BRUCELLOSIS IN HUMANS AND LIVESTOCK IN RURAL UGANDA: EPIDEMIOLOGY AND TOOLS FOR RESOURCE-LIMITED SETTINGS

By

RoseAnn Miller

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ABSTRACT

BRUCELLOSIS IN HUMANS AND LIVESTOCK IN RURAL UGANDA:
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Objectives: A cross-sectional study was undertaken to describe the epidemiology of zoonotic brucellosis in dairy farms in rural southwestern Uganda, and to evaluate the use of tools to address challenges to disease surveillance programs faced in rural sub-Saharan Africa and other resource-limited settings.

Methods: Blood samples were collected from cattle, goats and humans, and milk samples were collected from cows on 70 dairy farms in two sites in Uganda. Samples of blood and milk from livestock were collected and dried on laboratory-grade filter paper. Data describing each animal and human subject were recorded during sample collection, and herd-level risk factor data were collected by questionnaires on livestock health and management, human health and practices associated with increased brucellosis risk. Livestock blood and milk samples were tested using the Rose Bengal (RBT) and milk ring (MRT) tests. Human blood was tested using an immunochromatographic lateral flow assay (LFA). A qualitative real-time PCR (q-PCR) and nested PCR (n-PCR) were used to detect *Brucella* in DNA extracted from human, cattle and goat blood clots, cow milk, and human sera, and q-PCR was used to test DNA extracted from cattle and goat dried blood and milk samples. Multivariable regression was used to test associations between positive test results with subject characteristics and brucellosis risk factors.

Results: Tests for brucellosis were positive in 14% of 768 cattle sera, 29% of 635 bovine milk, 17% of 315 goat sera, and 11% of 236 human serum samples. Both q-PCR and n-PCR detected *B. abortus* DNA in cattle, q-PCR detected *B. abortus* in humans and goats, and n-PCR detected *B. melitensis* in cattle blood and milk, and evidence of *B. melitensis* in human blood. DNA was successfully extracted from blood and milk samples stored on filter papers after four years of storage at room temperature. Both *B. abortus* and *B. melitensis* DNA were detected in dried samples, and agreement was seen between results of screening tests with q-PCR results from dried samples. Increasing seroprevalences in goats was significantly ($p \leq 0.05$) associated with seropositivity in cattle (OR = 1.2, 95% CI = 1.1 – 1.3) and seropositivity in humans (OR = 1.2, 95% CI = 1.0 – 1.5). Improvements in farm biosecurity and hygiene decreased risk for positive MRT, positive RBT in goats, and positive LFA results in humans. Tick control in cattle reduced the risk of brucellosis (OR = 0.4, 95% CI = 0.2 – 0.7). Human seropositivity was associated with brucellosis in goats (OR = 1.2, 95% CI = 1.0 – 1.5), goat slaughtering (OR = 14. 2, 95% CI = 1.8 – 110.0), and acquiring goats from neighbors (OR = 5.1, 95% CI = 1.9 – 14.0).

Conclusions: Brucellosis is present in cattle, goats and humans on farms in southwestern Uganda. Although cattle are the focus of brucellosis control in Uganda, brucellosis in goats may be an important contributor to the epidemiology of the disease, and goats may be an important reservoir of *Brucella.* The associations between livestock brucellosis with wildlife contact, and tick control measures suggests that wildlife and arthropod vectors may play roles in the epidemiology of brucellosis on these farms. The LFA for human brucellosis, and dried sample storage on filter paper, met WHO criteria for use in resource-limited settings, and have potential for use in brucellosis surveillance and research.

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KEY TO ABBREVIATIONS

2-ME	2-mercaptoethanol test
AIC	Akiake Information Criteria score
ASSURED	WHO criteria for diagnostic test development: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable
BPAT	Buffered plate agglutination test
BSL-3	Biosecurity Level 3 laboratory facilities
CDC	Centers for Disease Control and Prevention
C-ELISA	Competitive ELISA
CFT	Complement fixation test
CI	Confidence Interval
Ct	Threshold cycle
DALY	Disability-adjusted life-years
DBS	Dried blood sample
df	Degrees of freedom
DMS	Dried milk sample
ELISA	Enzyme-linked immunosorbent assay
FMDV	Foot and mouth disease virus
FPA	Fluorescence polarization assay
FTA	Flinders Technology Associates
I-ELISA	Indirect ELISA
LFA	Lateral Flow Immunoassay

LMNP	Lake Mburo National Park
MAT	Serum microagglutination test
MLST	Multilocus sequence typing
MLVA	Multiple locus variable number tandem repeats analysis
MRT	Milk Ring Test
n-PCR	Nested PCR
OIE	Office Internationale d'Epizootie (World Organization for Animal Health)
OR	Odds Ratio
PFGE	Pulsed-field gel electrophoresis
QENP	Queen Elizabeth National Park
q-PCR	Qualitative real-time PCR
r	Correlation coefficient
RB51	*Brucella abortus* vaccine strain RB51
RBT	Rose Bengal Plate Test
RFLP	Restriction fraction length polymorphism
S19	*Brucella abortus* vaccine strain S19
SAT	Serum Agglutination Test
Se	Test sensitivity
SNP	Single nucleotide polymorphism
Sp	Test specificity
SST	Serum Separation Tube
TAT	Tube agglutination test

WGS	Whole genome sequencing
WHO	World Health Organization
X^2	Chi-square statistic

CHAPTER 1: Introduction

Brucellosis, or infection by *Brucella* species, is major zoonotic disease of ruminant livestock, and is responsible for human illness and loss of livestock in Uganda and other sub-Saharan countries (Akakpo et al., 2010; Seleem et al., 2010; McDermott et al., 2013). In animals, both domestic and wild, brucellosis is responsible for late-term abortion "storms" in pregnant females and epididymo-orchitis in intact males (Bardenstein & Banai, 2010; Schumaker, 2013). Human brucellosis can present with a variety of symptoms, and can be misdiagnosed as malaria, arthritis or other diseases (Reyburn et al., 2004; Franco et al., 2007; Dean et al., 2012b; Wang et al., 2012). The burden of disease attributable to brucellosis is not well-documented: lack of effective disease surveillance programs and the institutional challenges faced by veterinary and public health agencies in developing countries that make surveillance difficult result in underreporting of brucellosis in both animals and humans (Akakpo et al., 2010; Cutler et al., 2012; Dean et al., 2012a; McDermott et al., 2013; Ducrotoy et al., 2014). Brucellosis has significant economic impacts in developing countries, from decreases in production and mortality in livestock, to losses in farm income, and to public health costs for human brucellosis, including lost work or income due to illness, and costs for treatment (McDermott et al., 2013).

The epidemiology of zoonotic brucellosis in developing countries is complex, with several challenges to effective disease control and prevention programs. The disease ecology of brucellosis is complex (Muma et al., 2006; Godfroid et al., 2011; Gomo et al., 2012; Treanor, 2013), with several known reservoir hosts for *Brucella*, and the intracellular nature of infection can result in chronic brucellosis cases that are difficult to diagnose (Franco et al., 2007; Dean et al., 2012a; Fruchtman et al., 2005).

Existing control programs are normally based on experience in high- and middle-income countries, and are often not feasible or affordable in developing countries (McDermott et al., 2013). Many of the current gold-standard test for brucellosis in animals and humans require access to facilities and equipment that are not readily available or affordable (Kettler et al., 2004), from the ability to conduct high-throughput molecular typing for molecular epidemiological studies, down to the ability to collect and store samples through a cold chain with reliable electrical supply for freezers (HEEPI, 2011).

It is critical to develop disease control strategies in conjunction with local stakeholders to ensure that any intervention and control programs are effective, affordable, and culturally acceptable (Cascio et al., 2011; Montiel et al., 2015). This process requires baseline data on the extent and ecology of the disease in question, populations at risk, and an understanding of the resources available to support disease control programs of the affected region. The goal of this dissertation is to evaluate the extent and epidemiology of brucellosis in livestock and humans in rural southwestern Uganda, and evaluate alternatives for testing and biological sample storage that address the challenges to effective disease control strategies in developing countries.

Problem Statement:

- Although the presence of brucellosis in humans, livestock, and wildlife in developing countries has been acknowledged, there are gaps in the knowledge in the epidemiology of the disease:
    - The true burden of disease is not known in humans, livestock, and wildlife
        - Brucellosis is misdiagnosed and underreported in humans

        - Routine surveillance of livestock is not conducted in many countries
    - The dynamics of disease transmission in areas of high interaction between humans and livestock, including
        - *Brucella* spp. circulating between the groups
        - Alternative routes of disease transmission
        - Possible contributions of wildlife
    - The impact of chronic cases of brucellosis on transmission between humans, livestock, and wildlife
- Surveillance is a critical component of brucellosis control programs, but challenges exist to implementing programs:
    - Tests for surveillance programs that meet the WHO ASSURED criteria are not currently accepted by agencies establishing standards for programs that meet standards for international trade and public health
    - Alternatives to traditional sample collection and storage that meet ASSURED criteria have not been explored in the field in the context of livestock and human disease surveillance

The first goal of this dissertation was to provide data to describe the epidemiology of zoonotic brucellosis in dairy farms in rural southwestern Uganda, by describing the prevalence of cattle, goats, and humans on farms, identifying risk factors associated brucellosis test positivity, describing the species and strains of *Brucella* in samples from cattle, goats, and humans, and evaluating the potential impacts interaction between humans and livestock. The second goal of

the dissertation was to evaluate the use of diagnostic tests and sample storage techniques that can address the challenges to disease surveillance programs faced in rural sub-Saharan Africa.

Study Overview:

A cross-sectional study was conducted in two sites in southwestern Uganda, from July to November 2011. The study population consists of humans, cattle, and goats from farms in two different study areas in Uganda: Kiruhura District near Lake Mburo National Park (LMNP), and Bushenyi, Sheema, and Bunyaruguru Districts (from the old Bushenyi District) near Queen Elizabeth National Park (QENP) (Figure 1). Both areas are within the "cattle corridor" of Uganda, and differ in ecology, wildlife interaction, and animal husbandry practices. A stratified sampling approach was used to select dairy farms for participation in the study, stratified by site (LMNP, QENP), sub-county, and herd sizes.

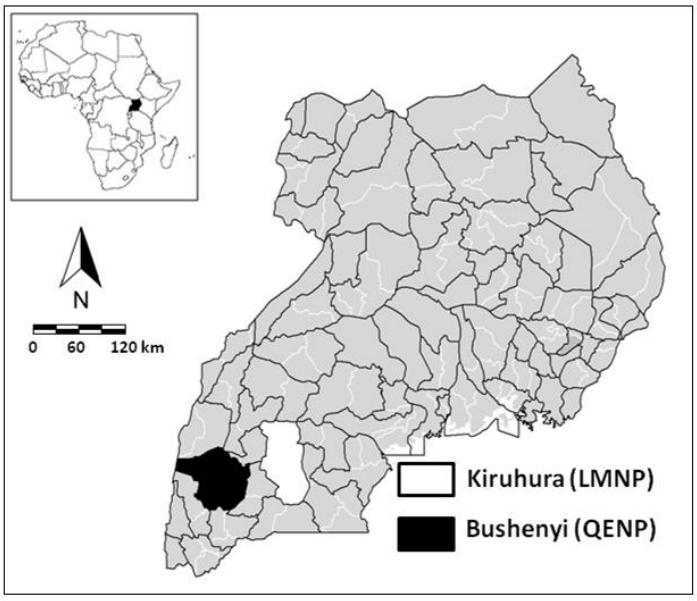

Figure 1.1 Map of study sites in southwestern Uganda

Samples were collected from 773 cattle (providing 635 milk samples), 315 goats, and 207 human subjects. Blood samples were collected from cattle, goats, and humans by venipuncture. Data describing each animal (breed, gender, age, date of most recent calving or kidding, abortion history) and human subject (age, gender, history of fever, raw milk consumption, livestock contacts) were collected during sample collection. Herd-level risk factor data were collected by in-person interviews using pre-tested questionnaires on livestock health and management, human health and practices perceived to be associated with increased brucellosis risk.

Primary Research Questions:

- What is the extent of brucellosis in livestock farming households in rural Uganda?
- What factors contribute to the inter-species transmission of brucellosis on the farm in Uganda?
- What practical options are available to reduce the impact of brucellosis by improving surveillance programs in Uganda?

Structure of the Dissertation:

To address the primary research questions, the dissertation was divided into separate but integrated studies:

Chapter 2: **Review of the Literature**

Chapter 3: **The Prevalence of Brucellosis in Humans, Cattle, and Goats in Uganda: a comparative study** (published in Transboundary and Emerging Diseases (Miller et al., 2015)

3.1. Hypothesis: Brucellosis is prevalent in humans, cattle, and goats in rural Uganda

3.1.1. Objective: Collect biological specimens and risk factor data from dairy farms in two different districts in southwestern Uganda

3.1.2. Objective: Conduct statistical analyses to identify risk factors associated with livestock brucellosis

3.2. Hypothesis: Human brucellosis is associated with interaction with infected livestock

3.2.1. Objective: Use screening tests for detection of brucellosis in specimens from humans, cattle and goats

3.2.2. Objective: Conduct statistical analyses to identify risk factors associated with human brucellosis

Chapter 4: **Detecting Human Brucellosis in Southwestern Uganda: Evaluation of a lateral flow assay for use in resource-limited settings** (submitted for publication in Emerging Infectious Diseases)

4.1. Hypothesis: The lateral flow assay (LFA) can be used to assess brucellosis exposure in humans in the field in rural southwestern Uganda.

4.1.1. Objective: Test sera from persons from dairy farms in Uganda using the LFA

4.1.2. Objective: Describe associations between LFA status and supporting test results (PCR, microagglutination) and risk factors for brucellosis

4.2. Hypothesis: The LFA is suitable for use in surveillance programs in resource-limited settings.

4.2.1. Objective: Evaluate the LFA as a tool for disease surveillance in resource-limited settings.

4.2.2. Objective: Evaluate the use of the LFA as a diagnostic tool to detect possible cases of brucellosis in resource-limited settings.

Chapter 5: **Validation of the use of dried blood and dried milk spots for detection of *Brucella* in cattle and goats for surveillance in resource-limited settings** (in preparation for publication in Zoonoses and Public Health)

5.1. Hypothesis: DNA for detection of *Brucella* can be extracted from DBS and DMS from cattle and goats.

5.1.1. Objective: Extract usable DNA from dried blood and milk samples.

5.2. Hypothesis: Results of PCR with DNA extracted from stored DBS and DMS are comparable to blood and milk screening tests for the detection of *Brucella* in cattle and goats.

5.2.1. Objective: Compare PCR results of DNA samples from dried samples with the results of screening tests.

5.3. Hypothesis: DBS and DMS on laboratory-grade filter paper can be used to store samples for epidemiological investigations in resource limited settings.

5.3.1. Objective: Utilize test results from dried samples to describe aspects of the epidemiology in livestock on dairy farms in rural Uganda

5.3.2. Objective: Evaluate DBS and DMS as tools for disease surveillance in resource-limited settings.

Chapter 6: **Use of molecular tools to detect *Brucella* in cattle, goats, and humans in Ugandan dairy farms**:

6.1. Hypothesis: Both *B. abortus* and *B. melitensis* are present in cattle, goats, and humans on dairy farms in rural Uganda.

6.1.1. Objective: Identify species of *Brucella* present in DNA extracted from cattle, goat, and human blood samples, and cattle milk samples, from dairy farms in southwestern Uganda, using a nested PCR (n-PCR) detect genetic markers for *Brucella* spp., and specific targets for *B. abortus*, and *B. melitensis*.

6.1.2. Objective: Identify species of *Brucella* present in DNA extracted from cattle, goat, and human blood samples, and cattle milk samples, from dairy farms in southwestern Uganda, using a qualitative real-time PCR (q-PCR) detect genetic markers for *Brucella* spp., and specific targets for *B. abortus*, and *B. melitensis*.

6.2. Hypothesis: Species of *Brucella* found in cattle and goats on dairy farms in rural Uganda are not host-specific.

6.2.1. Objective: Describe the distribution of *Brucella* species from human blood, bovine and caprine blood, and bovine milk samples, based on n-PCR and q-PCR.

6.2.2. Objective: Describe associations between n-PCR and q-PCR test results with results of screening tests and risk factors associated with brucellosis.

6.2.3. Objective: Utilize results from PCR to describe the epidemiology of brucellosis in cattle, goats, and humans from dairy farms in southwestern Uganda.

Chapter 7: **Summary and Conclusions**

CHAPTER 2: Review of the Literature

Introduction: Zoonotic Diseases

Emerging and re-emerging zoonotic diseases are global challenges to animal and public health (IOM & NRC, 2008). The human impact of zoonotic diseases is particularly devastating in developing countries, where the spread of disease is more likely and where public health resources are limited (Cascio et al., 2011). Not only do zoonotic diseases affect humans and domestic animals, but they can have significant impacts on ecosystems, such as reducing prey species numbers, which has negative effects on predators (Gortázar et al., 2007), and changing browsing and grazing pressure by livestock or wildlife, which can significantly affect ecosystem forage production (Prins & Van der Jeugd, 1993).

The economic costs of zoonotic diseases have been estimated to be over $20 billion in direct costs (World Bank, 2010a). Zoonotic diseases have negative consequences for livestock production: decreased milk production, reduced fertility, slower growth, animal mortality, and losses when the presence of disease restricts the markets for animal products. In many under-developed regions of the world, increasing smallholder animal husbandry has been targeted for improving farmer income and nutrition (Lai, 2007; Swanepoel et al., 2010; Jones et al., 2013), but as livestock production increases, opportunities for disease transmission between livestock and humans also increase (Randolph et al., 2007; IOM & NRC, 2009; Cascio et al., 2011).

Beyond the costs associated with the treatment of human disease, indirect costs of zoonoses are often overlooked. The impact of zoonoses in terms of disability-adjusted life-years (DALYs) can be quantified to describe the long-term impacts of disease on the human population: a cost-benefit analysis of vaccinating livestock in Mongolia for brucellosis found

that, when the impact of disease on DALYs was included, the estimated costs for vaccination (US$ 8.3 million) were exceeded by its overall benefit (US$ 26.6 million) (Roth et al., 2003). Economic losses from outbreaks of Nipah virus, West Nile Fever, SARS, HPAI, BSE, and RVF from 1997–2009 went at least US $80B: prevention would have avoided losses of $6.7 B/year (World Bank, 2010b), and cost-benefit analyses determined that interventions in animal populations to reduce levels of zoonotic diseases were cost effective; control of the animal diseases was less expensive than the costs of disease in humans (World Bank, 2010b).

Brucella: A Model Zoonotic Pathogen

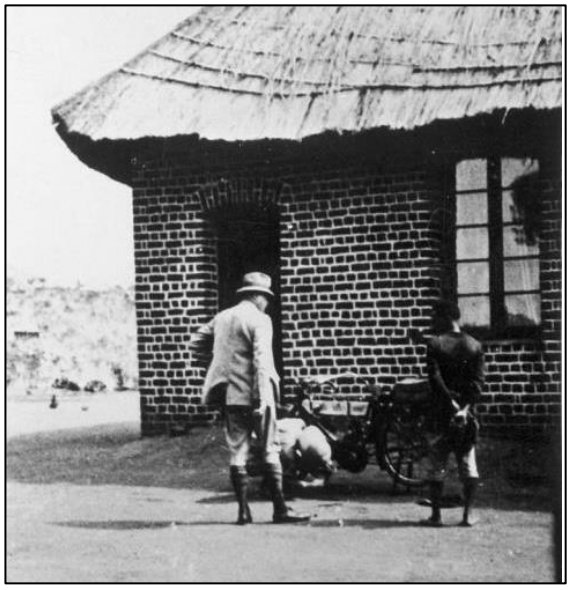

Figure 2.1 Sir David Bruce in Kazo, Uganda, 1912

Brucellosis: a brief history

Brucellosis (infection by *Brucella* species) is a neglected and ancient zoonotic disease of global importance (Franco et al., 2007; Holveck et al., 2007; Mauldin et al., 2009; WHO, 2007). The disease is primarily associated with ruminant livestock, and is responsible for human illness and fetal wastage/infertility of livestock in sub-Saharan Africa (Ndyabahinduka & Chu, 1984; Mutanda, 1998; Akakpo et al., 2010). The bacteria responsible for brucellosis was identified in the 1850s, and awareness of the disease in the western world has been credited to Sir David Bruce (Figure 2.1), who was able to isolate the parasite responsible for "Malta", or "remittent" fever (Bruce, 1891; Wyatt, 2013). In addition to the work by Bruce, for whom *Brucella* was named, Dr. Themistocles Zammit, a Maltese bacteriologist reported the infection in goats and determined that the organism was present in milk (Wyatt, 2013). Recent advances in paleoepidemiology and paleopathology have described evidence of brucellosis long before the work of the 19th century. Skeletal lesions suggestive of human brucellosis has been found in remains from the Bronze Age (Kay et al., 2014), the late Roman empire (Capasso, 1999), Europe from the 10th to the 19th century (Mutolo et al., 2012; Kay et al., 2014; Curate, 2006), and from 2.5 million year old hominids (*Australopithecus africanus*) (D'Anastasio et al., 2009). Lesions consistent with brucellosis have also been reported in skeletal lesions of horses (Bendrey, 2008; Janeczek et al., 2010) and goats (Kafil et al., 2014), but the approach for diagnosis via skeletal lesions is controversial (Bendrey et al., 2008).However, *Brucella* DNA has been identified from 10th – 14th century human remains (Mutolo et al., 2012; Kay et al., 2014), and from a woman and goat buried together in the late Bronze Age in Iran (Kafil et al., 2014). A draft genome sequence of a ~ 700 year old strain of *B. melitensis* from human remains from Sardinia was

found to genomically clustered with *B. melitensis* biovars 3 Ether (ATCC 23458), a strain currently circulating in Italy (Kay et al., 2014).

The *Brucellae*

The *Brucella* belong to the family Brucellaceae, order Rhizobiales, class α-Proteobacteria (von Bargen et al., 2012; Ruiz-Ranwez et al., 2013; Olsen & Palmer, 2014; Kämpfer et al., 2014), and have been speciated based on host specificity/preference, pathogenicity within a given host species, and phenotypic traits (e.g., smooth versus rough colony appearance on solid media; nitrate reduction (Al Dahouk et al., 2014; Ronneau et al., 2014), although recent genomic analyses have introduced some controversy in *Brucella* taxonomy (Whatmore, 2009; Godfroid et al., 2011; Al Dahouk et al., 2014; Olsen & Palmer, 2014).

The *Brucella* spp. are intracellular bacteria that invade and replicate in host cells, which protects them from host immune responses, which may play a role in their persistence and ability to cause chronic infection (Franco et al., 2007; Godfroid et al., 2011; Gomez et al., 2013). The initial entry of *Brucella* into the host occurs primarily through the mucosal epithelium (de Figueiredo et al., 2015). Once in the host, a transient bacteremia develops and *Brucella* preferentially invade and replicate in polymorphonuclear cells, macrophages, and dendritic cells, and have widespread distribution in lymphoid tissues and other preferred host cells (Franco et al., 2007; Baldi & Giambartolomei, 2013; Gomez et al., 2013; Scian et al., 2013). It has been suggested that *Brucella* is attracted by the erythritol present in reproductive tissues, and colonizes the trophoblasts in the chorionic villi of the placenta, resulting in abortion or

premature delivery (Lecuit et al., 2003; Aydın et al., 2013; Olsen & Palmer, 2014). In males, orchitis and epididymitis are common symptoms of infection (Olsen & Palmer, 2014).

Since *Brucellae* lack traditional pathogenic factors (e.g., exotoxin, exoprotease, cytolysin, exoenzymes) that result in direct damage to the host (Moreno & Moriyón, 2002; Baldi & Giambartolomei, 2013), pathogenic changes are associated with the inflammatory response in specific host tissues. As *Brucella* infection progresses to chronic brucellosis, focal infections in tissue induce inflammatory responses associated with histologic changes and clinical symptoms. The production of IgM occurs early in infection, followed by production of IgA and IgG later in infection (Marrodan et al., 2001; Corbel, 2006).

The host range of *Brucella* is broad, and *Brucella* have been detected in mammals, birds (Cadmus et al., 2010), and even fish (El-Tras et al., 2010) and amphibians (Eisenberg et al., 2012). There are host preferences in *Brucella*: *B. abortus* primarily associated with cattle; *B. melitensis* with goats; *B. ovis* with sheep, *B. suis* with pigs, and *B. canis* with dogs (Corbel, 1997). Other identified species of *Brucella* include *B. microti* (Scholz et al., 2008), *B. neotomae* (in desert rats)*, B. ceti*, which has different strains predominating in dolphins, porpoises, and humans (Guzmán-Verri et al., 2012), *B. pinnipedialis* (Guzmán-Verri et al., 2012), and *B. papionis* in baboons (Whatmore et al., 2014). There are also two related species of *Brucella* that have only been identified in human biopsy samples: *B. inoptinia* (classified as strain B01), was detected from a human infection after breast implant surgery (Scholz et al., 2010), and B02, which was isolated from a lung biopsy in a patient with chronic destructive pneumonia (Tiller et al., 2010a). Brucellosis has been detected in a variety of wildlife and feral species across the globe (García-Yoldi et al., 2007; Olsen, 2010; Truong et al., 2011; Godfroid et al., 2013; Fournier

et al., 2015), often in relation to the disease in domestic animals (Muma et al., 2007; Ramamoorthy et al., 2011; Godfroid et al., 2013; Kim et al., 2013; Fournier et al., 2015).

The most pathogenic species in humans, *B. melitensis, B. abortus,* and *B. suis,* the agents responsible for brucellosis in goats, cattle, and pigs, respectively, have a smooth phenotype (LPS with an O-polysaccharide chain) (Franco et al., 2007). The cell envelope of *Brucella* consists of bilayer phospholipid inner membrane, a periplasmic layer, and an outer membrane composed of lipopolysaccharide (LPS), a sugar-based outer and inner core; and the O-antigen, which is exposed to the environment (Haag et al., 2010; Kämpfer et al., 2014). The outer membrane of smooth strains (S-LPS) have all three components, but rough strains (R-LPS) lack the O-antigen (Haag et al., 2010; von Bargen et al., 2012; Kämpfer et al., 2014). The S-LPS *Brucella* are able to rapidly enter host cells by interacting with lipid rafts in the host cell plasma membranes and avoid protective responses from the host, (Gomez et al., 2013; Olsen & Palmer, 2014; de Figueiredo et al., 2015), whereas the R-LPS *Brucella* (*B. canis, B. ovis*), enter the host cells through phagocytosis and are exposed to more host cell defenses (Kämpfer et al., 2014; Moreno, 2014; Al Dahouk et al., 2014).

Although *Brucella* species primarily occur in the preferred host, they are not host-exclusive. Documentation of transmission of *B. abortus* from infected cattle to other species include goats (Leal-Klevezas et al., 2000; Adamu et al., 2012), dogs (Prior, 1976; Cadmus et al., 2011; Truong et al., 2011), cats (Truong et al., 2011), and rodents (Truong et al., 2011). Early studies reported the presence of *B. melitensis* in cattle milk by bacterial culture (Damon & Fagan, 1947). More recently, studies have reported the presence of *B. melitensis* in bovines by serology (Alvarez et al., 2011), immunohistochemical staining (Ahmed et al., 2012), and by PCR (Safarpoor Dehkordi

et al., 2014; Al-Mariri, 2015), *B. canis* in cattle (Baek et al., 2009), and dogs have acquired *B. suis* in an area where *B. suis* has become established in feral hogs (Ramamoorthy et al., 2011).

Brucellosis

The symptoms of early brucellosis infection are those associated the inflammatory response to systemic infection. In humans, the most commonly reported symptoms are a high, undulating fever and night sweats (Dean et al., 2012b), but in livestock infection is often asymptomatic, and only recognized after abortion, orchitis, or other reproductive-related problems (Glynn & Lynn, 2008).

Since fever is such a common symptom of human brucellosis, it is often mistakenly diagnosed and treated as malaria in regions where malaria is endemic (Franco, 2007; Nankabirwa et al., 2009; Dean et al., 2012a,b). This was recognized as early as 1891, with a commentary from Sir Bruce regarding the over-use of quinine (the treatment of the day for malaria) for cases of Malta fever (Bruce, 1891). In endemic areas, studies have reported that malaria was confirmed in only 25 – 32% of patients seeking treatment for self-diagnosed malaria, and in only 25% of presumptive diagnoses by health care workers in local clinics (Ndyomugyenyi et al., 2007). Reports have indicated that between 63% – 75% of clinic treatments for malaria have been given to patients without the disease (Nankabirwa et al., 2009; Ndyomugyenyi et al., 2007). Malarial misdiagnosis can result in consequences for patient health, reduce the effectiveness of malaria control programs (Amexo et al., 2004; Reyburn et al., 2004; Nankabirwa et al., 2009; Crump et al., 2013), and create conditions for drug resistance to develop (Wongsrichanalai et al., 2002). Given the similarities in case presentation and limited

diagnostic capacities in rural health care settings (Ndyabahinduka et al., 1978; Ndyabahinduka & Chu, 1984). Since the national malaria recommendations in Uganda call for presumptive diagnosis as malaria any patient presenting with fever in absence of danger signs, and prior correct use of artemisinin-based combination therapy, health officers mistakenly treat cases of brucellosis as malaria (Nankabirwa et al., 2009). Recognition of the misdiagnosis of brucellosis as malaria is increasing, and the testing of malaria patients for brucellosis has been recommended in cases where treatment for malaria has failed (Kunda et al., 2007).

