯ birenze kabiri ◯

4.18. Mukoresha uwuhe muti? ……

4.19. Mugurira hehe inka? Mu gihugu ◯ mu Bugande ◯ muri Tanzaniya ◯

4.20. Hari ikizamini wafashe mbere yo kugura? Yego ◯ Oya ◯

Niba ari yego ni ibihe? Amaraso ◯ niba hari ibindi ni ibihe……

4.21. Mujya mubona inka zambukiranya imipaka? Yego ◯ Oya ◯

Niba ari yego zambuka gute? Ziri mu modoka ◯ n' amaguru ◯

ICYITONDERWA: IBIBAZO BIRI MU IBARA RY'IKIGINA BIJYANYE N'IBIKORWA BISHOBORA GUTUMA ABANTU BANDURA AMAKORE Y'AMATUNGO CYANGWA IMISUHA.

Appendix 3. Questionnaire for abattoir workers

QUESTIONNAIRE FOR ABATTOIR WORKERS

This questionnaire (interview) is designed to collect information about the prevalence and risk factors of brucellosis, and tuberculosis in cattle at the abattoirs. These data will be used to inform policy makers to improve monitoring and surveillance of infectious diseases. This interview will take 5 min. The names of participants will be kept confidential.

This questionnaire has four (4) sections as shown below:

1. Background information about the interviewer
2. Background information about respondent
3. Closed ended questions
4. Information about past infections

Section 1: Schedule of the interview

2.1 Interview language: ----------
2.2 Interviewer name: ----------

	Day/month/year
Date of first interview attempt	
Date and time arranged for second interview attempt	
Date and time arranged for third interview attempt	
Date of interview	
Date form checked by supervisor	
Date entered in computer	

Section 2: Background information of the individual (to be filled in before interview)

2.1. Address of the Abattoir/GPS: ----------
2.2. Name of respondent (will be kept confidential):----------
2.3. Sex of respondent 1. Male ☐ 2. Female ☐ 3. Unknown ☐
2.4. Age of respondent 15-30 ☐
31-60 ☐
61-Above ☐
2.5. Marital status 1. Married ☐ 2. Single ☐ 3. Divorced ☐
2.6. Level of education 1. None ☐ 2. Primary ☐ 3. Secondary ☐ 4. Tertiary ☐
2.7. Job description
Butcher/slaughter man ☐
Inspector ☐
Transporter ☐
Others----------
2.8. How long have you worked in the abattoir?
One year ☐
Two years ☐
Three years and above ☐

What animal section do you work in?

Cattle ☐
Sheep/goat ☐
Pig ☐

Section 3: Information about practices by respondent

3.1 Do you eat raw meat at this abattoir? 1. Yes ☐ 2. No ☐
(*If yes*: how often do you eat raw meat *if no*: go to question 3.2)
Regularly ☐
Sometimes ☐

3.2 Do you ever consume uninspected beef or pork? 1. Yes ☐ 2. No ☐

3.3 Have you ever bought condemned meat from this abattoir? 1. Yes ☐ 2. No ☐

3.4 If yes, did you consume the meat well done? 1. Yes ☐ 2. No ☐

3.5 Do you have access to drinking water at your work place? 1. Yes ☐ 2. No ☐

3.6 If yes, is it REG tap water? 1. Yes ☐ 2. No ☐
If no, please specify: ……

3.7 Have you ever cut your hands during work 1. Yes ☐ 2. No ☐

3.8 Do you work when you have wound or injury? 1. Yes ☐ 2. No ☐

3.9 Do you sometimes have animal secretions splash on your face
1. Yes ☐ 2. No ☐

3.10 Do you wash your hand regularly during work 1. Yes ☐ 2. No ☐
(*If yes*: go to question 3.10.1...*if no* go to question 3.11)
3.10.1 How often
Once ☐
Twice ☐
Every time ☐

3.11 Do you wear face mask during work? 1. Yes ☐ 2. No ☐
(*If yes*: go to question 3.11.1 *if no* go to question 3.12)
3.11.1 How often
Sometimes ☐
Always ☐

3.12 Do you wear hand gloves during work? 1. Yes ☐ 2. No ☐
(*If yes*:go to question 3.12.1 *if no* go to question 3.13)
3.12.1 How often
Sometimes ☐
Always ☐

