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Brucellosis at a livestock/human/wildlife interface in South Africa understanding the epidemiology and control

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**Brucellosis at a livestock / human / wildlife interface in South Africa:
understanding the epidemiology and control**

**La brucellose à l'interface entre l'homme et l'animal domestique et sauvage en
Afrique du Sud: comprendre l'épidémiologie et le contrôle**

Dissertation présentée par
Gregory Simpson
en vue de l'obtention du grade
de Docteur en Sciences

2018

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Brucellosis at a livestock / human / wildlife interface in South Africa: understanding the epidemiology and control.

by Gregory Simpson

Summary

Brucellosis is seen as a neglected zoonosis that affects a wide variety of species. This research focuses on a South African “One Health” setting where there are wildlife, domestic animals and humans existing in close proximity. There are five studies to this research, the first is a systematic review of brucellosis in wildlife in Africa, which revealed that evidence for *Brucella* infection was found serologically in sixty-one wildlife species and identified the *Brucella* species (*Brucella abortus*, *Brucella melitensis*, *Brucella suis* and *Brucella inopinata*).

This research then took an in-depth look into the epidemiology of the *Brucella* infection at a human, domestic animal and wildlife interface in Eastern South Africa in a site that we saw as an example of a communal livestock ranching setting in the proximity of wildlife in Africa. Besides *B. abortus* infection, we identified *B. melitensis* infection in buffalo (*Syncerus caffer*) in the Kruger National Park. Yet, we found low serological evidence of infection cattle and goats and none in dogs and humans. The goats were deemed to not be infected by confirmatory tests (indirect enzyme-linked immunosorbent assay (iELISA) and brucellin skin test). The dogs were all classified negative by the RBT. The cattle were found to have a low serological prevalence (1.4%), decreasing serology with age and significantly less males positive than females, which is not indicative of a wildtype *B. abortus* infection. This indicated the serological positives could be due to residual vaccine antibodies, which was supported by our inability to identify the organism when doing further focused testing with serology, brucellin skin test and culture.

This led us to conduct a longitudinal cohort study to investigate the immune response to the state regulated “high dose” Strain 19 vaccination of heifers between 4 – 8 months.

We found a comprehensive and rapid serological response that peaked at 2 weeks for Rose-Bengal test (RBT) and 10 weeks for indirect Enzyme-Linked Immunosorbent Assay (iELISA). The response declined more sharply with RBT compared to iELISA (5% RBT positive and 17% iELISA positive between 10 and 14 months after vaccination). We found evidence of an immunological response four and a half years after vaccination. This serological persistence can, as hypothesized, interfere with disease control methods using serology for identification of infected animals. To reduce the risk of cross-reaction with disease surveillance and given the low incidence observed, a more appropriate vaccination method for this setting maybe a “low dose” or a subconjunctival means of administration, although it could be challenging to restrain heifers sufficiently in this setting for this type of administration.

These research outcomes are of significant relevance to disease control authorities trying to focus limited resources on brucellosis control in similar settings. The infected wildlife in close proximity is a risk to both humans and domestic animals. The identification of *B. melitensis* for the first time in buffalo in Africa is of significance as this suggests a spillover from a reservoir species, most likely small stock species, although it could have been from another unknown infected wildlife species. Yet, we found that the domestic animals and therefore their products at this site were free of *Brucella* infection and thus currently not a risk to the humans. Our research showed the effectivity of the vaccination program in eliciting an immune response in this African “One Health” setting, suggesting that the induced herd immunity would protect animals in the case of the introduction of a wild type *B. abortus* strain.

Future research in these settings should study the epidemiology of brucellosis in wildlife further, ideally with genetic testing of the bacteria to identify the sources and transmission of *Brucella* spp. between species, and other vaccination protocols in cattle that result in less serological persistence and therefore interfere less with herd disease categorisation.

Articles Information

The following chapters of the results have been published:

Chapter 3

Documenting the absence of brucellosis in cattle, goats and dogs in a “One Health” interface in the Mnisi community, Limpopo, South Africa

Gregory Simpson, Tanguy Marcotty, Elodie Rouille, Nelson Matekwe, Jean-Jacques Letesson, Jacques Godfroid: *Documenting the absence of brucellosis in cattle, goats and dogs in a “One Health” interface in the Mnisi community, Limpopo, South Africa*. Tropical Animal Health and Production 12/2017; 50(4)., DOI:10.1007/s11250-017-1495-1

Chapter 4

Investigation of brucellosis in humans and human risk factors at a human-wildlife-livestock interface in Mpumalanga Province, South Africa

Gregory J.G. Simpson, Vanessa Quan, John Frean, Darryn L. Knobel, Jennifer Rossouw, Jacqueline Weyer, Tanguy Marcotty, Jacques Godfroid, Lucille H. Blumberg: *Prevalence of Selected Zoonotic Diseases and Risk Factors at a Human-Wildlife-Livestock Interface in Mpumalanga Province, South Africa*. Vector Borne and Zoonotic Diseases 04/2018;, DOI:10.1089/vbz.2017.2158

Chapter 5

Immunological response to *Brucella abortus* strain 19 vaccination of cattle in a communal area in South Africa

Gregory J.G. Simpson, Tanguy Marcotty, Elodie Rouille, Abel Chilundo, Jean-Jacques Letesson, Jacques Godfroid: *Immunological response to Brucella abortus strain 19 vaccination of cattle in a communal area in South Africa*. Journal of the South African Veterinary Association 03/2018; 3(1)., DOI:10.4102/jsava.v89i0.1527

The following published article is in the appendix as it gives background information to the study:

Amanda M. Berrian, Jacques van Rooyen, Beatriz Martínez-López, Darryn Knobel, Gregory J.G. Simpson, Michael S. Wilkes, Patricia A. Conrad: *One Health profile of a community at the wildlife-domestic animal interface, Mpumalanga, South Africa*. Preventive Veterinary Medicine 08/2016; 130:119-128., DOI:10.1016/j.prevetmed.2016.06.007
See appendix I.

The following chapter of the results has been submitted:

Chapter 1

Brucellosis in wildlife in Africa: a systematic review and meta-analysis

The following chapter of the results is ready to be submitted:

Chapter 2

Isolation and identification of *Brucella melitensis* in buffalos in the Kruger national park

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ABBREVIATIONS

AFI	Acute febrile illness
AT	Agglutination test
BAT	Buffered <i>Brucella</i> antigen test
BPAT	Buffered plate agglutination test
CA	Contagious abortion
cELISA	complement Enzyme-linked immunosorbent assay
CFT	Complement fixation test
CFU	Colony forming units
CI	Confidence interval
DNA	Deoxyribonucleic acid
FPA	Fluorescence polarisation assay
FPSR	False positive serological reaction
GLTFCA	Greater Limpopo transfrontier conservation area
HHWRS	Hans Hoheisen wildlife research station
HIV	Human immunodeficiency virus
iELISA	indirect Enzyme-linked immunosorbent assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IGRA	Interferon gamma release assay
IQR	Interquartile range
KNP	Kruger national park
LMIC	Low to middle income countries
LNN	Lymph node
LPS	Lipopolysaccharide
MCP	Mnisi Community Program
MLST	Multilocus sequence typing
MLVA	Multiple loci variable number of tandem repeats analysis
MRT	Milk ring test

MVS	Mpumalanga veterinary services
MZN	Modified Ziehl Nielsen
OBP	Oderstepoort Biological Products
OPS	O specific polysaccharide
OR	Odds ratio
OVI	Onderstepoort Veterinary Institute
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RBT	Rose bengal test
S19	Strain 19
SANP	South African national parks
SAT	Serum agglutination test
SLPS	smooth lipopolysaccharides
ST	Brucellin skin test
SVL	Skukuza Veterinary Laboratory
TFCA	Transfrontier conservation area

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INTRODUCTION

This manuscript represents a body of work on the epidemiology and control of brucellosis at a wildlife/livestock/human interface setting in a communal pastoral farming area in southern Africa. This is an example of a South African “One Health” setting, where humans, domestic animals and wildlife interact directly and indirectly and exist in close proximity.

This introduction gives the background to the disease and the context relevant to the setting. In low and middle income countries (LMIC), with limited resources, control of the disease can be harder to achieve than upper income countries and greater awareness of its presence and the workings of cost effective control mechanisms will benefit brucellosis prevention, treatment and control in these resource limited settings.

In the body of this thesis a systematic review and meta-analysis then collates the published information on brucellosis in wildlife in Africa. The work then investigates the disease in domestic animals, humans and wildlife at the study site. These investigations use a variety of diagnostic techniques to ascertain the absence or presence and identification of *Brucella* spp.

We then investigate the immunological response to the vaccine at the study site that is used in heifers according to government regulations. This response has a confounding effect on using serology for disease diagnosis and control. Thereafter the conclusions and insights from all this work are discussed.

Aetiological agents of brucellosis

In 1860 Dr J.A. Maraston in Malta gave the first accurate description of brucellosis in humans calling it “Mediterranean gastric remittent fever”. In 1886 Dr David Bruce identified the responsible organism that was later to be called *Brucella melitensis*. Nineteen years later Dr Themistocles Zammit identified goats as the source of *B.*

melitensis. Brucellosis caused by bacteria of *Brucella* spp. is currently the commonest anthroponosis worldwide (Ariza et al. 2007) with significant economic, public health and veterinary importance (Pappas 2010). Since its identification over 120 years ago in humans it has been isolated in wide variety of animals and found to have a global distribution (Figure 1).

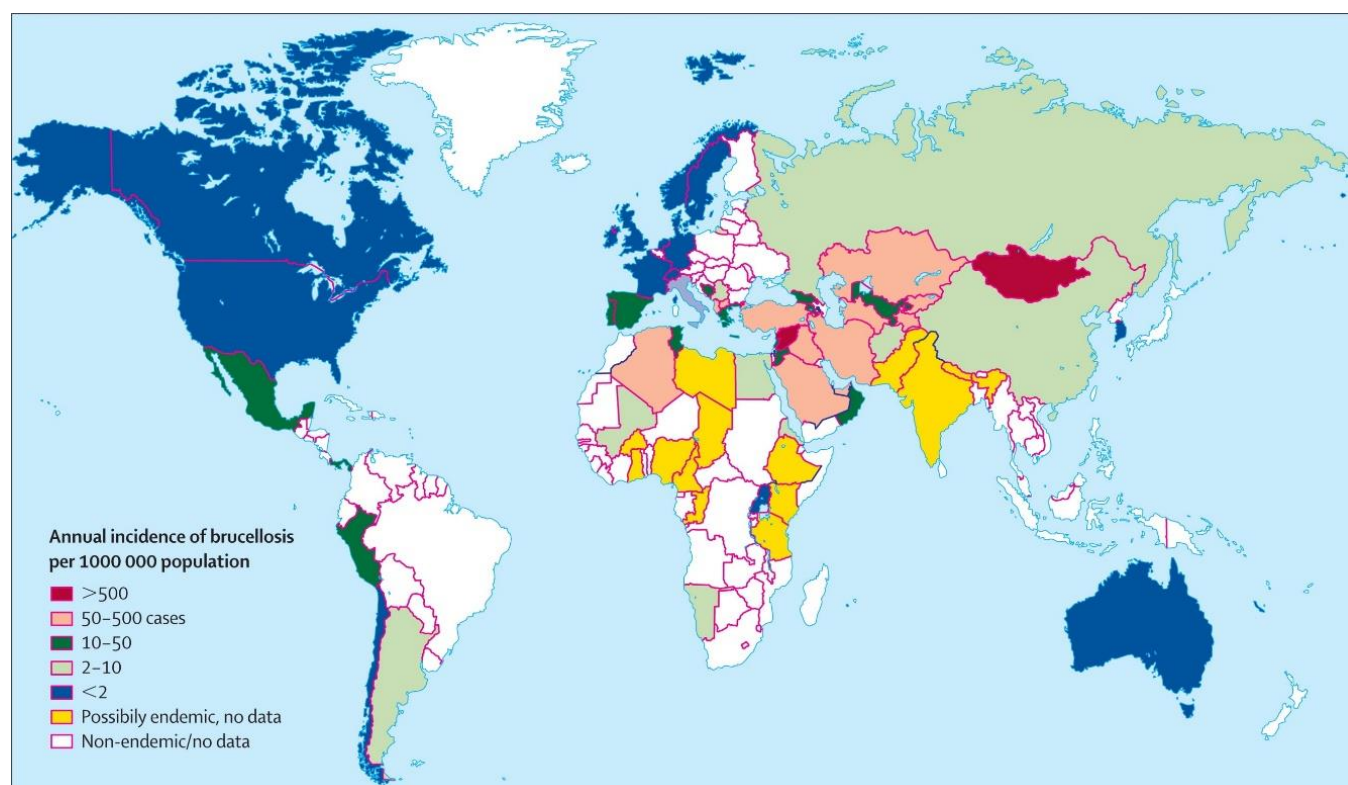


Figure 1. Worldwide incidence of human brucellosis (Pappas et al. 2006) (Reprinted with permission from Elsevier).

Brucella spp. are gram negative and facultative intracellular pathogens (Ghazaei 2016). The predominant pathological aetiological agents for humans are *Brucella abortus* (25% of cases) and *B. melitensis* (70% of cases) (Doganay et al. 1997), which are mainly carried by large and small ruminants respectively (Njeru et al. 2016). Over 500 000 human cases are estimated to occur annually (Pappas et al. 2006). There is also the other species *B. suis*, *B. canis* and *B. ceti* from animals that are known to infect humans (Figure 2 & Table 1).

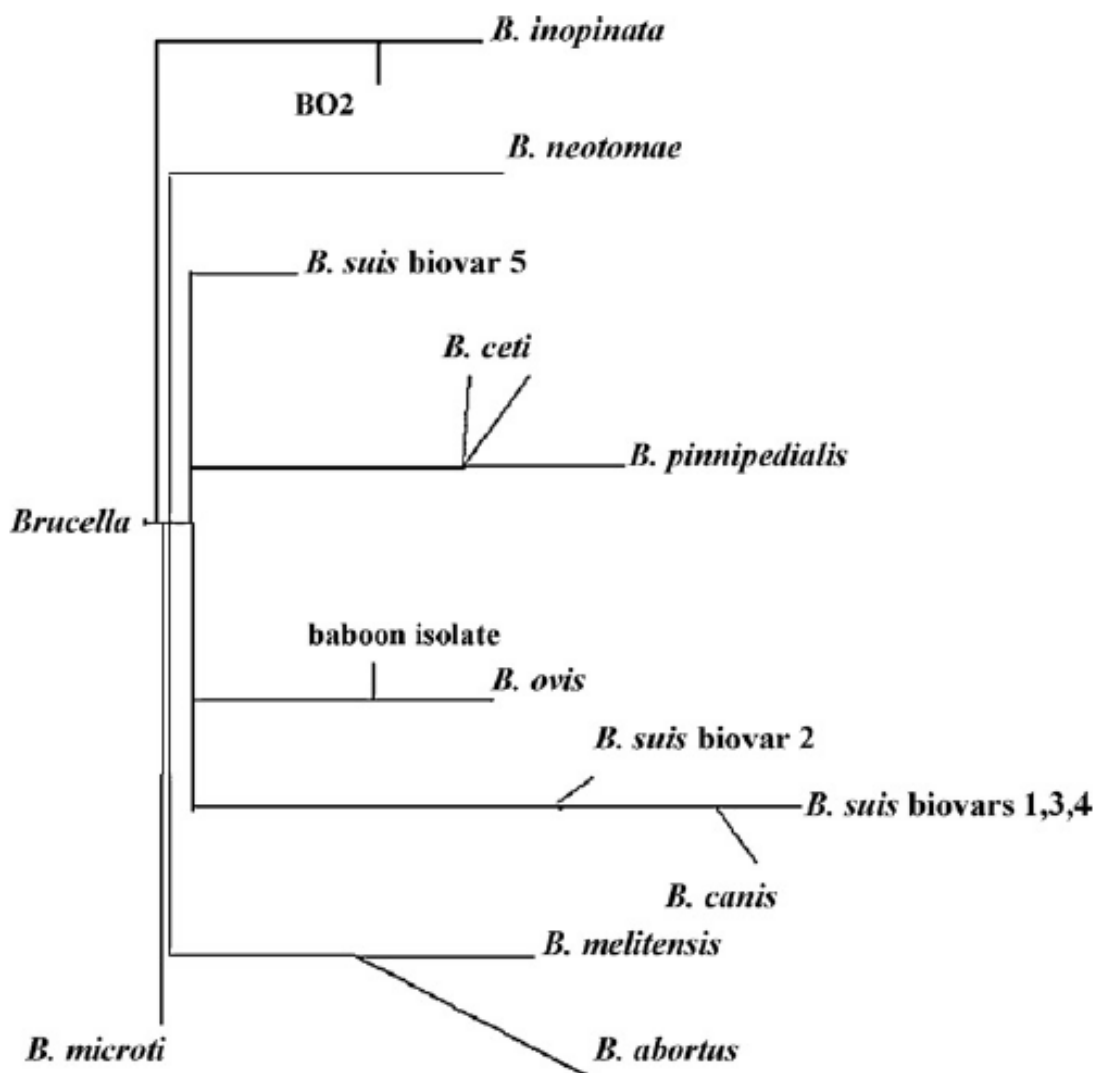


Figure 2. Phylogenetic relationships of the recognized *Brucella* species (Pappas 2010)(Reprinted with permission from Elsevier).

Table 1. A provisional *Brucella* spp. taxonomy with animal hosts and human disease (Pappas 2010; Marcotty et al. 2013; Godfroid et al. 2010; Whatmore et al. 2014)

Species	Biovar	Animal host	Human disease
Old species			
<i>B. melitensis</i>	1-3	Sheep, goats, camels	The most common cause of human brucellosis

<i>B. abortus</i>	1-6, 9	Cattle, buffalo, elk, yaks, camels	The second most common cause of human infection
<i>B. suis</i>	1,3	Domestic pigs	Pathogenic to humans
<i>B. suis</i>	2	Wild boar	Pathogenic to humans, of increasing interest with hunters of wild boar at risk
<i>B. suis</i>	4	Reindeer, caribou	Pathogenic to humans
<i>B. suis</i>	5	Rodents	Not reported
<i>B. canis</i>	-	Canines	Pathogenic to humans, increasing reports, particularly from South America, possibly understudied elsewhere
<i>B. ovis</i>	-	Sheep	Not reported
<i>B. neotomae</i>	-	Rodents	Pathogenic to humans
Novel species			
<i>B. ceti</i>	-	Porpoises, dolphins, whales	Reports of complicated disease (neurobrucellosis, spondylitis) and one laboratory infection
<i>B. pinnipedialis</i>	-	Seals	Not reported
<i>B. microti</i>	-	Red foxes, common voles (also isolated from soil)	Not reported
<i>B. inopinata</i>	-	Human	Isolated from a human case (prosthetic breast implant infection)
<i>B. papionis</i>		Baboons	Not reported

The lipopolysaccharide (LPS) on the surface of the *Brucella* bacteria is the most important virulence factor, antigenic and immunogenic structure (Kianmehr et al. 2015). It has three

domains: lipid A, the core oligosaccharide and the O-antigen / chain (Figure 2) (Cardoso et al. 2006). The LPS structure divides the *Brucella* species into two groups based on colony morphology: smooth (S-LPS), which have a O-antigen, and rough (R-LPS), without an O-antigen (Cardoso et al. 2006). The smooth group includes *B. abortus*, *B. melitensis*, *B. suis* and the rough includes *B. ovis* and *B. canis* (World Organisation for Animal Health 2016).

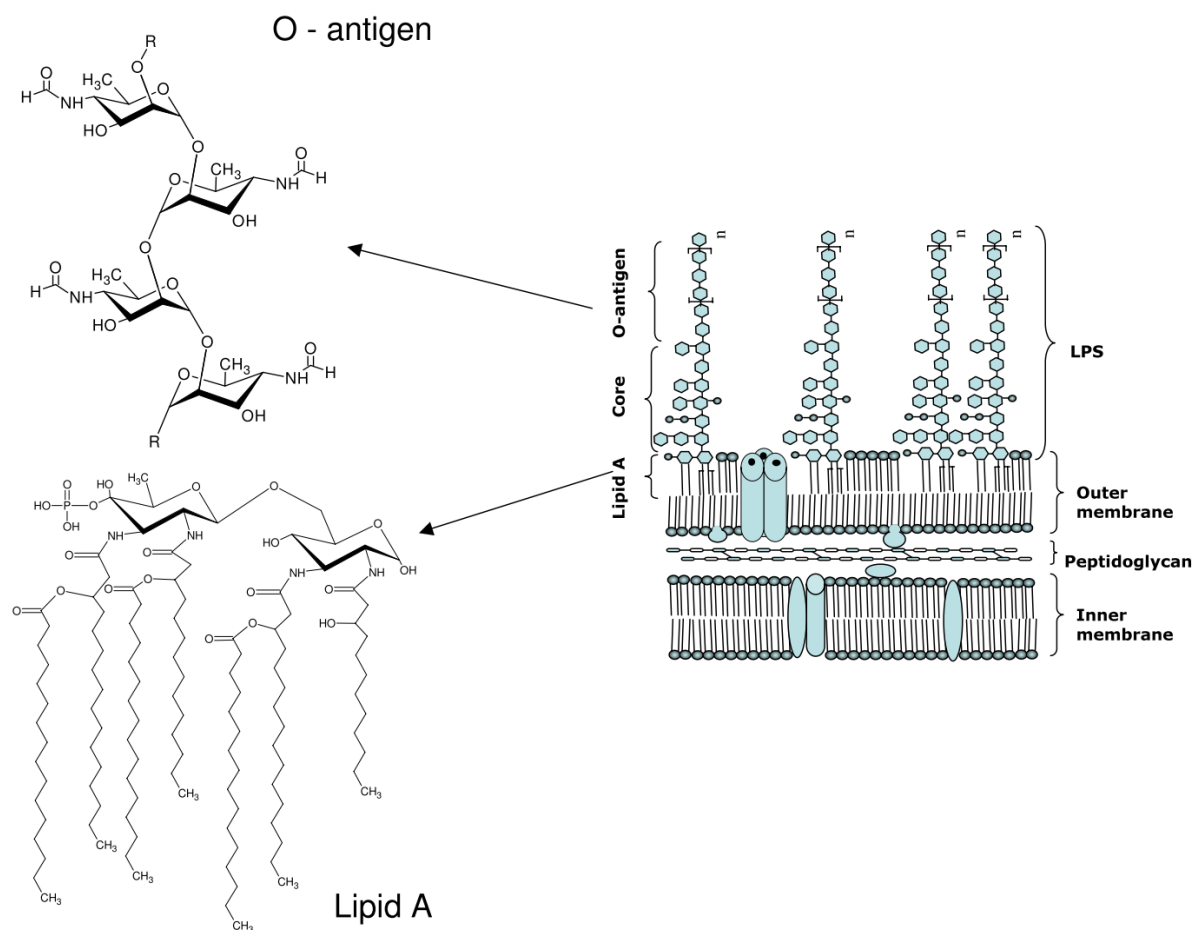


Figure 2. The structure of lipopolysaccharide of *Brucella* spp. (Cardoso et al. 2006) (Open access).

Pathogenesis of brucellosis

Brucellosis in humans can lead to severe disease characterised by fever, malaise, anorexia, arthralgia and back ache (Doganay et al. 1997); however often it presents atypically with non-specific symptoms, making it difficult to diagnose (Corbel 2006). There is a lack of a clear clinical algorithm that is predictive for brucellosis, which highlights the need for diagnostic and epidemiological information (Njeru et al. 2016). In Africa, acute febrile patients with brucellosis are often undiagnosed and treated for malaria (Njeru et al. 2016). Human brucellosis is considered a neglected disease (Pappas 2010; Marcotty et al. 2009) with fewer than 10% of human cases being diagnosed, treated or reported (Mantur et al. 2007). There is no vaccine available for humans.

Brucella spp. are well adapted to their hosts, they successfully bypass the bactericidal effects of phagocytes. The bacteria enters the body by ingestion, inhalation, penetration of intact skin, abrasions or conjunctival mucosa (Doganay et al. 1997). When ingested, the gastric environment is not favourable to the bacteria, providing some protection from infection, but once into the circulatory system the serum only has moderate anti-*Brucella* properties (Doganay et al. 1997). Neutrophils destroy some strains, but lack activity against *B. melitensis*. Virulent *Brucella* organisms can infect phagocytic and non-phagocytic cells where they reproduce in the endoplasmic reticulum and resulting in expansion and transmission to new cells (Doganay et al. 1997). *Brucella* spp. infection in humans results in formation of granulomas of epithelioid, polymorphonuclear leukocytes, lymphocytes and giant cells (Doganay et al. 1997). This is more characteristic of *B. abortus* than *B. melitensis* and *B. suis*.

Brucellosis is a systemic disease that can involve any organ system. It can be symptomatic or asymptomatic. Osteoarticular localisations are the most common complication in humans and the most severe is cardiac that results in bacterial endocarditis and death (Doganay et al. 1997).

The main sources of *Brucella* spp. for humans are animal products such as milk, meat, cheese, etc. and animal urine, blood and abortion material (Doganay et al. 1997). *B. abortus* in cattle spreads to humans through infected milk and contaminated tissue

(Mcdermott & Arimi 2002). Brucellosis in cattle can lead to abortion, lower milk production, infertility and hygromas and abscesses (Corbel 2006; J Godfroid et al. 2005). Bulls generally do not become functionally infertile, but their semen quality maybe affected and the semen can be infected (Bercovich 2000).

In pregnant animals *B. abortus* has a predilection for the gravid uterus of cattle. It localizes and replicates in the rough endoplasmic reticulum of the trophoblastic epithelium (Figure 3) (Detilleux 1989). This cell invasion and replication results in cell death leading to placentitis and abortion (Detilleux 1989).

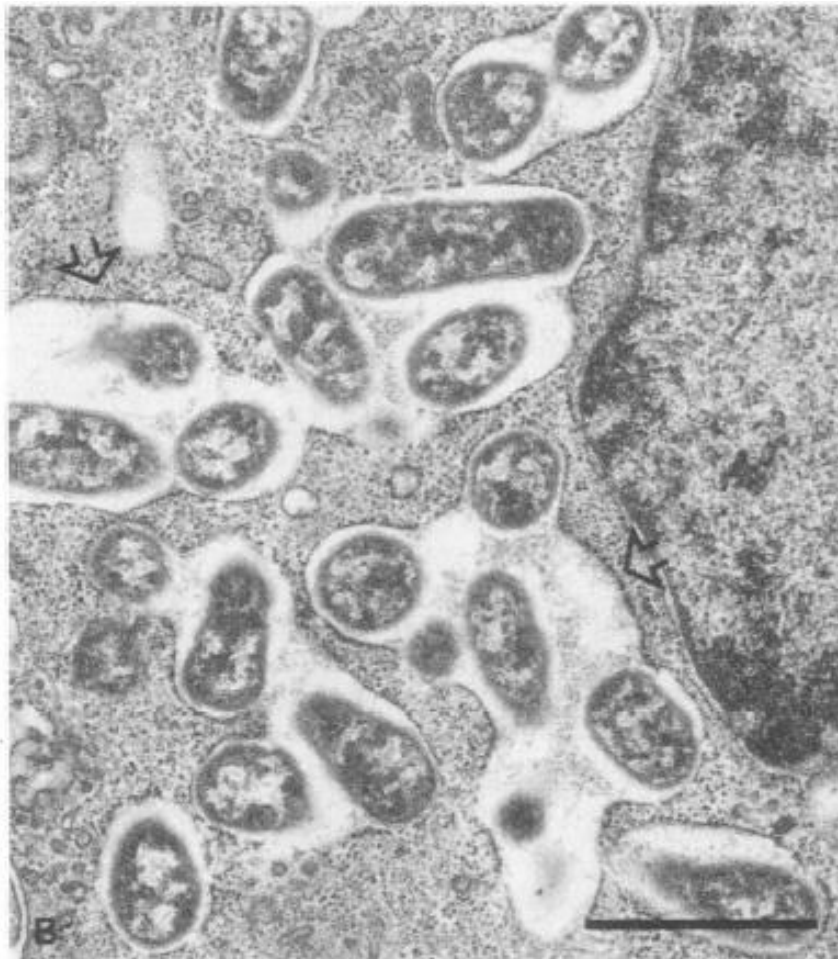


Figure 3. Electron micrograph of *B. abortus* located in cisternae of vero cell (Detilleux 1989) (Reprinted with permission from American Society for Microbiology).

Diagnosis of brucellosis

Diagnosis of infection and disease in humans and other animals is through a combination of history of exposure, clinical signs, serological responses, isolation and ideally identification. A combination of growth characteristics, serological, bacteriological or molecular methods are required for species distinction (World Organisation for Animal Health 2016; Cardoso et al. 2006). There are a variety of laboratory tests and each has its pros and cons (Table 2). Antigen isolation and identification is the most definitive as positive animals are shown to be infected by the bacteria. Yet, antigen isolation and identification is too costly and time consuming for routine surveillance. They are suitable for a smaller number of individuals to identify the organism and confirm what is shown by the other testing methods when an outbreak is suspected in a population.

Table 2. Tests methods available for *Brucella abortus*, *melitensis* and *suis* and their appropriate purposes (World Organisation for Animal Health 2016)(Open access).

TEST	PURPOSE				
	Population free from infection	Individual free from infection*	Used in parallel for eradication*	Confirmation of suspect or clinical cases***	Surveillance of herd/flock prevalence
Antigen test					
Staining	-	-	-	+	-
Culture	-	-	-	+++	-
PCR	-	-	-	-/++	-
Serological test					
BAT (RBT or BPAT)	+++	++	+++	+	+++
FPA	++	++	+	++	++

CFT	++	++	+++	++	+++
iELISA	+++	++	+++	++	+++
cELISA	++	+	+	+	++
SAT	++	+	+	-	+
NH and cytosol protein based tests	-	-	+	++	-
Milk tests	+++	-	+++	+	+++
Cellular test					
BST	++	-	+	+++	++

Legend: +++ = recommended method, ++ = suitable method, + = maybe used but application limited, - = not applicable. * this applies to herds/flock, countries or regions free from infection. ** to improve efficacy of eradication policies in infected herds/flocks it is advisable to use tests in parallel eg BBAT or FPA and CFT or iELISA and sensitivity is increased by adding BST. ***in low prevalence settings or almost free setting the predictive value of a serological positive will be low and agent identification is needed. Abbreviations: BAT-buffered *Brucella* antigen test, FPA- Fluorescence polarization assay, CFT- complement fixation test, iELISA-indirect Enzyme linked immunosorbent assay, cELISA-competitive Enzyme linked immunosorbent assay, SAT- Serum or slow agglutination test, MRT- milk ring test, BST- Brucellin skin test.

Humoral immunity responds to the exposure of an antigen that results in a primary and a secondary immune response. The primary response occurs the first time an antigen is encountered and the responding cells are naïve B or T cells and results in memory cells highly specific for the antigen (Figure 4). Antibodies, mainly IgM are produced, usually in low numbers and production peaks by seven to ten days. In the secondary immune response the memory cells producing many times the amount of antibodies compared to the primary response. The antibodies in the secondary immune response are primarily IgG, but also IgM and IgA. The antibody levels in the secondary immune response remain higher for longer and have a higher affinity for the antigen.

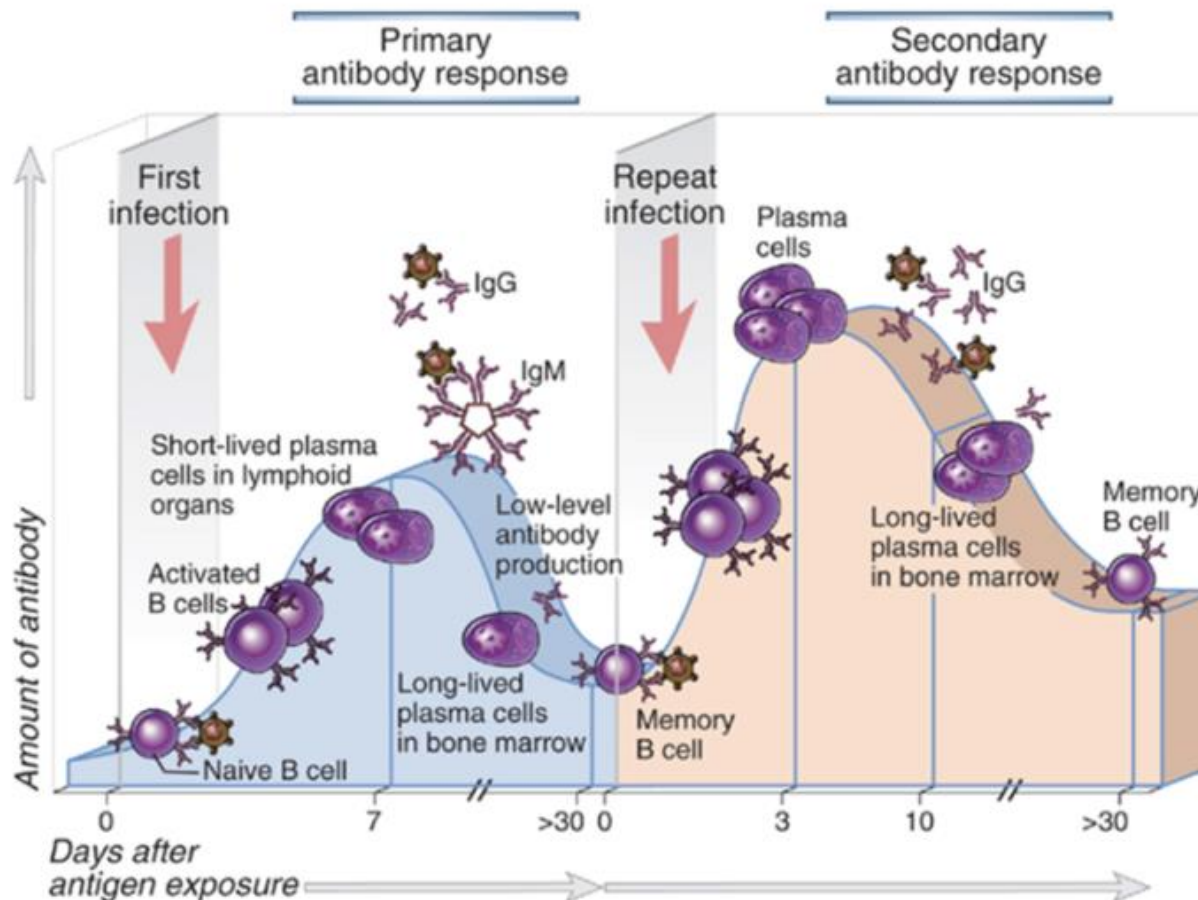


Figure 4. Primary and secondary immune response showing responsive immune cells and antibody production over time (Abbas, Abul, Lichtman, Andrew, Pillai 2011)(Reprinted with permission from Elsevier).

An infection with *Brucella* spp. induces both a humoral and a cellular immunity. The humoral antibodies appear to play a role in resistance to *Brucella* spp and the cell-mediated immunity appears to be the principal mechanism of recovery (Doganay et al. 1997). Identifying antibodies to *Brucella* spp. is an easier and more economical than isolation and identification. Serological antibody testing is therefore more suitable for infection screening. Samples can be taken from animals without causing detriment and they are simple, quick and cheap testing methods that can be done at small district or regional laboratories.

In brucellosis serology, the *Brucella* immunodominant antigens are associated with the surface “smooth” lipopolysaccharide (S-LPS) which is shared by all the naturally occurring “smooth” *Brucella* species. Besides being 100% sensitive and 100% specific, an ideal serological test should make it possible to differentiate infectious animals from infected or exposed ones. Unfortunately, such a test does not exist. Brucellosis serology has other drawbacks, among which is the impossibility to ascribe which *Brucella* species induced antibodies in the host and the impossibility (per definition) to detect “latent” infection, defined as a seronegative *Brucella*-infected animal.

There are differing sensitivities and specificities between the available tests and this must be taken in to account interpreting results (Table 3). In infected populations a positive serological result should be considered as confirmation in a clinical case and if there are no clinical signs the animal should be considered infected (World Organisation for Animal Health 2016). In low prevalence or almost free regions serological reactions must be confirmed by culture, PCR or ST and in free regions suspect animals must be positive to a screening and confirmatory serological test and may be confirmed by culture, PCR or ST (World Organisation for Animal Health 2016). There are standard operating procedures for disease status recognition for countries or compartments provided by the World Organisation for Animal Health.

Table 3. Sensitivity and specificity of indirect tests for the diagnosis of cattle brucellosis (Godfroid et al. 2010)(Open access).

TEST	Sensitivity %	Specificity %
Serological test		
BAT	87	97.8
FPA	96.6	99.1
CFT	90-91.8	99.7-99.9
iELISA	97.2	97.1-99.8
cELISA	95.2	99.7
SAT	81.5	98.9
Milk tests		

MRT	88.5	77.4
FPA	76.9	100
iELISA	98.6	99
Cellular test		
BST	78-93	99.8

Legend: Abbreviations: BAT-buffered *Brucella* antigen test, FPA- Fluorescence polarization assay, CFT- complement fixation test, iELISA-indirect Enzyme linked immunosorbent assay, cELISA-competitive Enzyme linked immunosorbent assay, SAT- Serum or slow agglutination test, MRT- milk ring test, ST- Brucellin skin test.

