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RESEARCH ARTICLE

Multiplex PCR Assay for the Simultaneous Detection of the Brucella Genus in Human Whole Blood and Serum

Mohsen Zamanian¹ , Elham Jahani²  and Hassan Mahmoudi^{3,*} 

¹Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

²Department of Pathobiology, Faculty of Veterinary Medicine, Bahonar University of Kerman, Kerman, Iran

³Department of Microbiology, Hamadan University of Medical Sciences, Hamadan, Iran

Abstract:

Background:

Brucellosis disease is a serious zoonosis worldwide and only 17 countries have been recognized as free of brucellosis. The World Health Organization has reported that the incidence of brucellosis is 500,000 cases in a year. Multiplex polymerase chain reaction (PCR) is an ideal method for the identification of brucellosis. The most common primers for the diagnosis of Brucella include B4/B5 and F4/R2. The advantages of multiplex PCR include targeting multiple sequences at the same time, and multiple results are produced in a single test run which saves time and the reagents simultaneously. The purpose of this investigation was to extend and optimize a multiplex PCR for the identification of genus Brucella from serum and whole blood samples.

Methods:

In this experimental and sectional study, blood samples of 25 suspected patients in the acute phase of brucellosis with serum titers higher than 1:80 were collected. Two pairs of specific primers of B4 and B5 the specific gene was amplified. PCR and Multiplex PCR were performed on blood and serum samples.

Results:

Among 25 blood samples, 15 cases (60%) and 9 cases (36%) and among 25 serum samples, 23 cases (92%) and 13 cases (52%) were positive for B4/B5 and F4/R2 in PCR, respectively. In multiplex PCR, among 25 blood samples, 5 cases (20%) showed both bands, 11 cases (44%) showed band 222bp, 4 cases (16%) showed band 905bp and 5 cases (20%) showed no bands. Among 25 serum samples, 6 cases (24%) showed both bands, 15 cases (60%) showed band 222bp, 3 cases (12%) showed band 905bp and 1 case (4%) showed no bands.

Conclusion:

The results of this study show that this multiplex PCR can be used for the diagnosis of brucellosis with high sensitivity in clinical laboratories routinely and it can serve as an alternative substitution for risky culture method and nonspecific serological methods.

Keywords: Brucella, Multiplex PCR, Whole blood, Serum, Brucellosis, World Health Organization (WHO).

Article History

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1. INTRODUCTION

Brucellosis disease is a serious zoonosis worldwide and only 17 countries have been recognized as free of brucellosis [1]. The World Health Organization (WHO) has reported that the incidence of brucellosis is 500,000 cases in a year [2 - 5]. The methods based on polymerase chain reaction (PCR) can

compensate the drawbacks of traditional techniques for the identification of Brucella (culturing and serology methods) [6]. PCR reduces the time of identification of the diseases and Multiplex PCR is an ideal method for identification of human brucellosis from different clinical samples, which can simultaneously detect and amplify several genomic sequences in one reaction, thereby saving time and costs [7 - 9]. Nowadays, this method has useful applications such as identification of different pathogens simultaneously, detection of genetic disorders, and assessing the quantity and quality of

* Address correspondence to this author at the Department of Microbiology, Hamadan University of Medical Sciences, Hamadan, Iran; Tel: +98-9189539458; E-mail: Hassanmahmoudi24@gmail.com