3.13 Do you wear over all clothing during work? 1. Yes ☐ 2. No ☐
(*If yes*: go to question 3.13.1 *if no* go to question 3.14)
3.13.1 How often?
Sometimes ☐
Always ☐

3.14 Do you wear gum boots during work? 1. Yes ☐ 2. No ☐
(*If yes*: go to question 3.14.1 *if no* go to question 3.15)
3.14.1 How often?
Sometimes ☐
Always ☐

3.15 Do you wear protective glasses during work? 1. Yes ☐ 2. No ☐

(*If yes*: go to question 3.15.1 *if no* go to Section 4)

3.15.1 How often?

Sometimes ☐
Always ☐

3.16 During tea break, do you eat with your protective clothing/overall on?

1. Yes ☐ 2. No ☐

Section 4: Information about past infections

4.1. Have you ever been sick from the time you are in the abattoir? 1. Yes ☐ 2. No ☐

(*If yes*: go to question 4.1.1 *if no* go to question 4.2)

4.1.1 What kind of illness? ----------

4.2. Can you get infected with brucellosis from animals? 1. Yes ☐ 2. No ☐

4.3. Can you get infected with tuberculosis from animals? 1. Yes ☐ 2. No ☐

4.4. Have you ever heard any of cysticercosis in animals or humans? 1. Yes ☐ 2. No ☐

4.5. Have you ever heard any of leptospirosis in animals or humans? 1. Yes ☐ 2. No

4.6. Have you ever heard any of Q-fever in animals or humans? 1. Yes ☐ 2. No ☐

4.7. Have you ever had a case of abortion / infertility / orchitis? 1. Yes ☐ 2. No ☐

4.8. Have you ever been seronegative to malaria while presenting fever? 1. Yes ☐ 2. No ☐

4.9. If yes, what was the diagnosis in case of seronegative malaria?----------

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4.10. Have you ever been diagnosed with illness from the abattoir? 1. Yes ☐ 2. No ☐

(*If yes*: go to question 4.10.1 *if no* go to question 4.11)

4.10.1 What was the diagnosis?

Brucellosis (febrile, joint pain, malaria negative) ☐
Leptospirosis (fever, headache, jaundice, chills, vomiting, diarrhea) ☐
Tuberculosis (cough for two weeks, emaciation) ☐
Cystircercosis (clumps in the muscles, vision problems, seizures, and headache) ☐
Q-Fever (fever, chills, cough, no-productive headache, tiredness, chest pain, stomach pain, weight loss, nausea, vomiting or diarrhoea) ☐
Do not know
Others----------

4.11. Do you think you can get sick from the abattoir? 1. Yes ☐ 2. No ☐

4.11.1. If yes, can you provide examples of the disease you can get from abattoirs?.....

.....

IKINYARWANDA

IBIBAZO BIGENEWE ABAKOZI BO MU IBAGIRO

Ibi bibazo bizifashishwa mu gushakisha amakuru ajyanye n'indwara y'amakore n' igituntu nka n' ihene. Aya makuru azafasha abashinzwe ubuhinzi n'ubworozi gukaza ingamba zo gukumira indwara zandura zifata abantu, amatungo, n'inyamaswa. Iki kiganiro kizamara iminota itanu (5). Amazina yanyu cyangwa azagirwa ibanga.

Ibi bibazo bigabanyijemo ibice bine (4) bikurikira;

1. Gahunda y'ikiganiro
2. Umwirondoro w'ubazwa
3. Ibibazo bifunguye
4. Amakuru ajyanye n'uburwayi bwo mu gihe cyahise

Igice cya 1: Gahunda y'ikiganiro

2.1 Ururmi: ----------

2.2 Izina ry' uyobora ikiganiro: ----------

	umunsi/ukwezi/umwaka
Itariki ya mbere y'ikiganiro	
Itariki n'isaha by' ikiganiro ku inshuro ya 2	
Itariki n'isaha by' ikiganiro ku inshuro ya 3	
Itariki n'isaha by' ikiganiro nyirizina	
Itariki ibi bibazo byasuzumwe na mwarimu	
Itariki ibibazo n'ibisubizo byinjiriye muri mudasobwa	

