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Article

Epidemiology of *Brucella* Species Circulating in Small Holder Dairy Cattle in Tanzania

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Abstract: Brucellosis is a zoonoses caused by bacteria of the genus *Brucella*, which causes economic losses in livestock and threatens public health. A cross-sectional study was conducted to determine the prevalence of *Brucella* species and risk factors in smallholder dairy cattle in six regions of Tanzania from July 2019 to October 2020. Dairy cattle (n=2048) were sampled from 1371 farms. DNA ex-tracted from blood and vaginal swabs was tested for *Brucella* using qPCR targeting the IS711 gene and positives were tested for the *alkB* marker for *B. abortus* and BMEI1172 marker for *B. melitensis*. The molecular prevalence was 3.5% (95% CI: 2.8-4.4) with the highest prevalence 8.1% (95%CI: 4.6-13.0) in Njombe region. *B. melitensis* was the predominant species detected (66.2%). Logistic regression revealed PCR positivity in dairy cattle was higher in the animals with a history of abortion (OR: 3.17, 95%CI: 1.05-9.62) and farms without dogs around (OR: 2.68, 95%CI: 1.12-5.90). Further studies are recommended to understand the source of *B. melitensis* and the implications to veterinary public health. Livestock keepers should be informed of the risks and biosecurity practices to reduce introduction and control *Brucella*. Cattle and small ruminant vaccination pro-grams could be implemented to control brucellosis in high-risk populations in the country.

Keywords: dairy cattle; brucellosis; qPCR; molecular prevalence; risk factors; *Brucella*; Tanzania

1. Introduction

Brucellosis is a zoonotic bacterial disease causing economic loss in dairy cattle production systems. Brucellosis is considered to be one of the most widespread zoonotic diseases globally [1]. The disease is caused by a bacterium of the Genus *Brucella*. Of the twelve *Brucella* species identified that affect mammals, the common species affecting domestic animals are *B. abortus* in cattle, *B. melitensis* in goats, *B. ovis* in sheep, *B. suis* in pigs and *B. canis* in dogs [2, 3]. *Brucella* spp. are somewhat host specific, however, recent studies highlighted the importance of cross-species infection [4-6].

Studies have found that, brucellosis in cattle can also be caused by *B. melitensis* or *B. suis* [2, 7, 8]. This renders eradication through vaccination with *B. abortus* derived vaccines ineffective, since the efficacy of the S.19 vaccine which is widely used in endemic areas has not been fully validated against *B. melitensis* and those vaccines which confer cross-protection may not be available especially in LMICs [9-11].

In Tanzania, the first isolates of *B. abortus* and *B. melitensis* from cattle and goats respectively was done in 1967 [12], however no typing of the isolates was done [12]. In 2015, *B. abortus* was isolated from aborted materials of dairy cattle in Njombe region and the first typing identified *B. abortus* biovar 3 [13]. Around the same time *B. abortus* biovar 1 was detected and typed from cow's milk [14]. Over recent years mixed farming practices have been reported to be associated with brucellosis reemergence in Tanzania [15, 16]. Research in neighboring countries have identified cattle infected with other *Brucella* species. Studies in Kenya, Uganda and Rwanda have identified *B. melitensis* and *B. abortus* from dairy cattle [6, 8, 17]. Furthermore, *B. melitensis* the most pathogenic among the classical *Brucella* species has been frequently isolated from febrile human patients in northern Tanzania [18], however the possible sources of infection in humans was not established. The authors, however concluded that to control human brucellosis, vaccination should also target small ruminants by using *B. melitensis* vaccine [18]. In Tanzania there have not been any reports of the isolation or molecular detection of *B. melitensis* in cattle. Therefore, the objective of the current study was to identify *Brucella* species circulating in smallholder dairy cattle populations in Tanzania by using molecular techniques and establish risk factors associated with brucellosis molecular positivity.

2. Materials and Methods

2.1. Study Area and Design

A cross-sectional study was conducted from July 2019 to October 2020 to determine *Brucella* species circulating in smallholder dairy farming systems in two agroecological zones comprising six administrative regions of Tanzania. The study was conducted in three regions in the northern zone (Kilimanjaro, Arusha and Tanga) with 252,554 head of dairy cattle and three regions in the southern highland zone (Iringa, Njombe and Mbeya) with 103,306 head of dairy cattle (Figure 1). These regions have the highest density of smallholder dairy cattle in Tanzania [19, 20]. According to the household budget survey of 2018, all six regions were above the food poverty line of TSH 33,748 9 (US\$13) per person per month [21]. All study regions practice mixed farming where dairy cattle interact with other domestic animals [16].