samples [9 - 11]. The most common primers for the identification of genus *Brucella* with the target of preserved regions in *Brucella* genome include B4/B5 and F4/R2 [12, 13]. Primer pair B4/B5 amplifies a 223 base pair part of the gene encoding a 31 KD cell surface protein in *Brucella abortus* (BCSP 31 gene) [5, 12]. Primer pair F4/R2 amplifies a 905 base pair (bp) part of the *16S rRNA* gene in *Brucella abortus* [13 - 15]. Both primer pairs (B4/B5/ F4/R2) are quite conserved in *Brucella* spp. and are able to detect almost all *Brucella* species [14]. Several studies have been performed on the detection of *Brucella* at the species level and partially at the biovar level utilizing multiplex PCR [8, 16 - 20]. However, until now, no study with this technique has been performed for the identification of *Brucella* at the genus level. There are PCR inhibitors in clinical samples that constrain DNA amplification [21, 22]. In whole blood, substances such as ethylenediaminetetraacetic acid (EDTA), sodium citrate or heparin as blood anticoagulants [23], the components of red blood cell lysis and in serum, substances such as proteins and immunoglobulin G are known as inhibitors [21, 23, 24]. As *Brucella* has the facultative intracellular nature, the bacterial count is low in a patient's body [25]. The presence of inhibitors in whole blood and serum reduces the sensitivity of primers used in the diagnosis of disease [14, 23, 24]. The purpose of this investigation was to extend and optimize a suitable method for the detection of genus *Brucella* by multiplex PCR in serum and whole blood samples which have low amounts of DNA and high amounts of inhibitors.

2. MATERIALS AND METHODS

2.1. Sample Collection and Processing

25 blood specimens were obtained from teaching hospital in Nahavand city. Samples were taken from patients with the acute phase of brucellosis which have been referred to a physician with symptoms of brucellosis. Serum was isolated from whole blood samples and stored at -20 °C for later uses after confirming brucellosis by using serological tests (Wright, 2-mercaptoethanol and coombs wright).

2.2. Isolation of DNA from Clinical Blood and Serum Samples

DNA was extracted from serum samples using boiling method. 200 µl of serum was poured in 1.5 ml tube and then was centrifuged at 15000×g for 15 minute. Supernatant was discarded and 200 µl of DNase and RNase free water was added to sediment, mixed, and centrifuged at 15000×g for 10 minute. Supernatant was discarded and 40 µl of DNase and RNase free water was added to sediment, mixed, and placed in water bath with temperature 95-97 °C for 10 min. Then immediately placed in the freezer (-20 °C) for 2 minutes and finally centrifuged at 15000×g for 10 seconds. DNA was recovered by centrifuging the samples at 15,000 3 g for 10 min; the pellets were rinsed with 1 ml of 70% ethanol, dried, and resuspended in 30 ml of water. Also, DNA was extracted from whole blood specimens utilizing the kit (Denazist Asia Iran). DNA extraction included washing several times with buffers and one step lysis using protease K. The concentration and purity of the DNA were determined spectrophotometrically by reading $A_{260/280}$.

2.3. Primer Design

Two primer pairs that are broadly used for the detection of genus *Brucella* were selected. The primer pairs used were B4/B5 and F4/R2 (Table 1). Primers were assessed using bioinformatic software including Basic Local Alignment Search Tool (BLAST), allele ID 7, base stacking TM online and primer premier 5. The strength and weakness points of two primer pairs were evaluated. As F4/R2 has more sensitivity than B4/B5 for the detection of *Brucella* in pure culture [14], we changed B4/B5 to match the sensitivity of F4/R2. After investigations of factors that cause a negative impact on amplification, sequences B4 and B5 were changed accordingly. Therefore, firstly, the annealing temperature of B4/B5 primer pair was made closer to that of F4/R2 primer pair; secondly, negative factors for amplification were removed as much as possible. Finally, AA in 3' end of B4 and CG in 5' end of B5 were removed. Primer pair B4/B5 with this modification amplifies a 222 bp fragment in the same gene. The primer pairs (10 pmol) were used in PCR and multiplex PCR.

Table 1. Primers and genes used in PCR and multiplex PCR.

Product	Gene	Sequence 5' → 3'	Primer
222bp	<i>BCSP31</i>	TGGCTCGGTTGCCAATATC CGCTTGCCITTCAGGTCTG	B4 B5
905bp	<i>16S rRNA</i>	TCGAGCGCCCGAAGGGG AACCATAGTGTCTCCACTAA	F4 R2

2.4. Amplification and Detection of *Brucella* DNA by PCR and Multiplex PCR

Multiplex PCR is a widespread molecular biology technique for the amplification of multiple targets in a single PCR experiment. In a multiplexing assay, more than one target sequence can be amplified by using multiple primer pairs in a reaction mixture. As an extension to the practical use of PCR, this technique has the potential considerably save time and effort in the laboratory without compromising the utility of the experiment. Single template PCR is a technique that uses a single template which can be a genomic DNA along with several pairs of forward and reverse primers to amplify specific regions within a template. To prepare the main solution for PCR and multiplex PCR with primers B4/B5 and F4/R2, ingredients were mixed in tubes 0.2 ml DNase free in a total volume of 25 µl. For positive control, DNA isolated by the boiling method from a pure culture of *Brucella abortus* strain S19 was used. In the negative control, sterile distilled water was used instead of DNA.

