SEROPREVALENCE AND CHARACTERIZATION OF *BRUCELLA* SPP. ISOLATED FROM HUMANS AND CATTLE IN SOUTHERN AND WESTERN PROVINCES OF ZAMBIA

By

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THE UNIVERSITY OF ZAMBIA

Lusaka

DEDICATION

This thesis is dedicated to my son Jonathan Thapewa P. Chisanga. May your life be paved with God's unmerited favour, grace and abundant blessings.

DECLARATION

I, **Ruth Lindizyani Mfune**, do hereby declare to the Senate of the University of Zambia that this thesis is my own original work and has not been submitted for any academic qualification or award of a degree to this or any other University.

..... ….....

Signature **Date**

APPROVAL

This thesis by **Ruth Lindizyani Mfune** has been approved as having fulfilled the requirements for the award of a Doctoral degree in Public Health (One Health-Zoonoses) by the University of Zambia.

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ABSTRACT

Brucellosis is one of the top seven neglected zoonoses endemic in livestock and humans in developing countries including Zambia. A cross-sectional study was conducted between May 2017 and August 2021 in Zambia in the Southern province in Choma, Monze and Namwala and in the Western province in Mongu and Senanga. The study aimed to investigate exposure to and characterise the circulating *Brucella* spp. in cattle and humans to gain insight into their public health importance. A total of 1,815 sera from 175 cattle herds were collected and screened against brucellosis. The Rose Bengal Test (RBT) and competitive Enzyme-Linked Immuno Assay (c-ELISA) were used in serial testing to detect antibodies against *Brucella* species. A total of 1,047 variable biological samples including ten hygroma fluid, 210 vaginal swabs, four foetal materials, 666 milk samples from cattle and 157 whole human blood samples, were cultured and analysed. Molecular analysis (16S rRNA PCR, Real-Time PCR, Multiplex Bruce Ladder PCR) was conducted to isolate, identify and characterize *Brucella spp*. The *Brucella* isolates were evaluated for susceptibility to six antimicrobials using the disk diffusion method, namely; rifampicin (5μg per disk), trimethoprim-sulfamethoxazole (23.75 μg), doxycycline (30 μg), tetracycline (30 μg) ciprofloxacin (5 μg), streptomycin (10 μg), gentamicin (10 μg) and chloramphenicol (30 μg). The breakpoints used have been established according to the 2020 Clinical Laboratory Standards Institute guidelines for slow-growing bacteria (*Haemophilus* spp.).

The herd-level and individual animal anti-*Brucella* antibody seroprevalences were 32 per cent (CI 95%: 25.0-38.9) and 9.92 per cent (CI 95%: 8.5-11.2). The Western province had a higher herd-level seroprevalence (32.3%, CI 95%: 20.7-43.8). Five isolates (3 human blood and 2 cattle milk isolates) were identified to *Brucella* genus level using 16S rRNA PCR and characterised by 16S rRNA sequencing. A similarity search by blastn of the sequences (identity of 99%)

identified them as *Brucella spp* and confirmed by Real-time PCR performed using IS*711* and *bcsp31* gene targets. On Bruce ladder Multiplex PCR, the *Brucella* strain had similar phenotypic characteristics as the *Brucella* vaccine strain S19. All isolates (3, 8 and 12) were resistant to trimethoprim-sulfamethoxazole, doxycycline, tetracycline and chloramphenicol but sensitive to rifampicin. All three isolates showed intermediate resistance to ciprofloxacin.

The overall brucellosis seroprevalence rates at the individual animal and herd levels were 9.92 per cent and 32 per cent respectively. *Brucella* species are circulating in human and bovine milk in the Southern province of Zambia. Molecular typing of the isolated *Brucella* spp. DNA indicates that they belong to the *Brucella abortus* S19 vaccine strain. While vaccination is the traditional and recommended method for controlling brucellosis, the current study findings show that the S19 vaccine that we are using continues to be detected not only in animals and animal products but also in humans long after it has been used in animals underscores it is zoonotic transmission potential from cattle to humans which seems to be a public health problem.

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ACRONYMS

%	-	Percentage
°C	-	Degrees Celcius
Ab	-	Antibodies
AMA	-	American Medical Association
AVMA	-	American Veterinary Medical Association
AMOS	-	Abortus Melitensis Ovis and Suis
AMST	-	Antimicrobial Susceptibility Test
bp	-	Base pairs
bv	-	Biovars
cELISA	-	Competitive-Enzyme Linked Immuno-sorbent Assay
CFT	-	Complement Fixation Test
CFU	-	Colony Forming Units
CITA	-	Centro de Investigaciòn y Tecnologia Agroalimentaria
CLSI	-	Clinical Laboratory Standards Institute
CO_2	-	Carbon Dioxide
CSO	-	Central Statistical Office
DNA	-	Deoxyribonucleic Acid
dNTP	-	Deoxyribonucleotide Triphosphate
ECDC	-	European Centre for Disease Prevention and Control
EEA	-	European Economic Area
EU	-	European Union
H_2 S	-	Hydrogen Sulphide

HCl	-	Hydrochloric Acid
HGDI	-	Hunter and Gaston Diversity Index
Ig	-	Immunoglobulin
iELISA	-	Indirect-Enzyme Linked Immuno-sorbent Assay
KCl	-	Potassium Chloride
Km^2	-	Kilometer Squared
Mab	-	Monoclonal Antibodies
MDR-TB	-	Multidrug-resistant *Mycobacterium tuberculosis*
ME	-	Mecaptoethanol
MFL	-	Ministry of Fisheries and Livestock
$MgCl_2$	-	Magnesium Chloride
MIC	-	Minimum Inhibitory Concentration
MLST	-	Multi Locus Sequence Typing
MLVA	-	Multiple-Locus Variable-Number Tandem- Repeat Analysis
mM	-	Milimoles
MST	-	Minimum Spanning Tree
ng	-	Nanograms
OD	-	Optical Density
OIE	-	World Organisation for Animal Health
PBS	-	Phosphate Buffer Saline
PCR	-	Polymerase Chain Reaction
PI	-	Percentage Inhibition
R-LPS	-	Rough Lipopolysaccharide

RBT	-	Rose Bengal Test
rpm	-	Revolutions Per Minute
rRNA	-	Ribosomal Ribonucleic Acid
RT-PCR	-	Real-Time Polymerase Chain Reaction
µl	-	Microlitres
µM	-	Micromolars
SAT	-	Serum Agglutination Test
SDA	-	Serum Dextrose Agar
S-LPS	-	Smooth Lipopolysaccharide
SNP	-	Single Nucleotide Polymorphism
U	-	Units
USD	-	United States Dollars
UNZA	-	University of Zambia
VNTR	-	Variable Number of Tandem Repeats
WGS	-	Whole Genome Sequencing
WHO	-	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 Background

Brucellosis is one of the top seven neglected zoonotic diseases that affect domestic livestock, wildlife and humans worldwide. This disease is caused by intracellular Gram-negative bacteria of the *Brucella* genus (Whatmore *et al.*, 2014) and is enzootic in low- and middle-income countries (LMICs) that heavily practice traditional livestock farming systems (Lokamar *et al.*, 2020). In a review of seventy-six animal diseases, brucellosis was ranked among the top ten that impact poor people and is commonly referred to as the "poor people's disease" (Dean *et al.*, 2012). Currently, twelve known species in the Brucella genus are pathogenic to and preferentially infect cattle *(B. abortus* biovar 1-6, 9), goats and sheep *(B. melitensis* biovar 1-3), sheep *(B. ovis),* pigs *(B. suis* biovar 1-3*), hare (Lepus europaeus) (B. suis biovar 2),* reindeer (*Rangifer tarandus*), hare (*Lepus europaeus* (*B. suis* biovar 4), dogs *(B. canis),* rodents *(B. neotomae* and *B. microti),* seals (*B. pinnipedialis)* and cetaceans (*B. ceti*), frogs and humans (*B. inopinata),* baboons (*B. papionis*) and red foxes (*B. vulpes*) (Scholz *et al.,* 2016).

Animal transmission is mainly through grazing of contaminated pasture or contact with abortive materials from *Brucella* infected animals (Poester et al., 2013). In animals, brucellosis is characterised by abortions, reduced milk production, calf mortality, hygromas and infertility (OIE, 2018). Animal to human transmission of *B. abortus* and *B. melitensis* occurs via contact and consumption of contaminated raw milk and dairy products. Occupationally exposed individuals commonly affected are veterinarians, abattoir workers, slaughterhouse personnel,

livestock keepers, herdsmen (shepherds) and livestock farmers through contact with infected animals or abortive materials (Corbel *et al.,* 2006; Ducrotoy *et al.,* 2017). *Brucella* infections in humans present with nonspecific symptoms, often becoming chronic and may relapse, even with treatment (Corbel *et al.,* 2006). Even though *Brucella* displays host species preferences, cross-species infections can occur in mixed husbandry systems or at the livestock-wildlife interface (Godfroid *et al.,* 2013). *B. melitensis*, *B. abortus* and *B. suis* are the most pathogenic to humans and livestock (OIE, 2018). *Brucella* has also been traditionally considered a biological weapon and remains on the category B priority pathogens list of the US Center for Disease Control and Prevention (CDC)(Pappas *et al.*, 2006a).

Brucellosis has yet been eradicated in most developed countries (Franc *et al.,* 2018). However, it remains endemic and neglected in low-income countries in Latin America, the Middle East, the Mediterranean, Asia, and Africa (Mcdermott *et al.,* 2013). In Africa, brucellosis has been reported in livestock, wildlife and humans using serological (Samaha *et al.,* 2009; Tumwine *et al.,* 2015; Asgedom *et al.,* 2016; Germeraad *et al.,* 2016; Njeru *et al.,* 2016; Awah-Ndukum *et al.,* 2018; Carugati *et al.,* 2018; Craighead *et al.,* 2018; Madut *et al.,* 2018; Ogugua *et al.,* 2018; Zerfu *et al.,* 2018) and molecular methods (Bertu *et al.,* 2015; Mathew *et al.,* 2015; El Hofy *et al.,* 2016; Tekle *et al.,* 2019). Several studies have reported brucellosis in South Africa (Kolo *et al.,* 2019; Simpson *et al.,* 2021), Zimbabwe (Matope *et al.,* 2011; Gomo *et al.,* 2012; Ledwaba *et al.,* 2019), Malawi (Tebug, 2012) and Tanzania (Sagamiko *et al.,* 2018; Ntirandekura *et al.,* 2020).

In Zambia, brucellosis has been reported in smallholder and pastoral cattle (Chimana *et al.,* 2010; Muma *et al.,* 2007). However, there is scarce data on the *Brucella* spp. circulating in humans and cattle in the Southern and Western provinces of Zambia. This study aimed to fill this

knowledge gap through isolation and molecular characterization of *Brucella spp*. circulating in humans and cattle.

1.2 Statement of the problem

Globally, more than 500,000 human brucellosis cases are reported annually (Pappas *et al.*, 2006b). The high disease seroprevalence in cattle and the fact that human infections are spillovers from animals indicates a high risk of human exposure to *Brucella* infections in traditional cattle rearing areas. Brucellosis has been reported in traditional cattle herds in Southern and Western provinces of Zambia at seroprevalence levels of 22.7 per cent (Muma *et al.,* 2013) and 22.2 per cent (Muma *et al.,* 2007), respectively. Human seroprevalence has been reported among occupationally exposed people at about one per cent and traditional farmers at 5.03 per cent (Muma *et al.,* 2008). However, there is a lack of culture collection of field *Brucella* spp. and the apparent absence of their molecular epidemiology. Given the low seroprevalence recorded in Zambia, it is possible that human cases are under-reported or misdiagnosed in febrile cases encountered in other infectious diseases like malaria or typhoid fever (Mcdermott & Arimi, 2002).

Furthermore, brucellosis affects livestock production and productivity through decreased milk production, abortions, infertility and mortalities. This results in economic losses to the livestock industry. In the year 2016, the overall total losses attributed to brucellosis-related calf mortality and milk losses in Southern and Western provinces were estimated at ZMW1,535,800 (USD $134,131) and ZMW77,700 (USD$ 6,786.02), respectively per farmer (Mwinyi *et al.,* 2016). These losses deprive farmers of their household income and affect economic growth at the

community and national level. Further, the antimicrobial susceptibility status of *Brucella* isolates is not documented. A study by Shevtsov *et al.* (2017) in Kazakhstan found 26.4 per cent rifampicin-resistant *Brucella* isolates from clinical patients, which was associated with a rise in the isolation of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB). This study will aim to fill this gap through isolation and molecular characterization of *Brucella spp*. circulating in humans and cattle.

1.3 Study Justification

More serological investigations on *Brucella* spp. have been documented in cattle and humans than molecular studies in Zambia. Although *B. abortus* is likely infecting cattle and transmitted to humans, isolation and molecular characterization of strains are essential to deciphering transmission at the livestock or human interface. Further, most of the research studies in Zambia have focused on seroprevalence in cattle and wildlife, while very little work has been done in humans. Therefore, this study will fill this knowledge gap through detection, isolation, and molecular characterization of *Brucella spp*. in humans and cattle.

Since human incidence is associated with animal infections, there is a need to understand the prevailing *Brucella* species infecting humans and the epidemiological situation. The information generated from this study will indicate the possible sources of infection and identify the level of exposure and the potential risks of human infections. The disease status in animals is of economic importance as it imposes restrictions on trade and movement of livestock locally and internationally (Godfroid, 2017). Data on the antibiotic resistance among *Brucella* strains circulating in Zambia will also help improve treatment efficacy in humans and prevent the spread

of resistant *Brucella* spp. This information will be beneficial to various stakeholders in developing specific interventions within the One Health framework and contribute to the development of the Zambian livestock industry.

1.4 Knowledge Gap in Zambia

Presently, the *Brucella* species circulating in cattle and humans in Zambia are not documented; there is a need to isolate, identify, and characterize them to provide information on the circulating *Brucella* spp. organisms. This data is critical in implementing prevention and control measures in both humans and animals. The study aimed to fill this knowledge gap through detection, isolation and molecular typing of *Brucella* spp. in humans and cattle.

1.5 General objective

This study aimed to investigate the *Brucella* species circulating in humans and cattle in the Southern and Western provinces of Zambia using serological and molecular tools to facilitate the informed implementation of prevention measures.

1.5.1 Specific objectives

1.5.1.1 To determine the *Brucella* seroprevalence in cattle

1.5.1.2 To isolate and identify the *Brucella* species circulating in humans and cattle

1.5.1.3 To determine the genetic diversity of *Brucella* species circulating in humans and cattle

1.5.1.4 To evaluate the antimicrobial susceptibility of *Brucella* species isolated from humans and cattle.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Brucella* characteristics

Brucellosis is an infectious zoonotic disease caused by gram-negative, facultative, intracellular non-motile, facultative coccobacilli bacteria of the genus *Brucella* (Moreno *et al.,* 2002). In humans, it is also known by other names such as Undulant fever, Crimean fever, Mediterranean fever, remitting fever, Maltese fever, while in cattle, it is commonly referred to as contagious abortion or Bang's disease (Godfroid *et al.*, 2005).

The *Brucella* organisms range from 0.5-0.7µm wide and 0.6-1.5µm long (OIE, 2018). Currently, there are twelve known *Brucella* species, each having a preferred primary host, namely; *B. abortus* (cattle), *B. melitensis* (sheep and goats), *B. suis* (pig), *B. canis* (dogs), *B. ovis* (sheep), *B. microti* and *B. neotomae* (rodents) (El-Sayed & Awad, 2018). Recently, additional species have been isolated from frogs and humans (*B. inopinata),* baboons (*B. papionis*), cetaceans (*B. ceti*), and seals *(B. pinnipedialis)*(Whatmore *et al.,* 2014) and red foxes (*B. vulpis*) (Scholz *et al.*, 2016). These novel species have contributed to the evolving knowledge of disease ecology.

The *Brucella abortus, B. melitensis* and *B. suis* have smooth lipopolysaccharide (S-LPS) on their outer cell membrane, while *B. ovis* and *B. canis* have a rough LPS. The smooth *Brucella* strains are more pathogenic in humans than wild strains (OIE, 2018). This pathogenicity is attributed to the O-antigen presence in the S-LPS, while it is either absent or reduced to only a few sugar residues in the R-LPS (Corbel *et al.,* 2006).

2.2 Human brucellosis

2.2.1 Transmission and clinical manifestations in humans

In humans, brucellosis is mainly caused by *B. melitensis*, followed by *B. suis, B. abortus* and less frequently by *B. canis* (Moreno *et al.*, 2014). The *Brucella* species can survive for long periods in dust, dung, water, aborted foetuses, soil, and air, and this plays a vital role in disease epidemiology (Ducrotoy *et al.,* 2017). Consumption of raw milk and dairy products is the primary route of transmission, while occupational exposure to contaminated biological materials or infected animal/carcasses or products can also lead to infection (Corbel *et al.,* 2006). Studies have shown that occupational groups at high risk of exposure are veterinarians, laboratory personnel, livestock farmers, herders, milkers and abattoir workers (Amegashie *et al.,* 2016; Awah-Ndukum *et al.,* 2018; Madut *et al.,* 2018).

Human brucellosis displays nonspecific acute symptoms such as intermittent fever, backache, headaches, anorexia, weight loss, weakness and arthralgia that may become chronic and affect other body systems (Pappas *et al.,* 2006b; Pereira *et al.,* 2020). Other documented manifestations include epididymo-orchitis, respiratory and neurological signs (Pappas *et al.,* 2006b; Dean *et al.,* 2012). Late-term abortions associated with *Brucella* infection have also been reported among pregnant women in Rwanda (Rujeni & Mbanzamihigo, 2014) and Saudi Arabia (Khan *et al.,* 2018). Furthermore, Ntirandekura *et al.* (2018) found an association between brucellosis and abortions in pregnant women in Africa. The disease symptoms are difficult to distinguish from other febrile diseases such as malaria and typhoid fever and can lead to misdiagnosis, wrongful treatment and under-reporting (Dean *et al.,* 2012; Ducrotoy *et al.,* 2017). Even in areas with good laboratory facilities, misdiagnosis can be attributed to the low levels of disease suspicion among

medical practitioners and the non-availability of *Brucella* rapid diagnostic test kits (Ntirandekura *et al.,* 2018).

