

# ***Brucella abortus* and *Brucella melitensis* in Iranian Bovine and Buffalo Semen Samples: The First Clinical Trial on Seasonal, Senile and Geographical Distribution Using Culture, Conventional and Real-time Polymerase Chain Reaction Assays <sup>[1]</sup>**

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## **Abstract**

Conventional and real-time PCR assays were developed for detection and identification of *Brucella* species in bovine and buffalo semen samples. Totally, 91 bovine and buffalo semen samples were collected from 4 major provinces of Iran in various seasons. The animals which their semen samples were collected for this study had less than 1 year old, 1-2 years old, 2-3 years old and more than 3 years old. Samples were cultured and DNA was extracted and novel primers have been designed using the IS711 target of *Brucella* species for conventional PCR. Positive results of PCR have been studied for presences of *Brucella abortus* and *Brucella melitensis*. Totally, 21.56% and 14.28% of bovine and buffalo semen samples, respectively, were positive for *Brucella* species. Khozestan had the highest while Sistan Va Balochestan had the lowest incidences of *Brucella* species in studied regions. Samples which were collected in spring season had the highest rate of infection. Also, samples which were collected from less than 1 year old bovine and buffalo semen samples had the highest incidence of *Brucella* species. Totally, 25 (24.50%) and 4 (3.92%) bovine semen samples and 14 (15.38%) and 1 (1.09%) buffalo semen samples were positive for *Brucella abortus* and *Brucella melitensis*.

**Keywords:** *Brucella abortus*, *Brucella melitensis*, Seasonal distribution, Senile distribution, Geographical distribution, Semen, Iran

## **İran'da Sığır ve Yaban Sığırları Semen Örneklerinde *Brucella abortus* ve *Brucella melitensis*: Kültür, Konvansiyonel ve Real-time Polimeraz Zincir Reaksiyonu Kullanılarak Mevsimsel, Yaşa Bağlı ve Bölgesel Dağılımı Üzerine İlk Klinik Çalışma**

### **Özet**

Sığır ve yaban sığırlarının semen örneklerinde *Brucella* türlerinin tespit ve tanımlanması amacıyla konvansiyonel yöntemler ve Gerçek Zamanlı Zincirleme Polimeraz Reaksiyonu kullanıldı. İran'da değişik mevsimlerde 4 ana bölgeden toplam 91 adet sığır ve yaban sığırları semen örnekleri toplandı. Semen örneklerinin toplandığı hayvanlar 1 yaş, 1-2 yaş, 2-3 yaş ve 3 yaş üzeri olarak gruplandırıldı. Örneklerden kültür hazırlandı, konvansiyonel PCR amacıyla DNA ekstraksiyonu yapılarak *Brucella* türleri için IS711 kullanılarak primerler dizayn edildi. Pozitif sonuç veren örnekler *Brucella abortus* ve *Brucella melitensis* yönünden incelendi. Sığırların %21.56'sında yaban sığırların %14.28'inde *Brucella* tespit edildi. Çalışılan bölgeler arasında Khozestan en yüksek Sistan Va Balochestan ise en düşük *Brucella* oranına sahipti. İlbaharda toplanan örneklerde oran en yüksek olarak bulundu. En yüksek *Brucella* oranı 1 yaşın altındaki hayvanlardan toplanan semen örneklerinde tespit edildi. *Brucella abortus* 25 sığırdada (%24.50) ve 14 yaban sığırlarında (%15,38) *Brucella melitensis* ise 4 sığırdada (%3.92) ve 1 yaban sığırlarında (%1.09) belirlendi.

**Anahtar sözcükler:** *Brucella abortus*, *Brucella melitensis*, Mevsimsel dağılım, Yaşa bağlı dağılım, Bölgesel Yayılım, Semen, İran



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## INTRODUCTION

Brucellosis is a highly contagious zoonotic bacterial disease of human and many species of animals worldwide caused by gram-negative, aerobic and facultative intracellular bacterium of the genus *Brucella*. It is an important public health problem in many parts of the worlds, such as the Mediterranean littoral, the Middle East and parts of Latin America [1]. *Brucella* species are classically classified into 6 main species 2 of which are *Brucella abortus* (*B. abortus*) and *Brucella melitensis* (*B. melitensis*) cause abortions in ruminants [2].