After the acute phase, symptoms of brucellosis are determined by the location of focal infection in the host. Chronic brucellosis often presents as arthritis-like musculoskeletal disease (Franco et al., 2007; D'Anastasio et al., 2011; Dean et al., 2012b), and osteoarticular brucellosis is the most common localized form seen in humans (Andriopoulos et al., 2007; Mehanic et al., 2012; Baldi & Giambartolomei, 2013; Šiširak & Hukić, 2014; Boskilovski, 2015; Fruchtman et al., 2015). The presence of hygromas in livestock, particularly in leg joints, has been considered to be an indicator of chronic bovine brucellosis in tropical regions, and *Brucella* have been found in hygroma fluids (OIE, 2012; Ducrotoy et al., 2015). Neurobrucellosis occurs when *Brucella* infect and replicate within astrocytes and microglia, causing neurological diseases in humans and marine mammals (Hernández-Mora et al., 2008; Guzmán-Verri et al., 2012; West et al., 2015). Pulmonary brucellosis can be caused by inhalation of the bacteria (Pappas et al., 2003; Águila et al., 2012; Erdem et al., 2014; de Figueiredo et al., 2015), and presents with symptoms similar to pulmonary tuberculosis, including cough, pneumonia, lung abscesses, and development of granulomas and nodules where *Brucella* can be sequestered and re-emerge (Águila et al., 2012; Erdem et al., 2014).

Invasive *Brucella* can enter remain in a dormant, non-reproductive phase in synoviocytes and other preferred host cells for very long periods of time (Deghelt et al., 2014; Celli, 2015), which may contribute to relapses of infection. Infected synovicytes have been shown to attract and increase adhesion of monocytes and neutrophils (Scian et al., 2013), which may increase opportunities for *Brucella* to spread from synovicytes to other parts of the host. Relapses of brucellosis have been reported in human patients with osteoarticular disease (Buzgan et al., 2010). In domestic animals, infected animals can shed *Brucella* long after the initial infection has been resolved (Olsen & Palmer, 2014). Chronic infection can result in long-lasting pro-inflammatory response, which has been associated with the development of endocarditis in humans, which is correlated with the infrequent occurrence of mortality (Kalaycioglu et al., 2005; Al Dahouk et al., 2006; Mehanic et al., 2012; Baldi & Giambartolomei, 2013).

The epidemiology of brucellosis

Transmission of *B. melitensis* and *B. abortus* can be through shedding in milk, or exposure to fluids and tissues associated with abortion or the birth of infected fetuses (Poulou et al., 2006; Makloski, 2011; Olsen & Palmer, 2014).Other strains of *Brucella* (*B. suis, B. canis*) are known to be shed in mill, semen, and urine, and also from fluids/secretions from mucosal surfaces (Olsen & Tatum, 2010; Makloski, 2011). Livestock are the primary sources of brucellosis for humans, with goats and cattle being the primary hosts of *B. melitensis* and *B. abortus*, respectively (Olsen & Tatum, 2010). Human cases of brucellosis are tied to the disease in livestock (Osoro et al., 2015; Tumwine et al., 2015), reductions of brucellosis in livestock have resulted in reducing the number of human cases of the disease (Roth et al., 2003; Glynn & Lynn, 2008), and in one

instance cases of human brucellosis increased after animal control programs were discontinued (Shemesh & Yagupsky, 2013).

In addition to traditional livestock hosts for brucellosis (e.g., cattle for *B. abortus*, goats for *B. melitensis*), wildlife can also serve as reservoir hosts including African buffalo (*Syncerus caffer*) as reservoirs for *B. abortus* in Africa (Waghel & Karstad, 1986; Godfroid et al., 2013). Contact with livestock infected with *Brucella* is considered to be the original source of infection in wildlife, but these alternative hosts have demonstrated the ability to maintain infection and transmit disease to other free-ranging populations and livestock (Waghel & Karstad, 1986; Rhyan & Spraker, 2010; Michel & Bengis, 2012; Olsen & Palmer, 2014). There is evidence that transmission occurs between livestock and wildlife where species interact (Jiwa et al., 1996; Muma et al., 2007; Wyckoff et al., 2009; Gomo et al., 2012; Motsi et al., 2013; Rhyan, 2013). Although humans can become infected with brucellosis, person-to-person transmission is uncommon, and humans are considered to be a "dead end" host for *Brucella* (Moreno, 2014).

Important routes of infection for brucellosis are through direct contact with infected animals, infected materials, and the ingestion of materials contaminated with viable *Brucella* (Alton & Gulaskeharam, 1974; Bernard et al., 2005; Andriopoulos et al., 2007; Franco et al., 2007; Makita et al., 2008; Al-Anazi & Al-Jasser, 2013; Kutlu et al., 2014; Moreno, 2014; Olsen & Palmer, 2014). A study of brucellosis in tuberculosis patients in Pakistan found higher seroprevalences of brucellosis in young girls in rural areas, in regions where women were primarily responsible for milking cows and cleaning stalls; activities which would expose them *Brucella* from contaminated livestock (Qazilbash & Bari, 1997). However, a study in Uganda found no strong associations between human brucellosis and cattle-keeping, and suggested

that direct animal contact was not the most significant route of transmission (Kabagambe et al., 2001).

In order for *Brucella* to be transmitted by indirect contact through contaminated foods or substrates, the organism must be capable of surviving outside a living host, particularly under cool, moist conditions protected from exposure to direct sunlight (Wray, 1975; Nielsen & Duncan, 1990). Studies have found that *B. abortus* may survive in liquid manure at lower temperatures, and was able to survive for at least 8 months at 15°C (Verger, 1981). Even though it is believed that fermentation may kill *Brucella*, one study found that *B. abortus* survived in fermented milk after 10 days in refrigeration at a pH below 4.0 (Estrada et al., 2005), and in soft cheeses made from sheep, goat, and cattle milk (Verraes et al., 2015).

Consumption of milk contaminated with *Brucella* is probably the most significant routes of infection for humans. Human infection is associated with shedding of *Brucella* in milk and potential transmission to their offspring (Palanduz et al., 2000; Apa et al., 2013). Livestock with chronic disease appear symptomatic, but are capable of shedding *Brucella* for years after acute infection. In developing countries, chronically infected animals may serve as persistent sources of infection for other animals, and pose a public health risk by producing contaminated milk destined for human consumption (Olsen & Palmer, 2014). Consumption of raw milk, or dairy products produced from unpasteurized or underpasteurized milk (Falenski et al., 2011), has been strongly associated with human brucellosis (Kabagambe et al., 2001; Asiimwe et al., 2015; Nasinyama et al., 2015; Tumwine et al., 2015), and consumption of sour milk prepared with unpasteurized milk has been associated with human brucellosis in Uganda (Asiimwe et al., 2015). Residents of urban areas in the Kampala region of Uganda have previously been

demonstrated to be at risk of *Brucella* exposure through consumption of raw milk transported from peri-urban or rural areas (Makita et al., 2008).

Contact with *Brucella*-contaminated fetal membranes and fluids after abortion is the primary route of transmission in livestock and wildlife (Bercovich, 1998; Radostits et al., 2006; Glynn & Lynn, 2008). Transmission of brucellosis from pregnant women to hospital staff and physicians has occurred through contact with contaminated blood and reproductive fluids (Adams, 2002; Bardenstein & Banai, 2010; Cutler et al., 2012). Vertical transmission of brucellosis has been demonstrated in cattle, goats, and sheep (Olsen & Tatum, 2010; Díaz-Aparicio, 2013), in humans (Glocwicz et al., 2010), and recently in a sperm whale (West et al., 2015). There is inconclusive evidence for vertical transmission in dogs (Makloski, 2011) and sheep (Grilló et al., 1999). Venereal transmission of brucellosis is documented in swine and dogs (Poester et al., 2013). In humans, sexual transmission was suspected in the early 20th century (Wyatt, 2013), and has been documented in humans (Kato et al., 2007; Meltzer et al., 2010).

Other routes of transmission include inhalation of aerosols or fomites contaminated with *Brucella* (Ollé-Goig & Canela-Soler, 1987; Mesner et al., 2007; Sam et al., 2012), direct penetration through the epidermis (cuts, needle-sticks, transfusions) (Al-Anazi & Al-Jasser, 2013; Olsen & Palmer, 2014), and through blood transfusion (Akçakuş et al., 2005) and bone marrow transplantation (Ertem et al., 2000). The infectious dose of aerosolized *Brucella* is relatively low which has resulted in classification of *Brucella* as a Category B pathogen for bioterrorism (Ollé-Goig & Canela-Soler, 1987; Yagupsky & Baron, 2005; de Figueiredo et al., 2015). Early reports of outbreaks of brucellosis on sailing ships in the late 1800s were believed

to be the result of respiratory transmission, given the cramped living quarters and lack of ventilation in sleeping quarters (Wyatt, 2013). However, *Brucella* are rarely found in sputum samples from cases of pulmonary brucellosis (Pappas et al., 2003), which lessens the possibility of transmission through inhalation.

Although not an important route of infection, transmission by arthropod vectors is theoretically possible, including face flies, ticks, sucking lice, and lungworms (Cheville et al., 1989; Dawson et al., 2008; Zhleudkov and Tsirelson, 2010; Neglia et al., 2013). *Brucella* have been isolated from helminths collected from marine mammals (Garner et al., 1997; Perrett et al., 2004), which may constitute a route of infection or reservoir of brucellosis for cetaceans (Guzmán-Verri et al., 2012). Older studies have experimentally demonstrated the capacity for different arthropods to serve as possible vectors of *Brucella* (Philip & Burgdorfer, 1961; Zheludkov & Tsirelson, 2010; Delaunay et al., 2011; Zorrilla-Vacca, 2014), and a recent study detected *B. abortus* DNA and RNA, and evidence of vertical transmission, in sucking lice (*Haematopinus tuberculatus*) from *Brucella*-infected water buffalo (Neglia et al., 2013). In addition, the stress of tick infestations are known to have negative effects on cattle growth and production (Okello-Onen et al., 2003; Jonsson, 2006; Rodriques & Leite, 2013; Tolleson et al., 2012), and may affect immune responses of infested animals (Ribiero et al., 1990; Inokuma et al., 1993; Guo et al., 2009), which could increase susceptibility of cattle to *Brucella* infection. Although consensus is that the role of arthropod vectors of brucellosis is insignificant in comparison with other routes of infection (Philip & Burgdorfer, 1961; USDA APHIS VS & Strickland, 1976; Zheludkov & Tsirelson, 2010), the contribution of arthropod vectors should be

considered in the epidemiology of brucellosis on farms (Zheludkov & Tsirelson, 2010; Neglia et al., 2013).

Several risk factors have been associated with brucellosis in livestock and humans. The reported risk factors for human brucellosis are strongly associated with zoonotic transmission of the disease, and include livestock contact, raw dairy foods consumption, and lack of access to health care (Bernard et al., 2005; El Sherbini et al., 2007; Franco et al., 2007; Makita et al., 2008; Samaha et al., 2008; Cutler et al., 2010). Risk factors for brucellosis in cattle include increasing herd size (Berhe et al., 2007; Ibrahim et al., 2010; Kadohira et al., 1997; Muma et al., 2007), herds that purchase cows (Muma et al., 2007; Matope et al., 2010), and cattle of exotic and mixed/local exotic breeds (Jiwa et al., 1996; Mekonnen et al., 2010). Herd management practices reported to be associated with increasing brucellosis risk include pasturing cattle (Berhe et al., 2007; Kadohira et al., 1997), intensive herd management (Berhe et al., 2007; Mekonnen et al., 2010; Ducrotoy et al., 2014), and a lack of biosecurity and exposure to wildlife (McDermott & Arimi, 2002; Muma et al., 2007). Increasing levels of brucellosis in cattle in Nigeria have been linked to an increasing intensive cattle industry (Ducrutoy et al., 2014). Geographic location has been reported to be associated with differing disease seroprevalences (Berhe et al., 2007; Ibrahim et al., 2010; Kadohira et al., 1997; Muma et al., 2007). Presence of brucellosis on a farm could imply infection in multiple species, as associations have been found between seropositivity in cattle and goats within a region (Kabagambe et al., 2001; Megersa et al., 2011).

Zoonotic Disease Control

The goal of zoonotic disease control programs is to eliminate or reduce the impact of the disease on human and animal populations. Disease control programs rely on the following steps: first, identifying infected populations and conducting surveillance to estimate the extent of disease; next, applying strategies to reduce levels of disease and spread of disease in the infected population, including vaccination, livestock test-and-slaughter, wildlife feeding bans, and vector control programs; and finally, ongoing surveillance and monitoring to capture any new cases of disease and measure the progress of the control programs (Meyer, 1956; Dowdle & Cochi, 2011; Narrod et al., 2012; Godfroid et al., 2013; McDermott et al., 2013). These programs have succeeded in virtually eradicating bovine brucellosis in the developed world, but have required years of dedicated adherence to aggressive surveillance and control programs (McDermott et al., 2013).

Even with access to the most advanced technologies and strong infrastructure support, brucellosis can become a nearly intractable disease problem in developed countries. Brucellosis control is difficult when wildlife reservoirs of disease are present and capable of re-infecting livestock (Cosivi et al., 1998; McDermott et al., 2013), as in the western U.S., where brucellosis has become an issue for livestock in an area where free-ranging bison and elk form a free-ranging reservoir of *B. abortus* (Higgins et al., 2012). In other regions, control of brucellosis in goats and other small ruminants is not a priority, given that the majority of small ruminants are owned by lower income farmers and are not considered to be a priority for disease control (Blasco & Molina-Flores, 2011). Brucellosis in small ruminants is a public health risk for humans

(Franco, 2007), and examples of *B. melitensis* in cattle (Alvarez et al., 2011; Ahmed et al., 2012; Safarpoor Dehkordi et al., 2014; Al-Mariri, 2015) indicate that eradication programs for cattle may not succeed without including disease control in goats and sheep.

An important cornerstone of successful disease control programs is the development of strategies that are appropriate to the socio-economic and cultural conditions of the affected region (Blasco & Marino-Flores, 2011; Narrod et al., 2012). Strategies should be sensitive to local customs and livestock management practices to make them more acceptable to affected stakeholders. Programs should take into consideration costs of implementing control programs, and weigh them against the benefits associated with control or eradication of diseases (Narrod et al., 2012). A clear understanding of the ecology of disease and its impacts on livestock production, food security, and influences on household economics are needed to fully assess the impacts of zoonotic diseases on affected households (Narrod et al., 2012; McDermott et al., 2013). When these and other indirect costs of zoonoses are recognized, cost-benefit analyses have determined that control of the animal diseases was less expensive than the costs of disease in humans (World Bank, 2010b). In a cost-benefit analysis of different vaccination and disease control strategies for cattle brucellosis in Turkey, investigators found that disease control measures were more cost-effective than allowing brucellosis to persist in the cattle population, and that consideration should be used in selecting disease control strategies that are not only cost-effective but technically feasible (Can & Yalçin, 2013).

An important component of disease control programs are the availability of reliable tests for detecting cases of disease, effective vaccines, and effective disease surveillance programs. In addition, data on the prevalence of disease, routes of transmission, and reservoirs of disease

are critical for the design of effective control programs. These may be available or achievable in developed countries, but often unachievable in resource-poor countries. Lack of facilities and trained personnel, funding, infrastructure, and even political and social awareness of the burden of zoonotic disease in developing countries make disease control programs difficult to implement and maintain (Godfroid et al., 2013).

Challenges in resource-limited settings

The implementation of effective disease surveillance and control programs are challenges in resource-limited settings, where finances, human resources, and infrastructure may not be readily available (Zinsstag et al., 2007). The difficulty of detection and the chronic nature of brucellosis have made the disease difficult to control in the developed world, and the difficulties become compounded in less developed regions of the world. In addition, lack of access to veterinary and public health resources makes it difficult to implement disease prevention, treatment, and control programs.

Lifestyles that promote zoonotic disease transmission: Traditional livestock management practices in developing countries, such as transhumance, communal grazing, or keeping livestock longer due to economic constraints, are associated with increased risk of zoonotic disease in cattle. However, control of disease in livestock can reduce risk for human infection by decreasing human exposure through livestock (World Bank, 2010a, b). Animal agriculture (particularly cattle and goats) is a major form of livelihood in rural communities in Uganda and other sub-Saharan countries, where brucellosis is responsible for human illness and economic losses in livestock (Ndyabahinduka & Chu, 1984; Mutanda, 1998). Agriculture is the primary

livelihood of 88.8% of all households in southwestern Uganda (Uganda Bureau of Statistics, 2006), with an estimated 1.4 million cattle and nearly 600,000 goats in this region. Residents interact with livestock on a daily basis and consume animal products such as unpasteurized milk, milk products, and meat (Makita et al., 2008). High levels of direct contact with livestock coupled with poor food and household hygiene make reducing disease transmission challenging within rural Ugandan communities.

The importance of ecology and climate to the epidemiology of zoonotic diseases, including brucellosis in wildlife and humans, is known and disproportionately impacts developing countries (Parkinson & Butler, 2005; Cross et al., 2007; McDermott et al., 2013; Rodríguez-Morales, 2013). Reductions in health (and immune responses) in humans and livestock associated with water and food insecurity could also contribute to the spread of zoonotic disease. Environmental/ecological conditions can promote contact between wildlife and livestock, which could increase transmission of zoonotic diseases at livestock – wildlife interfaces. Ecological changes, both natural and anthropogenic, can increase or concentrate wildlife populations and promote disease transmission or increase competition between wildlife and livestock for dwindling water and food sources. Wildlife disease surveillance is uncommon in Uganda, and the presence of wildlife reservoirs often goes unnoticed until domestic animals or humans develop symptoms, particularly for a chronic disease such as brucellosis. Control of disease in wildlife reservoirs has relied on population reduction through increased hunting, trapping, or poisoning and vaccination, with mixed success. Efforts to reduce wildlife populations for disease control can be difficult, and often criticized by the public.

Capacity to conduct disease surveillance: The importance of surveillance for brucellosis control has been well established (Shemesh & Yagupsky, 2013), but requires money, resources, and infrastructure that may not be readily available in developing countries. There are logistical challenges and access by veterinary and public health workers to remote areas to identify at-risk subjects is difficult. Travel by vehicle is difficult in areas where well-maintained road systems are unavailable, and transhumant human and livestock populations can be difficult to locate and contact. There may be no recent or accurate human or livestock census data available to identify populations for surveillance, and health records may not be readily accessible or available. There are logistic challenges for tracking/retesting subjects in remote areas after initial screening or in locations difficult for public health workers to access (Smits et al., 2003). Surveillance programs for zoonotic diseases need accurate livestock identification, safe and effective animal restraint systems, appropriate equipment for sample collection and storage, a reliable cold chain for vaccines and reagents, and transportation from farm to laboratories for samples (Ward et al., 2012).

The costs of diagnostic tests can also be prohibitive. Ongoing research into improving diagnostic tests for brucellosis and other zoonotic diseases has resulted in a growing array of highly sensitive and specific tests. Unfortunately, many of these tests, including enzyme-linked immunosorbent assay (ELISA) and tests to detect bacterial DNA in samples, require significant resource investment. These tests may require specialized equipment and infrastructure, expensive reagents with temperature requirements for storage, and/or trained professionals to execute and interpret test results. Although new tests have greater sensitivity and/or specificity, they are not feasible in resource-limited countries. Therefore, it has been suggested

that increased access to diagnostics will have greater impact on global health than improving currently available tests (Peeling & Mabey, 2010). The World Health Organization developed criteria to guide the development of diagnostics for resource-limited settings. These guidelines specific that tests should be Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable (ASSURED) (Kettler et al., 2004). The ASSURED criteria also favor the development of single-contact screening tests that can be administered in the field, which brings the tests to the populations where they are needed (Kettler et al., 2004; Peeling & Mabey, 2010).

While ASSURED has targeted the development of diagnostic tests in humans, the storage of biological samples is another technologic need for resource-limited settings. Sample collections, fluid or other materials stored in glass or plastic tubes, can become cumbersome when hundreds or thousands of samples need to be maintained. Long term storage of specimens requires access to freezers for storage, preferably capable of maintaining temperatures at or below -20° C, is expensive and may not be feasible in developing countries. Annual costs of electricity for single -20° C and -80° C freezers have been estimated at $550 and $665, respectively (HEEPI, 2011), while cost estimates to maintain a single -80° C for one year, including energy and maintenance costs, were $6,000 at the U.S. National Cancer Institute (Baker, 2012).

The use of dried specimen storage is re-emerging as a tool for research and surveillance. Dried samples provide several advantages over existing sample storage approaches: preparation of the dried samples and methods of storage are simple, dried samples require relatively small volumes for storage, dried samples are relatively stable and only require low

humidity for preservation, and the basic materials required are inexpensive and readily available in developing countries. Blood is the most frequently reported dried specimen, and the most commonly used substrate for dried sample collection is filter paper, and the use of dried swabs for storing blood samples collected from wild boar has been evaluated (Petrov et al., 2014). The use of dried blood spots (DBS) has become an accepted practice in human medicine (Mei et al., 2001), and the WHO has developed criteria for using DBS for collecting samples for HIV testing and other disease surveillance and control programs. Studies of human brucellosis and chikungunya virus in humans have used DBS to preserve samples for ELISA and PCR testing (Andriamandimby et al., 2013; Takkouche et al., 1995). In veterinary applications, DBS have been successfully used for storing blood for monitoring environmental toxicants in wildlife (Lehner et al., 2013), antibody detection (Curry et al., 2011; Greenwald et al., 2009), and PCR for pathogen detection (Aston et al., 2014; Knuuttila et al., 2014). Dried milk spots (DMS) been used to store human milk samples for ELISA detection of antibodies (Brown et al., 1982; Miller & McConnell, 2011), and have been used for antibody detection in bovine milk (Brown et al., 1982), and to detect bacterial DNA in mastitic milk and dairy food processing (Tilsala-Timisjärvi & Alatossava, 2004). Specialized substrates for dried specimens have emerged with the demand for better specimen storage, but are prohibitive in cost for resource-limited countries. The Flinders Technology Associates (FTA)™ card, a dried specimen storage system (Mullen & Howard, 2009), includes a cellulose-based substrate impregnated with proprietary cell lysate reagents to break down cells and chemically bind DNA and RNA to the substrate. Studies comparing the performance of FTA™ with qualitative filter papers have found that FTA™ performed better than laboratory grade filter paper for detection of virus by PCR

(Jagero, 2015), and detection of antibodies to *Toxoplasma gondii* by a modified agglutination test (Aston et al., 2014). However, the cost of basic FTA™ Classic cards is nearly 40 times as expensive as 90 mm Grade 1 qualitative filter papers, from $470 per 100 FTA™ cards versus $12 for a box of 100 filter papers, making the technology difficult to afford in resource-limited settings.

Acceptance of disease control measures: Acceptance of disease control measures can be difficult to acquire, particularly when stakeholders do not understand the disease control problem, or there are economic or social barriers to acceptance of control measures. It is critical to develop disease control strategies in conjunction with local stakeholders to ensure that intervention and control programs are effective, affordable, and culturally acceptable (Cascio et al., 2011), but in many cases programs are developed without stakeholder input and meet with resistance in the field. Studies in the Middle East and Africa found that knowledge about brucellosis and other zoonotic diseases in livestock keepers was low (Swai et al., 2010; Adesokan et al., 2013; Tebug, 2013; Kansiime et al., 2014; Tebug et al., 2014; Tebug et al., 2015), including awareness of vaccination for brucellosis (Kansiime et al., 2015a). In other instances, general knowledge regarding brucellosis was good, but individuals still participated in high-risk behaviors that increase their risk for acquiring brucellosis (Holt et al., 2011). Some disease prevention measures, such as vaccination campaigns, can also suffer due to non-compliance by local officials or leaders, which can result in poor adherence to control programs (Ward et al., 2012).

Control of livestock diseases in developed countries relies on surveillance test-and-cull policies for affected animals, but the socio-economic costs of this approach can be

economically impossible in developing countries and result in refusals by livestock owners to participate in disease control programs (Cosivi et al., 1998). Test and slaughter programs are not well-received by livestock owners (Zinsstag et al., 2007), and are economically unfeasible in many developing countries (McDermott et al., 2013). One study of brucellosis control programs in Spain determined that, when vaccination campaigns were used, depopulation did not increase the efficiency of the program in terms of the time from the initiation to completion of eradication (Saez et al., 2014), indicating that depopulation need not be a component of brucellosis control in developing countries.

Improving stakeholder awareness of the negative impacts of zoonotic diseases beyond their visible effects on livestock, and providing alternative strategies that do not require loss of livestock may increase stakeholder participation in disease control programs. Brucellosis is known to reduce livestock productivity through losses of calves/kids through abortion and lowered milk production, but due to under-reporting of human disease, economic costs related to brucellosis in humans is less characterized (McDermott et al., 2013).

Tools for disease control in resource-limited settings

Tests for brucellosis: Current surveillance programs rely on screening tests that require collection of milk, blood, and/or serum to detect the presence of pathogens (e.g., visualization of *Plasmodium falciparum* in blood smears (Ndao et al., 2004)), host immune responses to infection (Gilbert et al., 2013), or pathogen DNA (Sacchi et al., 2011).

The majority of screening tests for brucellosis in humans and animals are serological tests, designed to detect antibodies against *Brucella* S-LPS. However, tests for S-LPS can be positive

long after infection by *Brucella*, and many tests are incapable of distinguishing immune responses to infection from those to vaccination with smooth strains of *Brucella*. Tests for cytosolic proteins, such as counter-immunoelectrophoresis (CIEP), ELISA and western blotting, may be more indicative of active infection (Corbel, 2006), but may not be readily available.

Commonly used screening tests for brucellosis include the Rose Bengal test (RBT), serum agglutination test (SAT), buffered plate agglutination test (BPAT), and ELISA for IgG, IgM and IgA (Corbel, 2006; OIE, 2012). The inexpensive RBT, which has high test sensitivity (Se) but lower specificity (Sp), can be easily and rapidly conducted without significant equipment and resources (Al Dahouk et al., 2013), and meets many of the ASSURED criteria (Kettler et al., 2004). The milk ring test (MRT), for detection of *Brucella* antibodies in milk, is used as a screening test for cattle (OIE, 2012). Given the higher rates of false positives associated with the MRT, particularly with milk from very early and late stages of lactation (Ferguson & Robertson, 1960; Morgan, 1970; OIE, 2012), the MRT has been designated for use for bulk tank milk screening, followed by individual animal testing, by the OIE (OIE, 2012). Despite these issues associated with the MRT, recent investigators have suggest that the simplicity and cost-effectiveness of the MRT still make this test a viable means for screening small herds for brucellosis in resource-restricted areas (Mohamand et al., 2014). The MRT and RBT test for different antibodies (IgA and IgG, respectively), and differences between infection status and shedding in milk may result in inconsistent findings, which has been seen in field studies where both tests were used together (Cadmus et al., 2008). Recently, a simple and rapid lateral flow assay (LFA) for detection of IgG and IgM for *Brucella* was developed (Smits et al., 2003; Irmak et al., 2004; Zeytinoğlu et al., 2006; Franco et al., 2007; Mizanbayeva et al., 2009; Peeling &

Mabey, 2010; Krug et al., 2011; Román et al., 2013; Lobna et al., 2014; Nour et al., 2015). The test is a simplified version of *Brucella*-specific ELISA, meets many of the ASSURED criteria, and has been successfully used in disease outbreak investigations in Peru (Mendoza-Núñez et al., 2008), detection of brucellosis in family contacts of patients (Kose et al., 2006), and for serosurveys in Turkey and the Sudan (Regassa et al., 2009; Osman et al., 2015). Although the LFA has not designated as an official test for use in surveillance programs, it has demonstrated potential as a valuable tool in resource-limited settings.

Supplementary tests are used in conjunction with screening tests to confirm screening test findings, but are not considered to be diagnostic tests in themselves. Supplementary tests for brucellosis include the rivanol precipitation test, 2-mercaptoethanol (2-ME) test, milk ELISA, and the fluorescence polarization assay (FPA) (Corbel, 2006; OIE, 2012). The complement fixation test (CFT) and indirect ELISA (i-ELISA) are considered to be confirmatory tests, but the CFT is extremely complicated and difficult to conduct (Corbel, 2009). Since IgM presents early in acute infection, serological tests for acute brucellosis that are efficient at detection of IgM, such as the RBPT and SAT are preferred (Marrodan et al., 2001). For chronic or relapsing disease, when IgM is low or absent, the Coombs *Brucella* anti-globulin test is preferred (Corbel, 2006; Nouri et al., 2014). All of these tests require access to incubators, centrifugation, and can be complicated to conduct.

The gold standard for the diagnosis of active brucellosis infection is bacterial culture (Corbel, 2006; OIE, 2012), but *Brucella* are slow-growing and fastidious, and culture should only be handled under BSL-3 conditions to avoid laboratory infection (Corbel, 2006; OIE, 2009; Al Dahouk et al., 2014). The sensitivity of using bacterial culture to detect *Brucella* can be low and

can be influenced by the types of tissues tested, numbers of viable organisms present, antimicrobial drug use, and the stage of infection (Yagupsky, 1999; Franco, et al., 2007; Espinosa et al., 2009; O'Grady et al., 2014). Consequently, a combination of screening and confirmatory tests, with supporting epidemiological evidence, are more commonly used to diagnose the disease in humans and animals. In humans, the WHO defines human cases as either suspected (presence of clinical signs consistent with brucellosis, and the case is epidemiologically linked to suspected or confirmed animal cases or contaminated food products), probable (a suspected case with a positive presumptive RBT or SAT), and confirmed (a suspected or probable case with a positive confirmatory IgG ELISA or Coombs test, or bacterial culture) (Corbel, 2009). Despite challenges with recovery using bacterial culture, it allows identification of the species of *Brucella* in a subject, which can be important for patient treatment, is critical epidemiological information for regulatory and public health programs (Franco et al., 2007).