Studies show that the clinical picture of the disease in rural areas is quite different from that in urban areas. People in rural areas have little or no access to medical services; hence the disease is usually detected in late stages resulting in chronic form or misdiagnosis. On the other hand, the urban population can easily access medical services that help the early detection and treatment of the disease (Dean *et al.*, 2012).

2.3 Brucellosis in cattle

2.3.1 Transmission and risk factors

Cattle herds that graze on contaminated pastures or feed and drinking water get exposed to the organism orally, while pregnant cows that have recently aborted or calved down are a significant source of infection to other animals through contact and contaminated pasture (Poester *et al.,* 2013). In a latently infected cow, pregnancy reactivates the infection following erythritol production, which stimulates the multiplication of the *Brucella* organism in the placenta (Islam *et al.,* 2014). On the other hand, *Brucella* spp. infect cows through artificial insemination and natural breeding (Mai *et al.,* 2012). Factors that influence the transmission of *Brucella* spp. can be grouped into four: host, management, agro-ecological, and farmer factors. Host factors include the animal's age, sex, pregnancy status, history of abortion, retained placenta and herd size (Islam *et al.,* 2014). Some studies have found a significant association between *Brucella* seropositivity and age, sex (Asgedom *et al.,* 2016; Awah-Ndukum *et al.,* 2018). Husbandry practices such as communal grazing, extensive farming systems, natural breeding practices, large

herd sizes, type of housing and source of replacement stock can also influence the spread of infection. Mcdermott and Arimi (2002) reported a higher seroprevalence in animals kept under extensive systems and observed that large herd sizes and poor housing increased the risk of herd exposure to infection.

2.3.2 Clinical signs

In the infected herds, abortion in pregnant cows is a consistent clinical sign of brucellosis, while other signs include stillbirths, reduced milk production, the birth of weak offspring and infertility (Mcdermott & Arimi, 2002). Calves born from infected cows usually test seronegative but are latently infected. In susceptible herds, the rate of abortion may vary from 30 to 80 per cent while calves born weak may die shortly after birth (Khurana *et al.,* 2021). Such female calves may abort during their first pregnancy, thereby maintaining the infection in the herd (Corbel et al., 2006). In bulls, the organism is localized in the testis, epididymis and accessory glands, resulting in acute orchitis, epididymitis, seminal vesiculitis and consequently infertility (Muma *et al.,* 2007). Hygromas are commonly associated with chronic infection (Mcdermott & Arimi, 2002).

2.4 Laboratory diagnosis in cattle and humans

There are three main diagnostic tests for brucellosis; serological (indirect tests), bacteriological and molecular (direct tests) (Smirnova *et al.,* 2013). Bacterial isolation is the gold standard as it is peculiar and confirmatory compared to other methods. However, serological, bacteriological and molecular methods are needed for reliable identification and biotyping of *Brucella* (Ducrotoy *et al.,* 2017; OIE, 2018).

2.4.1 Serological Methods

These tests are based on the detection of antibodies against a Smooth LPS found on the outer cell walls of all *Brucella* species except for *B. ovis* and *B. canis,* which have rough strain LPS (Nielsen & Yu, 2010) and are antigenically similar to those found in bacteria such as *Yersinia enterocolitica, Escherichia coli, Salmonella*, *Stenotrophomonas multophilia* and *Vibrio cholerae.* Animals infected with *B. abortus* produce an early IgM antibody response, which appears five to fifteen days post-exposure. This is followed by subsequent production of IgG1, IgG2 and IgA antibodies. However, several other microorganisms have antigens with epitopes similar to those of the *Brucella* spp. This may lead to cross-reactions that mimic brucellosis serological reactions; therefore, measurement of IgM antibody may result in a false-positive reaction in serological tests which may reduce the test specificity (Godfroid *et al.,* 2010). Serology is used in the presumptive diagnosis of brucellosis in humans, individual animals and herds. It includes Serum Agglutination Test (SAT), Rose Bengal Test (RBT), Complement Fixation (CFT), Fluorescence polarization assay (FPA), Brucella IgM/IgG lateral flow assays, 2- Mercapteoethanol (2-ME) and primary binding immunoassays namely; Indirect Enzyme-Linked Immunosorbent Assays (iELISA) and cELISA (OIE, 2018). The OIE has discouraged the use of SAT due to its specificity and/or sensitivity challenges.

i) Rose Bengal Plate Test

This simple and rapid spot agglutination test detects specific antibodies and uses an antigen buffered at pH 3.65. It is more effective in detecting IgG1 than IgM and IgG2 antibody types. The principle of the test is that the IgM antibodies' ability to bind to antigens is significantly reduced at a low pH; this reduces nonspecific reactions by preventing agglutination of IgM and

enhances agglutination IgG1 (Díaz et al., 2011). A study by Chisi et al. (2017) in infected cattle herds in South Africa found that RBT had a high sensitivity (95.8%), followed by cELISA (93.9%). The tests showed the same level of sensitivity when they were applied to *Brucella* free herds. False-positive results may occur on RBT due to cross-reactions with antibodies from other bacteria, residual antibodies from *Brucella abortus* S19 vaccination and colostral antibodies in calves (Nielsen & Yu, 2010). In areas where animals are not routinely vaccinated, using the RBT can give a good indication of animal exposure to *Brucella* organisms (Kaltungo *et al.*, 2014).

ii) Enzyme Linked-immunosorbent Assay (ELISA)

ELISAs can test milk samples in animals apart from serum and cerebrospinal fluid samples in humans and animals. There are two types of ELISAs, namely, the indirect (iELISA) and competitive ELISA (cELISA) (OIE, 2018).

(a) Indirect ELISA

The iELISA is a two-step ELISA based on the specific binding of antibodies present in a sample to an immobilized antigen on a solid phase. This immune complex is then detected by an anti-globulin-enzyme conjugate which, in combination with a chromogenic substrate, gives a coloured reaction showing the presence of antibody in the sample (OIE, 2018). The iELISA test is a rapid, highly sensitive test that can detect *Brucella* specific IgG, IgM and IgA (Poester *et al.*, 2010). The disadvantage of iELISA is that its specificity is lowered because it cannot differentiate field infection antibodies from the *B. abortus* S19 vaccine antibodies or cross-reacting bacteria such as *Yersinia enterocolitica* and *E. coli* (Gall & Nielsen, 2004; Poester *et al.*, 2010).

(b) Competitive ELISA (cELISA)

According to Nielsen and Yu (2010), the cELISA assay was developed to overcome the iELISA shortfalls by using a monoclonal antibody (mAb) specific for one of the epitopes of the *Brucella* O-PS of the SLPS chains. The mAb competes with low-affinity antibodies present in the test serum and has a higher affinity for the antigen than the vaccine or cross-reacting antibody (Poester et al., 2010). This test can help distinguish vaccinated from naturally infected animals and those with cross-reacting organisms. This gives cELISA a higher specificity and lower sensitivity than iELISA (Kaltungo *et al.,* 2014). A study by Chisi *et al.* (2017) on infected cattle herds in South Africa showed that the cELISA diagnostic sensitivity and specificity were 93.9 per cent and 95 per cent whereas those for iELISA were 95.8 per cent and 92.8 per cent respectively. Therefore, the cELISA is an ideal test for large-scale screening of suspected herds and not individual animals (Godfroid *et al.,* 2010; Smirnova *et al.,* 2013). The *Brucella* seropositivity does not indicate the time or stage of infection but merely indicates the presence of exposure to *Brucella* spp.; hence the actual disease prevalence may be higher than that indicated by the diagnostic test. Furthermore, ELISA kits are more costly for developing countries than the RBT and CFT test kits.

A study by Chisi *et al.* (2017) assessed the performance of serology tests and found that the RBT and iELISA combination had the highest sensitivity (93.5%) when tested in series. In contrast, when tested in parallel, the iELISA and cELISA combination had the highest sensitivity (100%).

2.4.2 Bacteriological method: Culture and isolation, Identification and Typing

Bacterial isolation and identification are vital in determining the pathogenic *Brucella* species and understanding the disease epidemiology (Ducrotoy *et al.,* 2014). It is also considered the gold

standard because it is the only method that gives a definitive diagnosis for brucellosis in humans and animals (Ledwaba *et al.,* 2020).

In animals, the samples collected for culture are milk (Hoffman *et al.,* 2016), hygroma fluids (Sanogo *et al.,* 2012), vaginal swabs, aborted foetal tissues (spleen, stomach and lungs) and placenta up to six weeks post-abortion or parturition (OIE, 2018). The highest concentration of bacteria is found in placenta materials, followed by lymph nodes, then milk (Poester *et al.,* 2010). *Brucella* organisms are slow growers with a low rate of isolation from culture; hence the culture media needs to be enriched with horse serum or sheep blood and *Brucella*-antibiotic supplements (Kumar *et al.,* 2021). *Brucella* spp. have been successfully cultured using various selective *Brucella* culture media that include Farrell's medium (FM), Serum Dextrose Agar (SDA), modified Thayer Martin (mTM) and Modified Agrifood Research and Technology Center of Aragon (mCITA) media (Karagül & Ikiz, 2017; Ledwaba *et al.,* 2020; Mathew *et al.,* 2015). Farrell's medium can inhibit the growth of fungal and bacterial contaminants (Gerado *et al.,* 2015; Poester *et al.,* 2010). However, the nalidixic acid and bacitracin used in its formulation inhibit the growth of some strains of *B. abortus*, *B. melitensis* and *B. ovis* (Karagül & Ikiz, 2017). The mTM shows greater sensitivity to other species but does not inhibit contaminating microorganisms. Consequently, an mCITA medium was developed based on mTM with different antimicrobials and amphotericin B added to inhibit contaminants without impairing the growth of *Brucella* species. Studies show that diagnostic sensitivity is significantly increased when both Farrell's and modified Thayer-Martin's media are used concurrently (Karagül & Ikiz, 2017; Poester *et al.,* 2010). According to (Ledwaba *et al.,* 2020), mCITA and/or FM can be used in *Brucella* spp. isolation as mCITA is the optimum selective medium compared to mFM and FM.

In humans, whole blood is commonly used to culture and isolate the *brucellae* organisms (Poester *et al.,* 2010). Some studies have used bone marrow, lymph nodes, synovial fluid aspirate and liver tissue to increase the suboptimal recovery rate. Mantur *et al.* (2008) found that bone marrow culture identified 82.5 per cent of human brucellosis cases, while blood culture identified only 45.6 per cent. Currently, there are five blood culture methods commonly used and have been reviewed in several studies: manual monophasic, manual biphasic, lysis-based blood culture, blood clot culture and automated blood culture systems (Yagupsky, 2015). According to the review by Yagupsky. (2015), the manual monophasic method has a long incubation period and is labour intensive due to repeated subculturing, while the biphasic medium (Castaneda) uses a flask that contains solid agar and liquid culture broth. The inoculated flask is supplemented with 10 per cent CO_2, tilted then incubated. In the lysis centrifugation (LC) method, the blood cells are osmotically lysed with a detergent (Sodium polyethol sulphonate), centrifuged and spread on solid media. The automated blood culture system (ABC), such as Bactec 9000, detects metabolic changes in the positive blood culture vials. The LC and ABC methods have a shorter detection time than other methods, despite being too costly for developing countries. The blood clot culture is a cheap and straightforward method involving clotting a blood sample and seeding it on solid media.

Colony growth is seen within two to three days and is only considered negative after two to three weeks of incubation (OIE, 2018). The detection rate can be affected by several factors; the volume of the blood sample, detection method used, patient age and previous exposure to antibiotics (Yagupsky *et al.*, 2019).

Blood culture isolates are identified using Gram's stain, colony morphology, and biochemical tests. The culture colonies appear as single or paired Gram-negative coccobacilli. They are small

(0.5-1mm), punctate, non-pigmented and non-hemolytic on blood agar (Al Dahouk *et al.,* 2013). The *Brucella* organisms are urease, catalase and oxidase-positive, non-motile, and do not ferment sugars (OIE, 2018). Typing of *Brucella* isolates into biovars is based on the phenotypic differences of surface LPS antigens, sensitivity to dyes, CO_2 requirement, H_2S and urease production (Bertu *et al*., 2015). Other metabolic properties such as phage lysis, the ability to grow in alkaline fuchsine or thionine, sensitivity to erythritol and susceptibility to antibiotics, as described by Alton *et al*., 1988 (Morgan, 1990). The culture method for isolation and identification is time-consuming and costly due to the long incubation period of these slow-growing organisms, which can delay the diagnosis (Khurana *et al.,* 2021). Furthermore, this method requires using a biosafety laboratory level 3, during which the laboratory personnel are also exposed to the zoonotic and highly pathogenic *Brucella* species (Ledwaba *et al.,* 2020). Despite these limitations, the culture and isolation method is regarded as the gold standard (OIE, 2018).

2.4.3 Molecular methods

Polymerase Chain Reaction (PCR) is a simple molecular biology technique used to amplify and detect DNA sequences. PCR-based techniques can identify *Brucella* DNA to species and biovar levels (Poester et al., 2010). They detect specific sequences of *Brucella spp*. DNA by amplification of genomic targets. Studies show that brucellae share a high degree of DNA homology (>90% for all species) based on a DNA - DNA hybridisation (Ducrotoy *et al.,* 2014; Wang *et al.,* 2014). The PCR assays use primers that target specific *Brucella* DNA sequences such as the 16S rRNA operon, the *Brucella* 31 kDa outer membrane Protein (BCSP31) and

IS*711* insertion sequence genes, which are highly conserved among the *Brucella* species (Godfroid *et al.,* 2010).

2.4.3.1 Conventional PCR assays

PCR assays can identify *Brucella* organisms to species level, while others can partly identify biovar levels. For *Brucella* with high DNA homology among species, Real-time PCR using a hybridization probe (hybprobe) has shown higher sensitivity than conventional PCR and real-time PCR based on the Taqman probe (Kim *et al.,* 2015).

The AMOS (Abortus-Melitensis-Ovis-Suis) and Bruce-Ladder multiplex PCRs for *Brucella* identification that uses different primer combinations have been described in *Brucella* speciation (Yu & Nielsen, 2010). AMOS is a multiplex PCR assay based on the IS*711* related polymorphism of the *Brucella spp*. It uses a single reverse primer that targets the *Brucella* specific insertion element IS*711* and four different forward primers, each specific for a given species. The assay can differentiate *B. abortus* (biovars 1, 2 and 4), *B. melitensis* (biovars 1, 2, and 3), *B. ovis*, *B. suis* (biovar 1), as well as vaccines *B. abortus* S19 and RB 51 (Weiner *et al.,* 2012). Species are differentiated based on the different PCR fragment sizes amplified from the primers (Scholz *et al.,* 2016).The predicted amplicon size for *B. abortus, B. melitensis, B.ovis* and *B. suis. bv1* is 498,731,976 and 285 bp respectively. The AMOS-PCR assay has been modified over the years, with strain-specific primers incorporated into it to identify the RB51 and *B. abortus* S19 vaccine strains. The disadvantage of using AMOS PCR is that it cannot identify some species (*B. canis* and *B. neotomae* and some biovars within given species tend to give negative results (Lopez-Goni *et al.,* 2008; Yu & Nielsen, 2010).

The Bruce-ladder-PCR is another single-step multiplex PCR assay developed by García-Yoldi *et al.* (2006). It uses eight primer pairs in a single reaction to describe all *Brucella* species (at that time) to species and biovar levels. The assay can further discriminate *Brucella canis* and *Brucella microti* from *Brucella suis* strains and identify the vaccine strains *B. abortus* S19 and RB 51(García-Yoldi *et al.,* 2006; Kang *et al.,* 2011). An evaluative and comparative study of PCR based assays was done by (Lopez-Goni *et al.,* 2008; Weiner et al., 2012) respectively showed that Multiplex Bruce-ladder PCR could amplify five fragments of 1,682, 794, 587, 450 and 152 bp in *B. abortus* DNA, while an additional 1,071 bp fragment is amplified in *B. melitensis* DNA. DNA *for B.ovis* and *B. abortus* S19 lacks the 1,682 bp and 587 bp (common to all *Brucella* strains) fragments, respectively. Furthermore, *B. abortus* RB51 is distinguished by a specific additional 2,524 bp fragment and the absence of the 1,682 bp and 1,320 bp fragments, while *B.suis* has an additional 272 bp fragment (also present in *B. canis* and *B. neotomae).* This assay is done in one step compared to Multiple Locus Variable Number Tandem Repeat Analysis (MLVA). Itis a rapid, helpful tool that can be used in any reference or microbiology laboratory to identify *Brucella* strains from animal or human sources (Lopez-Goni *et al.,* 2008). Partial 16S rRNA gene sequencing is a reliable tool increasingly used to identify *Brucella* to genus level by targeting specific DNA sequences. The obtained 16S rRNA sequence can identify *Brucella* to the genus level but cannot discriminate against individual species (Gee *et al.,* 2004).

