Indian J Microbiol
https://doi.org/10.1007/s12088-023-01152-y

ORIGINAL RESEARCH ARTICLE

Bovine Brucellosis: First Comprehensive Evaluation from Hamedan, an Endemic Area in Iran

Maryam Adabi[1,2] · Jamal Gharekhani[3] · Saeed Alamian[4] · Marzieh Varasteh-Shams[1] · Maryam Fathi-Sheikhi[5] · Hossein Ghaderi[6,7] · Zahra Shivapour[5] · Zahra Valiei[8] · Salman khazaiee[9]

Received: 4 April 2023 / Accepted: 16 November 2023

Abstract Brucellosis is an infectious zoonotic disease. The disease is one of the major concerns in developing societies due to its great importance for public health and economic losses in the animal industry. The principal target of the study was to detect the prevalence of brucellosis and associated risk factors in cattle from Hamedan (western Iran) using different laboratory techniques. In 2020, blood samples from 900 cattle were obtained to detect brucellosis prevalence in the region. After screening by the modified Rose Bengal plate test, the positive samples were reevaluated using the Wright standard tube agglutination test (SAT), 2-Mercaptoethanol (2-ME), and Enzyme-linked immunosorbent assay. Serology-positive samples were confirmed by culturing bacteria from the lymph nodes and detecting *Brucella* DNA using specific primers, the BCSP31 target gene, and the IS711 locus. Brucellosis was detected in 1.88% (17/900, 95% CI 1–2.76%) of animals. The high prevalence of brucellosis was observed in female animals (2.77%, $p=0.947$), 2–4 years old animals (2.88%, $p=0.994$), Holsteins (5.69%, $p=0.989$), farm animals (6.49%, $p=0.999$), and animals with a history of vaccination against brucellosis (3.04%, $p=0.915$). In addition, there was no positive sample in October and December, and also the highest prevalence rate was found in September (5.33%, $p=0.970$). There was no statistically significant relationship between the variables and the rate of brucellosis. There were similar results between the different applied laboratory methods. The minimum and maximum levels of titer in the SAT method were + 2/80

✉ Jamal Gharekhani
gharekhani_76@yahoo.com

Maryam Adabi
m.adabi@aumsha.ac.ir

Saeed Alamian
s.alamian@wrvsri.ac.ir

Marzieh Varasteh-Shams
shams2424@gmail.com

Maryam Fathi-Sheikhi
Maryam_fathi67@yahoo.com

Hossein Ghaderi
ghaderih93@gmail.com

Zahra Shivapour
shivapoorzahra@yahoo.com

Zahra Valiei
vvaliei1266@gmail.com

Salman khazaiee
salmankhazaei61@gmail.com

[1] Infectious Diseases Research Center, Hamadan University of Medical Sciences, Hamadan, Iran

[2] Infectious Ophthalmologic Research Center, Imam Khomeini Hospital Clinical Research Development Unit, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

[3] Department of Laboratory Sciences, Central Veterinary Laboratory, Iranian Veterinary Organization (IVO), Hamedan Veterinary Office, Ayatollah-Rafsanjani Street, Hamedan, Iran

[4] Department of Brucellosis, Agriculture Research, Education and Extension Organization, Razi Vaccine and Serum Research Institute, Karaj, Iran

[5] Hamadan University of Medical Sciences, Hamadan, Iran

[6] Hamadan Veterinary Council (NGO), Hamadan, Iran

[7] Department of Microbiology, Faculty of Veterinary Sciences, Ilam University, Ilam, Iran

[8] Department of Microbiology, Faculty of Basic Sciences, Islamic Azad University, Hamadan Branch, Hamadan, Iran

[9] Research Center for Health Sciences, Hamadan University of Medical Sciences, Hamadan, Iran

Published online: 09 December 2023

and + 2/320, respectively. The rates for 2-ME were + 2/40 and + 4/160. Out of 17 positive samples, 2 were confirmed for *B. melitensis* and 15 for *B. abortus*. Notably, no sample showed co-infection of both *B. abortus* and *B. melitensis*. This study represents the first comprehensive evaluation of cattle brucellosis in Hamedan. Through molecular evaluation, the presence of *Brucella* spp. was identified in the seropositive samples. Among the cattle samples, the primary species isolated and confirmed was *B. abortus*. This finding shed light on the prevalence and distribution of *Brucella* species in the region, providing crucial insights for future disease management and control efforts. Considering the specificity of the used genes to detect bacteria, molecular biology can be a safe and rapid technique for diagnosing brucellosis, especially in cases without conclusive results. Regular screening of animals and culling seropositive animals are highly recommended; these affect the control of disease at the herd level.

Keywords Brucellosis · Cattle · Hamedan · PCR · Risk factor · Serology

Introduction

Bovine brucellosis caused by *Brucella abortus*, a Gram-negative intracellular coccobacillus, is a zoonotic disease with global prevalence [1]. Bernhard Bang identified *B. abortus* as the cause of cows' abortion for the first time in 1897. Recently, different biovars have been detected for *B. abortus* using molecular biology techniques [1]. Also, *B. melitensis* and *B. suis* play a role in brucellosis occurring in cattle [2].

Brucellosis is transmitted via both horizontal and vertical modes [3]. Aborted materials and genital discharges from infected animals have an important role in contaminating the environment as well as infecting other hosts [4]. In addition, milking from infected mothers can infect newborn calves [5]. The economic losses caused by brucellosis in the world's animal husbandry industry are irreversible, owing primarily to a decrease in milk production and abortions. For example, this rate has been estimated at US $600 million annually in the USA [6].

Brucellosis in humans has multiple manifestations [7]. While in livestock, the infection is often localized in the genital system [7], it mainly causes abortion in cows and infertility in bulls by involving the reproductive tract [8]. Granulomatous inflammation, lymphoid tissue disorders, and failure in the function of the mononuclear phagocytes are the predominant necropsy findings [9].

