



Animal Brucellosis: Seropositivity rates, Isolation and Molecular Detection in Southern and Central Ethiopia

Bayeta Senbata Wakjira ¹, Edilu Jorga ², Matios Lakew¹, Abebe Olani¹, Biniam Tadesse¹, Getachew Tuli¹, Redeat Belaineh¹, Shubisa Abera¹, Getachew Kinfel¹, Solomon Gebre¹

¹Animal Health Institute, Sebeta, Ethiopia; ²Ambo University, College of Agriculture and Veterinary Science, Ambo, Ethiopia

Correspondence: Bayeta Senbata Wakjira, Email didigabruma@gmail.com

Introduction: Brucellosis is a neglected bacterial zoonosis with serious veterinary and public health importance throughout the world. A cross-sectional study on animal brucellosis was conducted aiming to estimate seroprevalence and molecular detection.

Methods: Blood samples were collected from a total of 4274 individual animals (cattle, small ruminants and camel) from 241 herds/flocks for serology and PCR. Serum samples were tested using multispecies I-ELISA. Blood clots from seropositive animals were also tested for brucellosis via PCR. Additionally, 13 vaginal swab samples were collected from animals (2 from bovine and 11 from small ruminants) with recent abortion history for bacterial isolation and molecular detection.

Results: The overall individual animal and herd level seroprevalence was 3.95% (169/4274) and 18.26% (44/241) respectively. The animal level seroprevalence at species level was 1.58% (47/2982), 8.89% (97/1091) and 12.44% (25/201) in bovine, small ruminants (sheep and goat) and camel, respectively. Herd level seroprevalence were 5.43% (10/184), 52.08% (25/48) and 100% (9/9) in bovine, small ruminant and camel, respectively. The animal level seroprevalence of bovine from intensive and extensive systems was 1.10% (31/2808) and 2.87% (5/174) respectively. Blood clots tested for brucellosis via PCR were negative by RT-PCR. *Brucella* species was isolated from 6/13 (46.15%) vaginal swab samples cultured on *Brucella* selective agar, and shown to be *B. melitensis* using Real-Time PCR.

Conclusion: Overall, seropositivity for camels was higher than what has been reported previously. Also, there was a notable difference in this study in cattle seroprevalence when comparing extensive with intensive systems, with the extensive system having much greater seropositivity.

Keywords: *Brucella melitensis*, neglected bacterial diseases, camel, zoonosis

Introduction

Brucellosis is a bacterial disease of domestic and wild animals caused by the genus *Brucella* which has great public health importance globally.¹² Currently, 12 species of *Brucella* are recognized, including *B. abortus* (cattle), *B. melitensis* (sheep and goats), *B. ovis* (sheep), *B. suis* (pigs), and *B. canis* (dogs) in addition to seven other species found in various species of wild animals. It is well established that cattle can be infected with *B. melitensis* and that sheep/goats may harbor *B. abortus*. Either of these two *Brucella* species is capable of infecting camelids.¹⁷ Classically, detection and identification of *Brucella* spp. has been based on culture and phenotypic analysis (biotyping) and due to its potential for transmission via aerosol, it must always be handled in laboratories with biosafety level 3.

With the advent of molecular techniques, early PCRs for the genus were based on the 16S rRNA and *bcs31* genes.⁸ PCR methods based on the 16S rRNA amplify a DNA fragment common to all *Brucella* species but cross-react with members of the closely related genus *Ochrobactrum*.³¹ The IS711 element became the preferred target for general identification purposes due to its restricted occurrence in *Brucella* spp. and the presence of multiple copies, allowing for unparalleled sensitivity and direct testing on clinical samples.¹⁹

In Ethiopia, brucellosis is considered to be endemic. Vaccination of farm animals is not practiced. Reviews of several serological studies done on brucellosis have shown seroprevalence rates in different species ranging from 0% to 26.1%, 0.07% to 13.7%, and 0.53% to 9.6%, in bovine, small ruminant and camel, respectively.^{11,15,16,25,35,41} In pastoral communities, 34.1% human patients with febrile illness from Borana, 29.4% from Hammer and 3% patients from Metema areas were seropositive for *Brucella* spp. using the IgM/IgG Lateral Flow Assay,²⁹ suggesting high rates of transmission from animals to humans in pastoral areas.

Ethiopia is richly endowed with livestock, most of which are kept by small farmers. Unfortunately, the presence of endemic and transboundary animal diseases limits farmers' livelihoods as well as their health.