2.4.3.2 RT-PCR or qPCR Assays

Several Real-Time PCR assays have recently been developed using single-nucleotide polymorphisms (SNPs) that target either the IS*711* or *bcsp31* genes within the genome of the respective *Brucella* species or biovar (Bounaadja *et al.,* 2009; Foster *et al.,* 2008; Probert *et al.,*

2004; Redkar *et al.*, 2001). These assays can be designed for single or separate PCRs. A single RT-PCR species-specific assay for *Brucella abortus*, *Brucella melitensis* and *Brucella suis* was developed by Redkar *et al.* (2001), which uses the IS*711*-derived primer along with the *B. abortus* or *B. melitensis* or *B. suis*-specific primer. Similarly, Probert *et al.* (2004) developed a real-time triplex assay to rapidly detect *Brucella spp.*, *B. abortus* and *B. melitensis* isolates in a single test using primers and probe targeting the *bcsp31* gene. An evaluative study of various published real-time PCR assays targeting *bcsp31*, per, IS*711*, alkB/IS*711* and BMEI1162/IS*711* by Al Dahouk et al. (2013) revealed low detection limits among most assays. However, the study recommended using assays targeting the *bcsp31* gene in *Brucella* screening.

In contrast to the conventional PCR techniques, RT-PCRs are highly sensitive and specific rapid SNPs-based assays that have reduced DNA contamination and do not require electrophoretic analysis of PCR products (Yu & Nielsen, 2010). Studies have further shown that RT-PCR assays reduced time, human resources and reagent costs compared to conventional PCR assays (Probert *et al.*, 2004).

2.4.3.3 Specific high-resolution assays

Multilocus sequence-based molecular typing methods such as multilocus sequence typing (MLST) and multilocus-variable number of tandem repeats analysis (MLVA) have been used to characterize *Brucella* species (Ma *et al.*, 2016; Samaha *et al.*, 2008; Smirnova *et al.*, 2013). The MLVA assay used in epidemiological studies in human and animal brucellosis can trace the source of infection (Minharro et al., 2013). MLVA is an effective tool for cluster analysis of *Brucella* strains compared to MLST. However, when it comes to phylogenetic analysis, MLST is more commonly used than MLVA (Scholz & Vergnaud, 2013). These assays utilise the array-

length variations in tandem repeats which can be readily identified and tested for polymorphism. The technique highly discriminates among *Brucella* species, among previously characterised *Brucella* strains, and among unrelated field isolates that could not be differentiated by classical methods. However, this assay is not used for identification purposes at the species level because it requires additional selections of tandem repeats (Scholz & Vergnaud, 2013; Wang *et al.*, 2014). The MLVA assays have higher specificity, reproducibility and technical ease than the culture methods; hence combining these methods will achieve the best results. However, these assays are costly, especially for developing countries (Gall & Nielsen, 2004).

2.5 Global distribution and seroprevalence of brucellosis in humans and livestock

The current global burden of brucellosis ranges from one to 200 new human cases per ten million individuals per year (Dean *et al.*, 2012). Most European countries are brucellosis-free, with occasional human cases diagnosed in people from endemic areas (Pappas *et al.*, 2006b). In 2014, 18 EU/EEA countries reported 354 confirmed cases of brucellosis with an overall rate of 0.1 per 100,000 population (European Centre for Disease Prevention and Control, 2016). The disease is endemic in Mediterranean countries (Southern & Eastern Europe), Latin America, the Middle East, Central Asia and Africa. In Mexico, the annual human brucellosis cases are 28.7 per million the population (Dean *et al.*, 2012). The Middle East is home to five of the ten countries with the highest disease incidence in the world. These include Syria, with an annual disease incidence of 1,603 cases per million per year (Pappas *et al.*, 2006) and 3.1 per cent seroprevalence in cattle. The disease prevalence in India's 185 million cattle population is 5-26.6 per cent while data on human incidence is not well known. Mongolia has a seroprevalence of 2.3-27.3 per cent and 16.0 per cent in humans and cattle, respectively, while Egypt's brucellosis

status is endemic, with an estimated 0.28-70.0 human cases per 100,000 and 11 per cent seroprevalence in cattle (Dean *et al.,* 2012; Holt *et al.,* 2011). The true disease picture is hugely underestimated due to the non-specific nature of brucellosis in humans, frequent misdiagnosis and poor access to healthcare (Pappas *et al.,* 2006).

2.6 Seroprevalence of human and bovine brucellosis in Africa

Serological studies have been documented in occupationally-exposed humans (livestock farmers, abattoir workers, herdsmen, livestock professionals and pregnant women) and cattle in most Western, Eastern and Southern parts of Africa shown in Table 1. The serology tests used in these human studies were SAT, BAPA, RBT, cELISA and IgG ELISA. In animal studies, on the other hand, RBT and cELISA tests have been used for screening and confirmatory purposes, respectively.

Table 1: Seroprevalence of human and bovine brucellosis in some African countries

S/N	Country	Seroprevalence (study population)	Diagnostic test	Reference(s)
1.	Egypt	5.44%, 4.98% (individual animals) 6%, 8%, 8% (human)	BAPA, RBT SAT, BAPA, RBT	(Samaha *et al.*, 2008) (Samaha *et al.*, 2009)
2.	Nigeria	10.1% and 29.2% (individual traditional cattle and herds) 24.1% (abattoir workers)	RBT and cELISA RBT and cELISA	(Ogugua *et al.*, 2018) (Aworh *et al.*, 2013)
3.	Ivory Coast	10.3% (indigenous cattle)	RBT and iELISA	(Sanogo *et al.*, 2012)
4.	Cameron	3.4 and 5.93% (abattoir cattle) 5.6 and 12.15% (abattoir workers) 0.28% (Pregnant women)	RBT and cELISA RBTand IgG ELISA RBT and iELISA	(Awah-Ndukum *et al.*, 2018)
5.	Ethiopia	2.4 and 45.9% (individual and cattle herd) 1.3% and 4.7% (abattoir workers)	RBT and cELISA CFT & RBT	(Asgedom *et al.*, 2016) (Tsegay *et al.*, 2017)
6.	Tanzania	6.9% (indigenous cattle) 9.3 and 32% (individual animal and cattle herds) 5.52% (Pastoral farmers) 1.41% (occupationally exposed humans	RBT RBT and cELISA RBT cELISA	(Karimuribo *et al*., 2007) (Sagamiko *et al.*, 2018) (Swai & Schoonman, 2009) (Sagamiko *et al.*, 2020)

S/N	Country	Seroprevalence (study population)	Diagnostic test	Reference(s)
7..	Uganda	38.4% (pastoral cattle herds) 5.8% (cattle keepers), 9 (milk consumers) 1.2% (individual cattle) and 4.4% (human)	RBT STAT & cELISA iELISA and IgG iELISA	(Mugabi *et al.*, 2012) (Nasinyama *et al.*, 2014) (Nguna *et al.*, 2019)
8.	South Africa	1.45% (communal grazing cattle) 11 and 5.5% (abattoir cattle)	RBT and CFT RBT & iELISA	(Hesterberg *et al.*, 2008) (Kolo *et al.*, 2019)
9.	Zimbabwe	9.9% (cattle in Wildlife-livestock interface) 5.6% and 25.6% (individual & Smallholder dairy cattle herds)	RBT & cELISA RBT& cELISA	(Gomo *et al.*, 2012) (Matope *et al.*, 2011)
10.	Angola	14.96 and 40.10% (Individual & cattle herd) 15.6% (livestock professionals)	RBT RBT	(Mufinda *et al.*, 2015) (Mufinda *et al.*, 2017)
11.	Malawi	7.7% (individual cattle)	cELISA	(Tebug *et al.*, 2014)

2.7 Brucellosis in Zambia and the application of the One Health Concept

According to Tuchili (1988), brucellosis was first reported in Zambia in 1958 (Muma *et al*., 2007). Since then, the disease has become endemic in many parts of the country, including Western and Southern provinces, where serological studies have shown the presence of brucellosis in humans, cattle and wildlife. Available information indicates the distribution of brucellosis in Zambia as 5-20.7 per cent in the Southern province (Health *et al.,* 2011; Muma *et al.,* 2006), 7.9-18.7 per cent in Lusaka and Central provinces (Chimana *et al.,* 2010) and 17.9 - 22.18m per cent in the Western province (Muma *et al.,* 2007). The disease has been described in humans in Lusaka urban populations at one per cent (Orino *et al*., 1994) and traditional rural populations in the Southern province at 5.03 per cent (Muma *et al*., 2008). Human populations in rural areas are reportedly at higher risk of *Brucella* spp. compared with those in urban areas due to different lifestyles, occupations such as herding, milking and eating habits (Ducrotoy *et al.,* 2017).

From the studies that have been done in most African countries, it is evident that the pattern of the disease distribution in livestock varies depending on geographical regions, livestock production system, herd size, the close proximity of livestock to wildlife (animal-wildlife interface) and mixing of different livestock species (Mcdermott & Arimi, 2002). The disease also tends to be higher in large pastoral herds than in commercial and small confined herds (Bertu *et al.,* 2015).

The Zambian cattle population is estimated to be three million, 80 per cent of which is in the hands of traditional farmers, while 20 per cent is commercial. Commercial cattle production is carried out by large and medium-scale private farmers who keep exotic beef and dairy cattle

breeds of high production efficiency (Lubungu *et al.,* 2015). Commercial farmers supply milk and beef to urban areas and are found along the rail line between the Southern, Copperbelt and Central provinces (Chimana & Mwelwa, 2012). The traditional cattle population is highest in the Southern, Western, Eastern and Central provinces of Zambia, with an average herd size of forty (10 to 50) in most provinces except for the Southern province, where the average herd size is 100. Despite being the largest cattle population in Zambia, the traditional cattle system contributes minimally to the national beef and milk market (Lubungu *et al.,* 2015). This can be attributed to poor husbandry practices, low productivity and high mortality rates (Mumba *et al.*, 2018).

Traditional farmers rear their local cattle on communal land under an extensive grazing system. They are primarily of the Sanga and Zebu types crossed with Tonga, Barotse and Angoni breeds, but dairy crosses between exotic and traditional cattle are also used. Three linked herding patterns exist in areas like Kafue flats and the Zambezi basin: Village resident herds (always in the village), transhumance herds (move between the village and the floodplains), and interface herds permanently stay in the floodplains (Muma *et al.,* 2006; Mumba *et al.*, 2018). However, traditional cattle in other parts of Zambia are grazed as village resident herds in communal areas, and the distance they cover depends on the season and availability of water and pasture (Mumba *et al.,* 2018). Studies in Zambia have shown a higher disease seroprevalence in traditional cattle herds (Muma *et al.,* 2013) than in commercial herds (Chimana *et al.,* 2010). Brucellosis is assumedly the leading cause of abortions among traditional cattle in Zambia, mainly during the calving season (October to June) (Muma *et al.,* 2007). A strong association has also been observed between cattle abortion patterns and the distribution of human cases (Health *et al.,* 2011).

Brucellosis is endemic in Zambia and affects livestock production and productivity through decreased milk production, abortions, infertility and mortalities. This results in substantial economic losses among communities that depend on livestock rearing as the main economic activity. In the year 2016, the overall total losses attributed to brucellosis-related calf mortality and milk losses in Southern and Western provinces were estimated at ZMW1,535,800 (USD$134,131) and ZMW77,700 (USD$6,786.02) respectively per farmer (Mwinyi *et al.,* 2016). Unfortunately, Mwinyi *et al.* (2016) only looked at the socioeconomic impact. Animals are reservoirs of *Brucella* infection; hence its presence in animals suggests *Brucella* spp. will likely be transmitted to humans. It is possible that human cases are under-reported or mis-diagnosed as febrile cases encountered in other infectious diseases like malaria or typhoid fever.

Furthermore, brucellosis is not part of routine differential diagnosis. It will be more cost-effective to control brucellosis through a multisectoral One Health approach. This will improve the socio-economic well-being of livestock farmers. However, for this to happen, One Health programmes will need human capacity and financial support. Further, disease surveillance programmes will need to be implemented in Zambia.

2.8 *Brucella* species and biovars circulating globally and in Africa

Brucella spp. has been classified into several biovars, with *Brucella abortus* biovar 1 and *B. abortus* bv2 distributed worldwide, while *B. abortus* bv3 is mainlyfound in Europe, India and Africa (Minharro *et al.,* 2013). A study by Mustafa *et al.* (2017) in Kuwait genotyped the *Brucella melitensis* isolates into 10, 32 and 71 MLVA types, respectively. Furthermore, the combined MST analysis demonstrated that the Kuwait strains had their origin from the East Mediterranean region and closely resembled the UAE strains. In Brazil, *B. abortus* biovars 1, 2,

3, 4 and 6 have been isolated in cattle, with genotype 28 being the most frequent genotype (Minharro *et al.,* 2013).

B, abortus bv 3 has been reported in cattle in most West African countries (Bertu *et al.,* 2015; Sanogo *et al.,* 2017) but was initially reported as bv 1 in Nigeria (Sanogo *et al.,* 2017). The MLVA genotypic characterization of *Brucella* strains isolated from livestock detected heterogeneity within the Nigerian biovar 3a strains consistent with previous genetic analyses of seven strains from Ivory Coast, Gambia and Togo (Bertu *et al.,* 2015). Similarly, *Brucella* species have been characterized from bovine milk (Mathew *et al.,* 2015) and human sera (Ntirandekura *et al.,* 2020) in Tanzania using MLVA and 16S rRNA gene sequencing, respectively. The bovine isolates had typical *B. abortus* bv3 characteristics, which were genotypically related to strains from Europe and Asia. In contrast, the human sera isolates were phylogenetically grouped into two clades and three branches that were closer to *B. melitensis, B. abortus* and *B. suis* from the USA, Sudan, New Zealand, Germany and Egypt (Ntirandekura *et al.,* 2020).

In Uganda, *B. abortus* strains from cattle without biovar designation (atypical *B. abortus*) were isolated, which exhibited a single MLVA-16 pattern with high levels of genetic variation when compared to other African strains (Mugizi *et al.,* 2015). In Zimbabwe, *B. abortus* bv 1 and 2 have also been isolated in cattle using AMOS-PCR (Matope *et al.,* 2009. Another study used AMOS, Bruce-ladder, MLVA and whole-genome sequencing assays to characterize *Brucella* species isolated from different animals (Ledwaba *et al.,* 2019). These strains were identified as *B. ovis, B. abortus, B. canis* and *B. suis*, with *B. canis* being the first reported species in Zimbabwe. In South Africa, AMOS PCR and Bruce-ladder PCR were used to characterize

Brucella spp. isolates from cattle tissues and identified as *B. melitensis* biovars 2 and 3 and *B. abortus* biovar 1 (Kolo *et al.,* 2019).

2.9 Antimicrobial susceptibility of *Brucella* spp. isolates

Routine *in-vitro* antimicrobial susceptibility testing of *Brucella* spp. is not recommended due to the high risk of laboratory infection and biological safety level 3 requirements (Maves *et al.,* 2011). The Clinical Laboratory Standards Institute (CLSI) governs the standards and practices for the antimicrobial susceptibility testing (AST) of bacteria; however, there are no *Brucella*-specific CLSI guidelines. The breakpoints of *Brucella* against the tested antibiotics have been established according to the CLSI guidelines for slow-growing bacteria (*Haemophilus* spp.) (Morales-Estradaa *et al.,* 2016).

AST is based on determining the Minimum Inhibitory Concentration (MIC) by broth dilution, agar disc-diffusion, and antimicrobial gradient (E-test) methods. . The agar plates are inoculated with the organism of interest in the agar disc-diffusion test. Then, filter paper discs having the test compound in desired concentrations are placed on the agar surface. The Petri dishes are incubated under suitable conditions. The antimicrobial agent diffuses into the agar and inhibits the growth of the microorganism the diameters of inhibition growth zones are measured (Clinical and Laboratory Standards Institute, 2018). The broth dilution test involves two-fold antibiotic dilutions (e.g. 1, 2, 4, 8 and 16ug/ml) in a liquid growth medium, inoculated with a standard bacterial suspension of 1-5 x 105 CFU/ml incubated overnight at 35°C. The presence of turbidity in the test tubes indicates bacterial growth, and the lowest antibiotic concentration that prevents growth represents MIC. Even though this method produces quantitative results, it is tedious as it involves manual preparation of antibiotic solutions for each test (Jorgensen & Ferraro, 2009). The E-test is cheap, easy to do, and the results are easy to interpret. However, the number of

antimicrobial agents that have diffused into the agar medium cannot be quantified. The E-test combines the dilution and diffusion methods principles to determine the MIC values. A plastic or paper strip (impregnated with an antibiotic concentration gradient and a concentration scale on the upper surface for MIC determination) is placed on the agar plate that has been previously inoculated with the test microorganism (Spengler & Urb, 2017).

The recommended agar includes *Brucella* and Muller-Hinton agars supplemented with 5 per cent sheep blood. The zone of inhibition will be in an elliptical shape (hence the 'E' in the name of the test); hence the MIC is read at the point where the zone of inhibition intersects the strip (Spengler & Urb, 2017). The disk diffusion susceptibility method is simple and practical compared to the other methods. Firstly, the bacterial inoculum (1-2 x 10^8 CFU/ml) is streaked on the surface of the Mueller-Hinton agar plate; after that, commercially prepared antibiotic disks with fixed concentrations are inoculated on the agar surface. The plates are incubated overnight at 35°C, and the zone of inhibition is measured to the nearest millimetres. The zone diameter results are qualitative because they give the susceptibility category (i.e. susceptible, intermediate or resistant) instead of MIC values and are interpreted using the CLSI criteria. This method is simple, less costly, and does not need special equipment (Jorgensen & Ferraro, 2009). More advanced methods such as the Vitek 2 system (BioMerieux) are highly automated and use compact plastic reagent cards with antibiotics and test media in a 64-well format. However, these may be too costly for developing countries.