In some countries, especially in Europe and Asia (including Iran), where animals like camelids and buffaloes are kept in close contact with infected sheep, goat and cattle, infections and abortions can also be caused by *B. melitensis* and *B. abortus* [3,4].

In Iran, *B. abortus* was first isolated from a bovine fetus in 1944 [5], and *B. melitensis* was first isolated from a sheep in Isfahan Province in 1952 [6]; brucellosis has been reported from various parts of Iran ever since. In the majority of cases of brucellosis in Iran, *B. abortus* and *B. melitensis* are the main pathogens. Artificial insemination is used to induce fertility in livestock in Iran and other sites of the world. Therefore, the quality and hygiene of semen samples should be considered. There are various assays for diagnosis of brucellosis such as culture, serological and molecular methods. Culture methods require a living host and are both time consuming and hazardous [7]. Previous study showed the low accuracy of serological methods for detection of brucellosis [8]. The usual method for detection and segregation of *Brucella* spp. is based on phenotypic traits, but it is associated with a high risk of laboratory-acquired infections and very time consuming [7].

Therefore, in order to avoid these problems and despite the high degrees of genetic similarity of *Brucella* spp., several conventional and real-time Polymerase Chain Reaction (PCR) assays that are easier, faster, safer and more accurate than traditional methods have been developed [9,10].

It seems that several risk factors like seasons, age and geographical area have an effective role in epidemiology and prevalence of *Brucella* spp. in animal hosts [11,12] but they are unknown in Iran. Therefore, this present investigation was carried out in order to study the seasonal, senile and geographical distribution of *Brucella* spp. in bovine and buffalo semen samples and identification of *B. abortus* and *B. melitensis* in positive cases.

## MATERIAL and METHODS

### Samples

From January 2011 to January 2012 (in various seasons of the year), a total of 102 bovine and 91 buffalo semen

samples were collected randomly from 43 commercial herds in various parts of Iran (Table 1). Those were commercial herds producing semen. The animals from which semen samples were collected for this study were clinically healthy and were classified into 4 age groups (less than 1 year old, 1-2 years old, 2-3 years old and over 2 years old) (animal age has been obtained from the history taking). All of these animals have been classified into two groups (with and without histories of orchitis). From each animal, 10 ml of semen were collected with using an artificial vagina. All semen samples showed normal physical characteristics including color and density. All samples were collected under sterile hygienic conditions and were immediately transported at 4°C to the laboratory in a cooler with ice packs. All of them were cultured and then the remaining semen samples were kept at -20°C until processing.

### Brucella Culture Method

A trial of bacterial isolation from the samples was performed on blood agar base (Oxoid) supplemented with 5% defibrinated sheep erythrocytes and antibiotics (vancomycin, nalidixic acid, bacitracin, nystatin and cycloheximide at the dose recommended in the OIE manual, 2000). Cultures were incubated for 10 days with 5% CO<sub>2</sub> at 37°C. The isolated bacteria were identified according to the conventional procedures [13].

### DNA Extraction

For *Brucella* DNA extraction, previous method that was introduced by Consuelo Vanegas et al. [14] was used. Purification of DNA was achieved using a genomic DNA purification kit (Fermentas GmbH, St. Leon-Rot, Germany), and the total DNA was measured at an optical density of 260 nm according to the method described by Sambrook and Russell [15].

### Primer Designs and Conventional PCR

In the present study, the conventional PCR assay has been designed by the authors. This PCR to screen the *Brucella* spp. detected the DNA sequence of the gene coding the IS711 target reported for *Brucella* in the GenBank database of the National Center For Biotechnology Information (NCBI) (GenBank No: AF242533.1). In order to design primers, recorded sequences of the IS711 target have been gotten from the NCBI. The CLS sequence viewer software (Version 6/4) has been used for alignments of the IS711 target. Forward and reverse primers have been designed based on the protected area in these sequences. Thermodynamic properties of designed primers were studied using the Gene Runner software (Version 3.05). In order to ensure the specificity of designed primers, the Basic Logical Alignment Search Tool (BLAST) service, has been used. The forward primer sequence was 5'-GCGGTCAATGTTTTCTCGCA-3', and the reverse primer sequence was 5'-TGGGGCATGTCATTGCTGAT-3'.