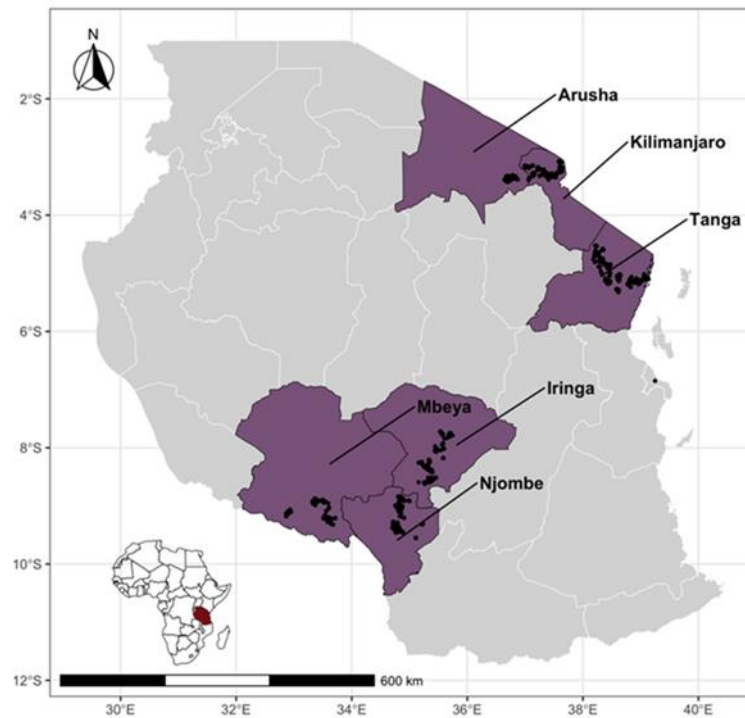


Figure 1. Map of Tanzania showing study regions (in purple color) with large populations of smallholder dairy cattle and unstudied regions in gray (right). Black dots indicate the locations of cattle sampled. Inset shows the location of Tanzania in Africa.

2.2. Study Population

Dairy cattle kept under smallholder farming systems were the target for the study. Dairy cattle in these regions were mainly crossbreds of Friesian, Ayrshire, and Jersey with Tanzanian Short Horn Zebu (TSHZ) and Friesian crosses comprising the largest proportion (80%) of breeds. The feeding management systems of these dairy cattle were two (1) an intensive management system in which pastures were cut and carried to the farm for them to feed and (2) an extensive system in which cattle were left to graze in private or communal land. Dairy cattle in this study were selected from a subset of the dairy cattle registry of the Africa Dairy Genetics Gains (ADGG) program (<https://data.ilri.org/portal/dataset/adgg-tanzania>, accessed on 1 June 2019). The ADGG project randomly registered over 52,500 cattle across the study regions in the database, furthermore 4000 dairy cattle were randomly selected and genotyped [22]. The sample for this study was selected from the genotyped animals, although at the time of the study not all the genotyped animals were available because of a high rate of animal take off (sale, natural death and slaughter) and the time that elapsed between genotyping and sampling.

2.3. Questionnaire Administration for Risk Factors Determination

A cross-sectional survey was conducted in 1371 farms and a questionnaire tool used to collect data on possible risk factors for brucellosis. The questionnaire covered details of the respondent and their brucellosis knowledge, farm location, and herd management practices such as feeding and watering, disease control, disposal of abortion material, mixed farming practices and cow or farm abortion history. The tool was developed and piloted before a final version was uploaded using the Open Data Kit (ODK) software platform on the International Livestock Research Institute (ILRI) server in Kenya (Supplementary Material S1). Then, the forms were downloaded for completion on farm from the Google play app store onto an Android tablet (Samsung, Suwon-si, Republic of Korea). Farmers or their representative were informed on the study aims and General Data Protection Regulation (GDPR) compliance. Farmers needed to sign a consent form before being interviewed in Kiswahili, and their cattle sampled.

2.4. Blood and Swab Sampling from Dairy Cattle and Samples Storage

A total of 2049 dairy cattle were sampled, and 5mls blood was collected aseptically by venipuncture into EDTA as was explained by others [23]. Animal identification number, date of collection and field barcode were labelled on each tube. In the field, all samples were kept in a cool box containing ice packs and transported daily to the field laboratory for storage. Similarly, vaginal swabs were collected aseptically following cleaning of the vulva using chlorhexidine-soaked paper towel. The vulva lips were opened by using fingers and a long shaft swab was inserted per vaginum and the mucosa was gently swabbed by rotating the swab shaft left and right while removing. After removing, the swab was then inserted in a cryovial tube containing sterile 1ml phosphate buffered saline (PBS) and squeezed on the tube wall while mixing, the shaft was then cut-off and thrown in a waste disposal bin leaving the swab tip in the PBS cryovial. The PBS cryovial was then labeled with collection date, barcoded, and scanned into the ODK form. Both EDTA blood tubes and PBS swab tubes were stored at -20°C in upright position until they were transported to Nelson Mandela African Institution of Science and Technology laboratory in Arusha for longer term storage.