Identification and typing

Identification of the *Brucella* spp. bacteria can be through a combination of staining to identify organism morphology, colony growth with associated characteristics and morphology and urease and oxidase tests where they stain positive (World Organisation for Animal Health 2016).

Staining methods

Brucella coccobacilli are normally arranged singly, non-motile, without spores or true capsules, no flagella or pili and their morphology is fairly constant (Figure 4) (World Organisation for Animal Health 2016). They are gram negative and are resistant to decolourisation to weak acids and therefore stain red with a blue background with Stamps modification of the Ziehl-Neelsen's method (Alton et al. 1988). A fluorochrome or peroxidase-labelled antibody conjugate based technique could also be used (World Organisation for Animal Health 2016). Weakly acid fast intracellular organisms of *Brucella* morphology is presumptive evidence of brucellosis (World Organisation for Animal Health 2016).

Culture

Culture can provide a definitive identification of bacterial species. Samples for culture can be collected from tissues (placenta, foetus, inguinal lymph nodes and epididymus), vaginal discharge, milk, dairy products and hygroma. Tissues are cut into small pieces

and macerated with a small amount of sterile PBS before being added to culture media. Bacterial isolation is slow, expensive and cumbersome, although often not considered sensitive, but should be done to confirm the disease and determine the species/biovar (World Organisation for Animal Health 2016). Isolation and culture is usually done on solid media. Antibiotics are added to suppress growth of organisms besides *Brucella* spp. Adding CO₂ to the atmosphere (5-10%) helps the culture of all *Brucella* spp. but is not needed for growth of *B. melitensis* or *B. ovis* (World Organisation for Animal Health 2016). Biovar identification or biotyping needs more complicated tests such as phage lysis and agglutination with anti-A, -M or -r monospecific sera (World Organisation for Animal Health 2016). Different biovars can have different preferred hosts so knowing the biovar can help understanding the epidemiology of an infection. Identifying the biotype is only relevant for smooth colonies of *B. abortus*, *B. melitensis* and *B. ovis*.

Vaccine strains can also be identified by their growth characteristics in culture (World Organisation for Animal Health 2016). *B. abortus* S19 has growth properties of *B. abortus* bv1, does not need CO₂, does not grow in benzyl-penicillin, thionin blue nor erythritol and uses L-glutamate, while Rev 1 vaccine grows like *B. melitensis*, but has smaller colonies, does not grow with fuschin, thionin, nor benzyl-penicillin, does not grow in streptomycin and *B. abortus* strain RB51 is different from smooth *B. abortus* biovar 1 as it has a rough morphology (Alton et al. 1988).

Nucleic acid recognition methods

Polymerase chain reaction

The polymerase chain reaction (PCR) is another method to detect and identify *Brucella* spp. Even though there is a degree of DNA homology within the *Brucella* genus methods such as PCR, PCR restriction fragment length polymorphism, Southern blot, field gel electrophoresis have been developed to identify different species and biovars (Bricker 2002; World Organisation for Animal Health 2016). A new multiplex PCR (Bruce ladder) that can identify and differentiate most biovars (*B. abortus* bv. 3, 5, 6, 9 and *B. suis* bv 2, 3, 4, 5) and vaccine strains (*B. abortus* S19 and RB51 and *B. melitensis* Rev 1) has been developed (World Organisation for Animal Health 2016). Another new multiplex PCR can

differentiate other *Brucella* organisms (*B. suis*, *B. canis* and *B. microti*), the vaccine strains and the marine species. The new PCRs are better than the first PCRs (e.g. AMOS) that could not identify all the biovars. There are other methods such as single nucleotide polymorphism, multilocus sequencing scheme that provide more information at the subspecies level (World Organisation for Animal Health 2016).

Multilocus sequencing and Multiple loci VNTR analysis

Multilocus sequencing has become the major approach applicable to studying the global epidemiology of bacteria and is also frequently used for phylogenetic studies. In its classical form multilocus sequence typing (MLST) involves the sequencing of short fragments (c. 500 bp) of a number of housekeeping genes which are subject to purifying selection and slow evolution and within which variation is nearly neutral. Nine discrete genomic loci corresponding to 4,396 bp of sequence were examined from 160 *Brucella* isolates. By assigning each distinct allele at a locus an arbitrary numerical designation the population was found to represent 27 distinct sequence types (STs) (Whatmore et al. 2007).

Multiple loci variable number of tandem repeats analysis (MLVA) uses the tandemly repeated DNA sequences to identify *Brucella* spp. on selected and characterised loci and can be used to trace the source of a particular strain, which can be useful for outbreak analysis. After amplification repetition can be analysed and the results compared to references to identify the spp., type and possibly source of the bacteria. This technique has identified 21 loci with over 100 sequence types for the whole *Brucella* spp. population structure (Vergnaud et al. 2018).

Both MLVA and MLST can assess genetic diversity of *Brucella* strains and identify newly emerged or atypical isolates as novel species, which is not possible on phenotypic characterization alone (Scholz & Vergnaud 2013). They are both robust and accurate and may replace the classical phenotyping scheme of *Brucella* spp. and biovar (Vergnaud et al. 2018).

Serological testing

Unfortunately, there is not one serological test that is appropriate in all situations as all tests have limitations especially when screening individual animals (World Organisation for Animal Health 2016). Vaccination with smooth *Brucella* spp. and infection with *Yersinia enterocolita* O:9 and other bacteria may create false positive reactions which interferes with accurate serological diagnosis (World Organisation for Animal Health 2016).

Serum agglutination tests (SAT) are generally regarded as being unsatisfactory for international trade, while the complement fixation test (CFT) is more specific and has a standardised system of unitage, but some enzyme linked immunosorbent assays (ELISAs) and the fluorescent polarisation assay are comparable to or better than the CFT with regards to diagnostic performance (World Organisation for Animal Health 2016). Different serological tests also react to different antibodies and so their results can depend on the antibody levels at the time of testing. Antibody IgM is produced first followed by IgG (Figure 5) and IgA, but IgG₁ can appear simultaneously with IgM (Bercovich 2000).

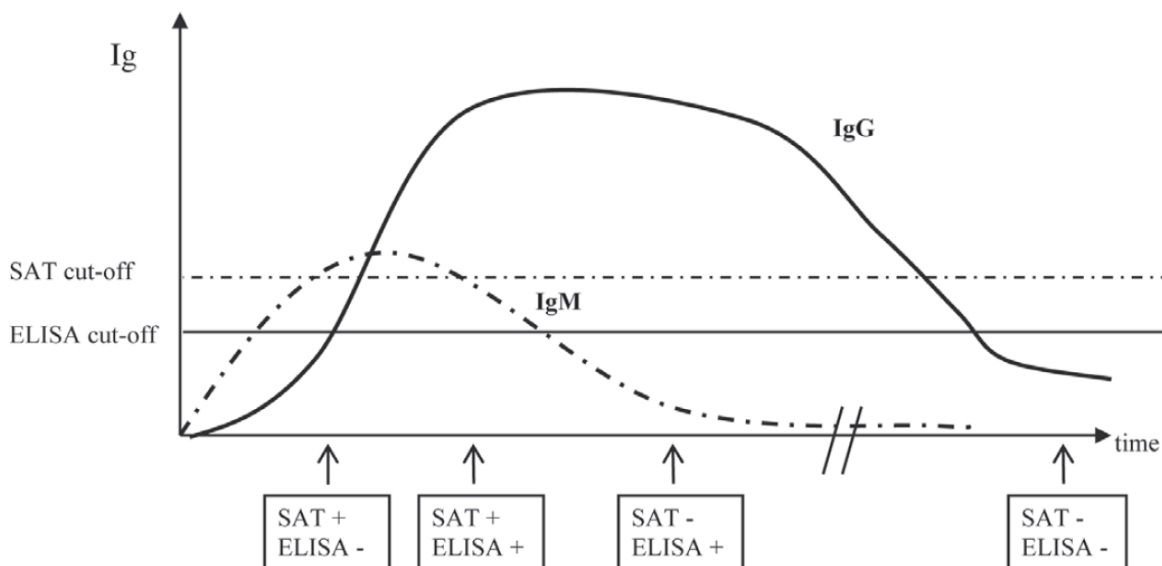


Figure 5. Outcome of serum or slow agglutination test and enzyme linked immunosorbent assay tests performed at different times post-infection (Godfroid et al. 2010)(Open access).

Below are the most commonly used serological diagnostic tests. OIE standardised reference sera should be used for tests requiring reference sera.

Buffered Brucella antigen tests (BAT)

Rose Bengal test (RBT)

This test produces an agglutination using a *B. abortus* S99 or S11119-3 antigen stained with a sodium salt called rose Bengal, which detects IgG₁ and to a lesser extent IgM and IgG₂ (Bercovich 2000). A positive result shows agglutination of antigen and antibody. It is very sensitive, but can give false positive serological reaction (FPSR) due to S19 or Rev 1 vaccination or other reasons so therefore it should be used with a confirmatory test (World Organisation for Animal Health 2016). RBT rarely produces false negative serological reaction. It is a simple and cheap test that can be done in a resource limited setting, which makes it ideal for screening cattle, sheep and goats in low to middle income countries (Ducrotoy & Bardosh 2017), to detect infected herds or guarantee absence of infection (World Organisation for Animal Health 2016).

Buffered plate agglutination tests (BPAT)

This test uses an antigen from *B. abortus* S11119-3 and two staining solutions of brilliant green and crystal violet. Like the RBT, it is a sensitive screening agglutination test for cattle.

Complement fixation test (CFT)

This test, although complex and needing good laboratory facilities and staff, is widely performed (World Organisation for Animal Health 2016). Accurate titration and maintenance of reagents is needed. It detects IgM and IgG₁ antibodies (Bercovich 2000) and enables the distinction between antibodies of vaccination and infection (Fensterbank

1986). The serum, antigen and complement are incubated resulting in antigen antibody complexes that bind to added complement protein, which stops the solution turning pink when sheep red blood cells with pre-bound antibodies are added (World Organisation for Animal Health 2016). The test can be quantified by setting up a series of dilutions of the sample serum to determine the highest dilution that will still yield a positive result. Animals vaccinated with S19 or Rev 1 are considered to be infected if they give a positive fixation result at a titre of 30 or greater at 18 months old or older (World Organisation for Animal Health 2016). It is deemed very specific, but less sensitive than RBT and ELISA (Alton et al. 1975).

Enzyme linked immunosorbent assay (ELISA)

Indirect ELISA (iELISA)

There are numerous variations of iELISA for antibody detection using different antigen preparations, antiglobulin-enzyme conjugates and substrate chromogens (World Organisation for Animal Health 2016). *B. abortus* S19 or S1119-3 or *B. melitensis* S16M can be used for antigens and whole cells, smooth lipopolysaccharide (S-LPS) or O specific polysaccharide (OPS) can be used as the antigens (World Organisation for Animal Health 2016).

Competitive ELISA (cELISA)

There are also several variations of cELISA for antibody detection using S-LPS or OPS as antigens from smooth *Brucella* strains, different antiglobulin-enzyme conjugates, substrate or chromagens (World Organisation for Animal Health 2016). The use of a monoclonal antibody (Mab) specific for one of the epitopes of the *Brucella* spp., which competes with antibodies from vaccination and infections of cross reacting bacteria (Ducrotoy & Bardosh 2017), usually results in less FPSR and is higher in specificity than BAT and iELISA, but tends to have less sensitivity (Muñoz et al. 2005).

Florescent polarisation assay (FPA)

The FPA is a simple and rapid test measuring antigen and antibody interaction using an OPS of *B. abortus* 1119-3 conjugated with a fluorescein isothiocyanate. The antibody

attaches to the antigen complex and its rotation is measured by the rate of light depolarisation and compared to when there is no antibody attached (Nielsen & Gall 2001). It can be performed in the field and laboratories using a homogenous assay and portable equipment. The accuracy should be equal to or greater than the BPAT, FPA, iELISA and cELISA (Nielsen & Gall 2001).

Serum agglutination test (SAT)

The antigen is a suspension of *B. abortus* S99 or *B. abortus* S1119-3 in phenol and saline (World Organisation for Animal Health 2016). This is mixed with dilutions of serum and agglutination is measured. It is for cattle only and has been well used in northern Europe but requires incubation. It detects antibodies of IgM, IgG₁, IgG₂ and IgA, so can detect acute infections (Bercovich 2000). This test has contributed to international harmonisation of brucellosis control and eradication, however it can give false positive and false negative reactions, so it is better for detection on a herd rather than individual level (Bercovich 2000). Cattle are considered infected when they test ≥ 30 international units (IU).

Native hapten and cytosol protein-based tests

These test are valuable when used with RBT in cattle that have been vaccinated with S19 (World Organisation for Animal Health 2016). They use a native hapten or *Brucella* cytosol that does not give FPSR due to *Yersinia enterocolitica* O:9, nor for heifers two months after vaccination and adults 4-5 months after vaccination (World Organisation for Animal Health 2016). Thus, in areas where S19 or Rev 1 vaccination occurs, this test can help differentiating antibodies due vaccination from those due to infection (World Organisation for Animal Health 2016).

Milk tests

Bulk milk testing is an efficient way to screen dairy herds that if showing positive reactions can then have individuals blood sampled for serological tests. They detect IgM and IgA bound to milk fat globules (Brothers et al. 2011)

Milk iELISA

Several commercial variations of this test is available and they tend to give less FSPR than the blood test (World Organisation for Animal Health 2016). It could be used in sheep and goats, but the cut-off needs to be properly validated (World Organisation for Animal Health 2016).

Milk ring test (MRT)

This test is applicable to bovine milk. The antigen used is *B. abortus* S99 or S1119-3, which is added to a staining solution that makes a blue ring if positive above the milk column (World Organisation for Animal Health 2016). False positive reactions may occur if cattle are vaccinated within 4 months before testing or if there is colostrum or mastitis (World Organisation for Animal Health 2016).

Test for cellular immunity

Brucellin skin test (ST)

An allergen is injected into the dermis and three days later in cattle or two days in goats the difference in skin thickness is measured, with a result greater than 1.1 mm increase being deemed positive (Saegerman et al. 1999). The ST measures the cell-mediated immune response as opposed to serological test that measure the humoral response (Godfroid et al. 2010). The brucellin skin test has such a high specificity such that positive unvaccinated animals that are serologically negative should be deemed positive (Pouillot et al. 1997). Yet, the test has been considered inadequate in the past due to the discrepancy that may occur between it and serological and bacteriological tests (Bercovich 2000). The brucellin is an LPS-free extract from rough *B. melitensis* B115, which does not result in antibodies reactive to serological tests (World Organisation for Animal Health 2009). However, as this rough strain contains *Brucella* OPS sugars, repeated inoculation of brucellin could elicit antibodies, interfering with other diagnostic tests (World Organisation for Animal Health 2016). The skin test response is less dramatic than when using the tuberculin antigen, but an allergic reaction in vaccinated animals could be detected up to four and a half years after vaccination and this test clearly excludes FSPR due to *Yersinia enterocolitica* O:9 (Saegerman et al. 1999).

Interferon gamma release assay (IGRA)

This test involves stimulation of lymphocytes in whole blood with a brucellin antigen that results with gamma interferon production that is detected with ELISA (World Organisation for Animal Health 2016). It can be seen as a rapid and convenient alternative to the ST and complementary to serological diagnosis (Weynants et al. 1995) as it is a useful test to distinguish FPSR, but more specific antigens are needed and protocol improvement (World Organisation for Animal Health 2016).

Control of brucellosis

The control and eventually the eradication of *B. abortus* includes several measures (J Godfroid et al. 2005). Control in livestock is by systematic vaccination (Rock et al. 2009), testing for disease and slaughtering of animals deemed positive (Zhang et al. 2018). Methods to prevent transmission to humans include pasteurization, boiling of milk, avoiding contact with abortion material and use of protective materials (Mantur et al. 2007). In South Africa, heifers between 4 and 8 months are vaccinated with Onderstepoort Biological Products *Brucella abortus* strain 19 vaccine (manufactured in Pretoria, South Africa). Males are not vaccinated because of the potential complication of orchitis (J Godfroid et al. 2005).

Immunity against *Brucella*

There are three commercially available vaccines for livestock. They are live cultures of *Brucella* spp. and can cause disease in humans.

Brucella abortus strain 19 vaccine

This smooth strain was first described in 1930, isolated from infected milk that was attenuated by being in room temperature for a year, and is the most widely used vaccine (Schurig et al. 2002). It is seen as the reference vaccine and this live attenuated vaccine

is normally given as single dose of $5-8 \times 10^{10}$ colony forming units (CFU) or viable organisms subcutaneously to heifers between 3 and 6 months of age producing good immunity against *B. abortus* and *B. melitensis* (World Organisation for Animal Health 2016). Although it is of low virulence for cattle it can cause abortions if used in pregnant cattle (Beckett & MacDiarmid 1985). A reduced dose of 3×10^8 to 5×10^9 CFUs can be given by subcutaneous to adult cattle, but may result in persistent antibody titres, abortion or excretion of the vaccine strain in milk (World Organisation for Animal Health 2016). A reduced dose given in conjunctival sac in one or two doses of 5×10^9 CFUs can be given at any age without these side effects (World Organisation for Animal Health 2016). The route of administration does not appear to affect the effectivity of the vaccine (Nicoletti 1984).

The presence of the LPS with an O chain results in the appearance and persistence of antibodies that are detected with serology making it difficult to differentiate vaccinated from infected cattle (Schurig et al. 2002).

Brucella abortus strain RB51 vaccine

This vaccine has become the official vaccine in several countries since 1996 although there is disagreement of its performance compared to S19 (World Organisation for Animal Health 2016). A dose of $1-3.4 \times 10^{10}$ CFU of live attenuated vaccine is given to calves subcutaneously between 4 and 12 months, with either no follow-up dose or a follow-up dose at 12 months or a reduced dose of $1-3 \times 10^9$ CFU (World Organisation for Animal Health 2016). It can cause abortion if given to pregnant cattle, but using the reduced dose in pregnant animals should not induce abortion. The reduced dose in calves does not create adequate protection (World Organisation for Animal Health 2016). (Schurig et al. 2002). It can also be safely used in mature males (Edmonds et al. 1999), unlike S19. This rough strain has almost no OPS and therefore does not induce antibodies causing FPSR to the standard tube agglutination tests as dose S19 (Schurig et al. 2002), but antibodies can be detected by ELISA for a few months post vaccination (Stevens, Olsen & Cheville 1995). Experimentation in mice indicates that RB51 is able to protect against infections with *B. abortus*, *B. melitensis*, *B. suis* and *B. ovis* (Winter et al. 1996), but in cattle the

protection against *B. melitensis* is unknown (World Organisation for Animal Health 2016). This vaccine is not effective for *B. melitensis* in sheep (World Organisation for Animal Health 2016). Oral administration of this vaccine to cattle provided a degree of protection and opens the possibility of this mode of administration as a model for wild ungulates (Elzer et al. 1998).

***Brucella melitensis* strain Rev 1 vaccine**

This live attenuated vaccine stimulates protection against infection with *B. melitensis* in sheep and goats and rams against *B. ovis* (Schurig et al. 2002). It can cause abortions if given during pregnancy, but is avirulent to rams (Schurig et al. 2002). There is still some discussion on the effectivity of the S19 vaccine against *B. melitensis*, but the Rev 1 may be able to control *B. melitensis* infection in cattle from small stock (World Organisation for Animal Health 2016).

There is also no vaccination against brucellosis in humans and in wildlife, although some degree of protection has been documented by the use of RB51 in bison in the Greater Yellowstone Conservation Area, USA (Olsen et al. 2006).

Brucellosis and the environment

Brucella spp. are an environmental contaminant. They contaminate the environment through foetal materials (the highest concentrations of bacteria), milk and other tissues. The survival of the bacteria outside of the host's body depends on environmental conditions, such as sunshine, and was found to be up to 60 days in water (Falenski et al. 2011) and 21-81 days foetal membranes, soil and tissue in the Yellowstone National Park, USA (Aune et al. 2012).

Study site

In sub-Saharan Africa, cattle and small stock are widespread across the continent, with lower densities in arid (North and South-west) and forested zones (Central Africa) (Figure

6). In Eastern Africa the incidence of brucellosis in animals was found to be the highest in pastoral or communal production systems (Mcdermott & Arimi 2002). A study in Tanzania, where livestock and wildlife graze together and share water points and there is human consumption of buffalo bush meat, found anti-*Brucella* spp. antibodies in 0.6% of humans, 6.8% of cattle, 1.6% of goats and 7.9% of buffalos (Assenga et al. 2015). It would be interesting to look deeper into the epidemiology of brucellosis in such a setting to understand the risk of transmission between these three categories.

The study site, in a Transfrontier Conservation Areas (TFCAs) in South-east Africa, has humans and their livestock living close to wildlife reserves. TFCAs are large areas covering multiple land uses, including protected conservation areas and one or more international boundaries (Anon 2018). TFCAs have humans, domesticated animals, and wildlife living in close proximity. Land adjacent to protected conservation areas commonly belongs to traditional authorities or is communal i.e. belongs to the local community and is used for housing, subsistence crop growing and grazing. Humans and animals often travel across protected conservation area boundaries that may or may not be fenced. The Greater Limpopo Transfrontier Conservation Area (Figure 7), which houses the study site, is the second largest TFCA in the world. It includes land in Mozambique, South Africa, and Zimbabwe (Figure 7) covering almost 100 000 square kilometres, with land uses ranging from core wilderness areas to communal areas and private nature reserves focused on wildlife utilisation (Cumming 2004).

The study site includes the Kruger National Park (KNP) and communal land to the west of the KNP (Figure 8 and 9). The KNP has near 40 000 buffalo. Brucellosis seroprevalence in these buffalo is estimated to be 13-38% (Chaparro et al. 1990; Herr & Marshall 1981). Evidence of brucellosis infection has been serologically identified in several wildlife species including African buffalo (*Syncerus caffer*), hippopotamus (*Hippopotamus amphibious*), zebra (*Equus burchellii*), eland (*Taurotragus oryx*), waterbuck (*Kobus elipsiprymnus*), and impala (*Aepyceros melampus*) (Chaparro et al. 1990). African buffalos and wildlife are considered a possible source of infection for cattle in the nearby communal areas (Gradwell et al. 1977; Muma, Samui, et al. 2007).

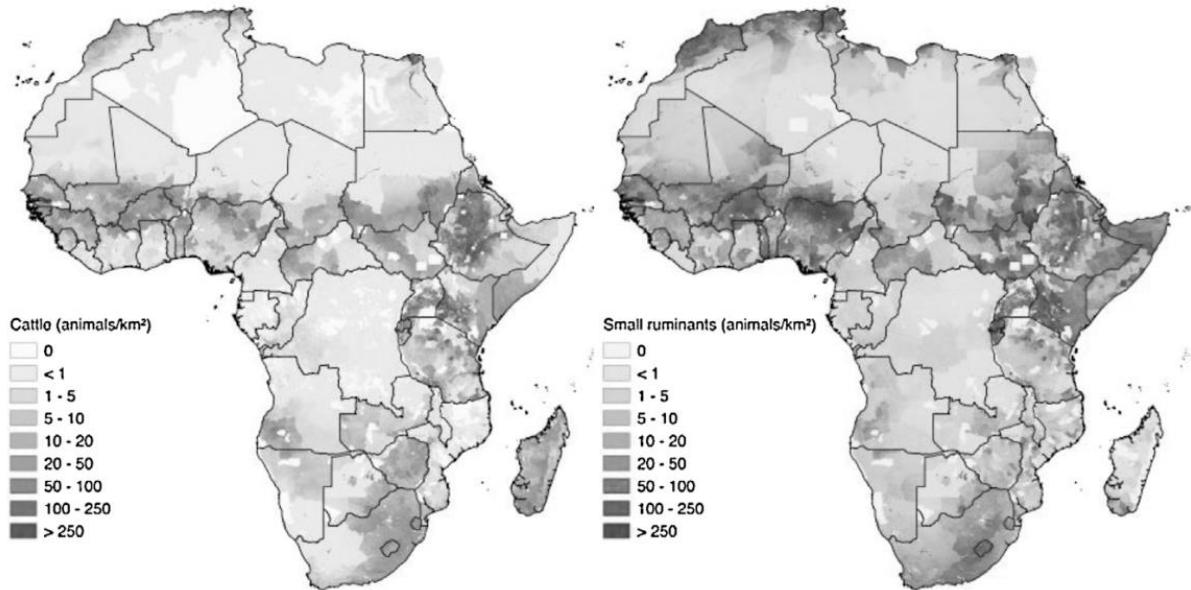


Fig. 6. Cattle and small ruminant densities in Africa. Livestock density is expressed as the number of cattle or small ruminants per square kilometre according to categories of different sizes in order to clearly differentiate between zones of low and high livestock density (Ducrotoy et al. 2017)(Open access).

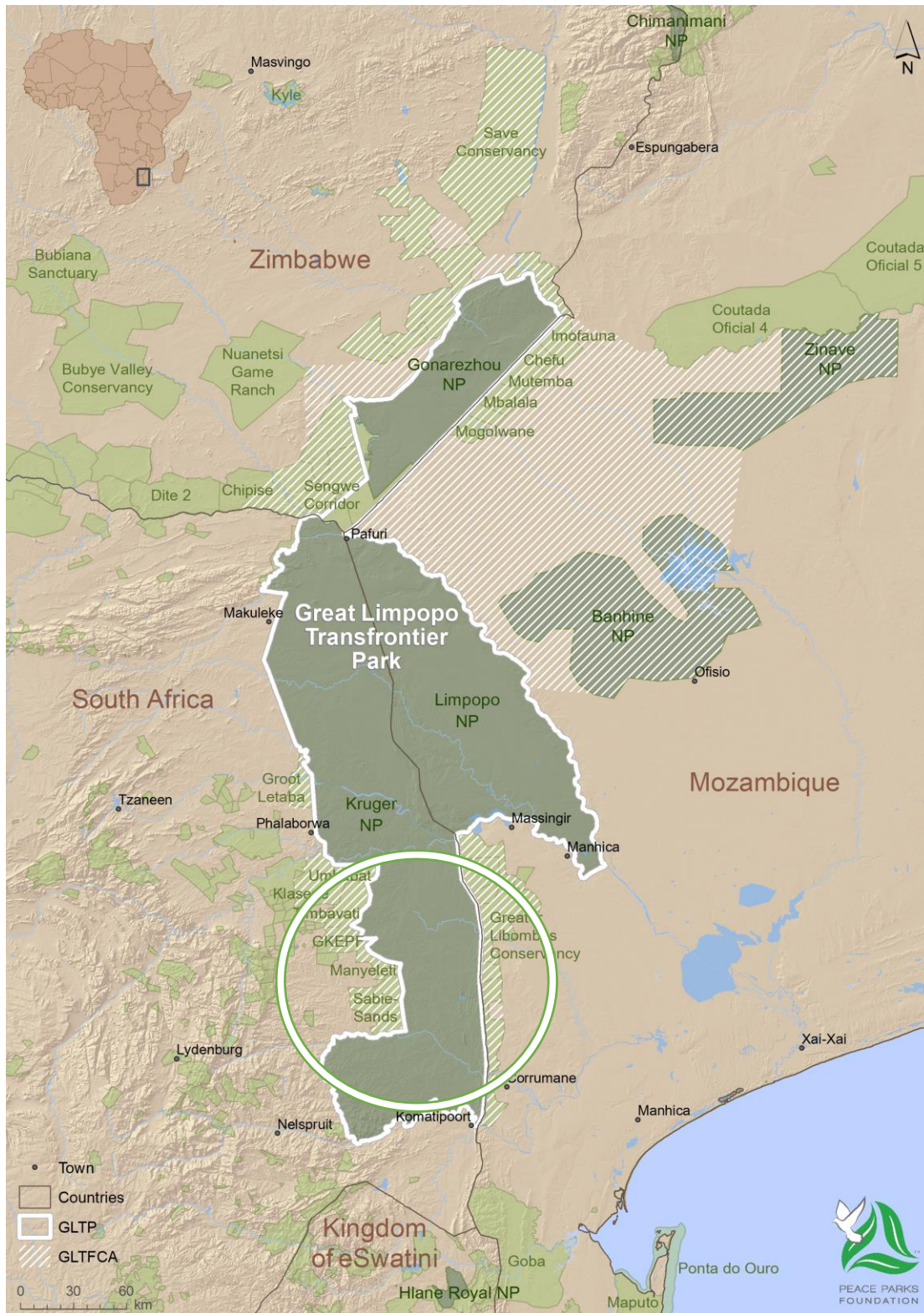


Figure 7. Map of the Great Limpopo Transfrontier Conservation Area with Great Limpopo Transfrontier Park and white ring showing research area (Peace Parks Foundation 2018).

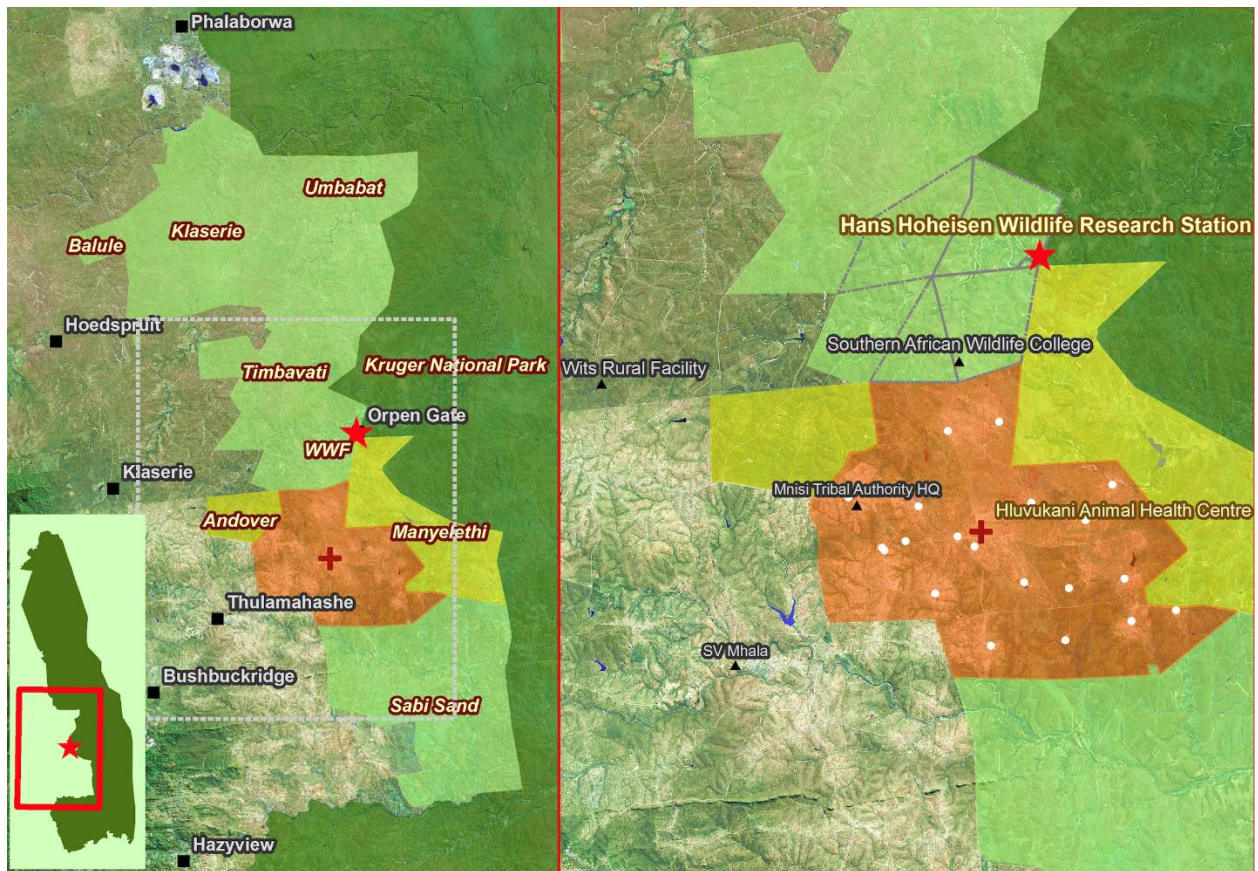


Figure 8. The study site is in the Kruger National Park (dark green) and Mnisi communal land (orange). The base for the study is the Hluvukani Animal Health Clinic/Centre and the Hans Hoheisen Wildlife Research Station. (Courtesy of Dr L van Schalkwyk).

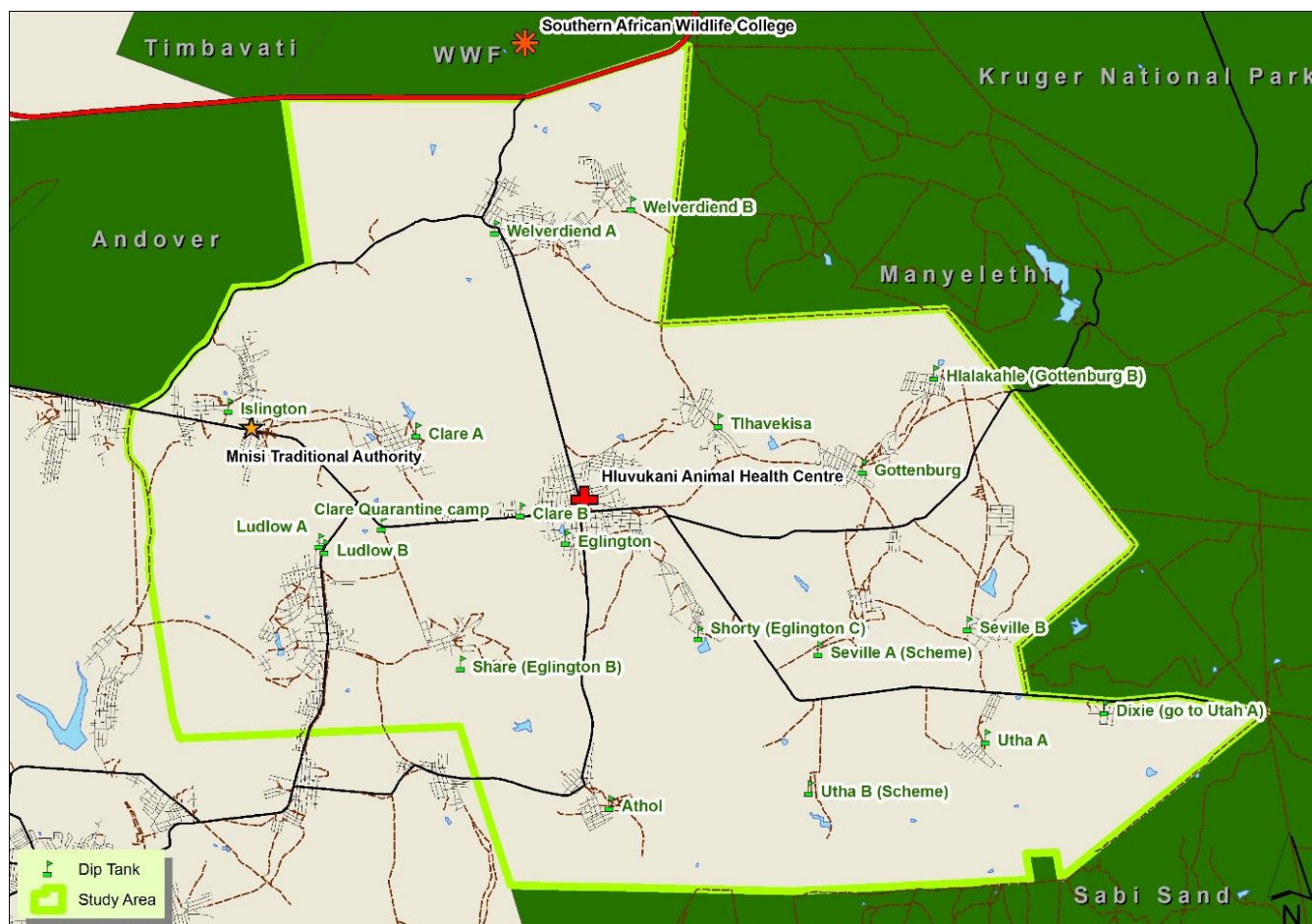


Figure 9. Mnisi communal land study site with diptanks (green flags). Image courtesy of Mnisi Community Programme, University of Pretoria.

Brucella abortus is the most abundant zoonotic *Brucella* species in South Africa. Cattle are the main domestic reservoir of *B. abortus* in South Africa (Mcdermott & Arimi 2002). In the province neighbouring the study site, communal cattle were found to have a prevalence of 0-15.6% (Hesterberg et al. 2008).

The Mnisi community (Figure 9), in our study, has 40 000 humans, 16 000 cattle, 3 500 goats and about 4000 dogs. Communal rangeland farming is practiced (Figure 10), where the cattle and goats are housed at night and sent out to graze or browse during the day. There is limited animal husbandry infrastructure at the households (Figure 11), normally just fencing to keep the animals housed at night. Each village will have one to two diptanks (Figure 12), which are maintained by the provincial state veterinary services. The

diptanks, where the cattle go to weekly, have wooden races to examine the cattle in large numbers for foot and mouth disease. The races are also used for cattle management procedures such as blood sampling and vaccination.