All of the semen samples were analyzed for presence of *Brucella* using the novel conventional PCR assay. The PCR reaction was performed in a total volume of 25  $\mu$ l containing 10  $\mu$ l DNA concentrated in 2  $\mu$ l of DNA sample, 0.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix, 0.8  $\mu$ M each primers and 0.5 U/reaction of Taq DNA polymerase. Reactions were initiated at 94°C for 5 min, followed by 30 cycles of 94°C for 50 sec, 57°C for 1 min, 72°C for 1 min and a final elongation step at 72°C for 5 min, with a final hold at 4°C in a DNA thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany). A negative control (sterile water), and a positive control DNA from *B. abortus* strain S19 (S19 vaccine strain) (Razi Institute, Karaj, Iran), were included in each amplification run.

### Gel Electrophoresis

The PCR-amplified products (IS711: 223 bp) were examined by electrophoresis (120 V/208 mA) in a 1.5% agarose gel, stained with a solution of ethidium bromide (0.004  $\mu$ g/ml) and examined under UV illumination.

### Real-Time PCR

The real-time PCR for species segregation was based on unique genetic loci of *B. melitensis* and *B. abortus*. The primer set (which was designed by the author) consisted of BMEI10466 (5'-TCGCATCGGCAGTTTCAA/CCAGCTTTTGGCCTTTTCC-3') (112 bp) with the Cy5-CCTCGGCATGGCCGCAA-BHQ-2 (5'Fluorophore $\rightarrow$ 3'Quencher) internal probe for *B. abortus* and BruAb2-0168 (5'-GCACACTCACCTTCCACAACAA/CCCCGTTCTGCACCAGACT-3') (222bp) with the FAM-TGGAACGACCTTTGCAGGCGAGATC-BHQ-1 internal probe for *B. melitensis*. In this study, the starting quantity of DNA from each serial dilution was plotted as a function of threshold cycle (CT) values to obtain a standard curve. On the other hand, the CT is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. All of the positive samples on conventional PCR assays were studied for presences of *B. abortus*, *B. melitensis*, both bacteria and other species of *Brucella*.

A typical 25  $\mu$ l reaction contained 12.5  $\mu$ l TaqMan<sup>®</sup> Universal PCR Master Mix (Foodproof<sup>®</sup> Brucella Detection Kit), a 300 nM concentration of each forward and reverse primer (Bioneer Corporation, Daejeon, South Korea), a 200 nM concentration of the probe (Bioneer Corporation, Daejeon, South Korea), and 2.5 ng of sample DNA. TaqMan Master Mix real-time PCR reactions were carried out using a Rotor-Gene 6000 instrument (Corbett Research, Mortlake, NSW, Australia). The reaction mixture was initially incubated for 10 min at 95°C. Amplification was performed with 45 cycles of denaturation at 95°C for 20 sec, annealing and extension at 62°C for 1 min. In this reaction, the Foodproof<sup>®</sup> Brucella Internal Control (White cap) and Foodproof<sup>®</sup> Brucella Control Template (Purple cap) were used as an internal and positive control, respectively.

### Sequencing

In order to confirm the PCR results, a sequencing method was used. For this reason, PCR products of some positive samples were purified with a High Pure PCR Product Purification Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's recommendations. Single DNA strands were sequenced with an ABI 3730 XL device and Sanger sequencing method (Macrogen, Seoul, South Korea). The sequence of each gene was aligned with the gene sequences recorded in the GenBank database on the NCBI.

### Statistical Analysis

Data were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using the Statistical Package for the Social Sciences (SPSS) 18.0 statistical software (SPSS Inc., Chicago, IL, USA), Analysis of Variance (ANOVA) test analyses were performed for study the differences between incidence of bacteria in various seasons and various ages, and differences were considered significant at values of  $P < 0.05$ . In this study, the distribution of CT values was compared between bovine and buffalo semen samples using an ANOVA test.

## RESULTS

The results of this present study showed that 22 out of 102 bovine semen samples (21.56%) and 13 out of 91 buffalo semen samples (14.28%) were positive for *Brucella* spp. [Table 1](#) presents the seasonal distribution of *Brucella* spp. in bovine and buffalo semen samples collected from various geographical regions.