2.5. DNA Extraction from EDTA Blood and PBS Swabs

EDTA tubes containing blood and PBS tubes containing a swab were allowed to thaw at room temperature on a table. After thawing each tube was briefly mixed by vortexing. Three hundred microliter (300µl) of blood/PBS was aliquoted and placed in a sample autoplade of TANBeads®. Total genomic DNA extraction was done by using TANBeads® Nucleic Acid Extraction Validation Kit (OptiPure Blood DNA Auto Plate) designed for use with the Maelstrom 9600 (Taiwan Advanced Nanotech Inc, Taoyuan City, Taiwan), a robotic system in ILRI laboratories in Nairobi, Kenya. The extraction kit was suitable for isolating DNA from whole blood including deep frozen blood and it used the silicone dioxide layer coated on the magnetic beads. A genomic DNA extract was provided in 100µl. After the extraction, random DNA samples were tested for quality and degradation by using Nanodrop spectrophotometer and 1% agar-rose gel electrophoresis respectively (Supplementary material S2).

2.6. Real Time PCR for *Brucella* Genus Detection and Species Characterization

The QuantiStudio 5 qPCR machine (Applied Biosystems, Woodlands, Singapore) with the 96 well plate format and 0.2ml block installed with QuantiStudio TM Design and Analysis software v1.5 was used for the analysis.

For *Brucella* genera detection, the DNA samples were tested for the presence of insertion sequence IS711 by using the IS711 primer pair and probe Table 1 in a uniplex assay. The qPCR conditions and reaction volumes used were adopted from Akoko et al., [5]. The reaction volumes were prepared as follows; 5µl of ready to use Master Mix (Lunar Universal Probe qPCR Master Mix, New England BioLabs, MA, USA), 1.25µl of IS711 Primer and Probe mix (Macrogen, Netherlands), 1.75µl molecular grade water and 3µl DNA template. The total reaction mix volume (cocktail) of 11µl was then thoroughly mixed up gentle vortexing for 1minute before setting the 96 wells PCR plate into the qPCR machine.

The thermo cycler conditions were set as for pretreatment with Uracil-DNA Glycosylases (UDGs) at 50°C for 2min, polymerase activation and DNA denaturation at 95°C for 10min, amplification step at 95°C for 15s, then 1min for annealing at 60 °C and 42 cycles.

Positive DNA samples based on the IS711 gene marker (positive to *Brucella* genus) were further characterized for *B. abortus* and *B. melitensis* using alkB and BMEI1172 primers and probe respectively Table 1.

Table 1. Oligonucleotide primers and probes used to perform qPCR assays.

Target	Targeted gene	Sequences of primers and probes (5' -3')		Fluorophore/ quencher	Reference
Genus <i>Brucella</i>	IS711	Probe:	AAG CCA ACA CCC GGC	FAM/- MGBNFQ	Matero, 2011
		Forward:	GGC CTA CCG CTG CGA AT		
<i>B. melitensis</i>	IS711 downstream of BMEI1162	Reverse:	TTG CGG ACA GTC ACC ATA ATG	Texas Red/BHQ2	Probert. 2004
		Probe:	CAGGAGTGTTCGGCTCAGAATAATCCACA		
		Forward	AACAAGCGGCACCCCTAAAA		
		Reverse	CATGCGCTATGATCTGGTTACG		
<i>B. abortus</i>	IS711 downstream of alkB	Probe:	CGCTCATGCTCGCCAGACTTCAATG	JOE/BHQ1	
		Forward:	GCGGCTTTTCTATCACGGTATTC		
		Reverse:	CATGCGCTATGATCTGGTTACG		

The reaction volumes used for *B. abortus* and *B. melitensis* specific assays were 7.5µl Master mix (Perfecta qPCR ToughMix UNG Low ROX), 0.75µl Primer and probe (species-specific), 1.75µl Molecular grade water, and 5µl of DNA template. The final reaction mix (cocktail) of 15µl was thoroughly mixed by vortexing for 1 minutes before setting the 96 well PCR plate into the qPCR machine.

The PCR conditions for *Brucella* species-specific assays were as follows; pretreatment stage with Uracil-N-Glycosylase (UNG- step) was set at 45°C for 5min to cleave all contaminating templates containing U bases, DNA denaturation at 95°C for 5min, amplification at 95°C for 15 seconds, annealing at 60°C for 30 seconds and 42 number of cycles.