Timely and valid detection of brucellosis in animals is essential to managing the herds' infection [10, 11]. The Rose Bengal Plate test (RBPT), Wright standard tube agglutination test (SAT), 2-Mercaptoethanol (2-ME), and Enzyme-linked immunosorbent assay (ELISA) are the most common serologic techniques which are using for detecting animal brucellosis in Iran [12]. Nowadays, ELISA with 100% sensitivity and 99.2% specificity has been used as a suitable alternative to culturing techniques [13]. SAT accounts for aggregated quantities of IgM and IgG, while IgG to *Brucella* infection is calculated using the treatment of sera samples. IgG tracing is important for determining the active stage of brucellosis [14].

Numerous brucellosis studies have been conducted in Iran [2, 3, 8, 15–17]. However, the prevalence of disease in wild animals, the main routes of infection transmission in the regions, *Brucella* diversity, disease management in animals and herds level, and controlling strategies are unclear. Brucellosis is an occupational disease [18], and unpasteurized dairy products significantly spread the infection [16]. Hamedan Province is one of the endemic regions for brucellosis in Iran (Fig. 1) [19]. In a study by Majzobi et al. [20], 4.1% of examined dairy products were positive for *Brucella* infection in Hamedan. At the same time, all of the examined raw cow's milk samples were negative in this area [21]. In Hamedan, the prevalence of brucellosis was reported 2.2–5.7% [3]. Regarding animal type, brucellosis was detected in 3.3%, 4.6%, and 3% of dogs, goats, and sheep, respectively [22, 23]. Also, no antibodies to *Brucella* infection were recognized in horses [24].

The principal aim of the current investigation was to detect the prevalence of brucellosis and associated risk factors in cattle from Hamedan, west of Iran using microbiology, serology, and molecular biology techniques.

Materials and Methods

Design of Study

This is part of a great cohort project on brucellosis in western Iran [12, 25]. In a cross-sectional, the blood samples of animals were screened for the presence of antibodies to *Brucella* infection using modified-RBPT (mRBPT). All positive samples were reevaluated using quantitative serology tests for brucellosis, SAT, 2-ME, and ELISA simultaneously. Also, supra-mammary lymph nodes were applied for microbiology and molecular biology evaluation.

Study Location

Hamedan is one of the provinces in western Iran (34.77° N and 48.58° E). It has warm and dry summers, cold semi-arid climates, and snowy winters (Fig. 1). The average temperature throughout the year is 11.3 °C. Agriculture and animal husbandry are the main occupations of the people in the

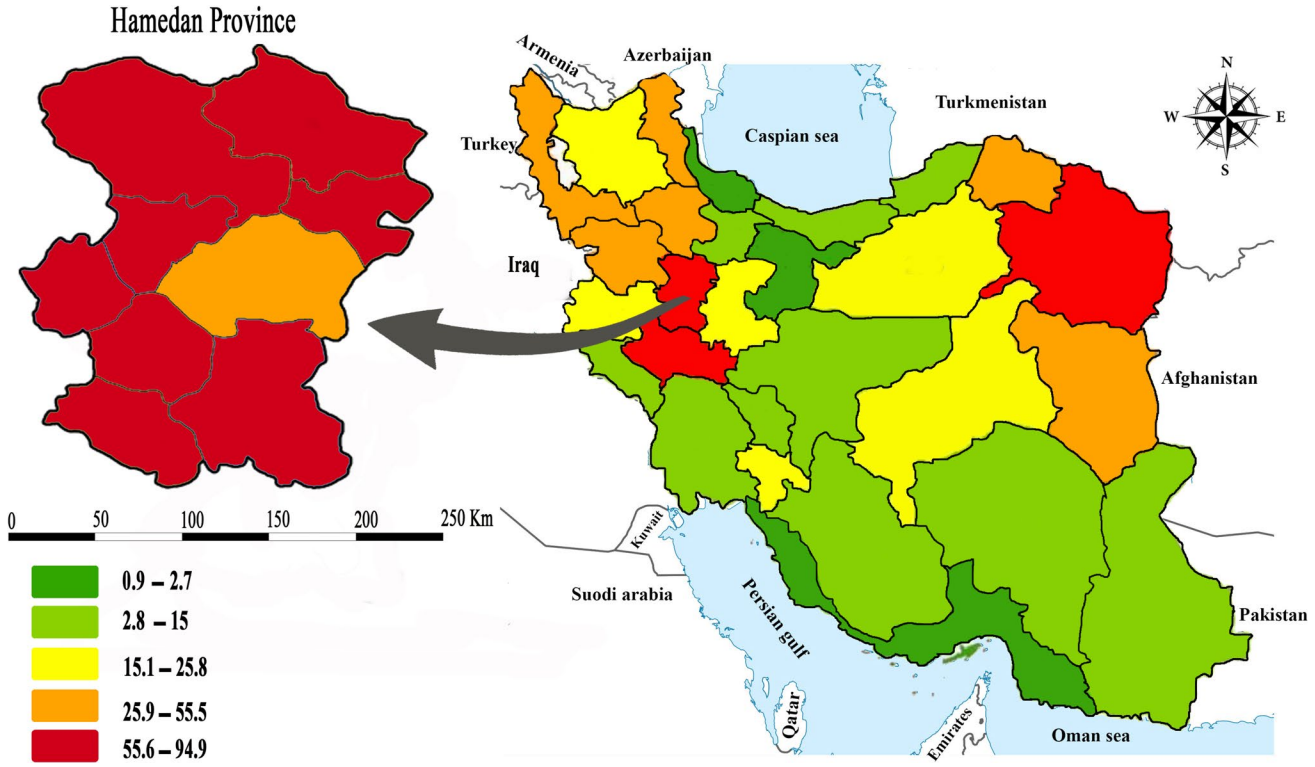

Fig. 1 Geographic distribution of human brucellosis in Hamedan (studied area) and Iran

region. The cattle population is 65,000 and 25,000 at rural and farm levels, respectively in this region.

Sample Collection

During six months (July–December 2020), blood samples from 900 cattle (on average: 10% of daily slaughtered or 150 samples each month) were obtained in Hamedan industrial slaughterhouse [26]. After randomly selecting animals, 5 ml of whole blood sample was collected from the coccygeal vein by using a disposable vacuum tube (Venoiect®, Iran). In addition, supra-mammary lymph nodes belonging to positive animals in mRBPT were sampled to confirm *Brucella* in the genus and species levels.

