

Serological, microbiological and molecular investigation of *Brucella* infection in milk and blood of Iranian horses

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Abstract

Background

Brucellosis is one of the most well-known and important zoonoses in the world, especially in Iran. Horse milk is a rare and expensive product with high nutrient ingredients in comparison to cow milk. In current study, a total of 164 blood and milk samples were randomly selected from 82 mares in Yazd city, Iran. Rose Bengal test (RBT) and milk ring test (MRT) methods were used as serological methods. A genus-specific PCR test targeting IS711 was performed using the primers *ISP1* and *ISP2* to identify *Brucella*. Specific primers were also used to identify *Brucella abortus* and *Brucella melitensis* at species level and then samples were sequenced. Positive PCR samples were cultured for bacterial isolation.

Results

Based on serological tests in milk and blood samples, no positive case was found. None of the blood samples were positive to identify *Brucella* genus, but 3 milk samples (3.66%) were positive in PCR assay. All 3 samples were identified as *Brucella abortus*. However, the microbiological culture of these samples did not lead into isolation of bacteria and no growth was observed. PCR can be applied as a sensitive method for diagnosis of *Brucella* infection in horses.

Conclusion

As the potential of *Brucella* infection confirmed by PCR in current study, special considerations should be taken to prevent close contact between horses and natural *Brucella* hosts particularly in regions where horse milk is consumed by people. Health authorities should be aware of possible transmission of brucellosis by horse milk consumption which should be considered in control measures.

Background

Brucellosis is considered as zoonotic diseases with worldwide distribution that can be transmitted through direct contact with infected animals or consumption of unpasteurized and contaminated milk (Mair and Divers, 2009). Iran is considered as endemic area for brucellosis by the human prevalence of 0.5% to 10.9% in different provinces (Sofian et al., 2008).

Brucellosis has been recognized in a wide range of animals such as goat (Gupta et al., 2006; Hamdy and Amin, 2002; Leal-Klevezas et al., 2000), cow (Hamdy and Amin, 2002; O'Leary et al., 2006; Salama, 2019), sheep (Addis and Desalegn, 2018; Hamdy and Amin, 2002; HASHEMI TABAR et al., 2017), horse (Tahamtan et al., 2010), and wild animals including fox (Scholz et al., 2016), white-eared opossum and coati (da Silva Batista et al., 2019), elk (Rhyan et al., 1997), and African buffalo (Madsen and Anderson, 1995). Although each species of *Brucella* has specific hosts, but horses could be infected by close

contact with other animals, including cow, sheep and goats in some livestock farms (Hamdy and Amin, 2002; Tahamtan et al., 2015). The horses brucellosis is associated with *Brucella abortus* and *Brucella suis* which cause infection in cattle and swine, respectively (Ghobadi and Salehi, 2013). According to studies, the prevalence of brucellosis of horses in Iran was reported from 0.5% to 2.5% (Ghobadi and Salehi, 2013; Tahamtan et al., 2015). It was reported that two species of *Brucella*, called *B. abortus* and *B. suis* biovars 1 and 3 have been isolated from horses (Badiei et al., 2013; Karthik et al., 2016). The supra spinous bursitis (fistulous withers) is known as the most common organ affected by the *Brucella* in horses. The inflammation of this bursitis causes significant pain. Also, infection of *Brucella abortus* causes infertility in horses and abortion in mares (Badiei et al., 2013; Ehizibolo et al., 2011). The transmission of brucellosis between horses and from horses to cow seems to be very unlikely (Badiei et al., 2013). Human infection is due to consumption of unpasteurized dairy products or as an occupational exposure to infected animals (Buhari et al., 2015).