Studies on antimicrobial susceptibility testing for the genus *Brucella* are scarce, and most involve using the E-test compared to the disk diffusion method. A study by Pauletti *et al.* (2015) in Brazil using the E-test found that a significant proportion of *Brucella* strains from cattle were sensitive to the common antimicrobials used to treat human brucellosis, while a considerable

proportion of strains showed reduced susceptibility to rifampicin and two strains were multidrug-resistant. Another study by Shevtsov *et al.* (2017) among seropositive patients in Kazakhstan found that 26.4 per cent and 97 per cent of the *Brucella* isolates were rifampicin-resistant and gentamicin sensitive, respectively, using the E-test. While all clinical isolates from a study in Peru were sensitive to the tested drugs using the E-test (Maves *et al.,* 2011). Another study by Morales-Estradaa *et al.* (2016) in Mexico found out that one isolate from goats was resistant to rifampicin and trimethoprim or sulfamethoxazole; one isolate from cow manure was resistant to ciprofloxacin, levofloxacin, and trimethoprim/sulfamethoxazole using the E-test. Similarly, a study by Abdel-Maksoud *et al.* (2012) among acute febrile patients in Egypt found probable resistance to rifampin (64%) and ceftriaxone (2%), respectively, using the E-test. Another study by Wareth *et al.* (2021) in Egypt reported that clinical and non-clinical *Brucella* strains were susceptible to doxycycline, tetracyclines, gentamicin, ciprofloxacin, levofloxacin, chloramphenicol, streptomycin and trimethoprim or sulfamethoxazole but had probable resistance to rifampicin and azithromycin using the broth microdilution and disk diffusion methods.

Despite the *Brucella* genome lacking the classical AMR genes, *Brucella*'s antimicrobial resistance is rising (Wareth *et al.,* 2021). The rpoB gene mutations have been associated with rifampin resistance in many bacterial species, including *Brucella*. However, when these rpoB genes were screened in some studies, they were found to have no mutations in isolates resistant to rifampicin. The resistance in *Brucella* spp. may develop concurrently with a rise in multidrug-resistant tuberculosis (MDR-TB) (Shevtsov *et al.,* 2017). It is essential to investigate the susceptibility profile of *Brucella* isolates since most antimicrobials used in the treatment of

human brucellosis are also used to control other infections such as TB, which increases the risk of the development of antimicrobial-resistant strains.

2.10 Treatment, Prevention and control of brucellosis

In human patients, treatment requires the use of antimicrobials that can penetrate the cells and survive the acidic environment (Pappas *et al.,* 2006b). The WHO recommended antibiotic regimen is 100 mg doxycycline twice a day for six weeks with 600-900 mg oral rifampicin daily for six weeks or streptomycin 1 gramme intramuscularly daily for two to three weeks to suppress bacterial replication (Corbel *et al.,* 2006). Some reviews showed that the triple regimen of the doxycycline-rifampicin-gentamicin combination was advantageous as the first treatment of choice over doxycycline-aminoglycoside and doxycycline-cotrimoxazole as alternative regimens (Alavi & Alavi, 2013; Skalsky *et al.,* 2008). These regimens are expensive with less than 100 per cent success rate, possibly due to poor compliance among patients, resulting in the development of antimicrobial resistance (Ducrotoy *et al.,* 2017). An eight-week regimen of two antibiotics gave high therapeutic success and minor treatment failure (Alavi & Alavi, 2013). Since human *Brucella* infection is a good indicator of the disease in the animals, prevention in humans greatly depends on eradicating the disease in animals coupled with occupational food hygienic practices such as boiling milk (Godfroid, 2017). Currently, there are no available commercial vaccines prevent human brucellosis. The primary source of disease transmission is contact with infected livestock and consumption of raw or unpasteurised milk from infected animals. Hence infections can be prevented by educating the pastoral communities on consuming boiled milk, pasteurised milk products and using personal protective wear when handling aborted and calving animals. Occupational groups like livestock farmers, abattoir workers, milkers, veterinarians and butchers

tend to have a high risk of exposure. These groups, therefore, need educational talks to improve their levels of knowledge, awareness and, consequently, practices towards brucellosis.

Treatment using antibiotics in animals is very costly, ineffectivecan, and generally not recommended; it is usually unsuccessful because the Brucella organism can survive and adapt inside the host macrophages.

Control of brucellosis in animals ideally involves surveillance testing of animals, quarantine, vaccination and culling of infected herds. Replacement stock should be selected from *Brucella*-free herds, and in cases where the prevalence of infected animals or herds is low, vaccination of replacement stock can be combined with test and slaughter (Ducrotoy *et al.*, 2017; OIE, 2018). Additionally, new animals should be quarantined and tested before being introduced into the herd.

Vaccinations are available to protect animals against infection. Currently, two vaccines are commonly used. These were prepared from two strains of *B. abortus*: strain 19, a smooth strain used as a live attenuated vaccine and the rough live attenuated vaccine from strain RB51 (OIE, 2018), which are both available on the Zambian market. According to Schuurman 1983 (Health *et al.*, 2011), the S19 vaccine has been used in animal vaccinations since the 1980s in state-owned and private commercial dairy farms in Zambia. However, a Brucella prevention programme does not exist; hence vaccinations are rarely done in traditional, and smallholder dairy production systems, except among a few individuals who can afford to buy vaccines (Health *et al.*, 2011).

Eradication of brucellosis requires mass tests and slaughter, a vaccination ban and restrictions on the import and export regulations for animals and animal products coming from endemic regions, and good hygiene measures to eliminate the risk of human infections (Corbel *et al.,* 2006).

2.11 The One Health Concept

More than two-thirds of all known infectious diseases and 75 per cent of emerging diseases are zoonotic (Corbel *et al.,* 2006). One Health is a multidisciplinary and international collaborative systems approach to optimise health at the animal-human ecosystem interface (Asokan, 2015). The health of humans, animals and ecosystems is interconnected; the "One Health" concept recognises this and advocates for coordinated, collaborative and interdisciplinary approaches to tackle health threats in the global community (Häsler *et al.,* 2014).

(a) History of One Health

The One Health concept can be traced to ancient civilizations but was only accepted during the 19th century. Robert Virchow, a German physician and father of modern pathology, coined the term "zoonosis" to describe naturally transmitted pathogens between animals and humans. However, Sir William Osler first used the term "one medicine." Later, during the 20th century, Calvin Schwabe revived the concept of "one medicine". Following the joint efforts of the American Veterinary Medical Association (AVMA) and the American Medical Association (AMA) in 2007, the term "One Health" finally emerged (Asokan, 2015). This concept was endorsed by international organizations such as the Food and Agriculture Organisation of the United Nations (FAO) and the World Organisation for Animal Health (OIE) in 2008. In the one health approach, veterinary medicine, human medicine, and other health-related disciplines work

together to achieve optimum health for animals, humans and the environment (Godfroid *et al.*, 2014).

(b) Benefits of One Health

The One Health (OH) approach increases efficiency and cost-effectiveness by reducing overlaps. This is achieved through shared control and detection efforts and results that benefit the health of humans, animals and the ecosystem. According to a review by Häsler *et al.* (2014), the benefits of the one health approach include:

(i) Early detection of public health threats and timely, effective or rapid response.

(ii) Improvement in human or animal health or well-being, e.g., reduces disease risk for humans and animals.

(iii) Higher quality of information and data and improved knowledge or skills are acquired, e.g. more information and insights through knowledge exchange.

(iv) Improved disease control measures, e.g., improved diagnosis of diseases through shared knowledge and facilities (e.g. in areas where raw milk consumption is high, brucellosis can be included as a differential diagnosis for malaria, typhoid fever and pyrexia of unknown origins).

(v) Economic benefit or increase in economic efficiency, e.g. investigating brucellosis in humans and animals as a single social system, results in more cost-effective control than in one sector only.

(a) Relevance of OH in control of zoonotic diseases

Brucellosis is recognised as an emerging and neglected bacterial zoonotic disease that affects animals and humans of all ages and sex groups. According to the World Health Organisation (WHO), half a million human cases are reported each year, with a quarter of cases estimated to go unreported (Corbel *et al.,* 2006). Controlling the disease in humans hugely depends on limiting the infection in animals. The One Health approach in tackling brucellosis is cardinal through accurate identification of the *Brucella* species that affect animals, understanding the evolving epidemiology and applying targeted control strategies in animals.

If human cases are mainly reported in certain occupational groups, public health control measures associated with milk and dairy products have been effectively implemented. If, on the other hand, most cases are reported in the general population, it shows that neither health measures nor control measures have been effectively employed (Godfroid *et al.,* 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study setting

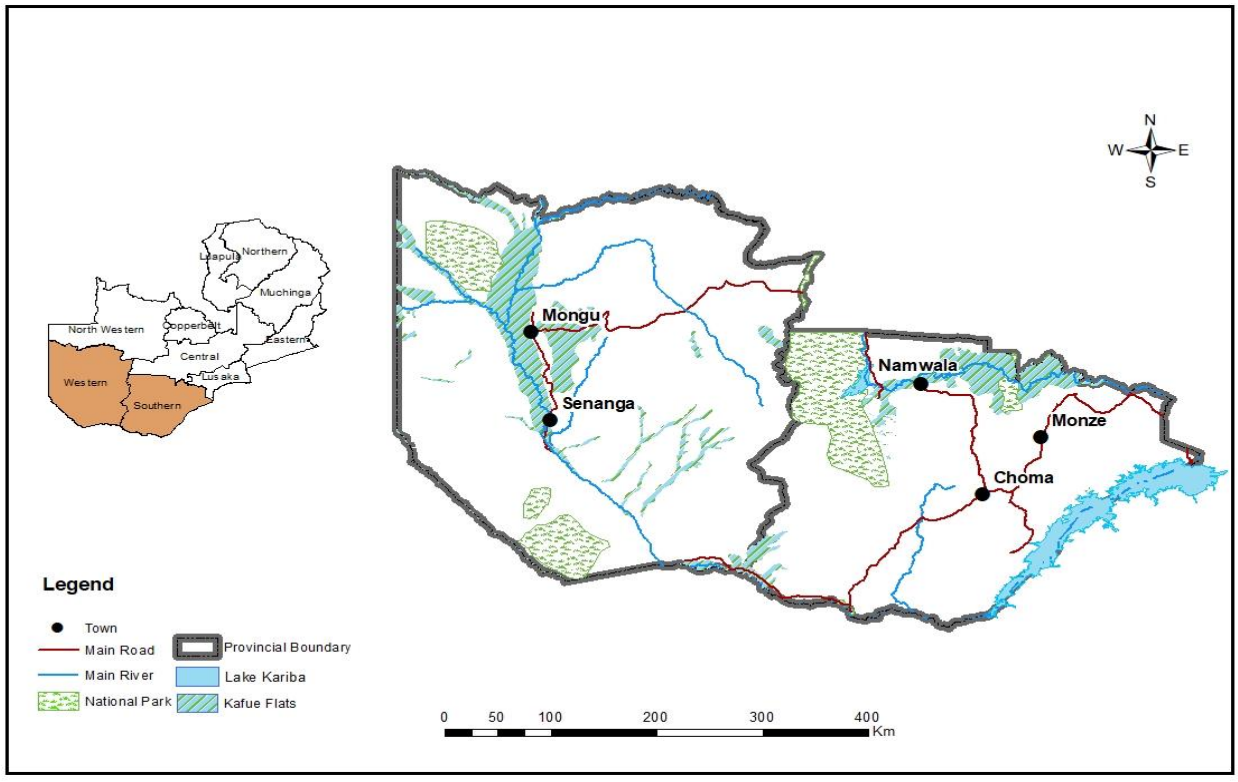
The study was carried out in five districts that were purposively selected from two provinces of Zambia namely Namwala, Choma and Monze in the Southern province and Senanga and Mongu in the Western province as shown in Figure 1. To generate the spatial map of our study area, ArcGIS version 10.7.1 (Environmental Systems Research Institute, Reddlands, CA) was used.

These provinces were selected because they have the highest cattle populations in Zambia (Lubungu *et al.,* 2015) and are endemic to brucellosis as documented by others (Muma *et al.,* 2007, 2013). The Southern province lies between latitudes $15^{\circ}14'$ S and $17^{\circ}42'$ S and longitudes 25° E and 28° S. It has a total land surface area of 85,283km^2 and shares borders with Botswana, Namibia and Zimbabwe. Choma is Southern province's provincial administrative capital, with eleven districts. Southern province has an estimated human population of 2,140,034 (Zamstat, 2021) and a cattle population of 2,105,891 (Ministry of Fisheries & Livestock, 2017). The traditional cattle breeds reared in the study areas are Tonga and Barotse in Southern and Western provinces respectively. The pastoral or nomadic cattle grazing system is practised, where animals are grazed in the Kafue flats or flood plains in the dry seasons and moved to upper areas during the wet season (Muma *et al.,* 2006). The Western province lies between latitudes 15^{o} S and 17^{o} S and longitudes 23^{o} E and 25^{o} E. It has a total land area of about 126,386 km^2 and borders Angola and Namibia. The province has seven districts with a rural population of approximately

1,079,115 (Zamstat, 2021) and an estimated cattle population of 890,288 (Ministry of Fisheries & Livestock, 2017).

The Western province has dominant sandy soils and the Barotse Floodplain of the Zambezi River that naturally waters the grasslands. Over three-quarters of the cattle in Western province are pastured in the floodplain. They are managed under a system of transhumance and move between the floodplains from January to July and adjacent uplands for the rest of the year (Sinkala *et al.*, 2014). Pastoral livestock farming is the mainstay of Western province's economy followed by fish and crop farming.

Figure 1: Map of Southern and Western provinces showing the study areas



Source: GIS Software

3.2 Study Design

This was a cross-sectional study of humans and cattle conducted in five districts in Southern and Western provinces. Namely Namwala, Choma and Monze in Southern province and Mongu and Senanga in Western provinces.

3.2 Study population

3.3.1 Animal and human study

The animal study consisted of the cattle and human populations in Namwala, Choma and Monze districts of Southern province while for Western province it was Mongu and Senanga districts. The individuals were grouped into five categories depending on their level of daily activities that could either lead to direct contact with suspected *Brucella*-infected animals or the use of infected animal products. These groupings ranged from herders, milk handlers, abattoir workers, butchers and animal handlers.

3.3 Sample size estimation and sampling

3.4.1 Cattle

The required cattle sample size was calculated using the formula;

$$n = Z^2 * p * (1 - p)/d^2$$

Where:

z = 1.96 at 95% Confidence interval

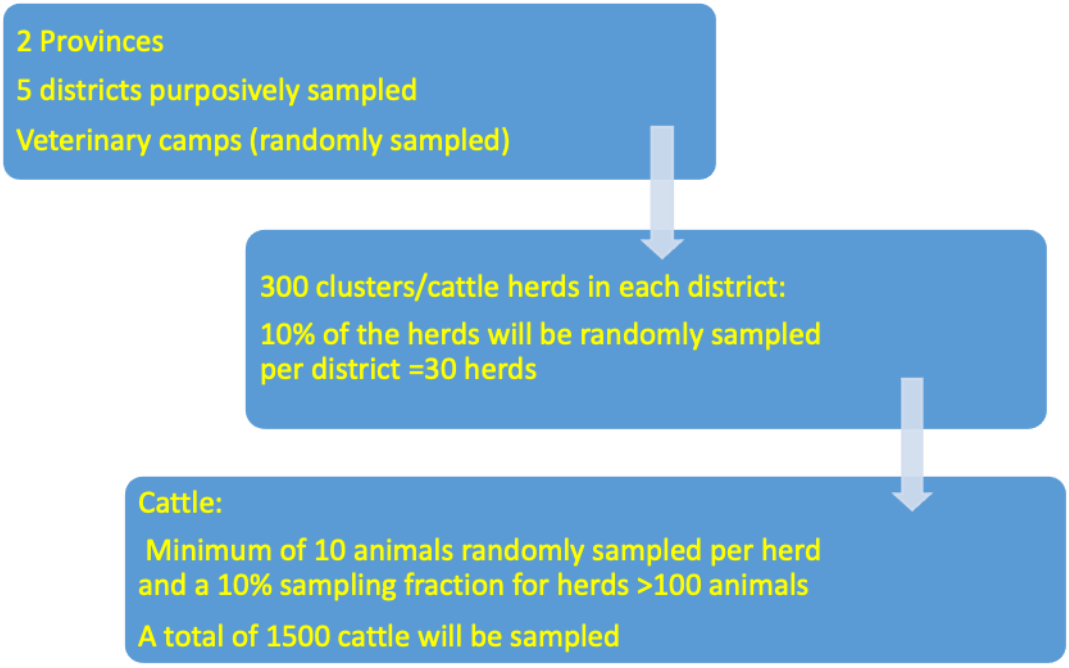
p = expected brucellosis herd seroprevalence of 32% (Sagamiko *et al.,* 2018)

d = the desired absolute precision of 10%

The resulting sample size of eighty-four was multiplied by the design effect (D) of 1.9, calculated using the formula $D = 1 + (b - 1)$ roh (Groves *et al.,* 2011). The average number of samples per cluster (b) was 10, and the intracluster correlation coefficient or rate of homogeneity (roh) was 0.1 (Zolzaya *et al.*, 2014). This gave a calculated sample size of 160 herds. To account for non-response, a 10 per cent adjustment was made, bringing the required minimum herd size to 176. A minimum of ten animals were randomly sampled per herd, and a 10 per cent sampling fraction for herds >100 animals. A total of 1,812 cattle from 175 herds were randomly sampled from five districts, namely Namwala, Choma and Monze in the Southern province; Senanga and Mongu in the Western province. This was performed by determining the sampling interval based on the number of herds available and the required sample size.

A multistage sampling strategy was used with the five study districts and cattle herds as primary and secondary sampling units. Livestock lists obtained from the veterinary offices were used to estimate the number of cattle sampled in veterinary camps of each study district in respective provinces.

Figure 2: Cattle sampling plan



Inclusion criteria: Only herds with a minimum of ten animals and only cattle from *Brucella* unvaccinated herds were included in the study. For bacteriological sampling, only cows with a recent history of abortion of two weeks or less, cows in the third trimester of pregnancy or those that had recently calved with a calf of less than three months were sampled.

