

## Molecular Detection of *Brucella* spp. in the Semen, Testis and Blood Samples of Cattle and Sheep

Faham Khamesipour<sup>1,3\*</sup>, Abbas Doosti<sup>2</sup> and Hamed Taheri<sup>3</sup>

<sup>1</sup>Young Researchers and Elite Club, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran.

<sup>2</sup>Biotechnology Research Center, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran.

<sup>3</sup>Under Graduated Student of Veterinary Medicine, College of Veterinary Medicine, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran.

(Received: 05 July 2013; accepted: 30 August 2013)

Brucellosis is a zoonotic disease, which involves both animals and human. Although the conventional methods have been widely used for its laboratory diagnosis, the polymerase chain reaction (PCR) techniques have proved to be useful due to specificity, sensitivity and the rapidness. The aim of this study was to detect *Brucella* spp. in semen, testis and blood samples of cattle and sheep. From December 2012 and February 2013, 45 sperm (cattle) and 84 testis (Bulls and Rams) and 315 (cattle and sheep) blood samples were collected. Samples were immediately transferred to the laboratory and DNA was extracted from all samples. PCR was performed using specific primers for *Brucella* DNA. From 130 samples (total 444) were positive by PCR method. Totally, 46 (total 173) and 84 (total 271) of sheep's and cattle different samples were positive for *Brucella* species; And 14 (31.11%) bulls semen samples, and 7 (15.21%) and 4 (10.52%) bulls and rams testis samples, and 63 (35%) and 42 (31.11%) cattle and sheep's blood samples were positive for *Brucella* spp. PCR method is sensitive and specific for diagnosis and detection of *Brucella species* in suspected cases. According to present findings, the examination of cattle's and sheep's for *Brucella* infection seems to be necessary for control and prevention of Brucellosis.

**Keywords:** *Brucella* spp; Cattle; Sheep; semen; blood; testis; polymerase chain reaction.

Brucellosis is caused by *Brucella* spp. Brucellosis remains one of the world's major and widespread zoonotic disease problems of great economic importance caused by facultative intracellular Gram-negative, coccobacilli, non-motile bacteria belonging to the genus *Brucella*; and can lead to reproductive problems in an amount of large livestock and other animals. Brucellosis in humans is associated with a broad spectrum of symptoms and can occasionally be fatal<sup>1,2</sup>.

Farmers, veterinarians, laboratory personnel and abattoir workers are subject to be infected by *Brucella*<sup>3</sup>. Brucellosis continues to be of great health concern and economic importance in many countries such as Iran, Mediterranean littoral, the middle east and parts of Latin America<sup>4</sup>.

*Brucella* spp. (*Brucella* species) are classically classified into 6 main species including *Brucella abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis* and *B. neotomae*<sup>5</sup>, and among these species, *B. abortus* and *B. melitensis* cause an abortion in ruminants<sup>6</sup>.

The main clinical signs of infection are abortion and mastitis in females, and orchitis and epididymitis with frequent sterility in males, due to the localization of *brucella* within the female and male reproductive organs. *Brucella ovis* reasons

\* To whom all correspondence should be addressed.  
Tel.: +989134132858;  
E-mail: Dr\_Faham@yahoo.com;

genital disease of sheep, characterized via testicular alterations and reduced fertility in rams, and by endometritis and occasional abortions in ewes<sup>7,8</sup>.

Brucellosis in cattle is typically asymptomatic in no pregnant females. Adult male cattle may develop orchitis and brucellosis may be a reason of infertility in both sexes<sup>9,10</sup>.

Shedding of *Brucella* in sheep and goats is also common in udder secretions and semen, and *Brucella* may be isolated from various tissues, such as lymph nodes from the head, spleen and organs associated with reproduction (uterus, epididymides and testes), and from arthritic lesions<sup>9</sup>.

The disease is transmitted via direct contact with infectious excretions, tissues, fluids of infected, via ingestion of milk, via the venereal route, or less commonly, via the conjunctiva or via inhalation<sup>11</sup>.

Serological findings and microbiological isolation, are the greatest dependable methods of diagnosis for brucellosis. Those procedures are not constantly successful; they are cumbersome, and represent a great risk of infection for laboratory technicians. Molecular detection methods have been widely used for *Brucella* diagnosis in the last decade. Polymerase chain reaction (PCR) technique provides a promising option for the diagnosis of brucellosis<sup>12,13</sup>.

PCR is a great tool for detection of DNA from different fastidious and noncultivable pathogens, having the additional compensations of allowing the detection of small numbers of microorganisms, existence reproducible and easily standardized, minimizing the risk of infection to laboratory workers, and having an entire processing time of about 2 to 3 hours. These

characteristics can be very important when rapid and accurate identification of *Brucella* spp. is required<sup>14,15,16</sup>.