Figure 10. Veterinary students conducting a farmer questionnaire on disease control showing the extensive pastoral farming nature of the study site.



Figure 11. The most common method of restraint of cattle for examination and sampling at the study site is tying the horns to a pole.



Figure 12. A cattle dip tank is a focal point for farmer education and disease control and where most of the sampling of domestic animals was conducted in the study site.

RATIONAL STATEMENT AND OBJECTIVES

Brucellosis is a neglected zoonosis according to the WHO and could be an important infection in “One Health” communities infecting numerous domestic and wild animal species and humans and a causing a significant health burden in humans and domestic animals that is going unnoticed. Communities living close to wildlife, with their domestic animals, might be at higher risk, than communities not near wildlife reservoirs.

The goal of this research is to better understand the epidemiology and transmission patterns of *Brucella* infections in animals and humans of the Mnisi community and wildlife in the neighbouring Kruger National Park, which are both in the Greater Limpopo Transfrontier Conservation Area.

This research is made up of five objectives (studies) to achieve this goal:

1. To review the literature on brucellosis in wildlife in Africa to collate information on wildlife infection and potential reservoir to domestic animals and humans.
2. To identify *Brucella* spp. infecting buffalo neighbouring the Mnisi community.
3. To assess the presence of *Brucella* spp. infection in cattle and if present identify the organism causing brucellosis in cattle in the Mnisi community.
4. To assess the presence of *Brucella* spp. infection in goats and dogs in the Mnisi community.
5. To explore the prevalence of brucellosis in humans.
6. To field test the *Brucella abortus* strain 19 vaccine using the current government vaccination protocol to monitor the emergence of specific antibodies and therefore understand the influence that may have on objective three above.

RESULTS

The results and discussions are divided into five chapters.

1. Chapter 1 is a systematic review and meta-analysis of brucellosis in wildlife in Africa. This review focuses on detection, isolation and prevalence studies of *Brucella* spp. in wildlife in Africa, but also includes control and prevention studies.
2. Chapter 2 is an investigation into the detection and identification of brucellosis in buffalo in the wildlife park neighbouring Mnisi community.
3. Chapter 3 refers to the studies on the detection, prevalence and isolation attempt of brucellosis in cattle and the detection studies in goats and dogs in the Mnisi community. These studies have been published.
4. Chapter 4 is a serological detection investigation in humans in the Mnisi community. The studies are of sick individuals presenting with fever at three government clinics and high-risk healthy individuals presenting at the government diptanks for cattle inspection for foot and mouth disease. These studies have been published as a more diverse study into eight zoonotic diseases in humans.
5. Chapter 5 is a study on the immunological response to S19 vaccination in case group of heifers versus a control group of male calves in the Mnisi community. This study has been published.

CHAPTER 1

Brucellosis in wildlife in Africa: a systematic review and meta-analysis

Abstract

This study aims to consolidate current knowledge of wildlife brucellosis in Africa and analyse available predictors of infection. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines were followed searching the Web of Science, Scopus, Cochrane library, Africa-wide Info and Wildlife and Ecology Studies Worldwide databases and Google scholar website. Information on species, test used, test results, area, rainfall, livestock and wildlife contact and year of study data were extracted. The systematic review revealed 42 prevalence studies, 9 disease control articles and 6 articles on epidemiology. *Brucella abortus*, *Brucella melitensis*, *Brucella inopinata* and *Brucella suis* were reported in wildlife in nine different articles. The prevalence studies revealed serological evidence of brucellosis in buffalo, antelope (positive in 14/28 species), carnivores (4/12) and other species (7/20) over the last five decades. A meta-analysis was performed, after eight studies were removed due to irrelevant species or insufficient information, on the remaining 34 prevalence studies. Pooled prevalence was estimated using an inverse variance heterogeneity model and meta-regression to identify factors associated with seroprevalence was done using a zero-inflated negative binomial model. Buffalo populations were more likely to be infected and had a higher seroprevalence than other species; the pooled seroprevalence was 13.7% (95% CI: 10.3-17.3%) in buffalo, 7.1% (95% CI: 1.1-15.5%) in carnivores and 2.1% (95% CI: 0.1-4.9%) in antelope. The pooled prevalence in the buffalo and antelope had a heterogeneity over 75%, which heterogeneity means the results must be interpreted with caution. There was a positive

prevalence association with high rainfall areas (≥ 800 mm) compared to low rainfall areas (< 500 mm) and studies published after 2000 had a positive prevalence association compared to before 1980. Domestic animal contact was found to have a positive prevalence association in antelope and carnivore species, but not in buffalo, suggesting buffalo may be a reservoir species able to sustain *Brucella* infection without the influx of bacteria from other host species. These results may facilitate more appropriate selection of preventive and control measures against brucellosis at the wildlife/livestock/human interface.

Introduction

Brucellosis caused by *Brucella* spp. is a disease of significant economic, public health and veterinary importance. Since its identification over 120 years ago in humans it has been isolated in wide variety of animals and found to have a global distribution. The predominant pathological aetiological agents in humans are *Brucella abortus* and *Brucella melitensis*, which are predominantly carried by large and small ruminants respectively.

Brucellosis in wild animals in Africa has been documented in a variety of countries since the early 1960's with serological studies and some *Brucella* isolations in many wildlife species (Bengis 1998). Most studies have been serological prevalence surveys to try to better understand the epidemiological situation in wildlife, with the understanding that wildlife infected with *Brucella* spp. may have implications to domestic animals and humans.

The objectives of this systematic review were to update our answers to the questions:

- I. Which wildlife species have been exposed to brucellosis and where are they found?
- II. Which *Brucella* species are infecting wildlife species?
- III. Are wildlife species a brucellosis risk to domestic animals and vice-versa?

Materials and Methods

Systematic review protocol

The guidelines made by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) were followed.

Literature search and data collection

We searched the Web of Science (1910 - June 2017), Scopus (1823 - June 2017), Cochrane library (June 2017), Google Scholar (June 2017), Africa-wide Info (June 2017) and Wildlife and Ecology Studies Worldwide (June 2017). We used the Boolean Operators “or” and “and” for our search and used the following terms, in free word test and topic or subject heading:

- I. “(Brucella OR brucellosis) AND
- II. (wildlife OR wild) AND
- III. Africa”.

No time limits were set. The databases search revealed 304 articles. The Web of Science (237), Scopus (20), Cochrane library (0), Africa-wide info (42) and Wildlife and ecology studies worldwide (5) (Figure 1).

Inclusion criteria, quality control and data extraction

The titles and abstracts or the full article were then screened by the primary author to ensure the following criteria:

- I. The article appeared in a peer-reviewed journal and books.
Conference proceedings and lay-media were excluded,
- II. The article was for a study partially or fully conducted in Africa,
- III. The article referred to brucellosis or *Brucella* spp.,
- IV. The article involved wildlife (undomesticated animals living in the wild),
- V. The article either provided information on prevalence, incidence or isolation of *Brucella* spp. or the article provided information on control, diagnosis, epidemiology or risk factors to brucellosis in wildlife in Africa.

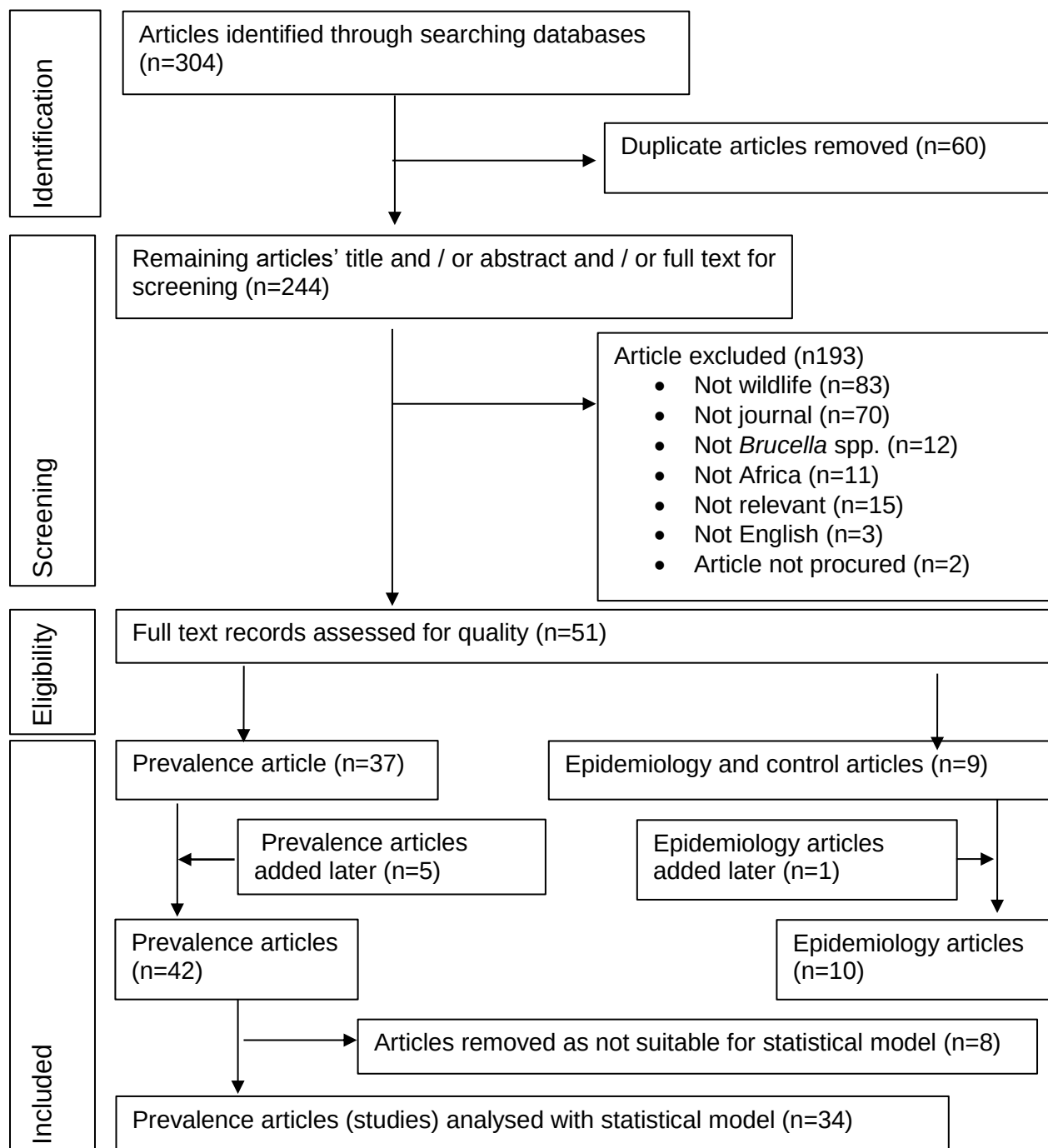
Duplicates removed 60 and screening with above criteria removed 193 articles. The remaining 51 articles, plus 6 added articles selected from references in the reviewed articles were separated into epidemiology and control (10) and bacteriological and prevalence (42) categories. The results were then analysed and reported in the results section. Bacteriological results were reported separately.

The prevalence study articles were grouped and the detail of the articles was assessed as to whether they had the following requirements for the meta-analysis:

- I. The species, study sites, sample sizes and results were described
- II. The species were terrestrial mammals, excluding rodents
- III. Serology were done and test described
- IV. Risk factors for brucellosis infection had estimates of association.

Prevalence studies that did not meet the above requirements were not included in the meta-analysis. Eight of the above studies were removed before statistical analysis for the following reasons: the study was of fish (El-Tras et al. 2010), dolphins (Lane et al. 2014), rats (Salem et al. 1974), no sample sizes given (Condy 1968; Condy & Vickers 1969; Bell et al. 1977), no serological tests were done (Gradwell et al. 1977) and one study used a card test not used by any other study leaving 34 studies for the meta-analysis.

Fig 1. Flow diagram of study methodology



The remaining prevalence study articles had the following variable extracted for the statistical model:

- I. Location of study
- II. Rainfall extrapolated from place of study
- III. Livestock wildlife contact (none, low degree, high degree, unknown)
- IV. Species involved
- V. Number of animals
- VI. Years of study
- VII. Type of study
- VIII. Diagnostics used
- IX. Test results.

The rainfall was calculated by using gridded mean annual precipitation data for the period ~1970-2000 (Fick & Hijmans 2017) obtained at 10-minute spatial resolution from www.worldclim.org (accessed 22 Sep 2017). Annual rainfall was recorded as the cell value for the location of the study if reported, or the mean of all the cell values contained within the geographic extent of the study location. For protected areas, shapefiles were obtained from www.protectedplanet.net (accessed 22 Sep 2017) and spatial overlays performed in ArcGIS 10.2 (Esri, Redlands, CA).

Statistical analysis

Group data were used for analysis, where a group represented data for a particular species and location that could be identified from a publication. Some publications therefore yielded several groups, each of which was therefore regarded as a separate

study to be included in the meta-analysis. Meta-analysis of prevalence was done for all groups combined, as well as separately for each species category, using the double arcsine transformation (Barendregt et al. 2013) in an inverse variance heterogeneity model, implemented in the MetaXL 5.3 add-in for Excel (EpiGear n.d.). In this model, each study is weighted by the inverse variance of its prevalence estimate, but the variance of the pooled estimate is inflated to account for the heterogeneity (Doi et al. 2015). Heterogeneity of estimates was assessed using the Higgins I^2 statistic (Higgins et al. 2003), with $I^2 > 75\%$ indicating high heterogeneity, and statistical significance of heterogeneity was assessed using the Cochrane's Q statistic. In order to visually represent the data, multiple groups within a publication were aggregated and separate meta-analyses of prevalence were done for each species category. Assessment of potential publication or selective reporting bias was done using funnel plots, overall and by species category.

To investigate the factors associated with variation in seroprevalence, i.e. heterogeneity, meta-regression was done using a zero-inflated negative binomial model. The number of animals tested was used as the exposure variable, thus adjusting for differences in sample size and also appropriately weighting each group in the meta-regression. The negative binomial component modelled the number of positive reactors (i.e. the seroprevalence within an infected population), while the inflation component accounted for the excess number of zero outcomes by modelling the odds of the outcome being zero (i.e. the population not being infected). Predictor variables assessed were species, year, annual rainfall, serological test used and the degree of contact with livestock. The model

was developed by backward elimination, with variables retained if significant ($P < 0.05$) or if they acted as confounders. Due to the likely differing nature of the wildlife-livestock interface for different wildlife species categories, the interaction term between species and livestock contact was also assessed. Robust (Huber-Eicker-White-sandwich) error variances were used to account for clustering. The suitability of the negative binomial model compared to a Poisson model was assessed using a likelihood ratio test of the null hypothesis that the overdispersion parameter (α) equals zero. The fit of the zero-inflated negative binomial model compared to the regular negative binomial model was assessed using Akaike's information criterion (AIC), with lower values indicating better fit. Meta-regression analysis was done using Stata 15.1 (StataCorp, College Station, TX, U.S.A.) and significance was assessed at $P < 0.05$.

Results

Epidemiology and disease control studies

The five epidemiological articles (Bengis & Erasmus 1988; Worthington & Bigalke 2001; Eisenberg et al. 2012; Mühldorfer et al. 2017; Schiemann & Staak 1971) and five articles (Roy et al. 2011; Munag'andu et al. 2006; Ducrotoy et al. 2017; Muma et al. 2014; Bekker et al. 2012) with a focus on control covered a variety of topics. These articles come to the following conclusions. Dynamical modelling using compartment models for infected and susceptible hosts such as wildlife and livestock could be integrated into strategic disease surveillance to tackle disease control and understand the multi-host pathogen system for diseases such as West Nile virus, Rift Valley fever and brucellosis (Kathleen A Alexander et al. 2012). But, reservoir dynamics in wildlife populations are complex and often without

sufficient knowledge about the specific role of the species in maintaining the pathogen in the system (Kathleen A Alexander et al. 2012). A brucellosis network modelling study excluded wildlife because of the difficulty in parameterising wildlife-related transmission rates needed for compartment models and suggested these rates are better modelled as random quantities (Roy et al. 2011).

Brucella abortus biovar 1 is reported to have limited impact on buffalo (*Syncerus caffer*) in the wild, even though it does cause abortions in buffalo (Worthington & Bigalke 2001). Few *in utero* deaths were found in more than 3000 pregnant buffalo culled and a 12-15% annual increase despite predation (Bengis & Erasmus 1988). Yet, in another study buffalo serologically positive for brucellosis had a lower body condition score, increased mortality, but did not affect fecundity and the population growth in infected herds was lower than uninfected herds (Gorsich et al. 2015).

Brucellosis appears to be a disease that has moved from domestic animals to wildlife, without wildlife being a source to cattle (Bengis & Erasmus 1988). Yet, wildlife in Africa was later seen as contributing to the re-emergence of the disease (Bekker et al. 2012). Interaction between Kafue lechwe antelope (*Kobus leche kafuensis*) and cattle was seen as an important risk factor for increased brucellosis in Zambia (Muma et al. 2014). Brucellosis may have originated from cattle and it has been suggested that it has now established itself in Kafue lechwe antelope, which could become a reservoir for other animals (Muma et al. 2011). Black lechwe antelope (*Kobus leche smithemani*), currently uninfected could be easily infected if cattle move into their area (Muma et al. 2011). There

is a cautious statement that gregarious wildlife species such as buffalo, eland (*Taurotragus oryx*), impala (*Aepyceros melampus*) and wildebeest (*Connochaetes taurinus*) were found to have a higher seroprevalence than more solitary animals such as black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros (Ducrotoy et al. 2017).

The creation of large landscapes under the transfrontier conservation initiatives have allowed sharing of ecological systems by humans, wildlife and domestic animals and may promote inter-species transmission of *Brucella* spp. (Ducrotoy et al. 2017). The presence of *Brucella* spp. in domestic animals and wildlife compounds the public health risk, especially to resource poor communities living in this ecological setting (Ducrotoy et al. 2017). The control of brucellosis in domestic animals is key to reducing the risk to humans. Infected bush meat can be a risk to humans in shared ecosystems (Kathleen Anne Alexander et al. 2012). Yet, the control in wildlife is hardly practical (Ducrotoy et al. 2017). Surveillance of wildlife may need to be added to routine domestic animal surveillance as wildlife could be a potential source of a direct source of infection for humans (Kathleen Anne Alexander et al. 2012). But, there is a need to investigate the performance of serological tests in wildlife (Ducrotoy et al. 2017). Atypical *Brucella* spp. have been found in frog species that are common in many parts of Africa and are used as human consumption as food or traditional medicine, indicating amphibians may play a bigger role in public health than thought (Mühldorfer et al. 2017).

Bacteriological studies

There were nine studies found to detail the identification of *Brucella* spp. from across the continent (Table 1). They were from a variety of species including buffalo, antelope, rat, fish and frog.

Table 1. *Brucella* spp. isolation studies in Africa with biovar, year of study, and country.

Species	Country	Year	Brucella	Biovar	Author
Rodents	Kenya	1963	<i>B. suis</i>	3 *	(Heisch et al. 1963)
Buffalo (<i>Syncerus caffer</i>)	Tanzania	1969	<i>B. abortus</i>	3	(Kalinin & Staak 1973)
Impala (<i>Aepyceros melampus</i>)	Tanzania	1971	<i>B. melitensis</i>	1	(Schiemann & Staak 1971)
Wild rats	Egypt	1974	<i>B. abortus</i>	3	(Salem et al. 1974)
Buffalo (<i>Syncerus caffer</i>)	South Africa	1977	<i>B. abortus</i>	1	(Gradwell et al. 1977)
Waterbuck (<i>Kobus ellipsiprymnus</i>)	Zimbabwe	1969	<i>B. abortus</i>	1	(Condy & Vickers 1969)

Eland (<i>Tragelaphus oryx</i>)	Zimbabwe	1972	<i>B. abortus</i>	1	(Condy & Vickers 1972)
Nile catfish (<i>Clarias gariepinus</i>)	Egypt	2008	<i>B. melitensis</i>	3	(El-Tras et al. 2010)
African bullfrogs (<i>Pyxicephalus edulis</i>)	Tanzania	2012	<i>B. inopinata</i>		(Eisenberg et al. 2012)

Table 1 legend.

* Possible misclassification because the species and the biovar were ascribed by phenotypic characterization only.

Spatial distribution of prevalence study results

The studies were predominantly in southern and eastern Africa. The most being in Zimbabwe (11), South Africa (9) and Tanzania (7) (Table 2).

Table 2. Prevalence studies per country in decreasing order with associated region (according to the African Union).

Country	No of Studies	Region
Zimbabwe	11	Southern
South Africa	9	Southern
Tanzania	7	Eastern
Zambia	4	Southern

Namibia	3	Southern
Uganda	2	Eastern
Kenya	2	Eastern
Egypt	2	Northern
Botswana	2	Southern
Democratic Republic of Congo	1	Central
Mozambique	1	Southern

Statistical analysis of prevalence studies

There were buffalo, 28 antelope, twelve carnivore and twenty other species tested for brucellosis in the selected prevalence studies (Table 4). The prevalence of positive results varied from zero to 100% but all the 100% positive studies only had one animal in the study. Buffalo had the greatest number of studies per species at 25, followed by 13 for impala, 11 for blue wildebeest (*Connochaetes taurinus*) and ten for giraffe (*Giraffa camelopardalis*). The studies used the RBT, the serum agglutination test, (SAT), the complement fixation test (CFT), indirect and competitive enzyme linked immunosorbent assays (ELISA) and the fluorescence polarization assay (FPA). The RBT and SAT were grouped in the analysis and labelled agglutination tests (AT).

Table 4. Range of serological prevalence results and number of studies by species arranged in groups: buffalo, antelope, carnivores and all other species.

Buffalo	No of studies	Seroprevalence (%)
Buffalo (<i>Syncerus caffer</i>)	25	0-53
Antelope		
Blesbok (<i>Damaliscus dorcas phillipsi</i>)	1	0
Bushbuck (<i>Tragelaphus scriptus</i>)	5	0-10
Dik dik (<i>Rhynchotragus kirkii</i>)	1	0
Duiker (<i>Silvicapra grimmia</i>)	2	0-2.7
Eland (<i>Taurotragus oryx</i>)	8	0-27.3
Gemsbok (<i>Oryx gazella</i>)	1	0
Grant's gazelle (<i>Nanger granti</i>)	2	0
Grysbok (<i>Raphicerus sharpei</i>)	1	4
Hartebees (<i>Alcelaphus buselaphus</i>)	3	0
Impala (<i>Aepyceros melampus</i>)	13	0-11.5
Impala black faced (<i>Aepyceros melampus petersi</i>)	1	0
Klipspringer (<i>Oreotragus oreotragus</i>)	1	0
Kudu (<i>Tragelaphus strepsiceros</i>)	8	0-3
Lechwe (<i>Kobus leche</i>)	2	0
Lechwe black (<i>Kobus leche smithemani</i>)	1	0
Lechwe Kafue (<i>Kobus leche kafuensis</i>)	3	10-42.3
Nyala (<i>Tragelaphus angasi</i>)	2	0
Reedbuck (<i>Redunca arundinum</i>)	5	0

Roan (<i>Hippotragus equinus</i>)	3	0
Sable (<i>Hippotragus niger</i>)	4	0-5.2
Springbok (<i>Antidorcas marsupialis</i>)	3	0
Steenbok (<i>Raphicerus campestris</i>)	3	0
Suni (<i>Nesotragus moschatus</i>)	1	0
Thomson's gazelle (<i>Eudorcas thomsonii</i>)	2	0-2.4
Topi (<i>Damaliscus korrigum</i>)	2	2.3
Tsessebe (<i>Damaliscus lunatus</i>)	5	0-11
Waterbuck (<i>Kobus ellipsiprymnus</i>)	7	0-100
Wildebeest blue (<i>Connochaetes taurinus</i>)	11	0-27.3
Carnivores		
Bat-eared fox (<i>Otocyon megalotis</i>)	1	0
Banded mongoose (<i>Mungos mungo</i>)	1	0
Black-backed jackal (<i>Canis mesomelas</i>)	1	42.9
Civets and genets (<i>Viverridae</i>)	2	0
Genet Cat (<i>Genetta genetta</i>)	2	0
Honey badger (<i>Mellivora capensis</i>)	1	0
Leopard (<i>Panthera pardus</i>)	1	0
Lion (<i>Panthera leo</i>)	5	0-50
Serval (<i>Felis serval</i>)	1	0
Spotted hyaena (<i>Crocuta crocuta</i>)	3	0-50
White tailed mongoose (<i>Ichneumia albicauda</i>)	1	0

Wild dog (<i>Lycaon pictus</i>)	1	33.3
Others		
Antbear (<i>Orycteropus affer</i>)	2	0
Baboon (<i>Papio ursinus</i>)	5	0-27
Bottle nose & Indo-pacific humpback dolphin (<i>Tursiops aduncus</i> & <i>Sousa plumbea</i>)	1	0
Bushpig (<i>Potamachoerus porcus</i>)	1	0
East African Hare (<i>Lepus capensis</i>)	1	0
Elephant (<i>Loxodonta africana</i>)	6	0
Giraffe (<i>Giraffa camelopardalis</i>)	10	0-100
Hippopotamus (<i>Hippopotamus amphibius</i>)	4	11.1-25.5
Jumping hare (<i>Pedetes surdaster</i>)	1	0
Nile catfish (<i>Clarias gariepinus</i>)	1	9.2
Porcupine (<i>Hystrix Africae-Australis</i>)	1	0
Primates (<i>Papio</i> spp., <i>Cercopithecus</i> spp.)	1	0
Rhinoceros white (<i>Ceratotherium simum</i>)	5	0
Rhinoceros black (<i>Diceros bicornus</i>)	3	0-6.3
Rockrabbit (<i>Procavia capensis</i>)	1	0
Rodents (<i>Pedetes</i>, <i>Lepus</i>, <i>Hystrix</i> spp.)	1	0
Spring hare (<i>Pedetes capensis</i>)	1	0

Tubulidentata or Antbears (<i>Orycteropus</i> spp.)	2	0
Warthog (<i>Phacochoerus aethiopicus</i>)	7	0-1.5
Zebra (<i>Equus burchelli</i>)	9	0-100

Univariate meta-analysis of prevalence

The overall pooled estimate of seroprevalence in all wildlife species combined was 4.6% (95%CI: 2.2-7.4%), with high heterogeneity ($I^2=87\%$; $P<0.001$). Pooled estimates of seroprevalence were highest in buffalo and lowest in antelope, and heterogeneity was high in all species categories except in carnivores (Table 5). Overall, the study that had the greatest weight and influence in the analysis was the report by Madsen (1995) of 1920 impala in Zimbabwe that all tested negative; omission of this study from the analysis resulted in a pooled prevalence estimate of 6.0% overall and 3.4% in antelope.

Table 5. Pooled prevalence and heterogeneity estimates in a meta-analysis of prevalence of brucellosis in African wildlife species.

				Heterogeneity	
Species	No. of prevalence reports	Pooled prevalence (%)	95% CI	Higgins' I^2	P-value
Buffalo	65	13.7	10.3 - 17.3	82%	<0.001
Antelope	115	2.1	0.1 - 4.9	85%	<0.001

Carnivores	19	7.1	1.1 - 15.5	28%	0.130
Other	72	2.8	0.6 - 5.6	74%	<0.001
Total	271	4.6	2.2 - 7.4	87%	<0.001

Seroprevalences aggregated by publication and in chronological order, are shown for African buffalo in Fig 2a, for antelope species in Fig 2b, for carnivore species in Fig 2c and for other species in Fig 2d. The pooled prevalence and heterogeneity estimates shown in the forest plots differ slightly from those in Table 5 as sub-studies within a publication were combined.

Fig 2a. African buffalo

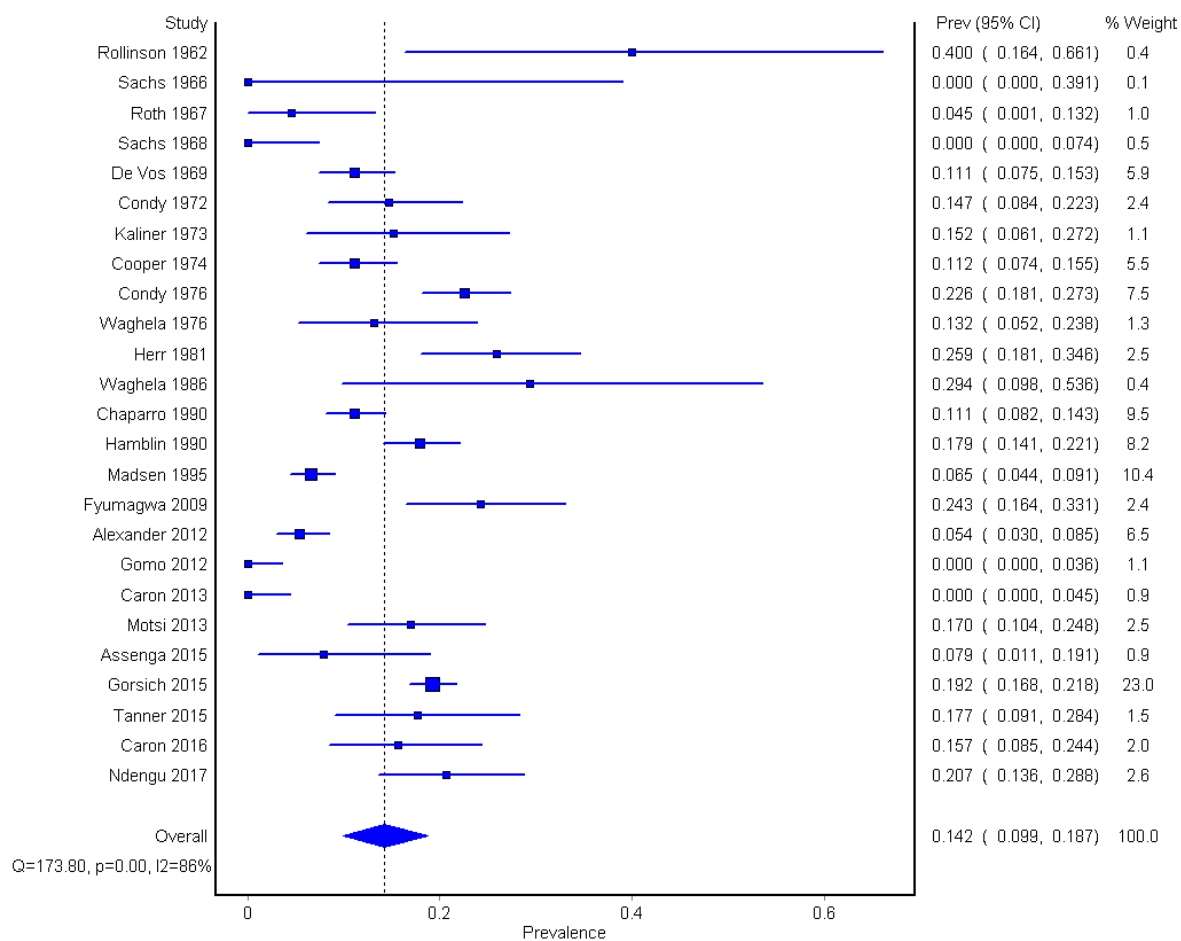


Fig 2b. African wild antelope species

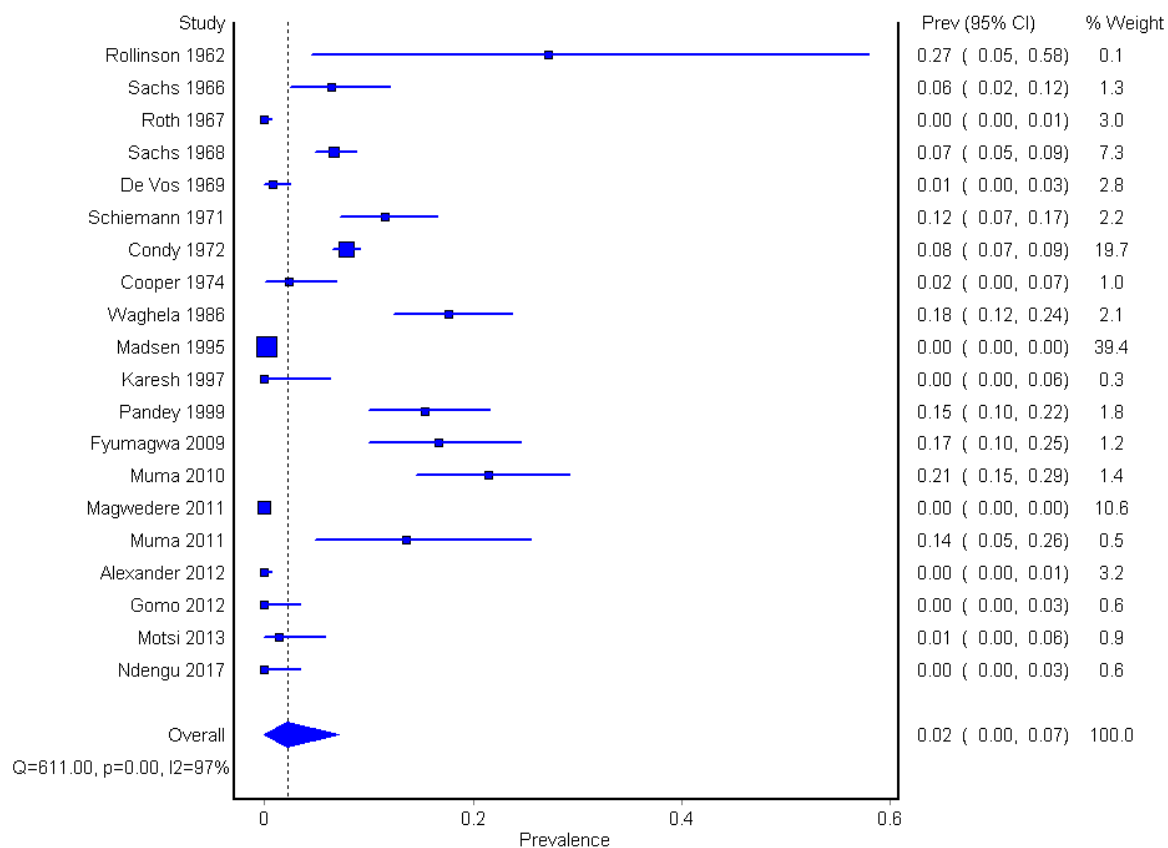


Fig 2c. African wild carnivore species

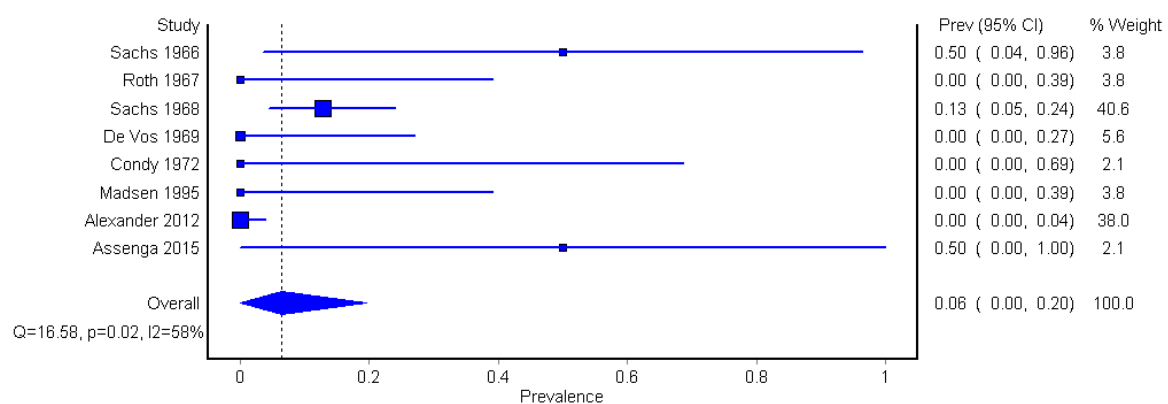
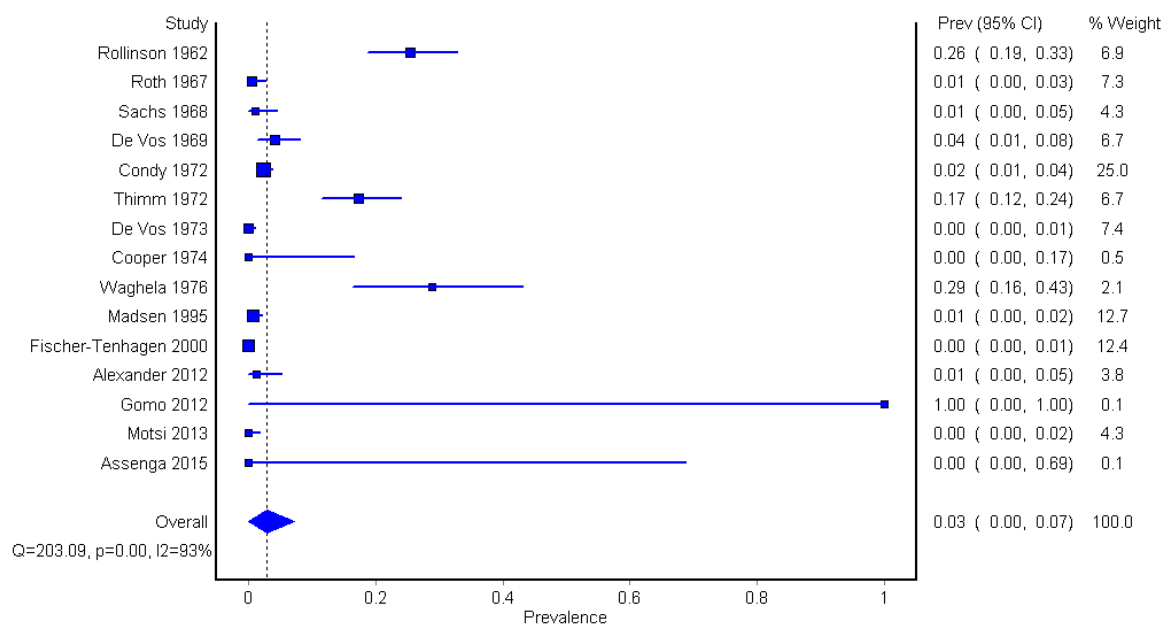


Fig 2d. African wildlife species excluding buffalo, antelope and carnivores



Figs 2a-d legend. Forest plot of *Brucella* seroprevalence in African buffalo (2a), African wild antelope species (2b), African wild carnivore species (2c) and African wildlife excluding buffalo, antelope and carnivores (2d) aggregated by publication, with weight contribution to pooled prevalence estimate, proportional to the inverse variance of each study's prevalence estimate. Studies are shown chronologically from top to bottom. Squares show point estimates, with size of square indicating sample size; horizontal lines indicate 95% confidence intervals; diamond shows point estimate and 95% confidence interval for pooled estimate. I² is the heterogeneity, indicating the proportion of variability between study results attributed to heterogeneity. Q is the Cochran's statistic used to test the null hypothesis that I²=0.