PCR and multiplex PCR were performed using two primer pairs on whole blood and serum samples. The conditions for PCR with primers B4/B5 were 40 cycles of initial denaturation at 90°C for 5 min, denaturation at 90°C for 60s, annealing at 53°C for 60s and extension at 72°C for 60s and a final extension at 72°C for 10 min. The conditions for PCR with primers F4/R2 were 40 cycles of initial denaturation at 90°C for 5 min, denaturation at 95°C for 30s, annealing at 54°C for 90s and extension at 72°C for 90s, and a final extension at 72°C for 6 min.

The condition for multiplex PCR with primers B4/B5 and

F4/R2 was including 40 cycles of initial denaturation 90°C for 5 min, denaturation at 90°C for 60s, annealing at 54°C for 60s and extension at 72°C for 60s, and a final extension at 72°C for 10 min.

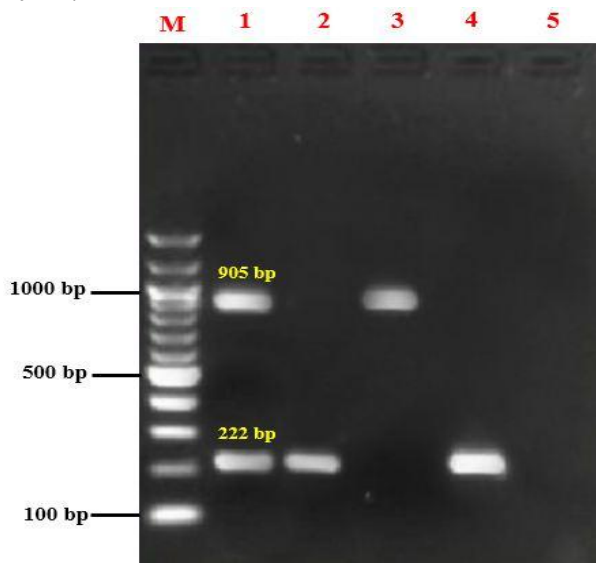


Fig. (1). The result of multiplex PCR and single PCR assay for detection of *Brucella* genus: Lane M: Marker DNA 100 bp, **Lane 1:** PCR product for B4/B5 and F4/R2, **Lane 2:** PCR product for B4/B5, **Lane 3:** PCR product for F4/R2, **Lane 4:** PCR product for B4/B5 (Positive control), **Lane 5:** Negative control.

3. RESULTS

Some modifications in the sequences of primers B4/B5 in comparison with the sequences designed by Baily et al [12] were done. The annealing temperature of this primer pair was changed from 56.8 ° C to 55.3 ° C (calculated by the base stacking T_m online) and was more close to the annealing temperature of primer pair F4/R2 (53 ° C). With this change, cross dimer was removed and the product rating was increased from 68 to 86. Also, non-specific connections in blast results were reduced greatly. From 25 whole blood samples which were examined by PCR, 15 cases (60%) with primers B4/B5 and 9 cases (36%) with primers F4/R2 were positive. The number of negative whole blood cases with primers B4/B5 was 10 cases (40%) and with primers F4/R2 was 16 cases (64%). From 25 serum samples which were examined by PCR, 23 cases (92%) with primers B4/B5 and 13 cases (52%) with primers F4/R2 were positive. The number of negative serum cases with primers B4/B5 was 2 cases (8%) and with primers F4/R2 was 12 cases (48%) (Table 2). In multiplex PCR with determined programs, bands were observed and results showed that DNA isolation and PCR were performed accurately. The 222 bp and 905 bp bands resulted from experiments with primers B4/B5 and F4/R2, respectively, are shown in Fig. (1). From 25 whole blood samples which were examined by multiplex PCR, 5 cases (20%) showed both bands, 11 cases (44%) showed the band resulted by primers B4/B5 and 4 cases (16%) showed the band resulted by primers F4/R2. In 5 cases (20%), no band was observed. From 25 serum samples which were tested by multiplex PCR, 6 cases (24%) showed both bands, 15 cases (60%) showed the band resulted by primers

B4/B5 and 3 cases (12%) showed the band resulted by primers F4/R2. In one case (4%), no band was visible (Table 3).