The aim of this study is to conduct a cross-sectional study, to estimate seropositivity of brucellosis in cattle, small ruminants and camels in southern and central Ethiopia and also to isolate and detect the circulating *Brucella* species in the study area using molecular methods.

Materials and Methods

Description of the Study Area

The study was conducted in southern and central areas of Ethiopia (Figure 1). From southern area, three different zones were sampled, with several districts in each. Specifically, in Wolayita Zone, the districts of Bolso Sore, Damote Sore, Damote Gale, and Soddo were studied. In South Omo Zone, the districts of Nyangatom, Hammer, Benatsemay, and Male were sampled. And in Borana Zone, the districts of Surupa, Arero, and Elowayu were sampled. All of these formed what is termed "southern area" in this paper. For the more central part of Ethiopia, West Shewa was sampled (Ambo district), and in East Shewa, four districts were sampled, including Adama, Lume, Batu, and Dugda. In addition, in the special

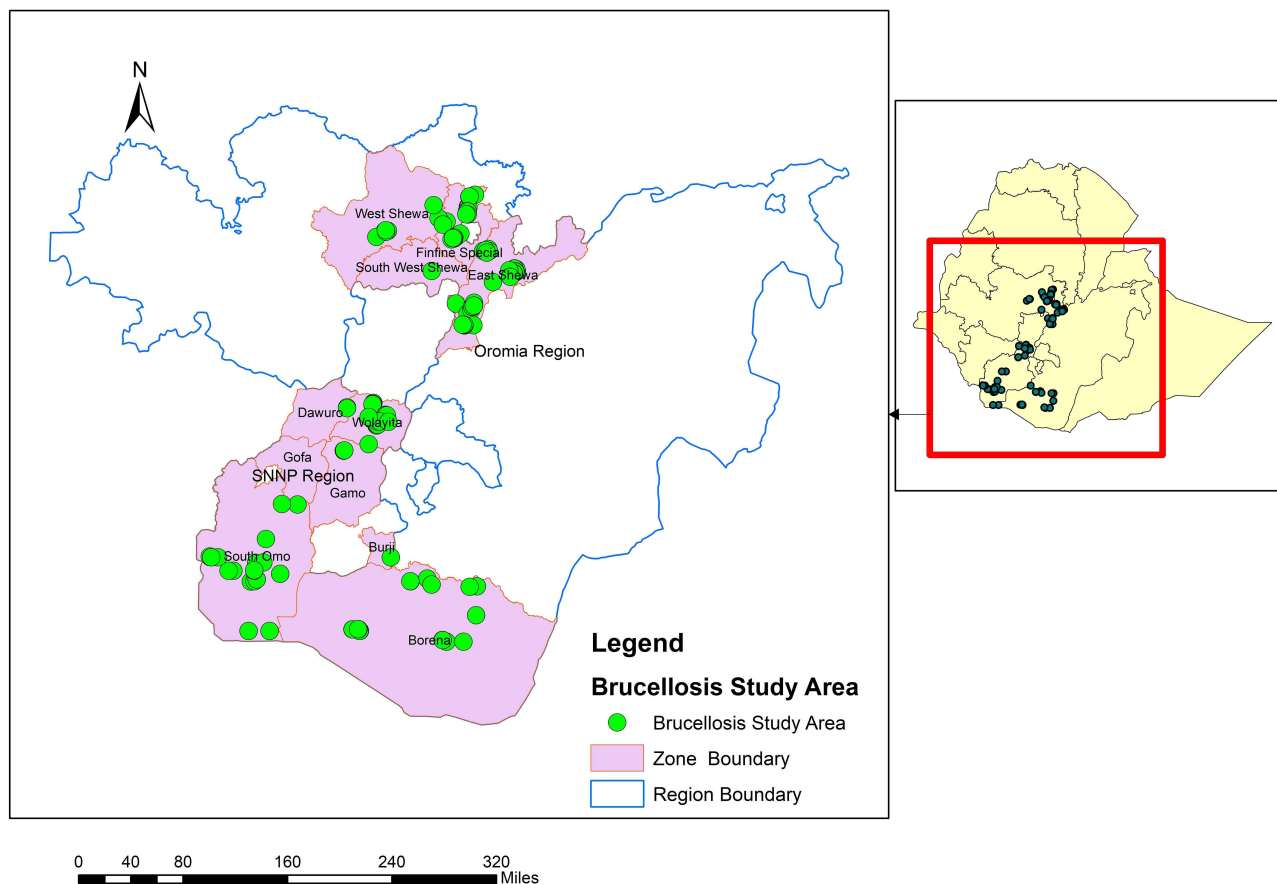


Figure 1 Map of the study areas and herds.

zone of Oromia surrounding Finfine, the districts of Holeta, Sululta, and Sebeta were sampled. These are considered “central area”.

