Isolation and identification of *Brucella abortus* and *B. melitensis* in ruminants with a history of abortion: the first report from Eritrea

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Abstract

Brucella species have never been isolated or documented in Eritrea. The present study was initiated to isolate and identify *Brucella* species that infect livestock in Eritrea. A total of 316 blood sera, 137 milk, 71 vaginal swabs, a placental cotyledon, and a fetal stomach content were sampled from 208 goat, 102 sheep, and 6 cattle. Series testing protocol starting with Rose Bengal plate test, followed by c-ELISA, culturing, and then multiplex AMOS-PCR was followed. The purpose-sampling strategy was applied, and animals with a history of abortion in the last three weeks were sampled to increase the probability of capturing *Brucella* species. Isolation was conducted on *Burcella* media agar, and *Brucella* broth was added with *Brucella* selective supplement containing 2,500IU polymyxin B, 12,500IU bacitracin, 50.0 mg cycloheximide, 2.5mg nalidixic acid, 50,000 IU nystatin, and 10.0 mg vancomycin. Accordingly, 41 animals were positive for a series of serological tests. The overall *Brucella* seropositive detection proportion was 13.0%, and goats constituted the majority 78% (n=32). The highest number of positive samples were from the *Maekel* region. Six *Brucella* organisms were successfully isolated from two milk samples, two vaginal swabs, one placental cotyledon, and one fetal stomach content obtained from seropositive animals. Among the *Brucella* isolates, five were *B. melitensis,* while the remaining one was *B. abortus* as per the multiplex AMOS- PCR assay. The present study documented the first *B. abortus* and *B. melitensis* isolates in Eritrea. Milk, vaginal discharges, placental cotyledon, and aborted fetus were identified as sources of *Brucella* for livestock and livestock keepers. Besides, the findings highlight a reasonable proportion of the reproductive disorders in cattle and small ruminants could be due to

brucellosis. This calls for public sector intervention to control the diseases in animals and to enhance community awareness of good practices such as drinking boiled milk and avoiding contact with infected tissues and body discharges to prevent potential public health risks from zoonosis.

Keywords: *Brucella;* Cattle; Eritrea; Goat; Sheep.

Introduction

Brucellosis is a reemerging but neglected zoonotic disease of the tropics (Lopez-Goni *et al.*, 2008; Ghanbari *et al.*, 2020). A global threat, brucellosis, affects both domestic and wild animals, inflicting significant economic damage on livestock production (Godfroid *et al.*, 2013). This bacterial disease inflicts disorder on the reproductive health of animals, leading to infertility, delayed breeding cycles, lost calves, decreased meat and milk yields, and forced culling (Pappas *et al.*, 2005; Gonzalez-Espinoza *et al.*, 2021). Unfortunately, humans are not spared either. Brucellosis in humans, almost invariably linked to infected animals or their products, poses a particular risk to those in close contact, including farmers, animal handlers, slaughterhouse workers, and veterinarians (Zinsstag *et al.*, 2005; Godfroid *et al.*, 2013; Ghanbari *et al.*, 2020).

The genus *Brucella* is responsible for causing brucellosis, with *B. melitensis*, *B. abortus*, and *B. suis* being the most significant species in public health as they pose a high risk of human infection (Alton and Forsyth, 1996). *Brucella* spp. are gram-negative, nonmotile, non-spore-forming, slow-growing, facultative intracellular bacteria. Laboratory diagnosis is based on direct diagnosis by culture, indirect diagnosis by serological tests, and direct rapid diagnosis by molecular methods (Di Bonaventura *et al.*, 2021). Unequivocal diagnosis requires the isolation of the organism; therefore, culture is considered the 'gold standard' in laboratory diagnosis of brucellosis due to its clinical and epidemiological relevance (Ghanbari *et al.*, 2020; Di Bonaventura *et al.*, 2021). Serological tests, although they lack specificity and provide results that may be difficult to interpret in individuals repeatedly exposed to *Brucella* organisms, nevertheless remain a diagnostic cornerstone in resource-poor countries (Yagupsky *et al.*, 2019). *Brucella* DNA detection by polymerase chain reaction (PCR) is a more valid and definitive diagnostic method, and PCR is performed by amplification of specific genomic sequences *in vitro* (Lopez-Goni *et al.*, 2008; Wang *et al.*, 2014).

Brucella species are very difficult to grow, and the process is tedious, time-consuming, requires expensive biosafety facilities, and poses significant risks to the operator and environment (Godfroid *et al.*, 2010; Mathew *et al.*, 2015). Identification of members of the *Brucella* genus at the species level is essential for epidemiological reasons due to the strong association between the individual species and their naturally occurring hosts (Al Dahouk *et al.*, 2010). Moreover, *Brucella* species isolation and identification in a specific area or country is of great importance, as the findings could help the concerned authorities, decision-makers, and experts to design and implement appropriate prevention and control strategies, particularly for potential vaccine design based on the circulating *Brucella* species and strains (Refai, 2002; Zinsstag *et al.*, 2005; Kurmanov *et al.*, 2022).