Table 2. The results of PCR on whole blood and serum samples.

F4/R2		B4/B5		Number	Samples
Negative	Positive	Negative	Positive		
16(64%)	9(36%)	10(40%)	15(60%)	25	Whole blood
12(48%)	13(52%)	2(8%)	23(92%)	25	Serum

Table 3. The results of multiplex PCR on whole blood and serum samples.

No band	B4/B5 and F4/R2 simultaneously	F4/R2 only	B4/B5 only	Number	Samples	
5(20%)	5(20%)	4(16%)	11(44%)	25	Whole blood	Multiplex PCR
1(4%)	6(24%)	3(12%)	15(60%)	25	Serum	

4. DISCUSSION

In this study, two primer pairs broadly used for the diagnosis of brucellosis were utilized. The goal of this investigation was to optimize a multiplex PCR technique for the identification of *Brucella* for utilizing in diagnostic laboratories. In the present study, changes were performed on the primers B4/B5 sequences. In a theoretical view, negative amplification factors for primers B4/B5 were reduced and also B4/B5 annealing temperature was closer to F4/R2 primers. In a practical view, this method was able to detect *Brucella* and bands of both primers were visible using gel electrophoresis. Modified primers B4/B5 with these changes amplified a 222 bp fragment in the same gene [*BCSP31* gene]. Queipo-Ortuno et al. found 100% sensitivity in peripheral blood using B4/B5 primer pair amplifying a 223 bp fragment of the *bcsp 31* gene [4]. Zerva et al. reported that the sensitivity of primers B4/B5 improved from 61% to 94% when serum was used instead of whole blood samples [5]. Baddour et al. [26] demonstrated that the sensitivity of primers B4/B5 and F4/R2 on peripheral human blood was 98% and 53.1%, respectively. Mitka et al. [27], showed that the sensitivity of primers B4/B5 on the buffy coat, whole blood and serum samples was 100%, 100% and 97%, respectively. Also, a few studies have evaluated the sensitivity of primers in clinical serum samples. It was observed that, in theoretical terms, modified sequences of this primer were more sensitive and more specific than sequences designed by Baily et al. [10], for the diagnosis of brucellosis because, in comparison of BLAST results, new sequences have negative amplification factors lesser than reference sequences. Also, both new sequences have identical annealing temperatures which increased the specificity of this primer pair. In this regard, further research is needed for evaluation and comparison of the new sequences of this primer with reference sequences to clarify which can be more efficient for the diagnosis of brucellosis in practice. Sifuentes-Rincon et al. designed four primers from *B. abortus* sequences at the well-conserved *Omp2* locus and amplified DNA of all six species of *Brucella* by using these primers [28]. Bricker et al. designed a

