Seroepidemiology, Molecular Detection and Risk Factors Associated with Brucellosis in Goats in Morogoro, Tanzania

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SUMMARY

Brucellosis is wide spread and an important re-emerging zoonosis causing a great socio-economic and public health concern especially in low income countries. The present study was performed to determine the prevalence and risk factors associated with brucellosis transmission in goats kept in three districts of Morogoro, Tanzania. A total of 478 goats sera were collected using multistage cluster random sampling from different herds. Two hundred and forty nine samples were collected from three wards of agro-pastoral farming system of Mvomero, 178 samples from three wards of Morogoro rural and 51 samples from Morogoro urban districts. Rose Bengal plate agglutination test (RBPT), indirect Enzyme Linked Immunosorbent Assay (iELISA) and real-time Polymerase Chain Reaction (RT-PCR) were applied for disease diagnosis. Structured questionnaire was used to assess for potential risk factors of the disease in the communities. Out of 478 samples, one serum (0.2%) tested positive to both RBPT and iELISA. *Brucella abortus* DNA was detected in 6.18% (n=11) and 2.81% (n=7) of investigated goats from Morogoro rural and Mvomero districts, respectively. Consumption of raw milk and/or blood, history of abortion and retained placenta, poor knowledge on brucellosis, and improper handling and disposal of fetal membranes and aborted foetuses were found to be statistically significant ($P<0.05$) disease determinant. This study has indicated the presence of *B. abortus* in goats reared in Morogoro region and risk factors perpetuating its spread. Therefore, there is a need for implementing control measures and raising public awareness to facilitate effective intervention of brucellosis.

Keywords: *Goats, Brucellosis, Seroprevalence, molecular diagnosis, Risk factors, Morogoro*

INTRODUCTION

Brucellosis is a contagious bacterial zoonotic disease that has been ranked second in importance by World health organization (WHO), the World animal Health organization (OIE) and the Food and Agriculture organization (FAO) causing considerable affects to both public health and animal production (WHO, 2006; Yilma *et al*., 2016).

Brucellosis has a worldwide distribution and affects economically important domestic livestock as well as a wide range of wild mammals (Zheludkov and Tsirelson, 2010; Godfroid *et al*., 2011; McDermott *et al*., 2013; Ducrotoy *et al*., 2017).

Serological studies done in different parts of Tanzania indicate that the infection is widely spread in domestic animals, wildlife and human beings (Karimuribo *et al*., 2007; Mellau *et al*., 2009; John *et al*., 2010; Swai and Schoonman, 2010; Lyimo, 2013; Assenga *et al*., 2015; Chatupila *et al*., 2015; Mathew *et al*., 2015; Shirima and Kunda, 2016; Sagamiko *et al*., 2018; Shirima *et al*., 2018).

The genus *Brucella* has 12 species of which the main pathogenic species worldwide are *B. abortus*, responsible for bovine brucellosis and small ruminants; *B. melitensis*, the main etiologic agent of ovine and caprine brucellosis; and *B. suis*, responsible for swine brucellosis (Samaha *et al.*, 2008; Godfroid *et al*., 2011; Zheludkov and Tsirelson, 2010). In domestic and wild animals, brucellosis causes abortion and infertility while in human causes a debilitating disease with unspecific symptoms comparable to other febrile conditions such as malaria (Akhvlediani et al., 2010; Muendo *et al*., 2012; Aparicio, 2013).

Although domestic animals are valuable assets to most people in rural areas, they pose more brucellosis risk to farm workers, veterinarians, ranchers, and meat-packing employees (Godfroid *et al*., 2013). Human acquire *Brucella* infection via direct contact with infected animals or consumption of their products, mostly milk and milk products especially cheese made from unpasteurized milk of sheep and goats and rennet from infected lambs and kids (Seleem *et al*., 2010).

However, the prevalence of brucellosis in humans depends upon several factors such as dietary habits, methods of processing milk and milk products, husbandry practices, and environmental hygiene.

The presumptive diagnosis of *Brucella* spp. infection in small ruminants mainly depends on Rose Bengal test (RBT), complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA) serological tests (Nielsen *et al.*, 2004; Nielsen *et al.*, 2005; Minas *et al*., 2008). However, the lack of diagnostic sensitivity of both the RBT and CFT makes implementation of a test and slaughter policy for brucellosis eradication in small ruminants less effective than in cattle (Ducrotoy *et al*., 2014). Furthermore, when the RBT and CFT are used singly or in combination (serially or in parallel), they are effective as flock screening tests, but they cannot detect all infected animals in a flock when used for individual testing (Minas *et al*., 2008). ELISA is regarded as more sensitive, specific and capable of differentiating acute from chronic infections (Biancifiori *et al*., 2000; Nielsen *et al*., 2005; Minas *et al*., 2008). However, ELISA cannot completely eliminate cross reactions from other bacteria like *Y. enterocolitica* O: 9 (OIE, 2004).

Molecular methods such as Polymerase chain reaction (PCR)-based techniques (conventional PCR, multiplex PCR and real-time PCR) are currently used for the diagnosis of *Brucella* pathogens and diagnosis of brucellosis because these techniques are rapid, sensitive and specific (Bounaadja *et al*., 2009; Kaden *et al*., 2017). PCR assays allow typing of *Brucella* DNA from clinical specimens like serum, whole blood and urine samples without to handle living *Brucella* organisms (Debeaumont *et al*., 2005; Queipo-Ortuño *et al*., 2008).

The quantitative or real-time polymerase chain reaction (qPCR) assay is specific, highly sensitive than the serological tests and is an appropriate method for the rapid, safe detection and further classification of *Brucella* at the species level (Marianelli *et al*., 2006; Al Dahouk *et al*., 2013; Mathew *et al*., 2015). However, a conclusive diagnosis requires isolation and identification of the organisms from various tissues (WHO, 2006; Wareth *et al*., 2015).

Despite the growing recognition of the importance of brucellosis, the disease is poorly understood and controlled in Tanzania. Successful control of brucellosis requires knowledge of its epidemiology in different animal species and the risk factors for its transmission. Limited information is available on the prevalence of brucellosis in small ruminants. Similarly assessment of risk factors influencing *Brucella* spp. transmission, livestock keeper's awareness, knowledge, attitude and practices regarding brucellosis in agro-pastoral areas has also not fully explored. This aim of this study was therefore to determine the seroprevalence of brucellosis in goats, to detect *Brucella* DNA at the species level using real-time PCR, and

identify risk factors influencing *Brucella* transmission in agro-pastoral communities.

METHODOLOGY

Study Area and study animals

The protocol for field studies and collection of animal materials was approved by Morogoro municipal, Mvomero and Morogoro rural Districts Veterinary Officers and Sokoine University of Agriculture. The study was carried out in three administrative districts in Morogoro region, Tanzania; namely Morogoro rural, Mvomero and Morogoro urban from November 2016 to July 2017. A total of 9 wards were selected, three from each district and three villages per ward a total of 27 villages) were chosen for sampling.

Geographically, the study areas cover latitude and longitude ranges of 5° 58" to 10° 0" South and 35° 25" to 35° 30" East. Livestock production system in the study area was generally predominated by extensive pastoral or agro-pastoral system, in which animals (cattle, goats and sheep) are allowed to forage freely in communal grazing land during daytime and kept in open enclosure 'Boma' during the night. Most of herds had a dynamic nature of keeping animals characterized by keeping diverse species of livestock with seasonal herd mobility. There was no history of vaccination against brucellosis in the study areas.

Study design and sample size determination

A cross-sectional multi-stage sampling was carried out. Selection of the study unit (district, ward and village) at each stage was based on a mixed design of convenience and random samplings. Districts were conveniently selected based on geographic localities / accessibility (Morogoro urban) and high number of goat population (Mvomero and Morogoro rural). Wards with large numbers of goats were randomly selected for sampling. An attempt was made to include at least three villages per ward whereas the selection of herds was based on the accessibility by vehicle or the proximity to roads and willingness of herd owners to cooperate. The number of goats from each herd was determined according to sample-size formula for detecting presence of disease in a population as described by Cannon and Roe, (1982) and reproduced in Thrusfield (1995).

In sample-size calculations, both the margin of error and the expected prevalence of brucellosis in the herd were set at 5%. Study animals include adult animals aged one year and above in a selected herd. A herds with <50 goats on the day of visit, about 50% of all adult goats were sampled while in a large herd (>50 goats) about 20% of all adult goats were included in the study.

Sample collection

Blood samples were collected and handled according to OIE guidelines (OIE, 2009). Goats were manually restrained and approximately 5.0 mL of blood samples was collected from the jugular vein using plain vacutainer tubes. Collected samples were kept cool at 4^{o}C in an a leak proof container with ice packs and transported to the laboratory at College of Veterinary Medicine and Biomedical Sciences, SUA for analysis. In the laboratory, samples were kept upright at 4^{o}C for a maximum of 24 hour. All tubes with blood were centrifuged at 5000 rpm for 5 min for serum separation. After centrifugation, the supernatants were collected in sterile Eppendorf tubes (1.5 mL) by pipettes and stored at –20^{o}C for further analysis.

Serological evaluation of serum samples

The Rose Bengal plate agglutination test was performed and interpreted according to the

guidelines of OIE (2012). Briefly, 25 µL of serum was mixed thoroughly with an equal volume of antigen preparation using clean applicator stick to produce a circular zone of approximately two centimeters in diameter. Any degree of agglutination was considered as positive reaction, whereas no reaction, i.e. failure to develop agglutination, was considered as negative and was an indication of the absence of anti-*Brucella* antibodies.

Indirect Enzyme-linked immunosorbent assay (iELISA)

Sera were tested with indirect ELISA (iELISA) (PrioCHECK® Brucella Ab 2.0) as described in the manufacturer's instructions. The 96-well polystyrene microtiter plates used in this study were pre-coated with purified extract of the *Brucella* spp. lipopolysaccharides (LPS) antigen. Briefly, 5 µL of test serum was dispensed in duplicate of antigen coated plate and quadruplicate for control sera. The plates were incubated for 1 hour at 37^{o}C, and the plates were washed 4 times with washing buffer.

The rabbit antihuman IgG HRP conjugate solution was then added to all wells and incubated for 1 hour at 37^{o}C. The plates were then washed (4 times) with washing buffer and treated with chromogen substrate solution for 10 minutes. Finally stopping solution was added per well and OD values were read at 450 nm using an ELISA Microtiter plate reader (Multiskan Go -Thermo Scientific).

Extraction of *Brucella* DNA

Genomic DNA was extracted and purified from sera using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines.

The genomic DNA was quantified spectrophotometrically using Nano-Drop (Nano-Drop Technologies, Wilmington, USA) and stored at – 20^{o}C until use.

Real-time PCR assay

Multiplex real-time PCR for simultaneous identification of *Brucella* genus (*bcsp31*), *B. abortus* (*alk*B), and *B. melitensis* (*BME*I1162) as previously described (Probert *et al*., 2004). The primers and probes were obtained from TIB Molbiol (Berlin, Germany) (Table 1). Briefly, each amplification reaction mixture was contained 0.75 µl of each primer (0.3 µM), 12.5 µl TaqMan™ Universal Master Mix (Applied Biosystems, USA), 0.25 µl probe (0.1 µM), 2 µl of DNA template (27.12 ng/µl) and was filled up to a total volumes of 25 µl with HPLC grade water. Positive controls that contained *Brucella* DNA and no template controls (NTC) that contained PCR-grade water instead of DNA were used in all assays. Real-time-PCR assays were performed with the following cycling conditions, decontamination at 50°C for 2 min, one cycle with initial denaturation at 95°C for 10 min, and 50 cycles with 95°C for 25 s and 57°C for 1 min.

All samples were tested in duplicates; a sample with a fluorescence signal 30 times greater than the mean standard deviation in all wells over cycles 2 through 10 was considered a positive result, whereas a sample yielding a fluorescence signal less than this threshold value was considered negative. Cycle threshold values below 40 cycles were interpreted as positive. The samples scored positive by the instrument were additionally confirmed by visual inspection of the graphical plots, which show cycle numbers against fluorescence values as previously described elsewhere (Fatima *et al*., 2016)

Questionnaire survey

The farmers were informed of the study and their verbal consent was sought prior to commencement of data collection. Semi structured questionnaire was designed and used to obtain information from the sampled respondents (animal owners or attendants. Relevant herd data was gathered to provide an insight on the exposure and risks factors. Information on goat management and husbandry practices, handling of aborted

materials, goat products consumption, public awareness, attitude and perception of brucellosis in the study area were collected.

Statistical analyses

Data from questionnaires and laboratory results were recorded in Microsoft Excel® 2007 and then exported to SPSS (statistical package for social scientists) Version 18 for statistical analysis. Descriptive statistics were computed for proportions of positive animals using RT-PCR results and their seroprevalence RBPT and iELISA recorded.

Table 1: Oligonucleotide primers and probes used in the multiplex real time PCR assay for the detection of *Brucella* spp., *B. abortus*, and *B. melitensis*

Target	Primer	Foward/Rev
Brucella spp.	5′GCTCGGTTGCCAATATCAATGC 3′	Forward
	5′GGGTAAAGCGTCGCCAGAAG 3′	Reverse
	FAM-AAATCTTCCACCTTGCCCTTGCCATCA-BHQ1	Probe
B. abortus	5′GCGGCTTTTCTATCACGGTATTC 3′	Forward
	5′CATGCGCTATGATCTGGTTACG 3′	Reverse
	HEX-CGCTCATGCTCGCCAGACTTCAATG-BHQ1	Probe
B. melitensis	5′AACAAGCGGCACCCCTAAAA 3′	Forward
	5′CATGCGCTATGATCTGGTTACG 3′	Reverse
	CY5-CAGGAGTGTTTCGGCTCAGAATAATCCACA-BHQ2	Probe

FAM carboxyfluorescein; HEX hexachlorofluorescein; BHQ1 Black Hole Quencher 1; BHQ2 Black Hole Quencher 2

RESULTS

Results from Serological and PCR tests

The prevalence of seropositivity to goat brucellosis in the different districts, wards and village is presented in Table 2. The results show that only one animal (0.21%) was positive for both RBPT and iELISA.

However, out of 478 samples tested, 18 (3.8 %) were positive for the qPCR of *Brucella* spp. Of these, 11 (6.2 %) were sera collected from Morogoro rural district ward while 7 (2.85 %) were from Mvomero district (Table 3). No significant differences (P> 0.05; 95% CI) were found between the two districts. Interestingly, all the 18 samples screened with PCR were detected to have both *Brucella* genus DNA (*bcsp*31) and specific *B. abortus* DNA (*alk*B).

Potential risk factors associated with Brucellosis transmission in the study area

Majority 96%) of correspondents uses communal grazing and share male animals for breeding. Seventy five percent admitted to have history of abortion and/or retained placenta in their herds, and a good number (60%) of them reported to either throwing away or feeding dogs the aborted fetuses and placenta. Seventy-four percent seek veterinary services when their animals are sick, a few (15%) were aware of brucellosis of which only 2% knew the transmission mechanism of the disease.

Sixty percent reported to consume undercooked meat (barbecued meat), raw milk and raw blood. The likely risk factors for disease transmission which was production systems and availability of veterinary services in villages and prevalence of Brucellosis was not significantly different between the three districts. Mixed keeping with other livestock, communal grazing, occurrence of abortion, handling of aborted fetuses and fetal membranes, consumption of raw milk and/or blood and people's awareness on Brucellosis were found to be statistically significant ($P<0.05$) disease prevalence determinant.

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Table 2 Distribution of seropositivity (RBPT and iELISA) to *Brucella* antigens in goats at different levels across the study areas

Study area		Village level		Herd level		Animal level	
District	Ward	No of villages	Prevalence; n (%)	n	Prevalence; n (%)	n	Prevalence; n (%)
Morogoro rural	Ngerengere	3	0 (0)	6	0 (0)	61	0 (0)
	Gwata	3	0 (0)	5	0 (0)	49	0 (0)
	Mikese	3	0 (0)	7	0 (0)	68	0 (0)
Mvomero	Mangae	3	0 (0)	8	0 (0)	100	0 (0)
	Dakawa	3	1 (3.7)	7	1 (2.2)	99	1 (0.21)
	Nyandira	3	0 (0)	5	0 (0)	50	0 (0)
Morogoro urban	Kihonda	3	0 (0)	1	0 (0)	5	0 (0)
	Misongeni	3	0 (0)	5	0 (0)	35	0 (0)
	Mkundi	3	0 (0)	2	0 (0)	11	0 (0)
Total		27	1 (3.7)	46	1 (2.2)	478	1 (0.21)

Table 3. Distribution of RT-PCR brucellosis positive samples from goats at different levels

Study area		Village level		Herd level		Animal level	
District	Ward	No of villages	Prevalence n (%)	n	Prevalence n(%)	n	Prevalence n (%)
Morogoro rural	Ngerengere	3	2(66.7)	6	2 (33.3)	61	5 (8.2)
	Gwata	3	1 (33.3)	5	2 (40.0)	49	5 (10.2)
	Mikese	3	1 (33.3)	7	1 (14.3)	68	1 (1.5)
Mvomero	Mangae	3	1 (33.3)	8	1 (12.5)	100	2 (2.0)
	Dakawa	3	1 (33.3)	7	2 (28.6)	99	5 (5.1)
	Nyandira	3	0 (0.0)	5	0 (0)	50	0 (0)
Morogoro urban	Kihonda	3	0 (0)	1	0 (0)	5	0 (0)
	Misongeni	3	0 (0)	5	0 (0)	35	0 (0)
	Mkundi	3	0 (0)	2	0 (0)	11	0 (0)
Total		27	6 (22.2)	46	1 (2.2)	478	18 (3.8)

DISCUSSION

Brucellosis is a zoonotic infectious bacterial disease, spreading worldwide and affecting animals or humans both in developed and developing countries. The disease is considered as one of the most important endemic zoonotic diseases in Tanzania because consumers and farmers are unaware of this disease, the possible risk, and appropriate countermeasures (Karimuribo *et al*., 2007; Mellau *et al*., 2009; John *et al*., 2010; Sawi and Schoonman, 2010; Lyimo, 2013; Assenga *et al*., 2015; Chatupila *et al*., 2015; Mathew *et al*., 2015; Shirima and Kunda, 2016; Sagamiko *et al*., 2018; Shirima *et al*., 2018).

Result from this work provides an epidemiological insight that could be utilized for enhanced management of such an important disease in animal production in the study area and similar settings in Tanzania.

Both RBT and iELISA are OIE prescribed screening tests for brucellosis (OIE, 2009). In the present study, low seroprevalence (0.21%) was detected by both RBP and iELISA. Low seroprevalence of brucellosis observed in this study is in line with those recorded by a study in Uganda (Nguna *et al*., 2019), Iran (Sharifi *et al*.; 2015), Spain (Reviriego *et al*., 2000) and Tanzania (Mathew *et al*., 2015).

In contrast, high seroprevalence of brucellosis in goats has been reported in Tanzania (Mellau *et al*., 2009; Assenga *et al*., 2015), Uganda (Kabagambe *et al*., 2001), Ethiopia (Bekele *et al*., 2011) and Egypt (Kaoud *et al*., 2010). Brucellosis seroprevalence was only detected in the pastoral communities in Mvomero and not in urban district of Morogoro. The absence of seropositive for *Brucella* in urban settings has been also reported in Uganda (Makita *et al*., 2011).

Low prevalence in urban and peri-urban areas is probably because animals in urban areas are more confined compared to pastoral and agro-pastoral areas. Moreover, the susceptibility of animals to brucellosis depends on many factors including grazing strategy (Muma *et al*., 2006), geographical variability (Stamiou *et al*., 2009), vaccination program (Mugabi, 2012) and population density (number of animals to land area) which is attributed to increased contact between susceptible and infected animals (Kaltungo *et al*., 2013). A perfect agreement between the two serological tests (RBPT and iELISA) reported in this study also observed elsewhere (Gwida *et al*., 2011). Although RBPT and ELISA are commonly used for diagnosis of Brucellosis, they have important limitation such as false positive and negative results, disconcordance between each other (Geresu and Kassa, 2016; Özdoğaç *et al*., 2018). Because of these limitations in serological tests, RT-PCR is an alternative to make a final decision (Surucuoglu *et al*., 2009).