[Table 2](#) presents the senile distribution of *Brucella* spp. in bovine and buffalo semen samples collected from various geographical regions.

Totally, 25 bovine (24.50%) and 17 buffalo (18.68%) of samples had the typical 223 bp fragment in gel electrophoresis ([Fig. 1](#)) which were positive for *Brucella* spp. positive conventional PCR results were studied using Taqman real-time PCR. Totally, 25 out of 102 (24.50%) and 4 out of 102 (3.92%) bovine semen samples were positive for *B. abortus* and *B. melitensis*, respectively. Also, 14 out of 91 (15.38%) and 1 out of 91 (1.09%) buffalo semen samples were positive for *B. abortus* and *B. melitensis*, respectively. Results showed that only a bovine semen sample were positive for presence of both *B. abortus* and *B. melitensis*. Also, 2 bovine semen samples (1.96%) and 2 buffalo semen samples (2.19%) were positive for other species of *Brucella* ([Table 3](#)).

The sensitivity and specificity of our novel primers for detection of *Brucella* spp. in bovine and buffalo semen samples were 100% and 96% and 100% and 94%, respectively ([Table 4](#)).

**Table 1.** Seasonal distribution of *Brucella* spp. in bovine and buffalo semen samples collected from various geographical regions**Tablo 1.** Değişik bölgelerdeki sığır ve yaban sığırlarından toplanan semen örneklerinde *Brucella* spp. mevsimsel dağılımı

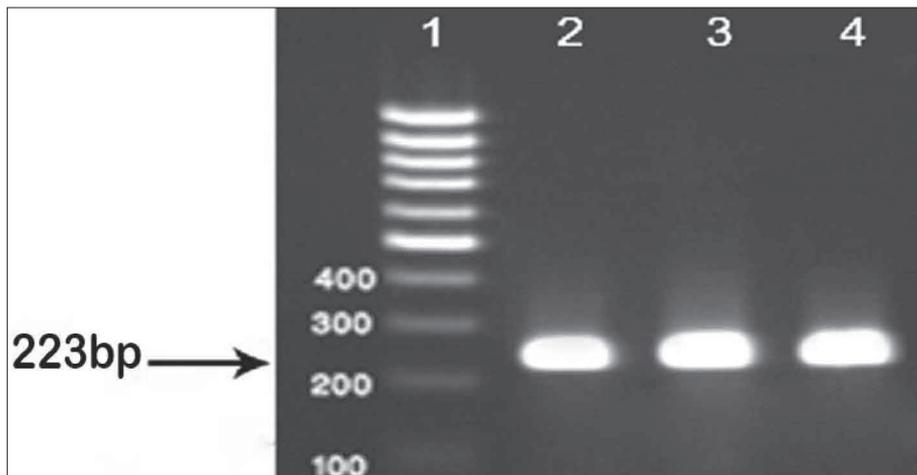
Provinces	No. of Samples		Culture Positive (%)		Seasonal Distribution (%)							
	Bovine	Buffalo	Bovine	Buffalo	Summer		Autumn		Winter		Spring	
					Bovine	Buffalo	Bovine	Buffalo	Bovine	Buffalo	Bovine	Buffalo
Khozestan	30	29	9 (30)	6 (20.68)	-	1 (16.66)*	2 (22.22)	2 (33.33)	1 (11.11)	-	6 (66.66)	3 (50)
Boshehr	27	21	6 (22.22)	3 (14.28)	1 (6.66)	-	1 (16.66)	1 (33.33)	-	-	4 (66.66)	2 (66.66)
Hormozgan	24	21	4 (16.66)	2 (9.52)	-	-	1 (25)	-	1 (25)	-	3 (75)	1 (50)
Sistan Va Balochestan	21	20	3 (14.28)	2 (10)	-	-	1 (33.33)	-	-	-	2 (66.66)	2 (100)
Total	102	91	22 (21.56)	13 (14.28)	1 (4.54)	1 (7.69)	5 (22.72)	3 (23.07)	2 (9.09)	-	15 (68.18)	8 (61.53)

\* Positive samples from a total of culture positive samples

**Table 2.** Senile distribution of *Brucella* spp. in bovine and buffalo semen samples collected from various geographical regions**Tablo 2.** Değişik bölgelerdeki sığır ve yaban sığırlarından toplanan semen örneklerinde *Brucella* spp. yaşa bağlı dağılımı