Study Population

The study population included cattle, small ruminants and camels, all over six months of age. All sampling was done in 2021, and all animals sampled appeared clinically healthy. From the southern areas, all the target populations were managed under extensive pastoral systems. In Walaita and Central Ethiopia, study populations were primarily dairy, and all were managed under intensive production systems. Extensive rearing refers to those herds and flocks managed primarily without housing and in which animals may move long distances for forage. Intensive production systems refers to herds reared in which animals within the herd are always in close contact. All of the intensive systems in this study were dairy farms.

Sample Size Determination and Sampling Methodology

The sample size was calculated separately for bovine, small ruminants and camels based on the previous reports of seroprevalence for the species in the study areas according to the following formula:⁴²

$$n = \frac{1.96^2 * Pexp(1 - Pexp)}{d^2}$$

In this formula, n equals required sample size, Pexp equals expected prevalence, and d equals desired absolute precision. The desired precision was 5%, which allows for a 95% confidence level. Accordingly, the sample size for cattle sampling for the nine districts of the Central regions was calculated as 153 for each district based on previous reports of 11.2% (124/1106) seroprevalence and for the Borana were calculated as 115 based on previous reports of 8.2%.^{13,51} As there were no published reports from intensive dairy farms from Wolaita zone, the sample size was calculated by considering the expected prevalence of 50% and hence the calculated sample size was 384. Small ruminant sample size was calculated based on previous reports of 8.1% (23/283) and 4.2% (16/384) for Borana (230) and South Omo (124), respectively.^{5,49} Sample size for camel was calculated as 47 based on a report of 3.1% (12/384) seroprevalence.¹

Based on the above calculation, 2144 animals from all districts of the study areas were determined to be included into the study. However, sample size was increased approximately two-fold so as to increase precision and reduce standard error. Accordingly, in the present study, a total of 4274 animals from all districts were selected to investigate brucellosis for this study purpose, as shown in Table 1.

Multistage sampling was used to get the required animal samples for the southern areas. Zones were selected purposively and districts (woreda) from the selected zones were randomly selected and in turn kebeles and villages were selected randomly from the districts. Accordingly, from each selected district four villages and from the selected villages, households/herd/flocks were selected by simple random sampling. All animals within the selected herds were sampled. Total number of samples required was distributed according to the animal population proportionally for each administrative category. The milk producing districts from the central part were selected purposively and the farms from selected districts (towns) were selected randomly and all cattle from the selected farms were sampled.

Blood Sample Collection

For serological and molecular analysis, blood samples were collected aseptically from the jugular vein of individual animals. Approximately 5–7mL of blood was collected from each study animal using new plain vacuum tubes and then the blood

Table 1 Summary of Calculated and Collected Sample Size for This Study

Species	Calculated Sample Size				Collected Sample			
	Central	Wolaita	Borana	South Omo	Central	Wolaita	Borana	South Omo
Bovine	1377	384	115	–	2392	416	174	–
Small ruminant	–	–	230	124	–	–	360	731
Camel	–	–	47	–	–	–	201	–

samples were kept overnight at room temperature to allow clotting. The separated sera were then carefully moved into cryovials. Harvested sera and blood clots were transported to the Animal Health Institute serology and molecular laboratory in an insulated box containing ice packs. All were stored at -20°C in the laboratory until processing.

Bacteriological Sample Collection

Vaginal swab samples were collected using Stuart transport medium from animals that had a history of recent abortion. This included two cows and 11 small ruminants. Swabs were transported under cool conditions to the bacteriology laboratory of the Animal Health Institute and stored at -20°C until processed.

Laboratory Diagnosis

Serological Tests

Commercial brucellosis serum indirect multi-species ELISA Kit (BRUS-MS-5P ID Screen Brucellosis Serum Indirect, Multispecies, lot number C35) was used to detect antibodies directed against *B. abortus*, *B. melitensis* and *B. suis* from 4274 sera samples and performed as per manufacturer's instructions. This commercial test is not yet validated for use in camels, and currently there are no serologic tests fully validated for use in camels.⁴⁷ However, the OIE supports the ELISA for screening of flocks/herds and individual animals in all livestock species, including camels.⁴⁰ Optical density was measured at 450nm. The kit was verified as per kit instructions and the positive cut-off point was calculated as:

Sample positivity percentage

$$(S/p\%) = \frac{OD_{\text{sample}} - OD_{\text{NTC}}}{OD_{\text{PC}} - OD_{\text{NC}}} \times 100$$

Accordingly, samples with a s/p% less than or equal to 110% were considered negative, greater than 110% and less than 120% were considered doubtful, and greater than or equal to 120% were considered positive.