The horse milk a popular gourmet food has long been used in central Asia. Horse milk has major nutritional and therapeutic properties which can be found in the diet of the elderly people, neonates and those who are in the recovery stage of diseases (Marcell Lopes Gomes Barreto et al., 2018; Smolders et al., 1990). In the advanced Western European countries, the horse milk is often supplied to organic food stores to maintain the health of people (Doreau and Martin-Rosset, 2011). The horse milk is highly digestible, rich in nutrients such as polyunsaturated fatty acids, high level of lactose, less casein content than cow's milk, and the good ratio of serum protein/caseins which is very close to human milk in terms of components (Businco et al., 2000; Curadi et al., 2001; Pieszka et al., 2015; Sharifi-Rad et al., 2013). Ascorbic acid values of horse milk are higher than those of human milk, while the value of vitamins A and E is lower than that of human milk (Doreau and Martin-Rosset, 2011). The horse milk is rich in lysozyme and lactoferrin (Doreau and Martin-Rosset, 2011; Pieszka et al., 2015). Clinical studies suggest that horse milk can be useful for children who are allergic to IgE-mediated and non IgE- mediated caused by cow milk (Businco et al., 2000; Renner, 1983). Therefore these unique characteristics of mare's milk make it a good source of food for the newborns, when the breastfeeding is not possible (Doreau et al., 1990; Madhusudan et al., 2017; Malacarne et al., 2002). In last year's, attention has been increasing in consumption of mare's milk for children as food and therapeutic purposes in Yazd, Iran. Due to the consumption of non-heat-treated mare's milk, the brucellosis can be transmitted to human by infected milk. By considering this fact that the most consumers of raw horse milk are neonates, it is necessary to investigate the prevalence of brucellosis in the mares of the Yazd.

Methods

Sample preparation

In current cross-sectional study, a total of 82 blood samples and 82 milk samples (mares of equestrian) were randomly collected from June to August 2018, Yazd, Iran. For each milk sample, about 15 ml of milk were taken in a labeled sterile tube and transferred to the laboratory immediately.

In addition, 10 ml of blood sample were taken from the jugular vein of horses. Each blood sample was divided into two parts, 5ml in a tube containing a clot activator in order to isolate the serum for Rose Bengal test and 5ml in an EDTA-containing tube for PCR test. The samples were stored in sterilized containers and transferred to the food hygiene and safety laboratory in a cold condition. The samples were stored at -20 °C until the test was performed. Information about the clinical status of horses, milk consumption, contact with domestic animals (cow, sheep and goat) and the history of abortion and age were obtained using a questionnaire. The horses were categorized in 3 age groups of 1–5 years (8 horses), 5–10 years (54 horses), > 10 years and more (20 horses).

Milk ring test (MRT)

The milk samples were stored at the refrigerator for one hour. Then, the milk and *B. abortus* antigen, manufactured by the Razi Vaccine and Serum Research Institute, kept at room temperature for one hour. Then, 1 ml of the samples was transferred to sterilized test tubes and one drop of ring test reagent was added. Then, it was gently mixed and incubated at 37 ° C. In the presence of antibody the fat band will change to blue (Kylla et al., 2018).

Rose Bengal Test (RBT)

Rose Bengal antigen and horse serum were kept in room temperature for about 20–30 minutes to reach the laboratory temperature. Then, a drop of serum (0.03 mL) was placed close to one drop of *Brucella* antigen on a glass plate and they were mixed thoroughly with a stick and it was dispersed at the size of a circle with diameter of about 2.5 cm. Then, the plate was moved on the rotator for four minutes (Tel et al., 2011).

PCR assay

DNA extraction: whole blood and milk samples

DNA extractions for whole blood and milk samples were performed by Iraizol® cat 1003 column-free DNA extraction kit (RNA Biotechnology Company). Quantitative and qualitative assessment of extracted DNA was performed using spectrophotometer (BioTek Instruments, USA) and 0.8% agarose gels, respectively. To identify the species and genus *Brucella*, IRIBA cow vaccine, Rev1 sheep vaccine and distilled water were used as positive and negative control, respectively.

Molecular detection

The PCR assay was performed on milk and blood samples to identify the *Brucella* genus bacteria using a primer targeting *IS6501* gene fragment which was performed by Ouahrani-Bettache et al (Ouahrani-Bettache et al., 1996). The information of primers is shown in Table 1. To identify *Brucella* species, *Brucella melitensis* and *Brucella abortus*, PCR was applied for positive *Brucella* genus samples using primers provided by Bricker and Halling (Bricker and Halling, 1994). The sequences of primers are shown in Table 2. PCR reactions were performed using a T100™ Thermal Cycler (Bio-Rad, USA).