Brucellosis is endemic to Eritrea (Efrem *et al.*, 2023). The presence of *Brucella* in Eritrea was first reported in 1943 (Cilli and Andolfato, 1943), and the pathogen persists both in livestock and human populations (Efrem *et al.*, 2023; Efrem *et al.*, 2022; Scacchia *et al.*, 2013; Omer *et al.*, 2002; Omer *et al.*, 2000;). All *Brucella* reports in Eritrea were based on indirect evidence generated through serology, except the study (Bereket *et al.*, 2021), which provided genetic evidence based on the PCR method. The present study was initiated to isolate and identify *Brucella* species that infect ruminants in the administrative regions of Maekel, Debub, Anseba, and the Northern Red Sea (NRS) of Eritrea.

Material and methods

Study area

The present study was conducted in four administrative regions of Eritrea, namely, Maekel, Debub, Anseba, and the Northern Red Sea (Figure 1). Eritrea is found in the Horn of Africa, bordering Ethiopia, Sudan, Djibouti, and the Red Sea. Eritrea is located between latitudes 12 ° 42’ N to 18° 2’ N and longitudes 36° 30’ E to 43° 20’ E. The country is divided into six administrative regions, namely Anseba, Debub, Gash-Barka, Maekel, Northern Red Sea (NRS), and Southern Red Sea (SRS). The four administrative regions were selected purposively due to the high population of livestock and history of brucellosis (Omer *et al.*, 2000; Omer *et al.*, 2002; Efrem *et al.*, 2022; Efrem *et al.*, 2023). The total estimated number of livestock species is approximately 3.15 million goats, 2.14 million sheep, 1.70 million local cattle, 0.22 million dairy cattle, 0.29 million donkeys, 0.19 million camels, and 0.002 million swine (MOA, 2019).

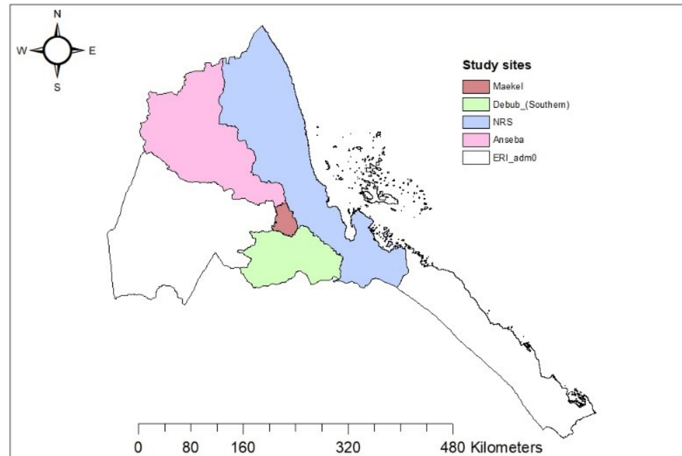

Figure 1. Map of Eritrea showing the study area

Study design and study population

The study was a case series study looking for livestock species with a recent history of reproductive disorder, *i.e.,* abortion/retained fetal membrane. Serological screening tests and bacteriological and molecular methods were deployed for the screening, isolation, and identification of *Brucella* species from the study population. The study population includes cattle, sheep, and goats with a history of reproductive disorder in intensive/semi-intensive and extensive farms of Maekel, Debub, Anseba, and NRS administrative region of Eritrea.

Sample size determination and sampling techniques

To specifically focus on animals potentially infected with *Bucella*, a purpose-sampling strategy was employed. During farm visits, livestock owners identified animals that had experienced late-term abortion or retained fetal membranes within the past three weeks. The animals were then recruited for specimen collection. Taking into account an expected prevalence of 30% based on Rose Bengal Test (RBT) data (Alsaad, 2022), the required sample size to detect *Brucella*-positive animals with a history of abortion was calculated using Epi InfoTM version 7.2.6 (CDC, USA). When a design effect of 1% was taken into account, a 5% error margin, and a 95% confidence level, the minimum sample size was determined to be 323; however, only 316 animals were

sampled for logistic reasons. In addition to serum samples, biological samples, including milk, vaginal swabs, placenta cotyledon, and abomasa content of aborted fetuses, were collected from 208 goats, 102 sheep, and 6 cattle. Ultimately, the collection consisted of 316 blood serum, 137 milk samples, 71 vaginal swabs, one placental cotyledon, and one fetal stomach content.

Sample collection and processing

For laboratory analysis, a 5 ml blood sample was collected in plain vacutainers. Serum separation was then performed using centrifugation. The resulting clear serum was harvested into cryo-tubes. The remaining whole blood and clear sera were stored at -20 °C until analysis. A pooled 10 ml of milk from the four quarters, a vaginal swab in 2 ml of PBS, placental cotyledon in 50 ml of sterile PBS container, and the content of fetal stomach (2 ml) were collected. All samples were adequately labeled with animal ID, sample type, and collection date. They were then transported in an ice box stored at + 4 °C until processed at the National Animal and Plant Health Laboratory (NAPHL) at the Ministry of Agriculture in Asmara.