multiplex method to recognize *B. abortus* strain S19 and RB51. Three new oligonucleotide primers in this study were added to the five-primer multiplex *Brucella* AMOS PCR assay [29]. Imaoka *et al.* [10], designed a combinatorial PCR with four primers to detect 4 main species of *Brucella*. Primers were prepared from BCSP31 and *Omp2* genes. In a study by Hinić *et al.*, a typical PCR with 7 primer pairs was performed to detect *Brucella* species [30]. Also, Sreevatsan *et al.* designed a multiplex approach for molecular identification of *B. abortus* and/or *Mycobacterium bovis* infection in cattle [31]. Probert *et al.* designed a Real-Time multiplex PCR method for the detection of *Brucella* spp., *B. abortus*, and *Brucella melitensis* in a single test [32]. In the studies mentioned above, multiplex PCR was performed for the detection of *Brucella* species using species-specific primers and no multiplex method has been adopted to detect *Brucella* at the genus level. In the present study, a multiplex PCR was designed to detect *Brucella* at the genus level. By using whole blood and serum samples, PCR inhibitors affect amplification which decreases the sensitivity of primers. Consequently, for determination of brucellosis from serum and whole blood, utilizing the primers B4/B5 is preferred. Also, for DNA isolation from serum samples boiling technique was used which decreases the quality than kit method, but using the serum instead of whole blood samples is preferred in the diagnosis of *Brucella*. The total positive cases of PCR for detection of *Brucella* from whole blood samples by using both primer pairs in 50 experiments was 24 cases (48%) and for serum samples was 36 cases (72%). While the number of positive cases in multiplex PCR for 25 whole blood samples by using both primer pairs was 20 cases (80%) and for 25 serum samples the number of positive cases was 24 cases (96%). Using this method, the problems related to molecular diagnosis of *Brucella* can be overcome and also detection can be done with maximum sensitivity. In the current study, only two primer pairs were used to detect *Brucella*. This method is easier and more sensitive than methods mentioned in other studies. If DNA isolation is optimized, the sensitivity of this method will increase for the detection of genus *Brucella*. Routine diagnosis of brucellosis by PCR assay has not been standardized yet. Therefore, this method can routinely be used for the diagnosis of brucellosis in clinical laboratories and also can be an alternative substitution for risky culture method and nonspecific serological methods.

CONCLUSION

The most cases of brucellosis in Iran are related to species *B. abortus* and *B. melitensis*, and primer pairs B4/B5 and F4/R2 have high sensitivity and specificity for these two species (Blast results). Also, using molecular methods in clinical laboratories for the detection of brucellosis has not been optimized; therefore this multiplex PCR can be useful in the diagnosis of brucellosis especially in Iran. This optimization has not been performed earlier and it was the main achievement of our study.

AUTHORS' CONTRIBUTIONS

HM and MZ designed the study. HM and MZ performed the experiments. HM and EJ performed the data analysis. MZ and HM drafted the manuscript. All authors contributed to the

interpretation of the results, provided critical feedback, contributed to the writing of the manuscript and have approved the final version

LIST OF ABBREVIATIONS

WHO	=	World Health Organization
PCR	=	Polymerase Chain Reaction
EDTA	=	Ethylenediaminetetraacetic Acid
BLAST	=	Basic Local Alignment Search Tool

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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REFERENCES

- [1] Nicoletti P. The epidemiology of bovine brucellosis. *Adv Vet Sci Comp Med* 1980; 24: 69-98. [PMID: 6779513]
- [2] Havas L. Problems and new developments in the treatment of acute and chronic brucellosis in man. *Acta Trop* 1980; 37(3): 281-6. [PMID: 6106369]
- [3] Morata P, Queipo-Ortuño MI, Reguera JM, García-Ordoñez MA, Cárdenas A, Colmenero JD. Development and evaluation of a PCR-enzyme-linked immunosorbent assay for diagnosis of human brucellosis. *J Clin Microbiol* 2003; 41(1): 144-8. [http://dx.doi.org/10.1128/JCM.41.1.144-148.2003] [PMID: 12517839]
- [4] Queipo-Ortuño MI, Morata P, Ocón P, Manchado P, Colmenero JD. Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. *J Clin Microbiol* 1997; 35(11): 2927-30. [http://dx.doi.org/10.1128/JCM.35.11.2927-2930.1997] [PMID: 9350761]
- [5] Zerva L, Bourantas K, Mitka S, Kansouzidou A, Legakis NJ. Serum is the preferred clinical specimen for diagnosis of human brucellosis by PCR. *J Clin Microbiol* 2001; 39(4): 1661-4. [http://dx.doi.org/10.1128/JCM.39.4.1661-1664.2001] [PMID: 11283112]
- [6] Morata P, Queipo-Ortuño MI, Reguera JM, Miralles F, Lopez-Gonzalez JJ, Colmenero JD. Diagnostic yield of a PCR assay in focal complications of brucellosis. *J Clin Microbiol* 2001; 39(10): 3743-6.