Table 1
Sequence of *ISP1* and *ISP2* primers

Primer name	Primer sequence	Product size	source
<i>ISP1</i>	GGTTGTAAAGGAGAACAGC	600 bp	(Ouahrani Bettache et al., 1996)
<i>ISP2</i>	GACGATAGCGTTTCAACTTG		

Table 2
Sequences of *B.abortus* and *B.melitensis* primers

species	Primer name	Primer sequence	Product size	source
<i>B. abortus</i>	B a-SP	5-GAC GAA CGG AAT TTT TCC AAT CCC- 3	498 bp	(Bricker and Halling, 1994)
	IS711SP	5-TGC CGA TCA CTT AAG GGC CTT CAT- 3		
<i>B.melitensis</i>	B m-SP	5-AAA-TCG-CGT-CCT-TGC-TGG- TCT-GA-3	731 bp	
	IS711SP	5-TGC CGA TCA CTT AAG GGC CTT CAT- 3		

¹*B. abortus* specific primer

²*B. melitensis* specific primer

Sequencing and molecular analysis:

The amplified DNA from PCR products were separated by gel electrophoresis and were sent for sequencing using an ABI 3730XL DNA Analyzer (Bioneer, Daejeon, Republic of Korea). Sequences edited, analyzed, and aligned with reference sequences from Gen Bank using Clustal (<http://www.clustalw.genome.jp>).

Bacteriological methods

The positive samples of milk were stored at -20 °C until the culture was done. Due to the high risk of *Brucella* bacteria and the need for high security conditions for identification, this bacterium was cultured at the Razi Vaccine and Serum Research Institute. First, the milk samples were centrifuged at 7,000 rpm for 15 minutes. Then, samples were achieved from the sediment at the bottom of the container and the cream, and cultured on the *Brucella* agar plates (BD, USA) containing 5% of the horse serum and the *Brucella* selective supplement (Oxoid, UK) according to the manufacturer's instructions. The plates were incubated for 10 days at 37 °C with atmospheres containing 10% carbon dioxide (Corbel, 1997).

Ethical consideration:

Furthermore, this study has ethical approval from the Ethics Committee of Shahid Sadoughi University of Medical Sciences (IR.SSU.SPH.REC.1396.35).

Statistical analysis

The results of the study were analyzed using SPSS16 and descriptive statistics (percentage of frequency) and Fisher's exact test at 95% confidence level were performed.

Results

Serologic tests

RBT was performed on 82 serum samples. According to Table 3, all serum samples were negative and no agglutination was observed in any of the samples. Milk samples were negative in MRT and no blue ring was seen in any of the samples.

Table 3
Distribution of brucellosis in blood and milk
using serological method

RBT	MRT	
Blood sample (%)	Milk sample (%)	
0	0	+
82	82	-

PCR assay

The totals of 3 milk samples (3.66%) were identified as *Brucella* genus using ISP2 and ISPI primers. The specific primers used to identify the *Brucella abortus* and *Brucella melitensis* and all 3 samples were identified as *Brucella abortus* (Figs. 1, 2 and 3). Comparison between the prevalence of *Brucella* genus in mare blood using Rose Bengal and PCR is shown in Table 4. Comparison of the prevalence of *Brucella* genus in mare's milk has no significant relationship according to MRT and PCR methods (Table 5). In the age group of 1 to 5 years, two positive cases in milk were reported by PCR. According to questionnaire the clinical statuses of mares were healthy and no history of abortion was reported. Although, the positive milk of horses did not have human consumption. In the age group of 10 years or more, one positive case was reported. It had no clinical manifestation and history of abortion and did not coexist with other animals and the milk was used by human. According to the results in Table 6, there is not any significant relationship between age, prevalence of brucellosis, history of animal abortion, coexistence with other animals and clinical manifestations ($P > 0.05$).

Table 4
 Comparison of *Brucella* genus in mare blood by
 Rose Bengaltest and PCR methods

RBT	PCR	
Blood sample (%)	Blood sample (%)	
0	0	+
82	82	-

Table 5
 Comparison of *Brucella* genus in mare'smilk by
 MRT and PCR methods

MRT	PCR	
Milk sample (%)	Milk sample (%)	
0	3 (3.66)	+
82 (100)	79 (96.34)	-
82 (100)	82 (100)	Total

Table 6
Frequency distribution of brucellosis in mare according to the results of the obtained variables in terms of percentage

Variable		Sample size	Positive (%)	Negative (%)
Milk consumption	yes	42	1 (2.4)	41 (97.6)
	no	40	2 (5)	38 (95)
Clinical status of animal	healthy	81	3 (3.7)	78 (96.3)
	ill	1	0 (0)	1 (100)
Coexistence with other animals	yes	4	0 (0)	4 (100)
	no	78	3 (3.8)	75 (96.2)
Age group	1–5	8	2 (25)	6 (75)
	5–10	54	0 (0)	54 (100)
	10 >	20	1 (5)	19 (95)
Abortion history	yea	5	0 (0)	5 (100)
	no	77	3 (3.9)	74 (96.1)
P value > 0.05				
variables in terms of percentage				

Sequencing and molecular analysis

The amplified DNA from secondary PCR products were separated by gel electrophoresis and were sent for sequencing using an ABI 3730XL DNA Analyzer at Bioneer Company (Daejeon, Republic of Korea).