Serological test

All sera samples were tested for *Brucella* using the RBPT kit (Animal Health and Veterinary Laboratory Agency; Surry, UK). The RBPT test was carried out following a standard protocol (Diaz *et al.*, 2011; Legesse *et al.*, 2023). In summary, 30µl of serum was mixed with an equal volume of antigen on a white tile that produced a zone of 2 cm in diameter. The mixture was gently agitated for 4 minutes at room temperature and then observed for agglutination. Consequently, serum with any visible agglutination was considered positive for *Brucella* antibodies and further confirmed by ELISA.

Blood sera screened as positive by RBPT were further tested using c-ELISA for confirmation following the protocol supplied with the reagent kit (INGNASA: Adeva Dela Institucion Libre de Ensenanza; Madrid, Spain), using a plate reader machine (Thermo, Multiscan Ascent). Each test result was validated as follows: (i) If the OD in the negative control wells (NC) was greater than 1 and the OD in the positive control wells (PC) was less than 0.35, then the result was valid for calculation. (ii) The percentage of inhibition (PI) of each sample was calculated as PI=100 × [1-(OD sample/OD negative control)]. (iii) Sera with PI≥ 40% were referred to as positive for *Brucella* antibody, while

sera samples with PI <40% were recorded as negative results. The sensitivity and specificity of the c-ELISA test were 95.2% and 99.7%, respectively.

Bacteriological procedures

Serologically positive blood samples and connected specimens were subjected to bacteriological procedures. Samples were processed in the BSC Class II Safety Cabinet (Telstar Bio-II-A) with strict application of personal protection equipment (PPE) for laboratory personnel biosafety. During this operation, access to the entire facility was restricted except for investigators—*Brucella* spp. Isolation and preliminary identification using biochemical tests were carried out as previously described in the WOAH manual (WOAH, 2018) and NAPHL standard operating protocol. The brief description of the bacteriological protocol is as follows:

Media preparation: *Brucella* isolation was performed using *Brucella* selective growth media. This includes *Brucella* media agar (CM 0169, Oxoid, England) and *Brucella* broth added with *Brucella* selective supplement (SR0083A, Oxoid, England) containing 2,500IU polymyxin B, 12,500IU bacitracin, 50.0 mg cycloheximide, 2.5 mg nalidixic acid, 50,000 IU nystatin, and 10.0 mg vancomycin, to inhibit other contaminates and enhance the growth of *Brucella* species. The medium was further enriched with filter-sterilized heat-inactivated (56 °C /30 min) 5% horse serum plus 0.5% dextrose and 0.5% methanol.

***Sample preparation and inoculation*:** Milk samples were centrifuged at 2000 g rpm for 15 minutes at 20 °C to obtain the sediment pellet at the bottom and the supernatant cream at the top. After sucking and removing the middle layer, the fatty layer and sediment were mixed using a sterile swab. Using the same swab, two *Brucella* agar plates were inoculated, and the remaining material was inoculated in 10 ml of *Brucella* broth. The vaginal swab was directly streaked on the prepared solid media (CM 0169, Oxoid, England) and inoculated into *Brucella* broth with supplement (*SR0083A,* Oxoid, England), similar to that of the milk sample. The surface of the cotyledon samples was flamed with a spatula, and the internal parts were cut into small pieces and homogenized using sterile mortar and pestle with little PBS. Then, from the homogenized sample, two drops were placed on each *Brucella* agar plate and spread using the streaking method, along with 1 ml to 10 ml of *Brucella* broth. Similarly, two drops and 1 ml of the fetal stomach content were inoculated into a *Brucella* agar plate and *Brucella* broth, respectively.

Incubation: *Brucella* media inoculated agar plates and broth bottles were incubated at 37 ^{0}C in the presence and absence of 5-10% CO_2 and checked after 48 hours, then daily for up to 7 days for both primary and consecutive subcultures. After seven days, plates and broth with no signs of growth were discarded. However, broths with signs of growth were cultured in agar plates and followed the same procedure as above to the end.

Brucella isolation: *Colonies:* Colonies with translucent, smooth margins and a light yellow color were suspected to be *Brucella* species. A typical colony was picked and examined using Gram's stain and modified Ziehl-Neelsen stain (MZN) methods. If gram-negative coccobacillus and red coccobacillus on a blue background in MZN staining were observed, then the suspected colony was further sub-cultured and examined.

Biochemical test: The grown subcultures were subjected to different phenotypic tests, including catalase, oxidase, urea hydrolysis, motility, growth in dyes (on 20 and 40 µg /ml of basic fuchsine and thionin), CO_2 requirement, H_2S production, and hemolysis tests were performed following standard protocols for each test. Plates showing positive growth for *Brucella* organism were sub-cultured and, after 48 hours, sent to the molecular biology laboratory for species-level identification.

Molecular protocols

***DNA extraction*:** The DNA genome was extracted from heat-inactivated pure *Brucella* culture using the High Pure template preparation kit (DNA HP kit, Roche Applied Sciences, Mannheim, Germany) according to the manufacturer's instructions.