Provinces	No. of Samples		Culture Positive (%)		Senile Distribution (%)							
	Bovine	Buffalo	Bovine	Buffalo	Bovine				Buffalo			
					1>	1-2	2-3	3<	1>	1-2	2-3	3<
Khozestan	30	29	9 (30)	6 (20.68)	5 (55.55) *	2 (22.22)	1 (11.11)	1 (11.11)	3 (50)	1 (16.66)	1 (16.66)	1 (16.66)
Boshehr	27	21	6 (22.22)	3 (14.28)	3 (50)	2 (33.33)	1 (16.66)	-	2 (66.66)	1 (33.33)	-	-
Hormozgan	24	21	4 (16.66)	2 (9.52)	3 (75)	1 (25)	-	-	1 (50)	1 (50)	-	-
Sistan Va Balochestan	21	20	3 (14.28)	2 (10)	2 (75)	1 (25)	-	-	2 (100)	-	-	-
Total	102	91	22 (21.56)	13 (14.28)	13 (59.09)	6 (27.27)	2 (9.09)	1 (4.54)	8 (36.36)	3 (23.07)	1 (4.54)	1 (7.69)

\* Positive samples from a total of culture positive samples

**Fig 1.** Novel conventional PCR for detection of *Brucella* spp. Lane 1 is a 100 bp ladder, lanes 2 and 3 are positive samples for bovine and buffalo semen samples (223 bp) and lane 4 is the positive control**Şekil 1.** *Brucella* spp.'nin PCR ile tespiti. 1. sıra 100 bp merdiveni, 2. ve 3. sıralar sırasıyla sığır ve yaban sığırı için pozitif örnekler (223 bp) ve 4. sıra pozitif kontrol

## DISCUSSION

Our study showed that the semen samples of infected bovine and a buffalo possibly plays an important role in distribution of brucellosis in Iran. Unfortunately, despite the high incidences of these bacteria in bovine and buffalo semen samples, very little research concerning detection of *Brucella* in semen samples has been performed.

In addition, in the majority of cases, the semen samples are not well screened for the presence of *Brucella* and other pathogens. When laboratories do screen for them, they commonly use traditional diagnostic methods like the enzyme-linked immunosorbent assay (ELISA). Diagnosis of brucellosis by the ELISA method is not recommended because it can be unspecific and subsensitive due to cross-reaction with other pathogens including *Yersinia*

**Table 3.** Incidences of *B. melitensis*, *B. abortus* and other species of *Brucella* in bovine and buffalo semen samples collected from various geographical regions**Table 3.** Değişik bölgelerdeki siğir ve yaban siğirlerinden toplanan semen örneklerinde *B. abortus*, *B. melitensis* ve diğer *Brucella* türlerinin yoğunluğu

Provinces	No. of Samples		Conventional PCR (%)		Novel Real-time PCR (%)							
	Bovine	Buffalo	Bovine	Buffalo	Bovine				Buffalo			
					<i>B. abortus</i>	<i>B. melitensis</i>	Unknown	Both bacteria	<i>B. abortus</i>	<i>B. melitensis</i>	Unknown	Both bacteria
Khuzestan	30	29	11 (36.66)	8 (27.58)	8 (26.66)*	2 (6.66)	1 (3.33)	1 (3.33)	6 (20.68)	-	2 (6.89)	-
Bushehr	27	21	7 (25.92)	4 (19.04)	6 (22.22)	-	1 (3.70)	-	3 (14.28)	1 (4.76)	-	-
Hormozgan	24	21	4 (16)	3 (14.28)	3 (12.5)	1 (4.16)	-	-	3 (14.28)	-	-	-
Sistan Va Baluchestan	21	20	3 (14.28)	2 (10)	2 (9.52)	1 (4.76)	-	-	2 (10)	-	-	-
Total	102	91	25 (24.50)	17 (18.68)	25 (24.50)	4 (3.92)	2 (1.96)	1 (0.98)	14 (15.38)	1 (1.09)	2 (2.19)	-