Igice cya 2: umwirondoro w' ubazwa (byuzuzwa mbere y' ikiganiro nyirizina)

2.1. Aderesi y'ibagiro / GPS: ----------

2.2. Izina ry' ubazwa (rizagirwa ibanga):----------

2.3. Igitsina cy'ubazwa 1. Gabo ☐ 2. Gore

2.4. Imyaka y' ubazwa 15-30 ☐
31-60 ☐
kurenza 61 ☐

2.5. Imiterere y'umuryango 1. Yarashatse ☐ 2. Ingarugu ☐ 3. Umupfakazi ☐

2.6. Amashuri 1. Ntayo ☐ 2. Abanza ☐ 3. Ayisumbuye ☐ 4. Kaminuza ☐

2.6. Akazi akora

Umubazi ☐
Upima inyama ☐
Umukarani ☐
Umushoferi ☐
Abandi----------

2.7. Umaze igihe kingana iki ukore muri iri bagiro? 253

Umwaka umwe

Imyaka ibiri ☐
Imyaka itatu kujyana hejuru ☐

2.8. Ukora ku buhe bwoko bw'amatungo?

Inka ☐
Ihene n'intama ☐
Ingurube ☐

Igice cya 3: Amakuru yo mu ibagiro

3.1. Ujya urira inyama muri iri bagiro? 1. Yego ☐ 2. Oya ☐

(*niba ari yego*: uzijya kangahe? *Niba ari oya*: jya ku kibazo 3.2)

Zokeje ☐
Zitogosheje ☐
Buri munsi ☐
Rimwe na rimwe ☐

3.2. Mujya murya inyama zitapimwe? 1. Yego ☐ 2. Oya ☐

3.3. Wigeze ugura inyama zajugunwe n'abazipima? 1. Yego ☐ 2. Oya ☐

3.4. Niba ari yego muzirya zokeje cyangwa zahiye neza? 1. Zokeje ☐ 2. Zitetse neza ☐

3.5. Mufite amazi meza yo kunywa hano ku ibagiro? 1. Yego ☐ 2. Oya ☐

3.6. Niba ari yego, ni amazi ya REG?1. Yego ☐ 2. Oya ☐

Niba ari oya, mwatubwira ayo mazi aho muyakura: …….

3.7. Wigeze ukomereka uri mu kazi? 1. Yego ☐ 2. Oya ☐

3.8. Iyo ufite igikomere uza ku kazi? 1. Yego☐ 2. Oya ☐

3.9. Hari ubwo amaraso aguratukira mu maso?

1. Yego ☐ 2. Oya ☐

3.10. Ujya ukaraba amaboko buri gihe iyo uri mu kazi? 1. Yego ☐ 2. Oya ☐

(*Niba ari yego*: jya ku kibazo 3.10.1...*niba ari oya* jya ku kibazo 3.11)

3.10.1. Kangahe?

Rimwe ☐
Kabiri ☐
Buri kanya ☐

3.11. Wambara masike ikingira amaso? 1. Yego ☐ 2. Oya ☐

(*niba ari yego*: komeza ku kibazo 3.11.1 niba ari oya komeza kuri 3.12)

3.11.1. Kangahe?

Rimwe na rimwe ☐
Buri gihe ☐

3.12. Wambara uturindantoki? 1. Yego ☐ 2. Oya ☐

(*niba ari yego*:?:komeza ku kibazo 3.12.1 *niba ari oya* komeza 3.13)

3.12.1. Kangahe?

Rimwe na rimwe ☐
Buri gihe ☐

3.13. Ujya wambara itaburiya ikingira umubiri wose uri mu kazi? 1. Yego ☐ 2. Oya ☐

(*niba ari yego*: komeza ku kibazo 3.13.1 niba ari oya, komeza ku kibazo 3.14)

3.13.1. Kangahe?

Rimwe na rimwe ☐
Buri gihe ☐

3.14. Wambara boti iyo uri mu kazi? 1. Yego ☐ 2. Oya ☐

(*niba ari yego*: komeza ku kibazo 3.14.1 *niba ari oya*, komeza kuri 3.15)