Brucella AMOS-PCR Assay: *Brucella* species identification was performed using the *Brucella* AMOS-PCR kit as per a published protocol (Bricker and Halling, 1994). The specific primers of *B. abortus, B. melitensis, B. ovis,* and *B. suis* are listed in Table 1.

Table 1. Primers used for the identification of *Brucella* species

PCR type: AMOS	Primer type	Sequence (5′ to 3′)	Target gene	Product length (bp)
	Forward	GAC GAA CGG AAT TTT TCC AAT CCC	IS711	498
B. abortus	Reverse	TGC CGATCA CTT AAG GGC CTT CAT		
B. melitensis	Forward	AAATCG CGT CCT TGC TGG TCT GA	IS711	731
	Reverse	TGC CGATCA CTT AAG GGC CTT CAT		
	Forward	CGG GTT CTG GCA CCATCG TCG	IS711	976
B. ovis	Reverse	TGC CGATCA CTT AAG GGC CTT CAT		
	Forward	GCG CGG TTT TCT GAA GGT TCA GG	IS711	285
B. suis	Reverse	TGC CGATCA CTT AAG GGC CTT CAT		

DNA amplification was performed using a conventional PCR machine (Eppendorf: Master cycler ingredient). The amplification cycles consisted of initial denaturation at 95 ◦C/5 min, followed by 35 cycles of denaturation at 95 ◦C /60 s, annealing at 58 ◦C for 2 min and then elongation at 72 ◦C for 2 min, and a final elongation step at 72 ◦C for 7min. PCR products were separated by gel electrophoresis using 1.5% agarose gels stained with ethidium bromide. Gel images were captured using UV light in a Gel-Imaging system (BIO-RADTM, USA) at 100 V for 60 min. A 100bp reference DNA marker ladder (BioLabsTM, New England) was used to determine the amplified band size corresponding to each Brucella spp. Furthermore, known strains of B. melitensis and water were used as positive and negative controls, respectively.

Data management and analysis

Laboratory results and related information were recorded in the Microsoft Excel 2016 worksheet. Descriptive statistics were used to report the proportion detected from various specimens. A proportion is a division of the total number of animals or samples determined as positive over the total number of animals or samples examined in a given diagnostic procedure multiplied by 100.

Ethical consideration

The study was approved ethically and authorized by the Ministry of Agriculture of the state of Eritrea. Informed consent was obtained from the herders after the necessary explanation of the study objectives provided by the research team. Participation was voluntary, and participants were informed to withdraw at any time if they decided to do so. Any information acquired from the

participants was kept strictly confidential. Trained animal health technicians and veterinary professionals collected samples.

Results

Serological analysis

Antibodies specific to the *Brucella* genus were detected in 45 (14.2 %) sera samples in RBPT, and 41 (13%) were confirmed positive on consecutive c-ELISA. The positive sera were from 32 of 208 goats (15.4%), 7 of 102 sheep (6.9%), and 2 of 6 cattle (33%). A relatively larger number of positive samples originated from Maekel (17.3%), followed by Debub (9.3 %) administrative regions. The detailed serological results are presented in Table 2.

Table 2. Serological analysis of blood samples from ruminants with a recent history of abortion

Region sampled	No . of seropositive/ total animal tested (%)			
	Goat	Sheep	Cattle	Sub-total
Maekel	25/118 (21.2)	6/62 (9.7)	0/2 (0)	31/182 (17.0)
Debub	7/70 (10.0)	1/35 (2.9)	2/4 (50.0)	10/108 (9.3)
Anseba	0/14 (0)	0/4 (0)	0	0/18 (0)
NRS	0/6(0)	0/2	0	0/8 (0)
Total	32/208 (15.4)	7/102 (6.9)	2/6(33.3)	41/316 (13)

NRS: North Red Sea

Detection and identification of *Brucella* spp.

Brucella suspected 253 samples were cultured in a selective medium, and six of the samples produced colonies that resemble *Brucella* spp (Figure 2). Therefore, the overall isolation rate of *Brucella* spp was 2.4%. The sources of positive samples include milk (1.5%), vaginal swabs (2.8%), a placental cotyledon, and one fetal stomach content (Table 3). All culture-positive samples were obtained from seropositive animals. However, none of the blood samples that were seropositive on c-ELISA (n =41) produced *Brucella* colonies. Furthermore, no samples from seronegative animals produced colonies. All samples of sheep origin were negative on the bacteriological protocol.

Table 3. Number and types of samples that produced *Brucella* colonies in culture

Sample source and type Goat	No. Positive samples/No. tested			
	Sheep	Cattle	Sub-total (%)	
Blood	0/32	0/7	0/2	0/41
Milk	2/110	0/25	0/2	2/137 (1.5)
Vaginal swab	2/47	0/24	0/2	2/73 (2.7)
Placental cotyledon	-	-	1/1	1/1
Fetal stomach content	-	-	1/1	1/1
Total (% positive)	**6/253 (2.4)**			

PCR-based *Brucella* species identification

Six *Brucella* spp colonies isolated and identified through biochemical tests were further processed for species-level identification using the published AMOS-PCR protocol (Bricker and Halling, 1994). Consequently, five of the six *Brucella* isolates were identified as *B. melitensis,* and the other isolate was *B. abortus*. Figure 3 depicts the PCR result for the *Brucella* species identification protocol.