The funnel plot for all publications combined (Fig. 3) shows marked asymmetry, with lower precision, i.e. smaller, studies tending to show higher prevalence estimates. This is most

likely due to publication bias, with smaller studies showing “negative” results less likely to have been published. This was seen in all species categories except buffalo, where only very minor asymmetry was observed, suggesting that studies performed on buffalo were likely to be published irrespective of outcome. The plot also shows a large horizontal spread of points due to high heterogeneity.

Figure 3. Funnel plot of published studies on *Brucella* seroprevalence in African wildlife showing study precision vs. transformed prevalence estimate.

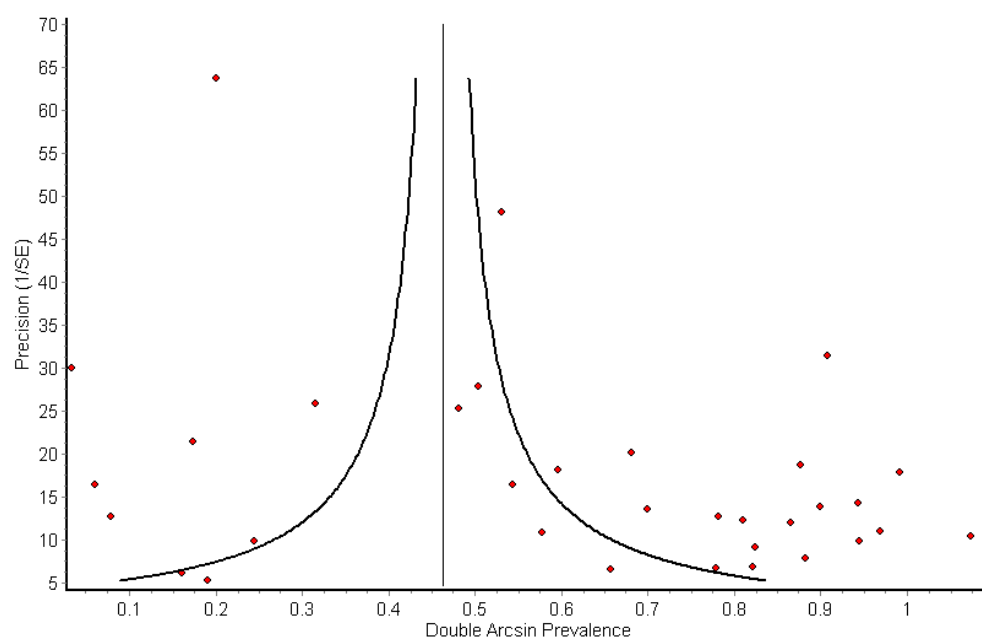


Fig 3 Legend

Curved lines indicate cut-off for statistically significant difference ($p < 0.05$) vs. pooled estimate (vertical line).

Multivariable meta-regression of selected prevalence studies

In the zero-inflated negative binomial meta-regression model (Table 6), the count model assessed factors associated with seroprevalence within infected populations, while the inflation model assessed factors associated with the population being non-infected. Several factors were associated both with the likelihood of a population being infected and with the seroprevalence within infected populations. The significance of the overdispersion parameter confirmed the suitability of the negative binomial vs. Poisson model and the AIC of 804 vs. 841 for the negative binomial model confirmed the suitability of the zero-inflated model. Note that the odds ratios (OR) in the inflation model in Table 6 refer to the odds of being negative, therefore their reciprocal, the odds ratios for being infected, are quoted in the summary below. In addition, the interaction between species and degree of livestock contact was significant in the count model ($P=0.041$), therefore the association of livestock contact with seroprevalence is shown separately for each species in Table 7.

Table 6. Meta-regression model of factors associated with seropositivity to *Brucella* in published studies of African wildlife.

Variable and category	Parameter estimate	95% confidence interval	P-value
Count model	Count ratio*		
Species			
Buffalo	1†	–	–
Antelope	0.42	0.25 - 0.68	0.001
Carnivore	1.19	0.53 - 2.71	0.673
Other	0.92	0.47 - 1.78	0.796
Year			
<1980	1†	–	–
1980-2000	1.38	0.84 - 2.28	0.208
>2000	1.73	1.17 - 2.56	0.006
Annual rainfall (mm)			
<500	1†	–	–
500-599	1.25	0.84 - 1.85	0.271
600-799	1.31	0.92 - 1.89	0.138
≥800	1.82	1.10 - 3.02	0.019
Serological test			
AT	1†	–	–

CFT	0.79	0.51 - 1.24	0.306
ELISA	1.06	0.64 - 1.75	0.815
FPA	0.36	0.19 - 0.68	0.001
Inflation model	Odds ratio†		
Species			
Buffalo	1†	–	–
Antelope	14.8	3.06 - 71.3	0.001
Carnivore	62.7	3.23 - >10 ³	0.006
Other	43.8	6.18 - 310	<0.001
Year			
<1980	1†	–	–
1980-2000	2.26	0.43 - 11.8	0.334
>2000	0.71	0.09 - 5.30	0.736
Annual rainfall (mm)			
<500	1†	–	–
500-599	0.25	0.03 - 1.82	0.171
600-799	0.51	0.11 - 2.48	0.405
≥800	0.07	0.00 - 0.91	0.043
Serological test			
AT	1†	–	–
CFT	3.45	0.77 - 15.4	0.104
ELISA	6.03	1.21 - 29.9	0.028

FPA	0.73	0.06 - 8.47	0.802
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Table 6 legend.

The AT (agglutination tests) are the RBT and SAT grouped together.

α (overdispersion parameter) = 0.37 (95% CI: 0.21-0.66; $P = 0.001$); AIC = 804

* Ratio of seroprevalence vs. reference category, within infected population

† Reference category

‡ Ratio of odds of population being non-infected vs. reference category

Table 7. Association between degree of livestock contact and seroprevalence to *Brucella* in different wildlife species categories

Level of livestock contact	Count ratio*	95% confidence interval	P-value
Buffalo			
None	1†	–	–
Low	0.92	0.49 - 1.71	0.781
High	1.05	0.63 - 1.75	0.863
Unknown	1.09	0.61 - 1.96	0.763
Antelope			
None	1†	–	–
Low	6.53	1.47 - 29.1	0.014
High	10.9	2.37 - 50.4	0.002

Unknown	8.25	1.86 - 36.6	0.006
Carnivore			
None	1†	–	–
Low	>10 ³	>10 ³ - >10 ³	<0.001
High	>10 ³	>10 ³ - >10 ³	<0.001
Unknown	0.25	0.02 - 3.04	0.278
Other			
None	1†	–	–
Low	1.24	0.36 - 4.34	0.733
High	0.17	0.03 - 0.96	0.045
Unknown	2.68	1.16 - 6.20	0.021

Table 7 legend.

* Ratio of seroprevalence vs. reference category, within infected population

† Reference category

Buffalo populations were the most likely to be infected, with antelope (OR=0.07; $P=0.001$), carnivores (OR=0.01; $P=0.006$) and other species (OR=0.02; $P<0.001$) less likely to be infected. Within infected populations, antelope showed a lower seroprevalence than buffalo (OR=0.42; $P=0.001$).

Seroprevalence appeared to increase over time, being significantly higher after 2000 than pre-1980 (OR=1.73; $P=0.006$). Although year was not significant in the inflation model

($P=0.137$), it was retained as a confounder, and populations did tend to be more likely to test positive post-2000 than during 1980-2000 (OR=3.19; 95% CI: 0.90-11.3; $P=0.071$).

Seroprevalence also had an association with rainfall; in areas with annual rainfall >800 mm populations were more likely to be infected (OR=14.3; $P=0.043$) and the seroprevalence was higher (OR=1.82; $P=0.019$).

The type of diagnostic test used was a significant source of variation in both components of the model ($P=0.022$); however, its inclusion was primarily in order to control for confounding.

The degree of livestock contact was associated with seroprevalence in antelope and carnivore species (Table 8), with a high degree of contact associated with the highest seroprevalence in antelope (OR=10.9; $P=0.001$). However, this was not seen in buffalo or in other species.

Discussion

This systematic review and meta-analysis aimed to collate the knowledge on which African wildlife species have been exposed to brucellosis by which *Brucella* species and to what extent wildlife species are able to sustain *Brucella* infections. This review found a large number of wildlife species infected or showing serological evidence of exposure to *Brucella* spp. However, the ability to maintain a sustainable infection within a given wildlife species without exposure from other sources has often not been addressed and remains

to be studied. In addition, if a population sustains a *Brucella* infection it does not mean it will transmit it to other species as that depends on many factors such as whether abortions occur, behaviour during parturition and management practices. Consequences of infected wildlife for public health depends on prevalence of *Brucella* infection and presence of bacteria as well as human activities related to infected wildlife species such as hunting, dressing of carcasses, meat handling, consumption, wildlife sampling and management in more intensive settings. The need for understanding cost-effectiveness and economic implications of a control program in low to middle income countries was highlighted in this review (Zinsstag et al. 2016). Disease control focus must be given to where the greatest health benefit will be attained, this has also been highlighted in foot and mouth disease control (Souley Kouato et al. 2018). This is difficult for a *Brucella* reservoir wildlife species as there is no vaccine available for brucellosis in wildlife (Godfroid et al. 2010).

Investigation of exposure to *Brucella* spp. in wildlife species is first done by serology. The RBT is a simple and reliable serological test recommended by the world organisation for animal health (OIE). However, when using it in wildlife, one is confronted with two problems interfering with the partly subjective reading of the agglutination, and these are likely to yield a biased result: 1) The presence of haemolysis (difficult for reading) and 2) fat (fat globules are wrongly identified as agglutinates) in the sera. Moreover, some false positive serological reaction can also be observed in agglutination tests (Erume et al. 2016). Serological studies for which only RBT has been used should always be analysed with caution and RBT should rather be compared to ELISA results and if there are large

discrepancies between results in both tests, a chloroform/centrifugation cleaning up of sera should be performed prior to RBT testing (Godfroid et al. 2016).

Although our meta-analysis allowed us to estimate a pooled prevalence for each species group, its primary aim was rather to assess the factors responsible for heterogeneity, i.e. the varying determinants of seropositivity within individuals as well as populations. The zero-inflated model allowed us to do this while correctly weighting the contribution of each study. The seroprevalence within infected populations was positively associated with studies after the year 2000 as compared to before 1980. This could be due to the disease spreading in wildlife populations recently, researchers focusing more on infected populations or it could be due to publication bias as studies with positive results are more likely to be published. The areas of higher rainfall, over 800mm, showed positive association with herds being infected with brucellosis and having a higher prevalence, which could be due the higher rainfall leading to an increased carrying capacity with a resultant increased transmission. Seropositivity in cattle in Zimbabwe was found to be progressively higher with increasing stocking density and herd size (Matope et al. 2010). In Uganda, a bimodal increase in prevalence was found in livestock associated with the rainfall seasons (Mwebe et al. 2011). This is thought to be due to the calving periods that occur during this time and hence the increased presence of bacteria due to parturition material and milk. It could also possibly be due to the fact that the bacteria survives longer in the environment in colder, wetter conditions with less sunlight as found in bison (*Bison bison*) in the USA (Aune et al. 2012). This is worth bearing in mind from a disease control point of view.

Serological studies only inform on possible exposure to *Brucella* spp. In order to identify which *Brucella* species is responsible for seroconversion, isolation of *Brucella* spp. or its DNA identification is necessary. In this respect, it is worth mentioning that recently, besides *Brucella abortus*, *B. melitensis* has been identified infecting water buffalo (*Bubalus bubalis*) in Egypt (Hamdy & Zaki 2018) and buffalo in South Africa (Henriette Van Heerden, personal communication). The latter raises questions on the origin of the infection, its sustainability in buffalo, its spillover potential to other wildlife species and its importance for veterinary public health. Additionally, molecular analysis will inform on the origin, the epidemiology and the transmission of *Brucella* spp. within and between different species but the veracity of this information is dependant to the regular molecular typing of the circulating strains of *Brucella* spp.

We found individual studies, which show proximity to wildlife reserves and porous nature of fences of wildlife reserves to be statistically significant risk factors for brucellosis in cattle (Enström et al. 2017; Ndengu et al. 2017). In our meta-analysis the livestock and wildlife contact was found to be positively associated with brucellosis infection in antelope and carnivore species and possibly other species, but not buffalo. This suggests that buffalo may be able to sustain *B. abortus* infections in infected herds without influx (inputs) from other sources. There has been a huge focus on buffalo due to other controlled diseases such as foot and mouth disease and tuberculosis for which they are also reservoir hosts. This particular scientific interest in buffalo may also explain why we found less evidence of publication bias for buffalo than in other species, i.e. in buffalo serological

results for *Brucella* were likely to be published irrespective of outcome, whereas in other species there was evidence that positive serological findings were more likely to be published than negative ones.

One of the hallmarks of *Brucella* infection is host specificity or preference given that specificity is not absolute. There is ample information in the literature highlighting that different wildlife species have been exposed to *Brucella* spp. However, very few studies address the sustainability of *Brucella* infection in wildlife. i.e. the ability to maintain the infection within a wildlife species without any external source of bacteria from a reservoir host species. The wildlife species are reservoir species, while those that cannot sustain the infection without recurrent contact with an external source (usually a reservoir species) are called spillover species (Godfroid 2002). It is of the utmost importance to be able to assess, in a given time, if a wildlife species is a reservoir or a spillover host as this has important implications for the control of the disease (Martin et al. 2011). Indeed, management/control measures should always be first implemented in reservoir species, not spillover species (Godfroid et al. 2013). In this respect, it is worth noting that the red deer (*Cervus elaphus*) is only a spillover host, rarely exposed to *B. abortus* infection in Europe, whereas the elk (*Cervus canadensis*) is considered to be a maintenance host in the Yellow Stone Greater Conservation Area and has nowadays replaced bison as a source of infection for cattle (Kamath et al. 2016). Spillback infection from elk to cattle is now of great concern and a cause of great controversy between wildlife managers, hunters and livestock owners. As there is currently no vaccine registered for wildlife

management options are currently based on spatio-temporal segregation between bison, elk and cattle (Kamath et al. 2016).

There is strong evidence that buffalo is a reservoir host for *B. abortus* (Gorsich et al. 2015). Although *B. abortus* infection has a significant impact on individual animals, it is not considered a direct threat to the sustainability of buffalo herds in Kruger National Park, South Africa (Gorsich et al. 2015; Bengis & Erasmus 1988). In a maintenance host like buffalo, higher herd infection rates and prevalences could be due to their herd dynamics of having large herds that maintain close contact within the group in comparison to other species. Tuberculosis, although a different disease, is seen to spread well between buffalo due to their social nature and large herd sizes, an average of 250 per herd in the Kruger National Park (Michel et al. 2006). This is likely to be the case for *B. abortus* infections too. It thus remains to be known if other wildlife species besides buffalo are maintenance hosts (as suggested for Kafue lechwe by Muma and Pandey (Pandey et al. 1999)) and if as such they can be potential sources of infection for other wildlife and livestock species (Godfroid 2002).

There could have been biases between studies analysed as there was not measurement conformity of contact between livestock and wildlife between the different studies. There were also assumptions made with regards to contact depending on the description of the study and place of the study sites. Ideally, in the future studies should be conducted with variables that could be compared easily e.g. distance between domestic and wildlife

species and settings well explained i.e. are there functioning fences between wildlife and domestic animals and what are the vaccination practices in livestock.

Most of the publications were from southern and eastern Africa, which leaves gaps in the knowledge for the whole continent. Also, smaller studies showing negative serological results are less likely to be published, except in the case of buffalo. It would be of benefit for more negative studies and studies from western and northern Africa to be published to give a more complete understanding of the epidemiological situation on the African continent. There is also a large gap in knowledge of brucellosis in domestic and wild suids in Africa. Although, it is thought that *Brucella suis* is prevalent in suids in Africa, its isolation from suids in sub Saharan Africa has not been reported in the scientific literature. Interestingly, it has been reported in cattle (Menshawy et al. 2014) and swine (Ibrahim 1996) in Egypt and in cattle in Zimbabwe (Ledwaba et al. 2014). *Brucella suis* is not a sustainable infection in cattle and the source of the infection has still to be identified in either domestic or wild/feral suids (Mathew et al. 2015; Fretin et al. 2013). In addition, the pig population distribution is influenced by religion taboos, which can partially explain the low numbers of isolation of *B. suis* in the Sahel countries. Lastly, new *Brucella* species have been described and the importance of *Brucella* exposure in marine mammals (Maurin 2005), ectotherms (such as frogs and fish (El-Tras et al. 2010; Eisenberg et al. 2012)) and primates (such as baboons (Whatmore et al. 2014)) needs to be assessed in the African context.

Conclusion

This systematic review highlights that exposure to *Brucella* spp. has been detected by serological studies in a wide variety of wildlife species and that brucellosis has been identified through culture in only a few wildlife species in Africa over the last five decades. The number of published studies on brucellosis in Africa has increased drastically since the year 2010, indicating a growing interest in research on brucellosis in wildlife. Although seroprevalences were higher in studies after the year 2000 compared to before the year 1980, it is not possible to ascribe this to an actual true biological trend. Epidemiological, serological and bacteriological evidences support the fact that buffalo is a reservoir species, able to sustain *B. abortus* infection without influx from other sources. Consequently, livestock and wildlife contact was found to be positively associated with brucellosis exposure for antelope and carnivore species (spillover species), but not for buffalo (reservoir species). Buffalo herds are more likely to be infected than any other species and show higher prevalences. As population growth drives wildlife habitat loss and increased contact between domestic animals and wildlife, understanding brucellosis epidemiology in wildlife is of increasing importance. Future research on brucellosis in Africa should focus on deciphering which wildlife species can sustain *Brucella* infections and what are the drivers for sustaining infections. In this respect, the recent isolation of *B. melitensis* in buffalo raises new questions about its sustainability in buffalo populations and highlights the importance of *Brucella* isolation to be able to identify changes in the epidemiology of brucellosis in wildlife.

CHAPTER 2

Isolation and identification of *Brucella abortus* and *B. melitensis* in buffalos in the Kruger national park

Abstract

Brucella spp. are known to infect a wide variety of domestic and wild animals globally. In Africa, serological studies overwhelm isolation studies, particularly for wildlife. The objective of this study is to isolate and identify *Brucella* spp. from buffalo (*Syncerus caffer*), the wildlife species found to have the highest brucellosis seropositivity in Africa. Opportunistic blood, tissue, amniotic fluid and milk samples were taken during the routine animal offtake operations in the Kruger National Park in South Africa. Blood samples were collected from ninety-six buffalo, of which 22% (21/96) were Rose Bengal Test (RBT) positive. Colonies compatible with *Brucella* spp. were isolated from tissue samples of six RBT positive animals on *Brucella* selective media. DNA was extracted and *Brucella* DNA was detected by the genus-specific 16S-23S rDNA interspacer region PCR. Besides *B. abortus*, *B. melitensis* was identified by AMOS PCR and confirmed by the Bruce ladder PCR. This is the first-time that *B. melitensis* has been identified in buffalo in the Kruger National Park. We need further research, ideally genetic testing, to identify the source of the spillover of the bacterium or if the buffalo are reservoir hosts of *B. melitensis*.

Introduction

Brucellosis in wild animals in Africa has been documented since the early 1960's with a variety of countries publishing serological studies and confirming positive isolations (Bengis 1998). Most brucellosis studies in wildlife have been serological surveys mainly to assess if wildlife infected with *Brucella* spp. may infect domestic animals (spillback) and humans.

Very few studies reporting the isolation of *Brucella* spp. in wildlife in Africa have been published, with most being in the 1960's and 1970's. Isolation and identification of *Brucella* spp. is needed to understanding the epidemiology of the infection across the

wildlife/livestock/interface (Simpson et al. 2018; Godfroid et al. 2018). In mammals, *Brucella abortus* has been identified in buffalo in Tanzania (Kalinier & Staak 1973) and Kruger National Park (KNP), South Africa (Gradwell et al. 1977), waterbuck (*Kobus ellipsiprymnus*) (Condy & Vickers 1969) and eland (*Tragelaphus oryx*) in Zimbabwe (Condy & Vickers 1972), while *B. melitensis* has been isolated in impala (*Aepyceros melampus*) in Tanzania (Schiemann & Staak 1971) and *B. suis* in rodents in Kenya (Heisch et al. 1963).

South Africa's largest national park, KNP, has over 37 000 buffalo with a brucellosis seroprevalence in populations varying from 8.7 to 47.6% (Gorsich et al. 2015). African buffalos and wildlife are considered a possible source of infection for domestic animals in the nearby communal areas (Muma, Samui, et al. 2007) and vice-versa. The predominant zoonotic *Brucella* species in South Africa is *Brucella abortus* with cattle as the main reservoir (Mcdermott & Arimi 2002). However, recently *Brucella melitensis* has also been isolated in goats in South Africa (Ribeiro et al. 1990), sable (Kriek 2018) and recently in cattle (Kolo et al. 2018). Interestingly, a case of human *B. melitensis* infection has been reported in a patient in the Western Cape province, South Africa resulting in the exposure of staff members at two medical microbiology laboratories (Wojno et al. 2016).

Methods

The animal sampling was done during the routine animal offtake in the KNP in 2017, according to the standard operating procedures of the South African National Parks. Carcasses were inspected for any gross pathology. Samples (blood, lymphnodes, spleen, testes, amniotic fluid and milk samples from lactating cows) were also collected. Rose Bengal tests (RBTs) were performed as described (Alton et al. 1988) using standardised *Brucella abortus* antigen obtained from Onderstepoort Biological Product (OBP), South Africa. The RBT positive animals then had their tissue samples subjected to culture colonies on *Brucella* selective CITA agar (Vicente et al. 2014; World Organisation for Animal Health 2016). DNA was extracted from cultures, and the *Brucella* DNA was detected by the genus-specific 16S-23S rDNA interspacer region PCR (Keid et al. 2007),

identified by AMOS PCR (Bricker & Halling 1995) and confirmed by the Bruce ladder PCR (García-Yoldi et al. 2006).

Results

Out of 96 animals that were tested by RBT, 22 were classified positive. Tissue samples from six RBT positive animals, including a milk sample from a lactating cow, yielded *Brucella* suspected colonies on *Brucella* selective CITA agar. Besides *Brucella abortus*, *B. melitensis* was identified by AMOS PCR and confirmed by the Bruce ladder PCR.

Discussion

The brucellosis seropositivity of buffalo in this study (22%) is comparable to the prevalence reported previously in the KNP (Gorsich et al. 2015). *Brucella abortus* was first isolated in 1977 in buffalo in the KNP (Gradwell et al. 1977). This study reports for the first-time *B. melitensis* infection in buffalo in the KNP and has not been reported elsewhere in Africa. However it has been identified previously in impala in Tanzania (Schiemann & Staak 1971) and recently in sable antelope (*Hippotragus niger*) in South Africa (Kriek 2018). *Brucella melitensis* has recently been isolated in cattle in South Africa (Kolo et al. 2018). Scientific literature suggests that cattle are not reservoir hosts but spillover hosts of *B. melitensis* (Muma, Godfroid, et al. 2007; J Godfroid et al. 2013). This is likely to be the case for buffalo too since they belong to the same subfamily Bovinae as bovines (*Bos taurus*). Altogether, this suggests that there is a reservoir host for *B. melitensis*, likely in livestock, i.e. sheep and/or goats in South Africa.

Brucella melitensis infection in goats was thought to have been eradicated in South Africa by 2002 (Emslie & Nel 2002). Following the human *B. melitensis* case reported in the Western Cape, South Africa, in 2016, it was noted that in 2014, the state veterinarian's investigation revealed that two other people who had lambed goats on a nearby farm had become ill and were subsequently diagnosed with brucellosis, based on serological testing. Of the 100 goats sampled on the farm in question, 44 tested positive for *Brucella* spp. on serological testing. The farm was quarantined but no attempt was made to isolate

the *Brucella* spp. (Wojno et al. 2016). These facts indicate that *B. melitensis* has not been eradicated in South Africa.

We thus hypothesize that *B. melitensis* has spilled over from its livestock reservoir to another livestock species (cattle) and to a wildlife species (buffalo). However, we cannot exclude that there may be an unknown *B. melitensis* infected wildlife species and that infection may have spilled over to buffalo. It is worth re-emphasizing that *B. melitensis* is the principal etiological agent of brucellosis in humans and is an important zoonotic disease with more than 500 000 new human cases per year.

In October 2017, a technical report entitled “Revisiting Brucellosis in the Greater Yellowstone Area (GYA)” was published by the US National Academies (National Academies of Sciences 2017). In its preface, the following is mentioned: “This report examines the changing dynamic of brucellosis in the GYA, providing a comprehensive update of what is new since the 1998 National Research Council report “*Brucellosis in the Greater Yellowstone Area*” and exploring various options for addressing the challenge of brucellosis disease management. Much has changed in the 19 years since the previous report. There is now clear evidence that transmission of *B. abortus* to domestic livestock in the GYA has come from infected elk, not bison, posing greater challenges for control of transmission to domestic species” and indicating that previous assumptions were incorrect.

Conclusion

Our findings provide new information on the epidemiology of brucellosis in buffalo. Besides *B. abortus* infection, *B. melitensis* infection has been identified in buffalo in the KNP. *Brucella melitensis* has likely spilled over from small stock, although we cannot exclude that other *B. melitensis* reservoir exists in other non-identified wildlife species. The fact that *B. melitensis* was cultured from buffalo milk, suggests that the infection could be transmitted in buffalo herds. There is thus a need to prevent *B. melitensis* spill over from livestock to buffalo populations to avoid *B. melitensis* infection in buffalo, or other

African wildlife species. We must fill the knowledge gaps in brucellosis infections at the wildlife/livestock interface in order to identify sources and transmission routes. We therefore call for action to decipher the epidemiology of *B. melitensis* infections in South African livestock.

CHAPTER 3

Documenting the absence of brucellosis in cattle, goats and dogs in a “One Health” interface in the Mnisi community, Limpopo, South Africa

Abstract

This study examines the status of the world's most common bacterial zoonoses caused by *Brucella abortus* and *Brucella melitensis* in cattle, goats and dogs in a communal rangeland farming community bordering wildlife reserves (with a buffalo *B. abortus* prevalence of 8.7-47.6%) in South Africa. Heifers aged 4 to 8 months are vaccinated against brucellosis with the live Strain 19 vaccine.

An apparent prevalence of 1.4% (21/1470) was observed in cattle sampled in a random manner and tested with the Rose-Bengal test (RBT). All cattle in herds with one positive animal were then tested with RBT (17/416 = 4.1% positive) and indirect enzyme linked immunosorbent assay (iELISA) (35/416 = 8.4% positive). Nine seropositive animals (6 RBT and iELISA positive and three only RBT positive) then had an intradermal Brucellin skin test and three were positive. The only skin test positive male was slaughtered and selected organs cultured for *Brucella* with negative results.

The cattle results showed a low seroprevalence, a decreasing seroprevalence with age after four years in females and a significant difference between the males and females. These characteristics are contrary to a wild-type infection. This suggests that *Brucella* seropositivity in cattle is due to S19 vaccination in less than one-year-old females and not natural infections. This conclusion is reinforced by a negative tissue culture result of the slaughtered Brucellin skin test positive male and the absence of *Brucella* seropositivity in goats (1/593 RBT positive, but iELISA and skin test negative) and dogs (0/315), which can be seen as potential spill over hosts. The close proximity of brucellosis-infected wildlife has not infected the domestic animals in this setting with vaccination, fencing between domestic animals and wildlife and cloven hoofed animal movement control.

Introduction

Transfrontier Conservation Areas (TFCAs) are large areas with land uses ranging from core wilderness areas and private nature reserves focused on wildlife utilisation to communal farming areas (Cumming 2004). In TFCAs the humans, domesticated animals and wildlife live in close proximity with the transfer of disease between different species of growing concern. Viral diseases such as rabies and foot and mouth disease have been shown to spill over from wildlife to domestic animals and bovine tuberculosis caused by *Mycobacterium bovis* has moved from cattle to a variety of wildlife species (Kock 2014). Another disease of public health importance is brucellosis, the world's most common bacterial zoonosis with over half a million new human cases annually (Franco et al. 2007). Brucellosis has been serologically identified in several herbivore wildlife species including African buffalo (*Syncerus caffer*) (Bengis et al. 2004; Chaparro et al. 1990; Schiemann & Staak 1971; Gradwell et al. 1977; Kaliner & Staak 1973). South Africa's largest national park, adjacent to the research site, has over 37 000 buffalo (South African National Parks 2011) with a brucellosis seroprevalence estimated to be 13-38% in 1981 (Chaparro et al. 1990; Herr & Marshall 1981) and 8.7-47.6% in 2015 (Gorsich et al. 2015). African buffalos and wildlife are considered a possible source of infection for domestic animals in the nearby communal areas (Gradwell et al. 1977; Muma, Samui, et al. 2007).

Brucella abortus is the most abundant zoonotic *Brucella* species in South Africa with cattle as the main domestic reservoir (Mcdermott & Arimi 2002). In the KwaZulu-Natal province, neighbouring the study site province, communal cattle were found to have a mean provincial prevalence of 1.45% (Hesterberg et al. 2008). Brucellosis is spread from domestic animals to humans through infected milk, milk products and contaminated tissue (Mcdermott & Arimi 2002). There is only anecdotal rare human to human transmission (Mesner et al. 2007; Meltzer et al. 2010; Vigeant et al. 1995). Brucellosis in humans is thus almost always from zoonotic origin and can lead to severe disease (Doganay et al. 1997). However, often it presents atypically with non-specific symptoms, making it difficult to diagnose (Corbel 2006). Human brucellosis is considered a neglected disease

(WHO/DFID-AHP et al. 2006), which is underdiagnosed and underreported (Franco et al. 2007), and there is no human vaccine (Jacques Godfroid et al. 2005).

There is a need to conduct studies on the prevalence of brucellosis (Gwida et al. 2010) in communities' livestock living alongside parks or wildlife reserves as they are thought to be at a greater risk of contracting brucellosis than communities not living next to wildlife (Bengis et al. 2004; Gomo, de Garine-Wichatitsky, et al. 2012; Mellau et al. 2009; Muma, Samui, et al. 2007).

Brucellosis in small ruminants is generally caused by *B. melitensis* but spillover of *B. abortus* from cattle to small ruminant is a possibility (Godfroid et al. 2011). *Brucella melitensis* has been reported in central South Africa in 1990 (Ribiero et al., 1990). Since then, there is no other report of infection in the scientific literature although infections have occurred intermittently in isolated herds of goats in central South Africa (Dr Jacob Pienaar 2015, personal communication, 3 September 2016). Although, none of these cases have occurred in the Mpumalanga province, the *B. melitensis* infection status in the Mnisi community still needs to be explored.

Dogs may also be infected by *Brucella* spp. when feeding on aborted materials and afterbirths (Cadmus et al. 2011; Wareth et al. 2017). Therefore, dogs could be an indicator of *B. abortus* and *B. melitensis* infection in cattle and goats.

The control of *B. abortus* includes several measures: systematic vaccination of heifers (Rock et al. 2009), calving management, testing for disease and slaughtering of animals deemed positive and movement control (Godfroid et al. 2004). In South Africa, heifers between 4 and 8 months are vaccinated with Onderstepoort Biological Products *Brucella abortus* strain 19 vaccine (manufactured in Pretoria, South Africa). This vaccine contains around 5×10^{10} Colony Forming Units (CFU) per 5 millilitres dose (R. Macdonald, Onderstepoort Biological Products, personal communication, September 3rd 2014) which is within the $5-8 \times 10^{10}$ CFU per dose (100 microliters), as recommended by the World

Organisation for Animal Health (World Organisation for Animal Health 2016). Males are not vaccinated because of the potential complication of orchitis (Godfroid et al. 2004).

The objectives of this study are to determine the presence of brucellosis in the domestic animal species and if present if the disease could have come from wildlife and whether there is a brucellosis public health risk in this community.

Materials and Methods

Research site

The Mnisi community research site, surrounding the Hluvukani Animal Clinic, consists of 34 000 hectares of bushveld savannah on the border of the Kruger National Park (KNP) in South Africa (Figure 1). The study area is surrounded on three sides by private and public nature reserves containing the 'big five' and associated wildlife. It is populated by around 40 000 people (Berrian et al. 2016) of whom 1497 are cattle owners with 16,418 cattle, 3350 goats (Mpumalanga Veterinary Services 2010) and an estimated 4000 dogs. There is a close association between animal handlers and their animals in this area. Cattle and goats are taken out daily to graze by humans and their dogs and brought back for overnight kraaling in the afternoon.

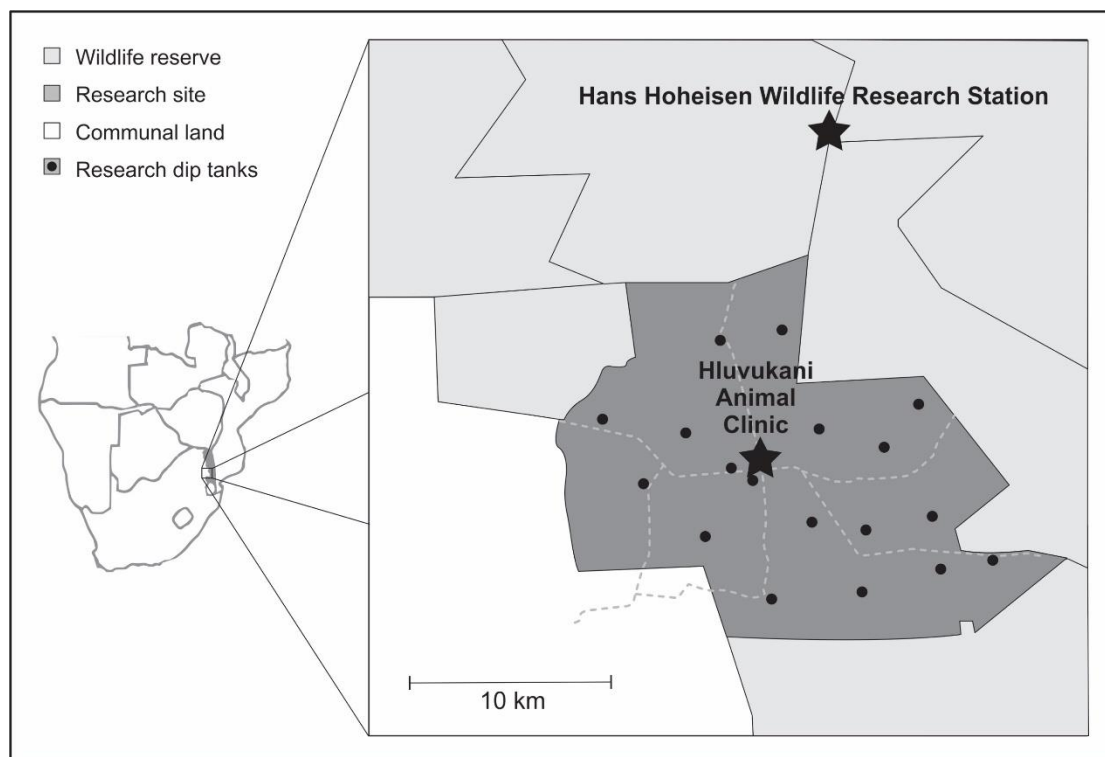


Figure 1. Research site in the Mnisi Community in South Africa. Black dots are research diptanks (see legend).