Bacteriological Test

Media Preparation and Culturing

Brucella selective media was prepared by suspending the required amount of *Brucella* medium base (CONDA Cat. 1374, Spain), in sterile 5% V/V inactivated horse serum (ie, horse serum held at 56°C for 30 minutes). Rehydrated contents of *Brucella* selective supplement (SR083A) were aseptically added to the sterilized *Brucella* basal medium and homogenized before plating and then 15 to 20 mL of the medium was poured into the Petri dish and allowed to solidify.³ The plates were incubated at 37°C for 48h for sterility check and no bacterial colony growths were considered as sterile and used for culture.

Thirteen vaginal swab samples were streaked directly from Stuart transport medium to the plate under Biosafety Level (BSL3) facilities with proper personal protections. Inoculated plates were incubated at 37°C aerobically. Duplicate samples were also incubated in the presence of 5%CO₂ (using anaerobic candle jar) for up to two weeks. The colonies were checked every 24h for *Brucella* species growth. *Brucella*-suspected colonies were characterized by their typical round, glistening, pinpoint and honey drop-like appearance according to standard methods.³

Microscopic Examination

Brucella suspected colonies were selected using a sterile plastic loop and mixed with a drop of sterile distilled water and smeared on a clean glass slide. The smear was heat fixed on the slide and air-dried. Identification of the organism was done by gram staining technique and Modified Ziehl-Neelsen staining technique.²⁴

Biochemical Test

Subsequent biochemical tests, including urea testing and lack of growth on MacConkey agar were also done.

Molecular Test

Genomic DNA Extraction from Blood Clots of Seropositive Animals

Following the result of serological test using ELISA kit, 169 blood clot samples from the seropositive animals were used for detection of *Brucella* nucleic acid using real time PCR. Genomic DNA was extracted using QIAMP DNA Mini Kit (QIAGEN GmbH strasse 1.40724 Hilden GERMANY) as per manufacturer's instructions.

Genomic DNA Extraction from Culture

DNA was extracted from solid media colonies by simple boiling method as described.²² Few colonies were removed and suspended in 500 μ L of sterile double distilled water in a 1.5mL micro-centrifuge tube and kept in a boiling water bath for 10 minutes. Five microliters of the supernatant were used for the PCR after centrifugation at 12000g for 3 minutes and the rest of the DNA sample was stored at -20°C .

Nanodrop DNA Examination

The extracted DNA was checked using Nanodrop spectrophotometer (THERMO, USA), which checks and measures the purity of DNA by reading the absorbance at (260/280 nm) and ng/ μ L concentration was calculated before PCR was performed using real time PCR.

Real-Time PCR

Real-Time PCR was performed for detection of *Brucella* spp. DNA from blood clot and culture samples by using the specific primers and TaqMan probe for IS711, *B. abortus* and *B. melitensis* sequence of forward and reverse as described in Table 2.

The thermocycler was run at 95°C for 10 min to denature double-stranded DNA, then amplification/extension occurred at 95°C for 15 second and 60°C for 1 minute for final extension. This process adjusted to run for 45 cycles. Finally, *Brucella* species was detected using species specific primers of *B. abortus* and *B. melitensis*. When the cycle threshold (CT) value of the samples were <45 , it was considered and evaluated as positive. If greater than 45, it was considered as negative.

Results

Serology

The seropositive distribution of *Brucella* spp. infection was assessed for both extensive and intensive production systems. The samples from the central area were all from bovine species and were all managed under intensive production system (intensive dairy farms). Samples from the southern area consisted of all species (bovine, small ruminant, and camel), and were all managed under extensive production systems except for cattle from Wolayita Zone where cattle were managed under intensive systems. The distribution of seropositive herds is presented in Figure 2.

The results of bovine seropositivity in the intensive system showed animal and herd level prevalence of 1.10% (31/2808) and 2.87% (5/174).