Exclusion criteria: Herds with less than ten animals, cattle from *Brucella* vaccinated herds, and cows less than six months pregnant were excluded from the study.

3.4.2 Humans

The study population included purposively sampled herdsmen/herdswomen, livestock keepers, abattoir workers and individuals who had direct contact with animals or animal products. For herdsmen and herdswomen and livestock keepers, at least one individual was purposively

sampled from each household. For abattoir workers, any individual who worked in the selected district abattoir and consented to participant in the study was sampled.

Inclusion and exclusion criteria

The inclusion criteria consisted of:

(i) Individuals who had direct contact with animals or animal products; livestock farmers, herdsmen, abattoir personnel and milk handlers

(ii) Individuals with a previous history of brucellosis

(iii) Young male and female individuals above 15 years old in any stated categories (on condition of parental consent).

Those who did not give consent were excluded from the study.

The sample size for humans was calculated using the Ausvet EpiTools software (http://epitools.ausvet.com.au/) based on the following assumptions:

(i) Expected seroprevalence of 5% (Muma *et al.*, 2006)

(ii) Desired absolute precision of 2%

(iii) Confidence level of 95%

Based on these assumptions, the calculated sample size was 153 humans. Sampling was stratified according to the study districts and weighted using the cattle population as a proxy weighting value for the persons to be sampled (Table 2).

Table 2: Human sample size weighted against cattle population per district

District	Cattle population	Weighting index (human population)	Number of persons to be sampled
Namwala	145,704	102,866	50
Monze	172,994	191,872	55
Choma	134,252	180,873	50
All areas	452,950	475,611	155

3.4 Data collection tools

A datasheet was used to collect information from each household head to capture human demographic and animal biodata (Annex V).

3.5 Sample Collection

3.6.1 Cattle

Samples of blood, milk, hygroma fluids and vaginal swabs were collected aseptically. Animals were restrained using a crush pen (where available) or restrained with care by animal handlers where the crush pen was not available. The study adhered to the requirements outlined in the OIE's Terrestrial Animal health code for handling research animals.

(i) Blood

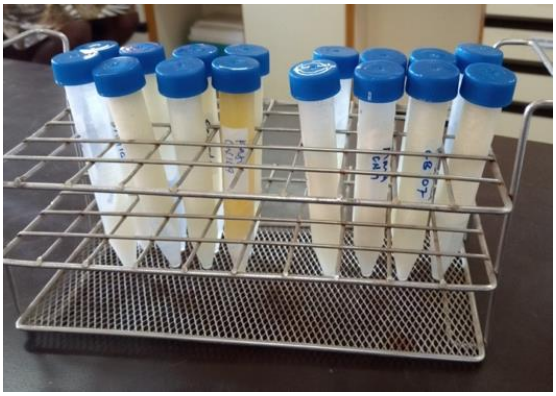
Five militres of blood was collected from the cow's jugular vein into labelled plain vacutainer tubes. These were kept tilted at room temperature then separated using a portable field centrifuge

the following day. Sera were stored in pre-labelled cryovial tubes at -20°C until further analysis at the University of Zambia) School of Veterinary Medicine laboratory (UNZAVET).

(ii) Milk samples

The udder was first washed and disinfected using cotton wool soaked in methylated spirit. The first stream of milk from the cow's teats was discarded. About 2ml of mid-stream milk was collected from each teat into pre-labelled sterile 15ml falcon tubes containing 9ml of Trypticase Soy broth (Oxoid, UK) and stored at -20°C until transported to UNZA School of Veterinary Medicine laboratory.

Figure 3: Milk samples in transport media



(iii) Vaginal swabs

Deep vaginal swabs were collected using sterile cotton swabs (Himedia, Mumbai, India). The swabs were immediately placed in collection tubes containing Amies transport medium (Himedia, Mumbai, India). The samples were stored at -20°C until transported to the UNZAVET laboratory for analysis.

(iv) Hygroma fluids

The hygromas were swabbed with cotton wool soaked in methylated spirit and dried for two minutes. After that, 2ml of the hygroma fluid was aseptically drawn using a 21gauge syringe into an airtight collecting tube containing 9ml of Trypticase Soy Broth (Oxoid, UK). The samples were stored at -20 °C until transported to the UNZAVET laboratory for analysis.

3.6.2 Human

Blood samples were collected by medical personnel from the health centres or clinics in respective districts. For culturing, 2ml of whole blood was collected using a sterile needle and syringe into a tube containing 8ml of Trypticase soy broth (Oxoid, UK) transport media. The samples were stored at -20°C until further analysis.

3.6 Laboratory analysis

Cattle sera were analyzed for anti-*Brucella* antibodies (serology), while the biological samples from cattle and whole human blood were subjected to bacterial culture. The cultured bacterial isolates were subjected to molecular analysis as described (Al Dahouk *et al.,* 2013; OIE, 2018).

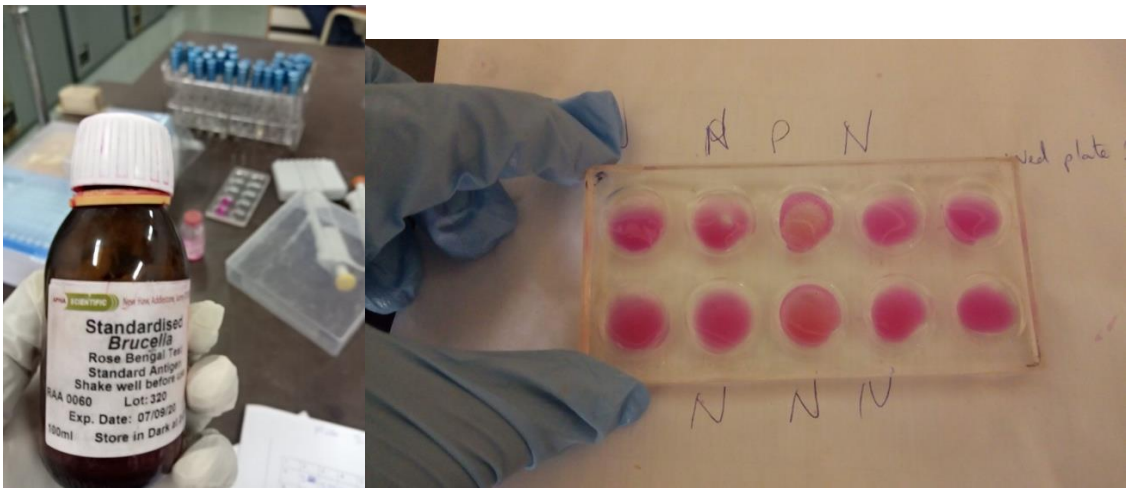
3.7.1 Serology

3.7.1.1 Rose Bengal plate test

The Rose Bengal plate test (RBT) kit manufactured by (Central Veterinary Laboratory, New Haw, Addlestone Surrey KT153NB, UK) was used to screen all cattle sera samples for anti-*Brucella* antibodies. The assay was conducted and interpreted according to the test procedure recommended by OIE (OIE, 2018). Briefly, 25µl of the test serum were placed in one well of the

glass plate containing an equal volume of RBPT antigen and mixed thoroughly. The slide was rocked gently from side to side for four minutes. After that, the sample was considered positive if any visible sign of agglutination was observed.

Figure 4: RBT antigen and screening of serum samples on Rose Bengal plate



Wells with visible clumping were positive while those without any clumping were negative

3.7.1.2 Enzyme-Linked Immunosorbent Assay (ELISA) test

Only sera samples that tested positive on RBPT were subjected to competitive Enzyme-Linked Immunosorbent Assay (cELISA) as a confirmatory test using the (INGEZIM BRUCELLA COMPAC 2.0, Spain) test kit. The assay was conducted and interpreted according to the protocol outlined by the manufacturer.

Briefly, 50uL of Mab-solution was added into all wells used for controls and samples (time difference between controls (A positive, weak positive, and negative control were included in 96-well plate.) Samples and Mab-solution addition did not exceed ten minutes. The plate was sealed, and the reagents were thoroughly mixed for five minutes. The plate was incubated at room temperature for thirty minutes and rinsed four times with PBS-Tween buffer. After that,

100uL of the conjugate solution was added to each well, and the plate was sealed and then incubated at room temperature for thirty minutes. Rinsing was repeated, as explained above. Then, 100uL of substrate solution was added to each well and incubated for ten minutes at room temperature (timing began after the first well was filled). The reaction was stopped by adding 50uL of stop solution in the same order as the substrate solution and mixed. Then the optic density (OD) of controls and samples was measured at 450nm in a microplate photometer (the air was used as blank). The OD was measured within fifteen minutes after adding the stop solution to prevent fluctuation in OD values. The OD of the positive control was the one with which the OD of each test serum was compared to establish the final result (negative or positive). Determination of the positive and negative tests using the cut off was provided in the cELISA kit guide. Negative results were determined by a per cent inhibition (PI) of < 30 per cent while positive results were ≥ 30 per cent.

3.7.2 Bacterial culturing and identification

Milk samples, hygroma fluids and vaginal swabs were cultured on *Brucella* selective media (Oxoid, UK) as described in the OIE Terrestrial Manual (OIE, 2018). Half of the plates were incubated in a normal incubator, while the other half were placed in a 5-10 per cent CO_2 incubator in the Biocontainment laboratory level II (BSL2) at the School of Veterinary Medicine at UNZA.

Culturing Method

The milk sample was centrifuged at 6,000–7,000g for fifteen minutes in sealed falcon tubes to obtain the milk cream and pellets. Using a sterile swab-stick, the milk cream and pellets were pipetted and spread on Farrell's selective media. The hygroma f1uids samples were pipetted and

spread on Farrell's selective medium using a sterile swabstick. Vaginal swabs were withdrawn from the protecting tube and rehydrated in PBS. The swab was after that, streaked over the surface of Farrell's selective media.

Incubation was done at 37.5°C for seven to fourteen days (OIE, 2018). The cultures were examined periodically up to the 14^{th} day, and suspected colonies typical of *Brucella* species were sub-cultured on blood agar (Oxoid, UK) and Nutrient media (Oxoid, UK). Briefly, Farrell's selective medium was prepared from *Brucella* medium base (Oxoid, UK) sterilised at 121°C for fifteen minutes. *Brucella* selective supplement (Oxoid, UK) and horse serum (ThermoFisher Scientific, Wilmington, USA) at 5 per cent were added according to the manufacturer's instructions. The isolates were studied for colony morphology, culture characteristics and Gram stained. The suspected isolates were subjected to biochemical tests for presumptive identification: oxidase, catalase, urease, CO_2 requirement, H_2 S production, and methyl red. This was done as described in the OIE Terrestrial Manual (OIE, 2018). Confirmation of the bacterial identification was done by PCR.

Figure 5: cELISA plate showing positive (colourless) and negative (yellow) wells

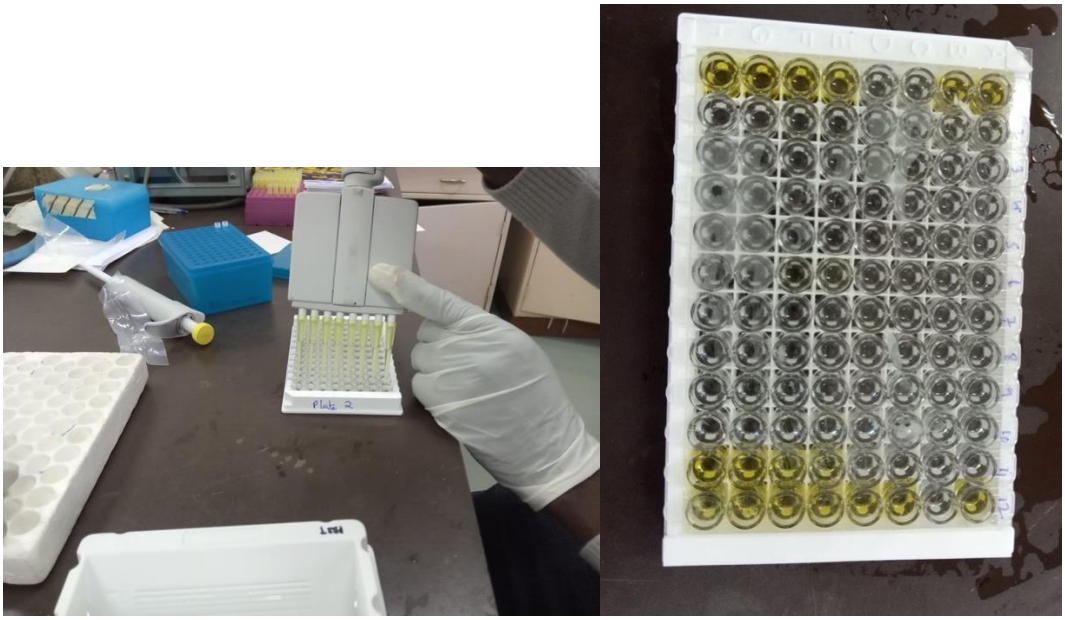


Figure 6:Colony growth of *Brucella* spp. on *Brucella* selective media prepared using sheep blood

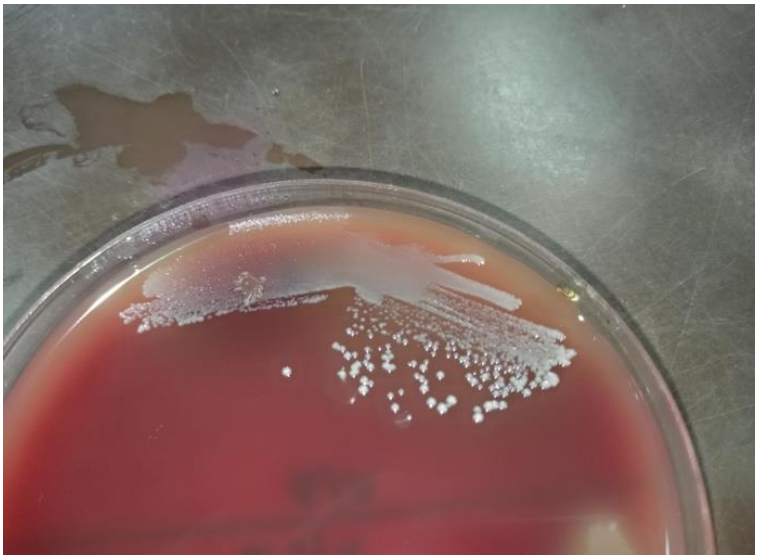
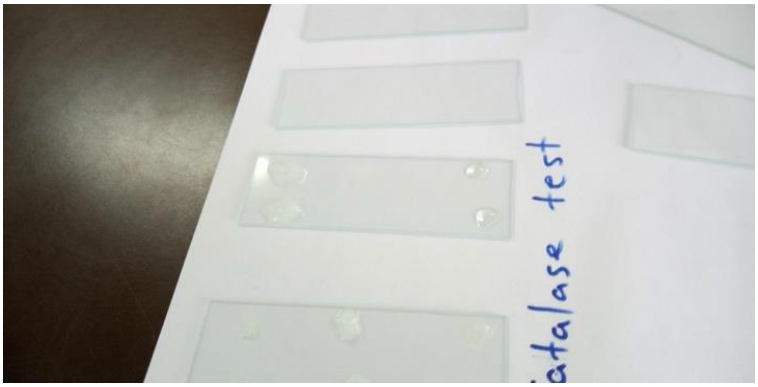


Figure 7:Urease test. The tubes showing a pink colour change are positive

Figure 8: Oxidase test using the oxidase discs. The purple colour indicates a positive result

Figure 9: Catalase test. Bubble formation indicates a positive result



3.7.3 AST of *Brucella* isolates using disk diffusion method

Three *Brucella* isolates (3, 8 & 12) were evaluated for susceptibility to six commonly used antimicrobials using the disk diffusion method. This method determined the disk diffusion susceptibility for the antibiotics; rifampin (5µg), trimethoprim-sulfamethoxazole (23.75µg), doxycycline (30µg), tetracycline (30µg), ciprofloxacin (5µg), streptomycin (10µg), gentamicin (10µg) and chloramphenicol (30µg).

A bacterial suspension was prepared from pure and fresh colonies for each isolate, and the tube turbidity was adjusted to the 0.5 McFarland turbidity standard. The suspensions were spread onto Muller-Hinton agar plates supplemented with 5 oer cent sheep's blood and incubated at 37°C in the presence of 5-10 per cent CO_2. Each antibiotic disc was individually placed on the inoculated agar and incubated at 35°C for forty-eight hours. All antibiotics were assessed in duplicate for all isolates, and the results were recorded after forty-eight hours of incubation. The breakpoints of *Brucella* against the tested antibiotics have been established according to the 2020 CLSI guidelines for slow-growing bacteria (*Haemophilus* spp.) (CLSI, 2020).

3.7.4 Molecular characterisation of *Brucella* isolates from cattle and human

3.7.4.1 Genomic DNA extraction using Quaigen kit

Genomic DNA was extracted from purified colonies using the Quaigen DNA extraction kit (Hilden, Germany) according to the manufacturer's protocol. Briefly, a loopful of culture colony was suspended in 200µl phosphate buffer saline (PBS). Twenty microlitres (20uL) of proteinase K was added to a 1.5 ml microcentrifuge tube, followed by a 200 µl Buffer AL. This was mixed thoroughly by vortexing and incubating at 56°C for 15 minutes. Two hundred microlitres (200 µl) of 96 per cent ethanol was added to the tube and mixed by vortexing. The mixture was

pipetted into a DNeasy MiniSpin column placed in 2ml collection tubes, and centrifuged at 6,000*xg* for one minute. The tube containing the filtrate was discarded, and the mini spin column was placed in a new 2ml collection tube, to which 500µl Buffer AW1 was added. The mixture was centrifuged at 6,000*xg* for one minute, and the tube containing the filtrate was discarded. Five hundred microlitre (500µl) Buffer AW2 was added to the Mini spin column and centrifuged at 20,000*xg*; for there minutes. The Mini spin column was placed in a new 2ml collection tube, the old collection tube was discarded, and one dry spin was run. After centrifuging at full speed for one minute, the Mini spin column was placed in a clean 1.5ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. 60µl Buffer AE was added to the Mini spin column and incubated at room temperature for 1one minute. It was after that centrifuged at 6,000*xg* for one minute. The extracted DNA was stored at -80°C until further use. DNA quantity and purity were determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, USA).