Study design

This was a cross-sectional study in cattle, goats, and dogs. The strategy in cattle was to estimate the seroprevalence, confirm brucellosis using a skin test and isolate *Brucella* spp. through organ culture of a skin test positive animal. The Rose Bengal Plate Agglutination test (RBT) was used as a cost effective and sensitive screening test to determine seroprevalence (Godfroid et al. 2010; World Organisation for Animal Health 2016). Indirect enzyme linked immunosorbent assay (iELISA), deemed more specific than RBT, and skin delayed-type hypersensitivity test (ST), the most specific test for the diagnosis of *B. abortus* infection in cattle (Godfroid et al. 2010) were then used on RBT seropositive animals as confirmatory tests. Organ culture was done on a male bovine that was deemed most likely to be infected with *Brucella* spp. by above testing regime and did not have a history of vaccination with S19. The strategy for goats and dogs was to establish the presence of *B. abortus* or *B. melitensis* infection using RBT and confirm with iELISA and also in goats the ST.

Cattle

The cattle study was conducted in two stages. The first stage was to calculate prevalence and identify infected herds of data collection and was conducted from May to June 2010. The second stage was to identify infected animals and identify the *Brucella* spp. and was conducted from April to September 2012.

The prevalence of brucellosis was expected to be 1% based on a prevalence reports and studies elsewhere in a similar context in South Africa (Hesterberg et al. 2008). The calculated sample size (1390) for calculation of prevalence corresponds to a population of 16000, an expected prevalence of 1%, a precision of 0.5% and 95% confidence (Thrusfield 1995). A herd was defined as all cattle belonging to one owner. There are 19 diptanks, 1-3 per village, each with 14-135 herds and each herd with one to over 100 cattle. Cattle owners are obliged by law and encouraged by free acaricide application to present their cattle weekly to the state veterinary services at the diptanks for foot and mouth disease inspection. This is generally well attended and statistics of cattle numbers fairly accurate. The aging of the animals was done by owners' knowledge and physical features of teeth, size and condition.

In the first stage every tenth animal in order of appearance at the diptank was sampled, with 1480 samples taken. Some of the diptanks took more than one visit as herds were missing the first sampling or due to time constraints. Ten millilitres of blood was collected from the jugular or tail vein. Blood was collected and demographic data including age, sex, and vaccination history were recorded. All sera were tested for brucellosis antibodies using the RBT. All RBT positive cattle were also tested with the iELISA. There was not sufficient funds to test all animals with iELISA so three out of five seronegative cattle (875 samples) were also tested with iELISA.

In the second stage, the entire herd of any subject seropositive by RBT or iELISA from the first stage was included in the study. All cattle were tested by RBT and iELISA. Animals that tested seropositive to both RBT and iELISA and were older than two years,

as the serological tests were deemed less likely to be false positive due to vaccination by strain 19 after two years (Plommet et al. 1976; Saegerman et al. 1999) were tested by the ST. A male that was seropositive and reactive to the intradermal skin test was slaughtered for *Brucella spp.* isolation.

Goats

During April and June 2012 goats from every diptank in the study site were sampled. A herd was again defined as all goats belonging to one owner. There are just over 600 owners with almost 3500 goats. The sample size was calculated to have a 95% chance to detect a minimum of one seropositive goat if the prevalence was 0.5 % (Lilenbaum et al. 2007). A minimum of 550 samples was necessary, which represents about 17 % of the goat population (Thrusfield 1995).

Due to the fact that goats do not present at diptanks in the same manner as cattle, herds were selected by random sampling using a table of random digits (random function of excel) and the whole herd sampled. Five hundred and ninety-three animals were sampled from 92 herds by visiting the home of the goat herd owner. Ten millilitres of blood was sampled from the jugular vein. RBT was used as a screening test, all positives and the rest of the herd were then tested with iELISA and ST.

Dogs

The dog population was estimated to be near 4000 as a household survey in two villages had given a ratio of humans to dogs as 10 to 1 and there are about 40.000 people. The sample size was calculated to have a 95% chance to detect *B. abortus* or *B. melitensis* in the dog population. We considered an expected prevalence of 1% although there is no relevant publication for this basis. A minimum of 288 samples was deemed necessary (Thrusfield 1995). *Brucella canis* was not looked for in this population.

There was no register of dog owners so opportunistic sampling was done at diptanks and Hluvukani Animal Clinic from April to July 2012. Blood was collected from 315 dog from the jugular or saphenous vein.

Disease diagnostics

Serology

All sera were separated by centrifugation at 1200g for 10 min, within 24 hours after blood collection and clot formation. One point four millilitres of each serum was stored in biological banking tubes (manufactured by Micronic, Lelystad, the Netherlands) at -20°C. The RBT (Onderstepoort Biological Products), the most suitable screening test for brucellosis was used (Corbel 2006; Godfroid et al. 2004). The RBT was performed as described (Alton et al. 1988) using standardised *B. abortus* antigen obtained from Onderstepoort Veterinary Institute, South Africa. The same cell concentration was used for both cattle and goats as recommended by the OIE. Any visible agglutination deemed the test as positive.

The indirect enzyme linked immunosorbent assay (iELISA) test is deemed more of a confirmatory test for *Brucella* spp. than the RBT (Godfroid et al. 2010). The iELISA used was the IDEXX Brucellosis serum antibody ELISA (Pourquier®) test kit (IDDEX, Montpellier, France). Samples with S/P percentage $\leq 110\%$ were considered negative for the presence of *Brucella* antibodies. Samples with S/P percentage $>110\%$ and $<120\%$ were considered suspicious positive. Samples with S/P percentage $\geq 120\%$ were considered positive for the presence of *Brucella* antibodies.

Brucellin skin test

The skin delayed-type hypersensitivity (ST) test utilising a *Brucella* antigen is more specific for *B. abortus* than a serological test (Saegerman et al. 1999; Godfroid et al. 2002; MacDiarmid & Hellstrom 1987) and is useful for monitoring brucellosis free herds (Doganay et al. 1997). This test is less likely to give false positives to other bacteria as the RBT and ELISA tests do (Bercovich 2000). The brucellin skin test was performed as

described (Saegerman et al., 1999) using standardized antigen, prepared from *B. melitensis* B115 rough strain (BRUCELLERGENE OCB®, Synbiotics Europe, France). In cattle one side of the neck skin was clipped and an average skin thickness was measured with a calliper before 100 µl of brucellin was injected intradermally. Seventy-two hours later the skin thickness at the site of injection was measured in the same manner again. If the average of the measurements three days later were more than 1.1 mm thicker than the initial measurements the test was viewed as positive. In goats the skin test was done just below the eyelid and the skin thickness measured again 48 hours later (World Organisation for Animal Health 2016). If the average of the measurements two days later were more than 1.1mm thicker than the initial measurements the test was viewed as positive.

Bacterial culture

Tissues for *Brucella* culture were obtained by sacrificing the animal. Pre-scapular lymph nodes, superficial inguinal lymph nodes, testes, epididymes and the spleen were sent to Onderstepoort Veterinary Institute Laboratory (Onderstepoort, South Africa) on ice for culture to identify *Brucella* spp. Farrell's medium has been used for bacterial culture, which contains antibiotics to inhibit the growth of other bacteria and the sample were checked from 5 to 10 days and identified according to characteristics for *Brucella* spp. (morphology, microscopy with Stamp's stain, agglutination with monospecific antisera, growth on media containing dyes, phage lysis, antimicrobial susceptibility testing). In the absence of any visible colony after 4 weeks, the results were considered as negative.

Data analysis

Serological data was analysed in logistic regressions (StataCorp. Version 11.0. 2009. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP) Sex was first used as only explanatory variable. Then, age was used as continuous explanatory variable in females only for lack of positive data in males. Design effects were evaluated but ignored since herds had no clustering effect on the data. Phase 1 ELISA data was weighted to compensate the fact that sampling fractions were different among RBT positive and RBT negative samples. In the absence of positives in a category, a Fisher

exact test was applied and 95% confidence interval was calculated using the exact method. P-values > 0.05 were considered statistically non-significant. Apparent prevalence estimates were transformed into true prevalence using published test sensitivity and specificity values. The specificity ascribed for the iELISA was higher for the estimation of the true prevalence at stage 2 of the study. The rationale for this is that animals belonging to herds that have been selected following the detection of positive RBT or iELISA individuals at stage 1, are more likely to be infected with *Brucella* spp. than the animals belonging to herds in which no seropositive animal has been detected during stage 1. We have chosen to ascribe the lower limit of specificity and the higher limit of sensitivity published in the literature for the iELISA, for stage 1 and stage 2 of our study, respectively (Godfroid et al. 2010).

Ethics approval

Ethics approval for the study was obtained from the University of Pretoria Animal Use and Care Committee (V026-12).

Results

Cattle

Stage 1

One thousand four hundred eighty cattle were sampled. Ten animals were excluded because of incomplete information, leaving 1470 for analysis. 1102 (75%) of cattle were female.

The RBT resulted in 21/1470 (1.43%) cattle being positive (Table 1). The female cattle had 19/1102 (1.72%) and the males had 2/368 (0.54%) RBT seropositive (Table 1). Almost all (20/21) of the RBT positive cattle were tested with iELISA with 12/20 (60%) being iELISA positive. Only 6/19 (32%) of the diptanks had at least one RBT seropositive animal. The RBT positive percentage per diptank varied from 0 to 4.8%. The RBT negative samples that were tested with iELISA had 13/875 (1.48%) positive. Twenty-two of the twenty-five (88%) iELISA positive samples from this stage were from females. The true prevalences were less than zero for both RBT and iELISA in males and females except when a greater specificity of 99.8% (true prevalence 2) was used which resulted in prevalences of 2.5% for females and 1% for males (Tables 1).

Table 1. Estimated apparent and true prevalences in phase 1 and phase 2 using RBT and iELISA as diagnostic tools (with 95% CI)

	Sex	N. obs.	N. pos.	Apparent prevalence (%)	True prevalence 1 (%)	True prevalence 2 (%)*
Phase 1						
RBT	Females	1102	19	1.7 (1.1 - 2.7)	-0.6 (-1.3 - 0.6)	
	Males	368	2	0.5 (0.1 - 2.1)	-2.0 (-2.4 - 0)	
iELISA	Females	673	22	2.7 (1.7 - 4.0)	-0.3 (-1.2 - 0.8)	2.5 (1.6 - 4.0)
	Males	223	3	1.2 (0.4 - 3.7)	-1.8 (-2.7 - 0.8)	1.0 (0.2 - 3.6)
Phase 2						

RBT	Females	345	16	4.6 (2.9 - 7.4)	2.9 (0.8 - 6.2)	
	Males	71	1	1.4 (0.2 - 9.3)	-0.9 (-2.4 - 8.4)	
iELISA	Females	345	35	10.1 (7.4 - 13.8)	7.7 (4.7 - 11.6)	10.3 (7.4 - 14.0)
	Males	71	0	0 (0 - 4.1)	-3.1 (-3.1 - 1.3)	-0.2 (-0.2 - 4.1)

* Using a 99.8% specificity for true prevalence 2 instead of 97.1% as for true prevalence

1

Stage Two

Twenty-four cattle herds with either an ELISA or RBT positive sample from stage 1 were included in this study. All the animals in the herds (416) were sampled. Of the 416 animals sampled 17 (4.1%) were RBT positive and 35/416 (8.4%) were iELISA positive (Table 2). RBT and iELISA positive results were found in 13/416 (3.1%) animals. The true prevalences for RBT were 2.9% females and -0.9% for males. The true prevalences for iELISA were 7.7% for females and -3.1% for males. When a greater specificity of 99.8% (true prevalence 2) was used, the iELISA results increased to 10.3% in females and -0.2% in males (Tables 1).

Six of the 13 animals that were RBT and iELISA positive were younger than two years and hence not eligible for the skin test as the serology was more likely to be influenced by vaccination (Saegerman et al., 1999). Another animal was not tested for logistical reason. This left 6 animals for the ST. A further three animals from stage two herds that were RBT positive and iELISA negative (one of these was male) were subjected to the ST. The difference in skin thickness varied from 0.42 to 2.5 millimetres three days after injection (Table 2). Three animals had differences of more than 1.1 mm (1.78, 1.89 and 2.5) (Figure 2), which is deemed the cut-off point for positives (Saegerman et al. 1999). Two of these three were RBT and iELISA seropositive, the other (1.78mm) was male and RBT seropositive only.

Table 2. Stage 2 cattle intradermal Brucellin test results with serological results. *Animal is the mother of **. Positive results are in bold and italics.

Herd	Sex	Age	RBT	ELISA	Skin thickness change in mm
1*	Female	6 years	POS	POS	<i>1.89</i>
1**	Female	4 years	POS	POS	0.46
2	Female	4 years	POS	POS	<i>2.5</i>
2	Male	3 years	POS	NEG	<i>1.78</i>
2	Female	4 years	POS	POS	0.94
3	Female	5-10 years	POS	NEG	0.36
4	Female	5-10 years	POS	POS	0.06
4	Female	8 years	POS	POS	0.16
5	Female	5-10 years	POS	NEG	0.42

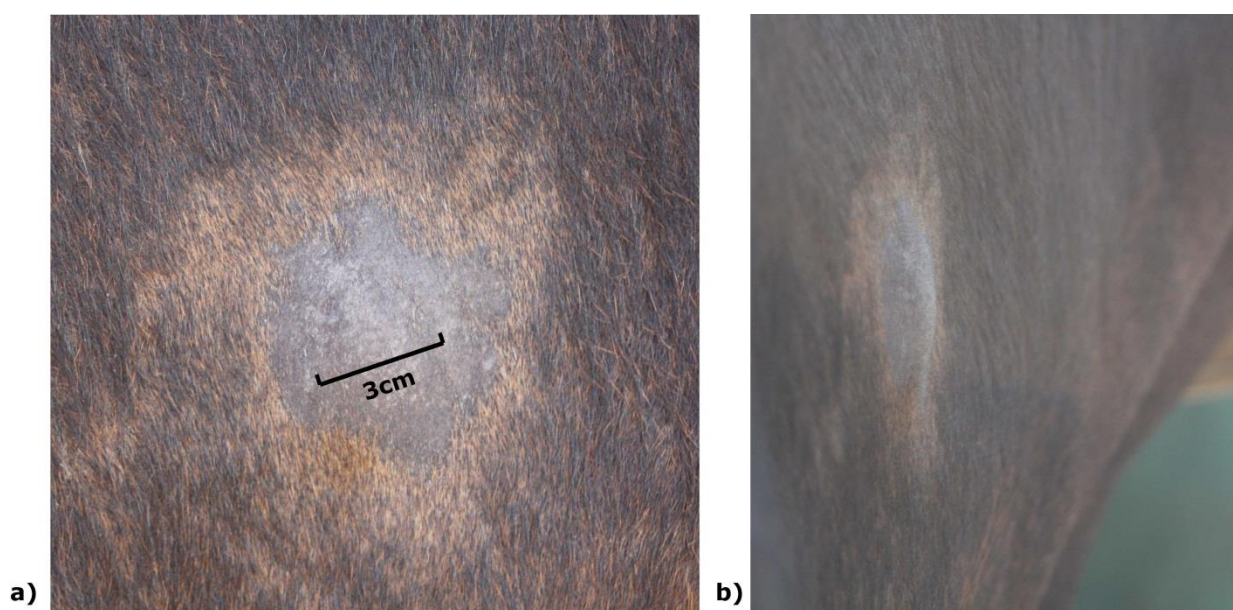


Figure 2: Positive skin test of the female in herd 2 with a 2.5 mm increase in skin thickness 72h after intra-dermal injection of 100 µl of brucellin; (a) front view; (b) profile view

In order to assess if the (male) animal that reacted in the ST was sensitised to *Brucella* spp. (wildtype or vaccine type) it was slaughtered and the pre-scapular lymph nodes, superficial inguinal lymph nodes, testes and epididymes and spleen were sampled for culture to isolate *Brucella* spp. The culture was found to be negative for *Brucella* spp.

Goats

A total of 593 goats from 92 herds were screened. The majority 490 (82.6 %) were female of different ages (aged from half a year to twelve years with a median age 3 years). An average of 39.5 (12-87) goats were sampled from each dipping tank of the study area, which represented an average of 17% of goat population in each dipping tank.

One in five hundred and ninety-three serum samples was positive for *Brucella* spp. by RBT, giving a prevalence of 0.17%. The seropositive goat was a female of more than 4 years. The rest of the herd (7 adult goats) were negative by RBT. The positive subject serum was iELISA negative. The RBT positive goat and two other herd members, all female, were tested with an ST. The average difference between the skin test readings, with a 48-hour gap, were 0.9 mm for the RBT positive goat and 0.46 mm and 0.14 for the other two goats. These results were deemed negative. As only one animal in herd was RBT positive and all herd animals were iELISA negative and the skin test was deemed negative the animal the RBT was seen as a false positive for brucellosis infection and the animal was not slaughtered for *Brucella* isolation attempt.

Dogs

Three hundred fifteen dogs (188 males and 127 females) of different ages (median age 2 years) were screened. A large majority (99 %) of the sampling was at the dipping tank, with a mean of 20 dogs per dipping tank, ranging from 12 to 44 dogs. Although the sampling was not completely random dogs have been sampled at every dipping tank, except in one (Utha Scheme) where there were no dogs.

No dog serum samples were positive by RBT. No iELISA test were done on dogs as there were no RBT positive serum samples.

Discussion

This study's objective was to assess the presence of *Brucella* spp. in a rural community's livestock that could have come from the neighbouring wild or domestic animals. As vaccination of heifers with S19 vaccine between the ages of 4 to 8 month occurs, it is challenging to make a serological distinction at low prevalences between true infection due to *Brucella* wildtype infection and vaccine related residual antibodies.

A wild type infection should show 1) an increasing seroprevalence with increasing age, 2) seropositivity in (non-vaccinated) males, 3) the ability to isolate the organism, 4) possible spill-over to other domestic animals and 5) human infections.

If we look at these factors individually:

- 1) An increasing seroprevalence with age is seen in unvaccinated buffalo in the Kruger National Park nearby (Gorsich et al. 2015; Motsi et al. 2013). Our result of a decreasing seroprevalence in female cattle older than 4 years is not supporting a wildtype infection (Gomo, de Garine-Wichatitsky, et al. 2012).
- 2) If there was wildtype infection male prevalence would be high and similar to the female as found in Zimbabwe in unvaccinated males and unvaccinated females (Gomo, de Garine-Wichatitsky, et al. 2012). The apparent prevalences in phase 1 are lower in males than females (male prevalence less than a third of female for RBT and less than half for iELISA), although not statistically significant. However, in phase 2 the male seroprevalence is very low and the female significantly higher (Fisher exact test: $p = 0.002$), especially with iELISA test. The true prevalence results in males are all less than zero, except when the higher iELISA specificity of 99.8% is used giving a prevalence of 1 in phase 1. The most likely explanation for the seropositivity in males is accidental S19 vaccination or false serological reactions. This highlights the

fact that there are residual seropositives in the females, most likely due to vaccination, that are not seen in the males.

- 3) Neither RBT nor ELISA tests are 100% sensitive or specific and although good, screening tests they should not be used alone (Matope et al. 2011). Both tests cross-react with *Yersinia enterocolitica* O:9 and other bacteria causing false positive results (Godfroid et al. 2002; Kittelberger et al. 1998; Garin-Bastuji et al. 1999). Towards the end of the study the confirmatory brucellin skin test, which is seen as more specific than the serological test although not as sensitive, was used and only positive in three of the nine cattle. The only bull that was RBT and skin test positive had its organs negative for culture of *Brucella* spp. We cannot exclude that this animal could have been vaccinated but had cleared the S19 vaccine strain or that these ST positive result is a false positive result, the ST not being 100% specific (Saegerman et al 1999).
- 4) The fact that only one goat (0.17%) was RBT positive and then iELISA and ST negative points to the likelihood of that RBT result being a false positive result and *Brucella* spp. not being present in this population of goats. These goats are thus likely not infected with *B. melitensis*. In other situations, spill over of *B. abortus* from cattle to goats was detected with a serum agglutination test (Kabagambe et al. 2001; Mellau et al. 2009). Dogs usually have close contacts with cattle afterbirths and have been seen eating them. Consequently, dogs were expected to act as a spill over species for the disease. The fact that they were all RBT seronegative strongly suggests the absence of wildtype *Brucella* spp. in the study area.
- 5) An unpublished study that sampled and tested 64 high risk humans (individuals that herd and handle livestock and veterinary staff) at 5 of the diptanks in this study found that there were no positive reactions with the human BrucellaCapt® agglutination test. Although this is a small sample size it is focussed on individuals that would have the highest risk to being infected from infected livestock (Hald et al. 2016).

The seroprevalence of brucellosis has recently been estimated in the neighbouring Kruger National Park buffalo herds to be from 8.7% to 46.7% (Gorsich et al. 2015). Our results indicate the absence of transmission of brucellosis from wildlife to livestock, in

spite of frequent fence transgressions by wildlife. The lack of brucellosis, both *B. abortus* and *B. melitensis* in the cattle, goats and dogs indicates that the control measures in cattle (strain 19 vaccination of heifers), movement restrictions for cattle and goats (due to foot and mouth disease control measures) and fencing between wildlife and domestic animals are adequate to keep brucellosis out of the domestic animal populations in this setting.

Having said the above, there are some limitations to our study. The interpretation of serological results is always difficult in the context of vaccination. We did not culture the S19 vaccine strain, which would have reinforced our conclusions. Our results strongly suggest that there is not a wildtype *Brucella* infection in livestock that could have originated from neighbouring wildlife or domestic animals. In the absence of clinical signs suggestive of brucellosis, the low number of seropositive results in cattle with decreasing antibodies with age allowed us to conclude that these antibody titres were due to vaccination rather than true wildtype infection.

The results of this study allow us to conclude that there is no risk of transmission of brucellosis to human beings through contact with livestock and dogs and no foodborne risk (mainly through the consumption of raw milk and milk products) in the area at the time of the study.

CHAPTER 4

Investigation of brucellosis in humans and human risk factors at a human-wildlife-livestock interface in Mpumalanga Province, South Africa

Introduction

Brucellosis is the commonest zoonotic disease worldwide with over half a million new cases annually (Pappas et al. 2006). Brucellosis is a febrile disease in humans that copies many other diseases, both infectious and non-infectious, and although it is rarely fatal it is severely debilitating (Franco et al. 2007). Fever is a physiological response to either a non-infectious (e.g. vasculitis or cancer) or an infectious cause (e.g. viral, bacterial, fungal or parasitic). Studies in sub-Saharan Africa investigating acute febrile illness (AFI) patients have found evidence of present or past zoonotic infections (Biggs et al. 2011; Prabhu et al. 2011). In a hospital based study in a pastoral community in Kenya 13.7% of acute febrile illness patients were found to be serologically positive for brucellosis (Njeru et al. 2016).

Humans contract brucellosis from infected animals and animal products. Infected wildlife can be a risk to hunters (Pappas 2010) and infected domestic animals to farmers and consumers of their animal products (Mcdermott & Arimi 2002). Brucellosis has a higher incidence in pastoral production systems (Mcdermott & Arimi 2002) similar to the study setting. *Brucella* spp. have been found in frog species that are common in many parts of Africa and are used as human consumption as food or traditional medicine (Mühldorfer et al. 2017). Amphibians may play a bigger role in public health than thought (Mühldorfer et al. 2017).

Despite the apparent burden of zoonotic disease, human epidemiological surveillance (Rodríguez-Prieto et al. 2015) and laboratory capacity remain suboptimal as early warning systems for (re)-emerging zoonotic diseases outbreaks or monitoring endemic disease. These deficiencies result in a negative feedback loop fostering lack of clinical awareness, which causes mis- and under-diagnosis and consequently mismanagement

of cases (John et al. 2008). In this light, the early detection of new and emerging human pathogens remains problematic (Zumla et al. 2016).

The motivation for this study is that the presence and proportion of brucellosis at this livestock-wildlife-human interface in Africa is unknown and therefore detecting if brucellosis is present in this community and whether the domestic animals or wildlife could be a source of infection is valuable.

The aims of the study were to: 1) detect whether brucellosis is present in humans in the area; 2) investigate the proportion of acute febrile illness patients and healthy cattle farmers that have evidence of brucellosis; 3) identify risk factors for brucellosis in this population and 4) provide input into the current public health surveillance, diagnostic and treatment protocols used in this setting.

This study reports laboratory results for an communal rangeland farming community, situated in the north-eastern Mpumalanga Province of South Africa, which is in close proximity to wildlife. Two groups were evaluated for exposure to brucellosis using a combination of immunological and molecular methods.

Prevalence of selected zoonotic diseases and risk factors at a human-wildlife-livestock interface in Mpumalanga Province, South Africa

Abstract

A lack of surveillance and diagnostics for zoonotic diseases in rural human clinics limits clinical awareness of these diseases. We assessed the prevalence of nine zoonotic pathogens in a pastoral, low-income, HIV-endemic community bordering wildlife reserves in South Africa. Two groups of participants were included: malaria-negative acute febrile illness (AFI) patients, called febrilers, at three clinics (n=74) and secondly farmers, herders and veterinary staff found at five government cattle dip-tanks, called dip-tanksters (n=64). Blood samples were tested using one PCR (*Bartonella* spp.) and eight antibody-ELISAs, and questionnaires were conducted to assess risk factors.

Seventy-seven percent of febrilers and 98% of dip-tanksters had at least one positive test. *Bartonella* spp. (PCR 9.5%), spotted fever group *Rickettsia* spp. (IgM 24.1%), *Coxiella burnetii*. (IgM 2.3%) and *Leptospira* spp. (IgM 6.8%) were present in febrilers and could have been the cause of their fever. Dip-tanksters and febrilers had evidence of past infection to *Rickettsia* spp. (IgG 92.2% and 63.4%, respectively) and *C. burnetii* (IgG 60.9% and 37.8%, respectively). No *Brucella* infection nor current *Bartonella* infection was found in the dip-tanksters, although they had higher levels of recent exposure to *Leptospira* spp. (IgM 21.9%) compared to the febrilers. Low levels of West Nile and Sindbis, and no Rift Valley fever virus exposure were found in either groups. The only risk factor found to be significant was attending dip-tanks in febrilers for Q fever (p=0.007).

Amoxicillin is the local standard treatment acute febrile illness, but would not be effective for *Bartonella* spp. infections, spotted fever group rickettsiosis, Q fever infections, nor the viral infections. There is a need to revise acute febrile illness treatment algorithms, educate medical and veterinary staff about these pathogens, especially spotted fever group rickettsiosis and Q fever, support disease surveillance systems and inform the population about reducing tick and surface water contact.

Introduction

Zoonotic diseases are caused by pathogens naturally transmitted directly or indirectly (e.g. by vectors) between animals (usually vertebrates) and humans. Of all currently known human pathogens, 61% are considered to be zoonotic, whilst 73% of all emerging and re-emerging infections are also zoonotic (Jones et al. 2008). An estimated 2.5 billion human cases related to zoonotic infections occur globally each year, causing 2.7 million deaths (Grace, Mutua, et al. 2012). Zoonoses are estimated to account for one quarter of the disease burden in low-income countries (Grace, Gilbert, et al. 2012). Poverty raises the risk of zoonotic disease in communities living in close contact with their livestock or with wildlife (Grace et al. 2017). Although, direct wildlife to human zoonotic transmission is rare (Kock 2014).

Studies in sub-Saharan Africa investigating acute febrile illness (AFI) patients have found evidence of present or past zoonotic infections (Table I). In populations with a high prevalence of HIV infection, this proportion of zoonotic infections may be even higher due to immunosuppression, HIV also increases the clinical severity of many zoonoses and results in a more protracted, chronic illness (Froberg et al. 2004; Biggs et al. 2011).

Table I. Selected studies of zoonotic aetiologies of acute febrile illness in sub-Saharan Africa.

Disease	Population	Result	Reference
Brucellosis	Febrile hospital patients with a history of animal contact, north-eastern Kenya (n=1067)	13.7% qPCR-positive for <i>Brucella abortus</i> , 11% ELISA seropositive for <i>Brucella</i> spp. IgM/IgG	(Njeru et al. 2016)
Leptospirosis		9% serologically confirmed acute	(Biggs et al. 2011; Prabhu et al. 2011)

	Febrile hospital inpatients (n=870), northern Tanzania	leptospirosis by MAT* (representing 20 antigen serovars)	
Spotted fever group rickettsiosis		8% serologically confirmed by ≥ 4 -fold increase in IgG to <i>Rickettsia conori</i> by IFA**	
Q fever		5% serologically confirmed acute Q fever by ≥ 4 -fold increase in IgG to <i>Coxiella burnetii</i> by IFA**	
Spotted fever group rickettsiosis	Population based infectious disease study (n=357) and febrile clinic patients (n=699), western Kenya	57% had detectable IgG against SFG antigen and 7% were PCR-positive (<i>gltA</i> assay) for <i>Rickettsia</i> spp. (<i>R. felis</i> DNA was identified)	(Maina et al. 2012)
Bartonellosis	Febrile patients, Democratic Republic of the Congo (n=155)	4.5% serologically positive for <i>Bartonella henselae</i> , <i>B. quintana</i> , or <i>B. clarridgeiae</i> spp. by IFA**.	(Laudisoit et al. 2011)

* Microscopic agglutination test; ** immunofluorescent assay

A lack of human surveillance for zoonotic diseases (Rodríguez-Prieto et al. 2015) and laboratory capacity causes a lack of clinical awareness, leading to under-diagnosis and consequently mismanagement of cases (John et al. 2008). In this light, the early detection of new and re-emerging human pathogens is also problematic (Zumla et al. 2016). Gaps in data on the burden of zoonotic infections are especially pressing in the study site, a pastoral setting where humans, their domestic animals and wildlife have frequent direct and indirect contact. The zoonoses selected for this study were based on prior information concerning infection prevalence and laboratory capacity. The study aimed to document the prevalence of nine zoonoses in this setting; investigate the proportion of acute febrile illness patients and high-risk cattle farmers, herders and veterinary staff that have evidence of exposure or infection to these zoonoses; and identify risk factors for these zoonoses. Implications for public health surveillance, diagnostic and treatment protocols used in this setting are explored.

Materials and methods

Study site

The study was implemented in three rural government primary health care clinics and five government cattle dip-tanks in Bushbuckridge Municipality, Mpumalanga Province, South Africa (Figure 1). The prevalence of HIV infection in the province is 14.5% and the municipality is situated in the Ehlanzeni District, where levels of HIV are amongst the highest of all districts in the country (between 16% and 22%) (Shisana et al. 2014).

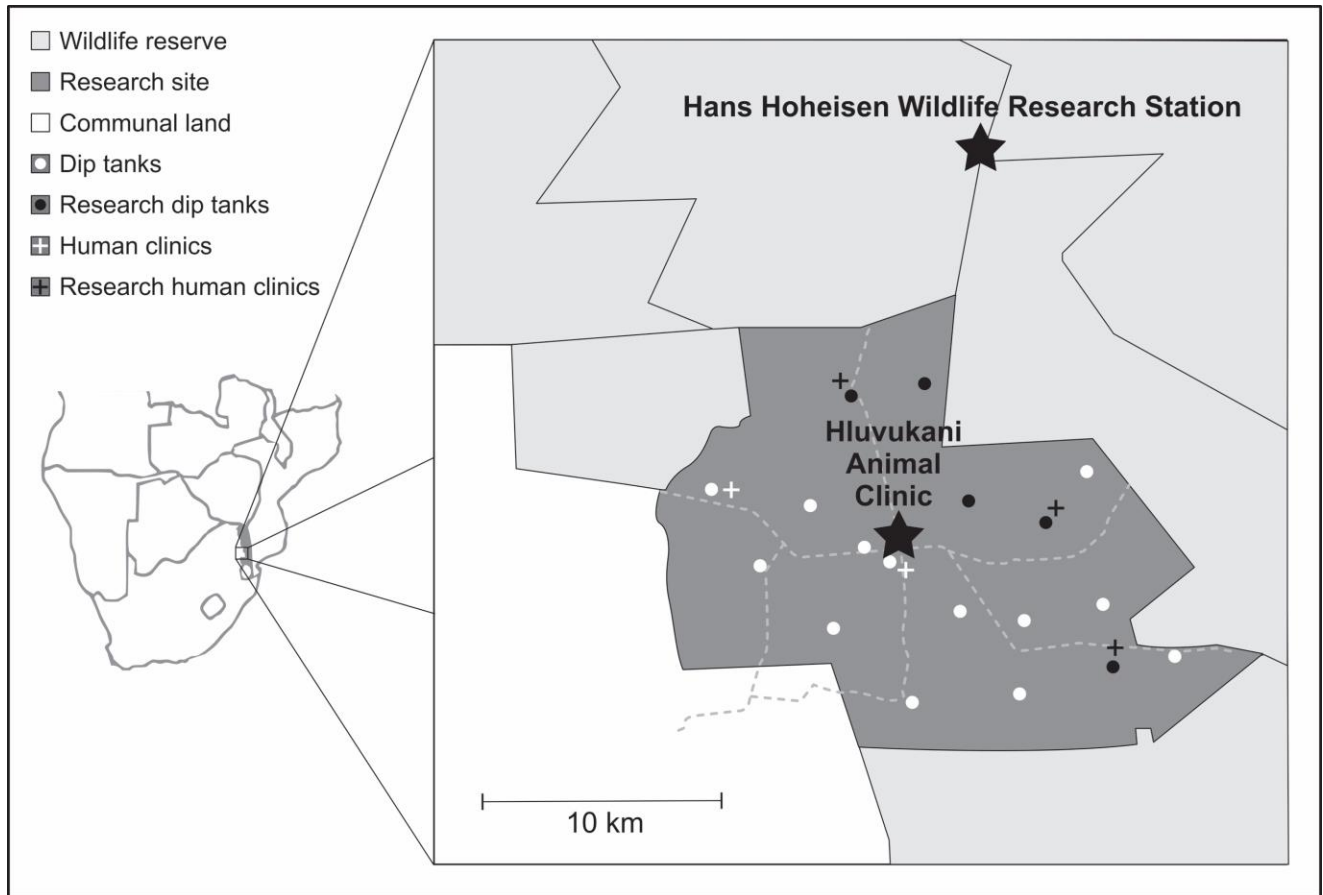


Figure 1. Location of the research site, Bushbuckridge Municipality, Mpumalanga Province, South Africa.

The study area was in the Mnisi Community Programme area, containing the Hans Hoheisen Wildlife Research Station and Hluvukani Animal Clinic, and is a joint initiative by the University of Pretoria with the Mpumalanga Veterinary Services and the Mnisi Traditional Authority, to promote sustainable livelihoods through research into human and animal health, animal production and natural resource utilisation. Approximately 40,000 people live in 8,500 households (Statistics South Africa (SSA) 2012), with 72% of all households owning at least one species of animal and agro-pastoralist farming being the primary subsistence activity (Berrian et al. 2016). Chickens are owned in 55% of households, cattle in 25%, goats in 16%, pigs in 5% and companion animals in 36% (Berrian et al. 2016). The community shares three-quarters of its boundary with wildlife reserves. In the communal area there are hares, small antelope, rodents, small carnivores and in the wildlife reserves, all wildlife typical of “big five” reserves in this

region. In these wildlife areas brucellosis has been detected in buffalo, other antelope, zebra, lions, hyenas and other animals, including monkeys (De Vos & Van Niekerk 1969; Gorsich et al. 2015). Malaria is endemic in the area, but the average annual incidence of the infection has decreased over the past 10 years, due to intensive control activities (Maharaj et al. 2013). The contribution of malaria to the syndrome of AFI has thus reduced.

Participant recruitment

The sample size calculation was done using an infinite population, where 59 samples are required to detect a disease with 95% chance when the actual prevalence of the disease is 5% (Thrusfield 1995). In addition, 64 samples give unilateral 95% confidence intervals of proportions of 12% maximum, which was deemed sufficient for the study.

The study involved two groups of participants. The first comprised patients diagnosed with AFI at the clinics, hereafter called febrilers. An AFI case was defined as a patient with documented axillary temperature $\geq 37.5^{\circ}\text{C}$, or a history of fever within the previous 72 hours, whom the clinic staff would routinely test for malaria, in terms of the clinic standard operating procedure. Patients who tested negative for malaria were referred to the study nurse to be enrolled in this study. All patients ≥ 18 years of age who met this case definition were eligible for enrolment from October 2012 to June 2013. Information was captured onto a standard case information form (CIF). This CIF inquired about a variety of symptoms and questions related to risk factors for zoonotic disease, and diagnosis, treatment and outcome. The selected risk factors were a variety of contacts (such as handling and consumption) with various domestic animals, presence at dip-tanks and tick bites. Blood samples (one coagulated for serum and one EDTA anticoagulated) for laboratory tests were drawn at the time of presentation (acute sample) and the patients were asked to return two to three weeks later to provide follow-up convalescent samples. A study nurse facilitated enrolment of volunteers at all the clinic sites.

The second group comprised cattle farmers, herders and government veterinary staff (animal health technicians and veterinarians), who attended any of five cattle dip-tanks

for weekly stock inspections and tick control in the area in one week in June 2013 (Figure 2). This group were called dip-tanksters. They were recruited for interviewing and provided blood samples as described above for the febrilers, but without follow-up samples. A CIF with the risk factors detailed above was also completed, but excluded the clinical symptoms, diagnosis or treatment and outcome information. All information was captured onto a Microsoft Excel database.