3.14.1. Kangahe?

Rimwe na rimwe ☐
Buri gihe ☐

3.15. Wambara amadorubindi yabugenewe iyo uri mu kazi?1. Yego ☐ 2. Yego ☐

(*If yes*: go to question 3.15.1 *if no* go to Section 4)

3.15.1. Kangahe?:

Rimwe na rimwe ☐
Buri gihe ☐

3.16. Mu masaha y'ikiruhuko murya mwambaye itaburiya yabugenewe?

1. Yego ☐ 2. Oya ☐

Igice cya 4: Amakuru ajyanye n' uburwayi mu gihe cyahise

4.1. Wigeze urwara mu gihe wakoraga muri iri bagiro? 1. Yego ☐ 2. Oya ☐

(*niba ari Yego*: komeza ku kibazo 4.1.1 *niba ri oya* komeza kuri 4.2)

4.1.1 Wari urwaye iki? Wari umerewe ute? ----------

4.2. Ushobora kurwara imisuha bitewe n'amakore? 1. Yego ☐ 2.Oya ☐
4.3. Ushobora kurwara igituntu kivuye ku matungo? 1. Yego ☐ 2. Oya ☐
4.4. Wigeze wumva Rushe mu matungo cyangwa abantu? 1. Yego ☐ 2. Oya ☐
4.5. Wigeze wumva Leptospirosis mu matungo cyangwa abantu?1. Yego☐ 2. Oya ☐
4.6. Wigeze wumva Q-Fever mu matungo cyangwa abantu? 1. Yego ☐ 2. Oya ☐
4.7. Wigeze ukuramo inda/ubura urubyaro/ urwara amabya? 1. Yego ☐ 2. Oya ☐
4.8. Bigeze bakuburamo Malariya kandi ufite umuriro? 1. Yego ☐ 2. Oya ☐
4.9. Niba ari yego, bakubwiye ko wari urwaye iki?.....
……
4.10. Muganga yigeze agusangana indwara wanduriye ku ibagiro? 1. Yego ☐ 2. Oya ☐

(*niba ari yego*: komeza ku kibazo 4.10. *niba ari oya* komeza kuri 4.11)

4.10.1. Iyo ndwara yari iyihe?

Imisuha (brucellosis, umuriro, kubabara amabya, kutabyara, umutwe)☐
Leptospirosis (umuriro, umutwe, kuruka, guhitwa, umuhondo) ☐
Igituntu (tuberculosis: gukorora, kunanuka) ☐
Rushe (cysticercosis: kuzungera, kwituna inzoka) ☐
Inzoka (Taeniosis: guhitwa, kwituma inzoka) ☐
Q-Fever: umuriro, umutwe, guhitwa, gukorora) ☐
simbizi ☐
izindi----------

4.11. Urabizi ko ushobora kwandura indwara y' amatungo kuri uri bagiro?

1. Yego ☐ 2. Oya ☐

4.11.1. Niba ari yego tanga urugero rw' indwara wakwandurira ku ibagiro……
……

UNIVERSITY OF RWANDA

COLLEGE OF AGRICULTURE, ANIMAL SCIENCES AND VETERINARY MEDICINE (CAVM)

Directorate of Research, Innovation and Post-Graduate Studies

Our ref: 096 ../DRIPGS/2017
07th November, 2017

Mr. Bosco NTIVUGURUZWA
Tel: 0788648909
E-mail: boscus2@gmail.com
School of Animal Science and Veterinary Medicine
UR-CAVM

Dear NTIVUGURUZWA,

RE: Ethical Clearance for your research "the prevalence and characterization of brucellosis and bovine tuberculosis in cattle in Rwanda"".

On behalf of the Research Screening and Ethical Clearance Committee of the College of Agriculture, Animal Sciences and Veterinary Medicine, University of Rwanda, I am pleased to inform you that your request for the ethical clearance for the project entitled **"the prevalence and characterization of brucellosis and bovine tuberculosis in cattle in Rwanda"** has been approved.

You are recommended to start the data collection after getting the approval from our Ministry of health and to follow the approved protocol during the conduct of your research; any amendment should be communicated to the Committee for review and approval. At the completion of the project you are requested to submit the report to Directorate of Research, Innovation and Post-Graduate Studies.