3.7.4.2 PCR amplification of 16S rRNA genes

The 700bp fragment of the 16S rRNA gene was amplified using the Ex-Taq HS PCR kit (Takara Bio USA, Inc.) and universal primers pairs P3mod-F (5'-ATT AGA TAC CCT GGT AGT CC-3') forward primer and P5-R (5'-GGT TAC CTT GTT ACG ACT TC-3') reverse primer designed by Tsen *et al.* (1998). This was done using the Veriti 96 well Thermocycler AB (Applied Biosystems, Grand Island, NY) according to the manufacturer's instructions.

Briefly, 2µl of DNA template was added to a final reaction volume of 20µl consisting of 2µl 10X Buffer, 1.6µl of dNTPs, 0.8 µl of each primer, 0.1 µl of Ex-Taq HS and 13.7µl of Nuclease free water. The PCR mixture contained positive (DNA isolated from the S19 vaccine) and negative

controls (nuclease-free water). The PCR conditions were as follows; initial denaturation at 95ºC for five minutes, followed by thirty-five cycles of denaturation at 95ºC for thirty seconds; annealing at 54ºC for ninety seconds, extension at 72ºC for the ninety seconds and a final extension at 72ºC for ten minutes. The PCR products were visualised on 1.5 per cent agarose gel stained with ethidium bromide after electrophoresis at 100 volts for thirty minutes. This was done according to the manufacturer's instructions and Unver *et al.* (2006) described. The amplicon's size was assessed based on comigration of standard DNA ladder of molecular weight in the range of 100-1000 bp for the amplification of 16S rRNA.

3.7.4.3 16S rRNA gene sequencing

DNA fragments for sequencing were prepared from PCR positive samples using the QIA quick Gel Extraction Kit (Qiagen Inc. Valencia, CA, USA) according to the manufacturers' recommendations. The PCR products were first purified using the Wizard ® SV Gel and PCR Clean-Up System according to the manufacturer's instructions. Sequencing was carried out using the BrilliantDye Terminator v3.1 cycle sequencing kit (Edge Biosystem™). The sequencing reactions had a final volume of 20µl: This consisted of 1µl of PCR product, 1µl BrilliantDye Terminator (Edge Biosystems™), 3.5µl of 5X~sequencing buffer (Applied Biosystems™, Foster City, CA, USA), 1µl of the appropriate sequencing primer and 13.5µl of deionised water. Sequencing primers were universal primer pairs P3mod-F (5'-ATT AGA TAC CCT GGT AGT CC-3') forward primer and P5-R (5'-GGT TAC CTT GTT ACG ACT TC-3') reverse primer. Sequencing was done in duplicate for each primer. The cycle conditions were 96°C for forty-five seconds, followed by twenty-five cycles of 96°C for ten seconds, 50°C for five seconds and 60°C for two minutes. Following the cycle-sequencing protocol, the reaction was purified using ethanol precipitation.

In the ethanol precipitation procedure, 2µl of 3M Sodium acetate (NaAOc) (Sigma-Aldrich) (pH 4.6), 2µl of 125mM EDTA and 90µl of 100 per cent ethanol were added to each reaction tube containing the sequencing products. After vortexing, the samples were centrifuged at 15,000 rpm for twenty minutes. The supernatant was aspirated, and 200µl of 70 per cent ethanol was added. The samples were centrifuged at 15,000rpm for five minutes. The supernatant was aspirated, and 200µl of 70 per cent ethanol was added. The samples were centrifuged at 15,000 rpm for five minutes and vacuum dried for ten minutes. The tubes were covered with aluminium foil before adding 20µl of HIDI formamide and vortexing for fifteen seconds. Denaturing was done at 95°C for two minutes using a heating block. The precipitated product was loaded in the sequencer called Genetic Analyzer 3500 (Applied Biosystems 3,500 series genetic analyzer (Thermo Fisher Scientific). The obtained results were analysed using ATGC software, and the nucleotide sequences obtained were blasted on PubMed blast searched at http:// www.ncbi.nlm.nih.gov/BLAST.

3.7.4.4 Real-time PCR

Real-Time PCR was performed using IS711 and bcsp31 target primers as described elsewhere (Matero *et al.*, 2011; Probert et al., 2004).

3.7.4.5 *Brucella spp*. identification using Multiplex Bruce Ladder PCR Assay

Samples that tested positive on PCR were further analysed using the multiplex PCR (Bruce-ladder) assay kit (Ingenasa, Spain). This was performed according to López-Goñi *et al.* (2011) using eight primer pairs in a single reaction which was performed according to the manufacturer's conditions.

Briefly, PCR was carried out in a 20-µl reaction with 0.5 µl of each primer in the cocktail of eight primer sets, 1.6µl of dNTPs, 0.1µl of Ex Taq polymerase HS (MBI, Fermentas, USA), 2µl of template DNA. The samples were subjected to amplification (35s at 95°C, 45s 64°C, 3 minutes at 72°C) twenty-five times with initial denaturation for seven minutes at 95°C and final extension for six minutes at 72°C in a thermocycler (Eppendorf, Germany). The PCR products were separated using 1.5 per cent agarose gel by electrophoresis through ethidium bromide-stained 1.5 per cent agarose gel.

3.8 Data analysis

Serology

Results for anti-*Brucella* antibodies seropositivity were entered in Microsoft Excel and then imported to STATA 13® statistical software (StataCorp, College Station, TX, USA) for analysis. Categorical variables were summarized as frequency and percentages; continuous variables were summarised as proportions. An animal was considered seropositive if it tested positive on both RBT and cELISA tests. A herd was defined as the total number of cattle belonging to the same household, and it was considered seropositive if at least one cattle tested positive on both RBPT and c-ELISA tests.

Bacteriology

The bacteriology results and MIC values for the antimicrobial susceptibility tests were recorded in the Microsoft Excel spreadsheet 2013 version.

Molecular typing

The obtained sequences were verified using BLAST analysis on the NCBI website (http:// www.ncbi.nlm.nih.gov/BLAST), followed by assembly and editing using the ATGC plug-in the Genetyx Ver. 12. Multiple sequence alignment of the obtained sequences together with the downloaded reference sequences from the Genbank were generated using CLUSTALW1.6. The multiple sequence alignment file was then utilized to generate a phylogenetic tree using MEGA ver. 6 with 1,000 bootstrap replicates as a level of confidence. All the sequences generated in this study have been deposited in the DNA Database of Japan.

3.10 Ethical Considerations

Ethical clearance was sought from Excellence in Research Ethics and Science (ERES CONVERGE) (Ref No. 2018-Dec-004) before the commencement of the study. Permission to conduct the study was sought from the National Health Research Authority, the Ministry of Health and the Ministry of Livestock and Fisheries. The aim of the study was clearly explained to the research assistants and individuals who were included in the study. Upon arrival at the households, the study objectives were explained to the household head, and permission to conduct the study was sought. Written informed consents were obtained prior to any interviews and sample collection. For participants of the age below 18 years, informed consent was obtained from their parents prior to the study. Participants were allowed the option of withdrawing from the study when they chose to do so without incurring any consequences. Confidentiality of the study results and anonymity were emphasised to the participants. Informed consent was also sought from the animal owners before sampling the animals. Medical personnel and Veterinary

assistants were used to collect samples from human and animal subjects, respectively, to reduce discomfort during the procedure.

CHAPTER FOUR

RESULTS

4.1.1 Serology

4.1.2 Seroprevalence of anti-*Brucella* antibodies in cattle

A total of 1815 sera from 175 herds were sampled from five districts in Southern and Western provinces. The herd-level and individual animal anti-*Brucella* antibody seroprevalence were 32 per cent (CI 95%: 25.0-38.9) and 9.92 per cent (CI 95%: 8.5-11.2), respectively (Tables 3 & 4). Mongu district had the highest herd-level seroprevalence (46.1%, CI 95%: 26.5-65.8) while Choma district had the lowest (8.7%, CI 95%: 3.16-20.5), although this was not statistically significant. At the provincial level, the Western province had a higher herd seroprevalence (32.3%, CI 95%: 20.7- 43.8); these results were, however, statistically insignificant. The differences in seroprevalence levels between the two provinces as well as between Namwala and Mongu districts were very minimal. The individual animal seropositivity was higher among cattle aged 11-16 years (18.9%), female animals (10.6%), pregnant cows (14.3%) and pregnant heifers (13.3%) despite not being statistically significant. (Table 5).* The seroprevalence was higher in Namwala district (OR: 8.56, CI: 2.66-27.50), among cattle aged 11-16 years (OR: 1.55, CI: 1.20-2.01), female cattle (OR: 1.81, CI: 1.06-3.07), pregnant cows (OR: 1.30, CI: 0.44-3.84) and pregnant heifers (OR: 1.2, CI: 0.71-2.02) as shown in Table 5. These results were not statistically significant.

Table 3: Cattle herd-level seroprevalence by district

District	Herd n	Seroprev. %	95% CI
Choma	23	8.7	3.16-20.5
Monze	34	38.2	21.5-54.9
Namwala	53	37.7	24.5-51.0
Mongu	26	46.1	26.5-65.8
Senanga	39	23.1	16.1-28.5
Total	**175**	**32.0**	**25.0-38.9**

Seroprev. —Seroprevalence; %—Percentage; CI—Confidence interval; n—Number

Table 4: Cattle herd-level seroprevalence by province

Province	Herd n	Herd Seroprev. %	95% CI
Southern	110	31.8	23.0-40.6
Western	65	32.3	20.7-43.8
Total	175	32.0	25.0-38.9

Seroprev. —Seroprevalence; %—Percentage; CI—Confidence interval; n—Number

Table 5: Association between animal characteristics and individual animal-level seropositivity assessed by logistic regression

Variable	Category	Pos/Tested	Seropositivity (%)	Odds Ratio	95% CI
Province	S/P*	98/1113	8.8	Ref	-
	W/P**	82/702	11.7	1.37	1.0-1.87
District	Choma	3/186	1.6	Ref	-
	Monze	21/326	6.4	4.19	1.23-14.28
	Namwala	74/601	12.3	8.56	2.66-27.50
	Mongu	34/302	11.3	7.73	2.34-25.5
	Senanga	48/400	12.0	8.31	2.55-27.07
	Total	**180/1815**	**9.92**		**8.5-11.2**
Age (yrs)	0–5	80/993	8.06	Ref	-
	6–10	86/748	11.5	1.48	1.07-2.04
	11-16	14/74	18.9	1.55	1.20-2.01
Sex	Male	16/262	6.1	Ref	-
	Female	164/1553	10.6	1.81	1.06-3.07
Reproductive status	Cow	83/731	11.4	Ref	-
	Lact cow	55/571	9.6	0.83	0.58-1.19
	Preg cow	4/28	14.3	1.30	0.44-3.84
	Preg heifer	20/150	13.3	1.20	0.71-2.02
	Bull	18/262	6.9	0.44	0.26-0.75
	Heifers	0/73	0	0	0

*Southern province, **western province

4.2 Bacterial culture and identification

A total of 1,047 samples were processed from both humans (157) and cattle (890) out of which five presumptive *Brucella* isolates were obtained (Table 6). The detected isolates were from Choma (2 human samples) and Namwala (2 cattle and 1 human sample) districts. No isolate was detected from Monze district samples. *Brucella* species were identified in two cattle milk (sample IDs 2 and 3) and three human whole blood samples (sample IDs 8,11 & 12) using 16S rRNA PCR. All isolates (2, 3, 8, 11 & 12) grew in the presence and absence of $C0_2$**.** Morphologically, the isolates were Gram-negative coccobacilli.

Table 6: The distribution of the biological samples and the numbers of *Brucella* species isolated on culture

Sample	Source	No of the samples tested	No of *Brucella* isolates
Whole blood	human	157	2
Vaginal swabs	Cattle	210	2
Milk	Cattle	666	2
Hygroma fluid	Cattle	10	3
Foetal material	Cattle	4	3
Total		1047	12

All isolates (2, 3, 8, 11 &12) were positive on catalase, urease and oxidase tests, negative on MacConkey agar and Methyl red.

4.3 Molecular characterization of *Brucella* isolates from cattle and humans

Brucella DNA was extracted from 5 out of the 12 suspected colonies. These were identified to genus level (Table 7). The positive control used was the *B. abortus* S19 vaccine.

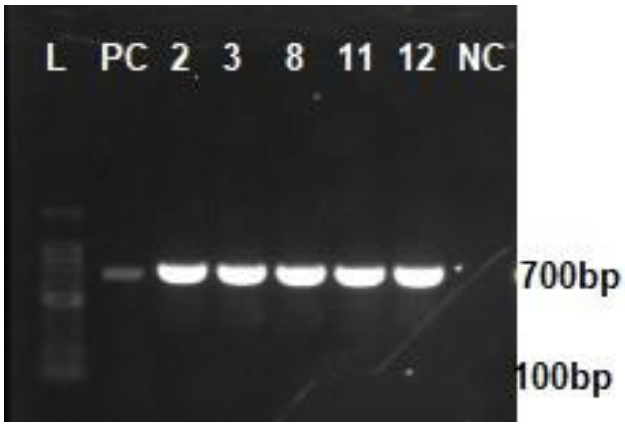
Table 7: 16S rRNA PCR summary of results

Sequence #	Species	Source of sample	Origin	16S rRNA
2	Human	blood	Choma	*Brucella* spp.
3	Human	blood	Choma	*Brucella* spp.
8	Cattle	milk	Namwala	*Brucella* spp.
11	Cattle	milk	Namwala	*Brucella* spp.
12	Human	blood	Namwala	*Brucella* spp.
positive control	S19 vaccine			*Brucella* spp.

4.3.1 PCR amplification of 16S rRNA genes

DNA was extracted from five out of the twelve suspected *Brucella* isolates. An expected 700bp PCR product was amplified in all 5 samples (Figure 10). The positive control used was the S19 *B. abortus* vaccine.

Figure 10: PCR Amplification of 16S rRNA gene in isolates from human and cattle



(L: A 100-bp DNA ladder, PC: Positive Control, S19 *B. abortus* vaccine, Samples 2, 3,8,11 &12, NC: Negative Control)

4.3.2 Real-time PCR

All five isolates were confirmed as *Brucella* spp., as shown in Table 8.

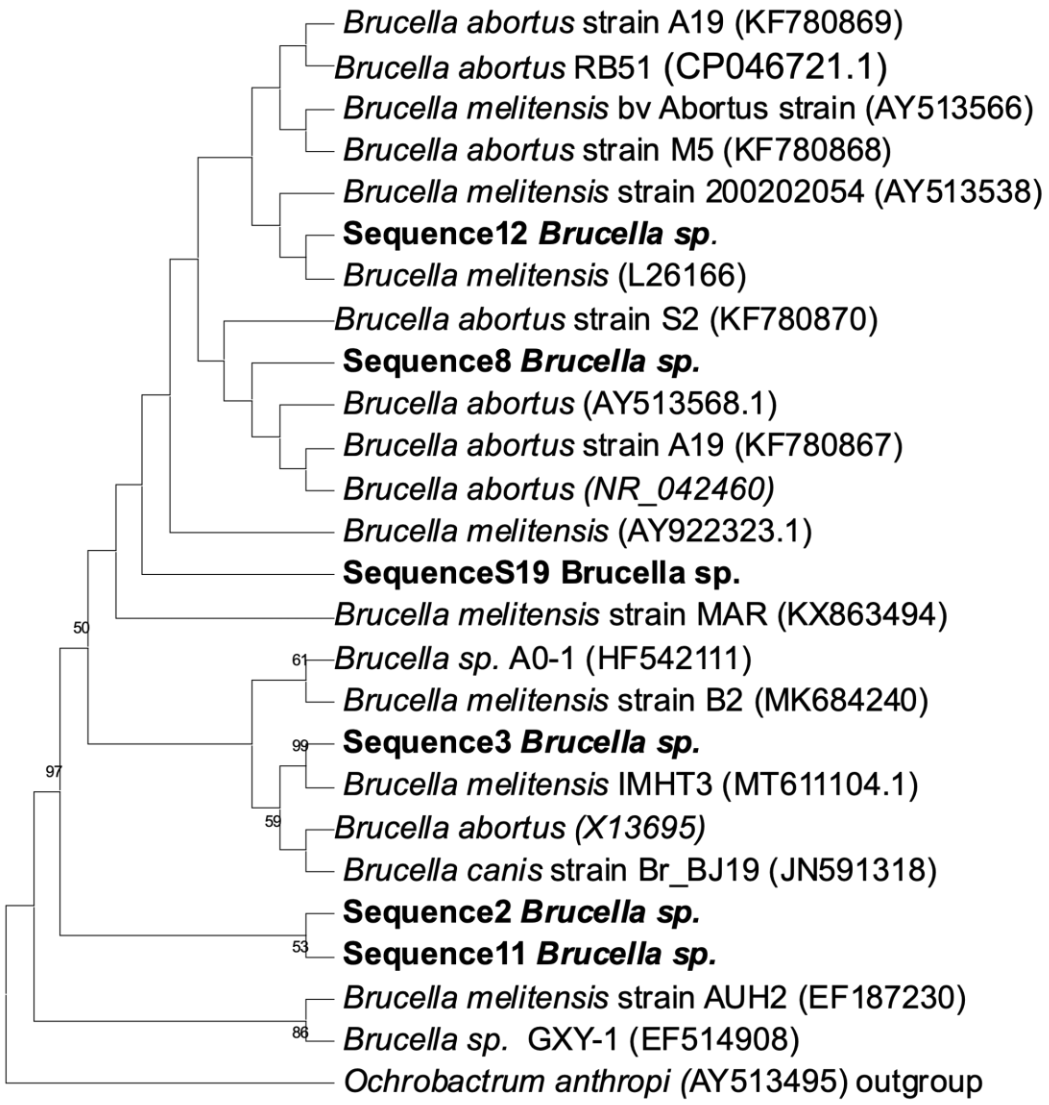
Table 8: Real-time PCR results

Sample ID	IS*711*	*bcsp*31	Interpretation
3	15.05	18.86	*Brucella* spp.
8	14.79	18.42	*Brucella* spp.
11	27.08	28.56	*Brucella* spp.
12	17.77	21.61	*Brucella* spp.
S19	14.26	18.11	*Brucella* spp.