Figure 2. Sampling of dip-tanksters at a dip-tank.

Laboratory methods

Laboratory testing for all samples was performed at the National Institute for Communicable Diseases. Tests for a panel of nine known endemic bacterial and viral zoonoses in South Africa were used in this study (Table 2).

Table 2. Panel of tests performed on blood samples from acute febrile patients and healthy subjects. Sensitivity and specificity are included if available.

Disease agent/s	Diagnostic assay	Samples tested	Interpretation of results	Reference
Bartonellosis	PCR*: <i>Bartonella</i> spp. 16S/23S reran internal transcribed spacer (ITS) region (in-house) and sequencing.	Acute whole blood samples from all participants. All positive amplicons were sequenced	Fragment sizes variable depending on species approximately 640 – 788 bp for outer primers and 481 – 573 bp for inner primers	(Trataris et al. 2012)
Brucellosis	Serology (total antibodies): Brucellacapt® assay (Vircell S.L., Spain)	Acute serum samples from all participants	Titres higher than 1:320 were deemed positive	96% sens. & 97.5% spec (Casao et al. 2004)
Chikungunya, Rift Valley fever, Sindbis fever and West Nile fever viruses	HAI:** in-house assay	Serum samples from all participants	Titres higher than 1:20 were deemed positive	(Lennette & Schmidt 1979)
Chikungunya, Rift Valley fever, Sindbis fever and West Nile fever viruses	IgM capture ELISA***: in-house assay	Serum samples that tested positive per arbovirus HAI	Percentage positivity values higher than the calculated run-based or population based cut-off values	RVF: 96.5% sens. & 99.4% spec. (Paweska et al. 2005)

Leptospirosis	IgM ELISA: Panbio® <i>Leptospira</i> (Standard Diagnostics Inc., Republic of Korea).	Convalescent serum samples or acute samples where convalescent samples not available	Index values calculated using run-based cut-off values as per manufacturer's recommendations	90.8% sens. & 55.1% spec. (Desakorn et al. 2012)
Spotted fever group <i>Rickettsiae</i> spp.	IgG IFA:**** <i>Rickettsia</i> <i>conorii</i> kits (Vircell S.L., Spain)	All AFI participants and all dip- tank participants	IgG: titer of 1:40 deemed positive	85% sens. & 100% spec. (Do et al., 2009)
	IgM IFA: <i>Rickettsia</i> <i>conorii</i> kits (Vircell S.L., Spain)	All AFI participants	IgM: titer of 1:192 or greater deemed positive, or fourfold rise in titer as per manufacturer's recommendations	94% sens. & 95% spec. (Do et al., 2009)
Q fever	IgG ELISA: Panbio® <i>Coxiella burnetii</i> (Q fever) (Standard Diagnostics Inc., Republic of Korea)	Convalescent serum samples, or acute samples where convalescent samples not available	Index values calculated using run-based cut-off values as per manufacturer's recommendations	71% sens. & 96% spec. (Field et al. 2002)

	IgM ELISA: Panbio® <i>Coxiella burnetii</i> (Q fever) (Standard Diagnostics Inc., Republic of Korea)	Acute serum samples if convalescent sample not available or if tested positive with IgG ELISA	Index values calculated using run-based cut-off values as per manufacturer's recommendations	99% sens. & 88% spec. (Field et al. 2000) ¹
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* PCR: polymerase chain reaction; ** HAI: haemagglutination inhibition assay; ***ELISA: enzyme-linked immunosorbent assay; ****IFA: indirect immunofluorescence assay

The DNA for the bartonella test was extracted according to Trataris et al. 2012, from packed red blood cells (obtained after spinning down EDTA tube) using the UltraClean® BloodSpin® DNA Isolation Kit (MoBio Laboratories, Czech Republic). The tests for leptospirosis, Q fever, brucellosis and HAI tests were not done in duplicate. The convalescent samples of the acute febrile illness patients were tested and, if found positive, then the corresponding acute samples were tested to detect seroconversion. Positive HAI samples were then tested with IgM ELISA.

Statistical analysis

Data were analysed in multivariable logistic regression models, separately for the clinic and dip-tank data. Test results of each test were used as binary responses variables (in different models). Backward selection of explanatory variables was applied using $p = 0.05$ as a cut-off. The tested explanatory variables were age (continuous), dip-tank attendance, eating wild animals, history of tick bites, herding of livestock, slaughtering of animals and eating livestock (binary) for both the clinic and dip-tank data. Confidence intervals (CI) of proportions >0 and <1 were calculated using logistic predictors whereas the exact method (assuming a binomial distribution) was used for proportions = 0 or 1.

Ethical clearance

Ethical approval was obtained from the University of the Witwatersrand Human Ethics Committee (certificate number M120667) and signed informed consent obtained from study participants. Further permissions were secured from the Mpumalanga Provincial Government for the study to be done at the three clinics.

Results

Participant demographics and symptoms

In the febrilers, of 119 eligible participants, 74 (62%) were enrolled (45 were excluded as they declined consent or the study nurse could not access them). There were twenty-three (16/23 were adult) AFI patients malaria test positive during the study period, who were not included in the study. The median age of the 74 febrilers was 34 years (interquartile range [IQR] 25-47 years), 46/74 (62%) were females, of which two were pregnant. About half were febrile on examination (38/74, 51%). The median duration of illness was 3 days (IQR 2-7 days). About 10% (6/74) were referred to hospital, and 62% (46/74) received an antibiotic. A quarter did not return for follow-up blood sampling (20/74; 27%). Only 4 febrilers had with no systemic symptoms other than fever, 36/74 (49%) presented with one other symptom, 20/74 (27%) with two and 4/74 (5%) with three other symptoms. The majority presented with muscle pain (85%), while fewer had respiratory symptoms (14%). A total of 64 dip-tanksters were recruited, of whom, 46 (72%) were male and a median 55 years old (IQR 42-68 years). The median duration of dip-tank attendance was 15 years (IQR 6-30 years).

Clinical and laboratory findings

In summary, 77% (57/74) of febrilers tested positive for at least one of the zoonotic pathogens included in the panel (Table 3). The most prevalent past infection was the spotted fever group (SFG) (*Rickettsia* spp.), with 63.4% (45/71, 95% CI 51.6-73.7%) testing positive for anti-*Rickettsia* IgG, while anti-*Rickettsia* IgM was positive in 24.5% (13/53, 95% CI 14.8-37.8%) of febrilers, indicating a more recent or current infection. Of the 13 anti-*Rickettsia* IgM positive patients, twelve were anti-*Rickettsia* IgG positive. Antibodies (IgG) to *Coxiella burnetii*, the causative agent of Q fever, were detected in

38.3% (28/73, 95% CI 28.0-49.9%) of patients. Other bacterial pathogens included bartonellosis with 9.5% PCR-positive (7/74, 95% CI 4.6-18.5%), that were identified as *Bartonella vinsonii* subspecies *berkoffii* (n=1) (which has a dog reservoir), *B. henselae* (n=2) (domestic cat reservoir), *B. quintana* (n=1) (human reservoir (Zeaiter et al. 2002)), *B. thailandensis* (n=1) (rodent reservoir (Saisongkorh et al. 2009)) and two *Bartonella* positives are unnamed, but rodent borne. Leptospirosis with 6.8% (5/74, 95% CI 2.8-15.2%) group-specific IgM antibodies and brucellosis with 1.4% (1/74, 95% CI 0.2-9.0%) group-specific total antibodies were also found. West Nile and Sindbis fever antibodies were detected in 4.1% (3/73, 95% CI 1.2-12.0%) and 1.4% (1/73, 95% CI 0.2-9.1%) of febrilers respectively. No chikungunya or Rift Valley fever (RVF) antibodies were found.

In the dip-tanksters, the proportion with anti-*Rickettsia* IgG antibodies was also high at 92.2 % (59/64, 95% CI 82.6-96.7%), and 60.9% (39/64, 95% CI 48.6-72.1%) had anti-*Coxiella* IgG antibodies. No evidence for exposure to *Brucella* spp. was detected, but 21.9% (14/64, 95% CI 13.4-33.6%) tested positive for *Leptospira* IgM antibodies. For viral aetiologies, 3.1% (2/64, 95% CI 0.8-11.7%) of dip-tanksters tested positive for West Nile virus IgM, 4.7% (3/64, 95% CI 1.5-13.6%) for chikungunya and 3.1% (2/64, 95% CI 0.8-11.7%) for Sindbis virus antibodies. No RVF antibodies were detected.

Table 3. Results of laboratory tests for selected zoonotic pathogens in AFI and diptank participants with 95% confidence interval

	Febrilers			Dip-tanksters		
	Positive	Sample size	% positive (95% CI)	Positive	Sample size	% positive (95% CI)
SFG IgG	45	71	63.4 (51.6-73.7)	59	64	92.2 (82.6-96.7)
SFG IgM*	13	53	24.5 (14.8-37.8)	-	-	-
Q fever IgG	28	73	38.3 (28.0-49.9)	39	64	60.9 (48.6-72.1)

Q fever IgM**	1	43	2.3 (0.3-14.8)	0	39	0 (0-7.4)
<i>Bartonella</i> spp. PCR	7	74	9.5 (4.6-18.5)	0	64	0 (0-4.6)
<i>Leptospira</i> IgM	5	74	6.8 (2.8-15.2)	14	64	21.9 (13.4-33.6)
<i>Brucella</i> Ig	1	74	1.4 (0.2-9.0)	0	64	0 (0-4.6)
Chikungunya Ig	0	73	0 (0-4.0)	3	64	4.7 (1.5-13.6)
West Nile virus Ig	3	73	4.1 (1.3-12.0)	2	64	3.1 (0.8-11.7)
Sindbis Ig	1	73	1.4 (0.2-9.1)	2	64	3.1 (0.8-11.7)
Rift Valley fever Ig	0	73	0 (0-4.0)	0	64	0 (0-4.6)

*SFG rickettsiosis IgM (recent) done on the 45 AFI participants who were positive on SFG rickettsiosis IgG (past) and 19 IgG negative and one IgG untested participants,

**IgM for Q fever done on acute specimens for which there was no convalescent specimen and in participants that were positive for IgG.

The AFI participant arbovirus HAI positive samples were retested with IgM ELISA and had the following results: the three WNV positive samples had two negative results and one untested and the Sindbis positive was also IgM positive. Amongst dip-tank participants: for chikungunya one of the three HAI-positive samples was also IgM positive, one was negative and the last untested; both WNV HAI-positive samples were IgM negative, and both Sindbis HAI-positive samples were also IgM positive.

The only risk factor that was statistically significantly associated with a disease was attending dip-tanks in febrilers for Q fever IgG ($p=0.007$). Thirteen of the 74 (18%) febrilers were found to have previously attended dip-tanks and of those tested 77% (10/13) were IgG positive compared to 31% (19/62). None of the risk factors for the dip-tanksters were associated with the test results.

Article discussion

Our study looked at both acutely febrile participants in clinics and cattle dip-tank participants at high risk for zoonoses. Undifferentiated acute febrile illness is among the most common presenting signs in patients seeking medical care in Africa (Perkins et al. 1997; Crump et al. 2003; Van Hemelrijck et al. 2009). Despite this, the syndrome is frequently misdiagnosed, and the aetiologies still poorly understood. Febrile illness is often misclassified as malaria, especially in adults living in areas with low to moderate malaria transmission (Reyburn 2004; Chandler et al. 2008; Nankabirwa et al. 2009). Misdiagnosis leads to delays in appropriate treatment and higher case-fatality rates (Chandler et al. 2008).

This study showed that 77% of febrilers and 98% of dip-tanksters had at least one positive test. Bartonellosis (PCR 9.5%), spotted fever (IgM 24.1%), and less likely, leptospirosis (IgM 6.8%) and Q fever (IgM 2.3%) could have been the cause of the acute febrile illness in the febrilers, as IgM and PCR suggest recent or current infection. This is a valuable finding, relevant to the management of clinic patients, as amoxicillin is the standard treatment for patients with AFI, but it would not be effective for these diseases. Therefore, the AFI treatment algorithms in this and similar settings need to be reconsidered and health staff educated on these infections.

The febrilers also showed a high past exposure (IgG) to SFG *Rickettsia* spp. (63.4%) and Q fever (37.8%), not previously described in this population. The high proportion of exposure to *Rickettsia* spp. is understandable, given that 70% of tick pools and 100% of flea pools taken from dogs in the area were positive for *Rickettsia* spp. (Kolo et al. 2016). Over 70% (50/71) of febrilers had either an SFG *Rickettsia* spp. or Q fever positive result, while 95% of dip-tanksters had either an SFG *Rickettsia* spp. or Q fever positive result. We expect veterinary staff or livestock herders and farmers presenting at the dip-tanks with their livestock to be at a higher risk for zoonotic diseases (especially for those diseases transmitted by vectors/reservoirs and associated with livestock) in this population. However, the only diseases where dip-tanksters had a confidence interval

higher than febrilers was spotted fever (IgG). There are differences between proportions of participants positive in the febrilers and dip-tanksters but this is not strongly statistically supported in this study.

Dip-tanksters are on average 21 years older than febrilers and therefore have had more time for exposure to pathogens. Although, the number of zoonotic illnesses per individual was not correlated with years of dip-tank attendance in the dip-tank group, contact with cattle dip-tanks was positively associated with Q fever exposure in AFI patients. The higher proportion of spotted fever IgG in dip-tank participants could be from cattle owners having higher levels of exposure to ticks, related to their dogs and other domestic animals that they will interact with daily. There was little evidence of other risk factors contributing to positive serology. The lack of positive associations may be due to a relatively small sample size and test-positives. Also, the environment is homogenous such that even if people say they do not own or work with animals, some contact is inevitable, due to the free-ranging nature of the animals and their close proximity to humans; for example, almost a fifth of the febrilers had some contact with the dip-tanks.

The higher bartonellosis test-positive proportion in febrilers could be as the test was detecting organisms and not antibodies, which would be more closely associated with patients presenting with fever than compared to dip-tanksters. *Bartonella* infection rate in other studies was also found to be high in HIV-infected individuals (Frean et al. 2002; Trataris et al. 2012), and although our participants were not tested for HIV, HIV prevalence may have been higher in the febrilers that had an average age of 34 years compared to the dip-tankster with an average age of 55 years, which correlates with the provincial age and HIV prevalence demographics (Shisana et al. 2014). The *Bartonella* species found in the febrilers were associated with humans (n=1) and domestic dogs and cats (n=3) and rodents (n=2). *Bartonella* infections have been found to be common in cats (23.5%) and dogs (9%) in a southern African study (Trataris et al. 2012), and in fleas (36.3%) elsewhere in Africa (Leulmi et al. 2014).

A review of leptospirosis in sub-Saharan Africa, noted very few human studies, with most data being from animals studies (De Vries et al. 2014). In our study evidence of leptospirosis was higher in the dip-tanksters, although with confidence intervals overlapping, which again makes sense given that transmission to humans is from the urine of infected animals, commonly rodents, pigs and other domestic animals. Cattle in a neighbouring province had a leptospirosis prevalence of almost 20% (Hesterberg et al. 2009) and a South African study found leptospirosis in 4.7% (25/530) of dogs tested (Roach et al. 2010). What is more difficult to understand is the dip-tanksters having a threefold higher IgM (which should indicate current or recent infection), than AFI patients. The dip-tank participants may be more consistently exposed to *Leptospira* spp., but not present with fever at the clinic. The *Leptospira* ELISA IgM is known to have a low specificity (55%) and diagnostic accuracy, which may influence the reliability of the results (Desakorn et al. 2012).

The brucellosis results are reflective of what has been found in domestic animals in the area. Although brucellosis is present in the region (Hesterberg et al. 2008), unpublished studies suggest that there is no brucellosis in the cattle, goats and dogs in the study area (Dr G Simpson, Pers. comm., 15th June 2017). This indicates that the control mechanisms of heifer vaccination and movement control may be keeping the disease at bay, even though there are high seroprevalences in buffalo (8.7-47.6%) across the neighbouring reserve fences (Gorsich et al. 2015).

The febrilers had 5.5% (4/73) showing at least one positive arbovirus positive result compared to the dip-tanksters with 10.9% (7/64). Little is known about the epidemiology of chikungunya virus in the region, although baboons (*Papio ursinus*) were previously found to be the primary vertebrate reservoir (Mcintosh et al. 1977). The vectors are tree canopy-associated *Aedes fucifer-taylori* group, which are less likely to be found in a peri-domestic situation, and which rarely feed on humans. The dip-tank participants may be more at risk as they take their cattle and goats into the surrounding bushveld for grazing. The presence of West Nile and Sindbis virus infections, although at low levels, is of

interest as patients are not routinely tested for these diseases. There are also veterinary implications associated with their presence.

Vectors can move pathogens between wildlife and domestic animals species (Bengis et al. 2004). This phenomenon is of growing concern with the expanding human populations, habitat destruction, intensification of wildlife and potentially greater contact between humans and wildlife (Bengis et al. 2004). The diseases investigated in this study can be transmitted to humans through vectors, food, contact or contamination of the environment, which has implications for vector, hygiene and environmental control. Malaria control in the area, for example, has had a negative impact on some mosquito populations, which may affect other mosquito-transmitted diseases. Further vector control, disease control in animals, as well as food and other hygiene improvements, would be beneficial in decreasing the burden of these diseases. There is also a need to educate the population about reducing tick and surface water contact.

A limitation of our study was that sample sizes were small. There was only one nurse covering three clinics for AFI patients, which made it difficult to access all the eligible patients. The dip-tank participants were opportunistically selected in a non-random manner. This could influence the extrapolation of the results to all the dip-tank attendees.

Article conclusions

Bartonella spp., *Rickettsia* spp., *Coxiella burnetii* and *Leptospira* spp. are present and could be undetected causes of fever and illness in a community bordering wildlife reserves in the north-east of South Africa. Some acute febrile illness patients (9.5%) were infected with *Bartonella* spp. associated with human, rodent and domestic dog and cat reservoirs. A high proportion of cattle dip-tank attendees and acute febrile illness patients had evidence of past infection or background exposure to *Rickettsia* spp. and *Coxiella burnetii*, which is of relevance to public and veterinary health. Current acute febrile illness treatment algorithms at the clinics prescribe amoxicillin, which would not be effective for these diseases. The low proportion of brucellosis and viral infections is encouraging, but

should continue to be monitored. The vector control strategies for livestock and public health should take into account ticks and fleas as they transmit *Bartonella* spp. and *Rickettsia* spp. Animal husbandry should be adapted to include means of infection prevention, such as disinfection, after contact with animal excretions to decrease Q fever, leptospirosis and potentially brucellosis. This is an opportunity for public and animal health organisations to discuss control programs to tackle common afflictions. Wider zoonotic surveillance should be continued, and veterinary and public health services should be aware of these diseases, their diagnoses and treatments, and educate their patients and owners in prevention methods and care seeking.

Discussion

Brucellosis is a cause of acute febrile illness. The results provide valuable insights on the presence of brucellosis in this area. Our study looked at evidence of brucellosis in both acutely ill (AFI) participants in clinics and healthy (diptank) participants at high risk for zoonoses. The brucellosis results indicate that the disease is absent or rare in the area. The fact that there are no positive results in the high-risk group indicate that the domestic animals in the area are not transmitting brucellosis to humans. The blood of the positive result in the clinic patient was haemolysed, which could have influenced the result or they could have contracted the disease outside of the study area in a neighbouring infected area. These results are reflective of what has been found in domestic animals in this area. Although, brucellosis is present in the region (Hesterberg et al. 2008), unpublished studies indicate there is no brucellosis in the cattle, goats and dogs in the study area (Dr G Simpson, Pers. comm., 15th June 2017). This indicates that the control mechanisms of heifer vaccination, movement control of cloven hoofed animals due to foot and mouth disease restrictions and fencing between wildlife and domestic animals may be keeping brucellosis at bay even though there are high seroprevalences in buffalo (8.7-47.6%) across the neighbouring reserve fences (Gorsich et al. 2015) and in domestic animals in other areas.

There was no statistical significant differences between proportions of participants positive in the AFI and high-risk healthy group (diptank participants). There was no evidence of any risk factors contributing to positive serology. The lack of positive associations between risk factors and disease may be due to the fact that the sample sizes and test-positive numbers are small and the environment is ubiquitous, such that even if people say they do not own or have contact with animals and their parasites, some contact is inevitable, due to the free-ranging nature of the animals and their close proximity to humans. Thirteen of the 74 (18%) of the clinic participants also had some contact with the diptanks.

A limitation of our study is that sample sizes were small. There was only one nurse covering three clinics for AFI patients, which made it difficult to access all the eligible patients, hence the high non-enrolment rate of 38%.

Conclusions

Our conclusion is that brucellosis is not being transmitted from the local domestic animals to humans. One acute febrile illness patient could have had a false positive or got the disease from surrounding areas with infected animals and wildlife. The current control strategies for brucellosis should be maintained and wider zoonotic surveillance should be continued to get a bigger epidemiological picture and veterinary and public health services should be aware of brucellosis diagnoses and treatments, and educate their patients and owners in prevention methods and care seeking.

CHAPTER 5

Immunological response to *Brucella abortus* strain 19 vaccination of cattle in a communal area in South Africa

Abstract

Brucellosis is of worldwide economic and public health importance. Heifer vaccination with live attenuated *Brucella abortus* strain 19 (S19) is the cornerstone of control in low and middle income countries. Antibody persistence induced by S19 is directly correlated with the number of Colony Forming Units (CFU) per dose. There are two vaccination methods: a “high” dose ($5-8 \times 10^{10}$ CFU) subcutaneously injected or one or two “low” doses (5×10^9 CFU) by the conjunctival route. Previous research in cattle at the study site found a low sero-prevalence of brucellosis and this study is to understand the influence of “high” dose vaccination on serological testing and therefore the possible implications on disease control.

This study included 58 female cases, vaccinated at Day 0, and 29 male controls. Sera was drawn repeatedly and tested for *Brucella* antibodies using the Rose Bengal Test (RBT) and an indirect Enzyme-Linked Immunosorbent Assay (iELISA).

The cases showed a rapid antibody response with a peak RBT positivity (98%) at two weeks and iELISA (95%) at eight weeks, then decreased in an inverse sigmoid curve to 14% RBT and 32 % iELISA positive at 59 weeks and at four and a half years 57% (4/7 cases) demonstrated a persistent immune response (RBT, iELISA or brucellin skin test) to *Brucella* spp.

Our study is the first of its kind documenting persistence of antibodies in an African communal farming setting for over a year to years after vaccination of a “high” dose S19, which can be difficult to differentiate from a wildtype *Brucella abortus* infection response using serology alone. A recommendation could be use a “low” dose or different route for

vaccination that produces a shorter period of antibody response with equivalent protection.

Introduction

Brucellosis caused by *Brucella abortus*, a gram-negative coccobacilli bacteria is a widely-distributed zoonosis of importance to public health (Corbel 2006). Animal brucellosis affects mammals including livestock and wildlife and commonly causes abortion in females and orchitis in males (Chaparro et al. 1990; Elaine M S Dorneles et al. 2015). In humans, symptoms include fever, malaise, orchitis and a variety of nonspecific symptoms (Doganay & Aygen 2003). The Kwazulu-Natal province in South Africa, with a setting similar to the study site, had an estimated prevalence of 0-1.5% (Hesterberg et al. 2008, p.15). In southern Africa, studies in pastoral production systems have shown the prevalence of brucellosis to be higher with larger herds, extensive movement of animals and co-mingling of herds at common grazing sites (Mcdermott & Arimi 2002).

In South Africa, where heifer vaccination is mandatory, cattle are seen to be the greatest source of outbreaks (Hesterberg et al. 2008). Detection of disease is done using the Rose Bengal test (RBT) as the serological screening test and the complement fixation test (CFT) as the confirmatory test. Both tests can give false positive reactions due to strain 19 (S19) vaccination (World Organisation for Animal Health 2009). Testing is voluntary, except for dairy cattle, where it is compulsory. The government has a voluntary Bovine Brucellosis Scheme to encourage animal owners to participate in eradicating brucellosis through testing and slaughtering of infected animals (South African Government Gazette 1988). Testing frequency is influenced by veterinary services resources and animal owners' motivation. Infected herds are quarantined, infected animals removed and the herd only deemed brucellosis-free after two negative tests at least three months apart (South African Government Gazette 1988). Although, in resource limited settings slaughter of positives is often not possible due to financial limitations (Moriyón et al. 2004).

Vaccination of heifers along with brucellosis testing and slaughter of positive animals is the foundation for control of brucellosis in cattle in endemic areas (Nicoletti 2010). Males are not vaccinated because of the potential complication of orchitis (Olsen & Palmer 2014) and the limited role they play in transmission (Olsen & Tatum 2010). Humans who work with these animals or consume their milk and meat are indirectly protected through the vaccination of cattle (Corbel 2006; Godfroid et al. 2011). Several strains are used for vaccination, where the disease endemic, as in many low to middle income countries (LMICs), S19 is preferred to RB51 (Moriyón et al. 2004).

The World Organisation for Animal Health (OIE) advises vaccinating $5-8 \times 10^{10}$ organisms (“high” dose) of S19 to heifers between 3 and 8 months of age (World Organisation for Animal Health 2016). S19 is effective at inducing an immunological response but, unlike RB51, this response interferes with the serological screening of natural infections (Schuurman 1983). S19 has a O-chain lipopolysaccharide, unlike RB51, that results in antibody persistence (Schurig et al. 2002). Little is known about the duration of the antibody response to S19 using the government recommended dose of 5×10^{10} organisms between 4 and 8 months in heifers and its interference in serological diagnostics in the longer term in the study site.

A reduced dose of 3×10^8 to 5×10^9 organisms (“low” dose) can be given by subcutaneous or conjunctival route to decrease the antibody response (World Organisation for Animal Health 2016; Nicoletti 1984). The downside is that this route is harder to administer, especially in a setting without practical and safe animal handling facilities to protect the animal and vaccinator from injury or self-vaccination. The only similar study in an African setting used a reduced dose of 3×10^9 organisms and found that in 92 adult communal cattle the RBT positive results decreased from 48% at one month post vaccination to 2.2 % at 9 months and disappeared within one year (Schuurman 1983). In Brazil, the same dose was given to adult animals and by 9 months the RBT positives dropped to 0% from 100% at 1 month and indirect enzyme-linked immunosorbent assay (iELISA) to 4% from 100% at 1 month (Poester et al. 2000). In Argentina, a greater but still reduced vaccination dose of 3×10^{10} organisms in dairy heifers resulted in 100% buffered plate agglutination

test (BPA) positive and 95% iELISA positive at 3 weeks, but 10% BPA positive and 0% iELISA positive at 50 weeks (Aguirre et al. 2002). Other studies have documented antibody and cell-mediated responses to vaccination (Elaine M.S. Dorneles et al. 2015; Nielsen & Duncan 1988; Saegerman et al. 1999; Stevens, Olsen & Cheville 1995), but it is difficult to use these studies to make inferences about using serological tests for disease control post vaccination in a communal farming setting as they looked at different indicators of the immune response. These studies also used different colonies forming units (CFU) vaccination dosages, ages of animals, breeds and settings.

The objective of this study was to follow the serological response to the *Brucella abortus* S19 vaccine (“high” dose) in cattle in a rural community bordered by wildlife protected areas using the current government vaccination protocol. We describe the proportion of cattle that seroconverted and the persistence of antibodies in the blood. These results will provide valuable information for understanding brucellosis screening in this study site and similar settings in Africa where “high” dose S19 vaccine is used.

Research methods and design

Setting

The Mnisi community consists of 30 000 hectares of savannah biome on the western border of the Kruger National Park in South Africa and is in the Greater Limpopo Transfrontier Conservation Area (Figure 1). The Hluvukani animal clinic, in the middle of the community was used as the base for the study. A quarter of households in the community, of around 40.000 people, possess cattle (Berrian et al. 2016). As there is a close contact between the cattle, their products, and their handlers, this community is at risk of contracting brucellosis if the disease was endemic in their livestock. The area is surrounded on three sides by private and public nature reserves containing elephant, rhino, buffalo, lion, leopard and associated wildlife, which is seen as a potential source of brucellosis (Muma et al. 2006). This study adds to previous research in the area that explored the brucellosis epidemiology in humans, cattle, goats and dogs and suggested that brucellosis was absent in these species at this site (Simpson et al. 2017). This

epidemiological situation allowed us to follow the serological responses induced by the S19 vaccination over time in the absence of a *B. abortus* wildtype infection, while in a rural communal livestock farming community bordering the Kruger National Park, South Africa.

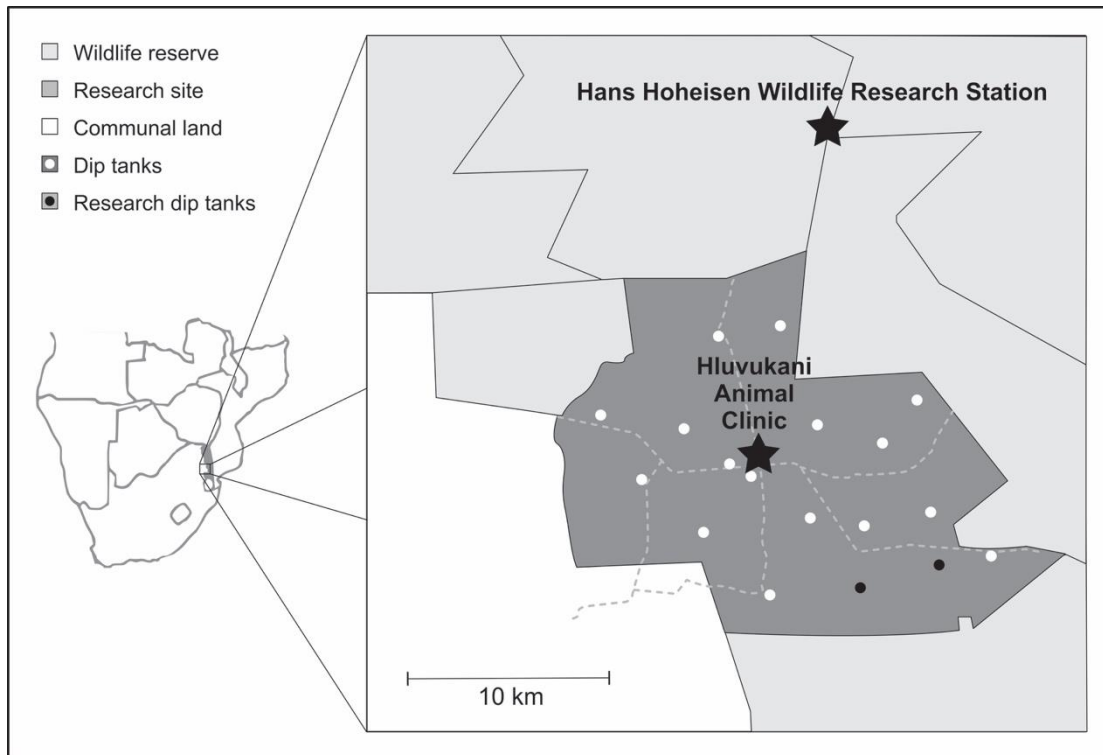


Figure 1. Map of the Mnisi research site. The sampling was done at the two research dip tanks indicated by black dots (see legend).

The Mnisi community has an estimated 16 000 cattle in the proximity of 19 dip tanks. Dip tanks were initially constructed to control tick-borne diseases and are used as administrative centres for livestock health surveillance. The study was discussed with the dip tank committees and six owners volunteered to have their herds enrolled in the study at the Athol and Utha Scheme diptanks. The cattle enrolled per owner varied from 2 to 35 according to their herd size.

Study design

This was a longitudinal cohort study following the routine government control programme. Heifers (n=58) with estimated age range between 3 and 12 months (median was 5 months) were vaccinated subcutaneously with 5 millilitres of Onderstepoort Biological Products (OBP), Pretoria, South Africa, *Brucella abortus* S19 vaccine. The OBP S19 vaccine has approximately 5×10^{10} organisms per dose of 5 ml (R. Macdonald, OBP, personal communication, September 3rd 2014). Twenty-nine unvaccinated males of 3 to 12 months (median was 6 months) in the same herds as the females served as controls. Eight percent of cases were estimated to be younger than 4 months and 8% were estimated to be older than 4 months.

The immunological response to vaccination was measured by two serological tests: RBT and iELISA. Blood was tested on the day of vaccination (Day 0) and subsequently on 2, 4, 10, 14, 19, 24, 32, 43, 51 and 59 weeks post vaccination. All animals had to be RBT negative at beginning to be included in the study. Four and half years later, the immunological response was again measured with the aid of these two serological tests and an intradermal brucellin skin test (ST) in as many cases that could be traced.

Sampling

Each animal was individually identified by ear tag and photograph. At sampling, 10 ml of blood was collected from the jugular or tail veins. The sera were separated by centrifuging at 1200 g for 10 min, within 24 hours after blood collection and 1.4 ml of each serum was stored in cryovials at -20°C.

Serological testing

The RBT was performed as described (Alton et al. 1988) and any visible agglutination deemed the test as positive. The sera taken on weeks 2, 4, 10, 14, 43, 51, 59 post vaccination were also tested with iELISA. The samples taken at 4.5 years post vaccination were tested with both RBT and iELISA. The iELISA used was the IDEXX Brucellosis antibody test kit (IDEXX, Montpellier, France).

Brucellin skin test (ST)

Four and a half years after their vaccination with the S19 vaccine, traced cases had a ST performed. The ST was performed as described (Saegerman et al. 1999, p.214) using standardized antigen, prepared from *B. melitensis* B115 rough strain (BRUCELLERGENE OCB®, Synbiotics Europe, France). The Brucellin was injected on day 0 and skin thickness measured on days 0 and 3.

Statistical analysis

RBT data were analysed in a mixed logistic regression using time and time² as continuous explanatory variables (StatCorp 2009). Individual animals were taken as random effects to account for repeated samplings of the same animals. iELISA data were analysed in a similar model, except for the explanatory variable. Here, a categorical variable was used: 2 months after vaccination (1-3 months) and 1 year after vaccination (10-14 months). The agreement between RBT and iELISA was evaluated using Cohen's kappa test.

In preliminary models, the effect of age at vaccination (continuous explanatory variable) and that of missing values (multiple imputation) on the serological responses were evaluated and found insignificant. Therefore, they were ignored in the statistical analyses presented in this article.

Results

A total of 691 samples were collected from the 58 cases and 29 controls. Sampling success at each sampling date differed through the study and went as low as 53% for cases and 52% for controls at week 22 (Figure 2).

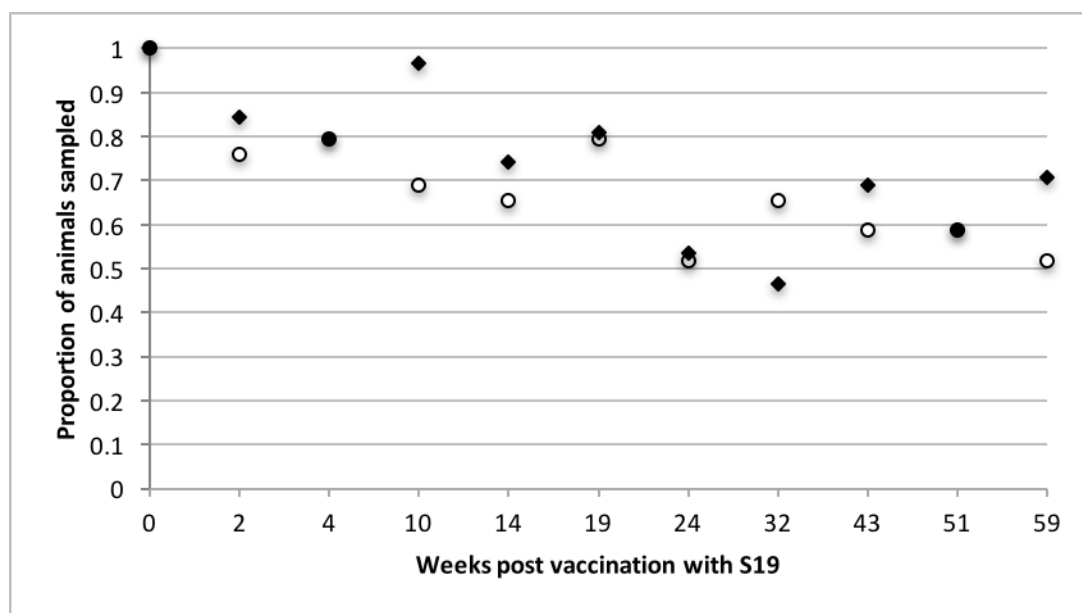


Figure 2. Proportion of animals sampled over time.

Key: *Black diamond*: are female/case proportion; *white circle*: male/control proportion; *black circle*: female and male proportion sampled are the same (51 weeks).

All cases and controls were RBT negative at the beginning of the study on the day of vaccination. One sample of a control animal was RBT positive during the study, but iELISA negative. There were no other control RBT or iELISA positive samples. All the control animal study results when corrected for sensitivity and specificity were found to be 0% (95% confidence interval (CI): 0.0- 10.4) (Thrusfield 1995).