Positive controls for *Brucella* strains, *B. melitensis* 16M and *B. abortus* 544 both sourced from Friedrich-Loeffler-Institute, Germany. For negative controls, a mixture of RNAase free molecular grade water and master mix was used. The assay efficiency statistics and limit of detection of the ten-fold serial dilution of the reference materials for genus and species detection are provided in the Supplementary material S3.

2.7. Data Analysis for Calculation of Molecular Prevalence and Risk Factors

Data analysis was carried out using R software (version 4.2.3) [24] with individual animals as primary sampling unit and district as the clustering unit rather than herd because so many herds were small and had only 1 animal sampled. The prevalence was estimated as ratio of PCR positives (numerator) and total number of animals tested (denominator) for overall and regional prevalences and *binom.test* function was used to generate the 95% binomial confidence interval. The design adjusted overall prevalence was estimated after incorporating the different sampling weights (Table 3) into the estimation using the *svydesign*, *confint* and *svyby* functions in the survey package [25]. This allowed the stratified study design to be accounted for in the prevalence estimates.

The univariable analysis was done by using the R packages *epitools* and *epitab* to calculate the odds ratio between PCR positivity (combined blood and swab results) and independent variables (risk factors) and the unadjusted 95% confidence interval and p-value. Independent variables with p value ≤0.2 were carried forward to multivariable analysis. None of the risk factors was significantly associated with the outcome of interest when the full data set for all six regions was used, therefore the dataset was sub-setted to include only four regions with the highest molecular prevalence (Njombe, Kilimanjaro, Mbeya and Arusha Regions). Collinearity was tested by calculating the Spearman’s correlation coefficient *rho* from a pair of continuous variables by using *cor.test* function of the *stat* package, highly correlated variables (*rho* >0.29) were not included (years of experience in dairy farming and animal age) in the models. A multivariable logistic regression was fitted using the R package *glmmTMB*, a saturated model was fitted, and variables selected by backward elimination to get the final model. Model comparisons were done using Akaike information criterion (AIC) (AIC function in the *MuMIn* package) and *lrttest* function (*lmttest* package) to estimate the best fitting model. The final model validation and diagnostics was assessed using the *DHARMA* package.

3. Results

3.1. Description of Sampled Dairy Cattle

A total of 2049 dairy cattle were sampled from 1371 farms across the six study regions. The median herd size was 2 cattle. The majority of sampled cattle were female (97.2%). The predominant breed was SHZ-Friesian crosses (68.7%), with other breeds being SHZ-Ayrshire (20.8%), SHZ-Jersey (6.9%) and indigenous breeds (3.6%).

3.2. Brucellosis Molecular Prevalence of Dairy Cattle in Selected Regions of Tanzania

There were 35/2046 blood and 37/1893 swab samples Brucella genus-positive (Table 2). There was no agreement between sample types.

Table 2. Regional distribution of Brucella genus positive blood and swab DNA samples from individual cattle in Tanzania.

Region	Total Sampled	AnimalNumber of samples (%)	positive_bloodNumber of samples (%)	positive_swab
Arusha	318	5/318 (1.6%)	10/294 (3.4%)	
Tanga	524	6/524 (1.0%)	1/412 (0.2%)	
Kilimanjaro	521	11/519 (2.1%)	8/513 (1.6%)	
Iringa	281	7/281 (2.5%)	1/273 (0.4%)	
Njombe	187	1/186 (1.1%)	14/186 (7.5%)	
Mbeya	218	5/218 (2.3%)	3/215 (1.4%)	
Total	2049	35/2046 (1.7%)	37/1893 (2.0%)	

The overall unadjusted animal molecular (PCR) prevalence (being positive by either blood or vagina swab) was 3.5% (95% CI: 2.8-4.4) and the overall design adjusted prevalence was 3.7% (96%CI: 2.7-3.7). Among the study regions Njombe region had the highest molecular (PCR) prevalence of 8.1%, followed by Arusha 4.7%, Mbeya 3.8% and Kilimanjaro region 3.7% (Table 3 and Figure 2).

Table 3. The combined molecular (PCR) prevalence based on Brucella genus positive results.