Individual animal level seroprevalence from extensive management system, for all species, is presented in Table 3 accordingly, the prevalence percentages were 9.19% (16/174), 8.89% (97/1091) and 12.44% (25/201) for cattle, small ruminant and camel, respectively. However, it needs to be noted that the ELISA, although proposed for adequate use in

Table 2 Primers and TaqMan®probes Used in This Study

Target Sequence	Forward Primer/Reverse Primer (5'→3')	Probe (5'Fluorophore→3'Quencher)
IS711	GCTTGAAGCTTGC GGACAGT/GGCCTACCGCTGCGAAT	FAM-AAGCCAACACCCGGCCATTATGGT-BHQ I
BMEI10466	TCGCATCGGCAGTTTCAA/CCAGCTTTTGGCCTTTTCC	FAM-CCTCGGCATGGCCCGCAA-BHQ-I
BruAb2_0168	GCACACTCACCTTCCACAACAA/CCCCGTTCTGCACCAGACT	FAM-TGGAACGACCTTTGCAGGCGAGATC-BHQ-I

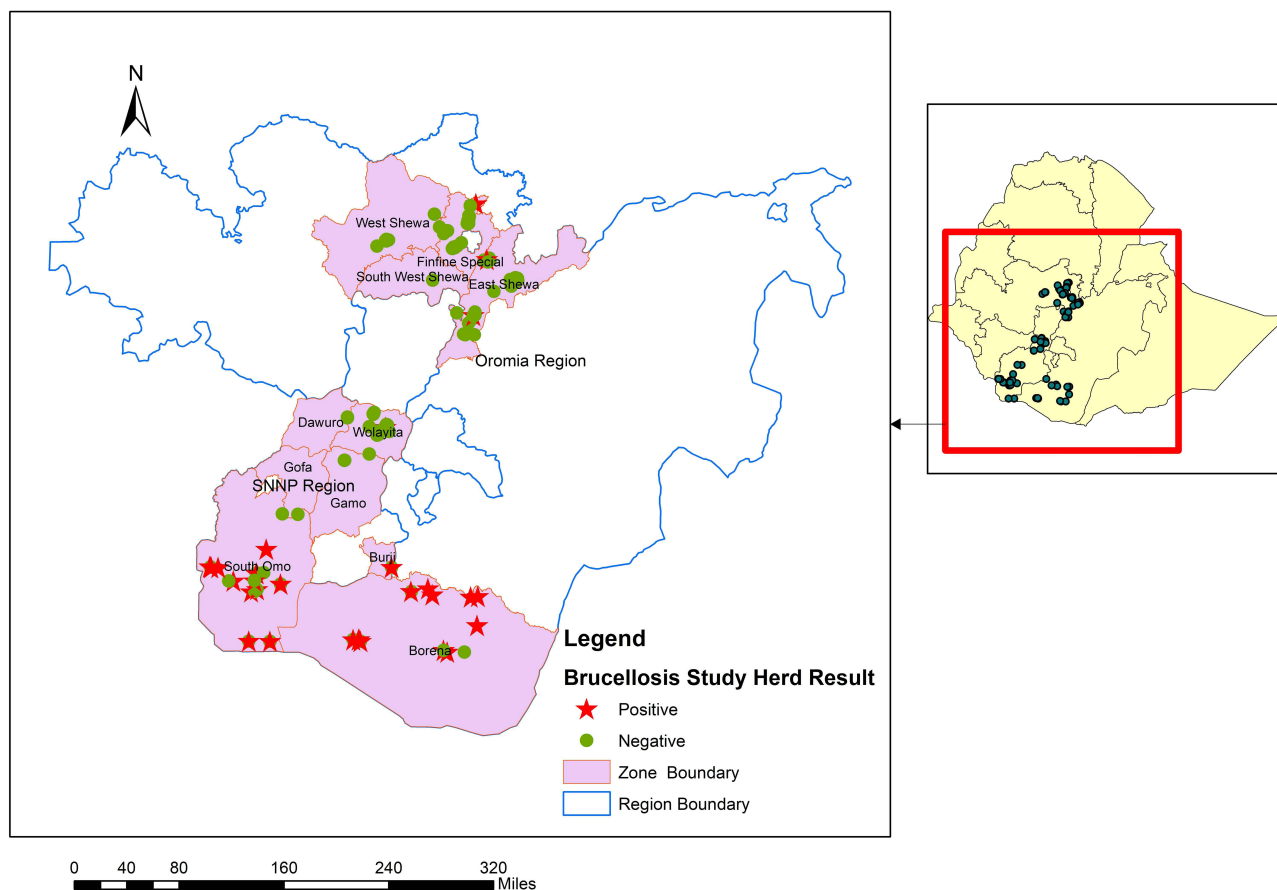


Figure 2 Distribution of *Brucella* seropositive herds.

camelids by the OIE, is not a validated test for that group of species, and so these results should be taken as presumptive and not definitive.⁴⁰

Isolation and Identification of *Brucella*

There was a total of 13 vaginal swabs collected in the field, all from animals with a recent history of abortion. These animals included 2 swabs from cattle, and 11 swabs from small ruminants. There were no vaginal swabs collected from camels. *Brucella* spp. were isolated from six of these, including both of the swabs from cattle and four of the 11 samples from small ruminants.