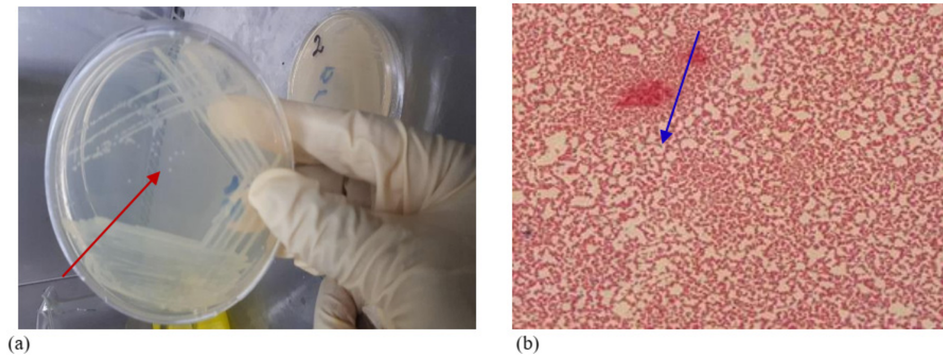

Figure 2. *Brucella* species isolated in Eritrea, 2023*:* (a) *B. melitensis* culture colony grown from goat vaginal swab sampled from a village (Beleza); (b) Gram-negative stained *B. abortus* from cow fetal stomach content sampled from a farm in Dekemhare.

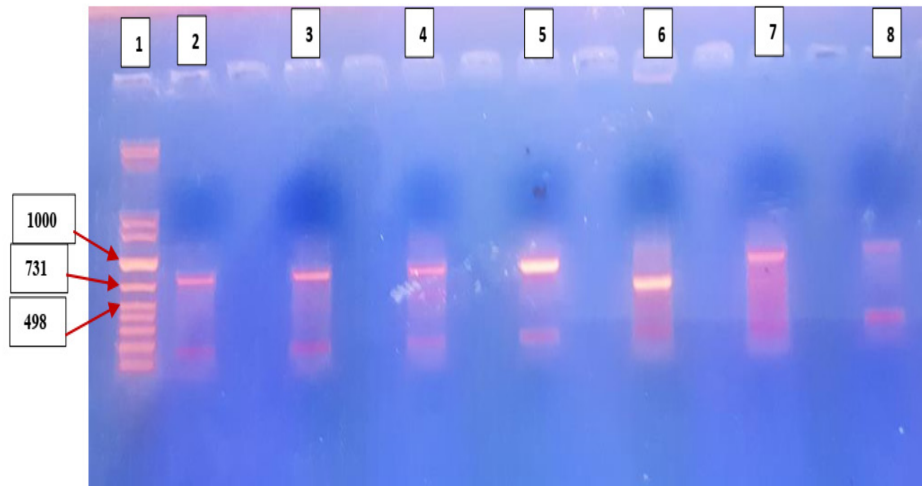

Figure 3. Agarose gel pictures show *Brucella* species-specific bands amplified using AMOS-PCR techniques. Lane 1: DNA marker (100bp); lane 2: *B. melitensis* reference strain 16M; lane 3,4,5,7 and 8 show the DNA band specific (731 bp) for *B. melitensis;* lane 6 showed the DNA band specific (498 bp) for *B. abortus*

Discussion

The present study detected 41 seropositive animals in a series of tests with RBPT and c-ELISA from the administrative regions of Maekel and Debub in Eritrea. The detection rate of *Brucella* in animals with a recent history of abortion was 13%, a relatively higher finding than the 5.6%, 4.3%, 1.4%, and 3.1% rate of detection in local cattle, goats, sheep, and camels, respectively (Omer *et al.*, 2000). A decade ago, a 2.8% seropositive report was made on dairy cattle that had a history of abortion in five regions of Eritrea (Scacchia *et al.,* 2013*).* Our previous study also detected a relatively lower proportion (1.1%) of dairy cattle in the Maekel and Debub regions of Eritrea (Efrem *et al.*, 2023). The disagreement with previous studies could be associated with differences in sampling strategy, or the disease might have advanced. In the present study, only animals with a history of late abortion were sampled to increase the probability of detecting *Brucella*-infected animals. On the other hand, in the previous studies, blood sera were collected from all animals; in most cases, random sampling was employed. Researchers who targeted *Brucella*-suspected goats in the Maekel region reported a comparable result (14.60%) to the present study (Bereket *et al.*, 2021).