Bacteriological methods

Finally, the positive samples in PCR assay were cultured. None of the 3 samples had growth and they were identified as *Brucella* negative.

Discussion

Horses are not considered as natural host for brucellosis but they can be infected as an by close contact with other animals, including cow, sheep and goats in some livestock farms (Hamdy and Amin, 2002; Tahamtan et al., 2015). Therefore, horses could be the carrier of infection without any clinical symptoms (Badiei et al., 2013). The *brucellosis* could be transmitted to human through direct contact with infected animals and their secretions, consumption of contaminated milk and dairy products or through inhalation of contaminated airborne particles (Tahamtan et al., 2010; Tuon et al., 2017). The mammary gland and accessory lymph nodes are considered as a target organ of *Brucella* localization, and viable bacterial cells may be excreted into milk (Shafei et al., 2012). In addition, during last year's by increasing the tendency to consume raw horse milk in neonates and adults and contact of individuals with the animal and the possibility of transmitting disease, the present study was conducted with the aim of evaluating

the prevalence of brucellosis in mare's milk and blood in Yazd city, Iran. In current study, 164 blood and milk samples screened by Rose Bengal and milk ring tests which were negative in terms of brucellosis. It was shown that 3 samples out of 82 milk samples were positive in PCR assay, while none of the blood samples was positive by PCR. The culture media of 3 positive samples in PCR showed no bacterial growth.

In a study conducted on 74 horses in Turkey in 2007, the sero-prevalence of anti-*Brucella* antibodies in horses was reported 9.5% using the Serum Tube Agglutination test (SAT) (Göz et al., 2007). Ghobadi et al (2013) investigated the prevalence of brucellosis antibodies in serum of 200 horses of Hamedan city by tube agglutination test and 2-mercaptoethanol test. The general prevalence in this study was reported 0.5 percent (only one sample was positive)(Ghobadi and Salehi, 2013). The prevalence of brucellosis in horses by Tel et al was reported 13.68% using the RBT method (Tel et al., 2011). In the another study prevalence of brucellosis in horses was reported 2.5% by RBT method (Tahamtan et al., 2010). Serologic tests are easy and fast, but false positive and negative results might be reported by these methods (Ilhan et al., 2008). In some studies, the prevalence of brucellosis was different from that of the present study, which might be due to the contamination of the horse after contact with brucellosis-infected animals and the transient and temporal presence of anti-*Brucella* antibodies in the horse, it has not had enough time to produce an antibody that can be at an acceptable level for the serological test (Rafiee, 2014).

Leary et al (2006) determined the *Brucella abortus* by PCR method in blood, milk, and lymphoid tissues of 21 positive cows. According to the results, the *Brucella* in the cow blood samples was not found. However, in the milk samples, 44% of positive cases were positive in culture method. Moreover, 75% of the extra-mammary lymph nodes and 66% of the retropharyngeal lymph nodes that were positive in culture were also reported to be positive by PCR. In present study, PCR assay did not show any superiority in comparison to conventional diagnostic methods in diagnosing the *Brucella* in blood samples. O'Leary et al suggested that one of the most important reasons for the inefficiency of PCR in their study was that the whole blood is not a good template for detection of *B. abortus* DNA (O'Leary et al., 2006). The results of the above-mentioned study are consistent with those of current study. Leal-Klevezas (2000) evaluated the efficiency of PCR method on blood and milk goat samples in comparison with milk culture, blood culture and Rose Bengal method. They showed that the sensitivity of the PCR method to culture and Rose Bengal test was higher and it could be used to diagnose the *Brucella* as a fast test (Leal-Klevezas et al., 2000). So far, there is not any research on the use of PCR in body fluids such as blood and milk of horse, simultaneously. In the current study, the PCR was used to evaluate the prevalence of brucellosis in milk and blood of the horse. The PCR method identified only 3 brucellosis positive in milk samples, although their blood samples were not positives. It should be noted that several factors can effect on the results of PCR and bacterial culture in clinical samples of the livestock, including the infection stage, the place of bacterium localized (depends on the stage of the infection) and the bacterial load present in the clinical sample, which may be one of the reasons for the differences between the results of the PCR assay in diagnosis of *Brucella* in serum and blood samples(Ilhan et al., 2008). Methods of DNA extraction are also effective in PCR ability to identification of bacteria. *Brucella* may be present in the collected samples at

very small amounts, so extraction of DNA in animal samples should be maximized to increase the possibility of isolation and identification(O'Leary et al., 2006).