\* Positive results of total samples

**Table 4.** Evaluation of sensitivity and specificity of conventional PCR for detection of *Brucella* spp. in the bovine (A) and buffalo (B) semen samples**Table 4.** Konvansiyonel PCR'in siğir (A) ve yaban siğirlerinde (B) *Brucella* spp. tespit etmedeki duyarlılığı ve özgüllüğü

A			
Sensitivity	Culture Positive	Culture Negative	Total
Conventional PCR positive	22 <sup>ab</sup>	3 <sup>c</sup>	25
Conventional PCR negative	- <sup>b</sup>	74 <sup>cd</sup>	74
Total	22 <sup>ab</sup>	77 <sup>cd</sup>	99
$\text{*Sensitivity: } \frac{a}{a+b} = 100\%$			
$\text{**Sensitivity: } \frac{d}{d+c} = 96\%$			
B			
Sensitivity	Culture Positive	Culture Negative	Total
Conventional PCR positive	13 <sup>ab</sup>	4 <sup>c</sup>	17
Conventional PCR negative	- <sup>b</sup>	74 <sup>cd</sup>	74
Total	13 <sup>ab</sup>	78 <sup>cd</sup>	91
$\text{*Sensitivity: } \frac{a}{a+b} = 100\%$			
$\text{**Sensitivity: } \frac{d}{d+c} = 94\%$			

*enterocolitica*, *Salmonella*, *Escherichia coli* O:157 and other *Brucella* spp.<sup>[16-18]</sup>. Therefore, this makes PCR as an accurate, safe, sensitive, fast and specific assay for detection and differentiation of *Brucella* spp., so essential in these cases. Furthermore, the real-time PCR assay has some advantages compared with the conventional PCR. It is an important diagnostic tool yielding reliable and reproducible results, does not require post-PCR analysis (gel electrophoresis, hybridization), and has a limited risk of cross contamination compared with the conventional method; however, real-time PCR is more expensive than conventional PCR. Many

studies have shown that the conventional method for detecting *Brucella* spp. is technically more time-consuming and labor-intensive than real-time PCR assay<sup>[8,19]</sup>.

To our best knowledge, this study has been introduced a pair of primer which had the high sensitivity and specificity for detection the IS711 target of *Brucella* spp. Also, this study is the first prevalence report of seasonal, senile and geographical distribution of *Brucella* spp. in bovine and buffalo semen samples in Iran. Our results showed that 24.50% of bovine and 18.68% of buffalo

semen samples were positive for *Brucella* spp. Also, 24.50% and 3.92% of bovine semen samples were positive for *B. abortus* and *B. melitensis*, respectively and 15.38% and 1.09% of buffalo semen samples were positive for *B. abortus* and *B. melitensis*, respectively. Statistical analysis showed significant differences ( $P < 0.01$ ) between *B. abortus* and *B. melitensis* in bovine and buffalo semen samples and between the presence of *Brucella* spp. in bovine with buffalo semen samples ( $P < 0.05$ ).

A previous study showed that Iran, Saudi Arabia, Jordan, Syria and Oman had the highest incidences of brucellosis among the countries of the Near East region [20]. Studies about brucellosis from various parts of Iran [12,21] and various species such as sheep [22], goats [23], cattle [24], buffaloes [21] and humans [6] indicated that brucellosis is one of the most important endemic zoonotic diseases in Iran. Brucellosis causes great economic losses in Iran. A previous report from Iran indicated that the prevalence of brucellosis was 0.037% in humans, 3.4% in sheep and goats and 0.56% in cattle in Eastern Iran during 2002-2006 [25]. The prevalence of *B. abortus* observed in bovine in this study (24.50%) is higher than those in Egypt (5.44%) [26], Ethiopia (4.9%) [27], and India (18.81%) [28], Punjab region (20.67%) [29] but lower than those in the Sokoto State (25.25%) [30] and Kenya (77.5%) [31]. The incidence of *B. abortus* observed in buffaloes in the present study (15.38%) is higher than those in Egypt (0.3%) [20] and the Punjab region (India) (13.4%) [10] but is lower than that in Africa (30%) [32]. It appears that there is no prevalence report of brucellosis caused by *B. melitensis* in bovine and buffaloes, making the present study is the first prevalence report of *B. melitensis* observed in bovine and buffalo semen samples in the world.