Laboratory Diagnosis

Microbiology

Lymph nodes were divided into smaller pieces by a sterile scissor. The materials were crushed using a laboratory stirrer until extracting the tissue juice, and cultured separately on both *Brucella* agar plus supplement and blood agar media (IBRESCO$^{@}$ Co., Iran). All cultured microbiology plates were incubated at 37 °C with 10% Co_2 for seven days. Then, the derived colonies were morphologically evaluated using Gram and modified Ziehl–Neelsen staining procedures simultaneously. Differential culture media and biochemical techniques such as catalase, oxidase, urea hydrolysis, nitrate reduction, H_2S production, and growth on thionin, as well as molecular biology methods were applied to confirm *Brucella*-colonies and also species of bacteria [21].

Serology

After centrifuging blood samples in 1200×*g* for 15 min, the sera samples were prepared for serology examinations. In serology, all positive samples in mRBPT were evaluated using SAT, 2-ME, and ELISA techniques. The Iranian Veterinary Organization guideline (Table 1) was used for the final interpretation of serology outputs based on SAT, 2-ME [12].

Table 1 The Iranian Veterinary Organization guidelines for serology diagnosis of brucellosis in cattle

RBPT	SAT	2-ME	Brucellosis result
Positive	≥ + 1/160	Each titer of the antibody	Positive
	+ 4/40 to + 4/80	≥ + 4/40	
	≤ + 1/20	≤ + 1/20	Negative

mRBPT

In this assay, 90 µl of sera and 30 µl of RBPT antigen (Vaccine and Serum Research Institute of Razi Co., Iran) were mixed on a rapid test white plate and shaken for 4 min. The appearance of any pink agglutination was recorded as a positive reaction [13].

SAT

In brief, 0.8 ml of phosphate buffer saline (PBS) was dispensed to the first agglutination tube; this volume was 0.5 ml for tubes 2–6th. Then, 0.2 ml of the sera sample was added to the first tube. Serial dilution was carried out by pipetting 0.5 ml of the first tube content into the following tubes. Then. 0.5 ml of the mixture was discarded from the last tube. Finally, 0.5 ml of 10% Wright antigen (Vaccine and Serum Research Institute of Razi Co., Iran) was added into all tubes and incubated at 37 °C for 24 h [12].

2-ME

The protocol was adjusted similarly to SAT. About 0.3 ml and 0.5 ml of PBS were poured into the first and resting tubes, respectively. Then, 0.2 ml of the sera sample was added to the first tube. In the next stage, 0.5 ml of 2-ME solution (Merck, Germany: 68 µl of 2-ME diluted in 5 ml distilled water) was added to the first tube, shaken, and incubated at 37 °C for 1 h. Serial dilution was carried out by pipetting 0.5 ml of the first tube's content into the following tubes. Then, 0.5 ml of solution from the last tube was discarded. Finally, 0.5 ml of 10% Wright antigen (Vaccine and Serum Research Institute of Razi Co., Iran) was added into all tubes and incubated at 37 °C for 24 h [12].

ELISA

We have used a commercial ELISA kit belonging to ID-Vet company, France (ID Screen® Brucellosis serum indirect multi-species, Lot No: 144) for detecting the antibodies in mRBPT-positive animals. All of the procedures are done point to point based on a manual suggested by the company. For all cases, the sample to a positive percentage (S/P%) was calculated using the optical density (OD) of the sample and controls (S/P% = OD sample-OD negative control/ OD positive control-OD negative control × 100). S/P% ≥ 120 was considered positive.

Molecular Diagnosis

DNA Extraction

Genomic materials from cultured bacteria of seropositive animals were extracted using a DNA purification commercial kit (Sinaclon, Iran, Lot No: PR881613), based on the supplier's manual. The extracted materials were analyzed qualitatively and quantitatively using electrophoresis and also NanoDrop (Eppendorf, Germany) by reading A260 and A280.

DNA Amplification

DNA amplification to recognize *Brucella* in genus and species levels using specific primers (Table 2) was done as reported earlier [9, 25]. BCSP31-B4 and BCSP31-B5 primers were applied for detecting the genus of *Brucella*. The total volume of BCSP31-PCR reactions was 12.5 µl including 6.25 µl 2x PCR master mixes (Amplicon, Denmark), 0.5 µl of each primer, 4 µl DNA template, and Distilled Water (D/W) up to 12.5 µl. Determination of *Brucella* species was accomplished using specific-species primers with amplicons of 498 and 731 bp for *B. abortus* and *B. melitensis*, respectively (Table 2). The forward primer of IS711 is unique to the detection of *Brucella* species, but the reverse primers are disparate and were derived from *B. abortus* and *B. melitensis* specific locus on Chromosomal DNA. IS711-PCR reaction was carried out in a total volume of 12.5 µl using a similar mixture of BCSP31-PCR. PCR profile was accomplished on a Thermal Cycler (MWG Biotech, Germany) by the following steps: primary denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 90 s, annealing at 64 °C for 1 min, and extension at 72 °C for 1 min with a final extension cycle at 72 °C for 5 min. The amplified products were evaluated using electrophoresis on 1.5% agarose gel. We used strains of *B. abortus* (ATCC 23455) and

Table 2 Information on primers regarding Gene type and product size used to detect *Brucella* in cattle from Hamedan

Product size	Primer	Gene
224 bp	Forward: 5′ tgg ctc ggt tgc caa tat caa 3′	*BCSP31-B4*
	Reverse: 5′ cgc gct tgc ctt tca ggt ctg 3′	*BCSP31-B5*
498 bp	Forward: 5′ tgc cga tca ctt aag ggc ctt cat 3′	IS711 *B.abortus*
	Reverse: 5′ gac gaa cgg aat ttt tcc aat ccc 3′	IS711 *B.abortus*
731 bp	Forward: 5′ tgc cga tca ctt aag ggc ctt cat 3′	IS711 *B. melitensis*
	Reverse: 5′ aaa tcg cgt ctt tgc tgg tct ga 3′	IS711 *B. melitensis*

B. melitensis (ATCC 23457) as positive controls and D/W as the negative control in the reactions.

Statistical Analysis

Chi-square ($\chi 2$) was applied for detecting the statistical relation between the seroprevalence of brucellosis and risk options which are presented in Tables 3 and 4 (SPSS 16.0, Chicago, IL, USA). A p-value ≤ 0.05 was considered significant. A 95% confidence interval (CI 95%) was estimated for the seroprevalence rate.

