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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Molecular detection of *Brucella* species among aborted small ruminants in southeast Iran

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Abstract

Brucellosis, caused by *Brucella* bacteria, is a common zoonotic infectious disease with various clinical manifestations in humans and animals. The disease is endemic in human and ruminant populations in Iran, with a particular prevalence in areas where humans have close interactions with livestock. Since domestic animals serve as the primary reservoir for brucellosis, this study aimed to identify the presence of *Brucella* spp. among aborted small ruminants in southeast Iran. Between 2021 and 2022, aborted fetuses of small ruminants (46 sheep and 4 goats) were collected from Zarand County in the Kerman province. Swab samples from the abomasum contents of these fetuses were obtained and subjected to DNA extraction. The samples were then tested for *Brucella* spp. detection using the polymerase chain reaction (PCR) method. Out of the 50 aborted fetuses examined, *Brucella* spp. was detected in 15 (30%) specimens, comprising 13 (28%) sheep and 2 (50%) goats. Species typing revealed the presence of *Brucella ovis* (6 sheep and 1 goat), *Brucella melitensis* (6 sheep), and *Brucella abortus* (1 sheep) among the positive specimens. This cross-sectional study highlights the high prevalence of various *Brucella* species in samples from small ruminant abortions in southeast Iran. Additionally, the identified *Brucella* species were not limited to their primary host livestock. These indicated potential cross-species transmission among small ruminants.

Keywords Brucellosis · Abortion · *Brucella* · Iran · Small ruminants · *B. melitensis* · *B. abortus* · *B. ovis*

Introduction

Brucellosis is a highly prevalent zoonotic disease that impacts domestic animals, humans, and wildlife. It is caused by various species within the *Brucella* genus. These slow-growth bacteria possess the ability to survive and multiply in different types of cells, including epithelial cells, trophoblasts of the placenta, dendritic cells, and macrophages [1, 2]. In humans, the most significant clinical manifestations of brucellosis include acute fever, chronic hepatomegaly, splenomegaly, and arthritis. In livestock, brucellosis primarily affects the organs of the reproductive system, leading to abortion, reduced fertility, and decreased milk production [3]. The conventional approach to diagnosing *Brucella* involves several steps, including clinical examination, cultivation of bacterial isolates from various biological samples, microscopy, biochemical tests (such as fermentation tests, catalase, oxidase, and urease), and serological tests (such as the Rose Bengal test, serum/latex agglutination test, complement fixation test, and enzyme-linked immunosorbent assay). These methods are usually time-consuming, often

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taking days to observe visible growth. Some methods are not very sensitive or specific, and they require trained personnel and a biosafety level 3 facility. In contrast, molecular techniques, such as the PCR method, have improved the fast and safe identification of *Brucella* [4].

Brucellosis is a public health concern worldwide, especially in endemic areas in the Mediterranean, North and East Africa, the Middle East, and parts of Latin America and Asia [5]. The high prevalence of brucellosis in livestock flocks with a history of abortion increases the risk of transmission to humans [6]. *Brucella* species, including *Brucella abortus, Brucella melitensis, Brucella suis, Brucella canis, and Brucella ovis,* mainly infect cattle, goats and sheep, swine, and dogs, respectively [7]. Among *Brucella* species, *B. abortus*, *B. suis*, and *B. melitensis* are the most pathogenic and invasive species for humans [7].

Brucellosis is a prevalent and endemic disease in both small ruminants (sheep and goat flocks) and humans in many regions of Iran. This disease not only affects the ruminant population but also poses a significant health concern for humans, as evident by the annual reports of clinical human cases nationwide. According to a study by Zeinali et al. [8] in 2022, the incidence rate of human brucellosis in Iran is estimated to be 29.83 cases per 100,000 population, and most of the cases were via contact with infected livestock (91%). From 2002 to 2006, in Iran's South Khorasan province, the occurrence rate of brucellosis reached 340 cases per 10,000 individuals in sheep and goats and 37 cases per 100,000 individuals in humans [9].

In Iran, brucellosis in small ruminants, primarily caused by biovar 1 of *B. melitensis*, imposes significant economic losses [10].

Due to the health and economic challenges arising from this disease, it is crucial to prioritize comprehensive research and implement effective control programs to address these infections in both animals and humans.

This study aimed to identify *Brucella* species in abortion samples collected from sheep and goat flocks in the southeast region of Iran between 2020 and 2021. The findings of this research can play a significant role in the control and prevention of this widespread disease.