As technology has progressed, a wide variety of methods have become available for molecular detection of *Brucella*, including pulsed-field gel electrophoresis (PFGE), PCR, restriction fraction length polymorphism (RFLP) analysis, single nucleotide polymorphism (SNP) analysis, multilocus sequence typing (MLST), multilocus variable number of tandem repeats analysis (MLVA), and whole genome sequencing (Whatmore, 2009; Scholz & Vergnaud, 2013). Studies have found that *Brucella* DNA persists after infection (Muñoz et al., 2005). The presence of DNA in seronegative samples may be due to chronic or early infection, when antibody levels are below detectable limits (Greiner & Gardner, 2000), or may represent fragmented DNA from *Brucella* that are no longer viable or have been effectively phagocytosed (Al Dahouk et al.,

2013). While these technologies are sophisticated and require significant investments in resources and capital, the data provided from molecular testing provides investigators with detailed information on circulating strains of *Brucella* in different populations, and provide insights into transmission dynamics of brucellosis (Sanogo et al., 2013a).

Results of any molecular analyses are highly dependent on the quality and quantity of genetic material extracted from samples, and several studies have indicated that DNA extraction methods significantly influence performance of different PCR assays (Matrone et al., 2009). There are a wide variety of approaches for each step in the PCR process: lysis can be conducted using mechanical processes (e.g., heating and freezing (Verjarano et al., 2013)) or a wide variety of chemical lysates (Matrone et al., 2009); removal of unwanted proteins using proteinases (e.g., proteinase K) and RNases; and a broad selection of DNA purification methods, including variations of phenol-chloroform extraction (Leal-Klevezas et al., 1995), ethanol precipitation, alkaline extraction (Al Mariri et al., 2010), and column purification. The process has been greatly expedited with the availability of a variety of DNA extraction kits, which have simplified the DNA extraction process and can be automated for high-volume throughput. Recently, the FTA™ card has been used to simplify the process of DNA and RNA extraction in conjunction with sample storage (Mullen & Howard, 2009).

The use of PCR to detect *Brucella* is gaining acceptance as a confirmatory test (Corbel, 2006; Yu & Nielsen, 2010; OIE, 2012), and as a valuable tool for molecular epidemiology. Standard PCRs (e.g., Bricker & Halling, 1994; Gupta et al., 2014), nested PCR (Rijpens et al., 1996; Al-Nakkas et al., 2002), and multiplex PCRs (Bhure et al., 2012) have been used to detect a variety of targets for *Brucella*. As technology has matured, multiplex PCRs, and quantitative and

qualitative real-time PCRs (rt-PCR) have been developed for detection of specific *Brucella* targets (Probert et al., 2004; García-Yoldi et al., 2006; Capparelli et al., 2008; Hinić et al., 2009; Bounaadja et al., 2009; Amoroso et al., 2011; Colmenero et al., 2011; López-Goñi et al., 2011; Safarpoor Dehkordi et al., 2014; Mugizi et al., 2015). Commonly used primer pairs for PCR target the IS711 insertion sequence (Halling et al., 1993), which appears in multiple copies in all *Brucella* (Whatmore, 2009),and genes encoding for different outer membrane proteins (e.g., *bcsp31*, *omp2a, omp2b, omp25*) (Cloeckaert et al., 1995; Yu & Nielsen, 2010; Guzmán-Verri et al., 2012; Scholz & Vergnaud, 2013). One of the first widely-used PCR for detection of the most common *Brucella* species was the AMOS (*abortus – melitensis – ovis – suis*) PCR developed at the USDA (Bricker & Halling, 1994; Scholz & Vergnaud, 2013). The Bruce-Ladder multiplex PCR utilizes eight pairs of primers to detect all known *Brucella* species (at the time) and the vaccine strains *B. abortus* S19, *B. abortus* RB51, and *B. melitensis* Rev1 (García-Yoldi et al., 2006). Several PCRs, including AMOS and Bruce-Ladder, has been modified over time to increase the numbers of biovars and species they can detect (Bricker & Halling, 1995; López-Goñi et al., 2011).The use of high-resolution post-amplification melt analysis of products from rt-PCR has been developed to improve test performance and the potential to identify unusual or novel *Brucella* (Winchell et al., 2010).

There are several methods used for the molecular biotyping of *Brucella.* Genotyping by MLVA has become a valuable tool for molecular epidemiological studies. One of the earlier and more commonly-used MLVA is the “HOOF-Prints” (Hypervariable Octameric Oligonucleotide Finger-Prints) assay, which relies on the detection of eight different loci (Bricker et al., 2003; Yu & Nielsen, 2010). As research has progressed, there are now a wide variety of MLVA assays

available, using different numbers of different loci in combination (Le Fléche et al., 2006; Tiller et al., 2009; Whatmore, 2009; Yu& Nielsen, 2010; Higgins et al., 2012), but there has yet to be a MLVA approach that has global acceptance. The MLVA approach is being used to develop a "lab on a chip", which would allow for a rapid, single-step process for *Brucella* genotyping (De Santis et al., 2009).

With the recent rapid improvements in technology, whole genome sequencing (WGS) has been applied to *Brucella* for studies to describe the evolution and divergence of *Brucella* (Chain et al., 2005; Foster et al., 2009; Tan et al., 2015), and to identify new species of *Brucella* (Tiller et al., 2010b; Whatmore et al., 2014). Results from WGS have been used for source tracking in epidemiological studies of brucellosis in dogs (Kaden et al., 2013), cattle (Garofolo et al., 2015) and humans (Quance et al., 2016), and as testing costs have decreased over time, WGS is being investigated as a diagnostic test for *Brucella* isolated from field samples (Shallom et al., 2012).

Vaccines for brucellosis: Currently, the only vaccines available for brucellosis are for use in livestock, due to their pathogenicity in humans (Corbel, 1997; Dorneles et al., 2015). There are live, attenuated vaccine strains that have been used in the former USSR (*B. abortus* 19-BA, *B. abortus* strain 82, *B. suis* 61) (Ivanov et al., 2011; Denisov et al., 2013) and China (*B. abortus* 104M, *B. suis* 52), but they are not available from sources with quality control standards that meet international requirements, and induce hypersensitivity with repeated doses (the duration of protection with 19-BA is only one year) (Corbel, 2006). Potential new vaccines for humans include vaccines based on phenol-insoluble residues of lipids extracted from *B. melitensis* and *B. abortus,* LPS-protein conjugates, recombinant proteins, DNA, and subunit gene targets (Corbel, 2006; Avila-Calderón et al., 2013; Dorneles et al., 2015).

Vaccines for use in livestock include the live attenuated *B. abortus* S19 and *B. abortus* RB51 for *B. abortus*, and *B. melitensis* Rev 1. The S19 vaccine was a naturally attenuated smooth mutant of *B. abortus* that has been widely used since the early 20th century (Avila-Calderón et al., 2013). It confers long-term immunity, but many screening tests (e.g., RBT, MRT) cannot distinguish vaccinated animals from infected animals, and (Avila-Calderón et al., 2013). The RB51 vaccine is a spontaneous rough mutant of *B. abortus* 2308, which has the benefit not inducing positive serological test results (Avila-Calderón et al., 2013). Although protection against *B. abortus* by S19 is excellent, it does not protect cattle against experimental infection with *B. melitensis* as well as it did for *B. abortus* challenge in an experimental study (Lucero et al., 2006). The *B. melitensis* Rev.1 is widely used in small ruminants, and has been recommended for vaccination of cattle in areas where *B. melitensis* is likely to infect cattle (Banai, 2002), although this is not in accordance with international guidelines for use of the Rev.1 vaccine (OIE, 2012).

Whole-herd and mass vaccination campaigns are recognized as an effective strategy for the control of brucellosis in developing countries (Blasco & Molina-Flores, 2011; Ward et al., 2012). The effectiveness of vaccination can be more important than test-and-slaughter programs: in one study of brucellosis control in a high-prevalence region of Spain, vaccination only with RB51, and S19 followed by RB51, was as effective as a combination of vaccination with a test-and-slaughter program, which would have important advantages in situations where removing animals from the farm is not acceptable to farm owners (Saez et al., 2014).

The One Health approach and strategies for zoonotic disease control in resource-limited settings

The One Health approach is the utilization of unified human and veterinary approaches to the control of zoonotic diseases (Schwabe, 1984). The foundations of One Health are grounded in veterinary medicine. The focus of early proponents of veterinary public health impacting human public health involved hazards of milk from diseased cows in the 1880s, from diseases including bovine tuberculosis, typhoid fever, diphtheria, and brucellosis (Steele, 2008). Actions to control milk-borne diseases included pasteurization after production, and control of brucellosis and bovine tuberculosis in cattle through Grade A milk requirements for cattle herd health status, which has resulted in the near eradication of these diseases as foodborne hazards in the US (Steele, 2008).

As the One Health concept has emerged as a combined approach to dealing with public and veterinary health, the scope of One Health has been expanding to encompass ecosystem health and sociology. Under One Health, human and animal health can be linked in different ways: economic benefits can be realized by the farm household when healthy livestock are more productive; nutritional benefits come from access to foods of animal origin from healthy stock; and zoonotic disease benefits resulting where healthy animals are less likely to transmit disease (Thumbi et al., 2015). Under One Health, control programs are designed to control disease in domestics (human and animal) and minimize the impact of both disease and control programs on the local/regional ecosystems and socio-economic needs. The interplay between humans, livestock, wildlife, and ecology in the epidemiology of zoonotic diseases, including brucellosis,

makes control of the zoonotic disease an ideal target for the application of the One Health approach.

Cooperation and the sharing of resources between public health and veterinary medical health through One Health approaches can address many of the challenges to zoonotic disease control efforts in resource-limited settings (Narrod et al., 2012). Sharing resources and facilities between public and animal health institutions and researchers takes advantage of existing infrastructure and reduces unnecessary duplication, and also has the shared benefit of increasing interaction between professionals in these disciplines. Interaction will raise awareness in all areas, including medical professionals, governmental agencies, and other stakeholders. Integrated human-animal disease control programs can have a synergistic effect in controlling disease and in program efficiencies (McDermott et al., 2013). In a review of the scientific literature describing surveillance programs for emerging zoonosis, a trend towards integrated human-animal surveillance systems embodies One Health principles (Rweyemamu et al., 2012). An additional component of the One Health approach, incorporating veterinary medical, ecological, public health, and sociological expertise, is to provide useful education programs to help stakeholders reduce risk of acquiring zoonotic disease. Culturally appropriate education and active engagement of livestock owners and other stakeholders in the development and execution of disease control programs is critical for their success (Cascio et al., 2011).

Combined public health and veterinary laboratory resources results in efficiency gains that reduce costs and improve access to health services, particularly in developing countries where zoonotic diseases like brucellosis are important issues and resources are limited (World Bank,

2010b). Simultaneous surveillance of human and animal populations, would reduce detection times, and is a recommended surveillance strategy for zoonotic disease in integrated human and animal programs (Cosivi et al., 1998). Successful joint vaccination programs have been conducted in Chad as part of a health care delivery system for livestock and humans (Bechir et al., 2004).

Recently, a One Health approach was used to develop joint syndromic disease surveillance for humans and livestock in western Kenya (Thumbi et al., 2015). Rather than relying on specific diagnostic tools, bi-weekly visits to farms collected data on human symptoms and animal signs, socio-economic data were collected every three months, and farmers were able to report livestock illness to a toll-free telephone number leading to an animal health team visiting the affected household within 24 hours. Data collected in the first year of this program established baseline values for human and animal health and household socio-economic status. Data analysis revealed positive associations between respiratory disease and gastrointestinal disease in humans and livestock, and clearly demonstrated health linkages between humans and animals. Data collection in this study entailed significant commitment of time and resources in travel and communication that a future cost-benefit analysis will determine the program's practicality and potential for use in other areas. As the use of mobile phones has increased in East Africa over time, this and other studies describing programs using phones for public health (Déglise et al., 2012; Brinkel et al., 2014), agriculture (Furuholt & Matotay, 2011), and animal health and management (Angello, 2015; Chenais et al., 2015; Mtema et al., 2016) demonstrate the potential of mobile phones to have significant positive impacts in resource-limited countries.

Summary

The impacts of zoonotic diseases take a significant toll on humans and animals in developing countries, where disease control programs are hampered by lack of resources and other challenges. The chronic nature of brucellosis makes infection difficult to detect, and similarities between symptoms of acute brucellosis in humans with symptoms of malaria contributes to underestimations of the extent and impact of brucellosis in developing countries. Control of brucellosis is complex, due to multiple reservoirs of disease. By utilizing disease control programs appropriate to resource-limited settings, and One Health approaches for integrated human health and animal health programs, effective disease control programs for brucellosis can be developed for use in developing countries.

CHAPTER 3:

The Prevalence of Brucellosis in Cattle, Goats and Humans in Rural Uganda: a Comparative Study

Structured Abstract

Introduction: Brucellosis is a widely recognized zoonotic infection and an important livestock disease in developing countries, but the extent of brucellosis is not well documented in Uganda, particularly in humans and non-bovine livestock.

Hypotheses:

- Brucellosis is prevalent in humans, cattle, and goats in rural Uganda.
- Human brucellosis is associated with interaction with infected livestock.

Objectives: A cross-sectional study was conducted to determine the presence of brucellosis in cattle, goats and humans in farms from southwestern Uganda, and identify risk factors associated with brucellosis in these three host groups.

Methods: Data and serum samples were collected from 768 cattle, 315 goats, and 236 humans, with 635 samples of bovine milk, from 70 farms in two different study areas in southwestern Uganda. Sera from livestock were tested with the Rose Bengal Plate test, using *B. abortus* and *B. melitensis* antigens, and human sera were tested with a commercial IgG/IgM lateral flow assay. Milk samples were tested using the OIE-approved milk ring test.

Results: Screening tests for brucellosis were positive in 14% of cattle serum, 29% of bovine milk, 17% of goat serum, and 11% of human serum samples. There were significant differences in the test prevalence of brucellosis by study site, with levels higher in the study area near Lake Mburo National Park than in the study area near Queen Elizabeth National Park. Positive associations were seen between increasing seropositivity of brucellosis in goats, cattle, and humans. Multivariable regression models identified risk factors associated with increasing test positivity at the individual and farm-levels for cattle, goats and for humans: improvements in farm biosecurity and hygiene, and tick control in cattle, reduced the risk of brucellosis.

Conclusions: Although cattle are the focus of brucellosis control in Uganda, the significant associations between seropositivity in humans and seropositivity in goats suggest that brucellosis in goats may be an important contributor to the epidemiology of the disease in the farm.

Key words: Livestock brucellosis; Human brucellosis; Comparative epidemiology; Rose Bengal Plate Test; Milk Ring Test; Lateral Flow Assay

Introduction

Brucellosis (infection by members of the bacterial genus *Brucella*), is a widely recognized zoonotic infection found in livestock, companion animals, and wildlife throughout the world (Glynn & Lynn, 2008; Lopes et al., 2010). While levels of brucellosis in livestock have declined in developed countries, where active vaccination and control programs have been in effect, the disease is still an important livestock disease problem in developing countries (McDermott & Arimi, 2002; Corbel, 2006). Animal agriculture (particularly cattle and goats) is a major form of livelihood in rural communities in sub-Saharan Africa, where *Brucella* infections have been shown to be responsible for human illness and loss of livestock (Mutanda, 1998; McDermott & Arimi, 2002). In Uganda, the prevalence of brucellosis has been reported to be 12 – 15.8% in cattle (Bernard et al., 2005; Mwebe et al., 2010), and 4% in goats (Kabagambe et al., 2001). The herd prevalence of brucellosis is much higher, with reports of 55% in cattle (Bernard et al., 2005) and 13 – 43% in goat herds (Kabagambe et al., 2001).

There have been spatial patterns in the occurrence of brucellosis in Uganda. Several studies have reported that cattle from eastern districts had lower herd- and animal-level prevalences of brucellosis than districts in the central and western parts of the country (Magona et al., 2009, Kashiwazaki et al., 2012). However, when looking at the test prevalence of brucellosis in samples submitted to referral laboratories in Uganda (Mwebe et al., 2010), the highest prevalences from districts submitting at least 100 samples were found in different districts in throughout the 'cattle corridor' in Uganda (including Kiruhura), while no brucellosis was found in other districts in the same areas. Some of these patterns have been attributed to cattle

management (Magona et al., 2009), while other studies found no significant differences in the prevalence of bovine brucellosis between different management strategies (Nizeyimana et al., 2013).

The epidemiology of brucellosis within domestic species (Bercovich, 1998; Mikolon et al., 1998), and between domestic animals and wildlife (Frölich et al., 2006), has extensively been investigated. Transmission among animals occurs primarily through direct contact with infected animals, contact with fluid or tissues from *Brucella*-induced abortions, or through the milk of infected dams (Glynn & Lynn, 2008). Although *B. abortus* is primarily associated with bovine brucellosis and *B. melitensis* primarily with caprine brucellosis, there have been reports of cattle with *B. melitensis* infections (Alvarez et al., 2011) and goats with *B. abortus* (Leal-Klevezas et al., 2000; Adamu et al., 2012). Human brucellosis has been associated with livestock contact, raw dairy foods consumption, and lack of access to health care (Franco et al., 2007; Bernard et al., 2005; Makita et al., 2008). There have also been instances of human-to-human transmission of brucellosis through breast milk of infected mothers to children (Palanduz et al., 2000; Apa et al., 2013) or through sexual intercourse (Kato et al., 2007). Human cases of brucellosis are tied to the disease in livestock, and reductions of brucellosis in livestock have resulted in reducing the number of human cases of the disease (Glynn & Lynn, 2008; Roth et al., 2003).

To investigate the epidemiology of brucellosis in livestock and humans in rural southwestern Uganda, a this study was conducted to test the **hypotheses** that brucellosis is prevalent in humans, cattle, and goats in rural Uganda; there are spatial patterns in the distribution of brucellosis in humans, cattle, and goats in rural Uganda; that both *B. abortus* and

B. melitensis circulate between livestock and humans within farms; and that human brucellosis is associated with interaction with infected livestock.

The **objectives** of the current study were to determine the seroprevalence of brucellosis, and associated risk factors, in cattle, goats, and humans from farms in rural southwestern Uganda. The specific aims of this study were to collect biological specimens and risk factor data from dairy farms in two different districts in southwestern Uganda; to use screening tests for detection of brucellosis in specimens from humans, cattle and goats; and to conduct statistical analyses to identify risk factors associated with brucellosis. Milk samples were tested for the presence of *Brucella* antibodies in milk from lactating cows to compare milk sample results with serological test results for individual cows.

Results from this study, coupled with information factors that can influence disease transmission, can make significant contributions to our understanding of how the dynamic interplay between livestock and humans influence the emergence and transmission of brucellosis and other zoonotic diseases, and can produce an integrated approach to reduction of disease risk in developing countries where humans and livestock commonly interact.

Materials and Methods

A cross-sectional study was conducted in two sites in southwestern Uganda, from July – November 2011. The two sites (Kiruhura District and the greater Bushenyi District), are located in the Ugandan "cattle corridor" (Figure 1.1, Chapter 1), areas with a rich variety of wildlife,

different forms of livestock management, and interaction between livestock, wildlife, and humans (NEMA, 2007). The Kiruhura District, near Lake Mburo National Park (LMNP), is primarily savannah grassland which has declined (due to overgrazing and human activities), with a resultant decline in grazing areas for livestock and wildlife (Ocaido et al., 2008). In contrast, the greater Bushenyi District, near Queen Elizabeth National Park (QENP), has a range of vegetation from open savannah to rainforest, scattered with papyrus swamps, crater lakes, and large open-water lakes. Both sites had histories of brucellosis in the past. Cattle farms in Kiruhura District are larger than those in Bushenyi, primarily due to the availability of pasture for larger numbers of animals.

Study populations

A stratified sampling approach was used to select dairy farms for participation in the study. Farms were stratified by site (LMNP, QENP), by sub-county within sites (7 within each site), and by relative herd sizes (small, medium and large). The sub-counties were selected based on their proximity to the national parks, from locations adjacent to the national park, to locations approximately 100 km from the park. Sample sizes were calculated using an expected individual animal prevalence of 10–15 % for both cattle and goats (Bernard et al., 2005; Kabagambe et al., 2001; Mwebe al., 2010), and cattle herd prevalence of 56% (Bernard et al., 2005). A minimum number of 276 individuals would be sufficient to detect an individual animal prevalence of 15% ± 10% for cattle and goats, and 66 farms would be sufficient to detect a herd prevalence of 50% ± 25%, all with 80% power and $\alpha = 0.05$. A total of five farms within each sub-county, including one small (10-30 head in LMNP, 5-10 in QENP), two medium (31-99 in LMNP, 11-29 in QENP),

and two large (100+ in LMNP, 30+ in QENP) farms, were enrolled, for a total of 35 farms within each site, and a total of 70 farms in the study. Cattle samples were collected by herd size stratum (five samples from small farms, 10 samples from medium farms, 15 samples from large farms), resulting in a target sample size of 770 cattle. Samples were collected from up to five individual goats and humans from each farm, resulting in a target sample size of 350 for each.

Sample and data collection

Individual dairy farmers that had at least five milking cows and were located in areas accessible by local transportation were contacted by local veterinary officers for possible participation in the study. After informed consent was obtained, a farm visit was arranged to collect biological samples from cattle (blood, milk), goats (blood), and humans (blood). Farm visits were conducted by veterinarians and veterinary students for animal specimen collection, and biomedical students and licensed Health Care Centre technicians for human specimen collection.

Animals for sample collection (preferably lactating females) were randomly selected by study investigators at the farm, manually restrained by the owner, and a 10 ml blood sample was collected by jugular venipuncture using Vacutainer® serum separation tubes. For cows in milk, a combined milk sample from all four quarters from each cow was collected aseptically in sterile 30 ml universal containers. Data describing each animal included a unique animal identification number, breed (local, exotic, mixed), gender, age, date of most recent calving or kidding, and abortion history (yes or no) (Appendix A).

For sample collection from human subjects, informed consent was sought from the farmer, farm workers, and family members. After consent was given, a 10 ml blood sample was collected by venipuncture, using Vacutainer® serum separation tubes. All samples were kept on ice until processed at local veterinary laboratory within 12 hrs of sample collection. In addition, information on each subject providing a sample was collected during the sample collection process. Data describing each human included age, gender, history of fever, consumption of raw milk, contact with livestock, contact with animals giving birth, and contact with slaughtered animals (Appendix B). In addition, samples were taken from veterinary medical officers and local village officials that requested the test at the time of the farm visits in their areas, after informed consent was obtained.

Risk factor data were collected by in-person interviews using pre-tested questionnaires to collect information on livestock health and management, human health and practices perceived to be associated with increased brucellosis risk (e.g. consumption of raw dairy products, handling animals after abortions). When necessary, veterinary and biomedical students and local veterinarian officers served as translators. The livestock questionnaire (Appendix C) collected data on the livestock inventory, including herd health history, livestock housing, pasturing, watering, and presence of wildlife in livestock areas. The human household questionnaire (Appendix D) included the household inventory, including contact with livestock (direct contact, contact with animals giving birth, and contact at slaughter) and wildlife, any family history of symptoms consistent with brucellosis, consumption of raw dairy products, sources of water, water treatment, and sharing of water with livestock.

Serological testing

Serological testing of samples from cattle, goats, and humans were conducted by trained veterinary and laboratory personnel under the supervision of one study researcher, to minimize issues associated with multiple observer error.

The Rose Bengal Plate test (RBT) was used to test bovine and caprine sera for brucellosis for *B. abortus* and *B. melitensis*, using techniques specified by the OIE (2012). Given the study hypothesis that both *B. abortus* and *B. melitensis* could be present in both cattle and goats, antigens for both *B. abortus* and *B. melitensis* were used to test samples from both cattle and goats. Earlier research suggested that the RBT performed better when antigens were matched to the species of *Brucella* infecting the host, and the RBT using *B. melitensis* may be more sensitive to M-epitope dominant biovars of *B. abortus (*Alton, et al., 1971; Corbel, 1985).

The RBT was conducted on serum samples within 12 hours of sample collection, and negative and positive controls for *B. abortus* and *B. melitensis* were tested at the beginning of every testing session. Approximately two 25 – 30 µl samples of clear serum were extracted from serum separation tubes using sterile disposable Pasteur pipettes, and placed on clean white ceramic tiles. Equal amounts of *B. abortus* and *B. melitensis* antigen were added separately to each sample, and mixed using sterile swab sticks. Each tile was rocked by hand for 10 minutes, and then examined for evidence of agglutination to each antigen. The results for each sample were recorded by strength of agglutination seen for each antigen: obvious and complete agglutination was recorded as a strong (+++) result, clear but not strong agglutination (agglutination present and clearly visible, but not complete) was recorded as a moderate result (++), and slight agglutination (present at the margins) was recorded as a weak (+) response.

A commercially available lateral flow assay (LFA) was used to test human serum samples for evidence of brucellosis, following protocols and test interpretation rules by the manufacturer (Royal Tropical Institute, RTI/KIT, Amsterdam, the Netherlands). Separate test cassettes, one each for IgG and IgM, was used for each human sample: approximately 5 µl of serum were applied to the sample window of the test cassette, 130 µl of running fluid was added, and the test was read after 10 minutes. Tests were considered valid if the control bar was present in the window of the cassette, and results were recorded as strong to weak, based on the color of the test stripe per manufacturer recommendations.

Milk testing

The Milk Ring test (MRT) was used to detect *Brucella* in cattle milk samples, using OIE protocols (OIE, 2012). Although the MRT is not recommended for use on individual animals due to the possibility of false-positive reactions in abnormal milk samples or samples with mastitis (OIE, 2012), the test was conducted to provide additional information regarding exposure to *Brucella* in milk from animals with serological test results. Creamy milk was mixed, and 1 ml transferred to an 11 x 100 mm test tube. One drop of milk ring antigen was added, the tube capped, and contents gently mixed by shaking and inverting the tube several times. After incubation for 1 hr at 37°C, the test was read and results recorded, then held at 4°C for 8 hours and read again, to improve test sensitivity (OIE, 2012). Milk ring tests where the cream layer was dark blue and the milk layer was completely white were recorded as a strong (+++) response, a moderate response had a distinct dark blue cream layer and pale blue milk layer (++), a weak response had a barely distinct darker blue cream layer and a blue milk layer (+),

and a negative response had no distinct color difference between the cream and milk, or a cream layer lighter than the milk layer.

Analytical approach

The apparent prevalence of brucellosis (with 95% confidence intervals) in each host species was calculated for each screening test (RBT for cattle and goat serum, MRT for cattle milk, LFA for human serum) by dividing the number of positive tests by the number of samples tested. The farm-level prevalence was calculated for each host species and test by dividing the number of farms with at least one test positive by the number of farms tested. The apparent prevalence rates were adjusted for the estimated sensitivity (Se) and specificity (Sp) of the RBT in cattle (70% and 99%, respectively), the RBT in goats (80% and 90%), the MRT in cattle (72% and 80%), and the LFA in humans (91% and 95%) using the approach described by Greiner and Gardner (2000). The reported Se and Sp of the RBT in cattle range from approximately 55-80% and 85% to nearly 100%, respectively (Gall & Nielsen, 2004; Sanogo et al., 2013), and a recent study found no significant differences between the RBT and I-ELISA for brucellosis in cattle in Uganda (Nizeyimana et al., 2013). In goats, the reported sensitivity and specificity of the RBT ranged from 75-90% and 80 to nearly 100%, respectively (Díaz-Aparicio et al., 1994; Mikolon et al., 1998; Nielsen et al., 2004).

Associations between brucellosis test prevalence and risk factors were assessed at both the individual and farm levels, using a significance level of $p \leq 0.05$, using SAS 9.3 (SAS Institute, Inc. Cary, NC, USA). For descriptive statistics, the association between brucellosis status (negative or positive) with risk factors and symptoms of brucellosis (cow or goat abortion history, human

subject history of fever within the last 12 months) were assessed using the Fisher's Exact two-tailed test, and the strength of association was reported as odds ratios (OR) with 95% confidence intervals (CI). To describe spatial patterns in brucellosis, choropleth maps of the mean herd prevalences of brucellosis in each host species were generated. Differences between prevalences between sub-counties of each district were assessed using the Fisher's Exact 2-tailed test at the individual subject level, and the Kruskal-Wallis Chi-square test (X^2) for differences in herd prevalences. Differences in risk management practices between the two sites (LMNP, QENP) were also assessed using the Fisher's Exact test.

Multivariable regression was used to describe the association between risk factors and brucellosis outcomes. Poisson regression was used for farm-level prevalence in cattle, goats and humans. Multinomial logistic regression models with random effects (to control for farm effects) were developed for individual-level responses (strong/weak/negative) to the RBT for *B. abortus* in cattle and *B. melitensis* cattle and goats, and the LFA in humans. First, all risk factor and signalment variables (gender, age, breed for cattle and goats), and the prevalence of brucellosis in other species on the same farm, were tested with univariable models. Univariable risk factor models with $p \leq 0.20$ were considered for inclusion in multivariable regression. Potential interaction between factors was assessed by using Spearman rank correlation to identify pairs of correlated variables, and any correlations with absolute values greater than 0.75 generated interaction terms for the multivariable model. Both forward and backward hierarchical stepwise model building was used to develop the final models, to find the model with the best combination of risk factor p-values ≤ 0.05 and lowest Akiake Information Criteria (AIC) score.

Results

Data were collected from 70 farms at the farm and animal level. Serum samples were collected from 768 cattle (384 each from LMNP and QENP), 315 goats (168 from LMNP, 147 from QENP), and 236 humans (159 from LMNP, 77 from QENP). Milk samples were collected from 356 cows in LMNP and 279 cows from QENP, for a total of 635 cows. Two farms in QENP did not have any goats, and no samples from humans were collected from three farms due to lack of cooperation from human subjects (one in LMNP, two in QENP). The average numbers of cattle reported in the herd inventories were significantly higher in LMNP than in QENP for herds in the small stratum (43.0 versus 14.4, respectively), medium stratum (73.7 versus 20.5, respectively), and large stratum (166.5 versus 44.4, respectively).