Table 4 Seroepidemiology of brucellosis in cattle in different sampling times from Hamedan, Iran

Sampling time	No. of sample	Seropositive	Statistical analysis
July	150	3 (2%)	$\chi 2 = 00.1$ $p = 0.970$
August	150	4 (2.66%)	
September	150	8 (5.33%)	
October	150	0	
November	150	2 (1.33%)	
December	150	0	
Total	900	17 (1.88%)	95% CI: ± 0.88

Results

Animals' Characteristics

Based on analysis of the derived information from a questionnaire, demographic and risk factors, including sex, age, race, source of animals (rural or farm), sampling time, and history of vaccination against brucellosis are demonstrated in Tables 3 and 4. Most of the animals were female, 2–4 years old, crossbred from rural areas, and had a history of vaccination against brucellosis.

Prevalence Rate

The prevalence rate of brucellosis in animals was estimated at 1.88% (95% CI 1–2.76%). Similar results were observed between different methods of serology and microbiology (Table 5). The minimum and maximum levels of titer in the SAT method were + 2/80 and + 2/320, respectively. This rate was + 2/40 and + 4/160 for 2-ME. In ELISA, S/P% was detected in the range 130–599 (Average = 346 ± 130) (Table 5). The level of antibodies found by ELISA increased parallel to SAT results (Fig. 2). All positive samples from serology and microbiology were confirmed by PCR. In the secondary stage of molecular detection on 17 positive samples, 2 (11.76%) and 15 (88.23%) samples were confirmed as *B. melitensis* and *B. abortus*, respectively. There was no sample with *B. abortus* and *B. melitensis* co-infection (Table 5). Both samples of *B. melitensis* positive (No. 11 and 13 in Table 5) belonged to non-vaccinated animals in rural areas.

Risk Factors

Complete data on risk options and the prevalence rate of brucellosis for different variables are presented in Table 4. But the highest rate of infection belonged to female animals (2.77%, $p = 0.947$), animals 2–4 years old (2.88%, $p = 0.994$), Holsteins (5.69%, $p = 0.989$), farm animals (6.49%, $p = 0.999$), and animals with a history of vaccination against brucellosis (3.04%, $p = 0.915$). There was no statistically significant correlation between the brucellosis rate and the variables (Table 3). Regarding sampling time, there was no positive sample in October and December. While a high

Table 3 Seroepidemiology of brucellosis in cattle in different risk factors from Hamedan, Iran using SAT and 2-ME methods

Risk factors		No. of sample	Seropositive	Statistical analysis
Sex	Male	288 (32%)	0	$\chi 2 = 0.004$, $p = 0.947$
	Female	612 (68%)	17 (2.77%)	
Age (year-old)	≤ 2	301 (33.45%)	1 (0.33%)	$\chi 2 = 0.011$, $p = 0.994$
	2–4	452 (50.22%)	13 (2.88%)	
	> 4	147 (16.33%)	3 (2.04%)	
Race	Native	280 (31.11%)	1 (0.36%)	$\chi 2 = 0.020$, $p = 0.989$
	Cross-breed	409 (45.44%)	4 (0.98%)	
	Holstein	211 (23.45)	12 (5.69%)	
Animals source	Rural areas	669 (74.33%)	2 (0.29%)	$\chi 2 = 2.480$, $p = 0.999$
	Farms	231 (25.67%)	15 (6.49%)	
Vaccination history	Yes	493 (54.78%)	15 (3.04%)	$\chi 2 = 0.011$, $p = 0.915$
	No	354 (39.33%)	2 (0.56%)	
	NI	53 (5.89%)	0	

NI, no information

Table 5 The results of *Brucella* infection in cattle from Hamedan regarding different diagnostic methods

No. of the positive sample	Serology results					Microbiology	PCR results		
	mRBPT	SAT	2-ME	ELISA			*Brucella* sp.	*B. melitensis*	*B. abortus*
				S/P%	Result				
1	+	+3/160	+2/160	370	+	+	+	−	+
2	+	+2/320	+2/80	599	+	+	+	−	+
3	+	+4/80	+4/80	141	+	+	+	−	+
4	+	+3/160	+3/80	355	+	+	+	−	+
5	+	+3/160	+4/80	361	+	+	+	−	+
6	+	+3/160	+4/80	341	+	+	+	−	+
7	+	+3/160	+4/80	320	+	+	+	−	+
8	+	+4/160	+4/160	410	+	+	+	−	+
9	+	+3/160	+4/80	299	+	+	+	−	+
10	+	+2/320	+3/80	566	+	+	+	−	+
11	+	+3/160	+3/80	350	+	+	+	+	−
12	+	+3/160	+1/160	291	+	+	+	−	+
13	+	+3/160	+2/80	280	+	+	+	+	−
14	+	+2/320	+2/160	550	+	+	+	−	+
15	+	+3/160	+4/80	270	+	+	+	−	+
16	+	+3/160	+4/80	261	+	+	+	−	+
17	+	+2/80	+2/40	130	+	+	+	−	+