Initially, the isolates were recognized on the basis of colony morphology as having a characteristic *Brucella* growth with very small, glistening, smooth, round and pin-point, honey-like colonies on *Brucella* selective agar plates after 4 days of incubation at 37°C, both aerobically, and in the presence of 5% CO₂.

Table 3 Animal and Herd Level Seroprevalance Result from Extensive Management System

Species	Animal Level			Herd/Flock Level		
	Number Examined	Number Positive	Prevalence in %	Farm Tested	Farm Positive	Prevalence In %
Bovine	174	16	9.19	10	5	50.00
Small ruminant	1091	97	8.89	48	25	52.08
Camel	201	25	12.44	9	9	100.00
Total	1466	138	9.41	67	39	58.21

Microscopic examination was carried out immediately after the primary isolation of *Brucella* selective agar and Gram stained cultures showed small gram negative coccobacilli arranged individually and in pairs. With Modified Ziehl-Neelsen (MZN) stain, the organisms of *Brucella* stained red on a blue background (Figure 3A). The suspected colonies hydrolyzed urea within 2 hours (Figure 3B). No growth was observed on MacConkey agar and the colonies were non-haemolytic on blood agar.

DNA was extracted from all six cultures that were morphologically and biochemically consistent with *Brucella* spp. The purity and concentration were measured by Nanodrop spectrophotometer giving 2.06, 2.01, 2.09, 1.7, 1.79 and 2.09 of DNA purity and 464.7ng/μL, 351.0ng/μL, 281.6ng/μL, 369.7ng/μL, 35.6ng/μL and 584.7ng/μL DNA concentration. All of these DNA samples were subjected to real-time PCR screening using the IS711 gene and subsequently for *B. melitensis* and *B. abortus*. All six isolates were identified as *B. melitensis* and none as *B. abortus* as showed in Figure 4.

PCR on Blood Clots

One hundred and sixty-nine blood clots from seropositive animals were subjected to DNA extraction before performing PCR. These samples were screened by using IS711 gene, and all samples were negative. There was no further analysis on these samples and a summary of animal brucellosis based on the three test methods is presented in Table 4.

Discussion

The results of this study have many similarities to previous studies on seroprevalence of brucellosis in Ethiopia. Many previous studies in Ethiopia have recorded individual animal prevalence and are results compare overall with similarity to those previously published results. In our study, overall individual animal level prevalence of 1.58% (47/2982) 8.89% (97/1091) and 12.44% (25/201) of brucellosis in bovine, small ruminant, and camel, respectively.

For individual animal prevalence in cattle, our study yielded 1.58% (47/2982) whereas other studies had ranges from 0.2% to 11.2%.^{2,7,9,14,17,18,20,21,26,27,38,43,45,50,52} Differences may be due to differing geographic areas or production systems sampled.

In the current study, key differences were observed in bovine brucellosis among individual cattle managed under intensive and extensive systems, with extensive having higher seroprevalence (9.19%) as compared to seroprevalence of individual cattle managed under intensive system (1.10%) as indicated in Table 5. This may be because in extensive management cattle are mixing with sheep and goats. Furthermore, in the intensive system, there is less opportunity for naïve cows to access infective placentas, as the animals are more closely monitored and often confined. Also, the