The aim of this study was to detect *Brucella* spp. in different samples from cattle and sheep.

## MATERIALS AND METHODS

### Sample collection

In this study, 84 bull's and Rams testis, 45 Cattle semen and 315 cattle and sheep blood specimens were collected randomly from slaughterhouses and Artificial Insemination Centre in various parts of Iran, between December 2012 and February 2013. All bull's and Ram's testis specimens those obtained from slaughterhouses, removed aseptically with sterile instruments, and were sent to the Biotechnology Research Center of Islamic Azad University, Shahrekord Branch in refrigerated boxes. All testis specimens were stored at -20°C for further use. Semen samples were obtained from Iran's Artificial Insemination Centre of Karaj, Iran. All blood samples with EDTA were taken from the caudal vein of the animals (Cattle and sheep) and immediately transported to the laboratory.

### Genomic DNA Extraction and Amplification

Genomic DNA was directly isolated from blood, semen and testis samples. To achieve this goal, DNP™ DNA extraction kit (CinnaGen, Iran) was used according to the manufacturer's protocol. The extracted DNA was immediately used or stored at -20°C until needed. The total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell (2001) (17).

The PCR assay was performed in 25 µL of reaction mixture containing 2.5 µL of 10X PCR

**Table 1.** Distribution of *Brucella* spp. in away samples

Animal	Specimens type	Total number	Positive	Negative
Sheep	Blood	135	42 (31.11%)	93 (68.89%)
Cattle	Blood	180	63 (35%)	117 (65%)
Bull's	Semen	45	14 (31.11%)	31 (68.88%)
	Testis	46	7 (15.21%)	39 (84.79%)
Ram's	Testis	38	4 (10.52%)	34 (89.48%)

buffer, 2 mM MgCl<sub>2</sub>, 200 μM dNTP, one units of Taq DNA polymerase, 20 pmol of each primer (Bru-F: 5'-CTATTA TCC GAT TGG TGG TCT G-3' and Bru-R: 5'-GGTAAAGCG TCG CCA GAA GG-3'), and 2 μL template DNA. The size of amplicons are 243 bp.

The PCR reaction mixtures were placed in a Corbett Palm-cycler (Corbett Research, Australia). The thermal profile involved an initial denaturation step at 95°C for 3 min followed by 32 cycles of denaturation at 94°C for 1 min, primer annealing at 57°C for 40 seconds, and extension at 72°C for 1 min. The cycling was followed by a final extension step at 72°C for 7 min. A negative control (sterile water), and a positive control DNA were included in each amplification run. Aliquots of amplified samples (15 μL) were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. A molecular weight marker with 100 bp increments (100bp ladder fermentas) was used as size standards.

#### Statistical analysis

Analysis of data was performed by the SPSS version 17.0 computer software (SPSS, Chicago, IL). Unadjusted prevalence estimates of *Brucella* spp. was calculated for the study

population as a whole. Cattle and sheep used in the analyses only appeared once. Significance was set at  $P \leq 0.05$ .

## RESULTS AND DISCUSSION

In the current study, were tested for *Brucella* spp. of Different Types of Samples, using a conventional PCR assay. Agarose gel electrophoresis of the amplification products showed the presence of 243 bp DNA fragment for *Brucella* spp. (Figure 1). The results of the prevalence of *Brucella* spp. in cattle and sheep from each sample are shown in Table 1 (presents the incidences of *Brucella* in bull's semen, bull's and ram's testis and cattle and sheep's blood samples collected).

Of the 444 samples tested 314 were totally negative, 130 resulted positive for *Brucella* spp. after PCR, and 14 out of 45 cattle semen samples (31.11%), and 63 out of 180 cattle blood samples (35%), and 42 out of 135 sheep's blood samples (31.11%), were positive for *Brucella* spp. Totally, 130 out of 444 (29.27%) samples, were positive for *Brucella* spp.

Rapid, definitive and accurate diagnosis



Agarose gel electrophoresis for identification of *Brucella* spp. DNA in cattle and sheep blood, testis and semen samples. ; Lane 1: are negative control; Lanes 2: are positive control; Lane 5: 100 bp DNA ladder (Fermentas, Germany); Lanes 3, 7, 8 and 9: positive samples (243 bp); Lanes 4, 6 and 10: negative samples.

**Fig. 1:** Ethidium bromide-stained agarose gel electrophoresis of PCR products (243bp) for detection of *Brucella* spp. in samples after PCR amplification

of brucellosis is very important for a positive outcome of eradication programmes<sup>18</sup>.