Region	Negative	Positive	Total	PCR Prevalence %	95% CI	Dairy cattle Population
Arusha	303	15	318	4.7	2.7-7.7	78,637
Tanga	517	7	524	1.3	0.5-2.7	41,639
Kilimanjaro	500	19	519	3.7	2.2-5.7	161,984
Iringa	273	8	281	2.8	1.2-5.5	7,081
Njombe	171	15	186	8.1	4.6-13.0	7,177
Mbeya	210	8	218	3.8	1.7-7.4	72,724
Total	1974	72	2046	3.5	2.8-4.4	369,242

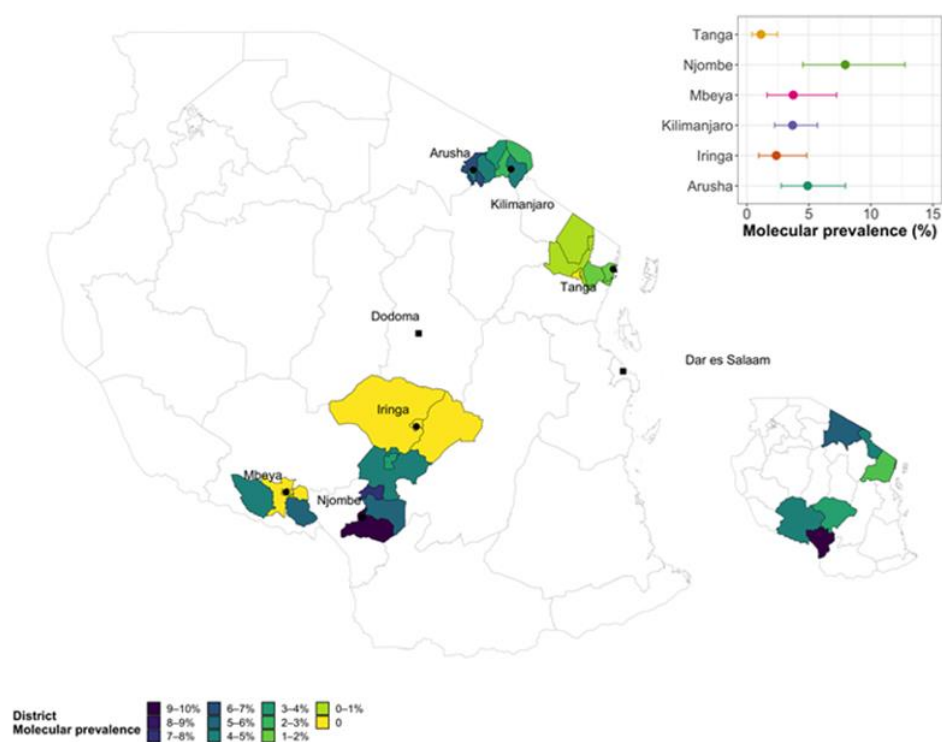


Figure 2. Choropleth map showing the regional molecular prevalence (insets) and the detailed molecular prevalence by local authority sampled in each region.

3.3. *Brucella Species Circulating in Dairy Cattle Population Identified from Brucella Genus Positive Swab and Blood Samples*

The majority of blood samples 19/35 (54.3%) and vaginal swabs 29/37 (78.4%) were PCR-positive for *B. melitensis* only, with a further 10/35 (28.6%) blood samples and 7/37 (18.9%) swabs PCR-positive for both (*B. melitensis* and *B. abortus*), meaning the vast majority of infections involved *B. melitensis* (Table 4). *B. abortus* occurred on its own in 2/35 (5.7%) blood samples. There were 4 blood and 1 swab samples where no species was identified (Table 4).

Table 4. Real-Time Polymerase chain reaction (qPCR) results for *Brucella* species identified from genus-positive swab and blood samples.

Region	Sample	<i>B. abortus</i>	<i>B. melitensis</i>	Mixed	Unidentified
Arusha	Blood n=5	0	3	2	0
	Swabs n=10	0	9	1	0
Kilimanjaro	Blood n=11	1	9	0	1
	Swabs n=8	0	6	2	0
Tanga	Blood n=6	0	2	3	1
	Swabs n=1	0	1	0	0
Njombe	Blood n=1	0	1	0	0
	Swabs n=14	0	11	2	1
Iringa	Blood n=7	0	3	2	2
	Swabs n=1	0	1	0	0
Mbeya	Blood n=5	1	1	3	0
	Swabs n=3	0	1	2	0
Total	Blood n=35	2	19	10	4
	Swabs n=37	0	29	7	1

Mixed=PCR-positive for both *B. abortus* and *B. melitensis*.

3.4. Brucellosis Hotspot Areas

Figure 2 is the spatial choropleth map of Tanzania (main) with the right bottom inset shows the study regions and their molecular prevalence with the highest prevalence in Njombe region (dark blue) and the main map shows molecular prevalence of the study districts (local authorities) for each study region. The main map shows that PCR-positive animals were clustered within a small number of local authorities in the Kilimanjaro, Mbeya, Arusha and Njombe regions.