At the second sampling, at two weeks, all except one of the 49 cases that turned were sampled were positive with RBT. All the 58 cases tested positive at least once with the RBT within 12 weeks. For three cases, it took 8 weeks to get the first post-vaccination positive result. The percentage RBT and iELISA positive decreased from their peak after two and eight weeks post vaccination respectively, but there were still positive animals, 14% for RBT and 32 % for iELISA, 59 weeks after vaccination (Figures 3 and 4).

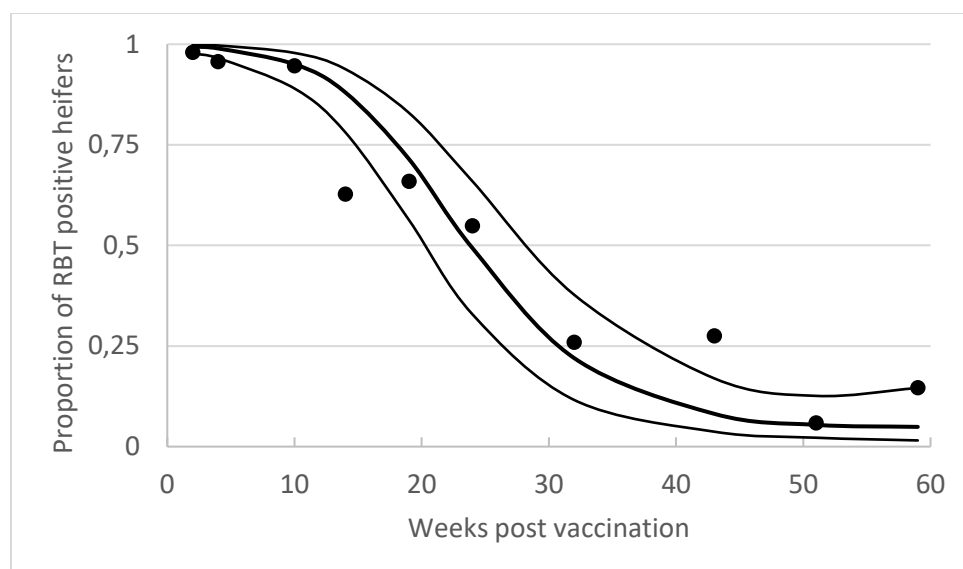


Figure 3. Observed proportion and prediction of RBT positive case results over time using quadratic logistic regression and with 95% confidence intervals.

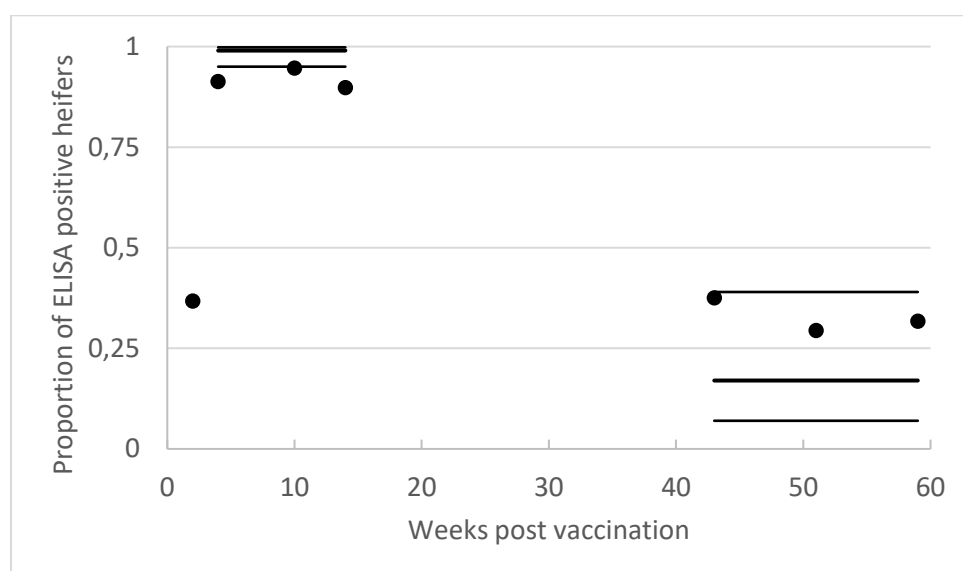


Figure 4. Observed proportion and prediction of iELISA positive case results over time using categorical logistic regression and with 95% confidence intervals.

Four and a half years after vaccination blood samples were taken from all cases that could be traced, which was only 7 (12%). Out of seven cases tested with RBT, ELISA, and Brucellin ST, one was positive with RBT and iELISA and three other cows were positive on ST only (Table 1).

Table 1. Table of serological and ST results of 7 cases four and a half years after vaccination.

ANIMAL	RBT	iELISA	ST
1	N	N	N
2	N	N	P
3	N	N	N
4	N	N	P
5	N	N	N
6	P	P	N
7	N	N	P

N = negative and **P** = positive.

Discussion

The RBT is seen as the best screening test for brucellosis in this context (Godfroid et al. 2010) and iELISA is seen as a confirmatory test as it is more specific than the RBT (Corbel 2006), both of which are recommended by the OIE. The ST is an assessment of the cell-mediated immunity as opposed to the humoral immunity assessed by the serological tests and is seen as more specific but less sensitive than the RBT and iELISA and more suitable to low prevalence settings (Godfroid et al. 2002; MacDiarmid & Hellstrom 1987; Saegerman et al. 1999).

The expected seroconversion of cases was 100% (Lord et al. 1998). A sample size of 58 cases that all seroconvert is required to obtain 95% as a lower limit of the 95% confidence interval (Thrusfield 1995). The results demonstrated a robust and rapid serological response to vaccination in 100% of animals. Ninety eight % (48/49) of cases seroconverted with RBT by week 2, which is reported in other publications (Stevens, Olsen & Pugh 1995), and all cases seroconverted using iELISA by 12 weeks. Cases took

longer to seroconvert to iELISA, but all seroconverted at least once to the iELISA within 12 weeks.

The proportion of RBT positive results decreased rapidly after 8 weeks. This decrease changed after 14 weeks to create an inverse sigmoid curve that plateaued after 43 weeks (Figure 3). This trend is linked to the significant quadratic term of time (time^2). According to the model, the median seroconversion sample date from RBT positive to negative (proportion of heifers that had turned negative) was 24 weeks post vaccination, whereas 5% of the heifers remained RBT seropositive a year after vaccination. The proportion of iELISA positive cases dropped from 99% (95% CI: 95 – 99.8%) 4 to 14 weeks post immunisation to 17% (95% CI: 7 – 39%) 43 to 59 weeks after vaccination (Figure 4). The decline in antibodies over time follows a sigmoidal curve. The response declines more sharply with RBT compared to iELISA (5% RBT positive and 17% iELISA positive cases between 10 and 14 months). The RBT is known to mainly detect immunoglobulins M (IgM) and immunoglobulins G (IgG) whereas the iELISA detects solely IgG (Nielsen et al. 2005). Hence, earlier RBT positivity and decline are expected since the IgM response to vaccination is quicker than the IgG response and does not last as long as the IgG (Godfroid et al. 2010). The apparent discrepancy between observed and fitted data in figure 4 and, to some extent, figure 3 is due to the random effect (animal) included in the mixed model. There is a substantial agreement between the RBT and iELISA results as shown with a kappa coefficient of 0.63, which indicates that the tests follow a similar pattern over time. The increase in positives from 51 to 59 weeks is due to cases that were not tested in week 51 and were tested in week 59 and found to be positive (1 RBT positive and 4 iELISA positive).

Four and a half years after vaccination out of seven cases, the rest were lost to follow-up due to slaughter, death from disease or movement, one animal (14%) was positive with RBT and iELISA and three other cows were positive (43%) with the ST only. The fact that one animal was positive on both serological tests and not on the ST and three were only ST positive reflects the different immunological responses detected by the different tests. The four positive animals at four and a half years were vaccinated at 3 (RBT and iELISA

positive), 4 (iELISA and skin test positive), 5 (skin test positive), and 8 (skin test positive) months. Therefore, their positivity was probably not due to being vaccinated after eight months of age, which has been suggested to be one of the main cause of persistence of an antibody response, although the age estimation is subjective.

This comprehensive response to the vaccine demonstrates the ability of the vaccine to induce a robust immunological response in an African savannah rangeland farming setting, which can be used to assess vaccine coverage assuming an absence of wild type infection. A concern during the study was that the animals would be subjected to wildtype infection. Vaccinated heifers that are infected by wildtype *B. abortus* will show a classical “boost effect”, which is the hallmark of a secondary immune response (Fensterbank & Plommet 1979). This was not seen in our study. As there was only one RBT positive sample in the controls and no iELISA positive controls over the 59 weeks follow-up, a wild type infection is unlikely to have interfered with the antibody responses in the cases. The fact that non-vaccinated bulls remained negative also strongly suggests that there was no circulation of wildtype *B. abortus* in this setting throughout the duration of the study. A companion study at the same time by the authors documents that there was indeed no wildtype infection (manuscript submitted for publication). A “boost effect” seen during disease surveillance, should always be thoroughly investigated by veterinary service.

Limitations of the study

The study had gaps in the sampling and testing of the animals as it was not always possible to sample an animal and due to financial constraints not all samples had an iELISA test. This gives an incomplete picture of the iELISA results curve. The study was undertaken by the primary authors and was as they experienced it. The RBT is subjective and therefore results could have bias.

Ageing of the animals may have been inaccurate because only visual indicators, such as size, condition, teeth present and questioning the owner were used. Combined with this inaccurate aging is the variation of genetics, feeding and husbandry between the herds

in the communal farming setting resulting in animals growing at different rates. These variations may result in heifers' age being underestimated and being vaccinated outside of the four to the eight-month window, which in turn may be responsible for a prolonging of the serological and allergenic positive responses as the older an animal is at vaccination the longer is the persistence of the ST reaction (Saegerman et al. 1999).

Recommendations

In South Africa where brucellosis is endemic, vaccination is compulsory but testing is not compulsory, it is important for owners and veterinary officials to know the status of animals. The fact that 17% of the cases had an iELISA positive response and 5% have RBT positive response around a year after vaccination and that four and a half years after vaccination there are still serological and allergenic test positives, shows that there can be immunological persistence to the vaccination for many years, which has been previously documented in dairy and beef cattle in Belgium (Saegerman et al. 1999). This is relevant for disease control as these positives may be seen and incorrectly interpreted as wild type infections and not vaccine responses as they are so many years post vaccination. If there is confusion over whether animals are reacting from vaccination or wildtype infection the infection history of herd, area, and animals brought into the herd is of importance. This antibody persistence after vaccination could also cause false positive reactions with the confirmatory CFT (World Organisation for Animal Health 2009) that is used in South Africa.

This necessitates a careful interpretation of serological results or the use of multiple tests (Abernethy et al. 2012), such as use of a competitive ELISA (cELISA), which gives less false positives from vaccination (Gall & Nielsen 2004), and ideally culturing of *B. abortus*, seen as the gold standard (World Organisation for Animal Health 2009) in order to confirm the presence of wildtype infection and not the vaccine type. The iELISA uses smooth lipopolysaccharides or O-polysaccharides as antigens, which is also sensitive for the antibodies produced by S19 vaccination, while the cELISA adds a monoclonal antibody that is specific for *Brucella* spp. O-polysaccharides that decreases false positives from

vaccination (World Organisation for Animal Health 2009). While disease status of individuals are being determined, it would be wise to identify and separate the animals that appear to be infected until their status can be assured or they are slaughtered.

This study used a high vaccine dose of 5×10^{10} organisms that is in line with the recommendations of the OIE ($5-8 \times 10^{10}$ organisms) (World Organisation for Animal Health 2016). It revealed a longer and more comprehensive antibody response than a similar study in Zambia that used a reduced dose of S19 (3×10^9 *Brucella* bacteria) and had no young animals (8-18 months at vaccination) RBT positive by 6 months (Schoorman 1983). This reduced dose will result in less interference with diagnostics, and has been shown to induce the same level of protection than high doses both experimentally (Fensterbank & Plommet 1979; Plommet et al. 1976) and in field work (Nicoletti et al. 1978).

Another option is to administer a reduced dose (5×10^9 organisms) via the conjunctival route that yields a lower serological response compared to subcutaneous injection (Nicoletti 1984; Plommet et al. 1976; World Organisation for Animal Health 2016). This will result in less interference with disease control by serological means, but is much more difficult and time consuming to administer in these settings and requires the animal to be well restrained to avoid unnecessary trauma to the animal or self-injection.

Conclusions

This study revealed a comprehensive and rapid antibody response in heifers vaccinated with S19 vaccine in this communal farming setting. Ninety-eight percent of animals seroconverted to the RBT by week 2 and all cases seroconverted to RBT and iELISA by 12 weeks. The antibody presence decreased in an inverse sigmoid fashion to half of the animals being RBT positive at 6 months and around 5% of animals RBT positive and 17% iELISA positive one year after vaccination. The serological effects of vaccination therefore can persist for more than a year and possibly several years as seen in one animal when tested four and a half years after vaccination. This can confuse evaluation of the disease

status of a herd by serological testing when there has been a history of vaccination with S19. This must be taken into consideration in making herd disease control recommendations after serological testing for brucellosis in this setting.

The constant decrease in serological titres in vaccinated heifers over time combined with the absence of seroconversion in non-vaccinated bulls and with the absence of clinical signs suggestive of brucellosis (i.e., high rate of abortion), allow us to suggest the absence of brucellosis in the presence of positive serological reactions induced exclusively by S19 vaccination (“high” dose). It may be advised to use a “low dose” vaccination that could result in less antibody persistence.

GENERAL CONCLUSIONS AND PERSPECTIVES

Brucellosis is believed to be a neglected zoonosis that infects domestic animals and wildlife as well as humans causing significant health and economic impacts. The overall goal of this body of research is to better understand the epidemiology of brucellosis in the Mnisi community, which is an African example of an “One Health” setting, where domestic animals, humans and wildlife live in close proximity and come into indirect and direct contact. To correctly understand the epidemiology at this site we explored the published literature on brucellosis in wildlife in Africa and then the evidence of brucellosis in buffalo, humans, goats, dogs, cattle and buffalo at the study site.

The systematic review of literature found that brucellosis had been serologically documented in sixty-one wildlife species and identified nine times in six species of wildlife and rodents in Africa. The identification of *Brucella* species was first made in the 1960's in Africa and has been made infrequently since. The identification of *Brucella* spp. in Egypt, Kenya, Tanzania, Zimbabwe and South Africa, indicates that brucellosis is spread widely across the continent and most likely is underdiagnosed and more ubiquitous than previously thought. Nine identification studies in over fifty years for the whole continent highlights the need to conduct more identification of *Brucella* species infecting populations so that we can better understand the epidemiological picture in Africa. There is also a gap in literature on *B. suis* infection in wild and domestic suids in Africa. It has been identified in cattle in Zimbabwe (Ledwaba et al. 2014), which indicates that there must be a source in another species, possibly wildlife, as cattle are spill over hosts (Fretin et al. 2013).

Wildlife is thought to have become infected from domestic animals and then certain wildlife species could have become a reservoir for infection. The Kafue lechwe in Zambia, for instance, were most likely infected from cattle that moved into their area in the 1970's and have remained infected either due to continued spill over from domestic animals or other species or have become a reservoir (Muma et al. 2011). Interestingly in Zimbabwe, in the Greater Limpopo Transfrontier Conservation Area, research found a positive sero-prevalence of 9.9% in cattle (n=1158) and giraffe (n=1) and yet, no sero-prevalence in

buffalos (n=47), impala (n=33) and kudu (n=16) (Gomo, Musari, et al. 2012). Even though the domestic animals and wildlife in this study are in close contact the disease had not yet spread to all susceptible species.

The majority of wildlife studies identified in the systematic review were opportunistic studies and the accuracy of using these results as a sero-prevalence is questionable as the population sizes would not have been known and true random sampling would not have been conducted. Yet, the studies do give us an indication of the epidemiology of the disease and evidence of its presence or not.

Bearing the above in mind, our meta-analysis found a positive association with livestock contact with antelope and carnivore species and not with buffalo. The independence of prevalence in buffalo from livestock contact indicates that buffalo maybe able to sustain *Brucella* infection independent of influx of bacteria from other host species and is thus a reservoir of *B. abortus* for other wildlife species and may also spill back to cattle. The meta-analysis also indicated that buffalo populations more likely to be infected, had a higher seroprevalence than other species.

Gregarious wildlife species such as buffalos, eland, impala and wildebeest are reported to have a higher seroprevalence than solitary animals such as rhinoceros (Ducrotoy et al. 2017). Although, that cannot be the only factor affecting seroprevalence. A driver for tuberculosis spread between buffalo is thought to be their social nature and large herd sizes, an average of 250 per herd in the Kruger National Park (Michel et al. 2006). The higher within herd infection rate and higher prevalence of brucellosis in buffalo populations could also be due to their social nature and large herd sizes.

Our meta-analysis of the results from the systematic review revealed an association with higher rainfall and more herds infected and infected populations showed a higher seroprevalence. This could be due to higher rainfall areas may have a higher density of animals and larger herds due to better grazing, which may result in higher transmission

rates and a higher likelihood of a herd being infected. The bacteria also survives for longer in wetter environments which may give more time for transmission to susceptible animals.

Our meta-analysis also found studies published after the year 2000 had higher seroprevalences than studies published before 1980. This may be due to bias in that there are more studies recently focusing on populations that are more likely to be infected, but it could also be due to the disease becoming more established in populations. The rising seroprevalences in the study settings will mean a greater risk to public and veterinary health that will need reliable control and prevention methods. It is harder to control a disease and rarely justified to do so in free ranging wildlife in comparison to domestic animals, but there are economical and effective control methods for domestic animals, such as vaccination, that can be used to induce immunity to infection from wildlife to domestic animals.

It is worth bearing in mind that serological tests for brucellosis in wildlife species are not validated so results must be interpreted with caution (International Office of Epizootics. Biological Standards Commission. 2012). This is certainly more relevant when comparing the same test used in different species as opposed to within one species. Although, it is also relevant when comparing different tests. Our meta-analysis of prevalence studies did not find convincingly significant difference between the agglutination and ELISA tests. There are methods to improve test reliability, for example RBT testing of wildlife sera has been found to have a better coherence with iELISA after a chloroform/centrifugation cleaning (Godfroid et al. 2016).

To understand our local situation better we sampled, tested and identified brucellosis in African buffalo in the study site in the Kruger National Park alongside the community we studied. Besides *B. abortus* infection, we identified *B. melitensis* infection in buffalo. This is an unexpected and worrisome information as *B. melitensis* has not been identified in African buffalo and not found in any animal in the Kruger National Park. *B. melitensis* has only been identified previously in impala antelope (*Aepyceros melampus*) (Schiemann & Staak 1971) in Tanzania and Nile catfish (*Clarias gariepinus*) in Egypt (El-Tras et al. 2010)

in published research. It has been identified in sable antelope (*Hippotragus niger*) in South Africa in 2007, 2015 and 2016 (National Department of Agriculture n.d.) outside of the KNP. These results suggest that *B. melitensis* has spilled over from a small stock population neighbouring the park. However, we cannot exclude or another wildlife species in the KNP that has been infected from the small stock. It is very unlikely that *B. melitensis* has become established in the buffalo populations and had not been detected until now. Control of the disease in wildlife in the park is currently not a possibility. But preventing the infection from going out of the park to uninfected domestic animals (spill back) is of importance. The results also have a public health implication as there is human consumption of wildlife from the park. This must be taken into consideration during processing of slaughtered wildlife to protect abattoir workers and wildlife products must be treated accordingly to prevent transmission to humans. It is also worth bearing in mind that although transmission from wildlife to humans has been documented as being rare there are borders in the Greater Limpopo Transfrontier Park where people go inside the park to hunt for “bushmeat”. In sub-Saharan Africa, no proof of direct transmission of *Brucella* spp. from wildlife to humans has been reported in the international scientific literature, although transmission from preparing and consuming buffalo bushmeat has been suggested in Botswana (Alexander et al. 2012). Nevertheless, education in these communities of the brucellosis risks from hunting wildlife may be of public health benefit.

In most settings in Africa, where there are domestic animals and wildlife in close proximity there are no or compromised fences and controlling brucellosis by testing with slaughter of livestock is not economically feasible. In these settings, there will most likely be more contact between domestic animals and wildlife than in our study site, which means the risk of inter-species transmission will be greater than in our study site. This will place more emphasis on the success of vaccination to protect domestic animals and then humans. Wider and continuous vaccination of livestock could be considered in these settings.

The isolation of *B. melitensis* in buffalo for the first time in Africa indicates the buffalo has become a spillover host. Goats were thought to be the reservoir of *B. melitensis*, but it has since been found in sable antelope (*Hippotragus niger*) in South Africa and now

buffalo, which indicates that the dynamics of *Brucella* infection at the wildlife/livestock interface is changing. We only tested buffalo so we cannot exclude the presence of undetected *B. melitensis* in other wildlife species. Unpublished research in the Limpopo National Park (LNP), Mozambique, where there were 25 000 people living in the park found that over 80% of households had goats and over 40% saw domestic animals and wildlife mixing (G. Simpson 2007). In this setting the only fencing between wildlife and domestic animals is when the livestock are brought home for the night. It is possible the buffalo were infected from these livestock and the disease moved through the park. A study of buffalo and cattle in the LNP found both infected with RBT and ELISA, but unfortunately not identification of species was made (Tanner et al. 2015).

Further molecular epidemiological studies (MLVA) should be done to establish the source of the bacteria to establish its transmission and assess which are reservoir species (vectors) and which are only spillover species (victims). This will have an impact on disease control and is of relevance in transfrontier conservation areas where wildlife can move across country boundaries and spread unnoticed with possible severe public health consequences if not controlled.

Research conducted at the study site found nearly three quarters of the households owning at least one animal, one quarter had cattle and 61% of respondents believed diseases of animals could be transmitted to humans, although brucellosis was not mentioned (Berrian et al. 2016). In order to fully understand the epidemiological situation in the study site we looked for evidence of infection in the domestic animals and humans. Sampling of dogs was included to the cattle and goats as the dogs ingest abortion materials in the field and carcasses of dead animals thereby potentially being exposed to brucellosis. Goats have been found to be infected with *B. melitensis* in South Africa (Ribeiro et al. 1990) and spillover of *B. abortus* from cattle to goats was detected with a serum agglutination test in a similar setting in Uganda (Kabagambe et al. 2001; Mellau et al. 2009). In our study site the lack of evidence of infection in goats and dogs strongly suggest that *B. melitensis* and *B. abortus* infections are absent in the animal reservoir in this community.

The cattle showed a low sero-prevalence of 1.4%. It is not straight forward making a serological distinction between true infection due to *Brucella* wild type infection and vaccine related residual antibodies at low sero-prevalences. Neither RBT nor ELISA tests are 100% sensitive or specific and although good screening tests they should not be used alone (Matope et al. 2011). Both tests cross-react with *Yersinia enterocolitica* O:9 and other bacteria causing false positive results (Godfroid et al. 2002; Kittelberger et al. 1998; Garin-Bastuji et al. 1999).

A wild type infection should show 1) an increasing seroprevalence with increasing age, 2) seropositivity in (non-vaccinated) males, 3) the ability to isolate the organism, 4) possible spill-over to other domestic animals and 5) human infections. We found the opposite with seroprevalence decreasing with age, the male true seroprevalence is zero and we were unable to isolate the organism indicating the absence of the disease in cattle.

To understand if the sero-prevalance pattern found was due to vaccination we conducted a longitudinal cohort study of vaccinated heifers and compared them to unvaccinated male calves. We found a comprehensive and rapid antibody response to RBT and iELISA by 12 weeks. The antibody presence decreased in an inverse sigmoid fashion to half of the animals being RBT positive at 6 months and around 5% of animals RBT positive and 17% iELISA positive one year after vaccination. The serological effects of vaccination therefore can persist for more than a year and possibly several years as seen in one animal when tested four and a half years after vaccination. This can explain the antibody picture found in the serological testing of the cattle.

The healthy immunological response to a high vaccine dose of 5×10^{10} organisms, that is in line with the recommendations of the OIE ($5-8 \times 10^{10}$ organisms) (World Organisation for Animal Health 2016), in the heifers in the field is both encouraging and confusing from a disease control perspective. The vaccine induce immunological response will lead to false positive tests results in disease control. Thus careful interpretation of serological results or the use of multiple tests (Abernethy et al. 2012), such as use of a competitive

ELISA (cELISA), which gives less false positives from vaccination (Gall & Nielsen 2004), and ideally culturing of *B. abortus*, seen as the gold standard (World Organisation for Animal Health 2009) is needed in order to confirm the presence of wildtype infection and not the vaccine type. The iELISA uses smooth lipopolysaccharides or O-polysaccharides as antigens, which is also sensitive for the antibodies produced by S19 vaccination, while the cELISA adds a monoclonal antibody that is specific for *Brucella* spp. O-polysaccharides that decreases false positives from vaccination (World Organisation for Animal Health 2009).

Another option would be to consider using a lower dose of vaccine or a different method of inoculation. In Zambia a lower dose (3×10^9 *Brucella* bacteria) was used in older animals (8-18 months at vaccination) and the serological results did not last longer than 6 months (Schoorman 1983). The reduced dose has shown the same level of protection as high doses both experimentally (Fensterbank & Plommet 1979; Plommet et al. 1976) and in the field (Nicoletti et al. 1978).

A reduced dose (5×10^9 organisms) could also be administered by a conjunctival route that will result in a lower serological response compared to subcutaneous injection (Nicoletti 1984; Plommet et al. 1976; World Organisation for Animal Health 2016). This will be more beneficial from a disease control perspective, but the conjunctival route is harder to administer, and more time consuming in these settings and requires the animal to be well restrained to avoid unnecessary trauma to the animal or self-injection, which could have a serious consequence. If there is a suitable head clamp or holding facilities it may be possible to use this route, but this is unlikely in this setting.

Brucellosis is an old and persistent disease that has adapted to infect a wide variety of species. Although, it causes significant morbidity and mortality the disease is not controlled in many “One Health” settings, especially if there are infected wild species. Our testing of high risk humans that were herding cattle through the cattle dip tank showed no sero-positive individuals. This correlates to what we found in the cattle and other domestic animals. As the cattle showed a low to negative true seroprevalence and absence of

isolation, the goats, dogs and humans free of brucellosis, we deemed the domestic animals and community free of infection with *B. abortus* and *B. melitensis*. Brucellosis has also not been introduced from infected domestic animals from out of the area. This means that, currently, there is no risk of transmission of brucellosis to humans through contact with livestock and dogs and no foodborne risk (mainly through raw milk or milk products). It is encouraging to know that in a setting where there is infected wildlife the domestic animals and humans can be free from disease. Transmission depends on a viable source of sufficient bacteria coming in contact with susceptible animals. It would be valuable to know the reasons for the absence of infection in the community. If the wildlife is infected and the domestic animals and humans are free of disease, this suggests that well maintained fences play a major role in avoiding contact between infected wildlife and non-infected livestock and thus avoiding that *Brucella* infection is transmitted across the fence from the wildlife.

There has been decades of similar stocking densities of domestic animals alongside these wildlife reserves. The lack of transmission across the fence could be due to the control methods of fencing between wildlife and domestic animals, vaccination and movement restrictions of cloven hoofed animals due to foot and mouth disease control measures, but it is not possible to make that deduction from the research. There are fence transgressions by wildlife from the park to the communal areas and vice-versa, although in buffalo the fence transgressions are more likely to be made by young males than aborting females. The environmental conditions of this setting may also not be conducive to transmission. Abortions in buffalo, if they follow a similar pattern to cattle, should occur at the end of the dry season and beginning of wet season. There is plenty of sunshine and although there may be rain the day time temperatures will be over thirty degrees, which is not conducive to bacterial survival. Transmission from wildlife to domestic animals may also be harder than we think and there may be a greater risk of brucellosis being brought into the area from infected domestic animals than from wildlife. The lack of brucellosis in domestic animals and humans could be also due to the other control method of vaccination in heifers that avoids having a susceptible population in the cattle. As the

domestic animals are free of the disease livestock should be tested for brucellosis before being imported into the area.

Our studies found that brucellosis was present in the wildlife but not in the domestic animals and people of the study site. The setting has a fence between wildlife and domestic animals, control through vaccination and movement restriction of cloven hoofed animals due to foot and mouth restrictions. These factors have played an important role in preventing the disease being transmitted into the community and should be maintained to avoid any introduction of *Brucella* spp. in the future.

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APPENDICES

Appendix I**One Health profile of a community at the wildlife-domestic animal interface, Mpumalanga, South Africa**

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Abstract

We used a community engagement approach to develop a One Health profile of an agro-pastoralist population at the interface of wildlife areas in eastern South Africa. Representatives from 262 randomly-selected households participated in an in-person, cross-sectional survey. Questions were designed to ascertain the participants' knowledge, attitudes, and practices with regard to human health, domestic animal health, and natural resources including wildlife and water. Surveys were conducted within four selected villages by a team of trained surveyors and translators over four weeks in July-August 2013. Questions were a combination of multiple choice (single answer), multiple selection, open-ended, and Likert scale.

The study found that nearly three-quarters of all households surveyed reported owning at least one animal (55% owned chickens, 31% dogs, 25% cattle, 16% goats, 9% cats, and 5% pigs). Among the animal-owning respondents, health concerns identified included dissatisfaction with government-run cattle dip facilities (97%) and frequent morbidity and mortality of chickens that had clinical signs consistent with Newcastle disease (49%). Sixty-one percent of participants believed that diseases of animals could be transmitted to humans. Ninety-six percent of respondents desired greater knowledge about animal diseases. With regard to human health issues, the primary barrier to health care access was related to transportation to/from the community health clinics. Environmental health issues revealed by the survey included disparities by village in drinking water reliability and frequent domiciliary rodent sightings positively associated with increased household size and chicken ownership. Attitudes towards conservation were generally favorable; however, the community demonstrated a strong preference for a dichotomous approach to wildlife management, one that separated wildlife from humans.

Due to the location of the community, which neighbors the Great Limpopo Transfrontier Conservation Area, and the livestock-dependent lifestyle of the resource-poor inhabitants, a One Health approach that takes into consideration the interconnectedness of human, animal, and environmental health is necessary. The community profile described in this study provides a foundation for health research and planning initiatives that are driven by community engagement and consider the multitude of factors affecting health at the human-domestic animal-wildlife interface. Furthermore,

it allows for the determination and quantification of the linkages between human, animal, and environmental health.

Introduction

Africa has experienced dramatic shifts in human and animal population dynamics over the last century. Armed conflict, changes in land use, and a rapid rise in the human population have forced the movement of people and the re-establishment of communities in previously uninhabited areas (Kock *et al.*, 2002). In southern Africa, another such change in land use was the development of transfrontier conservation areas (TFCAs) in the early 2000s. There are currently 13 TFCAs under active development in Southern Africa. TFCAs have been promoted as a way to reconcile conservation and development objectives through increased cross-border collaboration and ecotourism (de Garine-Wichatitsky *et al.*, 2013). TFCAs seek to limit barriers between countries, thus allowing wildlife to roam more freely and promoting regional peace, cooperation, and socio-economic development (Department of Environmental Affairs, 2015). However, the increased movement of animals across the boundaries of these re-connected conservation areas presents new challenges for animal health and, in turn, adjacent human communities (Bengis, 2005). With the growing recognition of the interconnectivity of the health of all species with that of their environment, mitigation of disease transmission at the human-wildlife-livestock interface has become a major development and conservation objective (de Garine-Wichatitsky *et al.*, 2013).

The land of the Mnisi Traditional Authority (MTA) within the Bushbuckridge Local Municipality, Mpumalanga, South Africa exemplifies this interface. The Municipality contains over 500,000 people, and its entire eastern boundary borders the Great Limpopo TFCA. The Great Limpopo TFCA is one of the largest TFCAs in Southern Africa with a land area of nearly 100,000 km². It spans three countries (South Africa, Zimbabwe, and Mozambique) and contains five national parks. The people who reside in the Bushbuckridge Local Municipality on the periphery of the Great Limpopo TFCA are characterized by a high degree of poverty and many rely on land-based activities such as agriculture and natural resource use for their livelihoods (Andersson *et al.*, 2013). In this

area, the co-existence of humans, domestic animals, and wildlife is required and critical to the sustainability of the parks and success of wildlife conservation efforts.

Within the MTA, livestock dependence, coupled with proximity to wildlife areas, makes health promotion and poverty alleviation a more complex problem. A One Health (OH) approach that recognizes the interconnectedness of human, animal, and ecosystem health and encourages collaboration between diverse disciplines is ideally suited to address these types of problems (Mazet *et al.*, 2009; Conrad *et al.*, 2013; Grace, 2014). Integrated animal-human health surveillance using simultaneous data collection is a method by which to better understand these complex relationships (Thumbi *et al.*, 2015).

The goal of our study was to establish a baseline profile of a selected area within the MTA in order to plan follow-up research and activities that use a community-engaged, OH approach for the purposes of informing disease prevention and mitigation efforts in both human and animal populations. A community-engaged approach seeks participation from inhabitants and draws on the capacities and resources of those people, rather than a top-down approach. Community engagement is a core element of any research effort involving communities and may enhance a community's ability to identify and address its own health needs, disparities, and goals. In addition, results of community-engaged research are used to guide the development of interventions, education, or policies (Ahmed *et al.*, 2010).

This study seeks to describe human and animal demography within the study area and the perspectives of the people in terms of how they view their health; whether they acknowledge a connection between their health, the health of their animals, and environmental factors; what they consider to be priority concerns for their community; how they perceive wildlife and conservation; their self-reported health history; and their satisfaction with health services. Triangulating knowledge, attitudes, and practices with self-reported health and disease history is an important step in community health research and planning that requires a community-engaged approach. Furthermore, quantifying linkages between livestock and human health remains a priority for developing sustainable poverty relief and public health interventions for livestock-dependent communities (Thumbi *et al.*, 2015). The result of this study will be a comprehensive OH profile of the study area. This profile can be used for subsequent health planning and

research aimed at improving health and livelihoods of community members and mitigating disease risks.

Materials and methods

Study area

The study was carried out in a selected area within the MTA, hereafter referred to as the “Mnisi study area,” located in the Mpumalanga Province in the Republic of South Africa. The Mnisi study area is situated in the northeastern corner of the Bushbuckridge Local Municipality and is the core research and engagement area of the Mnisi Community Programme, Faculty of Veterinary Science, University of Pretoria (UP). The study area contains approximately 29,500ha of communal land, of which more than 75% borders private and provincial conservation areas (Figure 1). The study area includes the adjacent Andover and Manyeleti provincial game reserves, of which Manyeleti has open access to the Kruger National Park and is thus part of the Great Limpopo TFCA.

Population and sample size

The total population in the Mnisi study area is estimated at 40,000-50,000 individuals in approximately 8,500 households (Statistics South Africa, 2012). This study involved a stratified random sample of households within four purposively-selected villages: Athol, Gottenburg, Thlavekisa, and Utha (Figure 1). These villages were selected based on their close proximity to private and provincial game reserves as well as the presence or absence of a community health clinic (two with and two without). Within each village, each household was numbered using Google Earth™ images (Google Inc., Mountain View, CA, USA) and randomly selected using a random number generator (Microsoft Excel, Redmond, WA, USA). Sample size was determined by the following parameters for a population survey with random sampling: 2,300 total estimated households within the four selected villages (unit of analysis), an expected difference between villages of 15%, 95% confidence level, and 90% power. As a result, a total of 256 surveys were determined to provide sufficient statistical power (WinEpi, 2010).