4.3.3 16S rRNA gene sequencing and phylogeny reconstruction

The 16S rRNA partial gene sequencing was successful for all 5 samples (2, 3, 8, 11 & 12); a blastn search gave a nucleotide identity of 99 per cent and the sequences were identified as belonging to *Brucella* spp. (Figure 11).

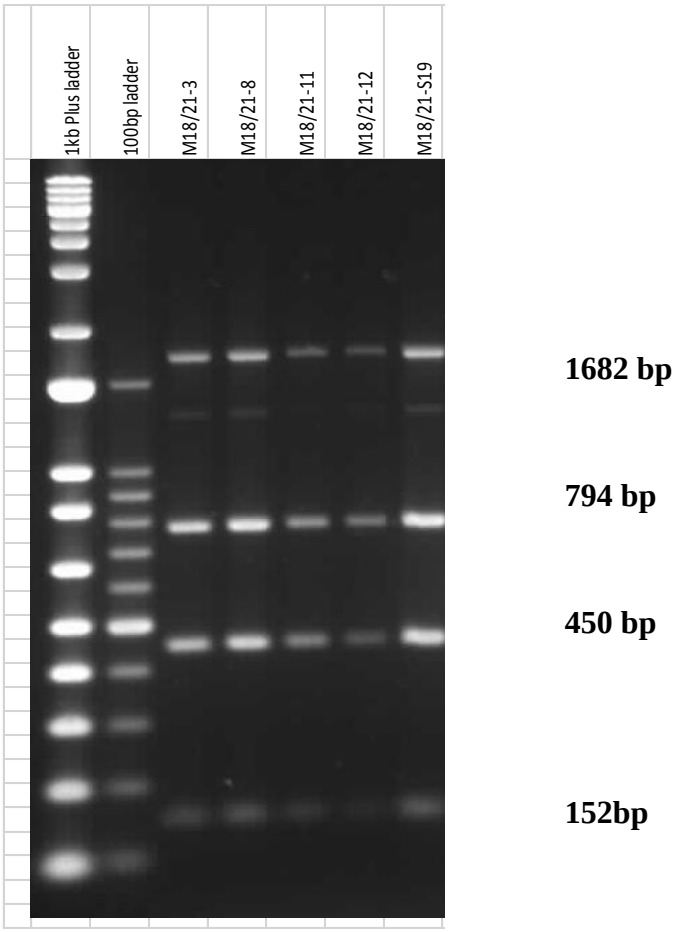
Figure 11: Phylogenetic tree analysis of 16S rRNA gene sequences of samples from Zambia in relation with other *Brucella* spp. downloaded from GenBank. *Brucella* spp. from Southern province, Zambia: Sequences 2 (human blood), 3 (human blood), 8 (cattle milk), 11(cattle milk) and 12 (human blood). *Ochrobactrum anthropi* was the outgroup organism used in this analysis



4.3.4 Bruce-ladder Multiplex PCR

All five isolates were identified as *Brucella abortus* S19 based on the presence of four (152bp, 450bp, 794bp & 1682bp) bands (Figure 12).

Figure 12: Bruce ladder multiplex PCR results for DNA from five *Brucella* isolates ***(L: 1 1kb-plus DNA ladder, Lane 2: 100bp ladder, Lanes 3, 4, 5 and 6: Samples 3, 8, 11 and 12 Lanes 7: positive control (*****B. abortus*** ***S19 vaccine)***



4.4 Antimicrobial susceptibility of *Brucella* isolates

Based on breakpoints outlined by the CLSI in 2020, all three isolates (3, 8 & 12) were resistant to trimethoprim-sulfamethoxazole, doxycycline, tetracycline and chloramphenicol but sensitive

to rifampicin. All three isolates showed intermediate resistance patterns toward ciprofloxacin (Table 9). Isolates 2 and 11 were not tested.

Table 9: Interpretation of inhibition zone (mm) of *Brucella* spp.

Antibiotic	**Concentration ug/disk**	**CLSI Breakpoints for sensitivity Range (mm)**			**Resistance pattern**		
		S	**I**	**R**	**S**	**I**	**R**
rifampicin	5 µg	> 20	17-19	<16	3	0	0
Trimethoprim-sulfamethoxazole	23.75 µg	>16	11-15	<	0	0	3
Doxycycline	30 µg	>29	26-28	<	0	0	3
Tetracycline	30 µg	>29	26-28	<	0	0	3
Ciprofloxacin	5 µg	>21	-	-	0	3	0
Streptomycin	10 µg			-	-	-	-
chloramphenicol	30 µg	>29	26-28	<25	0	0	3
Gentamicin	10 µg					-	-

S: susceptible; I: intermediate, R: resistant.

CHAPTER FIVE

DISCUSSION

The study aimed at determining the Brucella seroprevalence in cattle, isolate and identify the Brucella species circulating in humans and cattle and determine the genetic diversity of *Brucella* species circulating in humans and cattle. The study also aimed to evaluate the antimicrobial susceptibility of *Brucella* species isolated from humans and cattle.

5.1 Seroprevalence of anti-*Brucella* antibodies in cattle

The overall herd and animal seroprevalence levels were 32 per cent and 9.92 per cent, respectively. Mongu district had the highest herd-level seroprevalence (32.3%), although this was not statistically significant, while Choma district had the lowest herd seroprevalence (8.7%). At the provincial level, the difference in seroprevalence between Southern and Western provinces was very minimal which could be attributed to the similarities in livestock farming practices. The individual animal seroprevalence in this study was slightly higher than the 6 per cent reported in smallholder dairy cattle in Lusaka and Southern provinces (Muma *et al.*, 2012). Similarly, the herd seroprevalence was slightly higher than the previously reported 20.7 per cent in Southern province (Muma *et al.*, 2013) but lower than from the 46.2 to 74 per cent in the livestock andwildlife interface area of the Kafue flats among traditional cattle in Zambia (Muma et al., 2006). The odds ratio also suggested that animals in the Western province were more likely (OR = 1.37, CI: 1.0-1.87) to test *Brucella* positive compared to those from the Southern province whereas those from Namwala were more likely (OR: 8.56, 2.66-27.50) to test positive compared to those from Choma district. The observed high seropositivity at the individual

animal and herd levels could be attributed to the fact that both Southern and Western provinces have high traditional cattle populations which predominantly rely on communal grazing practices in the Kafue and Zambezi flood plains, respectively (Mumba *et al.,* 2018). The movement of cattle herds to the plains in search of greener pastures possibly results in interherd interactions and the consequent spread of infection (Sinkala *et al.,* 2014). Contact with the Kafue lechwe in the Kafue floodplains ecosystem has also been associated with *Brucella* infections (Muma *et al.,* 2007).

The slight increase observed in the herd and animal seroprevalences after a decade shows that the disease has possibly become stable over the years, thereby reaching endemic stability (Coleman *et al.,* 2001). The continuing lack of control measures for brucellosis in Zambia is evidenced by the government's priorities in disease control programs, where some livestock diseases are given more consideration (e.g., Foot & Mouth Disease, East Coast Fever & Contagious Bovine Pleuropneumonia) than others when it comes to controlling programmes (Animal Health Act, 2010). This is contributing to the unmitigated disease transmission and plateau state. The traditional cattle sector constitutes a significant proportion of the cattle production system in Zambia (Ogugua *et al.,* 2018; Sagamiko *et al.,* 2017); hence, this disease pressure is worrying due to the serious economic and public health risks it poses to pastoral communities.

Our herd seroprevalence is comparatively similar to the 29.2 per cent and 32 per cent reported in Nigeria and Tanzania, respectively (Ogugua *et al.,* 2018; Sagamiko *et al.,* 2018), but lower than the 45.9 per cent, 25.6 per cent, 40.1 per cent reported in Ethiopia, Cameroon and Angola, respectively (Asgedom *et al.,* 2016; Awah-Ndukum *et al.,* 2018; Mufinda *et al.,* 2015). In comparison to findings from studies in other countries in Southern Africa, our individual animal

seroprevalence are comparatively similar to the 9.9 per cent reported in Zimbabwe (Gomo *et al.,* 2012), higher than the 7.7 per cent in Northern Malawi (Tebug *et al.,* 2014) and 5.5 per cent in South Africa (Kolo *et al.,* 2019). The variations observed in these results may be due to several factors, including the sampling techniques and sample sizes, the different diagnostic tests and interpretations used and seasonal cattle movements in search of pastures amidst droughts.

The individual animal seropositivity and the likelihood of testing positive were higher among cattle aged 11-16 years (18.9%), female animals (10.6%), pregnant cows (14.3%) and pregnant heifers (13.3%) despite not being statistically significant. The lack of difference here seems to suggest suggests that brucella status is independent of age, pregnancy status and sex. This finding is similar to others (Madut *et al.,* 2018) and is consistent with the known relationship between age, sex and *Brucella* status. The chances of testing positive for brucellosis tend to be higher in female and older animals (Radostits *et al.,* 2000). As the animal reaches sexual maturity, the levels of growth-stimulating factors for *Brucella* organisms become high (Petersen *et al.,* 2013), while constant exposure to the *Brucella* organisms increases with age. The high number of females sampled compared to males may have also contributed to the high seropositivity observed among females. The high seropositivity among pregnant cattle can be explained by the elevated erythritol levels in the placental and fetal fluids during the third trimester (Petersen *et al.,* 2013). These high levels stimulate the growth and multiplication of the bacteria in the reproductive organs (Radostits *et al.,* 2000).

The combined use of RBT and cELISA tests serially in our study maximised the specificity of the test system while reducing labour and costs. The RBT was a cheap and easy-to-perform screening test with high sensitivity but limited specificity. To improve the specificity of the test system, only RBT-positive reactors were confirmed by the expensive and laborious but highly

sensitive and specific cELISA. Thus, as far as misclassification bias is concerned, the seroprevalence estimates in our study may be assumed to be unbiased.

5.2 Brucella isolation from cattle and humans

The identification and characterization of *Brucella spp.* isolates from cattle and humans were carried out. *Brucella* spp. were detected in two cattle milk samples. These findings are not in line with those from Tanzania (Ntirandekura *et al.,* 2020), Uganda (Hoffman *et al.,* 2016), Zimbabwe (Matope *et al.,* 2009) and Turkey (Arasoğlu *et al.,* 2013), where *B. abortus* wild-type was isolated from bovine milk samples. We isolated a *Brucella* strain with the same phenotypic characteristics as the *Brucella* vaccine strain S19. We isolated the same strain from human blood samples. *Brucella* spp. has also been isolated from human blood samples from milkers in Sudan (Osman *et al.,* 2015). Unpasteurised milk is commonly marketed and sold in informal markets in our study area and other parts of Zambia; hence, cases of human brucellosis are expected to be high. The low isolation rate of *Brucella* from human blood and cattle vaginal swabs and hygroma fluids can be due to several factors such as the use of transport media, the timing of sample collection and the low concentration of organisms in these samples as the infection progresses with time and the culturing method used. For human blood, the lysis centrifugation method has reduced detection time compared to other methods (Yagupsky *et al.,* 2019). It is also important to note that bacteraemia mainly occurs intermittently. The isolation rate could have been increased by sampling individuals with fever, especially those with acute febrile illness. According to OIE (2019), vaginal swabs should be collected shortly after abortion or calving to increase the recovery rate of the organisms.

5.3 Brucella genus and species identification and Phylogenetic analysis

Using 16S rRNA, a 700bp PCR product was amplified and sequenced in all 5 isolates, a blastn search gave a nucleotide identity of 99 per cent and the sequences were identified as belonging to *Brucella* spp. The only 16S rRNA gene sequences we could find for *Brucella* spp. from the Southern and Eastern regions of Africa was the recent one from Tanzania (Ntirandekura *et al.,* 2020). Real-time PCR targeting IS*711* and *bscp31* genes further confirmed that our isolates are *Brucella* spp. This report has to be compared to reports in Zimbabwe (Ledwaba *et al.,* 2019; Matope *et al.,* 2009), South Africa (Kolo *et al.,* 2018), and Tanzania (Mathew *et al.,* 2015) and Uganda (Hoffman *et al.,* 2016). It is important to note that sequencing of the 16S rRNA PCR does not allow the identification of *Brucella* strains to species level. The phylogenic tree construction merely showed the relationship between *Brucella* spp. from our study areas and those deposited in the Genbank.

Bruce Ladder multiplex PCR identified all five isolates as *Brucella abortus* S19 based on the absence of the distinct 587bp band and the presence of four (152bp, 450bp, 794bp & 1682bp) bands. These findings agree with those from Sudan (Osman *et al.,* 2015), where *Brucella abortus* S19 strain was reported in livestock and occupationally exposed individuals (milkers) in bovine milk and vaginal swabs in Turkey (Çelebi & Otlu, 2011)and South Africa (Ledwaba *et al.,* 2021). In contrast, studies from Uganda (Mugizi *et al.,* 2015) and Zimbabwe (Ledwaba *et al.,* 2019) have reported *B. abortus*, *B. ovis* and *B. suis*, respectively.

On the basis of the current study, the origin of the *B. abortus* S19 strain could not be ascertained due to insufficient data. However, it should be noted that the *B. abortus* S19 vaccine has been used widely in Zambia to control bovine brucellosis. Locally, the *B. abortus* S19 vaccine has

been produced by CVRI, has since stopped producing it, but private players were also licensed to import and use the same vaccine in the country. Routine vaccination against brucellosis is not mandatory in Zambia; a few farmers who can afford to buy the vaccines do so and vaccinate their animals. Indeed, *B. abort*us wild-type strains are more likely to circulate (and hence, be isolated) in the cattle population than the S19 strain, even more so if vaccination has been discontinued for some years, as is the case in Zambia. There are profound public health implications of the *B. abortus* S19 vaccine finding in our study, and therefore, our results need to be analysed cautiously. S19 is a live attenuated vaccine strain that can cause disease in humans through; close contact with vaccinated animals, accidental exposure of the veterinarian, farmer or animal personnel to the S19 vaccine during animal vaccination or consumption of milk from a vaccinated animal (Osman *et al.,* 2015). This further raises safety concerns when handling and administering the vaccine. In the case where the S19 vaccine is administered in cattle, it can be excreted in the vaccinated cow's milk at low frequencies but not for more than a year (Çelebi & Otlu, 2011). It can also be present in cows that are vaccinated at later stages than the recommended stages or those given low doses; hence, they abort and shed the vaccine in milk. According to the OIE, the the S19 vaccine is usually given to calves at 3-6 months. Therefore, the strains we isolated must be further characterized molecularly in order to be able to exclude laboratory contamination used S19 as a positive control in the tests), and further investigations have to be conducted to validate our findings. This can include sampling the rest of the herd from where the vaccine isolates were obtained and nearby herds. Furthermore, serological tests and bacterial cultures from slaughtered animals in these herds will trace the source of the *B. abortus* S19 identified in this study.

In humans, brucellosis is directly associated with infection in animals. A publication by Mubanga et al. (2021), which was part of our research project, documented an estimated human seroprevalence of 20.26 per cent among herdsmen and abattoir workers in the Southern province. These findings are similar to our cattle seropositivity findings. This shows that the infected animals in our study area are the source of human brucellosis. The cultural practice of consuming raw milk and unpasteurized milk products is dominant in the study areas which is a major risk factor for human brucellosis (Godfroid *et al.*, 2011). Many human cases may continue to be underreported, misdiagnosed and mistreated for other similar febrile diseases like Malaria (Njeru *et al.*, 2016). This can adversely affect the health outcomes of individuals. Brucellosis screening is not part of routine diagnosis in health centres among Zambian pastoral communities. A one health approach can play a significant role in controlling brucellosis in Zambia.

In cases where animals and patients are seronegative and asymptomatic, detection of *Brucella* DNA is instrumental in the definitive diagnosis and control. The molecular assays used in our study (16S rRNA and Bruce ladder PCR) are simple, rapid and more affordable for researchers in developing countries. These assays can differentiate between *Brucella* spp. to genus and species levels, thereby complementing the limitations of the bacteriological methods.

5.4 Antimicrobial susceptibility of *Brucella* isolates

Studies on antimicrobial susceptibility testing for the *Brucella* genus are scarce, and most involve using the E-test compared to the disk diffusion method. All three *Brucella* isolates were resistant to trimethoprim-sulfamethoxazole, similar to findings by Morales-Estradaa *et al.* (2016) in Mexico. The three isolates were also resistant to doxycycline, tetracycline and

chloramphenicol. All the 3 isolates were sensitive to rifampicin. This finding is contrary to those by Pauletti *et al.* (2015), Shevtsov *et al.* (2017) and Wareth *et al.* (2021) in Brazil, Kazakhstan and Egypt, respectively. It must be noted that in our study, we used the disk diffusion method which is simple, easy to use, and does not need special equipment (Jorgensen & Ferraro, 2009). The disk diffusion method was also used in the Egypt study in contrast to the E-test method used in Brazil and Kazakhstan studies. The study by Wareth *et al.* (2021) in Egypt, using the broth microdilution and disk diffusion methods, reported probable resistance to rifampicin and azithromycin of clinical and non-clinical *Brucella* strains. There are more advanced AST methods that can be used, such as the Vitek 2 system (BioMerieux), which is highly automated and use compact plastic reagent cards that have antibiotics and test media in a 64-well format. However, these may be too expensive for researchers in developing countries.