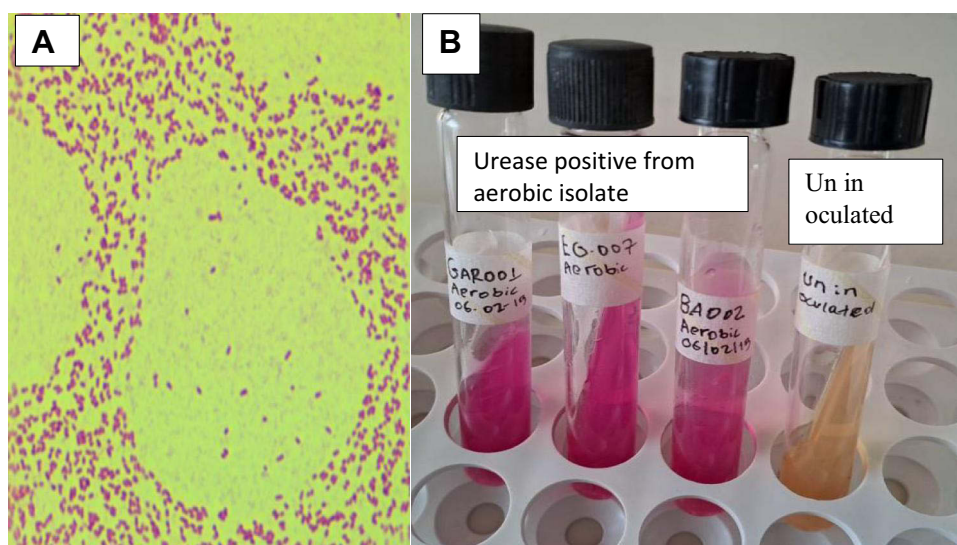
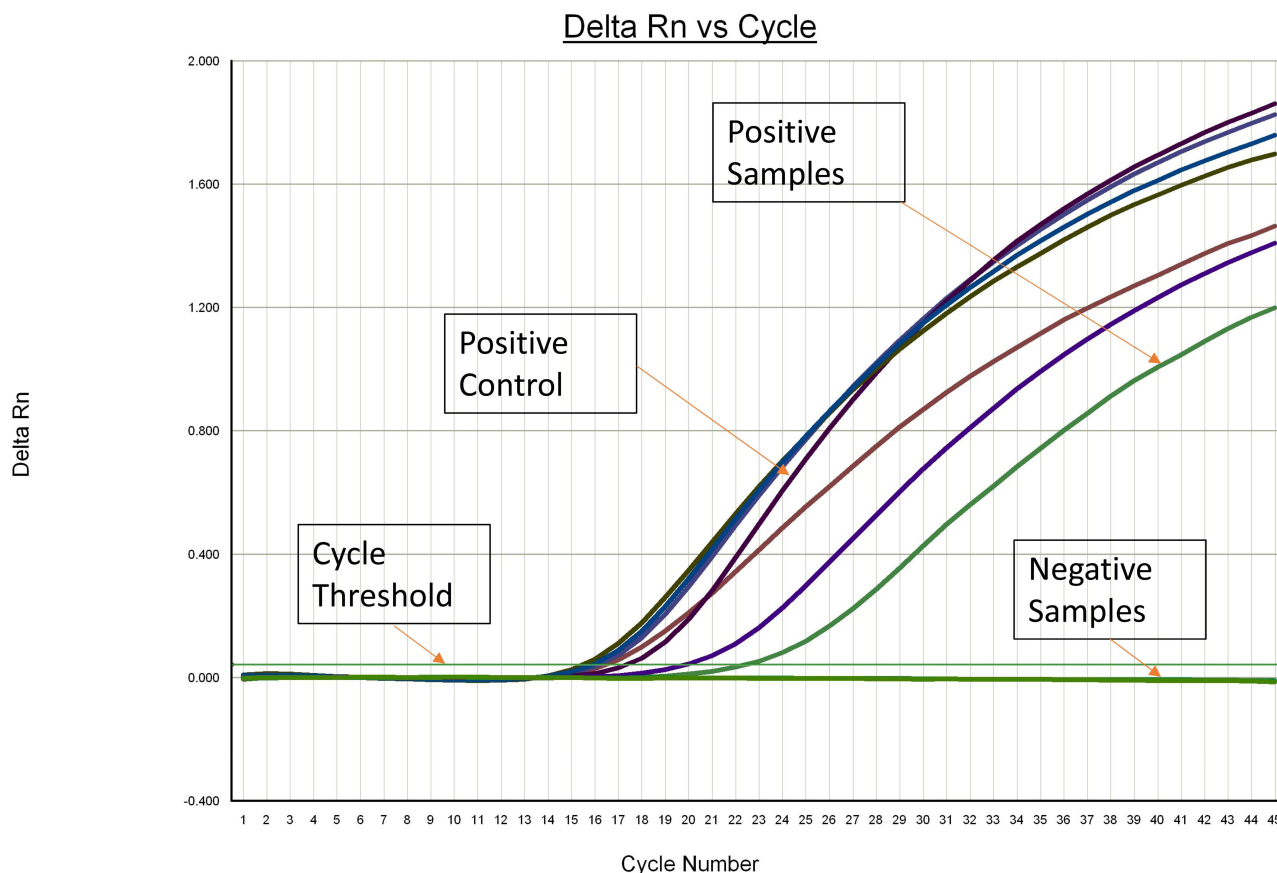


Figure 3 (A) Modified Ziehl-Neelsen (red-pink coccobacilli) stain of *Brucella* spp and (B) Urea Test Result within 2h in aerobic conditions.