Materials and methods

Area study and sample size

In this study, a completely random sampling method was used, and sampling was conducted based on the abortion reports among sheep and goat flocks from March 20, 2020, to March 20, 2021, in Zarand County, located in Kerman Province, southeast Iran. The specific study area is depicted in Fig. 1.

Based on official information provided by the Veterinary Department of Zarand County in Kerman province, the total livestock population (sheep and goats) in the city consisted of 290 sheep flocks and 46 goat flocks from March 20, 2020, to March 20, 2021. These flocks encompassed a total of 140,000 sheep and 30,000 goats.

During the sampling period, 50 flocks of sheep and goats (46 sheep flocks and 4 goat flocks) with abortion reports were identified. A total of 50 aborted fetus samples (46 sheep and 4 goats) were randomly collected, with one sample collected from each of these flocks.

Sampling method

In this study, samples were selected blindly without considering any previous tests, including serological examinations. To clarify, no tests were conducted prior to our sample selection process. The comprehensive data was collected for fifty aborted fetuses, comprising crucial details such as age, birth order, history of abortion, and vaccination records. Subsequently, these samples were transported under appropriate cold chain conditions to the Faculty of Veterinary Medicine at Shahid Bahonar University in Kerman. Upon opening the fetus carcass, the contents of the abomasum were carefully collected using a sterile swab and transferred into microtubes containing 200 µl of sterile physiological solution. To enable future research, the samples were then stored at a temperature of – 20 °C [11].

DNA extraction

In this study, DNA extraction was performed from abomasum swab samples obtained from the aborted sheep and goat fetuses. A commercial Tissue Genomic DNA Extraction Kit (Pars Tous Co., Iran) was utilized, following the manufacturer's instructions. The quantity and quality of the extracted DNA were assessed using a microplate spectrophotometer (Epoch bioTek, USA) at wavelengths of 260 nm and 280 nm. Subsequently, the DNA samples were stored at a temperature of – 20 °C for subsequent analysis.

The conventional polymerase chain reaction (PCR) and quantitative real-time polymerase chain reaction (qPCR) for detection of *Brucella spp*

To detect the presence of *Brucella* spp., a specific region (317 bp) of the IS711 insertion sequence belonging to the *Brucella* genus was amplified. The specific primers (Pishgam Biotechnology Co., Iran) used for amplification were as follows: forward primer (IS 711-F): 5′-GAGAATAAA GCCAACACCCG-3′ and reverse primer (IS 711-R): 5′-GAT GGACGAAACCCACGAAT-3′ [12].