A large number of animals (n = 275) with a recent history of late abortion were negative on serology. A possible explanation for this could be the history of abortion recorded in the data set was not necessarily caused by *Brucella* infection, other factors, and animal diseases that can induce abortion at different stages of the gestation period. For example, infectious agents (*Coxiella burnetii*, *Leptospira* spp, *Listeria monocytogenes,* viruses, protozoa like *Neospora caninum* and fungus); noninfectious agents (physical trauma, stress, toxemia, metabolic disorder, hormonal effect, nutritional deficiencies, hereditary factors, housing conditions, and other physical factors) can induce abortion in animals (Beuzón *et al.*, 1997; Vidic *et al.*, 2007; Clothier and Anderson, 2016). Bacteriological cultures (n =253) were carried out for both samples that came from seropositive and seronegative animals. However, *Brucella* colonies were isolated from only six seropositive animals. The remaining 35 seropositive animals did not form colonies in *Brucella* selective media. The detection rate differences observed between the serological test and culture methods could be caused by the cross-reactivity of antibodies against the *Brucella* lipopolysaccharide virulence factor, which is commonly used for the development of *Brucella* ELISA kit (Golchin *et al.*, 2023). Gram-negative bacteria such as *Escherichia coli* O157, *Vibrio cholera*, and *Yersinia enterocolitica* have been reported to result in cross-reactivity (Golchin *et al.*, 2022). Blood cultures from seropositive animals also did not yield *Brucella* colonies. Similar challenges of isolating *Brucella* from blood were reported elsewhere (Pappas and Papadimitriou, 2007; Patel *et al.*, 2017). Failure of isolation may be due to a low number of viable organisms (Warethc *et al.*, 2014), localization of *Brucella* in predilection tissues such as lymph nodes and uterus as an intracellular organism (Pappas *et al.*, 2005), or it could be associated with the intermittent availability of *Brucella* in peripheral blood (Yagupsky, 2015). Technical limitations could be an additional cause of the low isolation rate of *Brucella* from biological specimens in the present study.

Meanwhile, the highest number of isolates was recorded from goat species. Consequently, the highest number of *Brucella* seropositivity (n = 32) and the number of recovered *Brucella* isolates (n = 4) was observed in goats. Most likely, this was associated with the most significant number of goats accounting for the higher proportion of livestock population in the country. Additionally, goats are considered to be more susceptible to brucellosis (Rajala *et al.*, 2016). The detection of *Brucella* in goat milk has been reported elsewhere (Lonkar *et al.*, 2023). The presence of *Brucella* in milk is very concerning as it could

increase the risk of transmission to humans. In a previous community perceptions and practices study conducted on livestock owners in the NRS region, 90.3% (n = 575) of the participants reported that they handled and disposed of birth materials barehanded, which is a high-risk practice for brucellosis infection (Efrem *et al.*, 2022). Therefore, goat herders who assist in parturition without PPE and drink raw milk and dairy products should be aware of this risk and avoid such risky practices.

Five of the six *Brucella* isolates were identified as *B. melitensis* and one *B. abortus* using the AMOS-PCR method. The current result is in agreement with the recent finding in the same study area (Bereket *et al.*, 2021), who detected *B. melitensis* DNA from a vaginal swab of goats with a history of abortion using the duplex PCR method. It is important to note that in the current study, *B. melitensis* was isolated from the placental cotyledon of cattle. In most cases, such cross-infection to a nonspecific host occurred when different animal species were mixed and herded together. In Eritrea, especially in the highlands, rearing different animal species (cattle, sheep, goat, danker, dog) in the same herd/flock was a common practice. In addition, due to limited land area, different livestock species were housed together in a small room or yard, creating favorable conditions for the cross-transmission of *Brucella* species among the various animal species. Herding mixed populations of cattle, sheep, and goats is the main factors that contribute to the emergence and cross-infection of *B. melitensis* and *B. abortus* to nonspecific hosts, as explained elsewhere (Hegazy *et al.*, 2011). It is well documented that *B. melitensis* is the most virulent of all *Brucella* species for humans and animals, followed by *B. abortus* and *B. suis* (Alton and Forsyth, 1996; Mahmoud and Hamdy, 2018). However, the study did not provide molecular evidence for cross-infection to nonspecific hosts. Additionally, the limitation of the present study includes the use of a purposive sampling protocol that limits the extrapolation of the result to the epidemiology of brucellosis in Eritrea.

Conclusions

In this study, both *B. abortus* and *B. melitensis* were isolated and identified successfully for the first time in the country. *B. melitensis* was the most dominant *Brucella* species infecting goats and cattle. Milk, vaginal discharges, placental cotyledons, and fetal stomach content were confirmed as a source of *Brucella* isolates. National initiatives are recommended to commence control interventions in animals, strengthen community awareness, and promote good

practices, such as drinking boiled milk and proper disposal of aborted fetuses and the placenta. Further molecular epidemiological studies on the isolates to understand the cross infections and relatedness of Eritrean isolates with the global database are commendable.

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Data availability

Data are available upon request from Efrem G. (yafet1212@gmail.com)

Reference

Al Dahouk, S., Scholz, H.C., Tomaso, H., Bahn, P., Gollner, C., *et al.*, 2010. Differential phenotyping of Brucella species using a newly developed semi-automated metabolic system. *BMC Microbiol.*, 10, 269.

Alsaad, K.M., 2022. Detection of Brucella abortus in Cows Suffering From Some Reproductive Disorders, *Egypt. J. Vet.Sci.,* 53 (4), 605-612.