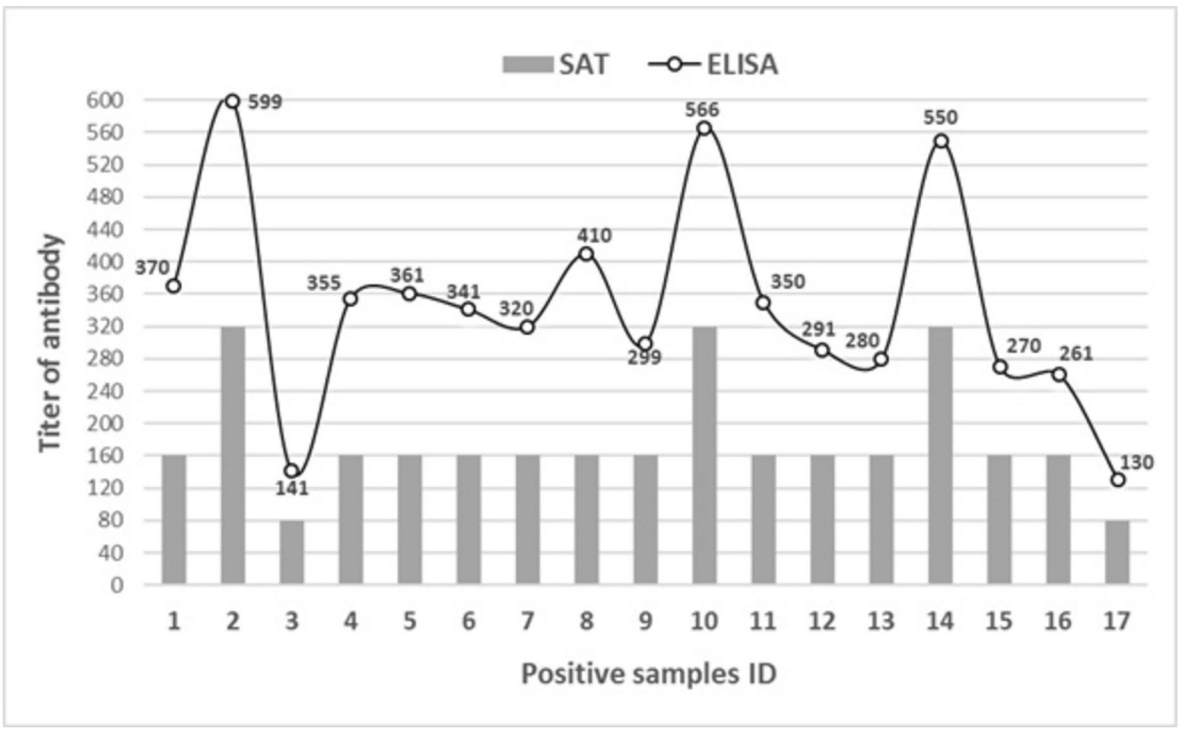

Fig. 2 Demonstration of the antibodies level by ELISA compared to SAT in positive samples

prevalence rate (5.33%) was found in September ($p = 0.970$, Table 4).

Discussion

Brucellosis is an endemic infectious disease in developing countries, especially Iran [3]. Human brucellosis has been reported to be more than 500,000 per year worldwide [17]. This rate was calculated to be 0.001% in Iran, with the highest incidence occurring in the western and northwest locations [27]. Khoshnood et al. [7] reported 15.53% of human brucellosis in a systematic review. In a report by Suresh et al. [28], the rate of livestock brucellosis was 8% in the Asian and African continents. Also in the Middle East, animals' brucellosis is estimated to be 0.85–23.3% [17]. This rate in Iranian livestock was 10.18% (14.66% in cattle) [3]. The disease is notifiable due to its public health consequences and substantial economic losses [6]. Knowledge of brucellosis prevalence and risk factors is critical for developing a regional disease control program [16].

In the current survey, we used different laboratory techniques to diagnose brucellosis in animals. Microbiological analysis is the gold standard to detect brucellosis [14]. But serology is a rapid and low-cost technique for monitoring and screening the disease in animals and herds level [11]. Detection of *Brucella* infection with high sensitivity and specificity has been provided by molecular biology methods [15]. For molecular evaluation, the spleen and lymph nodes are the most reliable samples in the post-mortem period [1]. Rechecking seropositive cases by molecular methods is an effective tool in epidemiology works in animals [3]. Some research is conducted by combining serology, bacteriology, and molecular assays to detect *Brucella* species [29].

In our study, 1.88% of animals were positive for brucellosis using different serology, microbiology, and molecular biology methods. In similar works using ELISA, 0.97% and 1.2% of positive cases were reported from the southeastern and western regions of Iran, respectively [30, 31]. In addition, bovine brucellosis was reported at 1.8% in Argentina [32], 1.9% in China [33], 2% in Tajikistan [34], 2.6% in Bangladesh [35], 3% in Ethiopia [36], 3.56% in Turkey [37], 8.7% in Pakistan [38], and 17% in India [39]. In a study by Alamian et al. [2] from different central and southern regions of Iran, 2808 blood samples and 157 lymph nodes of cattle were tested using serology and molecular biology. Antibodies to brucellosis were observed in 5.6%, 3.9%, and 4.9% of animals using RBPT, SAT, and ELISA, respectively. Additionally, the rate of disease in animals from rural regions was higher than in others. All seropositive animals were confirmed by molecular methods and were *B. abortus* (biovars 1 and 3). In the previous research by Dadar et al. [3], *B. abortus*, *B. melitensis*, and co-infection of *B. abortus* and *B. melitensis* were reported in Iranian livestock. *B. abortus* was isolated primarily from seropositive animals in the study by ZareBidaki et al. [4]. In our work, *B. melitensis* was rarely detected in cattle parallel to other researchers [3, 4, 15, 40]. In a recent study by Dadar et al. [9], *B. melitensis* was introduced as the most common isolate derived from cattle, sheep, goats, and camels in different regions of Iran. This is due to the rearing of various animal species together, which is common in Iran. Mixed farming with various species of livestock increases the risk of brucellosis where sheep and goats act as primary hosts for *B. melitensis* and cattle as spillover hosts [1].

A wide range of brucellosis was reported by researchers. The main reasons for the results differences are the different study design and protocol methods, sample size, diagnostic methods, herd size, animals' density, and farms' biosecurity [3].