- [http://dx.doi.org/10.1128/JCM.39.10.3743-3746.2001] [PMID: 11574607]
- [7] Queipo-Ortuño MI, Colmenero JD, Bermudez P, Bravo MJ, Morata P. Rapid differential diagnosis between extrapulmonary tuberculosis and focal complications of brucellosis using a multiplex real-time PCR assay. *PLoS One* 2009; 4(2):e4526 [http://dx.doi.org/10.1371/journal.pone.0004526] [PMID: 19225565]
- [8] Yu WL, Nielsen K. Review of detection of *Brucella* spp. by polymerase chain reaction. *Croat Med J* 2010; 51(4): 306-13. [http://dx.doi.org/10.3325/cmj.2010.51.306] [PMID: 20718083]
- [9] Mirnejad R, Doust RH, Kachuei R, Mortazavi SM, Khoobdel M, Ahamadi A. Simultaneous detection and differentiates of *Brucella abortus* and *Brucella melitensis* by combinatorial PCR. *Asian Pac J Trop Med* 2012; 5(1): 24-8. [http://dx.doi.org/10.1016/S1995-7645(11)60239-3] [PMID: 22182638]
- [10] Imaoka K, Kimura M, Suzuki M, Kamiyama T, Yamada A. Simultaneous detection of the genus *Brucella* by combinatorial PCR. *Jpn J Infect Dis* 2007; 60(2-3): 137-9. [PMID: 17515651]
- [11] Kumar S, Tuteja U, Sarika K, Singh D, Kumar A, Kumar O. Rapid multiplex PCR assay for the simultaneous detection of the *Brucella* Genus, *B. abortus*, *B. melitensis*, and *B. suis*. *J Microbiol Biotechnol* 2011; 21(1): 89-92. [http://dx.doi.org/10.4014/jmb.1007.07051] [PMID: 21301197]
- [12] Baily GG, Krahn JB, Drasar BS, Stoker NG. Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. *J Trop Med Hyg* 1992; 95(4): 271-5. [PMID: 1495123]
- [13] Romero C, Gamazo C, Pardo M, López-Goñi I. Specific detection of *Brucella* DNA by PCR. *J Clin Microbiol* 1995; 33(3): 615-7. [http://dx.doi.org/10.1128/JCM.33.3.615-617.1995] [PMID: 7538508]
- [14] Navarro E, Escribano J, Fernández J, Solera J. Comparison of three different PCR methods for detection of *Brucella* spp in human blood samples. *FEMS Immunol Med Microbiol* 2002; 34(2): 147-51. [http://dx.doi.org/10.1111/j.1574-695X.2002.tb00616.x] [PMID: 12381466]
- [15] Romero C, Pardo M, Grillo MJ, Diaz R, Blasco JM, Lopez-Goñi I. Evaluation of PCR and indirect enzyme-linked immunosorbent assay on milk samples for diagnosis of brucellosis in dairy cattle. *J Clin Microbiol* 1995; 33(12): 3198-200. [http://dx.doi.org/10.1128/JCM.33.12.3198-3200.1995] [PMID: 8586702]
- [16] Bricker BJ, Halling SM. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. *J Clin Microbiol* 1994; 32(11): 2660-6. [http://dx.doi.org/10.1128/JCM.32.11.2660-2666.1994] [PMID: 7852552]
- [17] Rees RK, Graves M, Caton N, Ely JM, Probert WS. Single tube identification and strain typing of *Brucella melitensis* by multiplex PCR. *J Microbiol Methods* 2009; 78(1): 66-70. [http://dx.doi.org/10.1016/j.mimet.2009.04.010] [PMID: 19410609]
- [18] Ferrão-Beck L, Cardoso R, Muñoz PM, *et al.* Development of a multiplex PCR assay for polymorphism analysis of *Brucella suis* biovars causing brucellosis in swine. *Vet Microbiol* 2006; 115(1-3): 269-77. [http://dx.doi.org/10.1016/j.vetmic.2006.02.002] [PMID: 16530357]
- [19] López-Goñi I, García-Yoldi D, Marín CM, *et al.* Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains. *J Clin Microbiol* 2008; 46(10): 3484-7. [http://dx.doi.org/10.1128/JCM.00837-08] [PMID: 18716225]