The high incidence of brucellosis in semen samples of an unspecific host (buffalo) in the present study possibly indicates that these animals were maintained in close association with infected sheep and cattle. In addition, the high prevalence of brucellosis in animals in Khozestan Province probably represents the low number of veterinary facilities in this province, geographical and climate conditions and importation of infected livestock from neighboring countries like Iraq.

The semen samples collected in spring seasons had the highest prevalence of *Brucella* spp. in bovine (68.18%) and buffalo (61.53%) semen samples. Statistical analyses were significant ( $P < 0.05$ ) for the prevalence of *Brucella* spp. between spring season and winter. The main reason for this finding is the fact that temperature and climate maybe have effect on the prevalence of *Brucella* spp. in bovine and buffalo semen samples. An explanation for the highest prevalence of *Brucella* spp. in spring season might be that during this time some climatic events such as heat, rain, and thunderstorms, as well as variation in barometric pressure might have occurred and may

have influence on the autonomic nervous system. These events is known to cause reduction in the levels of animal immunity. Therefore, several infections might also have been occurred.

Seasonal distribution of the brucellosis were expressed previously with the highest occurrence in May (15.9%), June (16.3%) and July (15.1%) (spring) [11], which was in agree with our results. In Germany, the largest number of cases was recorded in August and September [33]. In Central Greece, the largest number of outbreaks was reported from spring [34]. In countries with temperate or cold climates there is a marked seasonal variation in the incidence of acute brucellosis, with most cases occurring in spring and summer.

Also, the results from this study indicated that Khozestan had the highest while Sistan Va Balochestan had the lowest prevalence of *Brucella* spp. in bovine and buffalo semen samples. Statistical analysis were significant for the prevalence of *Brucella* spp. in both bovine and buffalo semen samples between Khozestan and while Sistan Va Balochestan provinces ( $P < 0.05$ ). After analyzing the average temperatures of these 4 seasons and provinces, it has been found that spring season and Khozestan province had the most constant temperature. The high prevalence of *Brucella* spp. seems reasonable in spring season and Khozestan province of Iran since this bacterium needs moderate temperature with high degree of moisture for growth and survival. Similarly, regional differences have also been reported previously from Azerbaijan [12].

Our results showed that there were strong age distributions for the incidence of *Brucella* spp. in bovine and buffalo semen samples in Iran. Results showed that 59.09% less than 1 year old bovine and 36.36% of less than 1 year old buffalo semen samples were positive for *Brucella* spp. Also, statistical analyses were significant for the prevalence of *Brucella* spp. between young bovines and buffaloes and old bovines and buffaloes ( $P < 0.05$ ). This may be explained by the fact that younger bovines and buffaloes have weaker immune system. Thus, several infection and illnesses maybe expected. Seasonal distributions of *Brucella* have been reported previously [33].

This study showed that both *B. abortus* and *B. melitensis*, can infect bovine and buffaloes, but the incidence of *B. abortus* was higher than *B. melitensis*. It appears that buffaloes are unlikely the primary hosts for *Brucella*, but they can be infected with both *B. abortus* and *B. melitensis*. Consequently, the prevalence of brucellosis in buffaloes is dependent on the infection rate of primary hosts being in contact with. On the other hand, spread of brucellosis in buffaloes depends on the *Brucella* spp. prevalence in other animals sharing their habitat and on the husbandry methods of the different species.

We also claim by this study that both bovine and buffalo can be important reservoirs for transmission of this zoonotic disease to humans in Iran. Our results revealed that bovines and buffaloes less than 1 year old in spring season and Khozestan province had the highest prevalence of *Brucella* spp. in Iran. Several control programs should be performed on Khozestan province in spring seasons especially on younger bovines and buffaloes. The present study shows that molecular methods such as conventional and real-time PCR are accurate, reliable and rapid assays for detection and identification of *B. abortus* and *B. melitensis* in bovine and buffalo semen samples but that the real-time PCR assay is better. It seems that this study is the first report of direct detection and segregation of *B. melitensis* and *B. abortus* by application of conventional and real-time PCR assays in bovine and buffalo semen samples in Iran. We hope that the real-time PCR method introduced in this study as an accurate, safe, fast, sensitive and specific assay for detection and segregation of *B. melitensis* and *B. abortus* in clinical samples.

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