Congratulations to you team, we are looking forward to a successful implementation of the project.

Sincerely,

Guillaume Nyagatare, PhD
Acting Director of Research, Innovation and Postgraduate Studies
Chairperson of the College Research Screening and Ethical Clearance Committee
UR-CAVM

Cc:
- Dean of the School of Animal Science and Veterinary Medicine

UNIVERSITY of RWANDA

COLLEGE OF MEDICINE AND HEALTH SCIENCES

DIRECTORATE OF RESEARCH & INNOVATION

CMHS INSTITUTIONAL REVIEW BOARD (IRB)

Kigali, the 28th /04/2020

NTIVUGURUZWA Jean Bosco
PhD Candidate
University of Pretoria

Notice of Renewal of Approval for Research Project: No 056/CMHS IRB/2020

Your Project title ***"Prevalence and Characterization Of Brucellosis And Bovine Tuberculosis In Cattle In Rwanda"*** hasbeen evaluated by CMHS Institutional Review Board.

Name of Members	Institute	Involved in the decision		
		Yes	No (Reason)	
			Absent	Withdrawn from the proceeding
Prof Kato J. Njunwa	UR-CMHS		X	
Prof Jean Bosco Gahutu	UR-CMHS	X		
Dr Brenda Asiimwe-Kateera	UR-CMHS	X		
Prof Ntaganira Joseph	UR-CMHS	X		
Dr Tumusiime K. David	UR-CMHS	X		
Dr Kayonga N. Egide	UR-CMHS	X		
Mr Kanyoni Maurice	UR-CMHS		X	
Prof Munyanshongore Cyprien	UR-CMHS	X		
Mrs Ruzindana Landrine	Kicukiro district		X	
Dr Gishoma Darius	UR-CMHS	X		
Dr Donatilla Mukamana	UR-CMHS	X		
Prof Kyamanywa Patrick	UR-CMHS		X	
Prof Condo Umutesi Jeannine	UR-CMHS		X	
Dr Nyirazinyoye Laetitia	UR-CMHS	X		
Dr Nkeramihigo Emmanuel	UR-CMHS		X	
Sr Maliboli Marie Josee	CHUK	X		
Dr Mudenge Charles	Centre Psycho-Social	X		

After reviewing your protocol, **Continuation of Approval has been granted to your study.**

Please note that approval of the protocol and consent form is valid for **12 months**.

Email: researchcenter@ur.ac.rw P.O Box 3286 Kigali, Rwanda www.ur.ac.rw

You are responsible for fulfilling the following requirements:

1. Changes, amendments, and addenda to the protocol or consent form must be submitted to the committee for review and approval, prior to activation of the changes.
2. Only approved consent forms are to be used in the enrollment of participants
3. All consent forms signed by subjects should be retained on file. The IRB may conduct audits of all study records, and consent documentation may be part of such audits.
4. A continuing review application must be submitted to the IRB in a timely fashion and before expiry of this approval.
5. Failure to submit a continuing review application will result in termination of the study.
6. Notify the Rwanda National Ethics committee once the study is finished.

Sincerely,

Date of Approval: April 28th2020
Expiration date: April 28th 2021

Professor GAHUTU Jean Bosco
Chairperson Institutional Review Board,
College of Medicine and Health Sciences, UR

Cc:

- Principal College of Medicine and Health Sciences, UR
- University Director of Research and Innovations, UR

Email: researchcenter@ur.ac.rw P.O Box 3286 Kigali, Rwanda www.ur.ac.rw

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

8 July 2020

Approval Certificate
Annual Renewal (EXT2)

AEC Reference No.: V004-18
Title: Prevalence and characterization of brucellosis and bovine tuberculosis in cattle in Rwanda
Researcher: Dr JB Ntivuguruzwa
Student's Supervisor: Prof H van Heerden

Dear Dr JB Ntivuguruzwa,

The **Annual Renewal** as supported by documents received between 2020-06-11 and 2020-07-03 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-07-03.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number Available
Bovine (Brucellosis)	1400
Bovine (Tuberculosis)	300

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-07-08.

3. Please remember to use your protocol number (V004-18) on any documents or correspondence with the AEC regarding your research.

4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8483
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa