microorganisms

MDPI

Article

Direct Detection of *Brucella* Species in Blood Clots from Livestock in Northern Tanzania

AbdulHamid S. Lukambagire[1, 2*], James M. Akoko[3, 4], Coletha Mathew[1], Rosamystica M. Sambu[5], Raphael R. Mwampashi[6], Richard Yapi[7, 8], Nelson B. Amani[9], Judith S. Njau[9], Bassirou Bonfoh[8], Gabriel M. Shirima[10], Blandina T. Mmbaga[2, 11, 12, 13], Christopher J. Kasanga[1], Robab Katani[14], Jo E.B. Halliday[15] and Rudovick R. Kazwala[1]

[1] College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, Morogoro, Tanzania; ASL - lukhamid@gmail.com; CM - colethamat@sua.ac.tz; CJK - chrisskasa@gmail.com; RRK - kazwala@gmail.com

[2] Kilimanjaro Clinical Research Institute, Moshi, Tanzania; ASL, BTM – b.mmbaga@kcri.ac.tz

[3] Department of Biomedical Sciences and Technology, Maseno University, Kisumu, Kenya; JMA - jamesakoko@yahoo.com

[4] International Livestock Research Institute, Nairobi, Kenya; JMA

[5] Ministry of Livestock and Fisheries, Zonal Veterinary Centre, Mwanza,Tanzania; RMS - mkula1969@yahoo.com

[6] Ministry of Livestock and Fisheries, Zonal Veterinary Centre, Arusha, Tanzania; RRM - rmwampashi@gmail.com

[7] Centre d'Entomologie Médicale et Vétérinaire Université Alassane Ouattara; Bouaké, Côte d'Ivoire; RY - ryrichardyx@gmail.com

[8] Centre Suisse de Recherches Scientifiques en Côte d'Ivoire, Abidjan, Côte d'Ivoire. RY; BB - bassirou.bonfoh@csrs.ci

[9] Kilimanjaro Clinical Research Institute - Biotechnology Laboratory, Moshi, Tanzania; NBA - nelisob3@yahoo.com; JSN – j.njau@kcri.ac.tz

[10] School of Life Sciences and Bioengineering, Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania; gabriel.shirima@nm-aist.ac.tz

[11] Kilimanjaro Christian Medical Centre, Moshi, Tanzania; BTM

[12] Duke Global Health Institute, Durham, NC, USA; BTM

[13] Kilimanjaro Christian Medical University College, Moshi, Tanzania; BTM

[14] Huck Institutes of Life Sciences, Pennsylvania State University, USA; RK – rxk104@psu.edu

[15] Institute of Biodiversity, Animal Health & Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK; JEBH - jo.halliday@glasgow.ac.uk

* Correspondence: A.S.L - lukhamid@gmail.com

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Microorganisms* **2022**, *10*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

Abstract: Brucellosis is an endemic zoonotic disease of public health priority in many sub-Saharan countries, where robust tools for detection of *Brucella* in animal populations are needed for surveillance. Blood collected from 501 animals at a ranch suspecting brucellosis was tested for *Brucella* infection using molecular and serological techniques. Information on animal species, sex and abortion history were recorded. Blood clot DNA extracts were tested using two *Brucella* spp. genus specific targets, IS*711* and *bcsp31*. Samples positive for both targets were subjected to a multiplex species-specific assay targeting *alkB* for *B. abortus* and *BMEI1162* for *B. melitensis*. All sera were tested using the Rose Bengal test. *Brucella* spp. DNA was detected by qPCR in a total of 58 (11.6%) of 501 blood samples. *B. abortus* was identified in 18 and *B. melitensis* in 22 of the 58 samples positive for *Brucella* spp. A total of 73 (14.6%) of 501 sera tested positive by RBT with poor agreement (kappa = 0.102) between the RBT and *Brucella* spp. qPCR assay results. *Brucella abortus* was found in cattle and goats, while *B. melitensis* was detected in cattle, sheep and goats. These findings support the use of molecular assays alongside serology in brucellosis surveillance programs.

Keywords: Brucellosis; Livestock; Blood clots; qPCR assays; Tanzania

1. Introduction

Brucellosis is an endemic, priority zoonosis of public health importance in many low- and middle-income countries (LMICs) [1–4]. The disease, caused by some species of the genus *Brucella*, affects a wide range of terrestrial and aquatic animals, with suspected evolutionary origins in wild animals [5]. The need for robust diagnostic tools and methods to support the routine surveillance of brucellosis in human and animal populations has been highlighted as a critical step in One Health control strategies in Tanzania [6]. Sero-prevalence studies conducted in Tanzanian livestock have revealed animal level prevalence of brucellosis varying from 0.9% [7] to 48% [8] of cattle in cross-sectional and targeted suspect farms respectively. Sero-prevalence studies in humans have reported higher prevalences among agro-pastoral (4.2%) and pastoral (58.1%) communities [9,10] compared to studies conducted in urban and peri-urban communities.

Twelve species of *Brucella*, with a range of strain and serotype variants, have been reported from different geographical areas [11,12]. The main species transmitted to humans and implicated in clinical illness include *B. melitensis*, *B. abortus*, and *B. suis*, with a number of studies showing host association of these species with small ruminants, cattle and pigs, respectively [8,13–15]. The small number of molecular epidemiology studies of brucellosis conducted in Tanzania have shown *B. abortus* and *B. melitensis* to be the main species affecting livestock and humans [8,14,16]. The identification of *Brucella* species is critical to the design of control strategies; the association of multiple hosts with different zoonotic *Brucella* species indicates the need for vaccination across livestock hosts [6]. The host-pathogen association can also provide information on what animal hosts are principally involved in the transmission of *Brucella* between animals and from animals to humans [17,18]. Breaking pathways of transmission of brucellosis from animals to humans can be achieved through careful handling of animal products, heat treatment of milk and use of protective equipment during assisted parturition [19]. Animal to animal transmission can be reduced by isolation and culling of infected animals [20]. However, other interventions including routine surveillance and vaccination are yet to be fully implemented in many resource-limited settings in the sub-Saharan Africa (sSA) [1–4]. Robust diagnostic tools that can rapidly detect and speciate *Brucella* are central to understanding the epidemiology of brucellosis, thus informing the design of brucellosis control and elimination strategies in resource-limited settings, where high burdens of brucellosis has been reported [1,2,4,21].

The Rose Bengal Test (RBT) is the World Organization for Animal Health (OIE) recommended test to screen for brucellosis in livestock [22].

The RBT is relatively cheap and easy to perform, ideal for testing large numbers of livestock, under field conditions. Serology-based tests offer an overall picture of the herd brucellosis status and can also be used to inform control measures [23,24]. However, no single serological test has perfect performance in all species and contexts and the OIE recommend that reactivity of samples testing positive in screening tests should be confirmed in many cases using an established confirmatory or complementary strategy [20]. In addition, most serological tests are limited in the inability to distinguish between vaccinated and naturally infected animals and serological tests cannot be used to identify the species of *Brucella* causing infection [23,24].

The World Health Organization and OIE recommend the speciation of infectious *Brucella* species by the PCR-based, molecular characterization from bacteria isolated on the culture media. PCR-based techniques can also be used for direct detection of *Brucella* spp. DNA in a range of diagnostic samples [17,19,20]. The performance of these approaches is not well characterized and sensitivity may be low due to the low concentration of bacteria in many samples and low sample volumes tested [20]. The performance of molecular diagnostic assays used may vary depending on the source sample, clinical condition and timing of sample collection, sample preparation as well as the choice of targets used [25,26]. Variations in the performance of molecular diagnostic assays may also be attributed to differences in reagent types, reaction platforms, and laboratory settings from those previously reported [22,27].

The insertion sequence IS*711* and the cell surface protein *bcsp31* gene targets are common to all species of *Brucella*, with IS*711* expressed in multiple copies (5 to 7) per genome, depending on the species in question [28]. Assays exploiting the detection of IS*711* and *bcsp31* have been widely used, and the combination of IS*711* and *bcsp31* targets has been successfully applied for genus level detection of *Brucella* spp. across a range of hosts [3,28–32]. For the purpose of species-level identification, an assay targeting *alkB* and *BMEI1162* sequences to distinguish *B. abortus* from *B. melitensis* has been applied [3,33]. Real-time PCR techniques can be used in surveillance programs for rapid pathogen detection of Brucella spp., although these are yet to be routinely applied in Tanzania and many similar LMICs [34,35].

A number of studies conducted in sub-Saharan Africa (sSA) have exploited molecular techniques for the detection of *Brucella* using whole blood, serum, amniotic fluid, and abortion materials [13,15,36,37]. However, the molecular detection of *Brucella* in DNA extracted directly from blood clots, a sample type readily accessible and routinely generated by any sero-surveillance work, has been minimally reported in the literature [38]. The goal of this study was to determine the prevalence and species of infecting *Brucella* in blood clot extracts from animals at a livestock ranch in northwestern Tanzania. The ranch population had a history of

abortions and reduced milk production and brucellosis was suspected in this population.

2. Materials and Methods

2.1. Study sample populations

A total of 501 blood samples were collected from cattle (n=451), goats (n=30), and sheep (n=20) from a ranch in Kagera region, situated in northern Tanzania. The ranch, which comprised 14 cattle herds, one goat and one sheep herd, reported a reduction in milk production and a history of abortions in its cattle herds, suspected due to brucellosis. Although the 16 herds were separated overnight, all were communally grazed. Assuming a 50% prevalence in the cattle population, a minimum sample size of 365 of the 6,400 cattle present on the ranch was considered sufficient to determine the prevalence of cattle brucellosis with a confidence level of 95% and precision of 0.05 [39,40]. To collect blood samples from the cattle population, animals in each of the 14 herds were guided through a crush and every fifth animal was sampled until a maximum of 35 animals (minimum of 20) were selected from each cattle herd. Sheep and goat herds were opportunistically sampled.

2.2. Sample collection

Field sample collection was conducted by ASL, JMA, CM, RMS and RRM. Blood samples were drawn from the jugular vein of animals into plain, plastic 10 ml Vacutainer tubes (BD Vacutainer, UK) and allowed to coagulate at room temperature before being stored on ice in a cool box for transportation to the testing site. Serum for testing with the RBT was decanted off into 1.5 ml screw cap micro-centrifuge tubes (Thomas Scientific, USA). Both the blood clots and serum were stored in liquid nitrogen for transportation to the testing laboratories.

2.3. DNA spiking experiments

Spiking experiments and clot manipulation were conducted at the at the Kilimanjaro Clinical Research Institute – Biotechnology Laboratory (KCRI-BL) by ASL, NBA and JSN. For the estimation of the yield of *Brucella* from blood clots, samples were experimentally spiked with ten-fold serial dilutions of 16M *B. melitensis* referene strain genomic DNA. Blood was collected from an adult femal goat on two occassions, two weeks apart. At the first instance blood was drawn from the jugularvein of the animal into four ml Vacutainer tubes; with sodium hepatin antiguagulant for culture and plain tubes for serum separation alongside vaginal swabs. The animal was screened for exposure to *Brucella* by the RBT, qPCR on serum and vaginal swab DNA extracts and also by culture of anticoagulated whole blood and vaginal swab smear. Two weeks later, 10ml of blood was drawn and proportioned as follows: four ml blood was

collected into a sodium heparin anticoagulated tube and six ml distributed into 12 plain Vacutainer tubes at 500 µl each. In each plain tube, the blood was mixed with two µl of *B. melitensis* genomic DNA serially diluted ten-fold down to $1*10^9$ dilution. One tube was not spiked as a negative control for the extraction experiments. All tubes were mixed thoroughly with a pipette and left to stand at room temperature for one hour. Any serum formed was decanted off and both serum and blood clot were stored at -80° C for 5 days before prepartion and extraction.

2.4. Clot preparation and DNA extraction

DNA was extracted from livestock blood clots using the Qiagen DNEasy extraction kit (Qiagen, Germany) as adapted from the manufacturer's instructions. The blood clots were disrupted as adapted from to achieve separation of remnant serum and white cell fractions from the packed red cells [41,42]. Briefly, 400 µl of sterile, distilled water was added to approximately 500 µl of frozen clot volume in the sample collection tube and mixed briefly on a shaker at 4° C for 20 seconds. An additional 200 µl of sterile phosphate-buffered saline (PBS) was then added to minimize haemolysis and the total volume stored upright overnight at 4° C . The next day, the liquid supernatant phase including the white cell layer visible in most samples was separated from the remnant solid clot and an additional 100 µl of PBS added to this liquid phase. The mixture was shaken for 20 seconds on ice then centrifuged at 14,000 g for 10 minutes and the supernatant separated off. This centrifugation step was repeated twice and the final liquid supernatant stored at -20 °C until extraction of DNA. For DNA extraction, the supernatant volume brought to room temperature and vortex mixed. A volume of 250 µL of the sample was digested with 25 µL proteinase K, mixed with lysis buffer, and incubated for 30 minutes at 56° C. After washing and separation steps in spin columns, two elution steps of 50 µL each were done. The resultant 100 µL eluate was then stored at -20° C until analysis. Negative controls were included in each extraction batch.

2.4. Brucella spp. detection assays

For *Brucella* spp. detection, the genus-specific assays targeting IS*711* and *bcsp31* were set up on a qPCR Rotor Gene 5-plex (Qiagen, Germany) platform as adapted from elsewhere [31,43]. Each sample was tested in parallel with primers for IS*711* and *bcsp31*. Details of the primer and probe sequences for the genus specific assays are summarised in the supplementary Table S1. All reaction mixtures (25 µl) were prepared by mixing 2.5 µl of DNA template with 0.25 µM of the fluorescent probe, 0.5 µM of each primer, 12.5 µl of the Quantinova *Taq* powermix (Qiagen, Germany), and 5 µl of nuclease-free water. All test runs were performed with the following conditions; pre-treatment at 50° C for 2 minutes, polymerase activation and DNA denaturation at 95° C for 10 minutes, fol-

lowed by 45 cycles at 95° C for 15 seconds of denaturation, then one minute of annealing at 57° C. A positive control (*B. melitensis* 16M) and no template control (nuclease-free water) were included in every run. All samples (including extraction controls) and controls were tested in duplicate with both IS*711* and *bcsp31* assays. A test run was classified as valid if amplification was observed for positive controls and no amplification was observed for the negative controls. All sample extracts that showed amplification and a cycle threshold (Ct) value below 40 in one or both duplicate wells for the IS*711* and *bcsp31* assays were considered assay positive. Samples positive in both the IS*711* and *bcsp31* assays were classified as *Brucella* spp positive.

2.5. Species-specific qPCR detection assays

Extracts from all samples classified as *Brucella* spp. positive were further tested using the speciation assay. The reaction mixture (25 μL) was run on the Rotor Gene 5-plex platform as adapted from Probert *et al*. [43]. Briefly, 2.5 μL of template DNA was mixed with primers for the *alkB* target for *Brucella abortus,* and *BMEI1162* target for *B. melitensis* under run conditions previously reported [43]. Details of the primer and probe sequences for the *Brucella* species-specific assays are given in the supplementary Table S1. All reaction mixtures (25 μl) were prepared by mixing 2.5 μl of DNA template with 0.25 μM of the fluorescent probe, 0.5 μM of each primer, 12.5 μl of the Quantinova *Taq* powermix (Qiagen, Germany), and 7.5 μl of nuclease-free water. Positive controls (*B. melitensis* 16M and *B. abortus* 544 strains) and negative template controls were included in each run. All sample extracts were tested in duplicate and samples that amplified at a cycle threshold (Ct) value below 40 in one or both duplicate wells for a given target were considered positive for *B. melitensis (BMEI1162) or B. abortus (alkB).*

2.6. Serology testing with the Rose Bengal Test

Serology testing of livestock samples was done by ASL, JMA, CM and RRM. Serum from all 501 animals was tested with the RBT on site at the time of sampling. The test for cattle sera was run as follows: RBT antigen and serum samples were brought to room temperature and 30 μl serum was mixed with an equal volume of antigen (Rose Bengal antigen, RA-0060, Animal and Plant Health Agency (APHA)-Scientific, Weybridge-UK) on a white tile using a sterile, wooden splint. For sheep and goat sera, an antigen to serum ratio of 1:3 (i.e., 25 μl of RBT antigen to 75 μl of serum) was used [22]. *Brucella abortus* positive control serum (APHA, RAB1003) and *Brucella abortus* negative control serum (APHA, RAB0701) were included on each plate, as controls. In all cases, the tile was gently rocked at room temperature for four minutes before observing agglutination under natural light. Any sample with visible agglutination was considered RBT positive [44].

2.7. Data analysis

The assay run data and corresponding metadata on the samples tested were analyzed using the R software [45]. The standard curve for Log concentration against cycle threshold of the spiked clot experiments was plotted using Microsoft Excel. Descriptive statistics were estimated for proportions of animals positive for *Brucella* spp. and metadata variables. Logistic regression models were run to estimate associations between three qPCR defined outcome variables (*Brucella* spp. positivity of individual animals, *Brucella abortus* positivity and *Brucella melitensis* positivity) and a set of potential explanatory variables; animal species, sex, RBT result, abortion history and history of retention of placenta. Significant associations were defined by a p-value $\leqslant 0.05$. For each model, maximal models were constructed and then simplified using likelihood ratio tests. The performance and level of agreement (Kappa score) of the *Brucella* spp. assay was evaluated by cross-tabulation with the RBT results. Data analysis and interpretation were done by ASL, CM, RY, G.M.S, CJK, JEBH and RRK.

2.8. Ethical consideration

This research was approved by Kilimanjaro Christian Medical University College (KCMUCo) Ethics Committee (698), the National Institute of Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/3102). All study protocols were carried out in accordance with institutional guidelines and regulations as stipulated in the study approvals. Questionnaire administration and animal sampling was done after obtaining written, informed consent from ranch owners. Test results were fed back to the ranch management and advice on transmission control was provided to the veterinarians and managers of the ranch.

3. Results

3.1. Population structure of livestock samples tested

The majority of samples, 90.0% (451/501), were collected from cattle, while 6.0% (30/501) were from goats and 4.0% (20/501) were from sheep. Of the samples tested, 96.2% (482/501) came from female animals. Three (0.6%) of the 451 cattle had a reported abortion history, and one (0.2%) had a retained placenta (Table 1).

Table 1. Summary of livestock population characteristics and *Brucella* spp. qPCR results. 95% CI (95% confidence interval) (N = 501).

Variable	Level	Number tested	*Brucella* spp. qPCR positive (%)	95% CI
Animal Species	Cattle	451	53 (11.8)	8.9 - 15.1
	Goats	30	4 (13.3)	3.8- 30.7
	Sheep	20	1 (5.0)	0.1 – 24.9
Sex	Female	482	58 (12.0)	9.3 – 15.3
	Male	19	0 (0.0)	0.0 – 17.6
Abortion	No	479	0 (0.0)	0.0 – 7.7
	Yes	3	3 (100)	29.2 – 100.0
	NA (males)	19	NA	NA
Retained Placenta	No	481	0 (0.0)	0.0 – 0.7
	Yes	1	1 (100)	25.0 – 100.0
	NA (males)	19	NA	NA

3.2. Extraction and spiking experiments on blood clots

Brucella DNA was detected by both *Brucella* Spp. qPCR assays in the serially spiked clots down to the $1*10^9$ dilution aproximately, 6*10e2 fg/ µl (Figure 2, Supplementary file). All sera separated from the spiked blood clots also tested positive on both *Brucella* Spp. assays, while the RBT screening done on all the spiked clot serum separated was negative. All tests done to screen the goat for exposure to *Brucella* were negative, including the culture on anticoagulated whole blood and vaginal swab smear.

3.3. Brucella spp. detection by qPCR in livestock blood clot extracts

The overall proportion of animals that tested positive for *Brucella* spp. by qPCR detection (*IS711* and *bcsp31* target positive) in this livestock population was 11.6% (CI, 8.9 - 14.7). Of the 501 DNA extracts, 58 that amplified with both the IS*711* and *bcsp31* targets were classified as *Brucella* spp. positive. These samples had a mean cycle threshold (Ct) of 34.5 (range 26.3 – 39.9) with the *IS711* assay and 37.8 (range 31.9 – 39.9) with the *bcsp31* assay. Of the Brucella spp. qPCR positive samples, 91.4% (53/58) were from cattle, 6.9% (4/58) from goats and 1.7% (1/58) were from sheep. An additional 16 extracts amplified in the IS*711* assay alone, while 14 were amplified in the *bcsp31* assay alone. These samples were conservatively classified as negative for *Brucella* spp. in this analysis. The three samples (100%) from the animals with a reported history of abortion and the single animal with a retained placenta were *Brucella* spp. qPCR positive (Table 1). The logistic regression modeling showed no significant association between any of the three outcomes evaluated (*Brucella* spp. positivity, *B. abortus* positivity and *B. melitensis* positivity) and the independent variables evaluated in this population.

3.4. Speciation of Brucella in livestock blood clots

The 58 clot extracts classified as *Brucella* spp. positive were also tested using the species-specific assays with *B. abortus* and *B. melitensis* specific targets. Of the 58 *Brucella* spp. positive extracts, 31.0% (18/58) were classified as positive for the *B. abortus* target, 38.0% (22/58) were classified as positive for the *B. melitensis* target, and the remaining 31.0% (18/58) did not amplify with either species-specific target. *Brucella abortus* was detected in 16 (30.2%) of the cattle samples, and two (50.0%) of the goat samples that were positive for *Brucella* spp. *B. melitensis* was detected in 19 (35.8%) of the *Brucella* spp. positive cattle samples, two (50.0%) goat samples and one (100.0%) sheep sample. The 18 samples that did not amplify in either target for *B. abortus* or *B. melitensis* were from cattle blood clots. All samples (100.0%) from cattle that had abortion history and retained placenta were positive in the *B. abortus* qPCR assays (Table 2).

Table 2. Summary of the characteristics and speciation of *Brucella* spp. qPCR positive samples (N = 58).

Variable	Level	Number tested	Speciation assays (%)		
			abortus (alk B)	***melitensis (BMEI1162)***	**Undetermined**
Animal Species	Cattle	53	16 (30.2)	19 (35.8)	18 (34.0)
	Goats	4	2 (50.0)	2 (50.0)	-
	Sheep	1	0 (0.0)	1 (100)	-
Sex	Female	58	18 (31.0)	22 (37.9)	18 (31.0)
	Male	0	-	-	-
RBT	Neg	52	15 (28.8)	19 (36.5)	18 (34.6)
	Pos	6	3 (50.0)	3 (50.0)	0 (0.0)
Abortion	Neg	55	-	-	-
	Pos	3	3 (100)	-	-
Retained Placenta	Neg	57	-	-	-
	Pos	1	1 (100)	-	-

The mean Ct from the IS*711 Brucella* spp. detection assay and animal species of origin of the samples that were characterized by the species-specific assays are shown in Figure 1. The IS*711* assay Ct range of the *Brucella* spp. positive samples that remained undetermined in the speciation assay (26.3 – 39.5) spanned the Ct range of samples that were classified as *B. abortus* (30.1 – 37.4) or *B. melitensis* (26.5 – 39.9) positive across the different animal host species.

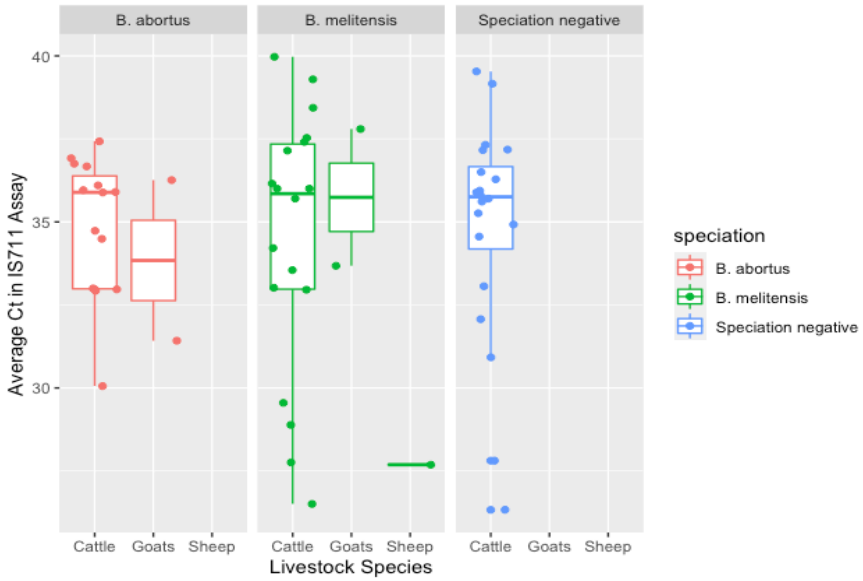

Figure 1. Box plot of animal species of origin, Ct of the IS*711* qPCR assay and result of the *Brucella* species-specific assays. Extracts that amplified in the *B. abortus* specific assay are shown in the left panel (red), *B. melitensis* specific assay in the middle panel (green), while samples that did not amplify for either of the species-specific targets (speciation negative) are shown in the right most panel (blue). Boxes show the median and interquartile range of the IS711 qPCR Ct values. Points show the observed average Ct values for tested samples.

3.5. RBT results and cross-tabulation with Brucella spp. qPCR detection assay

Of the 501 samples tested, 73 (14.6%) were positive by the RBT. This population of RBT positive animals comprised 95.9% (70/73) cattle, 2.7% (2/73) goats, and 1.4% (1/73) sheep. The proportion of RBT positive samples that were also qPCR positive was 8.2% (6/73). The characteristics of animals tested using the RBT are summarized in Table 3. Out of the 58 samples that were qPCR positive, six (10.3%) samples were also positive by the RBT. These six samples were further classified as three (50.0%) *B. abortus*, and the other three (50.0%), as *B. melitensis.* The 52 samples that were RBT negative but positive by qPCR were further classified using the species-specific assays as 15 (28.8%) *B. abortus* positive, 19 (36.5%) *B. melitensis* positive and 18 (34.6%) remained undetermined.

Table 3. Summary of livestock population characteristics and RBT test results. 95% CI (95% confidence interval) (N = 501).

Variable	Level	Number tested (%)	RBT positive (%)	95% CI
Animal Species	Cattle	451	70 (15.5)	12.3 – 19.2
	Goats	30	2 (6.7)	0.8 – 22.1
	Sheep	20	1 (5.0)	0.1 – 24.8
Sex	Female	482	71 (14.7)	11.7 – 18.2
	Male	19	2 (10.5)	1.3 – 33.1
Abortion	No	479	2 (0.4)	0.05 – 1.5
	Yes	3	1 (33.3)	0.8 – 90.6
	NA	19	-	-
Retained Placenta	No	481	1 (0.2)	0.0 – 1.2
	Yes	1	0 (0.0)	0.0 – 97.5
	NA	19	-	-

The overall level of agreement between the RBT and *Brucella* spp. qPCR for all sampled animals (n=501) was poor (kappa = 0.102). The details of the cross-tabulation between the RBT and *Brucella* spp. qPCR assays are summarized in Table 4.

Table 4. Cross-tabulation of the RBT against the *Brucella* spp. assay on livestock samples (N = 501).

RBT	*Brucella* spp. qPCR		
	Negative	Positive	Total (%)
Negative	376	52	428 (85.4)
Positive	67	6	73 (14.6)
Total	443 (88.4)	58 (11.6)	501 (100)
Kappa score		0.102	

4. Discussion

This study sought to determine the prevalence and species of infecting *Brucella* spp. using blood samples from livestock on a ranch situated in northern Tanzania. The overall proportion of animals positive by *Brucella* spp. qPCR was 11.6%. The proportion of *Brucella* spp. positive extracts in cattle was 11.8%, goats was 13.3% and sheep was 5.0%. Species-specific qPCR assays used to detect *B. abortus* and *B. melitensis* specific targets in genus positive extracts revealed *B. abortus* positive samples collected from cattle and goats while *B. melitensis* was detected in all three livestock species. The overall proportion of animals that were RBT positive in this population was 14.6% with 15.5% in cattle, 6.7% in goats, and 5.0% in sheep. There was poor agreement (kappa = 0.102) between the *Brucella* spp. infection status of animals as indicated by the qPCR assays on blood clot extracts and RBT on serum collected from the same animals.

This study exploited blood clots for DNA extraction and *Brucella* spp. detection. There have been very few reports of studies using this sample type for *Brucella* detection by molecular assays to date [38]. The direct ex-

traction of DNA from blood clots, as explored in this study, presents a means to exploit a commonly generated sample type that is often otherwise discarded. Livestock health surveillance programs routinely collect blood samples to determine exposure to or detection of multiple pathogens and could thus exploit the residual blood clots for *Brucella* testing as demonstrated in this study. Blood clots have been reported to give poor yield (low sensitivity) with bacterial isolation [46]. The reported low sensitivity *Brucella* detection from clots has been attributed to the typically short bacteremia caused by *Brucella* early in infection and at varying points during the course of untreated illness [46]. Future studies could focus on the quantification and optimization of different extraction techniques from blood clots, for the detection of *Brucella* DNA.

The findings of this study indicate higher proportions of blood clots testing positive by qPCR for *Brucella* spp. detection than might be expected from these previous studies. In this study, there were similar proportions of samples positive by the RBT (14.6%) and the *Brucella* spp. qPCR assays (11.6%). However, only six samples were positive in both assays. This observation of poor correspondence between these two diagnostic approaches has been reported in previous studies [34,47] and emphasizes the difference in information on infection status obtained by antibody detection with serological tests compared to DNA detection by molecular assays. In contrast to the findings of this study, a previous study in humans showed RBT positivity to be a statistically significant predictor of animal level positivity by the same *Brucella* spp. qPCR assays used in this study in mixed livestock and human ecosystems, although there were also discordant samples [15]. In combination, these findings suggest that serological and molecular assays for *Brucella* spp. detection, as used in this study, provide distinct and complementary information on individual exposure and infection status [48]. Serological status provides information on the past exposure of animals to *Brucella*, while the qPCR provides information on the presence and species classification of *Brucella* DNA in host tissues [49]. The interpretation of the results obtained from qPCR techniques directly applied to clinical and field samples to understand animal infection status remains unclear to date. DNA detection does not necessarily mean detection of viable *Brucella* in a sample matrix [50]. It is plausible that the level of agreement seen between the results of these different diagnostic approaches might vary based on the time course of infection in the sampled population. The population investigated for this study was selected based on the suspicion of brucellosis on the ranch. All samples that were collected from individual animals with a reported history of abortion and retained placenta tested positive by the *Brucella* spp. qPCR assays although not all were positive by the RBT (Table 1). Potentially, the animals testing qPCR positive and not also RBT positive were sampled early in the time course of infection, when the presence of *Brucella* DNA in the bloodstream is most likely and before the

development of antibodies detectable by RBT. Potentially, the animals positive by RBT and not also positive by qPCR may represent animals sampled later in the time course of infection, when the probability of detection of *Brucella* DNA in the bloodstream has declined but antibodies persist. One potential advantage of qPCR based assays for brucellosis case detection lies in their ability to detect DNA present in the bloodstream much earlier after infection than antibody development, making qPCR based diagnostics potentially more sensitive to detect early evidence of infection [49]. Although not exhaustively characterized, it is likely that antibodies against *Brucella* persist for long periods post-infection in livestock [51]. Antibody detection by serology and DNA detection by molecular diagnostic approaches may both give accurate indications of brucellosis exposure, but show poor agreement between the test results at animal level due to variation in the time course of infection within the sampled population and variable performance of these diagnostic approaches at different stages of infection. There have been very few studies to rigorously document the results of qPCR based *Brucella* spp. detection in different sample types over the course of infection in different species [48]. The performance and interpretation of qPCR assay results from different sample types when used alongside classical serology and bacteriological techniques still requires further investigation.

Seventy-three (14.6%) of the samples collected from the animals in this study were positive by the RBT screen test. This observed proportion is comparable to previously reported sero-prevalences obtained in Kagera region [14] and other parts of Tanzania using the RBT [8,15,52,53]. The *Brucella* spp. qPCR assay indicated *Brucella* detection in 11.6% of the sampled animals and species-specific classification of positive samples was achieved for 40 of these 58 samples (Table 2). Diagnostic assays that distinguish between infecting *Brucella* species can provide data that cannot be obtained using serological tests [27,54], but could be crucial in guiding decision-making processes for brucellosis control strategies [6,17,55]. The findings of this study therefore indicate the potential benefits of enriching serological surveillance data on brucellosis with the additional information that can be obtained using molecular diagnostics [48].

The *Brucella* species-specific assays utilized in this study detected *B. abortus* and *B. melitensis* [43] in samples positive by the *Brucella* spp. qPCR assays. Over 30% of the *Brucella* spp. positive samples were not positive in either of the species-specific assays. Previous studies from the region that have exploited the same assays observed similar patterns in terms of the proportion of samples that were species typed [15,36]. The inability to identify the *Brucella* species for all genus level positive samples could be due to the lower sensitivity of the IS*711*-based downstream targets for *B. abortus* and *B. melitensis* as compared to the genus level assay (with amplification of both *IS711* and *bcsp31* targets). In this study, the IS*711* assay Ct ranges for samples that were not classified by the species-specific as-

says overlapped the Ct range of samples that were classified as either *B. abort*us or *B. melitensis*. This could imply that in some cases at least the lack of amplification in either species-specific assay may not be due to the quantity or quality of DNA in the clot extracts. It is also plausible that other species of *Brucella* that would be detected in the *Brucella* spp. assay [8,43,56] but not the *B. abortus* and *B. melitensis* specific assays were present in this study population. This observation indicates the need for further investigation and genomic characterization of *Brucella* species and strains present in the region and assessment of the performance of diagnostic assays for these strains. In this study population, *Brucella abortus* was detected in cattle and goat samples while *B. melitensis* was detected in all three livestock species. Studies conducted previously in Tanzania and Kenya utilizing the same *Brucella* qPCR assays have also detected *B. abortus* and *B. melitensis* among different livestock hosts as well as wildlife species [13,15,36].

This study had a number of limitations. The low number of *Brucella* spp. positive samples limited the exploration of associations between qPCR outcomes and the livestock characteristics observed, including the animal host association with different species of *Brucella*. The *Brucella* speciation assay used in this study was limited to the classification of *B. abortus* and *B. melitensis* and could not detect any other *Brucella* species that may be present in this population. Only limited clinical data were available for the animal population investigated. Finally, the use of additional serological assays to compare with the RBT results and application of qPCR based approaches to other sample types to enable cross comparison of diagnostic findings at the animal level was beyond the scope of this study.

This study has demonstrated the use of qPCR assays to reveal *Brucella* spp. infection using blood clots collected from common livestock hosts of public health importance. Speciation of *Brucella* spp. positive samples detected *Brucella abortus* in cattle and goats while *B. melitensis* was detected in all three livestock species. There was poor agreement between RBT and *Brucella* spp. qPCR assay results. The findings from this study show potential for the use of blood clots in the detection and speciation of *Brucella* in livestock using molecular methods. The study findings also indicate the need for further evaluation of the performance of qPCR based and serological testing approaches when used in combination, with the potential to improve understanding of brucellosis epidemiology and thus improve brucellosis surveillance and control programs in much of sSA.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Summary of the primer and probe sequences for the *Brucella* spp. and species-specific qPCR targets.

Author Contributions: A.S.L., B.B., B.T.M, G.M.S, C.J.K., R.K., J.E.B.H, and R.R.K. - Conceptualization; A.S.L, B.B., B.T.M., J.E.B.H. and R.R.K. – Funding acquisition; A.S.L., J.M.A., S.R.M., C.M., Y.R., G.M.S., N.B.A, J.S.N. and J.E.B.H. – Methodology, resources and data curation; A.S.L, J.M.A,

R.M.S., R.R.M., C.M, N.B.A, J.S.N., Y.R., G.M.S., J.E.B.H. and R.R.K. - Investigations. A.S.L., C.J.K., R.K., J.E.B.H., and R.R.K. - Formal analysis. A.S.L., C.M., R.Y., G.M.S., C.J.K., J.E.B.H. and R.R.K. – writing, original draft preparation; ALL authors – writing, review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: A.S.L: A.J.M, C.M, S.R.M, R.R.M, Y.R, G.S.M, B.B, B.T.M and R.R.K are supported by the DELTAS Africa Initiative Afrique One-ASPIRE scholarship scheme (Afrique One-ASPIRE/DEL-15-008, http://afriqueone aspire.org). The study received additional laboratory capacity support provided by the University of Glasgow's GCRF Small Grants Fund, an initiative supported by an allocation of Global Challenges Research Fund from the Scottish Funding Council under agreement SFC/AN/12/2017.

Data Availability Statement: The supporting data for the analysis and deductions drawn in this paper will been made available through the Kilimanjaro Clinical Research Institute's Data Repository at: https://www.kcri/XXX/XXXX.

Acknowledgments: We would like to thank the staff and administration at the Kilimanjaro Clinical Research Institute-Biotechnology laboratory that facilitated and contributed to the development of the study assays over the years. The Kagera Sugar Limited Company and management of the Kitengule ranch in northern Tanzania, for facilitating the fieldwork that generated the samples used in this study.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

References:

1. Ducrotoy M, Bertu WJ, Matope G, Cadmus S, Conde-Álvarez R, Gusi AM, et al. Brucellosis in Sub-Saharan Africa: Current challenges for management, diagnosis and control. Acta Trop. 2017;165: 179–193. doi:10.1016/j.actatropica.2015.10.023
2. Rubach PM, Halliday JEB, Cleaveland S, Crump AJ. Brucellosis in low-income and middle-income countries. Curr Opin Infect Dis. 2014;26: 404–412. doi:10.1097/QCO.0b013e3283638104
3. Gwida M, Al-Dahouk S, Melzer F, Rösler U. Brucellosis - Regionally Emerging Zoonotic Disease? Croat Med J. 2010;51: 289–295. doi:10.3325/cmj.2010.51.289
4. Dean AS, Crump L, Greter H, Schelling E, Zinsstag J. Global Burden of Human Brucellosis: A Systematic Review of Disease Frequency. PLoS Negl Trop Dis. 2012;6. doi:10.1371/journal.pntd.0001865
5. Godfroid J. Brucellosis in wildlife. OIE Rev Sci Tech. 2002;21: 277–286. doi:10.20506/rst.21.2.1333
6. The United Republic of Tanzania (URT). National Strategy for Prevention and Control of Brucellosis in Humans and Animals 2018-2023. Prime Minist Off. 2018. Available: https://www.mifugouvuvi.go.tz/uploads/publications/sw1602245043-NATIONAL STRATEGY ON PREVENTION AND CONTROL OF BRUCELLOSIS IN HUMANS AND ANIMALS.pdf
7. Asakura S, Makingi G, Kazwala R, Makita K. Brucellosis Risk in Urban and Agro-pastoral Areas in Tanzania. Ecohealth. 2018;15: 41–51. doi:10.1007/s10393-017-1308-z
8. Mathew C, Stokstad M, Johansen TB, Klevar S, Mdegela RH, Mwamengele G, et al. First isolation, identification, phenotypic and genotypic characterization of Brucella abortus biovar 3 from dairy cattle in Tanzania. BMC Vet Res. 2015;11: 1–9. doi:10.1186/s12917-015-0476-8
9. Malamasha-Mtui N. Epidemiological study of brucellosis in humans and animals in Babati and Hanan'g districts of Tanzania. MVM dissertation Sokoine University of Agriculture (Tanzania). Sokoine University of Agriculture. 2001.
10. Mirambo MM, Mgode GF, Malima ZO, John M, Mngumi EB, Mhamphi GG, et al. Seroposotivity of Brucella spp. and Leptospira spp. antibodies among abattoir workers and meat vendors in the city of Mwanza, Tanzania: A call for one health approach control strategies. PLoS Negl Trop Dis. 2018;12: 39–52. doi:10.1371/journal.pntd.0006600
11. Whatmore AM, Koylass MS, Muchowski J, Edwards-Smallbone J, Gopaul KK, Perrett LL. Extended multilocus sequence

588 analysis to describe the global population structure of the genus Brucella: Phylogeography and relationship to biovars.
589 Front Microbiol. 2016;7: 1–14. doi:10.3389/fmicb.2016.02049
590 12. Whatmore AM, Perrett LL, MacMillan AP. Characterisation of the genetic diversity of Brucella by multilocus sequencing.
591 BMC Microbiol. 2007;7: 1–15. doi:10.1186/1471-2180-7-34
592 13. Akoko JM, Pelle R, Kivali V, Schelling E, Shirima G, Machuka E, et al. Serological and molecular evidence of Brucella
593 species in the rapidly growing pig sector in Kenya. BMC Vet Res. 2020;16: 1–7. doi:10.21203/rs.2.21914/v1
594 14. Ntirandekura J, Makene VA, Kasanga CJ, Matemba LE, Kimera SI, Muma JB, et al. Molecular characterization of Brucella
595 species detected in humans and domestic ruminants of pastoral areas in Kagera ecosystem, Tanzania. Vet Med ans Sci.
596 2020; 1–9. doi:10.1002/vms3.298
597 15. Akoko JM, Pelle R, Lukambagire AS, Machuka EM, Nthiwa D, Mathew C, et al. Molecular epidemiology of Brucella
598 species in mixed livestock-human ecosystems in Kenya. Sci Rep. 2021;11: 1–11.
599 doi:https://doi.org/10.1038/s41598-021-88327-z
600 16. Bodenham FR, Lukambagire SA, Ashford R, Buza JJ, Cash-Goldwasser S, Crump AJ, et al. Prevalence and speciation of
601 acute brucellosis in febrile patients from a pastoralist community of Tanzania. Sci Rep. 2020;10: 1–10.
602 doi:https://doi.org/10.1038/s41598-020-62849-4
603 17. World Health Organization (WHO). Integrated control of neglected zoonotic diseases in Africa: Applying the "One Health"
604 concept. WHO; 2009 Mar. doi:https://hdl.handle.net/10568/3378
605 18. Godfroid J. Brucellosis in livestock and wildlife : zoonotic diseases without pandemic potential in need of innovative one
606 health approaches. Arch Public Heal. 2017;75: 1–6. doi:10.1186/s13690-017-0207-7
607 19. Corbel MJ, Food and Agriculture Organization of the United Nations, World Health Organization, World Organisation for
608 Animal Health. Brucellosis in Humans and Animals. Corbel M, Elberg S, Cosivi O, editors. World Health Organization.
609 Geneva: WHO Press; 2006. Available: https://apps.who.int/iris/handle/10665/43597
610 20. Organisation International Epizoonoses (OIE). Chapter 3.1.4: Brucellosis (Brucella abortus, B. melitensis and B. suis)
611 (Infection with B. abortus, B. melitensis and B. suis). OIE Terrestrial Manual 2018. Geneva; 2018. pp. 355–398. Available:
612 http://www.the-icsp.org/subcoms/Brucella.htm
613 21. Franc KA, Krecek RC, Häsler BN, Arenas-Gamboa AM. Brucellosis remains a neglected disease in the developing world: A
614 call for interdisciplinary action. BMC Public Health. 2018;18: 1–9. doi:10.1186/s12889-017-5016-y
615 22. Organisation International Epizoonoses (OIE). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. World
616 Organ Anim Heal. Geneva; 2008.
617 23. Yang Z, Xu G, Reboud J, Ali SA, Kaur G, McGiven J, et al. Rapid Veterinary Diagnosis of Bovine Reproductive Infectious
618 Diseases from Semen Using Paper-Origami DNA Microfluidics. ACS Sensors. 2018;3: 403–409.
619 doi:10.1021/acssensors.7b00825
620 24. Musallam I, Ndour AP, Yempabou D, Ngong CAC, Dzousse MF, Mouiche-Mouliom MM, et al. Brucellosis in dairy herds:
621 A public health concern in the milk supply chains of West and Central Africa. Acta Trop. 2019;197: 105042.
622 doi:10.1016/j.actatropica.2019.105042
623 25. Tomaso H, Kattar M, Eickhoff M, Wernery U, Al Dahouk S, Straube E, et al. Comparison of commercial DNA preparation
624 kits for the detection of Brucellae in tissue using quantitative real-time PCR. BMC Infect Dis. 2010;10.
625 doi:10.1186/1471-2334-10-100
626 26. Al Dahouk S, Tomaso H, Nöckler K, Neubauer H, Frangoulidis D. Laboratory-based diagnosis of brucellosis--a review of
627 the literature. Part I: Techniques for direct detection and identification of Brucella spp. Clin Lab. 2003;49: 487–505. Available:
628 http://www.ncbi.nlm.nih.gov/pubmed/14572205
629 27. OIE. Chapter 1.1.2. - Principles and methods of validation of diagnostic assays for infectious diseases. 2019th ed. OIE

630 Manual of Diagnostic Tests for Aquatic Animals. 2019th ed. Geneva: OIE; 2019. pp. 1–18. Available:
631 http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.6.00_INTRODUCTION.pdf
632 28. Bounaadja L, Albert D, Chénais B, Hénault S, Zygmunt MS, Poliak S, et al. Real-time PCR for identification of Brucella spp.:
633 A comparative study of IS711, bcsp31 and per target genes. Vet Microbiol. 2009;137: 156–164.
634 doi:10.1016/j.vetmic.2008.12.023
635 29. Zerva L, Bourantas K, Mitka S, Kansouzidou A. Serum Is the Preferred Clinical Specimen for Diagnosis of Human
636 Brucellosis by PCR Serum Is the Preferred Clinical Specimen for Diagnosis of Human Brucellosis by PCR. J Clin Microbiol.
637 2001;39: 1661–1664. doi:10.1128/JCM.39.4.1661
638 30. Al-Dahouk S, Sprague LD, Neubauer H. New developments in the diagnostic procedures for zoonotic brucellosis in
639 humans. Sci Tech Rev Off Int des Epizoot. 2013;32: 177–188. doi:DOI: 10.20506/rst.32.1.2204
640 31. Matero P, Hemmilä H, Tomaso H, Piiparinen H, Rantakokko-Jalava K, Nuotio L, et al. Rapid field detection assays for
641 Bacillus anthracis, Brucella spp., Francisella tularensis and Yersinia pestis. Clin Microbiol Infect. 2011;17: 34–43.
642 doi:10.1111/j.1469-0691.2010.03178.x
643 32. Wareth G, Melzer F, Tomaso H, Roesler U, Neubauer H. Detection of Brucella abortus DNA in aborted goats and sheep in
644 Egypt by real-time PCR Veterinary Research. BMC Res Notes. 2015;8: 1–5. doi:10.1186/s13104-015-1173-1
645 33. Bricker BJ. PCR as a diagnostic tool for brucellosis. Vet Microbiol. 2002;90: 435–446. doi:10.1016/S0378-1135(02)00228-6
646 34. Thakur S, Bedi JS, Singh R, Gill JPS, Arora AK, Kashyap N, et al. Quantitative polymerase chain reaction based
647 quantification of Brucella DNA in serum of pre- and post-therapeutic occupationally exposed infected human population. J
648 Infect Public Health. 2018;11: 514–520. doi:https://doi.org/10.1016/j.jiph.2017.10.004
649 35. Debeaumont C, Falconnet PA, Maurin M. Real-time PCR for detection of Brucella spp. DNA in human serum samples. Eur
650 J Clin Microbiol Infect Dis. 2005;24: 842–845. doi:10.1007/s10096-005-0064-0
651 36. Sambu RM, Mathew C, Nonga HE, Lukambagire AS, Yapi RB, Akoko J, et al. Circulating Brucella species in wild animals
652 of the Serengeti ecosystem , Tanzania. BMC One Heal Outlook. 2021;3: 1–10. doi:https://doi.org/10.1186/s42522-021-00047-6
653 37. Muturi M, Akoko J, Nthiwa D, Chege B, Nyamota R, Mutiiria M, et al. Serological evidence of single and mixed infections
654 of Rift Valley fever virus , Brucella spp . and Coxiella burnetii in dromedary camels in Kenya. PLoS Negl Trop Dis. 2021;15:
655 1–12. doi:10.1371/journal.pntd.0009275
656 38. Wubishet Z, Getachew A, Redeat, Abde A, Bayata S, Chala G. Detection of Brucella Species from Seropositive Animal
657 Blood Clots using real time PCR assay. Ann Clin Cytol Pathol. 2018;4: 1127–1131.
658 39. Flahault A, Cadilhac M, Thomas G. Sample size calculation should be performed for design accuracy in diagnostic test
659 studies. J Clin Epidemiol. 2005;58: 859–862. doi:10.1016/j.jclinepi.2004.12.009
660 40. Dupont WD. Power Calculations for Matched Case-Control Studies. Biometrics. 1988;44: 1157. doi:10.2307/2531743
661 41. Wegner G, Kucera W, Toursel W, Lerche D. Deformability investigations of erythrocytes stored at different temperatures -
662 PubMed. Folia Haematol Int Mag Klin Morphol Blutforsch. 1987;114: 474–475. Available:
663 https://pubmed.ncbi.nlm.nih.gov/2446979/
664 42. Cree IA. Principles of cancer cell culture. Methods in Molecular Biology. 2011. doi:10.1007/978-1-61779-80-5_2
665 43. Probert WS, Schrader KN, Khuong NY, Bystrom SL, Graves MH. Real-Time Multiplex PCR Assay for Detection of Brucella
666 spp., B. abortus, and B. melitensis. J Clin Microbiol. 2004;42: 1290–1293. doi:10.1128/JCM.42.3.1290-1293.2004
667 44. Organisation International Epizoonoses (OIE). Definitions of scales of measurement IN OIE Terrestrial Manual 2018. 2018.
668 Available: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.02.05_STATISTICAL_VALIDATION.pdf
669 45. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical
670 Computing; 2020. Available: https://www.r-project.org/.
671 46. Alton GG, Jones LM, Pietz DE. Laboratory techniques in brucellosis. Monograph series. World Health Organization. 1975.

pp. 1–163. Available: https://apps.who.int/iris/bitstream/handle/10665/38676/WHO_MONO_55_part2.pdf

47. Shahad AA, Shouq TA, Alanoud ZA, Abrar TK. Do Brucella Antibody Titers Correlate with Clinical Outcomes and Culture Positivity of Brucellosis? BioRxiv. 2019;1. doi:https://doi.org/10.1101/534941
48. Batrinou A, Strati IF, Tsantes AG, Papaparaskevas J, Dimou I, Vourvidis D, et al. The Importance of Complementary PCR Analysis in Addition to Serological Testing for the Detection of Transmission Sources of Brucella spp. in Greek Ruminants. Vet Sci. 2022;9. doi:10.3390/vetsci9040193
49. Cheville FN, McCullough RD, Paulson RL. Brucellosis in the Greater Yellowstone Area. Brucellosis in the Greater Yellowstone Area. National Academies Press; 1998. p. 188. doi:10.17226/5957
50. Moreno E, Blasco J, Moriyón I. Facing the Human and Animal Brucellosis Conundrums : The Forgotten Lessons. Microorganisms. 2022;10: 1–30. doi:https://doi.org/10.3390/ microorganisms10050942
51. Organisation International Epizoonoses (OIE). Chapter 3.6.1 - Development and optimisation of antibody detection assays. OIE Validation Recommendations. Geneva; 2014. pp. 1–13. doi:https://doi.org/10.1787/c88edbcd-en
52. Gabriel SM. The epidemiology of brucellosis in animals and humans in Arusha and Manyara regions of Tanzania. The University of Glasgow. The University of Glasgow. 2005. Available: http://theses.gla.ac.uk/4826/1/2005shirimaphd.pdf
53. Muturi M, Bitek A, Mwatondo A, Osoro E, Marwanga D, Gura Z, et al. Risk factors for human brucellosis among a pastoralist community in South-West Kenya, 2015 11 Medical and Health Sciences 1117 Public Health and Health Services. BMC Res Notes. 2018;11: 1–6. doi:10.1186/s13104-018-3961-x
54. Gall D, Nielsen K. Serological diagnosis of bovine brucellosis: A review of test performance and cost comparison. OIE Rev Sci Tech. 2004;23: 989–1002. doi:10.20506/rst.23.3.1545
55. Viana M, Shirima GM, John KS, Fitzpatrick J, Kazwala RR, Buza JJ, et al. Integrating serological and genetic data to quantify cross-species transmission: Brucellosis as a case study. Parasitology. 2016;143: 821–834. doi:10.1017/S0031182016000044
56. Sanjuan-Jimenez R, Colmenero JD, Morata P. Lessons learned with molecular methods targeting the BCSP-31 membrane protein for diagnosis of human brucellosis. Clin Chim Acta. 2017;469: 1–9. doi:10.1016/j.cca.2017.03.014