Study participants

Cattle: Out of the 768 cattle tested, 97.4% were female and 93% were adult animals. The least commonly reported breed of cattle were local Ankole cattle (8.5%), while 42.3% and 46.8% of cattle were exotic breeds (Holstein-Friesian, Jersey) and mixed local/exotic breed cattle, respectively. There were significantly higher proportions of exotic breeds in QENP than in LMNP (62% versus 22.6%, respectively; $p < 0.0001$), and significantly higher proportions of mixed breed cattle in LMNP than QENP (61.3% versus 35.0%, respectively; $p < 0.0001$).

Goats: Of the 315 goats tested, over 95% were female and over 96% were at least 12 months of age. The majority of goats (57%) were local breeds, with exotic breeds and mixed

breeds making up only 13.7% and 29.3% of the study population, respectively. There were significantly higher proportions of exotic breed goats in LMNP than in QENP (24.2% and 0.7%; respectively; $p < 0.0001$), and significantly higher proportions of local breed goats in QENP than LMNP (74.8% and 42.4%, respectively; $p < 0.0001$). Abortions were reported in 7.0% of cattle in LMNP, which was higher than the 3.7% cattle in QENP ($p = 0.0501$). Only one goat sampled in this study has a history of abortion. At the farm level, there were significantly more farms reporting a history of cattle abortion in LMNP (51.4%) than in QENP (23.3%) ($p = 0.0251$), and more farms reported a history of abortion in goats from LMNP (61.8%) than QENP (12.9%) ($p = 0.0001$).

Humans: The majority of humans tested were males (73.9%). Subjects ranged from age 4 to 80, with a mean age of 33.4 years, and median age of 29.0 years. Females were older than males (39.4 years and 31.4 years, respectively). Only 20.9% of subjects reported drinking raw milk. Direct contact with cattle and goats (92.8% and 93.6%, respectively) and attending the birth of cattle and goats were common (75.7% and 75.3%, respectively), and 46.4% of study subjects participated in the slaughter of cattle and goats. A total of 59.1% subjects reported having fever within the last 12 months at the time of sample collection, and fever was reported more frequently in subjects from LMNP (69.0%) than subjects from QENP (41.9%) ($p = 0.0002$).

Livestock management: The majority of farms in the study raised their own cattle (97.1%) and goats (86.8%), used veterinary services for cattle (95.7%) and goats (89.2%), and treated cattle (92.8%) and goats (88.7%) for ticks. The majority of livestock grazed on private land (98.6% of cattle, 97% of goats). Most cattle were housed in fenced enclosures (94.2%), while most farms housed goats in sheds or barns (68.2%). The most common source of water

reported for livestock was from wells (69.1% for cattle, 66.1% for goats) and surface water (26.5% for cattle, 27.4% for goats), and 75% of farms reported a common source of water for both cattle and goats.

There were significant differences in livestock management practices between the two study areas (Table 3.1). In general, management practices that allowed contact between livestock and wildlife (grazing in wildlife areas, sharing water with wildlife, housing in areas with wildlife) and contact with livestock from other farms (sharing water) were significantly more common in LMNP than in QENP. Farms in QENP were less likely to introduce livestock to their farms from outside sources (from neighbors) and kept their goats in more secure facilities (sheds/barns) than LMNP farms. In addition, farms in LMNP reported more histories of abortion in their cattle herds and goats flocks than farms in QENP.

Screening tests for brucellosis

Livestock samples from both sites and herd size strata tested positive for brucellosis (Table 3.2, 3.3). In cattle, 63 out of 70 herds had at least one positive animal: the seven herds with no positive cattle were located in QENP. These farms had no positive goats, and in one farm, one of three human samples was IgG positive for brucellosis: this individual was a 16 year old male with a history of fever, direct contact with cattle and goats, and contact with animals giving birth. Only 23 out of 68 farms with goats tested positive for brucellosis, and all but one of these herds was located in LMNP.

Table 3.1 Significant differences in livestock management practices between study sites

	% of herds		
Risk Factor	LMNP	QENP	**Fisher's Exact 2-tailed p**
Cattle received from neighbors	14.3	0	0.0536
Goats raised by household	76.5	97.1	0.0272
Goats received from neighbors	20.59	0	0.0111
Cattle graze with wildlife	82.4	12.9	< 0.0001
Cattle housed with wildlife	41.2	5.7	0.0005
Cattle share water with wildlife	79.4	8.6	< 0.0001
Goats graze with wildlife	82.86	10.34	< 0.0001
Goats housed with wildlife	40.0	3.2	0.0003
Goats share water with wildlife	82.4	12.9	< 0.0001
Cattle share water with other herds	44.1	8.6	0.0009
Goats share water with other flocks	64.7	3.6	< 0.0001
Goats share water with cattle	97.0	51.6	< 0.0001
Goats housed in sheds/barns	51.4	87.1	0.0031
Goats housed in fenced pens	40.0	6.5	0.0016
Goats housed with other herds	40.0	6.9	0.0031

Table 3.2 Prevalence (with 95% CI) of brucellosis in cattle, adjusted for the sensitivity and specificity of the Rose Bengal Plate Test (RBT; Se = 70%, Sp = 99%) and the Milk Ring Test (MRT; Se = 72%, Sp = 80%)

			RBT with *B. abortus*			RBT with *B. melitensis*				Milk Ring Test		
				Prevalence			Prevalence				Prevalence	
Site	Herd Size	# Serum Samples	% Positive	Adjusted	95% CI	% Positive	Adjusted	95% CI	# Milk Samples	% Positive	Adjusted	95% CI
Lake Mburo National Park (LMNP)	10-30	36	22.2	30.8	11.1 – 50.4	33.3	46.9	24.5 – 69.2	31	25.8	11.2	0* - 40.8
	31-99	140	19.3	26.5	17.0 – 36.0	17.1	23.4	14.3 – 32.4	129	22.5	4.8	0* - 18.6
	100+	208	10.6	13.9	7.8 – 19.9	14.4	19.5	12.5 – 26.4	196	28.6	16.5	4.3 – 28.6
Queen Elizabeth National Park (QENP)	5-10	35	5.7	6.8	0* – 18.0	5.7	6.8	0* – 18.0	28	25.0	9.6	0* - 40.5
	11-29	140	2.1	1.7	0* - 5.1	3.6	3.7	0* – 8.2	103	29.1	17.6	0.7 – 34.4
	30+	209	6.7	8.3	3.3 – 13.2	8.6	11.0	5.5 – 16.5	148	35.1	29.1	14.3 – 43.9
All Sites		768	9.9	12.9	10.0 – 16.2	11.9	15.7	12.4 – 19.3	635	28.7	16.7	9.9 – 23.4

* computed value < 0.0

Table 3.3 Prevalence (with 95% CI) of brucellosis in goats, adjusted for the sensitivity and specificity of the Rose Bengal Plate Test (Se = 0.8, Sp = 0.9)

			B. abortus			*B. melitensis*		
				Prevalence			Prevalence	
Site	Herd Size	# Serum Samples	% Test Positive	Adjusted	95% CI	% Test Positive	Adjusted	95% CI
Lake Mburo National Park (LMNP)	Small	35	22.6	18.0	0* – 39.0	45.2	50.2	25.2 – 75.3
	Medium	70	15.9	8.5	0* – 20.8	17.4	10.6	0* – 23.3
	Large	70	23.5	8.8	0* – 21.3	38.2	10.9	0* – 23.9
Queen Elizabeth National Park (QENP)	Small	24	0	-	-	0	-	-
	Medium	60	0	-	-	1.7	0*	-
	Large	63	0	-	-	0	-	-
All sites		322	10.8	1.1	0* – 6.0	16.8	9.8	3.8 – 15.7

* computed value < 0.0

After adjustment of prevalence values for the sensitivity and specificity of each screening test, the seroprevalences in cattle using *B. abortus* and *B. melitensis* antigen were 12.9% and 15.7%, respectively, and the adjusted prevalence of brucellosis-positive milk samples was 16.1% (Table 3.2). In goats, the mean adjusted seroprevalences using *B. abortus* and *B. melitensis* were 1.1% and 9.8% (Table 3.3), respectively, and in humans the mean adjusted prevalence was 8.1% (Table 3.4). The unadjusted overall seroprevalence of brucellosis in cattle with a history of abortion was significantly higher than cattle reporting no abortions (47.5% and 30.3%, respectively; OR = 2.08, 95% CI = 1.09 – 3.94).

Table 3.4 Prevalence (with 95% CI) of brucellosis in humans, adjusted for the sensitivity and specificity of the Lateral Flow Assay Test (Se = 0.91, Sp = 0.95)

			IgG			IgM			Either IgG or IgM		
				Prevalence			Prevalence				
Site	Herd Size	# Serum Samples	% Test Positive	Adjusted	95% CI	% Test Positive	Adjusted	95% CI	% Test Positive	Adjusted	95% CI
Lake Mburo National Park (LMNP)	10-30	31	12.9	9.2	0* – 22.9	0	-	-	12.9	9.2	0* – 22.9
	31-99	60	13.3	9.7	0* – 19.7	0	-	-	13.3	9.7	0* – 19.7
	100+	60	20.3	17.8	5.9 – 29.8	5.1	0.1	0* – 6.6	22.0	19.8	7.5 – 32.1
Queen Elizabeth National Park (QENP)	5-10	13	7.7	3.1	0* – 20.0	0	-	-	7.7	3.1	0* – 20.0
	11-29	28	0	-	-	0	-	-	0	-	-
	30+	36	2.9	< 0	0* – 3.9	0	-	-	2.9	< 0	0* – 3.9
All sites		228	11.5	7.6	2.7 – 12.4	1.3	< 0	-	11.9	8.1	3.2 – 13.0

* computed value < 0.0

A total of 228 serum samples were collected from humans from 67 farms (Table 3.4). In the three farms with no human samples, 32% of cattle tested positive, and none of the herds had any goats that tested positive for brucellosis. A total of 21 farms had seropositive human samples, primarily for IgG: 17 farms were from LMNP, and 4 farms were from QENP. Three individuals tested positive by IgM: all were males from 24-39 years of age from large farms in LMNP, had histories of fever, and had direct contact with animals. In addition to study farms, additional samples were taken from veterinary medical officers and local village officials in LMNP, and two individuals tested positive for IgG: one female with a history of fever and direct contact with cattle and goats, and one male with a history of drinking raw milk and direct contact with cattle and goats. Since these samples came from individuals not connected to study farms, they were not included in further analyses.

Comparing test prevalences

There were significant differences in the seroprevalence of brucellosis between the two study sites. The prevalence of brucellosis in LMNP was significantly higher than in QENP for cattle and goats by overall seroprevalence ($p < 0.0001$), seroprevalence for *B. abortus* ($p < 0.0001$), and seroprevalence for *B. melitensis* ($p < 0.0001$). There were also significant differences in overall seroprevalence in humans (Fishers' Exact 2-tailed $p = 0.0018$).

There were significant positive associations between brucellosis seropositivity and positive MRT results in cattle. Overall, 45.0% of the 109 cattle that were seropositive to at least one

Brucella antigen had positive MRT results, while only 20.2 % of 659 seronegative cattle had positive MRT results ($p < 0.0001$; OR = 3.26, 95% CI = 2.14 – 4.98).

Significant associations were found between human seropositivity and goat seropositivity, and between cow seropositivity and goat seropositivity. Based on results of herd-level univariable Poisson regression models, the risk for a positive LFA test in humans increased with increasing seroprevalence to *B. abortus* (OR for 10% change in prevalence = 1.29, 95% CI = 1.12 – 1.49) and *B. melitensis* (OR = 1.21, 95% CI = 1.09 – 1.36) in goats. The seroprevalence to *B. abortus* and *B. melitensis,* and proportion of positive MRT results in cattle increased risk for *B. abortus* seropositivity in goats (OR = 1.24, 95% CI = 1.02 – 1.52; OR = 1.30, 95% CI = 1.11 – 1.53; OR = 1.63, 95% CI = 1.17 – 2.62; respectively). However, the only significant association between *B. melitensis* seroprevalence in goats was with the seroprevalence of *B. melitensis* in cattle (OR = 1.20, 95% CI = 1.04 – 1.39).

Associations between screening test results and possible symptoms of brucellosis (fever in humans, abortion in livestock) were evaluated (Table 3.5). In humans, subjects reporting fever demonstrated a slightly increased risk for brucellosis seropositivity by both IgG and IgM ($p = 0.2963$; OR = 1.71, 95% CI = 0.71 – 4.09), and by IgM alone ($p = 0.2746$, OR = 4.85, 95% CI = 0.25 – 95.12). At the household level, there were significant differences in the prevalence of brucellosis between households reporting fever lasting more than two days (n=35) and households not reporting fever (n=31): households with fever had higher prevalences of brucellosis in cattle (by serum and/or milk: 0.38 versus 0.25, $X^2 = 5.60$, $p = 0.0245$; by milk: 0.27 versus 0.18, $X^2 = 5.27$, $p = 0.0218$) and goats (RBT using *B. abortus* antigen: 0.15 versus 0.06, $X^2 = 4.47$, $p = 0.0344$). There were no significant associations found between individual cow's

abortion history and any screening tests, but there were significant positive associations between individual goat's abortion history and positive RBT test results.

Table 3.5 Associations between screening test results and history of symptoms of brucellosis

Host Species	Screening test	Level[a]	n	% with Symptoms	Fisher's Exact p	Odds Ratio OR	Odds Ratio 95% CI
Cattle	RBT with *B. abortus* antigen	Neg	669	5.23	0.5744	1.39	0.53 – 3.68
		Pos	70	7.14			
Cattle	RBT with *B. melitensis* antigen	Neg	652	5.21	0.4553	1.35	0.55 – 3.31
		Pos	87	6.90			
Cattle	Any RBT positive	Neg	637	5.18	0.4793	1.35	0.58 – 3.14
		Pos	102	6.86			
Cattle	MRT	Neg	575	4.9	0.3390	1.42	0.71 – 2.86
		Pos	177	6.8			
Goat	RBT with *B. abortus* antigen	Neg	256	8.98	< 0.0001	8.23	3.53 – 19.22
		Pos	29	44.83			
Goat	RBT with *B. melitensis* antigen	Neg	238	7.56	< 0.0001	7.59	3.55 – 16.21
		Pos	47	38.30			
Goat	Any RBT positive	Neg	238	7.56	< 0.0001	7.59	3.55 – 16.21
		Pos	47	38.30			
Human	LFA for IgG	Neg	205	59.5	0.6591	1.28	0.52 – 3.14
		Pos	23	65.2			
Human	LFA for IgM	Neg	226	59.7	0.2784	4.73	0.24 – 92.60
		Pos	3	100.0			
Human	Any LFA positive	Neg	203	59.1	0.3971	1.56	0.65 – 3.75
		Pos	26	69.2			

[a] Neg = negative; Pos = positive

Spatial patterns of brucellosis

There were significant differences in the prevalence of brucellosis between the two study sites. The prevalence of brucellosis in LMNP was significantly higher than in QENP for cattle by overall serology (X^2 = 12.71, 1 df, p = 0.0004), serology for *B. abortus* (X^2 = 12.07, 1 df, p = 0.0005), and serology for *B. melitensis* (X^2 = 11.80, 1 df, p = 0.0006). There were also significant differences in overall serology in humans (X^2 = 12.75, 1 df, p = 0.0004). At the individual level within LMNP (Figure 3.1), there were significant differences between sub-counties in the prevalence of brucellosis in cattle by overall serology (X^2 = 34.6, 6 df, p = < 0.0001), and by the MRT (X^2 = 24.0, 6 df, p = 0.0005), but differences between sub-counties were not significant for goats or humans. In LMNP, one sub-county (Kanoni) in the north had the highest levels of brucellosis in cattle, goats, and humans (50%, 40%, and 20%, respectively). The other LMNP sub-counties with higher prevalences in cattle were in the north, while the other sub-county with higher prevalences in both goats (40%) and humans (32%) was in a sub-county near the center of the district (Kikatsi). The sub-county with lowest prevalence in cattle (20%) was located in the center of the district (Kikatsi). The lowest herd prevalence for humans (5%) was in the north (Kazo), while for goats the lowest prevalence (16%) was in the southeast (Nyakashashara).

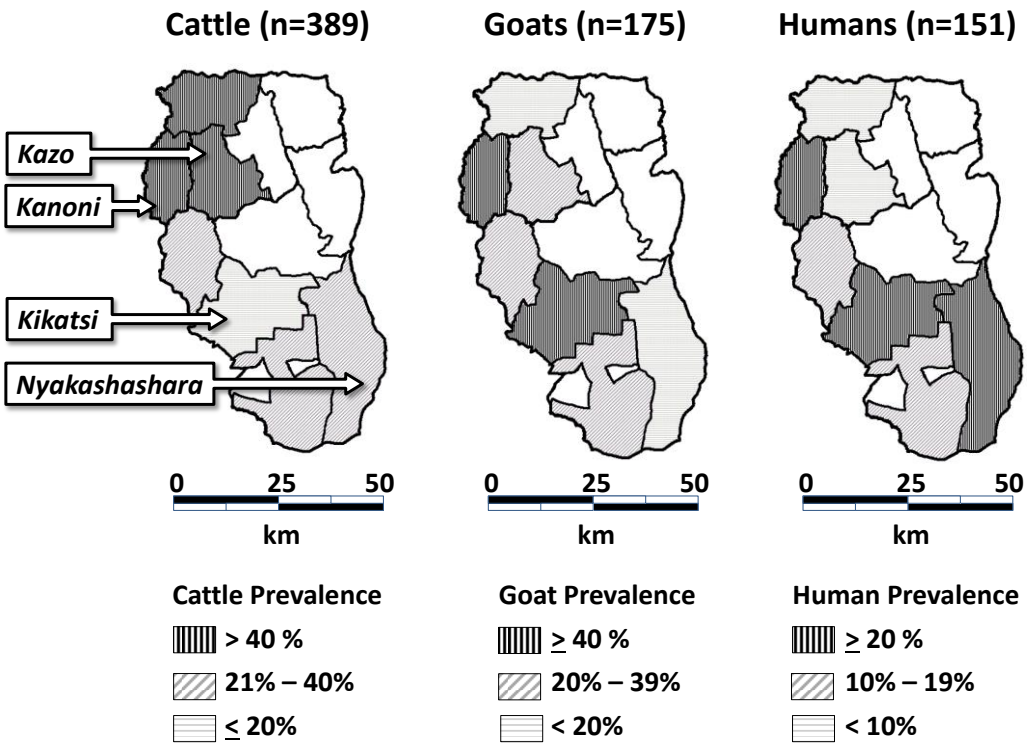

Figure 3.1 Choropleth map of individual-level screening test prevalence in cattle, goats, and humans in LMNP

Multivariable analyses

Several risk factors for consideration in the multivariable analyses were identified. Univariable risk factors associated with increasing risk for brucellosis test positivity at $p \leq 0.2$ included the LMNP study location, using cattle for meat, cattle or goats grazing with wildlife, cattle or goats housed in areas with wildlife, cattle or goats using surface water, cattle or goats sharing water sources with wildlife, cattle or goats sharing water sources with other herds, a history of abortion in goats on the farm, goats sharing housing with other flocks, and goats sharing water with cattle. Risk factors associated with decreasing risk included treating cattle for ticks, cattle or goats using well water, and housing goats in sheds or barns.

Cattle: Separate farm-level Poisson regression models were developed for the test prevalence of brucellosis in cattle for prevalence determined by the RBT and MRT (Table 3.6). Risk factors associated with increasing risk for brucellosis test positivity included cattle resident in the LMNP site, increasing levels of crossbred cattle in the herd, purchasing goats from markets, and the percentage of goat samples with positive RBT. Cattle water taken from wells was associated with decreasing risk for brucellosis.

At the individual animal level (Table 3.7), multinomial random-effects regression models were successfully developed for RBT results (strong positive, positive, negative) to *B. abortus* and *B. melitensis*. As in the farm-level models, the percentage of goat samples with positive RBT were associated with increasing risk for cattle test responses to *B. abortus* and *B. melitensis*, and treating cattle for ticks was associated with decreasing risk for brucellosis seropositivity for both *B. abortus* and *B. melitensis.*

Goats: The farm-level Poisson regression model for goat seropositivity by either *B. abortus* or *B. melitensis* (Table 3.8) identified risk factors associated with increasing brucellosis seropositivity: total number of goats on the farm, goats of mixed exotic/native breeds, goats sharing water with wildlife, goats using surface water, and goats sharing housing with other flocks. The individual-level multinomial model for *B. melitensis* identified several risk factors associated with increasing brucellosis seropositivity, including a flock history of abortion, goats using surface water and sharing water with wildlife, and the total number of cattle on the farm.

Table 3.6 Final farm-level multivariable Poisson regression models for brucellosis in cattle, by different screening test approaches

	Model Fit			Wald		Odds Ratio	
Model	**AIC**	**R^2**	**Risk Factor**	**X^2**	**P**	**O.R.**	**95% CI**
Serology (88+ of 701 tested)	189.64	24.73	% cattle of mixed breeds [a]	11.57	0.0007	1.09	1.04 – 1.15
			% goat samples with positive RBT for *B. abortus* [b]	9.67	0.0019	1.15	1.05 – 1.26
			Cattle treated for ticks	19.83	< 0.0001	0.18	0.08 – 0.38
			Goats have history of vaccinations	2.25	0.1340	2.13	0.79 – 5.72
Milk Ring Test (160+ of 676 tested)	248.16	14.45	Total cattle in household [c]	4.90	0.0269	1.03	1.00 – 1.06
			Cattle purchased from markets	3.41	0.0646	1.66	0.97 – 2.84
			Cattle water from wells	12.07	0.0005	0.51	0.35 – 0.75
			Goats share water with cattle	11.64	0.0006	0.47	0.31 – 0.73
All Tests Combined (212+ of 692 tested)	285.56	14.30	Cattle purchased from market	3.97	0.0463	1.68	1.01 – 2.79
			Cattle treated for ticks	7.81	0.0052	0.36	0.18 – 0.74
			Cattle water from wells	23.99	< 0.0001	0.42	0.30 – 0.60
			Goats have history of abortion	4.18	0.0409	1.43	1.02 – 2.01

[a] Odds ratio adjusted for 10% change in cattle breed composition

[b] Odds ratio adjusted for 10% change in positive tests

[c] Odds ratio adjusted for 10 unit change in number of cattle in the household

Table 3.7 Final individual-level random effects multinomial logistic regression models for results of Rose Bengal Plate test in cattle, by antigen

Model	AIC	Risk Factor	Test Response	Fixed Effects		Odds Ratio	
				t	P	O.R.	95% CI
B. abortus: Very strong & Strong (n=53); Weak (n=16); Negative (n=655)	7607.36	Percent of goat samples with positive RBT for *B. abortus* [a]	Strong	2.67	0.0077	1.24	1.06 – 1.45
			Weak	-0.16	0.8748	0.98	0.74 – 1.30
			Negative			Baseline	
		Cattle treated for ticks	Strong	-3.18	0.0016	0.09	0.02 – 0.40
			Weak	-3.30	0.0010	0.10	0.02 – 0.39
			Negative			Baseline	
B. melitensis: Very strong & Strong (n=65); Weak (n=17); Negative (n=660)	7643.30	Percent of goat samples with positive RBT for *B. melitensis* [a]	Strong	2.86	0.0044	1.19	1.06 – 1.33
			Weak	-0.40	0.6926	0.96	0.76 – 1.20
			Negative			Baseline	
		Cattle treated for ticks	Strong	-2.20	0.0282	0.22	0.06 – 0.85
			Weak	-2.33	0.0203	0.20	0.05 – 0.78
			Negative			Baseline	

[a] Odds ratio adjusted for 10% change in positive tests

Table 3.8 Final regression models for brucellosis in goats by serological testing

Model Fit				Individual	Wald		Odds Ratio	
Model	**AIC**	**R^2**	**Risk Factor**	**Test Response**	**X^2**	**P**	**O.R.**	**95% CI**
Farm-level Poisson regression (51+ of 269 tested)	139.22	40.81	Total number of goats[a]	–	2.46	0.1168	1.04	0.99 – 1.09
			Percent of goats of mixed breeds[b]	–	4.58	0.0323	1.08	1.01 – 1.18
			Goats share water with wildlife	–	12.36	0.0004	8.37	2.56 – 27.37
			Goats use surface water	–	10.72	0.0011	3.78	1.71 – 8.37
			Goats share housing with other flocks	–	5.16	0.0231	2.42	1.13 – 5.21
Individual-level multinomial random effects regression (Very strong & Strong =33; Weak =18; Negative =279)	3045.83		Total number of cows[a]	Strong	2.45	0.0154	1.14	1.13 – 1.16
				Weak	2.05	0.0418	1.12	1.01 – 1.26
				Negative			Baseline	
			Goat flock with history of abortion	Strong	2.42	0.0166	7.83	1.46 – 41.93
				Weak	1.29	0.1984	2.82	0.58 – 13.73
				Negative			Baseline	
			Goats use surface water	Strong	1.40	0.1629	2.99	0.64 – 13.94
				Weak	1.36	0.1744	3.18	0.60 – 16.90
				Negative			Baseline	

[a] Odds ratio adjusted for a change of 10 animals

[b] Odds ratio adjusted for 10% change in goat breed composition

Table 3.9 Final multivariable logistic regression models for brucellosis in humans, positive by IgG and/or IgM

Model	AIC	Risk Factor	Test Response [a]	Wald		Odds Ratio*	
				X^2	P	O.R.	95% CI
Farm-level Poisson regression (29+ of 220 tested)	79.56	Percent of goat samples with positive RBT for *B. abortus* [a]	-	6.10	0.0135	1.23	1.04 – 1.46
		Household members have direct contact with livestock	-	2.34	0.1263	1.13	0.97 – 1.31
		Household members slaughter goats	-	6.47	0.0110	14.22	1.84 – 109.94
		Goats acquired from neighbors	-	10.13	0.0015	5.12	1.87 – 13.98
Individual Level Multinomial Random Effects Logistic Regression	2180.65	Percent of goat samples with positive RBT for *B. abortus* [a]	Strong	1.62	0.1093	1.15	0.97 – 1.35
			Weak	2.53	0.0132	1.37	1.07 – 1.75
		Goats acquired from neighbors	Strong	3.38	0.0011	6.85	2.25 – 20.91
			Weak	1.78	0.0784	5.83	0.84 – 40.53

***** Baseline for odds ratios for the individual model: negative test response

[a] Odds ratio adjusted for 10% change in positive tests

Humans: In the farm-level regression model (Table 3.9), the percentage of goat samples with positive RBT and having household members that slaughtered goats were associated with increasing brucellosis risk. The association between seropositivity in humans and goats was also seen in the individual-level multinomial model for human brucellosis, and acquiring goats from neighbors was associated with increasing risk for brucellosis.

Discussion

Prevalence of brucellosis

This study was able to document the presence of brucellosis in cattle, goats and humans on farm in rural southwestern Uganda. The adjusted seroprevalence of bovine and caprine brucellosis found in this study, 12-15% and 1-10%, respectively, are comparable to those reported in earlier studies for cattle and goats (Bernard et al., 2005; Kabagambe et al., 2001; Mwebe et al., 2010). Our study found 29 humans testing positive for brucellosis using the LFA for IgG and IgM, for an adjusted prevalence of 8.1. This is in agreement with other studies of brucellosis in livestock owners (Samaha et al., 2008) and a recent study of abattoir workers in Uganda (Nabukenya et al., 2013). While the herd prevalence for brucellosis in goats in this study (33%) agrees with earlier studies (Kabagambe et al., 2001), the herd prevalence for cattle brucellosis in this study (90%) was higher than an earlier study of cattle farms in the same region of Uganda (55%, Bernard et al., 2005). The earlier study was broader in geographic range

and tested a larger variety of herds than the current study, which may account for the differences seen in herd prevalence.

This study relied on the use of screening tests for determination of brucellosis status in all three hosts, which is not as reliable or desirable as confirmation of infection by serial testing of individual or confirmation by bacterial culture, and the farms participating in the study were not a random sample of farms in the study areas. However, given the agreement between findings from this study and other researchers, results of analyses can be used to describe patterns in prevalence and associated risk factors. Findings from the molecular analysis of dried blood and milk samples from cattle and goats in Chapter 5 further support the results of these screening tests.

An important difference between the current study and past studies of brucellosis in Uganda was in how livestock serological testing was conducted: rather than use only *B. abortus* antigen in the RBT, we tested cattle and goat sera with both *B. abortus* and *B. melitensis*, which the earlier study did not indicate. Given that more cattle sera were positive for *B. melitensis* than *B. abortus* (11.7% versus 9.9%, respectively), and only 64.4% of *B. melitensis* samples were also positive for *B. abortus*, this testing strategy may have resulted in higher rates of detection in cattle. Although cross-reactivity cannot be excluded, 50% of the *B. melitensis*-positive cattle that provided milk samples were positive by the MRT, as were 50% of the *B. abortus*-positive cattle, and over 58% of cattle testing positive for both *B. abortus* and *B. melitensis*. There was also evidence for *B. abortus* in goats by the RBT, but at lower levels than *B. melitensis*: approximately 35% of seropositive goats were positive to *B. melitensis* alone, and of the 65% that were positive to both *B. melitensis* and *B. abortus*. Although cross-reactivity cannot be

excluded, 11 of the 52 goats (21%) that tested positive for both species of *Brucella* had very high agglutination to the *B. melitensis* antigen, while only 3 goat sera had strong agglutination to *B abortus*.