Vaccination has a significant role in the control of bovine brucellosis, especially in endemic regions through mass vaccine coverage in combination with a proper culling program. So, without control measures for brucellosis in cattle, bacteria may circulate in the farms for several years [11]. Vaccination with the attenuated *B. abortus* (RB51) strain is standard for cows in Iran. Also, full and reduced doses (RD) are used for heifers and adults. This vaccine can not be effective on *B. melitensis* in cattle due to the lack of cross-species protection. In a report from Brazil, 80% of positive isolates derived from dairy products belonged to the vaccine strain [41]. Therefore, in molecular studies, the differentiation of vaccine strains from field ones must be considered. In our findings, the brucellosis rate in animals with a history of vaccination was higher than in nonvaccinated animals, contrary to what was previously reported [12, 22]. It can be due to the sampling method and the farmer's incorrect information on vaccination time. Also, the potency and efficiency of the used vaccine can be considered. In our work, both *B. melitensis* belonged to non-vaccinated animals in rural areas. This is due to the keeping of sheep, goats and cattle in the same place and also the grazing of healthy animals beside the infected animals. *B. melitensis* is the most common cause of brucellosis in sheep and goats in Iran, which is achieving growing in cattle as an emergent zoonotic bacterium [15]. It is important to note that there are no highly protective, safe, and effective vaccines for bovines [2]. Also, vaccination against brucellosis is not mandatory in the rural areas of Iran and its coverage never reaches 100%; which is one of the limitations to control of the disease. So, designing a practical scientific program is needed for the possible control of *B. melitensis* in cattle.

In the present study, the rate of brucellosis in > 2-year-old animals was higher than in younger animals with no significant statistical correlation, similar to studies in Bangladesh and Ethiopia [35, 42]. In Robia and Gelalcha's [43] report,

the seroprevalence of brucellosis in older animals (> 6 years) was six-fold higher than in young animals ($p < 0.05$). Some researchers believe that a high rate of brucellosis can be observed because of low-level antibodies against infection and immune system defects [12]. Young animals are more resistant to brucellosis and can shed the infection more frequently. It is due to erythritol and reproductive hormones, which promote the growth of *Brucella* and whose concentration tends to rise with increasing age and sexual maturity [42]. On the other hand, the chance of contracting the disease increases with age [22]. Therefore, age plays an important role in brucellosis morbidity. All of the positive animals were female. Rahman et al. [35] reported a high rate of brucellosis in cows compared to bulls. Additionally, in Tilahun et al. [42] investigation, a similar rate of infection was reported in male (7.5%) and female (7.8%) animals. Males are kept in the herds for a longer period compared to females, thus their lifetime risk of infection increases [42]. *Brucella* has an increased tendency to be localized in the genital tract of female animals due to the level of erythritol, which stimulates the growth of *Brucella*. This is important as a potential reservoir for transmitting and propagating the pathogen [3].

Moosazadeh et al. [44] reported a direct statistical relation between season and prevalence of brucellosis. Temperature, period of sunshine, and evaporation all substantially impact seasonal fluctuations in brucellosis transmission [45]. According to Nyerere et al. [46], seasonal weather fluctuations have a significant influence on the dynamics of brucellosis transmission in both humans and animals. Thus, prompt action must be taken in response to changes to control the infection. We found the highest brucellosis rate (8 out of 17) in September. Similar findings were reported from Turkey, Germany, and Greece, with the highest prevalence in spring and summer [44]. The wide-scale research on the seasonal pattern of brucellosis demonstrates that the disease is more prevalent in the spring and summer seasons, which are the livestock's offspring seasons. The rate of brucellosis increases during the spring and summer due to some factors, including direct contact between farmers and aborted fetuses and/or infected materials, as well as consumption of contaminated dairy products [5, 10]. In contrast, the incidence of brucellosis significantly decreases in the second half of the year [11]. Furthermore, the disease incidence has a direct connection with decreased dairy and farm products during certain periods. In Iran, a meta-analysis confirmed that the highest incidence of brucellosis occurred in the spring and summer [44].

The animal breed is introduced as a risk factor for *Brucella* infection [43]. In our findings, brucellosis in Holstein cattle was higher than in crossbreed and native animals. The seroprevalence of brucellosis in native Zebu cattle was found 7.6% using ELISA [42]. In Robia and Gelalcha's [43] report, brucellosis in native cattle breeds was significantly ($p < 0.05$) higher than in crossbreeds. The updated management with high standards of biosecurity in industrial farms may be responsible for this difference compared to rural regions. Brucellosis in farms with intensive systems of breeding is higher than in extensive systems because of the close contact of the animals. Also, animals' susceptibility to infections differs regarding breeds [43].

The presence of different types of animals on a farm, the use of common calving pens, mating with bulls, manure disposal methods, sharing water sources for cattle within and outside farms, failure to comply with quarantine rules, and a low level of health standards, on the other hand, were reported as significant risk options in dairy cattle farms [10]. Regarding Barman et al. [39] report, the lack of effective vaccines and the problems associated with culling positive animals are the main gaps in the endemic regions. In Khurana et al. [1] research, the sex, and breed of animals, as well as the insemination method at the herd level, were introduced as being potentially related to brucellosis.

Knowledge of brucellosis signs in livestock, the prevalence rate and infection sources, common transmission routes, and preventive strategies to protect the public and planned control programs should be undertaken with perfect power [16]. In epidemiological investigations, the simultaneous use of laboratory methods increases the sensitivity, specificity, and positive predictive value. It reduces misdiagnoses and increases the chance of detecting the antibodies against brucellosis [14, 43]. We suggested keeping different species of livestock such as cattle, sheep, and goats, as well as sex separately for preventing the transmission of non-specific *Brucella* spp. Additionally, specific pastures should be considered for each herd and block the cross-move of animals between herds. It is essential to combine different laboratory methods such as serology, microbiology, and molecular biology for reducing the detection limits in the individual and herd levels.

The occurrence of brucellosis in cattle is influenced by significant vital points such as the fit certification of newly purchased cattle, their vaccination policy by using proper strains, and the confident culling of reservoir animals [10]. Mass vaccination is the best way to control brucellosis in endemic areas [1].

Conclusions

Our work was a comprehensive evaluation of cattle brucellosis in Hamedan for the first time. The findings indicated that the main species isolated from cattle was *B. abortus*. Molecular biology is considered a reliable technique in rapidly and precisely detecting *Brucella* infection in the genus and species levels, especially in cases with no serology

findings. The rate of brucellosis in animals was low compared to previous reports. Regular screening of animals and culling seropositive animals are highly recommended; these affect the control of disease at the herd level.