PCR technique is now easy to do, highly sensitive, and provide more specificity for detection of microorganisms. PCR are promising alternatives for the difficult culturing and identification of *Brucella* spp. via conventional methods. The results showed that PCR is a sensitive and specific method for detection and differentiation. PCR assays have been developed for the detection of *Brucella* in a wide diversity of clinical samples such as aborted fetuses<sup>19</sup>, lymphoid tissue<sup>20</sup>, semen<sup>21</sup>, blood<sup>22</sup> and milk<sup>23</sup>, and in all of these studies, PCR assays have been introduced as accurate and sensitive assays for detection of *Brucella* spp.

The results of this study show that the testis samples in 15.21% of Bulls and the testis samples in 10.52% of Rams served as a reservoir of disease in Iran. So it could be stated that the animal reservoirs rise the risk of potential spread of disease to other animals and specially humans, and this deserves special attention. And blood samples in 35% of cattle and the blood samples in 31.11% of sheep's served as a reservoir of disease in Iran. And semen samples in 31.11% of cattle served as a reservoir of disease in Iran.

In Iran, *B. melitensis* was first isolated from a sheep in Isfahan in 1950<sup>24</sup>, and the prevalence of brucellosis in animals reached 44% in 1956 and dropped to 5% in 1958. In 1980 and 1991, the prevalence rates were 6.4% and 10.18%<sup>25</sup>. The study results of Hamali *et al.*<sup>26</sup>, 6 out of 76 dams (7.8%) were seropositive to the *Brucella* spp. and 6 out of 76 aborted fetuses (7.8%) displayed positive reaction via the PCR tests.

An earlier study presented that Iran, Saudi Arabia, Jordan, Syria and Oman had the highest occurrences of brucellosis among the countries of the Near East region<sup>25</sup>.

The prevalence of human brucellosis in different parts of Iran varied from 1.5 up to 107.5 per 100,000 in 2003. The maximum levels of infection appeared in Zanjan with 67.1, Kurdistan with 83.5, Hamedan with 107.5, Azarbaijan Gharbi with 71.4 per 100,000 people<sup>27</sup>. The prevalence rate of brucellosis among horses in northeast Iran between May 2008 and April 2009 was 2.5%<sup>28</sup>. In the similar region, Doosti *et al.*, in 2011, brucellosis was found in 29.88% (127 number) of 452 total number of

samples cattle and the results of study propose that real-time PCR was extremely sensitive and specific for identification and differentiation of *B. melitensis* and *B. abortus* and that it could be a suitable tool for diagnosis of brucellosis<sup>29</sup>. Bokaie *et al.*, (2009) in northeast Iran reported a brucellosis prevalence of 0.56% in cattle and 3.4% in sheep and goats<sup>30</sup>. Extra study, 84 samples were positive by serological methods, Seventy three samples were positive by PCR method and 15 samples were positive by cultured method<sup>31</sup>.

In 2007, the prevalence of *B. melitensis* in aborted sheep in Turkey was 29.76%<sup>32</sup> and among cattle in Punjab (India), 18.26%<sup>33</sup>. *B. melitensis* was isolated from 14 out of 37 (38%) aborted sheep fetus samples examined in study of Unver *et al.*,<sup>34</sup>. The research results of Kaoud *et al.* In 2010 pointed out that brucellosis was found in 17.22%, 18.88% and 26.66% of cattle, goats and sheep herds, respectively<sup>35</sup>. Via ELISA, the seroprevalences of brucellosis between livestock and humans in western mountains region in Libya was 42% (cattle), 40% (humans) and 31% (goats) in 2008<sup>36</sup>. PCR assay by primers derived from the 16S rRNA sequence for detection of *Brucella* DNA were used by Romero *et al.*<sup>37</sup>.

Notwithstanding the advances made in control and surveillance, the prevalence of brucellosis is increasing in many developing countries due to various hygienic, socioeconomic, and political factors<sup>38</sup>. Under extensive management systems, the prevalence of brucellosis among various species of animals is low<sup>39</sup>.

In conclusion, the results of current study propose that PCR was highly sensitive and specific for identification and differentiation of *Brucella* spp. and that it could be a suitable tool for diagnosis of brucellosis. The results presented high presence of *Brucella* spp. in cattle and sheep samples and the conclusions of present study suggested that control and eradication programs for prevent and reduce of economic losses of brucellosis it seems to be necessary.

Consequently, it is essential to screen in all area frequently to prevent the spread of the disease and laboratory support is an significant tool in the diagnosis of the disease. Seemingly, PCR is one of the greatest ways to detect and characterize *Brucella* spp. as fast, less hazardous and sensitive method.

## ACKNOWLEDGMENTS

The authors would like to express their deep sense of gratitude and sincere thanks to the staff of the Biotechnology Research Center of Islamic Azad University of Shahrekord Branch in Iran.

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