Brucellosis risk factors

Risk factors for brucellosis in cattle reported in the literature included increasing herd size (Berhe et al., 2007; Ibrahim et al., 2010; Kadohira et al., 1997; Muma et al., 2007), and cattle of exotic and mixed/local exotic breeds (Jiwa et al., 1996; Mekonnen et al., 2010). This study found associations between increasing herd size in the farm-level model for cattle MRT tests, and the farm-level model for seropositivity in goats. We also found positive associations between brucellosis seropositivity and increasing percentages of exotic/crossbred (versus native Ankole) cattle and goats in stud farms. Since the overwhelming majority (over 90%) of cattle in this study were exotic and mixed/local exotic breed cattle, our analysis of the association between local breeds and brucellosis did not identify more significant associations with decreased brucellosis risk.

Other herd management practices reported to be associated with increasing brucellosis risk include pasturing cattle (Berhe et al., 2007; Kadohira et al., 1997), intensive herd management (Berhe et al., 2007; Mekonnen et al., 2010), and a lack of biosecurity (McDermott & Arimi, 2002). Herd management practices were very similar in all 70 farms: all cattle were kept for dairy purposes and none of the livestock on these farms were kept in confined housing, which did not allow for extensive analysis of the influence of pasturing cattle or intensive herd management in this study. Risk factors that indicated decreased biosecurity and hygiene (e.g.,

livestock sharing water sources with other farm livestock and/or wildlife, getting replacement cattle from neighbors), were associated with increasing brucellosis risk in this study, which agrees with other studies. The use of wells as sources for cattle and goat water describes a practice that reduces access to water by livestock from other herds and wildlife, and was associated with decreasing brucellosis risk.

Risk factors found to be significantly associated with cattle seropositivity did not match risk factors identified with positive MRT tests in this study. Cattle seropositivity was positively associated with seropositivity in goats, which has been reported in other studies (Kabagambe et al., 2001; Megersa et al., 2011). However, MRT positive tests were positively associated with larger herd inventories and herds that purchased cows, which has also been described by other investigators (Muma et al., 2007; Matope et al., 2010). The MRT and RBT test for different antibodies (IgA and IgG, respectively), and differences between infection status and shedding in milk may result in inconsistent findings, which has been seen in other field studies where both tests were used together (Cadmus et al., 2008) Given the higher rates of false positives associated with the MRT, particularly with milk from the very early and late stages of lactation (Ferguson & Robertson, 1960; Morgan, 1970; OIE, 2012), the lower specificity of this test may have introduced bias that would make comparisons with the RBT more difficult. Unfortunately, confirmatory testing of samples to determine the infection status of these cattle was not possible at the time of this study. Despite these issues associated with the MRT, recent investigators have suggest that the simplicity and cost-effectiveness of the MRT still make this test a viable means for screening small herds for brucellosis in resource-restricted areas (Mohamand et al., 2014).

There was a clear difference in the seroprevalence of brucellosis between the two study sites, and geographic location has been reported to be associated with differing disease rates (Berhe et al., 2007; Ibrahim et al., 2010; Kadohira et al., 1997; Muma et al., 2007; Kashiwazaki et al., 2012). Both selected study sites (QENP and LMNP) were chosen for their history of high levels of bovine brucellosis, but the seroprevalence of brucellosis in QENP were significantly lower than in LMNP. There were significant differences in livestock management practices between the two sites (Table 3.1), but the majority of these management factors were not significantly associated with test positivity in the univariable analyses, and were not retained in either the herd- or individual-level multivariable regression models for cattle. The lack of association between specific risk factors that differed by study site may be due to an insufficient sample size for detecting risk factor associations, and there may be other risk factors beyond the scope of this study that may be important factors for brucellosis in the study areas.

In addition to differences in prevalence between the two study sites, there were differences seen in the prevalence of brucellosis by sub-counties, especially in the sub-counties of LMNP. When comparing the choropleth maps of brucellosis between cattle, goats and humans, areas of the highest prevalences in goats and human were the same, but only one sub-county (Kanoni) had high prevalences in both cattle, goats and humans. While the total number herds in LMNP (n=35) did not allow for more robust spatial analyses, the patterns presented in the maps supports the results from the multivariable analyses that found positive associations between the prevalence of brucellosis in goats and humans, and strongly indicate that this association needs to be further investigated in dairy farms in Uganda.

One issue uncovered in this study was the reported history of vaccination in one of the study sites. Household members in QENP had been asked about the vaccination status of their herds at the time of sample collection, and all had indicated that the cattle being tested had not been vaccinated. There were no significant differences in the seroprevalence or MRT prevalence between farms that did or did not vaccinate cattle. However, when sample processing at the veterinary laboratory facilities in Bushenyi District began in the QENP site, study investigators found old boxes of brucellosis S-19 vaccine. Discussion with the local district veterinary officer revealed an extensive vaccination campaign in the region in 2007. Although vaccination is known to result in false positives with the RBT (OIE, 2012), the lower levels of seropositive cattle in QENP suggested that 2007 vaccination campaign did not contribute significant numbers of false positives in this study. Conversely, the higher prevalence of MRT-positive samples in QENP may be attributable to vaccination: *B. abortus* S19 has been found in supramammary lymph nodes after one year (Duffield et al., 1984), and MRT-positive results were reported to last four years after vaccination in dairy cows (Beckett & MacDiarmid, 1987). It is possible that the lower seroprevalence of brucellosis seen in QENP may be evidence of the effectiveness of these vaccination campaigns, but confirming this effect was beyond the scope of the current study.

The reported risk factors for human brucellosis are strongly associated with the zoonotic transmission of the disease, and include livestock contact, and consumption of raw dairy foods (Franco et al., 2007; Bernard et al., 2005; Makita et al., 2008; Samaha et al., 2008). In univariable analyses, there were positive associations between the prevalence of brucellosis in cattle and humans with the prevalence of brucellosis in goats, indicating that the epidemiology

of brucellosis in these households may involve all three host species. There were positive associations between slaughtering goats and brucellosis seropositivity in humans. The prevalence of human brucellosis was higher in households where raw dairy products (12.2% from 11 households) or sour milk (23.3% from 6 households) were consumed, but these products were not found to be significant risk factors for human brucellosis in the multivariable analyses. While only 8 of the 27 study subjects (29.6%) that tested positive by the LFA reported consuming raw milk (only 20% of LFA-negative subjects reported drinking raw milk), and only 4 or 18 households with positive humans reported that household members consumed raw milk: the study sample sizes (207 individuals from 70 farms) may not have been sufficient to demonstrate the associations between raw milk consumption and human brucellosis.

Associations between cattle, goat, and human seroprevalence

One of the goals of this study was to describe any associations between the seroprevalence of brucellosis in cattle, goats, and humans. The seroprevalence of brucellosis in goats was associated with increasing risk for brucellosis seropositivity in cattle and humans, albeit at low levels (OR ~ 1.2). This association was seen in the multivariable farm-level analyses of cattle (by serological testing) and humans, and in the individual-level models for both cattle and humans. However, there were no significant associations identified between brucellosis in goats with levels of brucellosis in cattle in the multivariable models, despite the positive association between increasing levels of *B. melitensis* seropositivity in cows with brucellosis in goats in the univariable models. This appears to suggest a unidirectional relationship: brucellosis in goats influences brucellosis in cattle, but brucellosis in cattle does not influence brucellosis in goats.

These findings strongly suggest that goats are an active component of the cycle of transmission of brucellosis on the farm.

Brucellosis has been identified in wildlife reservoirs in Africa, and there is evidence that transmission occurs between livestock and wildlife where these species interact (Gomo et al., 2012; Jiwa et al., 1996; Muma et al., 2007; Motsi et al., 2013). At the univariable level, there were associations seen between livestock sharing water and pastures with wildlife, and housing livestock in areas with wildlife. In the multivariable analyses, there were associations between wildlife contact and increasing brucellosis at the herd level for goats that shared water with wildlife, but no associations were seen for cattle in the farm- or individual-level multivariable models. Sampling local wildlife for brucellosis screening was beyond the scope of this study, but these findings suggest that further investigation into the role of wildlife in the epidemiology of brucellosis is warranted.

An unexpected factor with strong and consistent associations with positive screening test results was the protective effect of treating cattle for ticks in both farm-level and individual-level models for cattle. In the farm-level models, strong protective associations were seen between tick treatment and seropositivity (OR = 0.18), but not with the milk ring test. In the individual-level models, the protective effects of tick treatment were seen with seropositivity to *B. abortus* and *B. melitensis* antigens, and the protective effects in these models were stronger for stronger responses to each antigen. It is possible that the treatment of cattle for ticks may be a proxy measure for other cattle management practices not evaluated by this study, such as other cattle health practices, but there were no other strong associations between cattle seropositivity and other herd health practices (use of veterinary services, cattle receive

vaccinations). Another possibility for the associations between tick control and bovine brucellosis is that there may be a direct association between tick control and seropositivity. The stress of tick infestations are known to have negative effects on cattle growth and production (Okello-Onen et al., 2003; Jonsson, 2006; Rodriques & Leite, 2013; Tolleson et al., 2012), and may also affect the immune response of infested animals (Ribiero et al., 1990; Inokuma et al., 1993; Guo et al., 2009), which could contribute to increased susceptibility of cattle to *Brucella* infection. Additionally, ticks and other blood-feeding parasites may serve as vectors for brucellosis. Older studies have experimentally demonstrated the capacity for different arthropods to serve as possible vectors of *Brucella* (Philip & Burgdorfer, 1961; Zheludkov & Tsirelson, 2010; Delaunay et al., 2011; Zorrilla-Vacca, 2014), and a recent study detected *B. abortus* DNA and RNA, and evidence of vertical transmission, in sucking lice (*Haematopinus tuberculatus*) from *Brucella*-infected water buffalo (Neglia et al., 2013). Although the consensus is that the role of arthropod vectors of brucellosis is insignificant in comparison with other routes of infection (Philip & Burgdorfer, 1961; USDA APHIS VS & Strickland, 1976; Zheludkov & Tsirelson, 2010), this and other studies provide evidence that the contribution arthropod vectors should be considered in the epidemiology of brucellosis on farms (Zheludkov & Tsirelson, 2010; Neglia et al., 2013). The collection and testing of ticks from cattle and goats from study farms was beyond the scope of this study, but future studies of brucellosis in areas with high tick burdens should include testing these insects for *Brucella* to investigate their possible role as vectors for infection.

Conclusions

This study was able to determine the seroprevalence of brucellosis in cattle, goats and humans in farms from southwestern Uganda, described spatial patterns in the prevalence of brucellosis, and identified risk factors associated with brucellosis in these three host groups. The majority of the study findings support existing reports of factors associated with increasing brucellosis risk, and demonstrated the positive associations between increasing levels of positive screening test results for brucellosis in goats, cattle, and humans. Results of multivariable analyses suggest that improvements in farm biosecurity and hygiene may reduce the risk or brucellosis on the farm. In addition, this study found evidence suggesting the effectiveness of brucellosis control programs through a 'natural experiment': the seroprevalence of brucellosis in all three hosts were significantly lower in the QENP study area, where extensive vaccination campaigns had been conducted in the recent past. This contributes evidence to support the viewpoint that livestock vaccination can be successfully used to control zoonotic disease in humans, and provides additional impetus for expanding brucellosis vaccination in other regions with high prevalence in livestock.

Although cattle are the focus of brucellosis control in Uganda, the associations identified between human and caprine seroprevalence in this study suggest that brucellosis in goats may be an important contributor to the epidemiology of the disease on the farm. The highest correlations in prevalence between species were seen for human seroprevalence and goat seroprevalence, and results of multivariable analyses found were clear associations between household prevalence of brucellosis in goats with human seropositivity, and a weak association

between brucellosis in goats with positive milk tests in cattle. In addition, the finding of significant associations between cattle seropositivity and tick control measures suggests that these arthropods may play a role in the epidemiology of brucellosis on these farms. It was beyond the scope of the current study to identify specific routes of transmission of *Brucella* between hosts in Ugandan dairy farms, but additional work on the molecular epidemiology of brucellosis is under way on these farms, and should make important contributions to our understanding of the zoonotic impact of brucellosis in rural farming communities in East Africa.

CHAPTER 4:

Detecting Human Brucellosis in Southwestern Uganda:

Evaluation of a lateral flow assay for use in resource-limited settings

Structured Abstract

Introduction: Brucellosis is present in livestock, wildlife and humans in Uganda. In humans, acute brucellosis is often misdiagnosed and treated as malaria, and chronic and relapsing brucellosis are difficult to detect. The importance of surveillance for brucellosis control has been well established, but requires resources that may not be available in developing countries: the WHO recommends that diagnostic tests for resource-limited settings be affordable, sensitive, specific, user-friendly, rapid and robust, and equipment-free. Recently, a simple and rapid lateral flow assay (LFA) for detection of IgG and IgM for *Brucella* was developed that meets many of these criteria.

Hypotheses:

- The lateral flow assay (LFA) can be used to assess brucellosis exposure in humans in the field in rural southwestern Uganda.
- The LFA is suitable for use in surveillance programs in resource-limited settings.

Objectives: A cross-sectional study was conducted to evaluate a commercially-available lateral flow assay (LFA) for use as a screening test to detect *Brucella* IgG and IgM in farm workers and residents in resource-limited settings.

Methods: Serum samples, brucellosis risk factor data, and livestock brucellosis prevalences were collected for 237 subjects from 67 dairy farms in southwestern Uganda. Samples were tested by LFA, microagglutination, and qualitative PCR (q-PCR) for IS711 and *Brucella melitensis* and *abortus* species-specific targets.

Results: Brucellosis seroprevalence was 8.1% by LFA, and 24% by q-PCR. There was 40% and 8.3% agreement in paired LFA-q-PCR tests for negative and positive results, respectively. Risk factors for positive LFA results included brucellosis in goats, goat slaughtering, consuming soured milk, and sharing water with livestock.

Conclusions: The LFA met WHO criteria for screening tests for use in resource-limited settings, and for use in surveillance.

Introduction

Brucellosis is an ancient zoonotic disease of ruminant livestock, responsible for human illness and economic losses in sub-Saharan Africa (Akakpo et al., 2009), and is present in animals and humans in Uganda (Kabagambe et al., 2001; Makita et al., 2011; Nabukenya et al., 2013; Asiimwe et al., 2015; Kansiime et al., 2015b; Miller et al., 2015; Nasinyama et al., 2015; Tumwine et al., 2015). The prevalence of human disease has been linked to livestock disease (Osoro et al., 2015), and control of livestock brucellosis results in reduced prevalence of human disease (Shemesh & Yagupsky, 2013).

Acute infection is characterized by high levels of IgM, which develop in the first week of infection and can persist for months, but is absent in relapsing infections (Smits et al., 2003). After the first three weeks, IgG develops, and can persist for years in chronic or relapsing brucellosis (Smits et al., 2003; Al Dahouk et al., 2013). Chronic and relapsing brucellosis become focal infections that are difficult to detect, and present with a variety of symptoms related to the location of the focal infection (Franco et al., 2007; Dean et al., 2012a; Fruchtman et al., 2015).

The burden of human disease attributable to brucellosis in Uganda, which presents as an undulating fever often misdiagnosed as other febrile illness such as malaria (Nankabirwa et al., 2009), is not well-documented, and the disease is known to be underreported. Acute brucellosis often presents as an undulating fever that is often misdiagnosed and treated as malaria in malaria-endemic areas (Franco et al., 2007; Dean et al., 2012a), which can result in consequences for patient health and the effectiveness of malaria control programs (Crump et

al., 2013). Given the similarities in case presentation (Ndyabahinduka et al., 1978; Ndyabahinduka & Chu, 1984), health care workers often mistakenly treat cases of brucellosis as malaria. Studies have reported that malaria was confirmed in only 25 – 32% of patients seeking treatment for self-diagnosed malaria, and in only 25% of presumptive diagnoses by health care workers in local clinics (Ndyomugyenyi et al., 2007). Reports have indicated that between 63% – 75% of clinic treatments for malaria would have been given to patients without the disease (Nankabirwa et al., 2009; Ndyomugyenyi et al., 2007). Improper treatments for malaria results in unnecessary costs, and create conditions for drug resistance to develop (Wongsrichanalai et al., 2002): better understanding of the prevalence and epidemiology of brucellosis in malaria endemic countries like Uganda will have beneficial impacts on the treatment and control of both brucellosis and malaria.

Effective disease surveillance programs and accessible health care are challenges in resource-limited settings. Recognizing these needs, the World Health Organization established the ASSURED criteria to guide development of diagnostic tests for resource-limited settings: tests should be **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment-free, and **D**eliverable (Peeling & Mabey, 2010). The majority of screening tests for brucellosis rely on detection of *Brucella*-specific immunoglobulins in serum, including the Rose Bengal (RBT), serum agglutination (SAT), and Coombs tests (Al Dahouk et al., 2013). Confirmatory tests, which detect changes in different antibody levels over time, include the complement fixation test, *Brucella*-specific ELISA, and the SAT (Al Dahouk et al., 2013). Although some of these tests meet many of the ASSURED criteria (Irmak et al., 2004), including the RBT, many have poor specificity, or require additional testing to confirm infection (Smits et al., 2003). The importance

of surveillance for brucellosis control has been well established (Shemesh & Yagupsky, 2013), but requires money, resources, and infrastructure that may not be readily available in developing countries.

Recently, a simple and rapid lateral flow assay (LFA) for detection of IgG and IgM for *Brucella* was developed (Smits et al., 2003; Irmak et al., 2004; Zeytinoğlu et al., 2006; Franco et al., 2007; Mizanbayeva et al., 2009; Peeling & Mabey, 2010; Krug et al., 2011; Román et al., 2013; Lobna et al., 2014; Nour et al., 2015). The test is a simplified version of *Brucella*-specific ELISA, using a detection strip containing *B. abortus* 1119-3 lipopolysaccharide (LPS), using colloidal gold-conjugated anti-human IgM and IgG as detection reagents. The LFA meets many of the ASSURED criteria, and has been successfully used in disease outbreak investigations in Peru (Mendoza-Núñez et al., 2008), detection of brucellosis in family contacts of patients (Kose et al., 2006), and for serosurveys in Turkey and the Sudan (Regassa et al., 2009; Osman et al., 2015). The reported sensitivity (Se) and specificity (Sp) of the test is high, with a combined test Se of 96% and Sp of 99% (Smits et al., 2003; Zeytinoğlu et al., 2006; Román et al., 2013). Studies have determined that the stage of disease affected the LFA performance: the Se was 91-92% in acute brucellosis cases (Smits et al., 2003; Zeytinoğlu et al., 2006), 78% in chronic brucellosis (Zeytinoğlu et al., 2006), and 75% in recurrent cases (Zeytinoğlu et al., 2006). Several studies have investigated the LFA using hospital- or laboratory-based samples from subjects with clinical suspicion of brucellosis (Smits et al., 2003; Irmak et al., 2004; Zeytinoğlu et al., 2006; Mizanbayeva et al., 2009; Regassa et al., 2009; Lobna et al., 2014; Nour et al., 2015), and patterns in LFA IgG and IgM test positivity have been described for different forms of clinical disease (Zeytinoğlu et al., 2006). The potential for its use as a presumptive diagnostic tool in

resource-limited settings would be a significant benefit in countries where brucellosis is endemic in livestock and humans.

Research to investigate the potential of the *Brucella*-specific LFA as a rapid and reliable screening test for human disease surveillance has been limited. To determine if the LFA meets the ASSURED criteria for use in brucellosis surveillance in humans, the study was conducted to test following **hypotheses**: 1) The lateral flow assay (LFA) can be used to assess brucellosis exposure in humans in the field in rural southwestern Uganda; and 2) the LFA is suitable for use in surveillance programs in resource-limited settings. The specific aims of this study were to: 1) test sera from persons from dairy farms in Uganda using the LFA; 2) describe associations between LFA status and supporting test results (q-PCR, microagglutination) and risk factors for brucellosis; 3) evaluate the LFA as a tool for disease surveillance in resource-limited settings; and 4) evaluate the use of the LFA as a diagnostic tool to detect possible cases of brucellosis in resource-limited settings.

Materials and Methods

This study was part of a larger research project investigating the epidemiology of brucellosis in livestock and humans in sites located near Lake Mburo National Park (Kiruhura), and Queen Elizabeth National Park (Bushenyi) in rural southwestern Uganda (Miller et al., 2015). The two sites were selected for their large cattle populations and histories of livestock brucellosis, and differed in ecology and livestock management practices (Miller et al., 2015).

Human subjects were recruited from 67 farms participating in the livestock brucellosis prevalence study (Miller et al., 2015). Subjects recruited were apparently healthy residents or farm workers at least 12 years of age, that were willing to provide blood samples and complete questionnaires regarding subject and household health and known risk factors for brucellosis. Study activities involving human subjects were approved by institutional review boards from Michigan State University (MSU) and Makerere University College of Health Sciences, and informed consent was obtained from each subject.

Sample and data collection

A 5 ml blood sample was collected by regional health care technicians via venipuncture, and samples were placed in Vacutainer® serum separation tubes (SST) for transport to the regional veterinary laboratory in each site. During sample collection, participant age, gender, and their history of fever within the last year, raw milk consumption, and direct contact with cattle and goats on the farm were recorded. Farm-level risk factor data were collected by in-person interviews using pre-tested questionnaires to collect household and individual-level health histories, cattle and goat management practices, sources of household drinking water, and behaviors associated with brucellosis risk (e.g. raw dairy consumption, handling animals after abortions).

Levels of brucellosis in cattle and goats in study farms were determined in the previous study (Miller et al., 2015), and were included as possible risk factors for human brucellosis in the current study. Cattle and goat sera were tested by the RBT, using both *B. abortus* and *B. melitensis* antigens, and bovine milk samples were tested by the milk ring test (MRT), both

using protocols defined by the World Organization for Animal Health (OIE) (Miller et al., 2015). The prevalence of RBT-positive results was 14% in 773 cattle sera and 17% from 315 goat sera, and 29% of 635 bovine milk samples were positive by the MRT. The prevalence of abortion, calculated as the number of tested animals with a history of abortion divided by the total number of adult female animals tested on each farm, was 5.3% in cattle and 12.2% goats.

Laboratory techniques

LFA testing: The LFA tests were conducted within 12 hours of sample collection, according to manufacturer instructions (Smits et al., 2003). Two test cassettes were opened and each was labeled for IgG (G) or IgM (M). Five µl of serum from serum separation tubes was applied to the circular sample port of each test cassette, 130 µl of room-temperature IgG or IgM running fluid (PBS, 1.67% bovine serum albumin, 3% Tween 20, at pH 7.6) added, and the test was read after 10-15 minutes of incubation. Results for IgG and IgM were reported as scores from 0 (negative) to 4+ (strong positive) (Figure 4.1). The remaining samples were held in refrigeration until return to facilities at Makerere University, where they were stored at 0° C.

Figure 4.1 Example of lateral flow assay test results after 10 minutes: left end of the test window shows control band, and right end of window shows strong positive (4+) result in the upper cassette (IgG) and weak positive (1+) result in the lower cassette (IgM)

Microagglutination test (MAT): Frozen sera were shipped from Makerere University to MSU after frozen samples were thawed and held at pH 5.5 for 30 minutes to neutralize foot and mouth disease virus (Brown et al., 1981). Once at MSU, samples were held at -20°C until shipped to the Bacterial Special Pathogens Branch of the CDC for testing by MAT with minor modifications including the use of U-bottomed plates, incubation at 28°C, and discontinued use of safranin (Hinić et al., 2008). Sample brucellosis status was determined by cutpoint titer levels: a titer of $\geq$320 for positive results, 20–160 for borderline results, and titers <20 for negative results.

DNA extraction: Blood clots from LFA-positive individuals, and selected individuals from farms with high livestock seroprevalences from the previous study (Miller et al., 2015), were selected for DNA extraction at the BSL-3 laboratory facilities at the Microbiology Department of the Makerere University College of Health Sciences. Clotted blood was extracted from the SST by piercing the gel layer and using a disposable pipette to collect portions of the clot. Extraction of DNA from the clot sample was conducted using the QiaAmp DNA Mini Kit (QIAGEN, Valencia, CA, USA), and samples were stored at 0°C until shipment to the USA. DNA was extracted from human sera, using the QiaAmp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) at the CDC.

q-PCR for brucellosis: Qualitative real-time PCR (q-PCR) for the detection of *Brucella spp.*, *B. abortus*, and *B. melitensis* DNA was conducted on nucleic acid extracts from human blood and sera (Hinić et al., 2009). Samples were first screened using the IS711 pan-*Brucella* PCR for the detection of *Brucella* spp. DNA. Samples exhibiting Ct values less than or equal to 40 were considered positive, and were tested using the *B. melitensis* and *B. abortus* species specific PCRs. Real-time amplifications were carried out in a total reaction volume of 25 µl containing

12.5 µl TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, CA, USA), a 200 nM concentration primer and probes, and 5 µl of DNA extract. Amplification and real-time fluorescence detection was performed on the ABI 7500 DX Real Time PCR System (Applied Biosystems) using the following thermal cycling conditions: 10 minute denaturation at 95°C followed by 45 cycles of 95°C for 15s and 60°C for 1 minute. Samples with fluorescence before 40 cycles were considered positive.

Statistical analysis

Outcomes: The LFA outcomes for each subject were IgG positive ($\geq$ 1+), IgM positive ($\geq$ 1+), either IgG/IgM positive (IgG and/or IgM $\geq$ 1+), and q-PCR positive (IS711 positive). Results for the total antibody and IgG MAT were reported as positive (titer > 160) or negative. Using criteria based on studies of the LFA in subjects with diagnosed brucellosis (Zeytinoğlu et al., 2006), LFA cases were classified as possible 1) acute/subacute (IgM 2+ to 4+, IgG 0 or 1+); 2) chronic (IgM 1+, IgG 2+ to 3+); 3) recurrent (IgM 0, IgG 2+ to 4+), or 4) negative (IgM 0, IgG 0) cases. LFA-q-PCR cases were LFA positive and/or q-PCR positive. Prevalence for positive test outcomes (LFA, IgG, IgM, q-PCR) and possible case classification (acute, chronic, recurrent, negative) were calculated as the number of positives divided by the number of subjects tested, and prevalence for positive test outcomes was adjusted for LFA Se and Sp (Greiner & Gardner, 2000).

Descriptive statistics: Results of LFA and q-PCR were reported by gender within each study site. Associations between test outcomes and study site, subject gender, and categorical risk factors for brucellosis (raw dairy consumption, contact with livestock, sources of drinking

water) were assessed using the Fisher's exact test for categorical risk factors, and significant associations ($p < 0.05$) were reported as odds ratios (OR) with 95% confidence intervals (CI). The Kruskal-Wallis X^2 statistic of the Wilcoxon Rank-Sum test was used to evaluate associations between outcomes and continuous risk factors (subject age, livestock RBT seroprevalence, MRT prevalence).

Multivariable analyses: Multivariable logistic regression models for LFA positive results were developed to identify risk factors associated with test outcomes. Risk factors were screened for inclusion based on results of descriptive statistics and univariable logistic models, and risk factors with $p < 0.2$ were considered for inclusion. These variables were screened for potential interaction by evaluation of Spearman rank correlation, using $r > 0.75$ as an indicator of possible interaction. Stepwise and backward logistic regression approaches were used to develop models: the final model was based on the best combination of risk factor p-values (< 0.05) and lowest Akaike Information Criteria (AIC) score.

Results

Study participants

Serum samples for LFA were collected from 237 humans from 67 farms that provided animal samples for the previous study. After eliminating subjects with missing gender information, the study dataset of 226 subjects included 174 males and 61 females, and ranged in age from 12 to 82 years (mean 33.7, median 29.0). A history of fever within the last 12

months was reported by 60.3% of subjects, and 20.5% of subject reported drinking raw milk. Direct contact with cattle and goats was commonly reported (93.5% and 94.4%, respectively), as was attending the birth of cattle and goats (76.7% and 76.3%, respectively), and slaughtering cattle (47.2%) and goats (46.4%).

Test results

***LFA results*:** A total of 26 of the 226 samples tested by LFA were positive (Table 4.1), yielding a raw prevalence of 11.1, and an adjusted prevalence of 10.6 (95% CI = 6.4 – 14.8). Almost all positives were for IgG. There were significant differences in the numbers of positive subjects by study site, with higher levels of LFA-positive subjects in Kiruhura than Bushenyi.

Table 4.1 Results of LFA and qPCR test results, by study sites

			Study Site		Fisher's	Odds Ratio for Bushenyi	
Test	**n**	**Outcome**	**Kiruhura**	**Bushenyi**	**Exact P**	**O.R.**	**95% CI**
LFA	235	Positive	24	2	0.0033	0.15	0.03 – 0.65
		Negative	134	75			
LFA IgG	234	Positive	21	2	0.0093	0.18	0.04 – 0.77
		Negative	137	74			
LFA IgM	235	Positive	3	0	0.5528	0.29	0.01 – 5.62
		Negative	155	77			
IS711 q-PCR	60	Positive	9	12	0.0006	0.11	0.03 – 0.40
		Negative	5	34			
***B. abortus* q-PCR**	21	Positive	4	11	0.0464	13.75	1.21 – 156.65
		Negative	5	1			

Table 4.1 Results of LFA and qPCR test results, by study sites

Test	n	Outcome	Study Site		Fisher's Exact P	Odds Ratio for Bushenyi	
			Kiruhura	Bushenyi		O.R.	95% CI
***B. melitensis* q-PCR**	21	Positive	1	0	0.4286	0.23	0.01 – 6.25
		Negative	8	12			

***MAT results*:** Only one of 168 samples tested by MAT was positive by (total antibody 1:320), and one additional sample with total antibody titer (1:160) below the cutpoint for MAT positivity.

***q-PCR results*:** A total of 10 blood and 51 serum samples from 60 subjects were tested by q-PCR (Table 4.1). Of the 60 subjects, 21 (23.9%) were IS711 positive. *B. abortus* DNA was detected in 15 (71.4%) of the 21 samples, and one sample was positive for both *B. abortus* and *B. melitensis* DNA. The IS711 test prevalence was 100% of 10 blood samples, and 22% from 50 serum samples. There were significant differences in q-PCR prevalences between sites, with samples from Bushenyi having higher q-PCR prevalences of IS711 and *B. abortus*.