3.5. Univariable Analysis Results to Combined PCR Positivity

The univariable analysis is presented in Table 5. There was a significant association between keeping a bull and reduced Brucella PCR positivity ($p<0.05$). Dairy cattle kept on a farm that kept a bull were 0.46 times less likely to be Brucella PCR-positive (OR: 0.46, 95%CI: 0.21-1.03) when compared to dairy farms which did not keep bull. There was an association between dairy breeds and Brucella PCR-positivity, with Jersey and indigenous SHZ more likely to be Brucella PCR positive compared to Ayrshire (OR: 2.62, 95%CI: 0.97-7.16). There was a positive association between Brucella PCR positivity and history of abortion at the animal level (OR: 2.51, 95%CI: 0.86-7.38), and history of abortion on the farm level (OR: 1.51, 95%CI 0.58-3.89). Further associations were shown between Brucella PCR positivity and keeping pigs dogs or having pigs around and reduced risk of an animal being Brucella PCR positive ((OR: 0.55, 95%CI: 0.32-0.93), or with having contact with dogs (OR:0.5, 95%CI:0.25-0.99) and increased risk with introduction of new animals (OR: 1.53, 95%CI: 0.71-3.31). Regionally there does also appear to be a much higher risk of an animal being Brucella PCR positive if it is in Njombe compared to Arusha (OR:1.76, 95%CI: 0.84-3.69).

Table 5. Univariable analysis of combined blood and vaginal swab PCR positive dairy animals.

Variable	Level	Negative	Positive	OR	95% Confid. Interval		P_value
Farmer's gender	female	541	26	ref			1
	male	643	31	1.003	0.59	1.71	
Herd size	1-2animals	589	31	ref			0.341
	3-4animals	429	22	0.97	0.53	1.77	
	>4animals	163	4	0.47	0.12	1.35	
Farmer's experience in dairy farming	≤5years	105	6	ref			0.63
	>5years	1079	51	0.83	0.35	1.97	
Farmer's education	basic	969	49	ref			0.59
	secondary_plus	215	8	0.74	0.34	1.57	
Farm or neighbor keep goat	have_goat	795	38	ref			1
	no_goat	387	19	1.03	0.58	1.80	
Farm or neighbor keep dog	no_dog	773	36	ref			0.78
	have_dog	411	21	1.09	0.63	1.90	
Farm or neighbor keep sheep	have_sheep	271	12	ref			0.87
	no_sheep	911	45	1.11	0.58	2.14	
Farm or neighbor keep pig	no_pig	891	47	ref			0.26
	have_pig	293	10	0.65	0.32	1.29	
Keeping bull	no_bull	909	50	ref			0.05
	have_bull	275	7	0.46	0.21	1.03	
Body condition score	good	630	29	ref			0.78
	poor	554	28	1.09	0.64	1.86	
Introduction of new animal	no	1070	49	ref			0.26
	yes	114	8	1.53	0.71	3.31	
Placenta management	correct_dispose (bury/burn)	956	44	ref			

	Incorrect_dispose	228	13	1.24	0.65	2.34	0.49
Farm's distance	<100	946	41	ref			
	≥100	238	16	1.55	0.85	2.81	0.17
Cattle breed	SHZxAyrshire	264	10	ref			
	SHZxFriesian	850	40	1.24	0.6	2.8	0.550
	SHZxJersey+SHZ	70	7	2.62	0.82	7.95	0.058
Animal sex	female	1147	57	ref			
	male	37	0	0	0	NaN	0.41
Animal age	1-4years	501	21	ref			
	5-7years	488	27	1.32	0.71	2.49	0.351
	>7years	192	9	1.12	0.44	2.60	0.784
	Arusha	303	15	ref			
Region	Kilimanjaro	500	19	0.77	0.38	1.53	0.47
	Mbeya	210	8	1.77	0.32	1.85	0.66
	Njombe	172	15	1.80	0.85	3.71	0.17
History of abortion (Animal)	no	1049	53	ref			
	yes	32	4	2.51	0.86	7.38	0.056
History of abortion (Farm)	no	1110	52	ref			
	yes	71	5	1.51	0.58	3.89	0.39
Feeding management	pasture	166	6	ref			
	intensive	1915	51	1.39	0.59	3.29	0.452

3.6. Multivariable Logistic Regression Model for Association with Brucella PCR Positivity

The final logistic regression model is presented in Table 6. The model revealed that history of abortion of the animal was significantly associated with Brucella PCR positivity ($p>0.05$). Dairy cattle with a history of abortion were 3.17 times more likely to be Brucella PCR positive (OR: 3.17, 95%CI: 1.05-9.62) compared to those with no history of abortion. Dairy cattle in a farm with dogs around on the farm were less likely to be Brucella PCR positive (OR: 0.37, 95%CI: 0.18-0.77) than those in a farm which has dogs around on the farm or the neighbours farm. Even though there was no statistical significance ($p>0.05$), results showed that dairy cattle in a farm which introduced a new animal (dairy cattle) were 1.87 times more likely to be Brucella PCR positive (OR: 1.87, 95%CI: 0.82-4.24) than those in a farm which did not introduce a new animal. Keeping pigs or contact with neighbor's pigs (like with dogs) was protective with Brucella PCR positivity ($p<0.05$) and dairy cattle in a farm which keeps pigs or having pigs around were less likely to be Brucella PCR positive (OR: 0.46, 95%CI: 0.25-0.83) compared to those in a farm which do not keep pigs or have pigs around.