Alton, G., and Forsyth, J. 1996. “Brucella.” In *Medical Microbiology*, edited by Baron S. Galveston, Texas University of Texas Medical Branch.

Bereket, W., Mesfin, A., Beyene, N., Afewerki, Y., Abdelkadr, M., *et al.*, 2021. Investigation of brucellosis using molecular and serological tools in selected sites of Maekel Region, Eritrea. *Eur. J. Biol. Biotechnol.*, 2 (3), 92-96.

Beuzón, C., Schiaffino, A., Leori, G., Cappuccinelli, P., Rubino, S., and Casadesús, J., 1997. Identification of *Salmonella* Abortusovis by PCR amplification of a serovar-specific IS200 element. , *Appl. Environ. Microbiol.,* 63 (5), 2082–2085.

Bricker, B.J., and Halling, S.M., 1994. Differentiation of Brucella abortus bv. 1, 2, and 4, *Brucella melitensis, Brucella ovis,* and *Brucella suis* bv. 1 by PCR. *J. Clin. Microbiol.*, 32 (11), 2660-2666.

Cilli, V., and Andolfato, A., 1943. Primi dati sulla diffusione dell` infezione brucellare nel ovine e caprini del` Eritrea. *Bollentino Della Sociata' Italiana Di Medecina E Igiene Tropicale (Sezione Eritrea)-Asmara*, 2, 1-5.

Clothier, K., and Anderson, M., 2016. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. *Theriogenology*, 85 (5), 933-938.

Di Bonaventura, G., Angeletti, S., Ianni, A., Petitti, T., and Gherardi, G., 2021. Microbiological laboratory diagnosis of human brucellosis: An overview, *Pathogens*, 10 (12), 1623. doi: 10.3390/pathogens10121623.

Diaz, R., Casanova, A., Ariza, J., and Moriyon, I., 2011. The Rose Bengal Test in human brucellosis: a neglected test for the diagnosis of a neglected disease, *PLoS Negl. Trop. Dis.*, 5 (4), e950.

Efrem, G., Berhe, T., Fitsum, K., Samuel, W., Abraham, D., *et al.*, 2022. Community knowledge, attitude, practice and prevalence of brucellosis in livestock owners in Northern Red Sea Region. *J. Vet. Health Sci.*, 3 (2), 54-64.

Efrem, G.H., Mihreteab, B., Ghebremariam, M.K., Okbamichael, T., Ghebresilasie, Y., Mor, S.M., *et al.*, 2023. Prevalence of brucellosis and associated risk factors in dairy cattle in Maekel and Debub Regions, Eritrea, *Front Vet. Sci.*, 10 1177572.

Ghanbari, M.K., Gorji, H.A., Behzadifar, M., Sanee, N., Mehedi, N., and Bragazzi, N.L., 2020. One health approach to tackle brucellosis: a systematic review. *Trop. Med. Health*, 48, 86.

Godfroid, J., Al Dahouk, S., Pappas, G., Roth, F., Matope, G., *et al.*, 2013. A "One Health" surveillance and control of brucellosis in developing countries: moving away from improvisation. *Comp. Immunol. Microbiol. Infect. Dis.*, 36 (3), 241-248.

Godfroid, J., Nielsen, K., and Saegerman, C., 2010. Diagnosis of brucellosis in livestock and wildlife. *Croat Med. J.*, 51 (4), 296-305.

Golchin, M., Mollayi, S., Mohammadi, E., and Eskandarzade, N., 2022. Development of a diagnostic indirect ELISA test for detection of *Brucella* antibody using recombinant outer membrane protein 16 kDa (rOMP16). *Vet. Res. Forum*, 13 387–391.

Golchin, M., Mollayi, S., Mohammadi, E., and Eskandarzade, N., 2023. Serodiagnosis of human brucellosis by an indirect ELISA test using recombinant outer membrane protein 19 kDa (rOMP19) as an antigen. *BMC Biotechnol.,* 23, 46(2023). https://doi.org/10.1186/s12896-023-00817-2.

Gonzalez-Espinoza, G., Arce-Gorvel, V., Memet, S., and Gorvel, J.P., 2021. Brucella: Reservoirs and niches in animals and humans. *Pathogens*, 10 (2), 186. doi: 10.3390/pathogens10020186.

Hegazy, Y.M., Molina-Flores, B., Shafik, H., Ridler, A.L., and Guitian, F. J., 2011. Ruminant brucellosis in Upper Egypt (2005-2008). *Prev. Vet. Med.*, 101 (3-4), 173-1781.

Kurmanov, B., Zincke, D., Su, W., Hadfield, T.L., Aikimbayev, A*., et al.*, 2022. Assays for identification and differentiation of *Brucella* species: A review. *Microorganisms*, 10 (8), 1854.

Legesse, A., Mekuriaw, A., Gelaye, E., Abayneh, T., Getachew, B*., et al.*, 2023. Comparative evaluation of RBPT, I-ELISA, and CFT for the diagnosis of brucellosis and PCR detection of *Brucella* species from Ethiopian sheep, goats, and cattle sera, *BMC Microbiol.,* 23 (1), 216.