Classification of subjects by LFA test results: There were three chronic and 14 recurrent possible cases classified by the LFA. The three possible chronic cases included two with strong IgG (3+, 4+) and weak IgM (1+), and one with moderate IgG (2+) and trace IgM tests. One of the three chronic cases was tested by q-PCR and was negative. Of the 14 possible recurrent cases, one subject had the only positive BMAT test result (total antibody = 1:320, IgG = 1:160). Two recurrent cases were positive for IS711 and *B. abortus* from blood, and one was positive for IS711 from serum.

Statistical analyses

Comparison of LFA with MAT and q-PCR results: A total of 60 subjects were tested by both LFA and q-PCR (Table 4.2), of which 20 were LFA positive and 21 were q-PCR positive. Of these subjects, there was more agreement seen between samples with negative LFA and q-PCR findings (40% of samples tested) than in samples in agreement for LFA and q-PCR positive findings (8.3% of samples tested). There were higher levels of negative agreement in Kiruhura samples than Bushenyi samples, while higher levels of positive agreement seen in samples from Bushenyi than Kiruhura. The one sample that was positive by MAT was LFA IgG-positive (3+), LFA IgM-negative, and q-PCR-negative.

Table 4.2 Comparison of results of paired LFA and q-PCR tests, by study site and for all sites (n=60)

			qPCR Test Results		Fisher's	Odds Ratio for qPCR+	
Site	**Test**	**LFA Results**	**Positive**	**Negative**	**Exact P**	**O.R.**	**95% CI**
Kiruhura	Any LFA	Positive	3	15	0.7117	0.63	0.14 – 2.96
		Negative	6	19			
	IgG LFA	Positive	2	14	0.4455	0.41	0.07 – 2.26
		Negative	7	20			
Bushenyi	Any LFA	Positive	2	0	1.0	0.67	0.47 – 0.95
		Negative	10	5			
	IgG LFA	Positive	2	0	1.0	0.67	0.47 – 0.95
		Negative	10	5			
All Sites	Any LFA	Positive	5	15	0.3896	0.83	0.21 – 3.31
		Negative	16	24			
	IgG LFA	Positive	4	14	0.2414	0.62	0.14 – 2.80
		Negative	17	25			

Table 4.3 Significant ($p \leq 0.05$) associations between LFA test results and categorical risk factors

Categorical Risk Factors	LFA Test Results	LFA Test N	LFA Test % with RF	Fisher's Exact P	Odds Ratio O.R.	Odds Ratio 95% CI
Bringing goats to farm from outside sources	Positive	24	50.0	0.0364	2.53	1.08 – 5.97
	Negative	194	28.4			
Cattle exposed to wildlife	Positive	24	83.3	0.0247	3.5	1.15 – 10.64
	Negative	187	58.8			
Goats exposed to wildlife	Positive	23	87.0	0.0210	3.95	1.13 – 13.79
	Negative	188	62.8			
Household slaughtered cattle	Positive	24	83.3	0.0426	3.11	1.03 – 9.46
	Negative	198	61.6			
Household slaughtered goats	Positive	24	95.8	0.0018	12.3	1.63 – 93.05
	Negative	198	65.2			
Household consumed sour milk	Positive	24	33.3	0.0035	4.69	1.77 – 12.48
	Negative	187	9.6			
Household consumed raw dairy products	Positive	24	87.5	0.0018	5.83	1.68 – 20.23
	Negative	187	54.6			
Water shared with livestock, wildlife	Positive	22	86.4	0.0010	6.81	1.94 – 23.89
	Negative	166	48.2			

Risk factors associated with positive LFA and q-PCR results: Risk factors significantly associated with increasing odds for LFA-positive test results included livestock exposure to wildlife, household dairy consumption, and livestock exposure to wildlife (Table 4.3). Increasing levels of brucellosis in goats (by RBT) and abortions in cows were also significantly associated

with LFA-positive test results, while increasing brucellosis seroprevalence cows was associated with LFA-negative results (Table 4.4).

Table 4.4 Significant ($p \leq 0.05$) associations between LFA test results and continuous risk factors

Continuous Risk Factors	LFA Test			Kruskal Wallis		
	Results	N	Mean RF	X^2	df	P
Cattle Seroprevalence	Positive	24	9.1	5.64	1	0.0175
	Negative	202	19.8			
Goat Seroprevalence	Positive	24	36.7	7.43	1	0.0064
	Negative	197	20.0			
Cow Abortion Prevalence	Positive	24	8.2	7.19	1	0.0073
	Negative	197	4.7			

Multivariable analyses: A multivariable regression model was generated for LFA-positive status (Table 4.5). The seroprevalence of brucellosis in cattle and MRT prevalence were associated with decreasing odds for positive LFA results, while goat seroprevalence, the subject slaughtering goats, household consumption of soured milk, and water sources shared with livestock and wildlife were associated with increasing odds for LFA positive-results.

Table 4.5 Multivariable logistic regression models for LFA positive test results (IgG and/or IgM positive) (n=179, 22 LFA positive)

	Wald		Odds Ratio	
Risk Factor	**X^2**	**P**	**O.R.**	**95% CI**
Subject slaughtered goats	4.23	0.0398	3.29	1.06 – 10.21
Water shared with livestock and wildlife	3.79	0.0515	3.86	0.99 – 15.04
Household consumed soured milk	2.84	0.0921	3.09	0.84 – 11.45
Cattle seroprevalence	5.13	0.0236	0.61	0.40 – 0.94
Cattle MRT prevalence	4.60	0.0320	0.71	0.52 – 0.97
Goat seroprevalence	5.96	0.0149	1.23	1.04 – 1.45

Model R^2 = 32.21%, AIC = 114.23

Risk factors associated with subject classified by LFA test results: There was no significant association found between classifications of subjects as possible chronic cases by the LFA, but there were significant associations between risk factors and any subjects classified as chronic or recurrent cases (Table 4.6, 4.7). Subjects from households slaughtering goats, consuming sour milk, and residing in LMNP were significantly associated with increasing risk for classification as chronic/recurrent cases. Although not significant, there were trends ($p < 0.1$) found for increasing chronic/recurrent case risk with households sharing water sources with wildlife. The associations between recurrent cases and households slaughtering goats, consuming sour milk, and sharing water sources with and wildlife were present ($p < 0.1$), but not statistically significant.

Table 4.6 Associations ($p \leq 0.1$) between possible cases of brucellosis defined by the LFA and categorical risk factors

Categorical Risk Factors	Probable case status				Fisher's Exact P	Odds Ratio	
	Case	Status	N	% with RF		O.R.	95% CI
Household slaughtered goats	Recurrent	No	198	67.0	0.0673	5.91	0.75 – 46.42
		Yes	13	92.3			
Household consumed sour milk	Recurrent	No	198	11.1	0.0599	3.56	1.01 – 12.52
		Yes	26	30.8			
Water shared with livestock, wildlife	Recurrent	No	177	50.9	0.0618	4.35	0.91 – 20.71
		Yes	11	81.8			
District	Chronic/ Recurrent	No	218	11.8	0.0629	0.25*	0.06 – 1.14
		Yes	17	34.4			
Household slaughtered goats	Chronic/ Recurrent	No	206	66.5	0.0246	7.55	0.98 – 58.38
		Yes	16	93.8			
Household consumed sour milk	Chronic/ Recurrent	No	195	10.8	0.0324	3.77	1.19 – 11.90
		Yes	16	31.3			
Household consumed raw dairy products	Chronic/ Recurrent	No	195	56.4	0.0657	3.35	0.93 – 12.13
		Yes	16	81.3			
Water shared with livestock, wildlife	Chronic/ Recurrent	No	174	50.6	0.0531	3.58	0.97 – 13.29
		Yes	14	78.6			

* Odds ratio for residency in QENP

Table 4.7 Significant associations ($p \leq 0.05$) between possible cases of brucellosis defined by the LFA and continuous risk factors

Continuous Risk Factors	Probable case status				Kruskal Wallis		
	Case	Status	N	Mean RF	X^2	df	P
Cattle Seroprevalence	Recurrent	Yes	13	7.3	4.50	1	0.0339
		No	213	19.3			
Cow Abortion Prevalence	Recurrent	Yes	13	10.9	5.38	1	0.0203
		No	208	4.7			
Cow Abortion Prevalence	Chronic/ Recurrent	Yes	16	9.5	4.24	1	0.0395
		No	205	4.7			

Discussion

Results of LFA testing

The LFA was able to detect *Brucella* antibodies in humans in a serological surveillance context in this study. Most LFA positive tests were for IgG, with low numbers of positive LFA IgM tests, which has been reported in other studies using the LFA for serosurveys (Kose et al., 2006; Osman et al., 2015). Most studies using the LFA have focused on subjects tested for clinical brucellosis, and have reported higher IgM seropositivity (Smits et al., 2003; Irmak et al., 2004; Kose et al., 2006; Zeytinoğlu et al., 2006; Mizanbayeva et al., 2009; Román et al., 2013; Lobna et al., 2014; Nour et al., 2015). The LFA seroprevalences in this study were comparable to other surveys in Uganda of slaughterhouse workers (10%) and dairy farm families (6%) using the SAT (Nabukenya et al., 2013; Nasinyama et al., 2015), MAT (Nabukenya et al., 2013), and plate agglutination tests (Nasinyama et al., 2015). These seroprevalences were lower than

reported in clinic-based studies (17% – 31.8%) in Uganda, using patients suspected of having brucellosis (Kansiime et al., 2015b) or patients visiting clinics for other reasons (Nasinyama et al., 2015; Tumwine et al., 2015), which would increase the number of potential brucellosis cases in comparison to a regional serosurvey (Kansiime et al., 2015b; Tumwine et al., 2015).

Differences in the prevalence of brucellosis in different study sites seen in this study have been reported in other studies (Nabukenya et al., 2013; Nasinyama et al., 2015). Both sites were chosen for their history of high levels of bovine brucellosis, but investigators found that cattle vaccination with strain S19 had been conducted in Bushenyi in 2007 (Miller et al., 2015). The seroprevalence of brucellosis in cattle from Bushenyi (6.4%) was significantly lower than in Kiruhura (17.5%), suggesting that the vaccination did not result in false positives during RBT testing (Miller et al., 2015), and the lower seroprevalence of brucellosis in humans may be evidence of the effectiveness of vaccination in reducing both human and livestock brucellosis, as reported in other countries (Marei et al., 2011; Shemesh & Yagupsky, 2013).

The current study was able to describe associations between positive LFA test results and brucellosis risk factors. Positive associations between human and livestock seroprevalence have been reported (Tumwine et al., 2015), which agrees with the findings in goats in this study, but not with the decreasing risk for LFA-positive results with increasing brucellosis in cows. One study in Uganda found no strong associations between human brucellosis and cattle-keeping, and suggested that animal contact was not the most significant route of transmission (Kabagambe et al., 2001). Consumption of raw milk has been strongly associated with human brucellosis (Kabagambe et al., 2001; Asiimwe et al., 2015; Nasinyama et al., 2015; Tumwine et al., 2015), and household consumption of raw dairy and sour milk were associated with LFA-

positive subjects. Sour milk is commonly prepared with unpasteurized milk, and has been associated with human brucellosis in Uganda (Asiimwe et al., 2015).

This study found that LFA and q-PCR test results were in agreement in 48% of paired tests, primarily for negative test results. In comparison, one study using q-PCR and LFA on patients with clinical suspicion of brucellosis reported that 89% of 19 LFA positives were q-PCR positive, and all were confirmed by culture or a SAT titer $\geq$ 1:160 (Aroca et al., 2011). The differences in percentages of agreement may be attributable to the differences in study populations: higher levels of infection would be expected in subjects with clinical signs of brucellosis (Aroca et al., 2011) compared to the apparently healthy farm workers in the current study. The detection of *Brucella* DNA in subjects that were LFA seronegative was not surprising, and other studies have found that *Brucella* DNA persists after infection (Marei et al., 2011). The presence of DNA in seronegative samples may be due to chronic or early infection, with antibody levels below detectable limits (Greiner & Gardner, 2000a), or may represent fragmented DNA from *Brucella* that are no longer viable or have been effectively phagocytosed (Al Dahouk et al., 2013).

The current study was not able to confirm brucellosis in study participants and could not formulate estimates of the test sensitivity and specificity of the LFA. Since the sensitivity and specificity of the LFA are reported to be influenced by stage of infection (Smits et al., 2003; Zeytinoğlu et al., 2006), our inability to confirm brucellosis status in study subjects may have resulted in misclassification of subjects and biased study results. However, since our results were similar to others using the LFA for serosurveys (Kose et al., 2006; Osman et al., 2015), findings from this study can be used with the understanding that additional work is needed to confirm these results. Evaluating potential cross-reaction with *Yersinia enterocolitica* O:9

(Muñoz et al., 2005; Díaz et al., 2011) was beyond the scope of this study. Since the LFA is a simplified ELISA, using *B. abortus* 1119-3 LPS, levels of cross-reactivity due to *Y. enterocolitica* should be similar to those reported for other ELISAs using the same antigens. An earlier study evaluating the influence of *Y. enterocolitica* O: 9 on different serological tests in cattle reported that the Sp of competitive ELISA for *B. abortus* LPS was 85.7% in experimentally infected cattle, and 88.8% in herds with false positive reactors (Díaz et al., 2011).

In the current study, one LFA IgM-positive subject was reported to have a case of brucellosis three weeks before sampling. The diagnosis was based on results of plate agglutination (Makita et al., 2011) at the Mulago national referral hospital in Kampala. This individual came from a large farm in Kiruhura with seropositive cattle and goats, which provided epidemiological evidence to support zoonotic transmission of brucellosis on the farm, and reinforced the positive LFA test result. Confirmation by diagnostic testing at Mulago suggested that, when supported by epidemiological data, the LFA could be used as a screening test to detect cases of brucellosis. In addition to these findings, the subject had MAT test results just below the positive cutpoint (total antibody 1:160), and was q-PCR-positive for IS711, further strengthening the LFA test result.

Use of the LFA in resource-limited settings

There is a need for ASSURED compliant tests for brucellosis surveillance in resource-poor regions of the world, and the LFA was able to meet several of the ASSURED criteria. The LFA did not require any specialized equipment, facilities, or refrigeration, and was rapid (10 minutes from starting the test to reading results) and easy to use and interpret. Although this study was

not able to determine the brucellosis status of subjects tested by the LFA, other studies have found that the LFA has potential for use as a complementary or confirmatory test for human brucellosis in the field, and has been recommended for use when laboratory support is unavailable (Kose et al., 2006; Regassa et al., 2009; Marei et al., 2011; Nour et al., 2015; Osman et al., 2015). Since patterns of LFA IgG and IgM positivity have been associated with acute and chronic cases of brucellosis (Román et al., 2013), the LFA has been recommended as a complementary test to assess levels of IgG and IgM in RBT-positive subjects (Irmak et al., 2004).

One of the public health challenges for brucellosis control is the necessity of re-testing subjects to observe changes in *Brucella* titers over time, which could be addressed by a single point-of-contact screening test. There are logistic challenges and problems tracking subjects after initial screening tests in remote areas or in locations difficult to access by public health workers. The inexpensive RBT, which has high Se but has low Sp and antigens require refrigerated storage, can be easily and rapidly conducted without significant equipment and resources (Al Dahouk et al., 2013). If the cost of testing samples with the LFA was prohibitive, the LFA could be reserved for RBT-positive sera: serial application of the RBT and LFA will improve overall testing Sp, which is desirable serosurvey tests. The serial RBT-LFA could be employed as a single contact test: after a single blood sample is collected, the RBT and LFA could use the same sample, since the LFA requires a small quantity of serum (5 µl). The time to conduct the serial RBT-LFA would be less than 30 minutes, and could identify subjects with strong evidence of brucellosis. While this approach to brucellosis detection may lack the rigor of accepted confirmatory testing protocols (Al Dahouk et al., 2013), the serial RBT-LFA would

have the advantages of being an easily accessible, rapid, and relatively inexpensive diagnostic test that meets the ASSURED criteria for diagnostic tests (Peeling & Mabey, 2010).

Evaluation of the use of the results of a single use of the LFA to presumptively classify subjects as possible cases would be a significant finding, and improve the capacity of public health agencies to identify possible cases of brucellosis more rapidly than by relying on repeated serological testing or bacterial culture. Before use as a diagnostic tool, however, there have been a wide variety of LF IgG and IgM positive readings for even culture-confirmed brucellosis (Zeytinoğlu et al., 2006; Mizanbayeva et al., 2009). For more conclusive diagnosis using the LFA, changes in titer over time would be of great diagnostic value (Róman et al., 2013). Despite these limitations, the successful use of the LFA in conjunction with epidemiological information to detect the one case of acute brucellosis suggests the potential of this approach, and reinforces the need for public and veterinary health workers to collect information about possible sources of human brucellosis when conducting any disease surveillance programs.

Conclusions

In conclusion, the LFA was found to be a useful and practical tool for potential use for brucellosis surveillance in resource-limited settings, and test results were supported by risk factor data and results from q-PCR. Additional research is needed to evaluate the sensitivity and specificity of the LFA in the field. The possible use of serial RBT-LFA as a single point-of-

contact test for identification of potential cases of brucellosis should be pursued to provide public health workers with a valuable tool in disease control efforts in developing countries.

CHAPTER 5: Validation of the use of dried blood and dried milk spots for detection of *Brucella* in cattle and goats for surveillance in resource-limited settings

Structured Abstract

Introduction: Disease surveillance programs are needed in developing countries, but sample storage for effective surveillance programs are challenges in resource-limited settings. The use of dried specimens is re-emerging as a tool for research and surveillance, due to minimal equipment requirements, ease of sample preparation and storage, and the low cost for basic materials.

Hypotheses:

- DNA for detection of *Brucella* can be extracted from dried blood spots (DBS) and dried milk spots (DMS) from cattle and goats.
- Results of q-PCR assays with DNA extracted from stored DBS and DMS give data that is comparable to data from serologic and milk screening tests for detecting *Brucella* infection in cattle and goats.
- DBS and DMS on laboratory-grade filter paper can be used to store samples for epidemiological investigations in resource limited settings.

Objectives: To assess the potential of dried specimen storage for disease surveillance and epidemiological investigations, the specific aims of this study were to extract usable DNA from dried blood and milk samples from cattle and goats stored for four years; compare q-PCR results from dried samples with results of brucellosis screening tests; and utilize test results

from dried samples to characterize the epidemiology of brucellosis in livestock on dairy farms in rural Uganda.

Methods: Blood and milk samples were collected on #1 Whatman filter paper from cattle and goats in the field, air dried, and stored in sealed plastic bags with desiccant packs for four years at ambient room temperature. Samples were collected from dried filter paper using a hole punch, DNA was extracted using a commercially available kit, and a qualitative real-time PCR was used to detect IS711 and *Brucella melitensis* and *abortus* species-specific targets.

Results: Species-specific brucellosis DNA targets were successfully detected in dried blood and milk from cattle and goats, and PCR data correlated with results of the Rose Bengal and milk ring tests. In addition to detection of *B. abortus* in cattle, our data also detected *B. melitensis* in cattle and *B. abortus* in goats.

Conclusions: This study demonstrated the utility of using a laboratory grade filter paper for storage of blood and milk samples for later DNA extraction and q-PCR detection of *Brucella* .

Keywords

Dried blood spots, dried milk spots, q-PCR, *Brucella abortus*, *Brucella melitensis*, surveillance

Impacts

- Zoonotic disease surveillance and control programs are greatly needed in developing countries, where despite economic impact on livestock owners and public health implementation of effective surveillance programs are challenged by resource limitations. Maintaining high numbers of surveillance samples can require significant freezer space, something that is expensive to purchase and maintain, and which may not be viable in many developing countries.
- Use of dried specimens can be a valuable tool for research and surveillance in resource-limited settings. Dried specimens can be collected simultaneously with samples collected for screening tests, and are simple and affordable, especially when laboratory-grade filter papers are used. Dried samples takes up less storage space than tubes, are not subject to leaking or breakage, and are not as dependent on freezing for preservation.
- Dried blood and milk samples, stored at room temperature for four years on laboratory-grade filter paper, were successfully used to detect *Brucella* DNA in cattle and goats. Dried samples were easy to collect and store, and easy to transport. This study demonstrated that use of dried samples is a low-cost approach to sample storage that can be successfully applied in resource-limited settings.

Introduction

Disease surveillance and control programs are greatly needed in developing countries due to the impact of disease on populations, exposure to conditions that increase risk of zoonotic disease transmission, and economic impact on animal health. Unfortunately, implementation of effective disease surveillance programs and accessible health care are challenges in resource-limited settings, where finances, human resources, and infrastructure may not be readily available. The World Health Organization recently developed criteria to guide the development of diagnostics for resource-limited settings. Tests should be Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable (ASSURED) (Kettler et al., 2004). While these efforts are targeted toward the development of diagnostic tests in humans, collection and storage of biological samples is another area of concern for resource-limited settings.

Current livestock surveillance programs rely on screening tests that require collection of milk, blood, and/or serum to detect the presence of pathogens (e.g., visualization of *Plasmodium falciparum* in blood smears (Ndao et al., 2004)), host immune responses to infection (Gilbert et al., 2013), or pathogen DNA (Sacchi et al., 2011). Animal identification, animal restraint systems safe for animals and humans, equipment for sample collection and storage, and a reliable cold chain for preservation during transportation from farm to laboratory are necessities in surveillance programs. Sample collection results in materials stored in glass or plastic tubes, which can become cumbersome when hundreds or thousands of samples need to be maintained. Long term storage of these types of specimen requires

access to freezers, preferably those at temperatures at or below -20° C, which is expensive to purchase and maintain, and may be a challenge for developing countries. The annual costs of electricity for single -20° C and -80° C freezers have been estimated to be $550 and $665, respectively (HEEPI, 2011), while cost estimates to maintain a single -80° C for one year, including energy and maintenance costs, were $6,000 at the U.S. National Cancer Institute (Baker, 2012).

The use of dried specimen storage is re-emerging as a tool for research and surveillance. Dried samples provide several advantages over existing sample storage approaches: preparation of the dried samples and methods of storage is simple, dried samples occupy relatively small volumes for storage, dried samples are relatively stable and only require low humidity for preservation, and basic materials for producing dried samples are inexpensive and readily available. Blood is the most frequently reported dried specimen, and the most commonly used substrate for dried sample collection is filter paper, although the use of dried swabs for storing blood samples collected from wild boar has been evaluated (Petrov et al., 2014). The use of dried blood spots (DBS) has become an accepted practice in human medicine (Mei et al., 2001), and the WHO has developed criteria for using DBS for collecting samples for HIV testing and other disease surveillance and control programs. Studies of human brucellosis and chikungunya virus in humans have used DBS to preserve samples for ELISA and PCR testing (Andriamandimby et al., 2013; Takkouche et al., 1995). In veterinary applications, DBS have been successfully used for storing blood for monitoring environmental toxicants in wildlife (Lehner et al., 2013), antibody detection (Curry et al., 2011; Greenwald et al., 2009), and PCR for pathogen detection (Aston et al., 2014; Knuuttila et al., 2014). Dried milk spots (DMS) been

used to store human and bovine milk samples for detection of antibodies (Brown et al., 1982; Miller & McConnell, 2011), and to detect bacterial DNA in mastitic milk and dairy food processing (Tilsala-Timisjärvi & Alatossava, 2004).

Specialized substrates for specimens have emerged with the demand for better dried specimen storage, but may be cost-prohibitive in resource-limited countries. The most widely reported of the new dried specimen storage system is the Flinders Technology Associates (FTA)™ card (Mullen & Howard, 2009), which includes a cellulose-based substrate impregnated with proprietary cell lysate reagents to break down cells and chemically bind DNA and RNA to the substrate, which simplifies the process of DNA and RNA extraction (Mullen and Howard, 2009), neutralize viruses (Kraus et al., 2009), and has antimicrobial activities which prevent the growth of contaminants on the cards (GE Healthcare life Sciences, 2010). Studies comparing the performance of FTA™ with qualitative filter papers have found that FTA™ performed better than laboratory grade filter paper for detection of virus by PCR (Jagero, 2015), and detection of antibodies to *Toxoplasma gondii* by a modified agglutination test (Aston et al., 2014). However, the cost of basic FTA™ Classic cards is nearly 40 times as expensive as 90 mm Grade 1 qualitative filter papers, from $470 per 100 FTA™ cards versus $12 for a box of 100 filter papers, making use of FTA cards expensive in resource-limited settings.

Given the need for safe and affordable specimen storage to support surveillance programs in resource-limited settings, this study was conducted to test the following **hypotheses**, that 1) DNA for detection of *Brucella* can be extracted from DBS and DMS from cattle and goats; 2) Results of q-PCR with DNA extracted from stored DBS and DMS correlate with data from blood and milk screening tests for detecting *Brucella* in cattle and goats; and 3) DBS and DMS on

laboratory-grade filter paper can be used to store samples for epidemiological investigations in resource limited settings. The **specific aims** of the study were to extract DNA from dried blood and milk samples for analysis in a q-PCR assay for detecting *Brucella*; compare q-PCR results from dried samples with the results of screening tests of serum and milk; utilize test results to characterize the epidemiology of brucellosis in livestock on dairy farms in rural Uganda; and, evaluate the use of BS and DMS as tools for disease surveillance in resource-limited settings. The overall goal of the study is to demonstrate that dried blood and milk samples can be used to detect brucellosis in blood and milk from cattle and goats, and assess their potential for disease surveillance, epidemiological investigations, and research programs under resource-limited settings.

Materials and Methods

This study was part of a larger research program investigating the epidemiology of brucellosis in cattle, goats, and humans on dairy farms in Uganda (Miller et al., 2015). In brief, a cross-sectional study was conducted to determine the prevalence of brucellosis in cattle, goats and humans on 70 dairy farms in southwestern Uganda, using standard screening tests for livestock brucellosis (Rose Bengal Plate Test (RBT) and milk ring test (MRT)). Samples were collected from August to November, 2011.

Sample collection

Blood samples were taken by venipuncture from the jugular vein of cattle and goats using syringes and needles. After collection, blood was dropped onto #1 Whatman qualitative filter paper circles (9 cm diameter), labeled with the animal ID number, with the remainder of the sample placed into Vacutainer® serum separation tubes. Filter papers were hung to dry immediately after the sample was placed, using laundry hangers purchased from local department stores (Figure 5.1).

Figure 5.1 Blood and milk samples air drying prior to storage

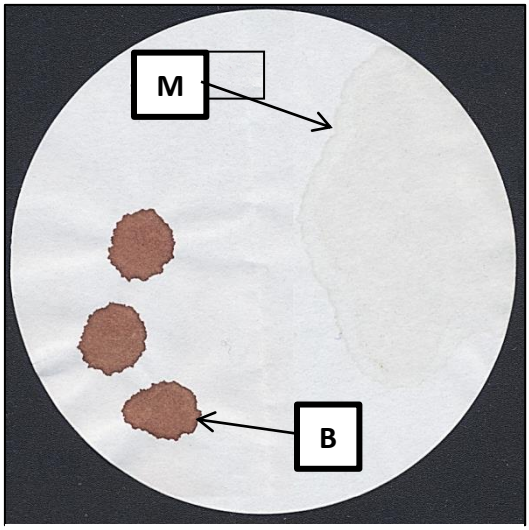

Figure 5.2 Dried blood (B) and milk (M) spots after 4 years of storage

Milk samples were collected from all lactating cows tested in the main study (Miller et al., 2015), and from goats on three farms that agreed to allow goat milk samples to be taken. A pooled sample of milk from all four quarters of the udder were taken from each animal after teats were wiped clean with alcohol-soaked cotton gauze and dried with paper towels. Samples were collected aseptically in sterile 30 ml universal containers, and kept on ice until processed at local veterinary laboratory within 12 hrs of sample collection. At the veterinary laboratory, a

sterile disposable 1.7 ml transfer pipette was used to place the milk sample onto a separate spot on the same #1 Whatman filter paper circle used to store the animal's dried blood sample. Filter papers were dried in a similar manner to blood samples (Figure 5.2), and when were completely dry, transferred to sealable plastic bags with desiccant packs. When the samples were returned to Makerere University, filter papers were placed in individual glassine or plastic bags, using pieces of silicone paper to separate each paper for storage, and stored in larger sealed plastic bags with desiccant packs.

Screening tests

Screening tests for brucellosis were conducted in local veterinary laboratories within 12 hours after sample collection. Serum from cattle and goats were extracted from the serum separation tubes and screened for brucellosis by the RBT using OIE protocols (OIE, 2012). Since earlier research suggested that the RBT performed better when antigens were matched to the species of *Brucella* infecting the host, and the RBT using *B. melitensis* may be more sensitive to M-epitope dominant biovars of *B. abortus* (Alton et al., 1975; Corbel, 1985), separate RBT tests using *B. abortus* antigen and *B. melitensis* antigen were conducted. The results for each sample were recorded by strength of agglutination seen for each antigen as strong, moderate, weak or negative. The MRT was conducted using OIE protocols (OIE, 2012), with results recorded as strong, moderate, weak or negative.

A subset of clotted blood and milk samples, held at 4°C, was selected shortly after sample collection for DNA extraction and q-PCR for *Brucella* (Hinić et al., 2009). Samples selected for DNA extraction in the first phase of the study were processed in a BSL-3 laboratory at Mulago

Hospital, Kampala, within one month of sample collection. For blood samples, clotted materials were extracted from the SST by piercing the gel layer and using a disposable pipette to collect portions of the clot. Extraction of DNA from the clot sample and milk samples were conducted using the QIAAmp DNA Mini Kit (QIAGEN, Valencia, CA, USA) in accordance with manufacturer directions using the automated QIAcube system. After extraction, DNA samples were stored at 0°C until shipment to the US in 2014.