Selected Detector: All
 Well(s): B1-B3,B5-B10
 Document: Bayeta reseach brucella 250419.sds (Standard Curve)

Figure 4 Real-time PCR amplification result of bacterial isolate using the *B. melitensis* primers.

biosecurity measures in the intensive management are higher than in extensive management systems. A previous study of dairy systems in Ethiopia, completed two decades ago, found a 0% seroprevalence for brucellosis.¹¹ The results from this study indicate that brucellosis is in quite a few dairy herds.

Table 4 Summary of Animal Brucellosis Using the Three Test Methods

Species	Total Number of Tested Sample			Test Result (ELISA, Culture and PCR)		
	ELISA	Culture	PCR	ELISA	PCR Result	
Bovine	2982	2	49	1.58 (47/2982)	From bacterial isolate 100% (2/2)	From Blood clot of sero positive animals All 47 were negative
Small ruminant	1091	11	4	8.89 (97/1091)	36.36%(4/11)	All 97 were negative
Camel	201	–	25	12.44 (25/201)	–	All 25 were negative
Total	4274	13	169	3.95 (169/4274)	46.15% (6/13)	All 169 were negative

Table 5 Sero-Prevalence of Bovine Brucellosis Among Cattle Reared Under Extensive and Intensive Management System

Management	Number Examined	Number Positive	Prevalence In %
Intensive	2808	31	1.10
Extensive	174	16	9.19
Total	2982	47	1.57

In the present study, for small ruminants, which were all under the extensive system, and all in the southern part of the country, the overall individual animal seroprevalence of brucellosis in small ruminants was 8.89% (97/1091). This is just in the mid-range of seroprevalences of small ruminant brucellosis reported elsewhere in Ethiopia, with ranges from 1.9% to 13.7%.^{4–6,16,23,28,33,35,37,39,46,49,50}

Regarding camels which were all in the southern part of Ethiopia and all under extensive management, the overall individual animal seroprevalence rate was 12.44% (25/201) which is slightly higher than what has been reported in previous studies, from 0.5% to 9.5%.^{1,10,13,15,16,30,34,36,44,48} Also, for camels, the herd seropositivity was 100%, but it should be noted that only nine herds were sampled. The results of this study regarding seropositivity in camels may indicate that camel brucellosis is increasing in Ethiopia. However, again, it needs to be noted that this ELISA test is not yet validated for camels. Additionally, none of the previously reported serologic studies for brucellosis in camels in Ethiopia were assessed using validated tests because none exist.

The variation in the animal and herd level prevalence among the reports might be attributable to the agro-ecology as a risk factor for brucellosis, the influence of the agro-ecological zone has a higher prevalence in dry zones.³² Since pasture areas are scarce in dry areas, animals must search for pastures in large areas that imply unrestricted animal-to-animal contact with potential transmissions. Similarly, in extensive farming system infected animals have the highest probability of close contact with healthy animals.

In this study, blood clots from the seropositive animals were assayed by RT-PCR for the presence of *Brucella* spp. All blood clots were negative. This is an indication that although animals are seropositive, and therefore likely infected at some time in the past, there was no *Brucella* spp. Circulating at the time of blood collection. At the initial infection, there is a bacteremia that is consistent, however, once infected, animals remain infected for life although the organisms are mostly sequestered within the lymphoid tissue. They do make periodic excursions from the lymph node to infect other areas, eg, placenta, but these potential periods of very low bacteremias are likely sporadic only.

Conclusions

The present study adds to the body of knowledge regarding the extent of *Brucella* infection in livestock in Ethiopia. In general, population levels of seropositivity were similar to previous studies done throughout the country, with the exception of camels, in which the rate of positivity was higher than seen in any previous study. More attention to public health measures surrounding camels and their products is indicated. Another notable finding was the higher seroprevalence in animals raised extensively, compared to intensive production, such as is seen in the dairy industry in Ethiopia.

Data Sharing Statement

All data generated and analyzed during this study are included in the manuscript. However, the raw data is available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

This study did not involve human participants. It only involved animal serum and swab samples. Research permit was provided by Animal Research Scientific and Ethics Review Committee of the Animal Health Institute, Sebeta, Ethiopia.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest in relation to this work.

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