In the present study, whole blood was used for DNA extraction. The DNA of bacterium might be not detected in blood by inhibitory agents; therefore use of serum instead of whole blood could cover the problem. However, it should be noted that the negative false results could be attributed to specific characteristic of *Brucella* which is intracellular, and DNA extraction at chronic phase of the disease which is found at small amounts(Ilhan et al., 2008). Different studies have shown various results by using different methods such as PCR, serologic and bacteriological tests(O'Leary et al., 2006). In addition, PCR is preferred because of high speed, proper repeatability, non-interference with serum cross reaction agents bacteria, high sensitivity to low bacterial levels, no need to living bacteria and low risk for laboratory personnel, performing the test with a minimum sample and not affecting the presence of other opportunistic objects in the clinical sample, although observing the appropriate conditions to prevent infection of reaction with bacterium DNA or products propagated in previous reactions in PCR is necessary(Ilhan et al., 2008). In present research, although 3 samples of milk were positive in the PCR test, no sample was diagnosed positive in the MRT serological test. This may be due to the high sensitivity of PCR to serological and microbial methods (Leal-Klevezas et al., 2000). The stage of infection (acute or chronic phases) is also involved in the bacterial diagnosis (Ilhan et al., 2008). Finally, positive milk samples in PCR were cultured and the culture results were negative. The negative result of bacterial culture is attributed to difficulty of isolating the *Brucella* bacteria and low sensitivity of bacterial recovery from milk(Leal-Klevezas et al., 2000). It should be noted that the bacterial excretion in milk is not continuous in livestock and may not be excreted at the time of sampling (Leal-Klevezas et al., 2000).

In the present study, although the number of horses on the range of 1 to 5 years was higher, no significant difference was found between the age groups of horses and the prevalence of brucellosis. Badiei et al. (2012) showed that, there was no significant difference among various age groups in terms of presence of anti-*Brucella* antibody. However, in the study conducted by Safirollah et al. (2012) on the prevalence of anti-*Brucella* antibodies in horses, donkeys and mules in Pakistan, the prevalence of antibody was significantly higher in older animals (5–11) than younger ones. In the present study, clinical symptoms of brucellosis were not observed in any of horses. Tajik et al reported no clinical symptom on the seroprevalence of anti-*Brucella* antibodies positive in Arabian horses(Rafiee, 2014). In the present study, no significant difference was found between the prevalence of brucellosis and coexistence with other animals (cow, sheep and goats). This result is consistent with the result of the study conducted by Badiei et al. (Badiei et al., 2013). However, Tahmtan et al who studied (2010) the prevalence of brucellosis in horses of Mashhad province showed the highest prevalence in horses due to the coexistence with other animals including cows, sheep and goats(Tahamtan et al., 2010). Unfortunately, horses may be infected with Brucellosis such as other species without any clinical manifestation which the reason is not clear. MacMillan et al infected horse with *Brucella abortus* to investigate the clinical changes. They showed that infected horses had not any sign of disease except for changes in rectal temperature. In biochemical observation the total serum amino acids, IgM and to lesser extent IgG were changed. They reported that

the tendency of *B. abortus* toward localization in the lymph nodes was compatible with other species (MacMillan et al., 1982).

Conclusions

The results of this study provide information on the status of mare infection with *Brucella* spp. Based on the positive cases identified by the molecular method, the probability of infection with *Brucella* in Yazd mare was revealed. As a result, there is the risk of infection with brucellosis in the people consuming raw milk in Yazd and the people who are in contact with this animal. The presence of brucellosis in mare is a concern for general health, since it can be considered as a health risk for individuals, especially children, the elderly and those with immune deficiency. Preventing the contact between bacteria-carrier livestock and horses, establishment of healthy farm to produce horse milk, observing bio-security in equestrian clubs and taking the possibility of infection of horses in brucellosis control programs are necessary. The results indicated that the PCR method can be used as a more sensitive method for diagnosis and identification of *Brucella* species in comparison with serological tests and classical bacteriological culture methods.