Dried sample processing

DNA extraction was conducted using a commercial kit (QIAamp DNA Mini Kit 250), following manufacturer instructions. A total of six 3 mm punches were taken from each dried blood spot and dried milk spot using a hand-held punch (the expected DNA extraction for untreated blood from three punches was expected to be 75 ng). Punched samples were placed in microcentrifuge tubes and extracted in accordance with manufacturer protocols before storage of extracted DNA at -20° C.

Qualitative real-time PCR (q-PCR) for the detection of *Brucella* spp., *B. abortus*, and *B. melitensis* DNA was conducted on cattle and goat blood and milk extracts (Hinić et al., 2009). Samples were first screened using the IS711 pan-*Brucella* PCR for the detection of *Brucella* spp. DNA. Samples exhibiting Ct values less than or equal to 38 were considered positive, and were tested using *B. melitensis* and *B. abortus* species specific PCRs (Table 5.1). Real-time amplifications were carried out in a total reaction volume of 25 µl containing 12.5 µl TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, CA, USA), a 200 nM concentration primer and probes, and 5 µl of DNA extract. Amplification and real-time

fluorescence detection was performed on the ABI 7500 DX Real Time PCR System (Applied Biosystems) using the following thermal cycling conditions: 10 minute denaturation at 95°C followed by 45 cycles of 95°C for 15s and 60°C for 1 minute. Samples with fluorescence before 40 cycles were considered positive.

Table 5.1. q-PCR Target sequences with Forward primer/Reverse primer (5'→3') and Probe (5'Fluorophore→3'Quencher)

IS711	F/R Primer	GCTTGAAGCTTGCGGACAGT/GGCCTACCGCTGCGAAT
	Probe	FAM-AAGCCAACACCCGGCCATTATGGT-TAMRA
BMEII0466	F/R Primer	TCGCATCGGCAGTTTCAA/CCAGCTTTTGGCCTTTTCC
	Probe	Cy5-CCTCGGCATGGCCCGCAA-BHQ-2
BruAb2_0168	F/R Primer	GCACACTCACCTTCCACAACAA/CCCCGTTCTGCACCAGACT
	Probe	FAM-TGGAACGACCTTTGCAGGCGAGATC-BHQ-1
BR0952	F/R Primer	CCTGCAAAAAGCAGGAACCA/CCTCCGCCAGTCGTGAAA
	Probe	FAM-ATATGGCCGGCTATCCGCGTTCG-BHQ-1
BOV_A0504	F/R Primer	CGCTATCGATGGCGTAGTTG/CCCTGATTTCAAGCCATTCC
	Probe	FAM-TGGCCTGACGGACGCGCTTATC-BHQ-1
BMEII0635–0636	F/R Primer	AAAATGCGGATCGGCCTT/TCCCGGCGCATTGCT
	Probe	Cy5-CCACGGCTTTCGCTCGGGC-BHQ-2
BMEII0986–0988	F/R Primer	ATGCGGATGCCCGTTTC/AACCTGGCGTCTTTGTCTATCACT
	Probe	FAM-TTGCCAGCCTGCCGCGAA-BHQ-1

Statistical analyses

Numbers of samples q-PCR-positive and q-PCR-suspect for *Brucella* (IS711 positive), and each *Brucella* species (*B. abortus* and *B. melitensis)* were tabulated by host species and type of sample (DBS, DMS), and PCR results were compared to data from serologic and milk screening

tests. Differences in categorical host characteristics (district, gender, breed) with different categorical q-PCR outcomes were evaluated by the Fisher's Exact 2-tailed test, and the strength and direction of associations between characteristics and q-PCR outcomes were reported as odds ratios (OR) with 95% confidence intervals (CI). Associations between positive/negative results of screening tests (RBT, *B. abortus* RBT, *B. melitensis* RBT, MRT) and results from q-PCR were described using Gwet's first-order agreement coefficient (AC1) statistic (Gwet, 2002), which takes into consideration chance agreement between tests (Wongpakaran et al., 2013). Values of the AC1 between 0.21 and 0.4 were classified as fair agreement, between 0.44 and 0.6 as moderate, between 0.61 and 0.8 as good, and values greater than 0.8 were classified as very good agreement (Landis & Koch, 1977).

Results

A total of 237 DNA samples were analyzed by q-PCR for IS711, consisting of 124 cattle and 18 goat DBS from 11 farms, and 91 cattle and 4 goat DMS from nine farms (Table 5.2). Within analyzed samples, 72.6% of 125 cattle and 22.2% of 18 goats had both DBS and DMS available for analysis.

Detection of *Brucella* DNA by q-PCR

The q-PCR was able to detect *Brucella* DNA from 23 DBS and 22 DMS samples obtained from goats and cattle (Table 5.2). All 11 farms in the study had at least one DBS positive sample, and

eight of nine farms had at least one DMS positive sample. Nine of 11 farms had DBS with *B. abortus* DNA, and in three of nine farms with DMS. *Brucella melitensis* DNA was detected in only 2 of 11 farms: one in a cow DMS from one medium farm in LMNP, and from one bovine DBS and one goat DMS from one large farm in LMNP. No DBS or DMS were positive for both *B. abortus* and *B. melitensis* DNA.

Table 5.2. Number of DNA samples extracted from dried blood (DBS) and milk (DMS) from cattle and goats, classified by q-PCR results

Sample	Species	N	q-PCR target	Positive (Ct < 38)	Not positive (Ct ≥ 38)	Negative
DBS	Cattle	124	IS711	20	37	67
		20	*B. abortus*	4	10	6
		20	*B. melitensis*	1	0	19
	Goats	18	IS711	3	9	6
		3	*B. abortus*	0	2	1
		3	*B. melitensis*	0	0	3
DMS	Cattle	91	IS711	20	24	47
		20	*B. abortus*	3	7	10
		20	*B. melitensis*	0	1	19
	Goats	4	IS711	2	2	0
		2	*B. abortus*	0	0	2
		2	*B. melitensis*	1	1	0

Association between screening tests and dried samples

Agreement statistics were good to very good agreement (AC1 > 0.6) for cattle screening tests and q-PCR positive samples, and between q-PCR for *B. melitensis* and RBT test results using either *B. abortus* or *B. melitensis*. Moderate agreement (AC1 = 0.44 to 0.6) was found

between q-PCR with other RBT test results (Table 5.3). There was only fair agreement (AC1 = 0.21 to 0.45) between MRT positive status and q-PCR for IS711 and *B. abortus*. The only significant association ($p \leq 0.05$) seen between RBT and q-PCR test results was between cattle MRT results and q-PCR for IS711 in DMS (Table 5.3).

Table 5.3. Associations between screening test results and results of q-PCR for IS711, *B. abortus*, and *B. melitensis* for cattle DBS and DMS

Screening Test	q-PCR Target	q-PCR Result	Screening test		Fisher's Exact p	AC1	Odds Ratio	
			Negative	Positive			OR	95% CI
RBT	IS711	Negative	78	17	0.4024	0.49	0.53	0.14 – 1.95
		Positive	26	3				
	B. abortus	Negative	13	4	1.0	0.51	0.43	0.02 – 10.0
		Positive	3	0				
	B. melitensis	Negative	13	4	1.0	0.51	0.43	0.02 – 10.0
		Positive	3	0				
B. abortus RBT	IS711	Negative	86	18	0.5256	0.60	0.53	0.11 – 2.49
		Positive	18	2				
	B. abortus	Negative	14	4	1.0	0.60	0.64	0.03 – 16.05
		Positive	2	0				
	B. melitensis	Negative	17	1	1.0	0.83	2.33	0.07 – 74.54
		Positive	2	0				
B. melitensis RBT	IS711	Negative	81	17	0.5643	0.54	0.62	0.17 – 2.31
		Positive	23	3				
	B. abortus	Negative	13	4	1.0	0.51	0.43	0.02 – 10.0
		Positive	3	0				
	B. melitensis	Negative	16	1	1.0	0.76	1.57	0.05 – 47.19
		Positive	3	0				

Table 5.3. Associations between screening test results and results of q-PCR for IS711, *B. abortus*, and *B. melitensis* for cattle DBS and DMS

Screening Test	q-PCR Target	q-PCR Result	Screening test		Fisher's Exact p	Odds Ratio		
			Negative	Positive		AC1	OR	95% CI
MRT	IS711	Negative	45	5	0.0043	0.39	5.19	1.69 – 15.94
		Positive	26	15				
	B. abortus	Negative	4	1	1.0	-0.39	0.62	0.04 – 8.70
		Positive	13	2				
	B. melitensis	Negative	50	0	-	-	-	-
		Positive	41	0				

Agreement between serological screening tests and q-PCR was limited to DBS for goats, since no screening tests for goat milk were conducted in this study (Table 5.4). There were no statistically significant associations between RBT test results and DBS q-PCR, and agreement was poor between screening tests and q-PCR results.

Table 5.4. Associations between screening test results and results of q-PCR for IS711[1] for goat DBS[2]

Screening Test	IS711 q-PCR	Screening test		Fisher's Exact p	Odds Ratio		
		Negative	Positive		AC1	OR	95% CI
RBT	Negative	7	3	0.2157	-0.06	0.13	0.01 – 2.86
	Positive	8	0				
B. abortus RBT	Negative	9	3	0.5147	0.20	0.21	0.01 – 4.76
	Positive	6	0				
B. melitensis RBT	Negative	7	3	0.2157	-0.06	0.13	0.01 – 2.86
	Positive	8	0				

[1] No positive q-PCR test results for *B. abortus* or *B. melitensis* in goat DBS samples
[2] MRT not applied to goat milk samples

Discussion

Results from this study demonstrates that the use of filter paper to stabilize blood or milk samples allows detection of *Brucella* up to four years after sample collection, holding samples under ambient conditions and using desiccant packs. Species-specific DNA targets were successfully detected in DBS and DMS from cattle and goats: *B. abortus* was detected in four of 20 cattle DBS, and one of 20 cattle DMS. Successful extraction of DNA has been conducted using other dried specimens stored for long periods of time, including extraction of human DNA from a blood clot on dried fabric after four years (Gill et al., 1985), and 10-13 year old bone marrow slides held at room temperature (Fey et al., 1987). Storing samples at -20° C with desiccants has been reported to increase the time that samples remain viable (Mei et al., 2011), and one study was able to recover whole-genome amplified DNA from DBS stored from 15 to 25 years at -24° C (Hollegaard et al., 2009). Studies using filter paper for preservation of antigens and other biomarkers have indicated that specimens should be used within weeks (Grüner et al., 2015) or months after collection (Joseph & Melrose, 2010; Mei et al., 2011; Miller & McConnell, 2011). It should also be noted that, in a study investigating *Toxoplasma gondii* in the Peruvian Amazon, IgG was successfully detected in elutes from DBS after samples were stored up to four months in high temperature (22–36° C) and relative humidity (80–100%) (Aston et al., 2014). Increasing the storage time for DBS and DMS by freezing specimens is desirable, but may not be possible in resource-limited settings.

In outbreak investigations or disease surveillance where sample testing will be conducted as quickly as possible, DBS and DMS could be an effective method for sample storage and

transportation. Testing samples within one month of collection should be achievable in most settings, even if this involves shipping samples from the field to diagnostic facilities in different countries. When shipping samples to international diagnostic laboratories is necessary, DBS and DMS have considerable advantages over the shipment of fluid samples: there is no requirement to maintain the cold chain during shipping, there are no concerns for breakage during shipping, and the shipment weight of dried samples will be lower than the weight of fluid or tissue samples that are properly wrapped for safe shipment. An additional advantage is that DBS and DMS can be easily treated to meet import requirements for shipment. In the current study, dried samples had to undergo foot and mouth disease virus (FMDV) inactivation before the samples could be brought from Uganda. Based on OIE protocols for FMDV inactivation required by the USDA Animal and Plant Health Inspection Services (OIE, 2012), dried samples were easily treated by holding the samples at 70°C for 30 min. This was simpler than inactivation steps used for fluid samples, which require holding samples at pH 5.5 for 30 minutes to neutralize FMDV (OIE, 2012).

Our findings indicate that the DMS is a viable method for storing milk samples for brucellosis testing. There was a strong association between results of q-PCR and screening tests in milk samples from cows. Both PCR and the MRT are tests known for high specificity, which explains the high level of agreement in negative test samples. The sensitivity and specificity of the MRT have been reported to be 72–88% and 85–90%, respectively (Salman & El Nasri, 2012; Vanzini et al., 2001), and the sensitivity and specificity of PCR on fluid milk samples has been reported as 87.5% and 100%, respectively (Romero et al., 1995). Although it was beyond the scope of this study, future research into the use of DMS with other accepted testing modalities,

including the MRT and indirect ELISA (OIE, 2012), should be pursued. If *Brucella* antibodies can be detected in eluates from DMS, this will make sample storage simpler, by obviating the need for refrigeration of samples that cannot be processed immediately.

This study was able to document high and moderate levels of agreement between DBS q-PCR and RBT test results in samples from cattle. Although there were no statistically significant associations between the RBT and q-PCR, there were trends in agreement between the RBT with q-PCR for *B. melitensis*, followed by RBT with IS711 q-PCR. The majority of agreement was seen in negative samples: 82% of samples negative to the RBT using *B. abortus* or *B. melitensis* antigen were also PCR-negative for IS711. As seen in DMS, the lower sensitivity and higher specificity of the RBT in cattle and goats (Díaz-Aparicio et al., 1994; Gall & Nielsen, 2004; Mikolon et al., 1998; Sanogo et al., 2013b) and high specificity of PCR contributed to the higher levels of agreement between the DBS and RBT. The higher levels of agreement between q-PCR for *B. abortus* with the RBT is intriguing, but the low number of samples that were tested with species-specific q-PCR (n=20) made it difficult to document significant associations between these samples. Likewise, the lack of associations between DBS and RBT in goats was not unexpected given the low number of samples (n=18) available in this study. Additional work should be conducted to expand the numbers of paired fluid and dried blood and/or serum samples for analysis to improve study power for associations suggested by our results.

There was evidence of the presence of *B. melitensis* in cattle samples and *B. abortus* in goat samples in this study. Other studies have reported *B. abortus* in goat samples, attributed to the transmission of common strains of *Brucella* between livestock on the same farm (Adamu et al., 2012; Leal-Klevezas et al., 2000). Studies have also reported the presence of *B. melitensis* in

cattle by serology (Alvarez et al., 2011) and in cattle and buffalo semen by PCR (Safarpoor Dehkordi et al., 2014). Given that the two *B. melitensis* DBS and DMS samples came from different species from the same farm, these findings and reports by other researchers support the concept that *B. melitensis* and *B. abortus* can be actively transmitted between livestock species on the farm.

Conclusions

This study was able to demonstrate the utility of using a laboratory grade filter paper for storage of blood and milk samples for DNA extraction and for subsequent detection of *Brucella*. In addition, *Brucella* species-specific DNA targets were successfully detected in DBS and DMS from cattle and goats, which is valuable information for epidemiological investigations.

Collection and storage of DBS and DMS meets the WHO ASSURED criteria for technologies that are affordable, user-friendly, equipment-free, and deliverable, and could be simultaneously when samples are collected for screening. The cost of filter papers are significantly less expensive than the use of FTA™ sample cards, making collection and storage of larger numbers of samples affordable in regions where financial resources are limited. Combining dried specimen collection with screening test sample collection could provide investigators with a simple and inexpensive storage medium that would allow subsequent submission of samples testing modalities that are more sensitive and specific or, too expensive or difficult to conduct on large numbers of samples.

Additional research is needed to further investigate the viability of samples stored on filter paper for use in research and diseases surveillance. Larger studies, using verified control and spiked blood and milk samples, should be conducted to quantify the storage time for dried samples, and longitudinal studies of should be conducted to describe and/or quantify the loss of DNA in dried samples over time. Future research on the ability to recover antibodies or other disease biomarkers from filter paper DBS and DMS should be pursued as a method for reducing or replacing freezer storage of fluid samples.

CHAPTER 6: Use of molecular tools to detect *Brucella* in cattle, goats, and humans in Ugandan dairy farms

Structured Abstract:

Introduction: Brucellosis is a widely recognized zoonotic disease found in livestock, companion animals, and wildlife throughout the world, and is an important livestock disease problem in developing countries. Traditionally, *Brucellae* have been speciated by host specificity and/or preferences, pathogenicity within a given host species, and phenotypic traits. Detection of *Brucella* species in natural hosts has been used to determine reservoirs of infection for human disease, but the host specificity of *Brucella* may not be a conclusive determinant of the reservoir host.

Hypotheses:

- Both *B. abortus* and *B. melitensis* are present in cattle, goats, and humans on dairy farms in rural Uganda.
- Species of *Brucella* found in cattle and goats on dairy farms in rural Uganda are not host-specific.

Objectives: The objectives of the study were to 1) detect and identify species of *Brucella* using qualitative real-time PCR (q-PCR) and nested PCR (n-PCR); 2) describe the distribution of *Brucella* species from human blood, bovine and caprine blood, and bovine milk samples; 3) describe associations between PCR with results of screening tests and brucellosis risk factors;

and 4) utilize PCR results to describe the epidemiology of brucellosis in cattle, goats, and humans on Ugandan dairy farms.

Methods: DNA from Blood and milk samples, results of screening tests, and brucellosis risk factor data, were collected from 35 cows, 15 goats and 11 human subjects from four farms in rural Uganda. The q-PCR and n-PCR were conducted for detection of insertion sequence IS711, and species-specific targets for *B. abortus* and *B. melitensis*. Samples were classified as negative, strong positive, or weak positive.

Results: Both q-PCR and n-PCR assays detected IS711, *B. abortus*, and *B. melitensis* DNA in samples from all three hosts. Both PCR assays were able to detect *B. abortus* DNA in cattle, and q-PCR detected *B. abortus* in humans, and goats. The n-PCR detected *B. melitensis* DNA in cattle blood and milk, and evidence of *B. melitensis* in a human sample positive for *B. abortus.*

Conclusions: Results from this study demonstrated the presence of *B. abortus* DNA in samples from humans, cattle, milk and goats, and indicated that cattle were probably the primary reservoir of brucellosis on Uganda dairy farms. However, the presence of *B. melitensis* in cattle samples, and association between the seroprevalence of brucellosis in goats with human brucellosis in this study suggest that goats contribute to human brucellosis, and may pose a public health threat in rural Uganda. Disease control efforts should be modified to include control of brucellosis in goats, and vaccination of cattle with vaccines effective against both *B. abortus* and *B. melitensis.*

Introduction

Brucellosis (infection by members of the bacterial genus *Brucella*), is a widely recognized zoonotic disease found in livestock, companion animals, and wildlife throughout the world, and is an important livestock disease problem in developing countries (Glynn & Lynn, 2008; McDermott et al., 2013; Al Dahouk et al., 2014). Transmission between animals occurs primarily through direct contact with infected animals, contact with fluid or tissues from *Brucella*-induced abortions, or through the milk of infected mothers (Glynn & Lynn, 2008). The most important routes of infection for human brucellosis are through direct contact with infected animals, and the ingestion of materials contaminated with viable *Brucella,* particularly through the consumption of raw milk or dairy products produced from unpasteurized or underpasteurized milk (Bernard et al., 2005; Glynn & Lynn, 2008; Makita et al., 2008; Al Dahouk et al., 2014; Olsen & Palmer, 2014; Tumwine et al., 2015). Control of brucellosis in livestock through vaccination has resulted in decreases in human disease (Roth et al., 2003; Shemesh & Yagupsky, 2013).

Traditionally, *Brucellae* have been speciated according to host specificity and/or preferences, pathogenicity within given host species, and phenotypic traits (Al Dahouk et al., 2014). The most pathogenic species in humans are *B. melitensis, B. abortus,* and *B. suis* (Al Dahouk et al., 2014). Although *B. abortus* is primarily associated with bovine brucellosis and *B. melitensis* primarily with caprine brucellosis, there have been reports of cattle with *B. melitensis* infections (Álvarez et al., 2011) and goats with *B. abortus* (Leal-Klevezas et al., 2000; Adamu et al., 2012), and recent genomic analyses have introduced some controversy in the

taxonomy of the species (Godfroid et al., 2011; Al Dahouk et al., 2014; Olsen & Palmer, 2014). Regardless of these issues, it is still critical to identify brucellosis reservoir so that interventions to reduce levels of disease and spread of disease in the infected population can be developed (Godfroid et al., 2013). However, the diagnostic utility of PCR has yet to be fully validated, as the majority of studies reporting the sensitivity and specificity of PCR used DNA extracted from cultured bacteria, which does not consider other factors that could influence detection from field isolates or samples (Yu & Nielsen, 2010),

A cross-sectional study was conducted to describe the epidemiology of zoonotic brucellosis in dairy farms in rural southwestern Uganda, by describing the prevalence of cattle, goats, and humans on farms, and identifying risk factors associated with brucellosis test positivity (Miller et al., 2015). In this study, positive screening tests for brucellosis were seen in cattle sera and milk, goat sera, and human sera, and assessment of risk factors associated with positive screening tests found significant associations between seroprevalences of brucellosis in humans and goats, suggesting that goats may be an important source of infection for humans.

To further investigate the circulation of *Brucella* on these farms, this study was conducted to address the following **hypotheses**:

- Both *B. abortus* and *B. melitensis* are present in cattle, goats, and humans on dairy farms in rural Uganda.
- Species of *Brucella* found in cattle and goats on dairy farms in rural Uganda are not host-specific.

The **specific aims** of the study were to 1) identify species of *Brucella* present in dairy farms in southwestern Uganda, using a qualitative real-time PCR (q-PCR) and a nested PCR to detect

genetic markers for *Brucella* spp., and specific targets for *B. abortus*, and *B. melitensis*, known pathogens in cattle, goats, and humans; 2) describe the distribution of *Brucella* species from human blood, bovine and caprine blood, and bovine milk samples, based on n-PCR and q-PCR; 3) describe associations between PCR test results with results of screening tests and risk factors associated with brucellosis; and 4) utilize results from PCR to describe the epidemiology of brucellosis in cattle, goats, and humans from dairy farms in southwestern Uganda.

Materials and Methods

Study design

This study was part of a larger cross-sectional study designed to describe the prevalence and epidemiology of brucellosis in cattle, goats and humans from dairy farms in southwestern Uganda (Miller et al., 2015). DNA was extracted from cattle blood and milk, goat blood, and human blood from four farms where there were strong evidence of *Brucella* infection, based on results of screening tests. Data and serum samples were collected from 773 cattle, 315 goats, and 207 humans, and 635 samples of bovine milk were collected from 70 farms in two different study areas in southwestern Uganda. Sera from livestock were tested with the Rose Bengal Plate test, using *B. abortus* and *B. melitensis* antigens, human sera were tested with a commercial IgG/IgM lateral flow assay (LFA) (Test-it™ Lateral Flow Assays, LifeAssay Diagnostics (Pty) Ltd., Cape Town, South Africa), and milk samples were tested using the OIE-approved milk

ring test (OIE, 2012). These screening tests yielded positive results in 14% of cattle sera, 29% of bovine milk, 17% of goat sera, and 11% of human sera (Miller et al., 2015).

Three of the 70 dairy farms in the study were selected for molecular testing based on the following criteria: 1) at least one human subject was positive by the LFA; and 2) samples from cows and goats were collected. Within these criteria, farms were selected for variety in livestock prevalences: one farm had RBT-positive cattle, MRT-positive milk samples, and no RBT-positive goats; one farm had no RBT or MRT positive livestock; and one farm had high levels of RBT-positive cattle and goats, and high MRT-positive milk samples.

Sample collection and processing

Individual dairy farmers that had at least five milking cows were contacted by local veterinary officers for possible participation in the study. After informed consent was obtained, a farm visit was arranged to collect biological samples from cows (blood, milk), goats (blood), and humans (blood). Farm visits were conducted by veterinarians and veterinary students for animal specimen collection, and biomedical students and licensed Health Care Centre technicians for human specimen collection.

Animals for sample collection (preferably lactating females) were randomly selected by investigators at the farm, manually restrained by the owner, and a 10 ml blood sample was collected by jugular venipuncture using Vacutainer® serum separation tubes (SST). For cows in milk, a combined milk sample from all four quarters from each cow was collected aseptically in sterile 30 ml universal containers. For sample collection from human subjects, informed consent was sought from the farmer, farm workers, and family members, and after consent

was given, a 10 ml blood sample was collected by venipuncture, using SSTs. All samples were kept on ice until processed at local veterinary laboratory within 12 hours of sample collection.

DNA extraction

Samples selected for DNA extraction in the first phase of the study were processed at the College of Medical Sciences BSL-3 laboratory facilities at Mulago Hospital, Kampala, held at 4°C, within one month of sample collection. For blood samples from humans, cattle, and goats, clotted materials were extracted from the SST by piercing the gel layer and using a disposable pipette to collect portions of the clot. Milk samples for DNA extraction were collected directly from the sterile universal containers where the samples were collected. Extraction of DNA from the clot sample and milk samples were conducted using the QIAAmp DNA Mini Kit (QIAGEN, Valencia, CA, USA) at the College of Medical Sciences BSL-3 facilities using the automated QIAcube system. After extraction, DNA samples were stored at 0°C until shipment to the U.S. in 2014. In the second phase, DNA was extracted from human sera were tested by the CDC in 2014, using the QIAAmp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA).

q-PCR

Qualitative real-time PCR (q-PCR) for *Brucella* IS711, *B. abortus*, and *B. melitensis* was conducted (Hinić et al., 2008). Real-time amplifications were carried out in a total reaction volume of 25 µl containing 12.5 µl TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, CA, USA), a 200 nM concentration of primer and probes (Table 6.1) (Microsynth, Balgach, Switzerland), 0.5 µl TaqMan® Exogenous Internal Positive Control

(IPC; Applied Biosystems) mix, 0.5× IPC template, and 5 µl µl of DNA extract. Amplification and real-time fluorescence detection was performed on the ABI 7500 DX Real Time PCR System (Applied Biosystems) using the following thermal cycling conditions: 10 minute denaturation at 95°C followed by 45 cycles of 95°C for 15s and 60°C for 1 minute. Samples with fluorescence before 38 cycles were classified as strong positive, and samples with fluorescence between 38 and 40 were classified as weak positive/suspect. Negative and positive controls (*B. abortus, B. melitensis*) were run with field samples.

Table 6.1 q-PCR Target sequences with Forward primer/reverse primer (5'→3') and Probe (5'Fluorophore→3'Quencher)

	Forward / Reverse primer	Probe
IS711	GCTTGAAGCTTGCGGACAGT/ GGCCTACCGCTGCGAAT	FAM-AAGCCAACACCCGGCCATTATGGT-TAMRA
BMEII0466	TCGCATCGGCAGTTTCAA/ CCAGCTTTTGGCCTTTTCC	Cy5-CCTCGGCATGGCCCGCAA-BHQ-2
BruAb2_0168	GCACACTCACCTTCCACAACAA/ CCCCGTTCTGCACCAGACT	FAM-TGGAACGACCTTTGCAGGCGAGATC-BHQ-1
BR0952	CCTGCAAAAAGCAGGAACCA/ CCTCCGCCAGTCGTGAAA	FAM-ATATGGCCGGCTATCCGCGTTCG-BHQ-1
BOV_A0504	CGCTATCGATGGCGTAGTTG/ CCCTGATTTCAAGCCATTCC	FAM-TGGCCTGACGGACGCGCTTATC-BHQ-1
BMEII0635–0636	AAAATGCGGATCGGCCTT/ TCCCGGCGCATTGCT	Cy5-CCACGGCTTTCGCTCGGGC-BHQ-2
BMEII0986–0988	ATGCGGATGCCCGTTTC/ AACCTGGCGTCTTTGTCTATCACT	FAM-TTGCCAGCCTGCCGCGAA-BHQ-1

Nested PCR

Nested PCRs for *Brucella* IS711, and *B. abortus* and *B. melitensis* species detection were conducted using primers specific for *B. abortus* and *B. melitensis* (Table 6.2). For detection of the IS711 sequence in study samples, the first round of PCR used an IS711-specific single primer (Bricker & Halling, 1994) to generate an 842 bp amplicon, and second round PCR used the products from the first round with 267-F and 544-R, producing a 300 bp amplicion for IS711. Negative controls and positive controls (*B. abortus, B. melitensis, B. suis*) were run with field samples.

Table 6.2 Primers used in nested PCR

Target	Primer	Primer Sequence (5'→3')
Brucella IS711 1st Round		TGC CGA TCA CTT AAG GGC CTT CAT
Brucella IS711 2nd Round	267–F	GCA TCA GCA ATG ACA TGC CCC ACA CCC T
	544–R	GGT CGC ACG CCG GTG TAT GGG AAA GG
B. melitensis 1st Round	mel-Out-F	GCT TGG CTC CGG TGC TGT GTT TTT GGG
	mel-Out-R	GCT TAG AGG GTG TGG GGC ATG TCA TTG CTG
B. melitensis 2nd Round	mel-In-F	CGG ATA TGA ATC TGA ACC AGC TTA
	mel-In-R	ACG CCC TAG GGG TGA ATC TG
B. abortus 1st Round	all-AbF-0	GGT GCC GAG ACC TGG GAC ATA TTG G
	all-AbR-0	GTG AGC CCT TCG CCC AGT GGA GC
B. abortus 2nd Round	all-AbF1	CGT CTC GTC AAG CGT CAA G
	all-AbR1	TTC ATG CCT CGT TTT TGT CG
B. abortus 2nd Round Alternate Inner pair	all-AbF2	CCA TAT TGA TGC AAG CCA CG
	all-AbR2	CCA ACG GTT ACA TTG CAG C

Two n-PCR assays, using two sets of primers, were conducted for *B. abortus*. In the first PCR, the first round used primers All-AbF-O and All-AbR-O to generate putative *B. abortus* amplicons of 1,841 bp, and the second round PCR used primers All-AbF-1 and All-AbFR-1, which generated *B. abortus* amplicons of 680 bp, and 3,435 bp amplicons for other *Brucella* spp. The second n-PCR assay used the same first round primers (All-AbF-O, All-AbR-O,) to generate1,841 bp amplicons, and used second round primers All-AbF-2 and All-AbFR-2 to generate *B. abortus* amplicons of 924 bp, and 3,660 bp amplicons for other *Brucella* spp.