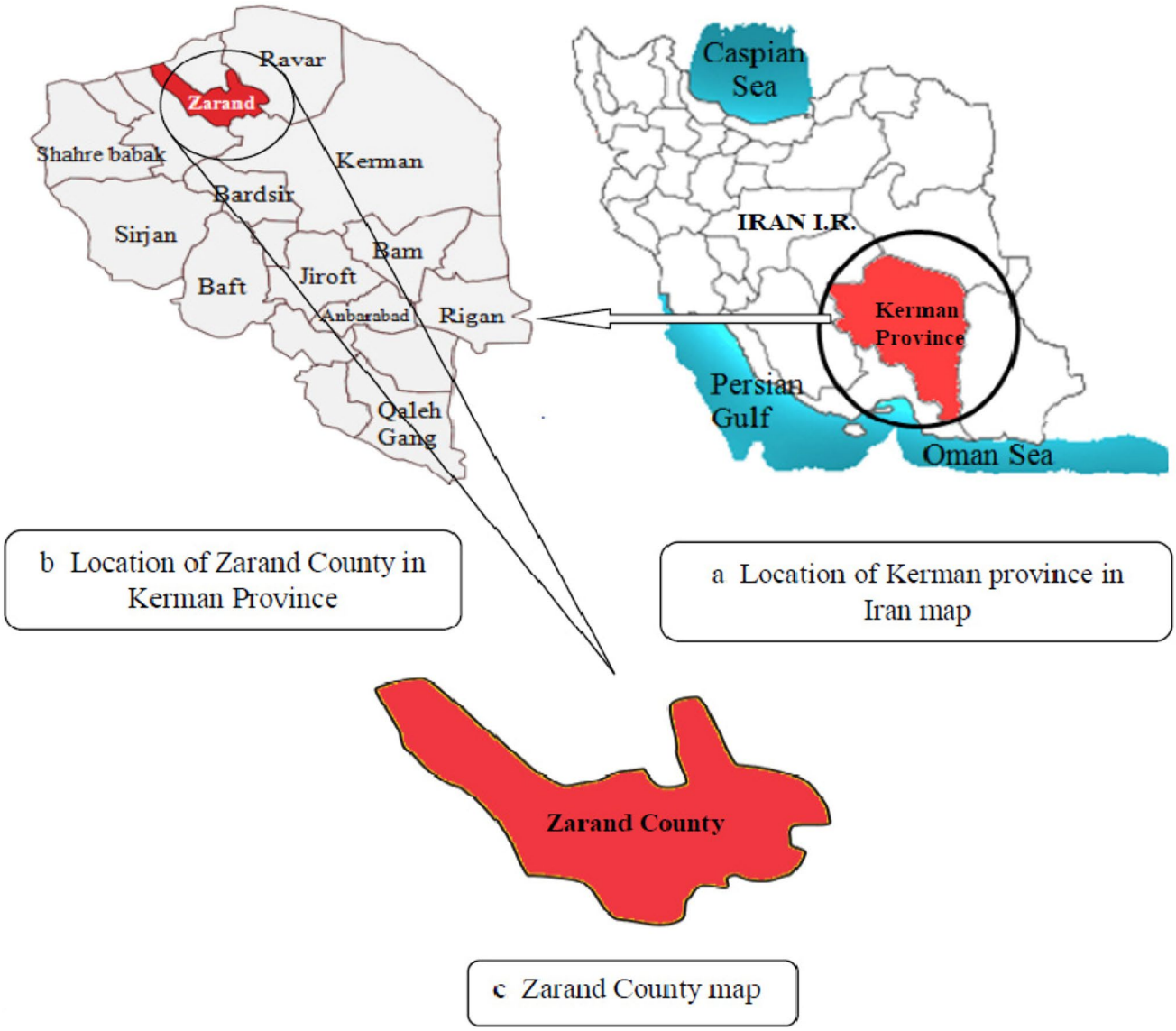

Fig. 1 The sampling area in the present study. The samples were collected from Zarand County, situated in Kerman Province in the southeast of Iran

For the PCR reaction, a mixture was prepared to contain 2.5 µl of DNA sample (1 µg), 12.5 µl of X Taq PCR Master Mix2 (Pars Tous Co., Iran), and 1 µl (1 uM) of each forward and reverse primer. The final volume of the reaction was adjusted to 25 µl with deionized sterile distilled water. *Brucella melitensis* strain Rev1 was used as the positive control, while sterile distilled water served as the negative control.

The PCR amplification was performed using the following temperature program in the thermal cycler machine: predenaturation: 95 °C for 3 min, denaturation: 95 °C for 30 s, annealing: 58 °C for 30 s, extension: 72 °C for 60 s, and final extension: 72 °C for 10 min [12]. After amplification, the DNA products were analyzed by agarose gel electrophoresis, and the bands were visualized using a UV transilluminator.

To confirm and identify the species of *Brucella*, the positive samples were sent to the National Reference Laboratory for Plague, Tularemia, and Q fever at the Pasteur Institute of Iran.

For the confirmation of positive *Brucella* spp., the qPCR method targeting the IS711 of the *Brucella* genus was employed. The qPCR used specific probe and primers with the following sequences: probe: FAM-AAGCCAACACCC GGCCATTATGGT-TAMRA (IS 711-probe), forward primers: 5′- GCTTGAAGCTTGCGGACAGT-3′ (IS 711-F), and reverse primer: 5′-GGCCTACCGCTGCGAAT-3′ (IS 711-R) [12]. The qPCR mixture consisted of the following components: 4 µl of the extracted DNA sample, 10 µl of RaelQ plus Master Mix2x (Ampliqon Co., Denmark), 200 nM of

the probe, and 900 nM of the forward and reverse primers. To reach a total volume of 20 µl, deionized sterile distilled water was added to the mixture. The Rotor-Gene system 6000 Corbett (Corbett, Victoria, Australia) was utilized for the qPCR. The following thermal cycling conditions were programmed: initial denaturation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 60 s [12]. Ten consecutive dilutions (10-folds) were used for the standard positive control stock standard curve. Quantification of the approximate bacterial load and the quantification cycle (Cq) values were achieved using this standard. Finally, all samples were tested against the standard curve, and Cq values for all samples were determined. The Real Time-TaqMan PCR results and Cq were analyzed using the Rotor-Gene® Q 2.3.5 software from QIAGEN.