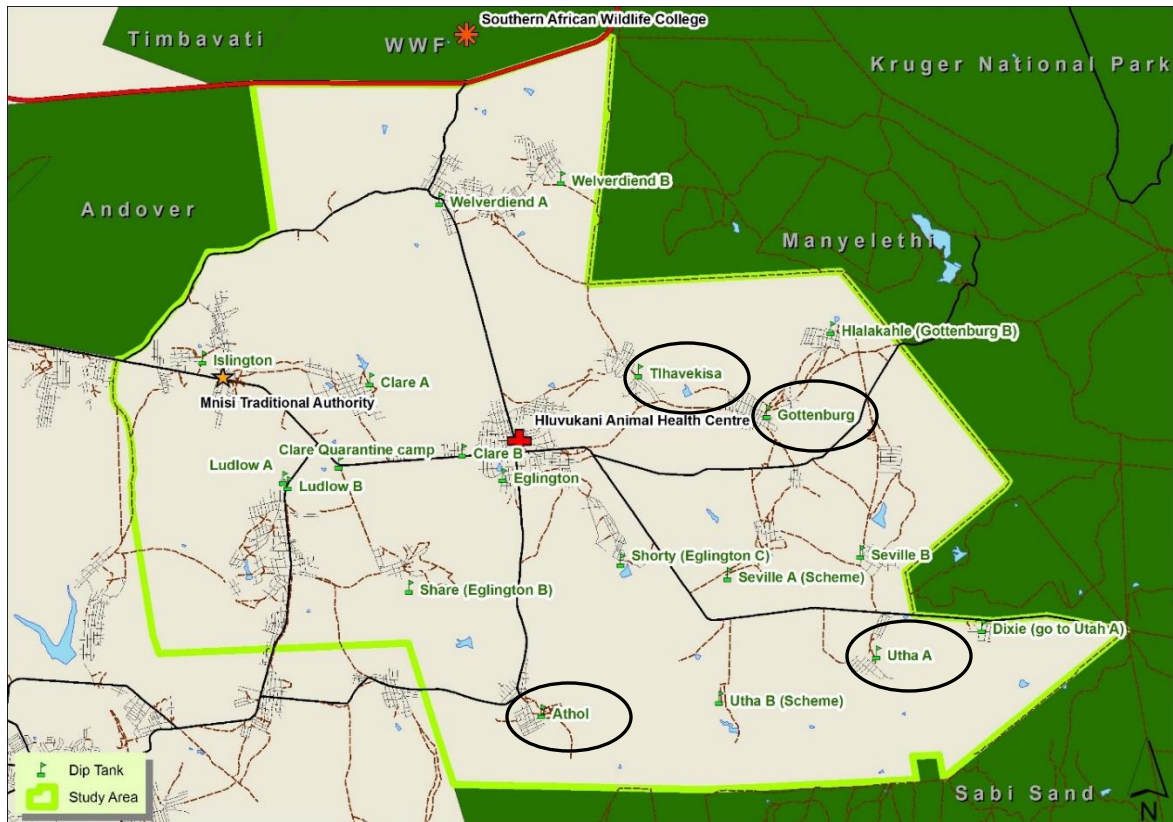


Figure 1. Map of the Mnisi study area (outlined in light green), Mpumalanga Province, Republic of South Africa, highlighting the location of the four selected villages (circles). Image courtesy of Mnisi Community Programme, University of Pretoria

Data collection method

A cross-sectional, in-person survey using a structured questionnaire administered in the participants' household was used to obtain information from selected individuals. Responses were collected on personal digital assistants (PDAs) using SurveyToGo mobile survey software (Dooblo Ltd., Kfar Sava, Israel), which was previously deployed in the study area with success (Madder *et al.*, 2012). The questionnaire underwent content validation by subject matter experts and community representatives to ensure appropriateness, relevancy, and cultural sensitivity of questions. A pilot questionnaire was trialed in a region not included in the final study site and adapted to maximize participant comprehension. All questionnaires were completed over four weeks between July and August 2013 by surveyors accompanied by a trained Shangaan-English translator employed by the Mnisi Community Programme. Potential participants were provided with

an oral description of the study goals, the voluntary and confidential nature of their participation, and a time estimate for their involvement. Only those participants aged 18 years or older from whom consent was obtained were surveyed. This study was reviewed by the University of California, Davis Institutional Review Board and determined to be exempt from human subjects regulations. Ethics approval was also obtained through the University of Pretoria Research Ethics Committee, Faculty Humanities and permissions were granted by the Mnisi Traditional Council as well as village leaders. The information obtained from the questionnaires was recorded by the investigators in such a manner that subjects could not be personally identified, either directly or through identifiers linked to the subjects.

Survey design

The survey was designed to collect data on human and domestic animal population demographics, animal health and services, human health and services, wildlife, and environmental health (Figure 2). The survey employed a structured questionnaire, which included a combination of multiple choice (single answer), multiple selection, open-ended, and Likert scale questions. The first section, which focused on participant demographics, was designed to determine study eligibility and preferred language as well as to classify participants by education level, status within the household, occupation, and household size.

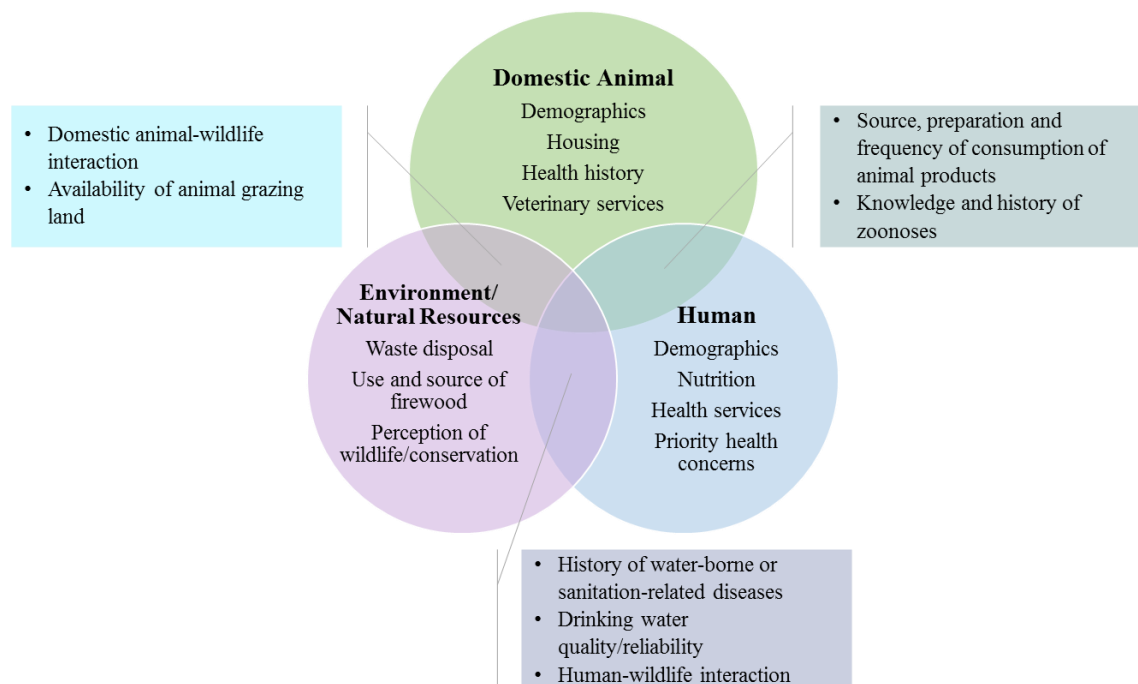


Figure 2. Schematic representation of the One Health framework used to design the topics to be included in the Mnisi study area questionnaire

The second section aimed at describing the domestic animal composition of the household, the health priorities and history of those animals as described by the participant, the participant's perception of veterinary services in the community, and his/her knowledge of animal diseases and transmission. Participants were also asked about their consumption of animal products and the preparation of those products.

The third section of the questionnaire focused on human health factors, including nutrition, access to and satisfaction with available health care, and priority health concerns in adults and children as described by the participant. Participants were also asked about their household's health history, specifically the occurrence of diseases linked to animals, sanitation, and water.

The final section of the questionnaire focused on natural resources and environmental health, and included topics such as waste disposal, interactions with wildlife including rodents, source of and satisfaction with water, and participants' perceptions of conservation efforts in and around their community.

Data analysis

Descriptive statistics were used to summarize the main features of the data collected. Summary statistics including mean, median, standard deviation (SD), range, and maximum values for continuous variables and frequency and proportions for categorical variables were calculated. The chi-square test for homogeneity was used to test for village-level differences. Exploratory results suggested the importance of rodent sightings around the home. For that reason, and because rodent-borne zoonoses have been identified to be prevalent and a major concern in the community (Quan *et al.*, 2014), we were particularly interested in identifying factors contributing to rodent presence at the household level. For this purpose, we used a multilevel logistic regression model with village as a random effect to identify factors contributing to observation or not of rodents at the household level (coded as 1/0, respectively). Rodent observation was considered as an ordinal variable with three response categories (daily, less than daily, never), but we found this categorization did not result in an improved model. The model is expressed as follows:

$$y_{ij} \sim \text{Bernoulli}(\pi_{ij}); \text{logit}(\pi_{ij}) = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{2ij} + \dots + \beta_k X_{kij} + u_j$$

where y_{ij} is the binary dependent variable—observation of rodents yes/no—for household i in village j ; π_{ij} is the expected probability of rodent observation within the household; β_0 is the intercept; $\beta_{1ij}, \beta_{2ij}, \dots, \beta_{kij}$ are the slopes; and u_j is the random effect, which accounts for clustering of households within villages. Model construction was initiated with a univariate analysis of hypothesized risk factors using a liberal p -value < 0.2 . Then a multilevel model was evaluated using forward selection. Quantitative variables were tested as predictors in their quantitative and binomial form, after being transformed using the median as the cut-off point. The best fitting multivariable model was assumed to be the one with the lower Akaike information criterion (AIC) and containing statistically significant predictors (p -value < 0.05). We also evaluated the effect of livestock ownership on the frequency of consumption of animal products by using a simple linear regression model. Statistical analyses were performed in SAS version 9.3 (SAS Institute, Cary, NC).

Results

Unemployed, <i>n</i> (%)	58 (81)	40 (66)	45 (70)	47 (72)	190 (73)
Total surveyed, <i>n</i> (%)	72 (27)	61 (23)	64 (24)	65 (25)	262

†Total number of people living in the same dwelling space for at least 3 months and providing jointly for purposes of food

‡Self-identification as the head of the household

SD = standard deviation

Domestic animal demography and health

Domestic animal ownership statistics are described in Table 2. Seventy-two percent of all households surveyed reported owning at least one animal ($n = 189$). Sixty-eight percent of all households owned livestock species, including cattle, goats, pigs, or chickens; and 36% of all households included a dog or cat. Among the animal-owning households, the most frequently owned animals were chickens (76%), followed by dogs (42%), cattle (35%), goats (22%), cats (12%), pigs (7%), ducks (<1%), and pigeons (<1%). Respondents with no formal education were more likely to own animals (one-sided chi-square test, $p = 0.0285$).

Livestock-owning participants were asked to identify the major health problems in their animals. Forty-nine percent described a condition in chickens characterized by swollen eyes, diarrhea, and sudden death. These clinical signs, used along with the Shangaan term “*mzungu*,” was consistent with Newcastle disease, a viral infection of domestic poultry caused by avian paramyxovirus type 1 that is endemic to parts of Africa (Ashraf *et al.*, 2014). Other major health problems identified in livestock included ticks and tick-borne diseases (11%), foot and mouth disease (FMD) (5%), rabies (1%), and lumpy skin disease (1%). With regard to preventive health, 42% of participants from livestock-owning households reported not administering or receiving vaccinations for their animals in the past 12 months. Cattle-owning participants ($n = 66$) were asked about health practices specific to cattle including the dipping or topical application of acaricides. With the exception of one individual, all participants reported that they dip their cattle for ticks. However, when asked how many times in the last month their cattle were dipped for ticks,

23 (35%) reported no dipping. Forty-three cattle-owning households (65%) reported that they use their own dip supplies. Using a five-point Likert scale, animal-owning participants ($n = 189$) were asked about their satisfaction with veterinary services in their communities; results are shown in Figure 3.

Table 2. Frequency and proportion of households surveyed in the Mnisi study area, Mpumalanga Province, Republic of South Africa ($n = 262$) reporting domestic animal ownership, by species, and the summary statistics for number of animals per household

Domestic animal ownership	Frequency (%)	Mean	Median	Maximum
Own at least one species	189 (72)			
Livestock	177 (68)			
Chickens	144 (55)	13.4	12	40
Cattle	66 (25)	12.4	7	78
Goats	42 (16)	6.9	6	17
Pigs	14 (5)	3.1	2.5	8
Ducks†	1 (0.4)	n/a	n/a	n/a
Pigeons†	1 (0.4)	n/a	n/a	n/a
Companion	95 (36)			
Dogs	80 (31)	2	1.5	6
Cats	23 (9)	2.3	2	5

†Number of animals was not collected for households owning ducks and pigeons

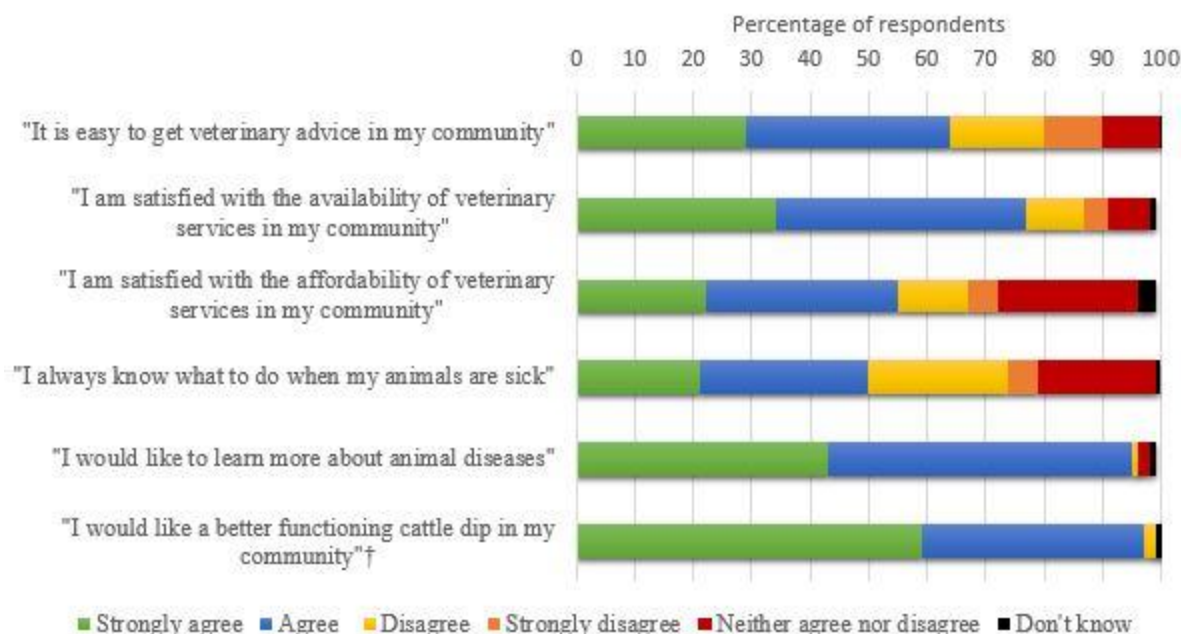


Figure 3. Stacked chart illustrating the perceptions of veterinary services among animal-owning survey participants in the Mnisi study area, Mpumalanga Province, Republic of South Africa ($n = 189$). †Pertains only to cattle-owning participants ($n = 66$)

Satisfaction with human health services

Participants were asked whether they or a member of their household had utilized a community health clinic in the past 12 months. For those who did access a clinic ($n = 210$, 80%), individuals were asked whether they were satisfied with the quality of care they or the member of their household received. Ninety-four percent responded favorably. In addition, 85% of participants responded that they were satisfied with the accessibility of the clinic. The proportion of respondents who were satisfied with health clinic accessibility did vary by village (two-sided chi-square test, $p = 0.0002$). Those respondents who resided in villages without a community health clinic were more likely to be dissatisfied with clinic accessibility than respondents from villages with a clinic (24% vs. 6%). The primary barriers to health clinic access identified by respondents included cost of transportation to the clinic followed by being too ill/debilitated to travel.

Human health and nutrition

Priority health problems for both adults and children, as perceived by the surveyed participants, are summarized in Table 3. “Flu” was the most commonly reported health problem for both children and adults; symptoms described included coughing, sneezing, runny nose, fever, and/or chills.

Only 3% of participants ($n = 9$) reported a history of an illness linked to animals in either themselves or a household member. When asked to describe the illness, surveyed individuals reported internal parasites, tuberculosis, rabies, malaria, and pruritus/skin lesions. Participants were not asked to differentiate between *Mycobacterium tuberculosis* and *Mycobacterium bovis*. Eight percent ($n = 22$) reported a history of an illness linked to poor water quality; symptoms included diarrhea, vomiting, headaches, and swollen hands and feet.

Table 3. Priority health problems in children and adults in the Mnisi study area, Mpumalanga Province, Republic of South Africa as identified by survey participants ($n = 262$)

Children (n)	Adults (n)
Flu† (215)	Flu (93)
Diarrhea (82)	Hypertension (69)
Chickenpox (35)	Back/joint pain (68)
Itchy scalp (19)	HIV (62)
Vomiting (11)	Headaches (51)
Fever (11)	Diabetes (49)
Wounds (9)	Tuberculosis (43)
Respiratory (coughing, asthma) (8)	Diarrhea (17)
Headaches (7)	Cancer (13)

†Participants used “flu” to describe an illness with coughing, sneezing, runny nose, fever, and/or chills. Etiology was not confirmed.

All surveyed individuals were asked about their diets and frequency of consumption of selected food items. The most frequently consumed food item was *pap*, a traditional

ground maize porridge, eaten on average 13.3 ± 4.4 times per week. Bread was consumed 5.9 ± 2.9 times per week, followed by poultry at 4.1 ± 2.8 times per week, and eggs and fruit both consumed 3.1 ± 2.7 times per week. Less common sources of protein included fish, beef, goat, and wild animals (predominantly impala, duikers, and scrub hares). A small, yet statistically significant relationship existed between poultry and red meat (beef, goat, pork) consumption and the total number of chickens and cattle owned, respectively. Household poultry consumption increased 0.04 times per week for every increase by one in the number of chickens owned ($p = 0.0244$); more frequent red meat consumption in a household, defined as more than monthly, increased by 4% for every increase by one in the number of cattle owned ($p = 0.037$). A statistically significant relationship between consumption of eggs and milk did not exist between poultry and cattle ownership, respectively.

Zoonotic disease knowledge and practices

All participants were asked about their perception of disease transfer from animals to humans. Sixty-one percent of those surveyed thought that some diseases of animals could be passed to humans. For these participants, open responses regarding what diseases could be passed from animals to humans included: rabies, food-borne diseases, foot and mouth disease, Newcastle disease, skin conditions consisting of pruritus and alopecia, tuberculosis, influenza, chickenpox, and joint problems from a new “fast-growing breed” of chickens; the most common response was “I don’t know.” The animal species of most concern with regard to disease transmission to humans was dogs ($n = 125$), followed by cattle ($n = 25$), and cats ($n = 11$).

Forty-eight percent of respondents thought that how you handle or prepare food products from animals could determine whether you got sick. Among those participants who reported owning milking animals, such as cattle or goats ($n = 92$), 36% drank the milk from their animals. Of those individuals, 24% preferred to drink their milk fresh (not boiled). For those participants who reported boiling their milk, their reasons for boiling included to “kill germs” and to “get rid of the smell.”

Eighty-five percent ($n = 224$) of those surveyed believed that shared water sources among people, livestock, and wild animals could be a health risk. However, 118 (45%) of

individuals reported that they collect water for their household from places where animals are known to use the water. A similar proportion reported washing clothes where animals (domestic or wildlife) drink the water. There was not a statistically significant relationship between knowledge of potential health risks of shared water and water collection behaviors (two-sided chi-square test, $p = 0.877$).

Environmental health

Individuals were asked about their perception of drinking water reliability and safety using a five-point Likert scale. The percentage of respondents who perceived water reliability favorably differed by village (two-sided chi-square test, $p = 0.0033$). Utha community members perceived water reliability least favorably; Gottenburg residents perceived water reliability most favorably. Water safety was generally recognized as favorable across all villages with 117 (45%) responding “strongly agree” and 131 (50%) responding “agree.”

All participants were asked about their utilization of disposable diapers. For those that had a young child in the household, 80% reported that they used disposable diapers. Among those individuals, the disposal methods included: burning (44%), throwing in pit toilet (22%), burying (21%), throwing in the bush (10%), and throwing in/around the river (6%).

Firewood was used for cooking energy in 94% of households. The primary source of firewood was self-collection from the bush (96%). Forty percent ($n = 106$) of households reported using cattle manure for either fire or fertilizer.

Wildlife (including rodents) and conservation

Seventy-six percent ($n = 198$) of those surveyed reported seeing rodents in and around their home; of those, 62% reported daily observations, 27% reported observations 1-6 times per week, and 9% reported monthly observations. Control techniques included rodenticides, glue traps, cats, beating with sticks, and chasing away. Significant risk factors for rodent sightings included high household size (defined as ≥ 6 persons per household) (OR = 2.742, 95% CI = 1.473 – 5.104, $p = 0.0016$) and chicken ownership (OR = 2.059, 95% CI = 1.113 – 3.811, $p = 0.0217$). Cat ownership was associated with a

decreased risk of rodent observations in and around the home (OR = 0.287, 95% CI = 0.107 – 0.767, $p = 0.0131$) (Table 4). No interaction terms were found to be significant.

Participants were asked about wildlife interactions with household members, domestic animals, and crops. Human-wildlife interactions were predominantly associated with hunting or with trapping/killing rodents. Livestock-wildlife interactions included shared or neighboring grazing areas with antelope species, buffalo, and wildebeest; predation of smaller livestock such as goats and calves by cheetah, hyenas, and lions; and snake bites on chickens. Companion animal-wildlife interactions included predation on rodents by cats and dogs used for hunting small mammals such as scrub hares and duikers. Thirty-eight percent ($n = 99$) of those surveyed reported wildlife contact with crops, predominantly by elephants ($n = 24$), rodents and hares ($n = 21$), baboons ($n = 16$), and birds ($n = 13$).

Table 4. Independent predictors, beta coefficients, odds ratios (OR) and 95% confidence intervals (CI) obtained for univariable and multivariable multilevel logistic regression model (with village as random effect) of domiciliary rodent observation (yes/no) in households within the Mnisi study area, Mpumalanga Province, Republic of South Africa

Predictors (n)*	β	Univariable OR	95% CI	p - value	β	Multivariable OR†	95% CI	p - value
X ₁ ‡. Household dwellers coded as 1 - ≥ 6 (median) (136), 0 - < 6 (126)	0.9520	2.591	1.567 – 5.031	0.0015	1.0056	2.734	1.461 – 5.114	0.0018
X ₆ . Chicken ownership coded as 1 – yes (144), 0 – no (118)	0.6828	1.979	1.118 – 3.505	0.0191	0.7213	2.057	1.108 – 3.818	0.0224
X ₈ . Cat ownership - coded as 1- yes (23), 0 – no (239)	0.7658	0.465	0.191 – 1.132	0.0917	-	0.289	0.107 – 0.777	0.0141
Fit statistics for multivariable model								
σ_j^2 (SE)						0.1264 (0.1596)		

AUC (ROC)

0.71

*Number of observations in each class of the predictor variable

†Final multivariable model included only those variables that were significant ($p < 0.2$) in the univariable model and significant ($p < 0.05$) in the multivariable model

‡Variable X_1 was tested both as continuous and binomial (0 = value < median; 1 = value $\geq$ median) variables to determine the best model fit

Model fit statistics: σ_j^2 = variance of the random effect; SE = standard error; AUC = area under curve; ROC = receiver operating characteristics

Seventy-four percent of participants agreed that diseases affecting wild animals could be transmitted to domestic animals; of the remaining, an equal proportion disagreed and responded, “I don’t know.” Participants’ perceptions of wildlife and neighboring conservation areas are shown in Figure 4. When asked about advantages of adjacent conservation areas, the primary responses included employment opportunities, recreation, wildlife conservation, protection from wildlife, educational opportunities, and donations. Disadvantages included wildlife damage to crops and predation of livestock, safety risks to community members, unfair employment practices, wildlife poaching/crime, and restricted access to natural resources such as grazing land and firewood.

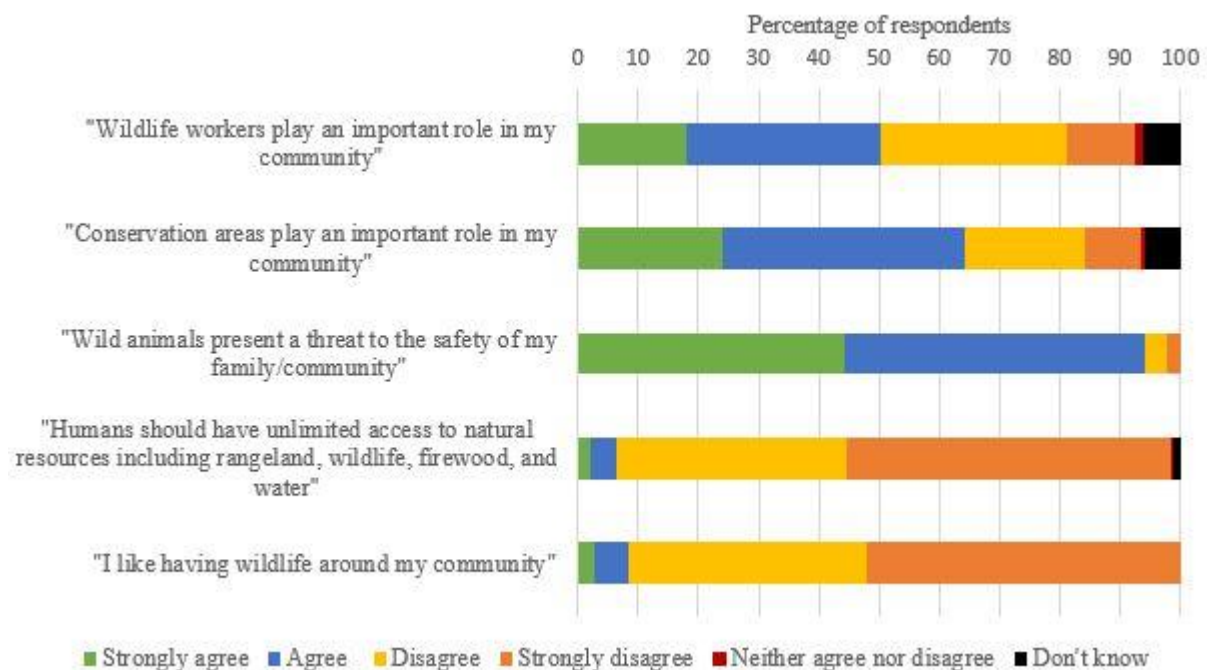


Figure 4. Stacked chart illustrating the perceptions of wildlife and conservation among survey participants in the Mnisi study area, Mpumalanga Province, Republic of South Africa ($n = 262$)

Discussion

This study describes a method by which to initiate integrated health research and planning at the community level that combines community engagement and the OH approach. The result is a comprehensive profile of animal and human demographics

and health priorities and a description of environmental practices including waste disposal, water collection as well as wildlife-domestic animal-human interactions. With regard to animal demography, 72% of all surveyed households reported owning at least one animal and 68% reported owning livestock. This proportion of livestock ownership is consistent with similar rural, resource-poor communities (Livestock in Development, 1999; Pica-Ciamarra *et al.*, 2011). The majority of animal-owning respondents were satisfied with the availability and affordability of veterinary services within the community. In the Mnisi study area, there is no privatized animal health care. Veterinary care is made available through the state veterinary service and the UP-run Hluvukani Animal Health Clinic which provides clinical services at a subsidized rate as part of student training. Cattle-owning participants across all villages, however, revealed a distinct need for a better-functioning cattle plunge dip for the administration of acaricides to prevent the transmission of tick-borne diseases and injury produced by tick bites. In this region, tick-borne diseases of cattle, including babesiosis, anaplasmosis, and heartwater, are prevalent (Masika *et al.*, 1997; Rikhotso *et al.*, 2005). The economic impact of ticks and tick-borne diseases is great and can be expressed in terms of mortality, production loss, cost of control, and, in some cases, movement restrictions of animals (Mbatia *et al.*, 2002). In addition, specific cattle ticks (*Amblyomma hebraeum*) can carry African tick bite fever, the most prevalent rickettsial disease in South Africa among humans (Frean *et al.*, 2008). The Mnisi study area is in a FMD protection zone with vaccination, and the South African government (Mpumalanga Veterinary Services) mandates weekly inspection of cattle at registered facilities. At this inspection, cattle are typically exposed to an acaricide by plunge dipping, but sometimes pour-on treatment is used in the absence of a dip tank at the inspection point. Frequent dipping was introduced to prevent cattle losses from the buffalo-borne corridor disease (*Theileria parva*) which is transmitted mainly by the brown ear tick (*Rhipicephalus appendiculatus*), as well as to serve as an incentive for farmers to participate in the weekly inspections related to FMD control policy. However, due to government budget restraints affecting water supply infrastructure to plunge dip tanks and even villages in especially the dry season, weekly dipping frequency is sometimes interrupted. The survey revealed that one-third of cattle owners had not dipped their cattle for ticks in the past month. While the survey was conducted in the winter months when ticks are less prevalent, the dissatisfaction with cattle dip facilities is likely due, in part, to this lack of consistency in dipping frequency.

Prior studies in the area have shown that intensive acaricide application may be unnecessary and that a strategic regimen, one that considers relative tick abundance, may be effective in producing endemic stability of tick-borne diseases in communally-grazed cattle (Rikhotso *et al.*, 2005). If well-communicated, a strategic tick control regimen could be favored among farmers in the Mnisi study area due to some of the documented disadvantages of intensive cattle dipping, including physical injuries, stress, and labor costs (Rikhotso *et al.*, 2005).

Among the livestock-owning respondents, a disease of poultry consistent with Newcastle disease was the most prevalent health concern of food-producing animals described. Chickens are a valuable protein source for resource-poor South Africans as they are inexpensive to keep. However, given the free-range nature of chickens in this region, the risk of infectious disease outbreaks among poultry is high and difficult to control without proper interventions (Thekiso *et al.*, 2004). Newcastle disease is a viral disease, and although it is endemic to South Africa, poultry losses are largely preventable with proper vaccination (Thekiso *et al.*, 2004). Preventive health measures such as vaccination and segregation of sick animals could be effective tools in these communities. Improved poultry health could have significant implications for public health and nutrition given the reliance on eggs and poultry for protein in the Mnisi study area.

This study identified a linkage between human nutrition and animal ownership. Households owning larger flocks of chickens and herds of cattle reported an increased consumption of poultry and red meat, respectively. The association between animal ownership and likelihood of consuming animal source foods has been previously demonstrated by Thumbi *et al.* (2015), albeit with egg and milk consumption. Our study did not show a statistically significant relationship between livestock ownership and egg or milk consumption. This finding may suggest a difference in dietary preferences or that these animal products are used in an alternative way, such as an income source. Although we identified an association between human nutrition and animal ownership, it is important to consider the possible confounding effect of household socioeconomic status, including non-livestock income sources, which we did not measure in our study and which may be positively associated with both animal ownership and household consumption of animal products (Thumbi *et al.*, 2015). Future surveys in the study area should consider including household income as it is a possible explanatory or confounding variable.

This survey highlighted many opportunities to improve animal health education in this community, not only pertaining to animal diseases for the sake of improving production and perhaps livelihoods, but also for improving public health. Zoonotic diseases such as rabies, tuberculosis, and brucellosis continue to pose a threat to public health in many parts of South Africa, including the Mnisi study area (Marcotty *et al.*, 2009; Conan *et al.*, 2015). Few households reported a history of an illness that could be linked to animals; however, zoonoses are likely underdiagnosed and misinterpreted in this community (Quan *et al.*, 2014). The perception by the survey respondents of malaria as a human disease linked to animals may indicate a misunderstanding of the zoonotic potential of malarial parasites and, thus, an opportunity for health education in this community. Additionally, in this study, one-quarter of those who drank the milk from their own animals preferred to drink the milk fresh as opposed to boiling first. The handling and consumption of fresh milk can be a source of zoonotic tuberculosis and brucellosis (Marcotty *et al.*, 2009). This health risk is preventable with proper education. Increased education about animal diseases was desired by 96% of animal-owning survey respondents. In this study, animal-owning individuals were more likely to have no formal education; thus, health education targeted towards animal-owning community members may be a sound approach to addressing critical infectious disease control points within the Mnisi study area.

With some public health risks, such as the collection and household utilization of water from sources shared by domestic animals and wildlife, it was evident that knowledge of health risks was not sufficient to alter behavior. Therefore, it is likely that, with regard to this particular public health issue, there are other factors to consider, including availability of water by established taps or proximity to such taps. Participants from Gottenburg revealed the highest satisfaction with water reliability, therefore follow-up studies could evaluate location-specific factors affecting water supply.

Prior to this survey, there was no knowledge on the frequency of utilization of disposable diapers or disposal practices. This study revealed that the majority of households with young children utilized disposable diapers and that nearly half of these households opted for burning the waste; however, throwing the waste in the bush or near the river was also described. Not only is the degradation process for disposable diapers slow, the contamination of waterways by human feces is a public health concern. As the frequency of these practices is likely to increase, future

research that focuses on evaluating water quality in relation to waste disposal may become a priority.

With regard to satisfaction with available public health services, overall the respondents were very satisfied with the community health clinics; however, the survey revealed a statistically significant difference between those respondents residing in villages with a community health clinic versus those without a clinic. Those individuals from villages without a clinic who cannot afford transport or who are too ill to travel may not be able to access care. Despite the health services being provided by the government at no cost, if an individual cannot access the clinic, they are not able to benefit from the services. The survey revealed a need for enhanced transportation options or mobile health service delivery to improve access to disadvantaged members of the community.

Due to the random selection of surveyed households and the high participation rate, results of this study are likely highly generalizable to other households within the four villages. However, it is probable to assume that our survey demographics likely over-represented unemployed, less educated household members. The survey included a skewed distribution of respondents by sex, education, and employment status. Surveys were conducted during the working hours of 8 a.m. – 5 p.m. on weekdays when most employed household members would not be available for questioning. In addition, it is likely that these individuals would be less aware of household practices pertaining to livestock care which are typically performed by males. Also, as this study relies on self-reported disease and health data, responses were subject to recall bias. Correlating these data with human and animal medical records would be one method by which to validate participant responses. Although the objective of the current study was largely descriptive of a single defined population, a lack of a comparison group may be considered a limitation. Follow-up studies should consider evaluating significant associations in additional communities.

In this study, “flu” was described as a priority health problem in both children and adults. It is important to acknowledge that responses may have been influenced by the seasonality of data collection as all surveys were conducted during the winter months. Future studies could evaluate the effect of temporality on human and animal health priorities. Further, participants described flu symptoms and not necessarily a diagnosis of influenza. Participants used “flu” to describe illness with coughing, sneezing, runny nose, fever, and/or chills. Etiology was not confirmed.

Diagnostic capability at community clinics is limited, therefore, a targeted effort should be made to determine the etiology of observed human and animal illnesses and the concurrent collection of economic, social, and biological determinants of health that have proven to be significant in this study. Zoonoses are likely underdiagnosed, therefore, additional studies focused on quantifying the burden of zoonotic diseases in this population are warranted.

Wildlife are an important potential reservoir for zoonoses, particularly peri-domestic wildlife such as rodents which were observed in three-quarters of all surveyed households. The observation of rodents around the home has been shown to be an independent risk factor for zoonotic diseases such as leptospirosis, regardless of direct contact (Sarkar *et al.*, 2002). In the current study, household rodent sightings were frequent across all villages and significantly associated with increased household size and chicken ownership, presumably due to increased food availability and/or more suitable rodent habitat (*i.e.*, poor external hygiene). Bonner *et al.* (2007) demonstrated that poor external hygiene may act as a risk factor for domiciliary rodent infestation and the transmission of rodent-borne zoonoses. Another possible explanation for the observed association between household size and rodent sightings is the increased likelihood of witnessing and reporting of rodent observations by household occupants to the adult survey respondent. The presence of a cat in the household significantly reduced the risk of household rodent sightings in our study. Endemic zoonoses such as leptospirosis can present with non-specific symptoms, thus posing a challenge to human and veterinary clinicians in resource-poor areas (Halliday *et al.*, 2015). Thus, regionally-specific risk factors such as these may be very helpful in refining diagnostic and treatment algorithms, leading to improved disease management.

Over 90% of study participants were in favor of the regulation of access to natural resources, including rangeland, wildlife, firewood, and water, indicating a strong sense of environmentally sustainable practices. In addition, the majority of participants believed the conservation areas played an important role in their community. Advantages of the adjacent conservation areas included economic (*e.g.*, employment opportunities, donations) and non-economic (*e.g.*, recreation, education, wildlife protection) factors. Prior studies involving communities adjacent to parks indicate that perceptions of biodiversity conservation are strongly related to locally-perceived benefits (Anthony, 2007; Vodouhê *et al.*, 2010). Thus, an environmentally aware,

conservation-conscious community is more likely to arise from one that benefits from the activities directly. Despite this appreciation for wildlife conservation, most survey participants indicated a dislike for wildlife around their community and a perceived threat by wildlife to personal safety. Together, these results suggest a preference by the community for a dichotomous approach to wildlife management, one that separates wildlife from humans, as opposed to one that is more integrative. This paradox has been previously described in communities adjacent to Kruger National Park (Anthony, 2007). It must be added that perceptions regarding models for the potential integration of, for instance, livestock and wildlife, in parts of the landscape where it might be feasible, were not explicitly tested. These findings, however, demonstrate the complexity of the landscape and the need for a broad, social-ecological approach to health.

Conclusion

In low-income countries, the linkages between livestock keeping and human health are complex and include both positive (e.g., animal source food availability) and negative (e.g., zoonotic and food-borne diseases) effects (Nicholson *et al.*, 2003; Randolph *et al.*, 2007). To improve health at the community level, the goal must be to maximize the positive linkages while minimizing the negative effects of the human-animal connections. Future steps should include the identification of priority diseases in humans and domestic animals, the quantification of the burden of these diseases, and the identification of their risk factors. This type of detailed data which summarizes agricultural practices, food consumption and water-use habits, illness in animals and people, and access to health care can be used to tailor education efforts for priority diseases and pandemic prevention (Mazet *et al.*, 2009). A healthier Mnisi community, part of a region delicately balancing on the fringe of the Great Limpopo TFCA of eastern South Africa, would provide benefits not only within the boundaries of the community but beyond the fences, potentially impacting health and conservation efforts in the regional network of protected areas.

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