Lonkar, S., Thorat, V., Gandge, R., Pharande, R., and Gaikwad, S., 2023. Detection of *Brucella melitensis* in milk and serum samples of goats by serological and molecular techniques. *Indian J. Anim. Sci.,* 93(8), 764–769.

Lopez-Goni, I., Garcia-Yoldi, D., Marin, C.M., de Miguel, M.J., Munoz, P.M*., et al.*, 2008. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains. *J. Clin. Microbiol.*, 46 (10), 3484-3487.

Mahmoud, E.R., and Hamdy, H., 2018. Detection of virulence-associated genes in *Brucella melitensis* biovar 3, the prevalent field strain in different animal species in Egypt. *Open Vet. J.*, 8 (1), 112-117.

Mathew, C., Stokstad, M., Johansen, T.B., Klevar, S., Mdegela, R.H*., et al.*, 2015. First isolation, identification, phenotypic, and genotypic characterization of *Brucella abortus* biovar 3 from dairy cattle in Tanzania, *BMC Vet. Res.*, 11, 156.

MOA. 2019. "Annual report." In, edited by Animal Health Division Agricultural Extension Department. Asmara, Eritrea: Eritrean Ministry of Agriculture.

Omer, M.K., Assefaw, T., Skjerve, E., Tekleghiorghis, T., and Woldehiwet, Z., 2002. Prevalence of antibodies to *Brucella* spp. and risk factors related to high-risk occupational groups in Eritrea. *Epidemiol. Infect.,* 129 (1), 85-91.

Omer, M.K., Skjerve, E., Holstad, G., Woldehiwet, Z., and Macmillan, A.P., 2000. Prevalence of antibodies to *Brucella* spp. in cattle, sheep, goats, horses and camels in the State of Eritrea; influence of husbandry systems. *Epidemiol. Infect.*, 125 (2), 447-453.

Pappas, G., Akritidis, N., Bosilkovski, M., and Tsianos, E., 2005. Brucellosis. *N. Engl. J. Med.*, 352 (22), 2325-2336.

Pappas, G., and Papadimitriou, P., 2007. Challenges in *Brucella* bacteremia. *Int. J. Antimicrob. Agents*, 30 Suppl 1 S29-31.

Patel, K.B., Chauhan, H.C., Patel, B.K., Patel, S.S., Shrimali, M.D., *et al.*, 2017. Isolation, identification, and molecular detection of *Brucella abortus* from buffaloes in Gujarat, India. *Int. J. Curr. Microbiol. App. Sci.*, 6 (4), 1787-1797.

Rajala, E.L., Grahn, C., Ljung, I., Sattorov, N., Boqvist, S., and Magnusson, U., 2016. Prevalence and risk factors for *Brucella* seropositivity among sheep and goats in a peri-urban region of Tajikistan. *Trop. Anim. Health Prod.*, 48 (3), 553-558.

Refai, M., 2002. Incidence and control of brucellosis in the Near East region. *Vet. Microbiol.*, 90 (1-4), 81-110.

Scacchia, M., Di Provvido, A., Ippoliti, C., Kefle, U., Sebhatu, T.T., D'Angelo, A., and De Massis, F., 2013. Prevalence of brucellosis in dairy cattle from the main dairy farming regions of Eritrea. *Onderstepoort J. Vet. Res.*, 80 (1), 448.

Vidic, B., Savic-Jevdjenic, S., Grgic, Z., Bugarski, D., and Maljkovic, M., 2007. Infectious abortion in sheep. *Biotechnol. Anim. Husb.*, 23 (5), 383–389.

Wang, Y., Wang, Z., Zhang, Y., Bai, L., Zhao, Y., *et al.*, 2014. Polymerase chain reaction-based assays for the diagnosis of human brucellosis. *Ann. Clin. Microbiol. Antimicrob.*, 13, 31.

Warethc, G., Melzer, F., Elschner, M.C., Neubauer, H., and Roesler, U., 2014. Detection of *Brucella melitensis* in bovine milk and milk products from apparently healthy animals in Egypt by real-time PCR. *J. Infect. Dev. Ctries.*, 8, 1339-1343.

WOAH. 2018. "Manual of diagnostic tests and vaccines for terrestrial animals " In *Brucellosis* (*Brucella abortus, B. melitensis, B. suis*). 355–395. Rome, Italy: World Organization for Animal Health.

Yagupsky, P. 2015. "Blood Cultures for the Diagnosis of Human Brucellosis " In *Updates on Brucellosis*, edited by Manal Mohammad Baddour. InTech.

Yagupsky, P., Morata, P., and Colmenero, J.D., 2019. Laboratory Diagnosis of Human Brucellosis. *Clin. Microbiol. Rev.*, 33 (1). e00073-19. doi: 10.1128/CMR.00073-19.

Zinsstag, J., Roth, F., Orkhon, D., Chimed-Ochir, G., Nansalmaa, M., Kolar, J., and Vounatsou, P., 2005. A model of animal-human brucellosis transmission in Mongolia. *Prev. Vet. Med.*, 69 (1-2), 77-95.