Conventional PCR for *Brucella* species identification

The confirmed positive samples underwent analysis to identify the specific *Brucella* species present. The following *Brucella* species were targeted for identification: *B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae*.

In a previous study, seven PCR tests (PCR2-7) were specifically designed to differentiate between these *Brucella* species (*B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae*) [12]. PCR2 yields positive results for *melitensis*, *B. ovis*, and *B. neotomae*. PCR3, PCR5, and PCR7 yield positive results for *B. abortus*, *B. ovis*, and *B. neotomae*, respectively. PCR4 detects positivity in *B. suis*, *B. canis*, and *B. neotomae*, while PCR6 detects positivity in *B. suis* and *B. canis* [12]. In the current study, we employed this specific protocol for differentiating between *Brucella* species. In this study, the PCR2 test was initially conducted. If PCR2 produced positive results (indicating *B. melitensis*, *B. ovis*, and *B. neotomae*), subsequent PCR5 and PCR7 tests were performed to determine the species of *B. ovis* and *B. neotomae*, respectively. If PCR2 was positive but PCR5 or PCR7 was negative, the species was identified as *B. melitensis*. In cases where PCR2 yielded negative results, PCR3 (specific for *B. abortus*) was carried out.

Since all samples in this study were successfully classified into a specific species using PCRs 2, 3, 5, and 7, additional PCR tests (PCR4 and PCR6) were not performed.

The PCR reaction mixture used in this study comprised 4 µl of DNA sample (1 µg), 10 µl of Taq DNA Polymerase 2 × Master Mix RED (Ampliqon Co., Denmark), and 0.7 µl (1 uM) of forward and reverse primers as indicated in Table 1. The final volume of the reaction mixture was adjusted to 20 µl using deionized sterile distilled water. The PCR reaction was carried out in a thermal cycler machine using the following temperature program: an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 56–61 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 10 min [12].

Table 1 Primers used for *Brucella* species identification via conventional PCR [12]

PCR	Target sequence	Forward (F) and Reverse (R) primers
2	BMEII0466	F: TCGCATCGGCAGTTTCAA R: CCAGCTTTTGGCCTTTTCC
3	BruAb2_0168	F: GCACACTCACCTTCCACAACAA R: CCCCGTTCTGCACCAGACT
5	BMEII0986–0988	F: ATGCGGATGCCCGTTTC R: AACCTGGCGTCTTTGTCTATCACT
7	BMEII0986–0988	F: ATGCGGATGCCCGTTTC R: AACCTGGCGTCTTTGTCTATCACT

Statistical analysis

For the statistical analysis of this study's data, SPSS software version 18 (SPSS Inc., Chicago, IL, USA) was used. A *p*-value below 0.05 was regarded as statistically significant in determining associations between the variables.

Results

Sample characteristics

The average age of the 50 included cases of aborted small ruminants (46 sheep and 4 goats) in this study was 2.92 years (minimum 1 year and maximum 6 years). In total, 8 (16%) of all animals (8 of 46 sheep and none of 4 goats) had at least one previous abortion history. The median of parturitions was 2 times (minimum 1 time and maximum 4 times). All livestock included in this study were vaccinated (FD Rev1) against brucellosis as part of a national control program.

Brucella spp. detection in the samples

In the present study, the *Brucella* spp. was detected in 15 samples (30%) using conventional PCR. The results of conventional PCR were confirmed by the qPCR method. Among these positive samples, 13 (26%) were from sheep and 2 (4%) were from goats. There were no significant differences ($p > 0.05$) in *Brucella* infection rates based on animal type (goats vs. sheep), age, history of abortion, or number of parturitions (Table 2).