Acknowledgements The authors appreciate the staff of Hamedan veterinary service for technical support.

Authors' Contributions Conceptualization: JG and MA; Methodology: MV and SA; Formal analysis and investigation: SK, HG, ZV, and MV; Writing-original draft preparation: JG, MF, HG, ZS, and ZV; Writing-review and editing: MA, JG, and SA; Resources: JG and SA; JG prepared Figs. 1 and 2. All authors reviewed the manuscript.

Funding This study was supported by grant No. 9808216031 from the deputy of research and technology, at Hamedan University of medical sciences.

Data and Code Availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical Approval This study involved a questionnaire-based survey of farmers as well as blood sampling from their animals. The study protocol was assessed and approved by the Animal Welfare Committee of Hamedan University of Medical Sciences (IR. UMSHA. REC.1398.575). The farmers provided their verbal informed consent for animal sampling as well as for the related survey questions. The collection of samples was carried out by veterinarians adhering to the regulations and guidelines on animal husbandry and welfare. The ethical principles were also followed closely during the data analysis and article submission.

Consent to Participate All of the animals in sampled regions have owners; the consents of owners are documented and available (in the local language).

Consent for Publication Not applicable.

References

1. Khurana SK, Sehrawat A, Tiwari R, Prasad M, Gulati B, Shabbir MZ (2021) Bovine brucellosis: a comprehensive review. Vet Q 41:61–88
2. Alamian S, Amiry K, Bahreinipour A, Etemadi A, Tebianian M, Mehrabadi MH, Dadar M (2021) *Brucella* species circulating in rural and periurban dairy cattle farms: a comparative study in an endemic area. Trop Anim Health Prod 53:1–9
3. Dadar M, Shahali Y, Fakhri Y (2021) Brucellosis in Iranian livestock: a meta-epidemiological study. Microb Pathog 155:104921
4. ZareBidaki M, Allahyari E, Zeinali T, Asgharzadeh M (2022) Occurrence and risk factors of brucellosis among domestic animals: an artificial neural network approach. Trop Anim Health Prod 54:1–13
5. Dadar M, Fakhri Y, Shahali Y, Mousavi Khaneghah A (2020) Contamination of milk and dairy products by Brucella species: a global systematic review and meta-analysis. Food Res Int 128:108775
6. Angara PE, Ismail AA, Ibrahim AM, Osman SZ (2016) Assessment of the economic losses due to bovine brucellosis in Khartoum state, Sudan. Int J Tech Res Appl 4:85–90
7. Khoshnood S, Pakzad R, Koupaei M, Shirani M, Araghi A, Irani GM, Moradi M, Pakzad I, Sadeghifard N, Heidary M (2022) Prevalence, diagnosis, and manifestations of brucellosis: a systematic review and meta-analysis. Front Vet Sci 9:976215
8. Dadar M, Alamian S, Tadayon K, Ashford RT, Whatmore AM (2022) Molecular characterization of zoonotic *Brucella* species isolated from animal and human samples in Iran. Acta Trop 229:106363
9. Dadar M, Alamian S, Brangsch H, Elbadawy M, Elkharsawi AR, Neubauer H, Wareth G (2023) Determination of virulence-associated genes and antimicrobial resistance profiles in *Brucella* isolates recovered from humans and animals in Iran using NGS technology. Pathogens 12:82
10. Dadar M, Godfroid J (2021) Main risk factors associated with small and large ruminant brucellosis. India J Anim Sci 91:885–890
11. Golshani M, Buozari S (2017) A review of brucellosis in Iran: epidemiology, risk factors, diagnosis, control, and prevention. Iran Biomed J 21:349
12. Adabi M, Khazaiee S, Sadeghi-Nasab A, Alamian S, Arabestani MR, Valiei Z, Gharekhani J (2022) Brucellosis in livestock: first study on seroepidemiology, risk factors, and preventive strategies to manage the disease in Famenin. Iran Vet World 15:2102–2110
13. Ferreira A, Cardoso R, Travassos Dias I, Mariano I, Belo A, Rolão Preto I, Manteigas A, Fonseca A, Corrêa De Sá M (2003) Evaluation of a modified Rose Bengal test and an indirect enzyme-linked immunosorbent assay for the diagnosis of *Brucella melitensis* infection in sheep. Vet Res 34:297–305
14. Al Dahouk S, Nöckler K (2011) Implications of laboratory diagnosis on brucellosis therapy. Expert Rev Anti Infect Ther 9:833–845
15. Dadar M, Alamian S, Behrozikhah AM, Yazdani F, Kalantari A, Etemadi A (2019) Molecular identification of *Brucella* species and biovars associated with animal and human infection in Iran. Vet Res Forum 10:315–321
16. Dadar M, Shahali Y, Whatmore AM (2019) Human brucellosis caused by raw dairy products: a review on the occurrence, major risk factors, and prevention. Int J Food Microbiol 292:39–47
17. Bahmani N, Bahmani A (2022) A review of brucellosis in the Middle East and control of animal brucellosis in an Iranian experience. Rev Med Microbial 33:63–69
18. Dadar M, Tabibi R, Alamian S, Caraballo-Arias Y, Jonathan Mrema E, Mlimbila J, Chandrasekar S, Dzhusupov K, Sulaimanova C, Alekesheva L, Manar S, Toguzbayeva K, Wickramatillake A, Mirzaei B (2022) Safety concerns and potential hazards of occupational brucellosis in developing countries: a review. J Public Health. https://doi.org/10.1007/s10389-022-01732-0
19. Nematollahi S, Ayubi E, Karami M, Khazaei S, Shojaeian M, Zamani R (2017) Epidemiological characteristics of human brucellosis in Hamadan province during 2009–2015: results from the national notifiable diseases surveillance system. Int J Infect Dis 61:56–61
20. Majzobi MM, Karami P, Khodavirdipour A, Alikhani MY (2022) Brucellosis in humans with the approach of *Brucella* species contamination in unpasteurized milk and dairy products from Hamadan. Iran Iran J Med Microbiol 16:282–287
21. Gharekhani J, Yakhchali M, Afshari A, Adabi M (2021) Herd-level contamination of *Neospora caninum*, *Toxoplasma gondii* and Brucella in milk of Iranian dairy farms. Food Microbiol 100:103873