Real-time PCR proved to be a valuable diagnostic tool when culture fails or serological results are inconclusive in human brucellosis (Probert *et al*., 2004).In this study, 18 out of 478 goat sera subjected to RT-PCR were positive to *Brucella* DNA. Real time PCR assay was able to detect *Brucella* DNA in some seronegative goats. Similar findings have been reported elsewhere in pigs (Hinić *et al*., 2009) and cattle (Lindahl-Rajala *et al.*, 2017).

The discrepancy between the serology and PCR results observed in the current study might indicate that the true number of *Brucella* infected animals within the study area could be underestimated by serology screening if used alone. However, PCR results may be an overestimation of the true positive goats as PCR detects presence of DNA not necessarily the infection.

Seronegative animals in the current study, which tested positive by RT-PCR, probably had been previously exposed to *Brucella* and turned seronegative after a certain time period (Godfroid *et al*., 2010). Alternatively, sampling might have done at an early stage of the infection, i.e. within the first 14 days; the

humoral immune response has not yet induced detectable levels of antibodies in the host (Gardner *et al*., 2000). Furthermore, individuals infected in utero or in the early post-natal period can become latently infected and hence never become seropositive (Corbel, 2006). The potentially significant number of serological false negative individuals observed in in this study highlights the importance of simultaneous use of both serology and molecular techniques to overcome limitations of misdiagnosis in *Brucella* surveillance, control, and eradication programs.

Although ruminants are susceptible to *B. abortus*, the infection in small ruminants is rare (Aparicio, 2013). In this study, *B. abortus* was the only *Brucella* spp found to infect the small ruminants. Similar findings have been reported in Tanzania (Mathew *et al*., 2015), Egypt (Wareth *et al*., 2015) and Mexico (Morales-Estrada *et al*., 2016). Infection of small ruminants with *B. abortus* can occur as result of natural exposure to infected materials from another species or indirectly through contact with soil contaminated with abortion secrets.

Brucellae can survive up to 15–25 days on a pasture depending on environmental conditions e.g. intensity of UV-light (Richomme *et al*., 2006). *Brucella* species are generally host-specific but cross-species infections commonly occur when different species of animals share the same stables, pasture, or facilities. Due to existence of mixed livestock farming and uncontrolled animal flock movements in in the study area, it is likely that the goats had contact with either the fetus or infective fluids from cattle abortion.

Improvement of knowledge, attitudes and practices among urban livestock farmers has a significant impact on the reduction of many zoonotic infections in urban farming. This study investigated the weak areas in knowledge, attitudes and practices with regards to goat brucellosis among agro-pastoral farmers in selected areas of Tanzania in order to generate information essential for control programmes and public health interventions. The majority of the respondents had never heard of the disease brucellosis. Similar results have been shown in a study from Kenya (Kang'ethe *et al*., 2007) and Tajikistan (Lindahl *et al*., 2015).

Fifty percent of correspondents admitted to consume undercooked meat, raw milk and raw blood on regular basis. Consumption of unpasteurized dairy products is known to be an important risk factor for human brucellosis (Sofian *et al*., 2008; Earhart *et al*., 2009). Sixty percent admitted to have improper handling and disposal of fetal membranes and aborted fetuses. One explanation for this could be poor knowledge of the risk with this practice, and this practice is a known risk factor for humans (Earhart *et al*., 2009).

The majority of correspondents used communal grazing and sharing of breeding male animals. The use of communal pastures allows frequent contact between animals, and provides increased opportunity for environmental exposure to infectious materials, for instance arising from parturition. Previous studies have reported that multiple livestock species herding especially keeping of small ruminants along with cattle has been reported as a risk factor for *Brucella* infection (Al-Majali *et al*., 2007; Megersa *et al*., 2012).

The study shows that *Brucella* infection is prevalent in goats, and different explanatory variables were found to be associated with prevalence. The presence of brucellosis in animals certainly poses a threat to the public health of pastoral communities. Hence, the need for investigating feasible control measures in animals and raising public awareness of prevention methods of human exposure to *Brucella* infection is becoming more evident.

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