Brucella species identification in the samples

Out of the 13 positive *Brucella* samples obtained from sheep abortions, 6 samples (46%) were identified as *B. ovis*, 6 samples (46%) were classified as *B. melitensis*, and 1 sample (8%) was classified as *B. abortus*. Among the two positive goat samples,

Table 2 The association between various characteristics and the presence of *Brucella* infection in the present study

Characteristics	Studied classes	No. of samples (%)	No. of positive for *Brucella* (%)	*P*-value
Animal type	Goat	4 (8.0)	2 (50.0)	0.42
	Sheep	46 (92.0)	13 (28.3)	
Age (years)	1	2 (4.0)	1 (50.0)	0.67
	2	13 (26.0)	4 (30.8)	Reference
	3	25 (50.0)	7 (28.0)	0.86
	4	8 (16.0)	2 (25.0)	0.81
	5	1 (2.0)	0 (0.0)	0.71
	6	1 (2.0)	1 (100)	0.36
History of abortion	Yes	8 (16.0)	1 (12.5)	0.27
	No	42 (84.0)	14 (33.3)	
Parturitions (times)	1	12 (24.0)	1 (8.3)	Reference
	2	29 (58.0)	11 (37.9)	0.08
	3	7 (14.0)	2 (28.6)	0.37
	4	2 (4)	1 (50.0)	0.31

one sample (50%) was identified as *B. ovis* and the other sample (50%) was identified as *B. abortus*. In total, *B. ovis* was detected in 7 (14%) samples, *B. melitensis* in 6 (12%) samples, and *B. abortus* in 1 (4%) sample in the present study (Table 3).

Discussion

Various factors, such as infectious and noninfectious factors, can contribute to abortion in small ruminants [13–15]. According to a comprehensive review study conducted in 2019, it was recognized that the most prevalent bacteria responsible for sheep and goat abortion in Iran were *Brucella*, *Toxoplasma*, *Chlamydophila*, *Campylobacter*, and *Salmonella* species [15]. Therefore, our study specifically highlighted the molecular detection of *Brucella* spp. as one of the significant infections contributing to abortions in Iranian small ruminants in samples from aborted goats and sheep. The findings of this research confirmed that *Brucella* infection is one of the potential causes of abortion within the studied population in a specific region of Southeast Iran. However, it would be advantageous to offer a more

Table 3 The results of PCR identification of*Brucella* species among positive samples

Sample ID	Infected animal	Cq in qPCR1 (*Brucella* spp.)	PCR2 (specific for *B. melitensis*; *B. ovis*; *B. neotomae*)	PCR3 (specific for *B. abortus*)	PCR5 (specific for *B. ovis*)	PCR7 (specific for *B. neotomae*)	Final identification
5	sheep	28.04	+	-	+	nt*	*B. ovis*
6	sheep	30.05	+	-	-	-	*B. melitensis*
13	sheep	21.47	+	-	-	-	*B. melitensis*
14	sheep	22.75	+	-	+	nt	*B. ovis*
15	sheep	27.59	+	-	+	nt	*B. ovis*
18	sheep	27.50	+	-	-	-	*B. melitensis*
19	sheep	21.63	+	-	+	nt	*B. ovis*
33	sheep	32.63	-	+	-	-	*B. abortus*
45	sheep	22	+	-	-	-	*B. melitensis*
12	goat	33.54	-	+		nt	*B. abortus*
17	goat	34.39	+	-	+	nt	*B. ovis*
31	sheep	34.21	+	-	-	nt	*B. melitensis*
32	sheep	35.93	+	-	+	nt	*B. ovis*
35	sheep	33.27	+	-	-	nt	*B. melitensis*
40	sheep	33.92	+	-	+	nt	*B. ovis*

*nt: not tested

comprehensive outlook on the causes of other infectious abortions in sheep and goats across Iran.