The antimicrobial resistance pattern observed among the *Brucella* isolates can firstly be attributed to possible misuse of antimicrobials by individuals who wrongfully self-diagnose and treat themselves. Secondly, the continued absence of routine diagnostic tests for brucellosis in hospitals and health centres in Zambia can lead to increased misdiagnosis cases and inappropriate treatment. Most of the antimicrobials used to treat human brucellosis are also used to treat other infections. This increases the risk of the development of antimicrobial-resistant *Brucella* strains.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The overall brucellosis seroprevalence rates at the individual animal and herd levels were 9.92 per cent and 32 per cent respectively. *Brucella* species are circulating in human and bovine milk in the Southern province of Zambia. Molecular typing of the isolated *Brucella* spp. DNA indicates that they belong to the *Brucella abortus* S19 vaccine strain. While vaccination is the traditional and recommended method for controlling brucellosis, the current study findings suggest that the S19 vaccine that we were using continues to be detected not only in animals and animal products but also in humans long after it has been used in animals. Pending confirmation of our results, this underscores it is zoonotic transmission potential from cattle to humans, which seems to be a public health problem.

Recommendations

1. Further studies are required to re-evaluate the use of vaccines for the control of bovine brucellosis.
2. Further studies are recommended to determine the public health safety of using the *B. abortus* S19 vaccine in cattle.
3. Further epidemiological and molecular analyses are needed to validate our results. Additional molecular analysis using WGS and multi-locus VNTR analysis (MLVA) is needed to provide further confirmation and enable comparison with previously published data.

4. WGS-AST analysis is also recommended for *Brucella* isolates to ascertain their antimicrobial susceptibility status.
5. Further studies are needed to investigate the presence of *Brucella* in different livestock species in Zambia and to determine the prevalence of brucellosis in different risk groups and hospitalized febrile patients.
6. There is a need for knowledge, attitude and practices (KAP) analysis among occupationally exposed groups, which can help formulate tailor-made public health education programs on brucellosis prevention.
7. Government should consider introducing rapid and simple point-of-care tests such as the *Brucella* IgM/IgG lateral flow assay to provide medical services to hard-to-reach rural populations. This will improve early case detection and correct treatment.
8. There is a need to implement the One Health surveillance and control strategies to minimise the disease burden in animals and thus prevent it in humans.

Study implications

Based on our study findings, concerning the Government should reconsider the animal health policy concerning the current non-mandatory stance on brucellosis vaccinations and the lack of routine rapid diagnostic tests (RDTs) for human brucellosis in health centres. Furthermore, caution must be taken in using S19 vaccines in cattle and the vaccination schedule should be revised in the disease control strategies. The AST findings will further shed more light on antimicrobial stewardship in the public health system in Zambia.

Study limitations

The study only focused on five districts in the Southern and Western provinces of Zambia, which were purposively sampled hence affecting the generalisation of our results. We had challenges during human and animal sampling due to the outbreak of FMD in the study areas in the Southern province. Human sampling in the Western province was not done at the time of animal sampling due to financial limitations. Our study had a low rate of *Brucella* isolation. This can be attributed to the *Brucella* organism being a fastidious and slow grower that requires certain conditions to grow.

Misclassification bias in our seroprevalence estimates was minimised by serially using RBT and cELISA tests. Our results related to the *Brucella* strains we have isolated need to be further validated to exclude possible laboratory contamination and make epidemiological solid inferences.

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APPENDICES

Appendix 4

Information Sheet and Informed Consent Form

INFORMATION AND CONSENT FORM FOR FARMERS, ABATTOIR WORKERS, HERDSMEN AND BUTCHERED

RESEARCH TITLE: Characterization of human and bovine *brucella* species in Southern and Western provinces of Zambia

This Informed consent form has two parts:

- Information Sheet (to share information about the research with you)
- Certificate of consent (for signatures if you agree to take part).

You will be given a copy of the full Informed consent form.

PART I: INFORMATION SHEET

Introduction

This is a study being done by Dr Ruth L. Mfune, who is currently pursuing her PhD programme, and Prof John B. Muma (Principal supervisor) of the University of Zambia, School of Veterinary Medicine. The study aims to identify the *Brucella* species that are dominant in humans and cattle in the Southern and Western provinces of Zambia.

We are doing research on brucellosis, a bacterial disease that affects cattle and can be transmitted to humans through the handling of infected animals and drinking untreated milk. You have been selected to take part in this study because you fit our study selection criteria and/or you are the owner or duly authorized agent for the owner of the selected cattle. Your

participation and/or that of your cattle herd is completely voluntary. Before agreeing to take part in the study, we request you to read this form, or we will go through it with you. The form describes the purpose of the study, the risks, benefits and alternatives to participating in the study, **and you are free to ask any questions.**

When all your questions have been answered, you can decide to either participate in the research or not. You can talk to anyone you feel comfortable with about the research before you make the final decision.

Purpose of the research

The aim of this laboratory-based study is to describe the *Brucella* bacteria to species level in humans and cattle and determine their genetic features. We want to know if the *Brucella* species that we will find in cattle are related to those in humans or not. The study will also investigate whether the isolated *Brucella* are susceptible to the commonly used antimicrobials in humans and cattle in the Southern and Western provinces of Zambia. **The selected participants for this study are livestock farmers, abattoir workers, herdsmen and butchered.**

Procedures

If you fit our selection criteria and volunteer to participate in the study, you will be requested to bring your cattle herd to a central place for sampling. We will request to collect samples from some of your cattle that fit our selection criteria. These will be 5ml of blood, 20ml of milk, vaginal swabs, foetal tissues from cows that have aborted and 10ml of fluids from swollen knee joints**. If any of your cattle test positive for *Brucella,* or you don't keep cattle but still fit the selection criteria (abattoir workers, herdsmen, butchermen)**, you will be asked to provide us with 10ml of blood to screen for brucellosis. This will be done after your consent to take part and private counselling from the health personnel. The samples will be processed and screened for

brucellosis and molecular analysis. No other samples will be required from you or your animals for this study. The processed sample from you and your animals will be used only for the purposes explained to you in this form. They will be kept for the entire period of the study and as long as they are still available.

Will my participation in this study be kept confidential?

We will do our best to keep your personal information and other information pertaining to your animal and cattle herd confidential. To help protect your confidentiality, your name or other details that identify you will not appear with the information pertaining to the results of our tests. We will not put names on any information collected from you. Instead, we will use serial numbers for identification. Only authorised research staff will have access to information bearing your name. The number will not be shared with or given to anyone except the medical doctor in charge in case you are found with the disease under study. We would like to assure you that the information you share with us will not be shared with anyone outside the research team and will be kept confidential.

What are the risks of this research?

There will be slight discomfort that your animals may feel during blood sample collection. However, this is a routine process in veterinary practice, and registered veterinary personnel will do the blood collection. The research team will do everything possible to minimise any possible discomfort or harm to your animal. **As a participant in this study, some of the risks that you may encounter as we collect blood from you include temporary discomfort from the needle stick, bruising and infection. In order to minimise these risks, experienced health personnel from the health center will carry out the blood collection. We will use sterile gloves, needles**

and syringes to avoid the risk of infections. The health personnel in our research team will treat any bruises or infections you will encounter during the procedure.

What are the benefits of this research?

The direct benefit of this research to you is that if you test positive for the disease, you will be offered treatment at the health centres. By allowing your cattle herd to participate in this study, you provide the opportunity to gain more understanding of the burden of the disease in your district and the *Brucella* species circulating in humans and cattle. This information will be passed on to Ministries of Health and Livestock, who will implement the appropriate treatment and disease control strategies in humans and animals, respectively. This will benefit the local people and improve livestock production.

Voluntary participation

Your participation in this research is voluntary. You have the right to choose whether or not to participate in the study. This will not affect your rights as a cattle owner. You will remain entitled to the same veterinary services as other livestock owners and still receive all the services offered at the health centre. In case you change your mind, you are free to leave the study even if you had agreed earlier.

Incentives and/or Compensation

There will be no incentives or money given to anyone for taking part in this study. However, by participating in the study, you will be treated if found with the disease, and you will also contribute to information concerning the disease.

Dissemination of results

We would like to share the genetic information on *Brucella* obtained from your blood sample and that of your animals with other researchers around the world at the end of the study. The

benefit is that many researchers can use the same information for different research projects. If you participate in this study, you will also need to agree to share your cattle's genetic information with other researchers in the future. We will publish the results of this study in journals and might present them in seminars.

Right to refuse or to withdraw

You are free to decide whether you want to take part in this study or not, to leave at any point during the interview. You can also decline to answer any questions that you feel are not comfortable. You are also free to withhold your blood specimen from us. This will not bring any problem to you as it is your choice, and all your rights will be respected.

Who to contact?

Dr Ruth L. Mfune and Prof. John B. Muma (Principal supervisor) of the University of Zambia, School of Veterinary Medicine, are conducting this research. Should you have any questions regarding this study and your rights as a research participant or if you wish to report any problems you have experienced related to the study, please contact:

Dr Ruth L. Mfune

Department of Disease Control,

School of Veterinary Medicine,

The University of Zambia,

PO Box 32379, Lusaka.

Mobile: 0976 723 234/0953 772 375

Email: lindizyani@gmail.com

Prof. John Bwalya Muma (Principal supervisor)

Department of Disease Control,

School of Veterinary Medicine,

The University of Zambia,

P.O BOX 32379, LUSAKA.

Tel: +260-211-293727 Email: jbwalya@lycos.com

ERES CONVERGE IRB

33 Joseph Mwilwa Road

Rhodes Park, LUSAKA

Tel: 0955 155633/4

E-mail: eresconverge@yahoo.co.uk

If you have questions about your rights as a study participant or feel that you have not been treated fairly or have other concerns, you can contact the ERES CONVERGE IRB office using the contact number +260955155633/4.

PART II: CERTIFICATE OF CONSENT

I have read and understood the information that has been presented to me both in vernacular and English languages. I have had all my questions answered to my satisfaction. I have been asked to participate in the above study by presenting myself and my cattle herd for sampling and hereby give free consent by signing this form. My consent to participate is voluntary, and I may withdraw from the study at any time. I am further aware that the information that will be obtained from me, including my animals, will be treated in confidence, and I will not be personally identified. I also understand that some risks always exist when animal handling and procedures are performed.

Signature or Participant _____

Date _____

Day/month/year

If illiterate

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the chance to ask questions. I confirm that the individual has given consent freely.

Signature of witness _____ AND Thumb print of participant

Date _____

Day/month/year

Statement by the researcher or person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability, making sure that the participant understands that the following will be done:

1. Blood samples will be collected from the participant.
2. If I have livestock, blood, milk, vaginal swabs, fetal tissues and hygroma samples will be collected from the participant's cattle.

I confirm that the participant was given a chance to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this Informed Consent Form has been provided to the participant.

Name of Researcher/ person taking the consent _____

Signature of Researcher/ Person taking the consent _____

Date _____

Day/month/year

INFORMED CONSENT FORM FOR STUDY PARTICIPATION (FOR PARENT OR LEGAL GUARDIAN)

TITLE OF STUDY: Characterization of *Brucella* species in human and cattle in Southern and Western provinces of Zambia

Principal Investigator: Dr Ruth L. Mfune

Co-Principal Investigator: Prof. John B. Muma

We are conducting a study on human and bovine *brucella* species in the Southern and Western provinces of Zambia.

We are conducting a study on the characterisation of human and bovine *brucella* species in the Southern and Western provinces of Zambia.

You child or dependant has been selected as one of the participants in our study and we would like to ask you for some of your time to explain the work that we are doing and to request for your permission to allow him or her to participate in the study. As we discuss the information below, please feel free to ask any questions.

Brief description of the Study

Many diseases in humans are caused by bacteria and are transmitted from animals to humans through handling infected animals or animal products. We are doing research on brucellosis, a bacterial disease that affects cattle and can be transmitted to humans through the handling of infected animals and drinking untreated milk.

The aim of this laboratory-based study is to describe the *Brucella* bacteria to species level in humans and cattle and determine their genetic features. We want to know if the *Brucella* species that we will find in cattle are related to those in humans or not. The study will also investigate

whether the isolated *Brucella* are susceptible to the commonly used antimicrobials in humans and cattle in the Southern and Western provinces of Zambia. **The selected participants for this study are livestock farmers, abattoir workers, herdsmen and butchered.**

The information gained from this study will be used to know if the type of *Brucella* causing disease in animals is the same as that in humans. This will help us design intervention programs, eventually improving food safety and human health.

If you agree to take part in this study, the only thing that we will request from your child is to provide us with 10ml of blood. This will be done after your parental consent and the child's consent to take part in the study. This will be done by medical personnel from the health center. The blood samples will be processed and screened for brucellosis and molecular analysis. No other samples will be required from him or her. The processed sample will only be used for the purposes explained to you in this form. They will be kept for the entire period of the study and as long as they are still available. That will be used for laboratory analysis.

If your child does not join the study, he or she will continue to receive all the health care services. You may choose to leave the study at any time you like without penalty.

This exercise will take about 15-20 minutes. There should be no risks to you or your child if you agree to take part in the study.

Your child will not directly benefit from this study. His or her treatment in the hospital will not change if you agree that he or she may take part in the study. The information we collect will be used to try to develop intervention measures against the brucellosis disease among people who handle animals.

Once we have completed the testing for this study, the samples will be stored in a secure deep freezer at the Disease Control Department in the School of Veterinary Medicine at University of Zambia. The sample will not have a name on it, so that it cannot be linked to you or your child.

If you have questions, complaints, or problems as a result of participating in this study, you may call Ruth L. Mfune, Department of Disease Control, School of Veterinary Medicine, The University of Zambia (+26 0976 723 234 or lindizyania@gmail.com). All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject, you may contact, anonymously if you wish, ERES CONVERGE IRB office at 33 Joseph Mwilwa Road in Rhodes Park, Lusaka. The ERES CONVERGE IRB office contact numbers are +260955155633/4 4, and the email address is eresconverge@yahoo.co.uk.

What does your signature (or thumbprint/mark) on this consent form mean?

Your signature (**or thumbprint/mark)** on this form means:

- You have been informed about this study's purpose, procedures, possible benefits and risks.
- You have been given a chance to ask questions before you sign.
- You have voluntarily agreed to participate in this study.

Please indicate Yes or No

- I agree to allow my child to take part in this study ☐Yes ☐No
- I agree to allow my child's blood samples to be used in this study ☐Yes ☐No

Name of parent or legal guardian: _____

_____ _____

Signature of parent or legal guardian or Thumb print Date

In signing here, I agree that I have read and understood the agreement/consent form and agree to my child to participate in the study.

Name of Witness: _____

_____ _____

Signature of Witness Date

_____ _____

Signature of the recipient of form Date

_____ _____

Signature of Witness Date

The signature of the witness above means that another person has observed the consenting of the parent or legal guardian. The witness must be impartial and not part of the study staff.

Appendix 5

ERES Converge Ethical Approval Letter

ERES CONVERGE

33 Joseph Mwilwa Road
Rhodes Park, Lusaka
Tel: +260 955 155 633
+260 955 155 634
Cell: +260 966 765 503
Email: eresconverge@yahoo.co.uk

I.R.B. No. 00005948
F.W.A. No. 00011697

28th January, 2019

Ref. No. 2018-Dec-004

The Principal Investigator
Dr. Ruth L. Mfune
The University of Zambia
School of Veterinary Medicine
Dept. of Disease Control
P.O. Box 32379,
LUSAKA.

Dear Dr. Mfune,

RE: CHARACTERIZATION OF HUMAN AND BOVINE BRUCELLA SPECIES IN SOUTHERN AND WESTERN PROVINCES OF ZAMBIA.

Reference is made to your corrections dated 23rd January, 2019. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2018-Dec-004**
Approval and Expiry Date	Approval Date: 28th January, 2019	Expiry Date: 27th January, 2020
Protocol Version and Date	Version - Nil.	27th January, 2020
Information Sheet, Consent Forms and Dates	• English.	27th January, 2020
Consent form ID and Date	Version - Nil	27th January, 2020
Recruitment Materials	Nil	27th January, 2020
Other Study Documents	Data Collection Forms.	27th January, 2020
Number of participants approved for study	-	27th January, 2020

Appendix 6

NHRA Approval Letter

THE NATIONAL HEALTH RESEARCH AUTHORITY
Paediatric Centre of Excellence
University Teaching Hospital
P.O Box 30075
LUSAKA

Telephone: +260 211 250309 | Mobile: +260 95 5632726

4th March, 2019

Dr. Ruth L. Mfune
University of Zambia
School of Veterinary Medicine
P.O Box 32379,
Lusaka, Zambia

Re: Request for Authority to Conduct Research

The National Health Research Ethics Board (NHREB) is in receipt of your request for authority to conduct research titled **"Characterization of Human and Bovine Brucella species in Southern and Western provinces of Zambia"**.

I wish to inform you that following submission of your request to the Board, the review of the same and in view of the ethical clearance, the Board has no objection to the study on condition that:

1. **A Material Transfer Agreement is obtained and cleared by the National Health Research Ethics Board should there be any need for samples to be sent outside the country for analysis.**
2. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
3. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
4. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
5. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents.

Yours sincerely,

Prof. Patrick Musonda
Chairperson
National Health Research Ethics Board

All correspondences should be addressed to the Director and Chief Executive Officer