Few dairy farms keep bulls, the regression model revealed that, dairy cattle in farms which kept a bull were less likely (OR:0.39, 95%CI: 0.16-0.96) to be Brucella PCR positive compared to those farms which did not keep a bull. Njombe region was significantly associated with Brucella PCR positivity, and dairy cattle in Njombe was 2.72 times more likely of being Brucella PCR positive (OR: 2.72, 95%CI: 1.21-6.10) than those in Arusha region.

Table 6. Final multivariable logistic regression model for Brucella PCR-positivity for dairy cattle in Tanzania.

Variable	Level	OR	Lower	Upper	P-value
History of abortion (Animal)	no	ref			
	yes	3.17	1.05	9.62	0.04
Farm or neighbour keep Dog	have dog	ref			
	no dog	2.68	1.30	5.55	0.008
Introduction of new animal	no	ref			
	yes	1.87	0.82	4.24	0.14

Farm or neighbor keep pig	have_pig	ref			
	no_pig	2.19	1.21	3.98	0.01
Keeping bull	no	ref			
	yes	0.39	0.16	0.96	0.04
Region	Arusha	ref			
	Kilimanjaro	1.20	0.56	2.56	0.6
	Mbeya	1.39	0.50	3.86	0.5
	Njombe	2.72	1.21	6.10	0.016

4. Discussion

Brucellosis is a globally neglected bacterial zoonosis. It was characterized for the first time in Tanzania from domestic animals in 1967 and later in 2015 [12-14]. In Tanzania, brucellosis in dairy cattle is endemic and has been affecting dairy production and public health apart from short period in the late 1990’s when it was controlled [26]. In Tanzania, most brucellosis studies have depended on the use of serological tests to make recommendations and conclusions on the best way to control the disease in animals and have assumed *Brucella* host-specificity due to lack of serological tools to differentiate them [27]. However, recent studies have shown that host-specificity of *Brucella* species no longer applies, as cross-infections have been recently reported globally [6, 28]. Therefore, molecular characterization of *Brucella* species is becoming increasingly important to understand the different epidemiology and make appropriate recommendations for control and eradication [29].

The current study reports the overall animal level adjusted PCR prevalence (molecular prevalence) of 3.5% across the study regions. The molecular prevalence reported in this study is lower than the molecular prevalence of 18.9% reported in Kenya [5] and similar to the 5.6% reported in Rwanda [6]. The discrepancy in prevalence is likely due to differences in study population and study locations as brucellosis prevalence has been reported to be lower in highland areas in Kenya [30] which have a similar agroecology to the areas in this study. To control the disease in the country, high-risk regions such as Njombe, Kilimanjaro, Mbeya and Arusha need to be prioritized for disease interventions [16].

The current study has identified that *B. abortus*, *B. melitensis* and unidentified *Brucella* species are circulating in this dairy cattle population. The current study revealed that dairy cattle are predominantly PCR-positive with *B. melitensis*, which is generally considered to be a pathogen of sheep and goats. Although, we did not find a statistically significant association between keeping goats and *Brucella* PCR positivity in this study, our previous work has demonstrated an association with seropositivity, with the odds of cattle being seropositive on a farm that keeps goats being 3.02 times compared to cattle on a farm that do not keep goats [16]. The role of goats in the epidemiology of cattle brucellosis in SSA has also been reported in previous studies [15].

The current study also reports dairy cattle PCR-positive for two *Brucella* species, *B. abortus* and *B. melitensis* and an unidentified *Brucella* species. Co-infections with more than one *Brucella* species have been reported in neighboring Rwanda and other African countries [6, 28, 31, 32]. Furthermore, PCR-positivity for two *Brucella* species was attributed to the mingling of cattle and small ruminants [33, 34]. The detection of an unidentified *Brucella* species highlights the potential of infection with other *Brucella* species such as *B. ovis*, *B. canis*, and *B. suis* which have been identified in dairy cattle following natural infection in different countries [35-37] and may be related to the presence of other domestic animals such as sheep, dogs and pigs in the dairy farms.