The nested PCR for *B. melitensis* used primers Mel-Out-F and Mel-In-F to generate 1,347 amplicons, followed by PCR using Mel-In-F and Mel-In-R primers to generate a 659 bp amplicon for *B. melitensis.* PCR reactions were run using TETRAD thermal cycler (BioRad) using the following thermal cycling conditions: 35 cycles of 10s at 98°C, 60° C for 5s, 72°C for 3 minutes 45s, followed by 75°C for 10 minutes.

Statistical analysis

Study outcomes were sample status by q-PCR and n-PCR for IS711 and species-specific targets, and screening tests. The numbers of samples positive and suspect for IS711*, B. abortus* and *B. melitensis* by n-PCR and q-PCR were reported by host species, type of sample (blood, milk), and results of screening tests. Associations between positive/negative results of screening tests (RBT, *B. abortus* RBT, *B. melitensis* RBT, MRT) and PCR results were described using Gwet's first-order agreement coefficient (AC1) statistic (Gwet, 2002), which takes into consideration chance agreement between tests (Wongpakaran et al., 2013). Values of the AC1 between 0.21 and 0.4 were classified as fair agreement, between 0.44 and 0.6 as moderate,

between 0.61 and 0.8 as good, and values greater than 0.8 were classified as very good agreement (Landis & Koch, 1977). Differences in categorical host characteristics with outcomes were evaluated by the Fisher's Exact 2-tailed test, and the strength and direction of associations between host characteristics and outcomes were reported as odds ratios (OR) with 95% confidence intervals (CI).

Results

A total of 92 DNA samples from 35 cows, 15 goats, and 11 humans from three farms were tested by screening tests, nested PCR and q-PCR (Table 6.3). For all hosts and samples, both PCRs detected positive samples more frequently than the screening tests. The samples tested for *B. abortus* and *B. melitensis*-specific targets by q-PCR were reduced due to the lack of sample volume: when the species-specific results from n-PCR were limited to only those with species-specific results from q-PCR, 2 of 19 cattle sera were positive for *B. abortus* and 4 of 23 were positive for *B. melitensis*, and 4 cattle milk samples were positive for *B. abortus* and *B. melitensis* by n-PCR.

Table 6.3 Number and percent positive (+) and suspect test (s) results for screening tests [Rose Bengal Test (RBT) for cattle and goat sera, the Milk Ring Test (MRT) for cattle milk, and the Lateral Flow Immunoassay (LFA) for human sera] and the nested PCR (n-PCR) and qualitative PCR (q-PCR), by host and type of sample

	Cattle						Goat			Human		
	Blood			Milk			Blood			Blood		
Test	N	% Pos	% Susp	N	% Pos	% Susp	N	% Pos	% Susp	N	% Pos	% Susp
RBT	32	9.4	0	–	–	–	15	26.7	0	–	–	–
MRT	–	–	–	34	17.6	0	–	–	–	–	–	–
LFA	–	–	–	–	–	–	–	–	–	11	27.2	0
n-PCR for IS711	32	40.6	6.3	31	19.4	3.2	15	26.7	6.7	11	36.4	0
n-PCR for *B. abortus*	32	6.3	0	31	16.1	3.2	15	0	0	11	0	0
n-PCR for *B. melitensis*	32	15.6	6.3	31	12.9	9.7	15	0	0	11	9.1	0
q-PCR for IS711	32	50.0	15.6	31	48.4	29.0	15	46.7	6.7	11	72.7	18.2
q-PCR for *B. abortus*	19*	57.9	21.1	25*	16.0	20.0	8*	37.5	12.8	8*	62.5	37.5
q-PCR for *B. melitensis*	18*	0	0	23*	0	0	5*	0	0	6*	0	16.7

* Number tested decreased due to low initial sample volume

There were no statistically significant ($p \leq 0.05$) associations between PCR-positive test results with screening tests (Table 6.4). Agreement between screening tests and q-PCR was poor for all sample types, screening tests, and hosts (AC1 < 0.2), but fair and moderate agreement between n-PCR for cattle blood with the RBT (AC1 = 0.27) and cattle milk with the MRT (AC1 = 0.50), respectively.

Table 6.4 Association between screening tests with PCR for IS711, by host and type of sample

Host	Sample Type	PCR	Screening Test Type	Screening Test Level	PCR Results Neg	PCR Results Pos	Fisher's Exact P	AC1	Odds Ratio OR	Odds Ratio 95% CI
Cattle	Blood	n-PCR	RBT	Neg	16	11	1.0	0.27	0.97	0.14 – 6.79
				Pos	3	2				
		q-PCR	RBT	Neg	14	13	1.0	0.16	1.62	0.23 – 11.26
				Pos	2	3				
		Either	RBT	Neg	12	15	1.0	0.02	1.20	0.17 – 8.38
				Pos	2	3				
Cattle	Milk	n-PCR	MRT	Neg	22	6	0.5615	0.50	0.27	0.01 – 5.38
				Pos	6	0				
Cattle	Milk	q-PCR	MRT	Neg	13	15	1.0	0.03	0.87	0.15 – 5.06
				Pos	3	3				
		Either	MRT	Neg	11	17	0.6722	-0.11	0.65	0.11 – 3.80
				Pos	3	3				

Table 6.4 (cont'd.)

Host	Sample Type	PCR	Screening Test Type	Screening Test Level	PCR Results Neg	PCR Results Pos	Fisher's Exact P	AC1	Odds Ratio OR	Odds Ratio 95% CI
Goat	Blood	n-PCR	RBT	Neg	7	4	0.5165	0.12	0.19	0.01 – 4.31
				Pos	4	0				
		q-PCR	RBT	Neg	6	5	1.0	0.13	1.20	0.12 – 11.87
				Pos	2	2				
Goat	Blood	Either	RBT	Neg	4	7	1.0	-0.18	0.57	0.06 – 5.77
				Pos	2	2				
Human	Blood	n-PCR	LFA	Neg	4	4	0.2364	-0.12	0.14	0.01 – 3.64
				Pos	3	0				
		q-PCR	LFA	Neg	3	5	0.4909	0.09	4.45	0.17 – 115.13
				Pos	0	3				
		Either	LFA	Neg	0	4	1.0	-0.08	2.69	0.10 – 73.20
				Pos	2	5				

There was only one significant association between PCR test results with risk factors, between n-PCR-positive cattle milk with goat biosecurity (herds that replaced goats from sources outside the herd and allowed cattle and goats to share water) (Table 6.5). There were non-significant associations for cattle blood DNA positive by n-PCR with goat biosecurity ($p = 0.0641$), and between human blood positive by n-PCR with subject history of fever within the last 12 months ($p = 0.0879$).

Table 6.5 Associations between PCR for IS711 with risk factors ($p \leq 0.1$), by host and sample type

Host	Sample Type	PCR	Risk Factor	Level	PCR Results Neg	PCR Results Pos	Fisher's Exact P	Odds Ratio OR	Odds Ratio 95% CI
Cattle	Blood	n-PCR	Goat Biosecurity[a]	No	5	0	0.0641	10.24	0.52 – 203.34
				Yes	14	13			
		q-PCR	Goat Biosecurity[a]	No	4	1	0.3326	5.00	0.49 – 50.83
				Yes	12	15			
Cattle	Milk	n-PCR	Livestock abortion history	No	13	6	0.0239	0.07	0.00 – 1.30
				Yes	16	0			
		q-PCR	Livestock abortion history	No	7	12	0.2998	0.39	0.10 – 1.56
				Yes	9	6			
Human	Blood	n-PCR	Subject with history of fever	No	1	3	0.0879	0.06	0.0 – 1.23
				Yes	6	1			
		q-PCR	Subject with history of fever	No	1	3	1.0	0.83	0.05 – 13.63
				Yes	2	5			

[a] Goat biosecurity: herds that replaced goats from sources outside the herd, shared water between cattle and goats

Discussion

Results from this study have demonstrated the presence of *B. abortus* DNA in samples from humans, cattle, milk and goats. Studies have found that PCR has been more effective at detecting brucellosis than screening tests for bovine milk and serum (Saleha et al., 2013). However, in one study in the Sudan (Abdalla et al., 2012), the MRT detected more positive

animals than IS711 PCR in suspect dairy cows: this may have been due to the low sensitivity (37%) of the MRT in the study. The prevalence of *B. abortus* DNA in human specimens by q-PCR (62.5%) was much higher than the reported 6 – 18% seroprevalence of brucellosis in humans in Uganda (Makita et al., 2011; Nabukenya et al., 2013; Nasinyama et al., 2015; Tumwine et al., 2015), but samples used in this study were selected based upon their likelihood to be *Brucella*-positive. This biased the likelihood of positivity in human samples in this study, when compared to the earlier seroprevalence studies utilizing plate agglutination, tube agglutination, and c-ELISA to detect *Brucella* antibodies. However, other studies have indicated that PCR was able to detect *Brucella* DNA in samples from seronegative or culture-negative subjects (Leal-Klevezas et al., 2000; Hinić et al., 2009; Osman et al., 2015).

This study was able to document the presence of *B. abortus* in goats on dairy farms in Uganda. An earlier study of brucellosis in goats in Uganda had reported the herd prevalence of *B. abortus* in goats to be 13%, with an individual animal prevalence of 4%, but these findings were based on the use of serological tests (Kabagambe et al., 2001). The card agglutination test was used to identify goats infected with *B. abortus*, and the tube agglutination test (TAT) was used to identify *B. melitensis* infection. The authors reported that 16% of the 19 card test-positive herds were TAT-negative, and 37% of the 126 card-test negative herds were TAT positive, suggesting that these herds were infected with primarily *B. abortus* and *B. melitensis*, respectively (Kabagambe et al., 2001). Results from q-PCR in this study support these findings of *B. abortus* in a significant proportion of goats on dairy farms with molecular evidence, and clearly demonstrates that goats may play a significant role in the circulation of *B. abortus* on dairy farms. The presence of *B. abortus* in goats has also been reported in other studies using

molecular tools (Leal-Klevezas et al., 2000; Wareth et al., 2015) and serological tests (Lilenbaum et al., 2007). In both studies using serological tests to detect *B. abortus* (Kabagambe et al., 2001; Lilenbaum et al., 2007), the presence of *B. abortus* was associated with goats housed with cattle, and in one study *B. abortus* seropositive goats had a history of nursing from cows (Lilenbaum et al., 2007). Risk factors associated with seropositivity in goats included increasing numbers of cattle on the farm, sharing water sources with wildlife, and housing goats with animals from other flocks, and factors associated with positive tests in cattle included the seroprevalence of brucellosis in goats and treating cattle for ticks, (Miller et al., 2015). These findings further support the hypothesis that, regardless of bacterial species, *Brucella* can actively circulate between livestock species on farms, and that control of livestock brucellosis cannot be limited to control strategies focused on a single host species. Additional phylogenetic analysis of samples from this study should be conducted to provide more detailed information about species, biovars and types of *Brucella* circulating in livestock and humans on dairy farms in rural Uganda.

The q-PCR detected the presence of *B. melitensis* in cattle blood and milk in this study. There have been several reports of *B. melitensis* detected in cattle using different PCR methods and targets (Wareth et al., 2014; Safarpoor Dehkordi et al., 2014; Al-Mariri, 2015), and in a study using multiple locus variable number tandem repeats analysis (MLVA) (Alvarez et al., 2011). The putative reservoirs for *B. melitensis* are goats and sheep, and livestock husbandry that allows cattle to come into contact with infected goats is considered to be the route of transmission (Al-Mariri, 2015). The establishment of *B. melitensis* in cattle has consequences for

any proposed vaccination programs, since some studies have found that the S19 vaccine does not confer effective immunity against *B. melitensis* in cattle (Lucero et al., 2006).

The lack of agreement between results of screening tests and results from PCR in this study have been reported by several other studies. The detection of *Brucella* DNA in subjects that were negative on antibody detection tests was not surprising, and other studies have found that *Brucella* DNA persists after infection (Muñoz et al., 2005). The presence of DNA in seronegative samples may be due to chronic or early infection, with antibody levels below detectable limits (Greiner & Gardner, 2000a), or may represent fragmented DNA from *Brucella* that are no longer viable or have been effectively phagocytosed (Al Dahouk et al., 2013).

The lack of *B. melitensis* DNA in goats was not expected. The seroprevalence of brucellosis in goats in Uganda has been reported to be from 12–16% (Bernard et al., 2005; Mwebe al., 2010), and the absence of *B. melitensis* by q-PCR may be due to the small number of goat samples (n=15) submitted for q-PCR. The detection of *B. melitensis* in one human sample from a farm with seropositive goats in this study suggests that the bacterium is present on the farm, and that testing more animals would likely result in detecting *B. melitensis* in goats. Human co-infection with *B. abortus* and *B. melitensis* has been reported infrequently, and a recent study in Khartoum found *B. melitensis* and *B. abortus* S19 in cattle workers, and noted that cattle on the farm had been vaccinated with S19 (Osman et al., 2015). Although *B. melitensis* was not found in cattle in this study, it has been documented by VNTR in cattle in Spain (Álvarez et al., 2011), and by q-PCR in bovine milk (Wareth et al., 2014). Testing other samples from goats and cattle on these farms is ongoing, and should be able to provide more evidence of *B. melitensis* on these farms.

Conclusions

Results from this study have demonstrated the widespread presence of *B. abortus* DNA in samples from humans, cattle, milk and goats, indicating that cattle are the primary reservoir of brucellosis on these farms. However, the presence of *B. melitensis* in cattle samples, and associations between the seroprevalence of brucellosis in goats with human brucellosis in this study, indicate that goats also contribute to brucellosis in dairy farms in rural Uganda, and may pose more of a public health threat that previously realized. Disease control efforts should be modified to include control of brucellosis in goats, and vaccination of cattle with vaccines effective against both *B. abortus* and *B. melitensis.*

CHAPTER 7: Overall Summary, Conclusions, and Recommendations

This dissertation was undertaken to address gaps in the knowledge in the epidemiology of brucellosis in humans and livestock in rural Uganda, as a model of the challenges and solutions for zoonotic disease control in developing countries. The first goal of the study was to provide data to describe the epidemiology of zoonotic brucellosis in dairy farms in rural southwestern Uganda, by describing the prevalence of cattle, goats, and humans on farms, identifying risk factors associated brucellosis test positivity, describing the species and strains of *Brucella* in samples from cattle, goats, and humans, and evaluating the potential impacts of interaction between humans and livestock. The second goal of the dissertation was to evaluate the use of diagnostic tests and sample storage techniques that can address the challenges to disease surveillance programs faced in rural sub-Saharan Africa and other resource-limited settings.

Summary of Findings

This study was able to determine the seroprevalence of brucellosis in cattle, goats and humans in farms from southwestern Uganda, and identified risk factors associated with brucellosis in these three host groups. The majority of the study findings support existing reports of factors associated with increasing brucellosis risk, and demonstrated the positive associations between increasing levels of positive screening test results for brucellosis in goats, cattle, and humans. Results of multivariable analyses suggest that improvements in farm biosecurity and hygiene may reduce the risk or brucellosis on the farm. The finding of

significant associations between livestock brucellosis with wildlife contact, and cattle seropositivity with tick control measures suggests that wildlife and arthropod vectors may play roles in the epidemiology of brucellosis on these farms.

Results from this study have demonstrated the widespread presence of *B. abortus* DNA in samples from humans, cattle, milk and goats, indicating that cattle are the primary reservoir of brucellosis on these farms. Reports from the literature suggest that goats are spillover hosts of *B. abortus,* but in instances where brucellosis control measures are only applied to cattle, goats may serve as a reservoir of *B. abortus* for cattle. The highest correlations in prevalence between species were seen for human seroprevalence and goat seroprevalence, and results of multivariable analyses found were clear associations between household prevalence of brucellosis in goats with human seropositivity, and a weak association between brucellosis in goats with positive milk tests in cattle. The presence of *B. melitensis* in cattle samples, and associations between the seroprevalence of brucellosis in goats with human brucellosis in this study, indicate that goats also contribute to brucellosis in dairy farms in rural Uganda, and may pose more of a public health threat that previously realized.

The effectiveness of brucellosis control programs through a 'natural experiment': the seroprevalence of brucellosis in all three hosts were significantly lower in the QENP study area, where extensive vaccination campaigns had been conducted in the recent past. This contributes evidence to support the viewpoint that livestock vaccination can be successfully used to control zoonotic disease in humans, and provides additional impetus for expanding brucellosis vaccination in other regions with high prevalence in livestock.

This study was able to demonstrate that the LFA was a useful and practical tool for potential use for brucellosis surveillance in resource-limited settings, with test results were supported by risk factor data and results from PCR. The utility of using a laboratory grade filter paper for storage of blood and milk samples for DNA extraction and detection of *Brucella* after four years of storage was also demonstrated. Species-specific DNA targets were successfully detected in DBS and DMS from cattle and goats, which can provide valuable information in describing the transmission of brucellosis within farms.

Conclusions

Brucellosis is present in cattle and goats on farms in southwestern Uganda. Although cattle are the focus of brucellosis control in Uganda and much of sub-Saharan Africa, the significant associations between seropositivity in humans and seropositivity in goats and the presence of *B. melitensis* in humans, cattle serum and cattle milk suggest that brucellosis in goats may be an important contributor to the epidemiology of the disease in the farm, and goats may be an important reservoir of *Brucella.*

In addition to the better-known routes of infection for livestock and humans, evidence suggests that there are other sources of *Brucella* that warrant additional focus. The role of wildlife reservoirs has been well established in the United States, and research supporting the role of wildlife as reservoirs for traditional livestock diseases is emerging. There is evidence for the possibility of exposure to *Brucella* through arthropod vectors in the field. Finally, the

association between positive screening test results and water sources has been reported in other zoonotic diseases, but has not been fully explored for brucellosis.

When considering disease surveillance and control programs in developing countries, this study was able to demonstrate that the LFA for human brucellosis, and dried sample storage on filter paper, met many of the WHO criteria for use in resource-limited settings, and have potential for use in brucellosis surveillance and research. The development of an effective point-of-care test for brucellosis would provide public health workers with a valuable tool in disease control efforts in developing countries, where patient contact is often difficult, and rapid diagnoses is necessary to ensure that patients receive treatment appropriate to the pathogen creating illness. Such a point-of-care diagnostic test could reduce the costs of brucellosis control programs in regions where mortality, morbidity, and socio-economic losses due to disease have a significant but under-appreciated impact on human health and well-being.

Recommendations

- Awareness of goats as a reservoir for brucellosis needs to be increased in all stakeholder groups, from farm residents and workers to veterinary medical professionals and health care workers.
- Disease control efforts should be modified to include control of brucellosis in goats, and vaccination of cattle with vaccines effective against both *B. abortus* and *B. melitensis.*

- Additional work on the molecular epidemiology of brucellosis should be conducted in this region, to make important contributions to our understanding of the zoonotic impact of brucellosis in rural farming communities in East Africa.
- Targeted surveillance of wildlife in regions were brucellosis is endemic in livestock should be conducted to minimize impacts on wildlife, and allow control of the disease in livestock.
- The role of ticks and other blood-feeding parasites should be explored, particularly to determine if *Brucella* can be vector-borne, and if this constitutes a route of transmission in nature.
- Further investigation is needed to describe the underlying causes of the association between positive brucellosis screening test results and water sources.
- Additional research in the use of LFA, alone or combined with other simple screening tests (e.g., RBT), as a single point-of-contact test for identification of potential cases of brucellosis should be pursued.
- Studies comparing the performance of dried filter paper samples to other standard storage methodologies, including FTA™ cards, should be conducted to provide investigators with the information needed to select sample storage appropriate to their needs and means.
- Experimental studies should be conducted to explicitly determine the storage conditions necessary to maintain dried filter paper samples for use in research, and the time, humidity, and temperature limits of dried filter papers.

- Future research on the ability to recover antibodies or other disease biomarkers from filter paper DBS and DMS should be pursued as a method for reducing or replacing the storage of fluid samples that requires access to freezer storage.

APPENDICES

APPENDIX A

Livestock sample data collection form

Appendix 1 contains the form used to document animal signalment data during sample collection, and was used to record results of screening test results in the regional veterinary laboratories.

Goat data was collected on the same form, with the substitution of the word “Goat” for “Cattle”, and “Birthing data” for “Calving date”.

The Prevalence of Brucellosis in Cattle, Goats and Humans in Southwestern Uganda

CATTLE SAMPLE from FARM NUMBER: _____

Sub-County: _____ Date: _____

ID Number	Sex	Breed (circle one)	Age	Last Calving date	History of abortion?	Samples
1		Local Mixed Exotic			Yes No	Blood
						Milk
						Swab
2		Local Mixed Exotic			Yes No	Blood
						Milk
						Swab
3		Local Mixed Exotic			Yes No	Blood
						Milk
						Swab
4		Local Mixed Exotic			Yes No	Blood
						Milk
						Swab
5		Local Mixed Exotic			Yes No	Blood
						Milk
						Swab

Test Results		
ID	**Sample**	**Results**
1	Blood	
	Milk	
	Swab	
2	Blood	
	Milk	
	Swab	
3	Blood	
	Milk	
	Swab	
4	Blood	
	Milk	
	Swab	
5	Blood	
	Milk	
	Swab	

APPENDIX B

Human sample data collection form

Appendix 2 contains the form used to document signalment data during sample collection, and was used to record results of screening test results in the regional veterinary laboratories.

Prevalence of Brucellosis in Cattle, Goats and Humans in Southwestern Uganda

FARM NUMBER: _____ **Date: _____**

HUMAN SAMPLES

					Direct contact		At Animal Birth		At Slaughter			Test results	
ID	**Age**	**Sex**	**History of Fever?**	**Drink raw milk?**	**Cattle**	**Goats**	**Cattle**	**Goats**	**Cattle**	**Goat**	**Wildlife**	**IgG**	**IgM**
H1													
H2													
H3													
H4													
H5													

APPENDIX C

Livestock Management Questionnaire

Appendix 3 contains the questionnaire documenting livestock management practices and inventories for each household. This questionnaire was completed through in-person interviews of the head of household or the farm livestock manager, and was conducted by study researchers at the time of sample collection.

Since the original questionnaire was printed on A4 paper (8.27” x 11.69”), the line spacing of this version of the questionnaire has been adjusted for the current (8.5” x 11”) page size.

The Prevalence of Brucellosis in Cattle, Goats and Humans in Southwestern Uganda

FARM NUMBER: _____ Sub-County Number: _____ Date: _____

1. Information about the Cattle Herd

a. How many cattle do you currently own?

Breed	Suckling Calves	Weaned calves	Young Stock	Cows	Bulls	Castrated Males
Native						
Exotic						
Mixed Native and Exotic						

b. What are their uses? (check all that apply, and circle the most important answer)
□ Milk only □ Meat only □ Mixed Use

c. Where do you get your cattle? (check all that apply, and circle the most important answer)
□ Raise your own □ From neighbors □ From other family members
□ From other sources, please describe: _____

d. Do you vaccinate your cattle? □ Yes □ No
If so, what was the date of last vaccination? _____

e. Do you use a dip for your cattle? □ Yes □ No
If so, what was the date of last dip? _____

f. Do you use any veterinary services for your cattle? □ Yes □ No
If so, describe the type of service and date of last service:
_____

g. Have there been any abortions in your herd recently? □ Yes □ No
If so, please describe when and how many cows were affected:
_____

h. Where do your cattle graze? (check all that apply, and circle the most important answer)
□ Private grassland □ Shared grassland □ Not grazed on pasture
□ Other sources, please describe: _____

i. Are wildlife present in areas where your cattle graze? □ Yes □ No

The Prevalence of Brucellosis in Cattle, Goats and Humans in Southwestern Uganda

FARM NUMBER: _____ Sub-County Number: _____ Date: _____

j. Where do you house your cattle? (check all that apply, and circle the most important answer)
□ In barn or building □ Fenced enclosure □ Not confined (kept on pasture)
□ Other, please describe: _____

k. Are wildlife present in areas where your cattle are housed? □ Yes □ No
l. Are other herds present in areas where your cattle are housed? □ Yes □ No

m. Where do cattle get drinking water? (check all that apply, and circle the most important answer)
□ Open well □ Borehole □ Public tap
□ Rain water □ Surface water (spring, pond, river, etc.)

n. Do wildlife share these water sources with your cattle? □ Yes □ No
o. Do other herds share these water sources with your cattle? □ Yes □ No

2. Information about the Goat Herd

a. How many goats do you currently own?

Breed	Suckling kids	Weaned kids	Does (she-goats)	Bucks (male goats)
Native				
Exotic				
Mixed Native and Exotic				

b. What are their uses? (check all that apply, and circle the most important answer)
□ Milk only □ Meat only □ Mixed Use

c. Where do you get your goats? (check all that apply, and circle the most important answer)
□ Raise your own □ From neighbors □ From other family members
□ From other sources, please describe: _____

d. Do you vaccinate your goats? □ Yes □ No
If so, what was the date of last vaccination? _____

The Prevalence of Brucellosis in Cattle, Goats and Humans in Southwestern Uganda

FARM NUMBER: _____ Sub-County Number: _____ Date: _____

e. Do you use a dip for your goats? □ Yes □ No
If so, what was the date of last dip? _____

f. Do you use any veterinary services for your goats? □ Yes □ No
If so, describe the type of service and date of last service:
_____

g. Have there been any abortions in your goats recently? □ Yes □ No
If so, please describe when and how many goats were affected:

_____

h. Where do your goats graze? (check all that apply, and circle the most important answer)
□ Private grassland □ Shared grassland □ Not grazed on pasture
□ Other sources, please describe: _____

i. Are wildlife present in areas where your goats graze? □ Yes □ No

j. Where do you house your goats? (check all that apply, and circle the most important answer)
□ In barn or building □ Fenced enclosure □ Not confined
□ Other, please describe: _____

k. Are wildlife present in areas where your goats are housed? □ Yes □ No
l. Are other goats herds present in areas where your goats are housed? □ Yes □ No
m. Do your cattle and goats share housing? □ Yes □ No

n. Where do goats get drinking water? (check all that apply, and circle the most important answer)
□ Open well □ Borehole □ Public tap
□ Rain water □ Surface water (spring, pond, river, etc.)

o. Do wildlife share these water sources with your goats? □ Yes □ No
p. Do other goat herds share these water sources with your goats? □ Yes □ No
q. Do your cattle and goats share these water sources? □ Yes □ No
r. Do you own any other animals? □ Yes □ No
If so, please list them and describe how many of each you own:

_____

_____

Appendix D

Household Questionnaire

Appendix 4 contains the questionnaire documenting household characteristics, behaviors associated with increasing brucellosis risk, and brief health history of household members. This questionnaire was completed through in-person interviews of the head of household or the farm livestock manager, and was conducted by study researchers at the time of sample collection.

Since the original questionnaire was printed on A4 paper (8.27” x 11.69”), the line spacing of this version of the questionnaire has been adjusted for the current (8.5” x 11”) page size.

Information about Household Members

a. How many people live in your household? _____

b. How many of these people have direct contact with livestock? _____

i. Do household members tend to any of these animals? Tending is caring for, feeding or removing waste (check all that apply)
□ Cattle □ Sheep □ Goats □ Pigs
□ Other animals, please describe: _____

ii. Has any household member assisted with the birthing of any animals in the past month?
□ Yes □ No

iii. Has any household member slaughtered or butchered (or assisted in slaughtering or butchering) any livestock or domesticated animals in the past month? (check all that apply)
□ Cattle □ Sheep □ Goats □ Pigs
□ Other animals, please describe: _____

iv. Has any household member hunted or butchered (or assisted in hunting or butchering of) any wild animals in the past month? (check all that apply)
□ Feral swine □ Waterbuck □ Buffalo
□ Other, please describe: _____

c. Has anyone been ill with any of the following symptoms within the last 6 months? Check all that apply:

□ Fever lasting more than 2 days with no identified cause (e.g., infection, pneumonia)
□ Fever over 38.5°C □ Night sweats
□ Fatigue □ Anorexia
□ Weight loss □ Headache
□ Arthralgia □ Generalized aching

i. On what date did symptoms first occur? _____
ii. Was the patient diagnosed with a specific condition? □ Yes □ No

If so, what diagnostic tests were used to identify the cause of disease?

_____

iii. Has the patient received any treatment? □ Yes □ No
iv. Has the patient recovered? □ Yes □ No
If so, on what date was the patient considered to be recovered? _____

v. Does the patient have any other illnesses? Check all that apply:
□ Malaria □ Tuberculosis □ HIV/AIDS
□ Other health problems: _____

vi. Have any other household members had similar symptoms? □ Yes □ No
   1. If so, on what date did their symptoms begin? _____

   2. If so, on what date did the household member recover? _____

d. Have you eaten any of the following foods in the last 6 months? (check all that apply)
   □ Raw milk □ Sour milk □ Undercooked meat
   □ Yoghurt, butter, or other products made from raw milk

e. Where did you get your drinking water in the last month? (check all that apply, and circle the most important answer)
   □ Piped water □ Open well □ Covered well or borehole
   □ Public tap □ Rain water □ Bottled water
   □ Surface water (spring, pond, river, etc.)

   i. Do you treat your drinking water by any of these methods? (check all that apply)
   □ Boiling □ Filtering □ Chlorinating □ Other methods

   ii. Does livestock or wildlife have access to any of these water sources? □ Yes □ No

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