22. Gharekhani J, Rasouli M, Abbasi-Doulatshahi E, Bahrami M, Hemati Z, Rezaei A (2016) Seroepidemiological survey of brucellosis in small ruminants in Hamedan province. Iran J Adv Vet Anim Res 3:399–405
23. Gharekhani J, Sazmand A (2019) Detection of *Brucella* antibodies in dogs from rural regions of Hamedan, Iran. Avicenna J Clin Microbiol Infect 6:122–126
24. Gharekhani J, Barati E, Doulatshahi EA, Talaei M, Rasouli MR (2020) *Brucella* infection in horses from Hamedan Province, Iran. Avicenna J Clin Microbiol Infect 7:60–62
25. Adabi M, Karami M, Keramat F, Alikhani MY, Bakhtiari S (2021) Serological and molecular investigation of human brucellosis in participants of *Famenin brucellosis* cohort study, Hamadan, Iran. Iran J Microbiol 13:319–324
26. Thrusfield M (2018) Veterinary epidemiology. Wiley
27. Mirnejad R, Masjedian Jazi F, Mostafaei S, Sedighi M (2017) Epidemiology of brucellosis in Iran: a comprehensive systematic review and meta-analysis study. Microb Pathog 109:239–247
28. Suresh KP, Patil SS, Nayak A, Dhanze H, Rajamani S, Shivamallu C, Cull CA, Amachawadi RG (2022) Prevalence of brucellosis in livestock of African and Asian continents: a systematic review and meta-analysis. Front Vet Sci 9:923657
29. Hinić V, Brodard I, Thomann A, Holub M, Miserez R, Abril C (2009) IS711-based real-time PCR assay as a tool for detection of *Brucella* spp. in wild boars and comparison with bacterial isolation and serology. BMC Vet Res 5:22
30. Ghasemi A, Hajinezhad M, Esmaeili S, Mostafavi E (2018) Seroprevalence of Q fever and brucellosis in domestic and imported cattle of Southeastern Iran. J Med Microbiol Infect Dis 6:48–52
31. Aghamohammad S, Rastin M, Mostafavi E, Hajijafari Anaraki A, Rahravani M, Sadaf R, Moravedji M, Rohani M (2023) Determination of seroprevalence of brucellosis in livestock and high-risk population in Kurdistan, Western Iran. Comp Immunol Microbiol Infect Dis 93:101942
32. Aznar M, Linares F, Cosentino B, Sago A, La Sala L, León E, Duffy S, Perez A (2015) Prevalence and spatial distribution of bovine brucellosis in San Luis and La Pampa. Argent BMC Vet Res 11:209
33. Ran X, Cheng J, Wang M, Chen X, Wang H, Ge Y, Ni H, Zhang X, Wen X (2019) Brucellosis seroprevalence in dairy cattle in China during 2008–2018: a systematic review and meta-analysis. Acta Trop 189:117–123
34. Lindahl E, Sattorov N, Boqvist S, Sattori I, Magnusson U (2014) Seropositivity and risk factors for *Brucella* in dairy cows in urban and peri-urban small-scale farming in Tajikistan. Trop Anim Health Prod 46:563–569
35. Rahman MS, Faruk MO, Her M, Kim JY, Kang SI, Jung SC (2011) Prevalence of brucellosis in ruminants in Bangladesh. Vet Med 56:379–385
36. Tesfaye A, Dejene H, Admassegn T, Asfaw D, Dagnaw G, Bitew A (2021) Seroprevalence of bovine brucellosis in Ethiopia: systematic review and meta-analysis. Net Med Res Report 12:1–6
37. Yumuk Z, O'Callaghan D (2012) Brucellosis in Turkey-an overview. Int J Infect Dis 16:e228–e235
38. Arif S, Thomson P, Hernandez-Jover M, McGill D, Warriach H, Hayat K, Heller J (2019) Bovine brucellosis in Pakistan; an analysis of engagement with risk factors in smallholder farmer settings. Vet Med Sci 5:390–401
39. Barman N, Patil S, Kurli R, Deka P, Bora DP, Deka G, Ranjitha KM, Shivaranjini C, Roy P, Suresh KP (2020) Meta-analysis of the prevalence of livestock diseases in North Eastern Region of India. Vet World 13:80–91
40. Mirkalantari S, Masjedian F, Amraee F (2021) Determination of investigation of the link between human and animal *Brucella* isolates in Iran using multiple-locus variable number tandem repeat method comprising 16 loci (MLVA-16). Braz J Infect Dis 25:101043
41. Miyashiro S, Scarcelli E, Piatti R, Campos F, Vialta A, Borges Keid L, Augusto Dias R, Genovez M (2007) Detection of *Brucella abortus* DNA in illegal cheese from São Paulo and Minas Gerais and differentiation of B19 vaccinal strain by means of the polymerase chain reaction (PCR). Braz J Microbiol 38:17–22
42. Tilahun A, Kegno S, Adugna T, Mamuye D (2022) A Seroprevalence study of Brucellosis in Boran (Zebu) breeds of pastoral area. Vet Med Res Rep 13:91–99
43. Robia DT, Gelalcha BD (2020) Epidemiological investigation of brucellosis in breeding female cattle under the traditional production system of Jimma zone in Ethiopia. Vet Anim Sci 9:100117
44. Moosazadeh M, Abedi Gh, Kheradmand M, Safiri S, Nikaeen R (2016) Seasonal pattern of Brucellosis in Iran: a systematic review and meta-analysis. Iran J Health Sci 4:62–72
45. Liu K, Yang Z, Liang W, Guo T, Long Y, Shao Z (2020) Effect of climatic factors on the seasonal fluctuation of human brucellosis in Yulin, northern China. BMC Public Health 20:506
46. Nyerere N, Luboobi L, Mpeshe S, Shirima G (2020) Modeling the impact of seasonal weather variations on the infectiology of Brucellosis. Comput Math Method Med 8972063:19