Abbreviations

Not applicable

Declarations

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Figures

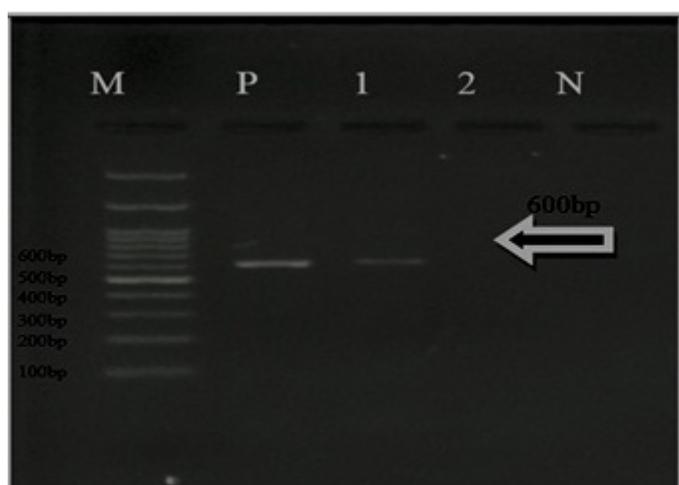


Figure 1

Electrophoresis of PCR products derived from IS6501 gene proliferation in horse milk and blood samples
 Well M: Marker 100 bp DNA ladder Well P: Positive Control (IRIBA vaccine) Well 1: Milk sample Well 2: Blood sample Well N: Negative Control (Sterilized Distilled Water)

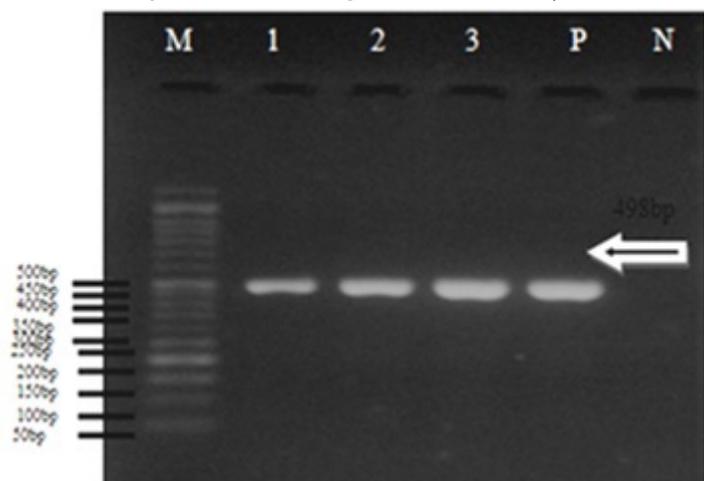


Figure 2

Electrophoresis of PCR products derived from Ba-SP and IS711-SP gene proliferation in horse milk samples Well M: Marker 50 bp DNA ladder Well P: Positive Control (IRIBA vaccine) Well 1-3: Milk sample Well N: Negative Control (Sterilized Distilled Water)

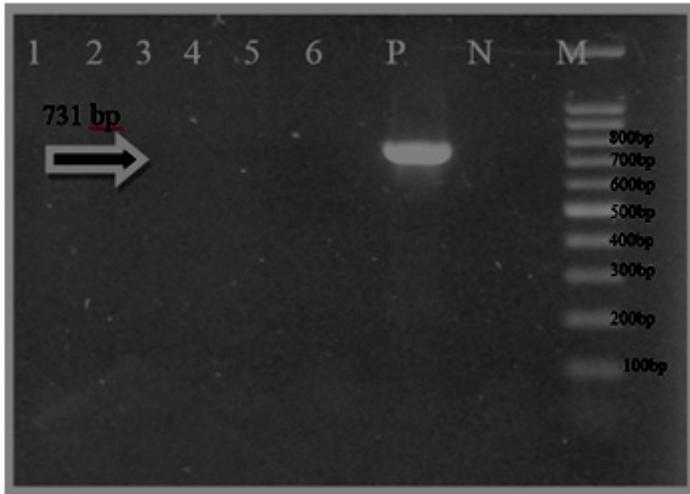


Figure 3

Electrophoresis of PCR products derived from Bm-SP and IS711-SP gene proliferation in horse milk samples Well M: Marker 100 bp DNA ladder Well P: Positive Control (Rev1 vaccine) Well 1-6: Milk sample Well N: Negative Control (Sterilized Distilled Water)

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