In the present study, we observed that 46.7% of brucellosis-induced abortions were attributed to *B. ovis*, while *B. melitensis* and *B. abortus* accounted for 40% and 13.3%, respectively. In a study conducted in the Sistan and Baluchistan province of eastern Iran in 2016, *B. melitensis* was identified in approximately 2.19% of the abomasum and spleen samples collected from aborted sheep fetuses [16]. Additionally, a study conducted from 2016 to 2019 in Iran revealed that all *Brucella* isolates obtained from abortion samples of sheep and goats were *B. melitensis* (8.99%) and *B. abortus* (0.52%) [17]. In the present study, we observed distinct *Brucella* species in the positive samples obtained from sheep abortions. Specifically, the identified species were *B. ovis*, *B. melitensis*, and *B. abortus*. This distribution of *Brucella* species in the sheep abortion cases provides important insights into the prevalence and diversity of *Brucella* infections in our study population. Additionally, out of two positive bacteria in the goat samples, one *Brucella* spp. was identified as *B. ovis* and the other as *B. abortus*. In our study, we found *B. abortus* in sheep and goats despite it being considered the principal host in cattle [18]. Several studies have explored cross-species transmission between small ruminants, providing valuable insights for comparison. For instance, a similar investigation in a neighboring region found a considerable number of sheep infected with *B. abortus* due to mixed farming, where small and large ruminants are raised together and share the same pasture, along with the presence of reservoir hosts on a farm [19]. Another study conducted in Egypt in 2015 [17] showed that *B. abortus* (principal host: cattle) could be transmitted from infected cattle to other livestock species raised in close contact with them. Furthermore, although *B. ovis* typically infects sheep [20], the present study identified this species in both sheep and goat aborted fetuses. Lastly, while goats are the natural host of *B. melitensis* [21], it was only identified in sheep in the present study. Our results confirm infections with non-specific species in sheep and goats and highlight the importance of considering cross-species transmission and infection dynamics in brucellosis epidemiology. Moreover, the presence of *B. ovis* and *B. abortus* in aborted goat fetuses indicated that these species are not limited to their principal livestock host. This understanding the mechanisms and factors involved in cross-species transmission is crucial for designing effective control strategies [22, 23]. Further investigation is needed to assess the specific pathways through which species spread between different hosts. Comparative genomics studies analyzing strains from cattle, sheep, and goats could provide insights into genetic adaptations that allow species to infect multiple hosts [24, 25]. In addition, the results of the present study demonstrate that *Brucella* infection in non-principal hosts not only disrupts disease control and prevention systems in the livestock population but also could facilitate the transmission of more significant species, such as *B. melitensis* and *B. abortus*, to humans. In a study conducted in various regions of Iran from 2016 to 2019, it was found that a *B. melitensis* vaccine strain Rev1 was detected in a small number of sheep and goat abortion cases [17]. However, it should be noted that the present study did not include any additional tests to distinguish the *B. melitensis* Rev.1 vaccine strain from other *B. melitensis* strains. This represents a limitation of our study. In Iran, vaccination practices involve the use of RB51 strains of *B. abortus* in cattle and Rev1 *B. melitensis* strain in smaller livestock populations such as sheep and goats. Therefore, these strains should also be taken into consideration when assessing the potential causes of abortion. Additionally, since *B. abortus* species was observed in sheep and goats, further research is crucial to explore the usage of recombinant vaccines that can effectively target all *Brucella* species across different livestock species.

In conclusion, this study highlighted the significant occurrence of diverse *Brucella* species in abortion samples from sheep and goat flocks in southeast Iran. While our study focused on *Brucella* species detection, considering the multifactorial nature of abortion, we acknowledge that it is essential to conduct further research to establish any causal relationship and explore the involvement of other potential causes of abortion in small ruminants in this region, in conjunction with *Brucella* species. Moreover, the study revealed infections in livestock with non-principal species of *Brucella*, such as *B. abortus* in sheep and goats, *B. ovis* in goats, and *B. melitensis* in sheep. This suggests that keeping and raising livestock together, even nearby, could contribute to the spread of non-principal species infections among different livestock species. This poses challenges for epidemiological studies and reduces the effectiveness of preventive measures like vaccination. To address these challenges, future studies should focus on identifying *Brucella* species in livestock to understand the mechanisms and factors that contribute to cross-species transmission. Additionally, it is crucial to provide training to livestock farmers, emphasizing adherence to livestock and health management principles. Such efforts, when combined with accurate species identification and the utilization of suitable vaccine strains, can immensely contribute to the control and prevention of brucellosis.

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Author contribution **AA**: conceptualization, data curation, investigation, methodology, software, validation, visualization, writing – original draft.

MKh: conceptualization, data curation, funding acquisition, investigation, project administration, resources, supervision, validation, visualization. **NB**: investigation, methodology, writing – original draft, writing – review and editing. **SE:** conceptualization, investigation, resources, validation, writing – review and editing. **EM.d:** methodology. **SK**: methodology. All authors have read and agreed to the published version of the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval This study was approved by the Research Ethics Committee of the Shahid Bahonar University of Kerman and Pasteur Institute of Iran (No. IR.PII.REC.1398.05). All methods and instructions were performed under institutional guidelines and regulations and were reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) and the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Farmers permitted (with informed consent) their animal samples to be included in this study.

Competing interests The authors declare no competing interests.

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