The multivariable model developed for the regions with the highest number of PCR positive animals (Njombe, Kilimanjaro, Mbeya and Arusha) demonstrated that the odds of *Brucella* PCR positivity in a dairy farm with dogs was 0.37 times when compared to farms that do not have dogs around. Earlier studies reported that feeding dogs fetal membranes and aborted material was found to be a common practice in 62.5% of cattle farmers [38]. This is further evidenced by detection of *B.*

melitensis and *B. abortus* from herding dogs [39, 40]. It is likely that dogs eating placenta and other aborted material reduce the chance to spread the bacteria in the environment, however this finding was contrary to the finding of a recent study in India which found that the odds of disease was 2.55 times in a farm that have dogs around compared to those with no dogs around [41]. This study also revealed that the odds of *Brucella* PCR positivity in a farms that do have pigs was 0.46 times when compared to the farms not having pigs around. This finding disagreed with the finding of other studies which found that presence of pigs in a farm was associated with the risk of brucellosis in cattle [42]. Although dogs and pigs may clean up the infected placenta and aborted materials and hence reduce the risk of infection to dairy cattle, this should not be encouraged and alternative control methods such as burning or burying the placenta are preferred.

History of abortion of an animal was significantly associated with *Brucella* PCR positivity, the odds of *Brucella* PCR positivity was 3.17 in dairy cattle with a history of abortion when compared to animals with no history of abortion. This finding was in agreement with the finding of other studies that the odds of the disease was higher in cattle with history of abortion [43-45]. Brucellosis is characterized by late-term abortion, even though is not pathognomonic sign, abortion may still be indication to implement strategies for brucellosis control in a dairy farm. Even though it was not statistically significant, introductions of new animals (cattle) in a farm was associated with *Brucella* PCR positivity in dairy cattle. However, other studies found that introduction of new animals to a farm was significantly associated with the risk of brucellosis in cattle [46, 47] regardless of the source of the animal [48]. Testing new animals for brucellosis should be prioritized as an effort toward the control brucellosis in the region. This study revealed that the odds of *Brucella* PCR positivity of a dairy cattle on a farm that kept its own bull was 0.39 times less than dairy cattle on a farm that did not keep a bull. This finding was in agreement with the finding of other studies which found that farms which used own bull were at low risk of brucellosis [46]. Unlike borrowing a bull for breeding, keeping a bull reduces the risk of *Brucella* infections among dairy cows on a farm. Njombe region was significantly associated with *Brucella* PCR positivity, and dairy cattle in Njombe was 2.72 times more likely to be *Brucella* PCR positive than dairy cattle in Arusha. This suggests that Njombe is a brucellosis high-risk region and should be a focus for rolling-out strategies to control the disease.

The presence of *B. melitensis* and mixed *B. abortus* and *B. melitensis* PCR positives in dairy cattle pose a challenge to controlling the disease by vaccination. The monovalent *B. abortus* S19 vaccine which is produced in Tanzania may not be effective in controlling the disease under these scenarios (*B. melitensis* and co-infections) as the vaccine has not been fully validated for conferring cross-protection and alternative vaccines which may confer protection may not be available for use in LMICs [9-11]. Further validation of the currently available vaccines under this scenario is required, furthermore production of a bivalent vaccine (containing *B. abortus* and *B. melitensis*) might assist in control and eradication of the disease in cattle [49, 50].

There were limitations of the study as there was no agreement in PCR positivity between vaginal swab and blood from the same animal which could be attributed by the short and transient bacteremia in cattle and that shedding of bacteria in vaginal samples tends to be post calving. Furthermore, poor agreement could have been attributed by long storage of samples for over one year in a deep freezing temperature (-20°C) which is likely to degrade the samples. There are no studies that have looked at the different sample types in cattle, however, a study in dogs compared blood and vaginal swabs and found moderate agreement between vaginal swab PCR and blood PCR, in addition there were more blood PCR positive and no vaginal swab PCR positive in a bacteremic group of bitches [51]. Finally, there was not sufficient DNA to allow sequencing for further characterization of the pathogens.

5. Conclusions

The current study confirms that bacteria of the genus *Brucella* are circulating in smallholder dairy cattle suggesting that brucellosis is present and likely to be causing clinical disease in dairy cattle. The importance of *B. melitensis* infections in smallholder dairy cattle is not clear and further understanding of the clinical significance and veterinary public health implications is needed.

This study recommends further studies on *Brucella* species circulating in dairy cattle and studies on the roles of small ruminants and other domestic animals in the epidemiology of brucellosis in dairy cattle. Training of farmers in good biosecurity and control methods is recommended, as well as vaccination of dairy cattle in high-risk populations.

6. Patents

This section is not mandatory but may be added if there are patents resulting from the work reported in this manuscript.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Informed Consent Statement: Consent forms were signed by cattle owners prior to the interview and sample collection.

Data Availability Statement: All relevant data are presented within the manuscript.

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