- [20] Mayer-Scholl A, Draeger A, Göllner C, Scholz HC, Nöckler K. Advancement of a multiplex PCR for the differentiation of all currently described *Brucella* species. *J Microbiol Methods* 2010; 80(1): 112-4. [http://dx.doi.org/10.1016/j.mimet.2009.10.015] [PMID: 19887090]
- [21] Morata P, Queipo-Ortuño MI, de Dios Colmenero J. Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis. *J Clin Microbiol* 1998; 36(9): 2443-6. [http://dx.doi.org/10.1128/JCM.36.9.2443-2446.1998] [PMID: 9705371]
- [22] Queipo-Ortuño MI, De Dios Colmenero J, Macias M, Bravo MJ, Morata P. Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis. *Clin Vaccine Immunol* 2008; 15(2): 293-6. [http://dx.doi.org/10.1128/CVI.00270-07] [PMID: 18077622]
- [23] Navarro E, Casao MA, Solera J. Diagnosis of human brucellosis using PCR. *Expert Rev Mol Diagn* 2004; 4(1): 115-23. [http://dx.doi.org/10.1586/14737159.4.1.115] [PMID: 14711354]
- [24] Matrone M, Keid LB, Rocha VCM, *et al.* Evaluation of DNA extraction protocols for *Brucella abortus* per detection in aborted fetuses or calves born from cows experimentally infected with strain 2308. *Braz J Microbiol* 2009; 40(3): 480-9. [http://dx.doi.org/10.1590/S1517-83822009000300010] [PMID: 24031391]
- [25] Gamazo C, Vitas AI, López-Goñi I, Díaz R, Moriyón I. Factors affecting detection of *Brucella melitensis* by BACTEC NR730, a nonradiometric system for hemocultures. *J Clin Microbiol* 1993; 31(12): 3200-3. [http://dx.doi.org/10.1128/JCM.31.12.3200-3203.1993] [PMID: 8308111]
- [26] Baddour MM, Alkhalifa DH. Evaluation of three polymerase chain reaction techniques for detection of *Brucella* DNA in peripheral human blood. *Can J Microbiol* 2008; 54(5): 352-7. [http://dx.doi.org/10.1139/W08-017] [PMID: 18449219]
- [27] Mitka S, Anetakis C, Souliou E, Diza E, Kansouzidou A. Evaluation of different PCR assays for early detection of acute and relapsing brucellosis in humans in comparison with conventional methods. *J Clin Microbiol* 2007; 45(4): 1211-8. [http://dx.doi.org/10.1128/JCM.00010-06] [PMID: 17267626]
- [28] Sifuentes-Rincón AM, Revol A, Barrera-Saldaña HA. Detection and differentiation of the six *Brucella* species by polymerase chain reaction. *Mol Med* 1997; 3(11): 734-9. [http://dx.doi.org/10.1007/BF03401711] [PMID: 9407549]
- [29] Bricker BJ, Halling SM. Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. *J Clin Microbiol* 1995; 33(6): 1640-2. [http://dx.doi.org/10.1128/JCM.33.6.1640-1642.1995] [PMID: 7650203]
- [30] Hinić V, Brodard I, Thomann A, *et al.* Novel identification and differentiation of *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* suitable for both conventional and real-time PCR systems. *J Microbiol Methods* 2008; 75(2): 375-8. [http://dx.doi.org/10.1016/j.mimet.2008.07.002] [PMID: 18675856]
- [31] Sreevatsan S, Bookout JB, Ringpis F, *et al.* A multiplex approach to molecular detection of *Brucella abortus* and/or *Mycobacterium bovis* infection in cattle. *J Clin Microbiol* 2000; 38(7): 2602-10. [http://dx.doi.org/10.1128/JCM.38.7.2602-2610.2000] [PMID: 10878051]
- [32] Probert WS, Schrader KN, Khuong NY, Bystrom SL, Graves MH. Real-time multiplex PCR assay for detection of *Brucella* spp., *B. abortus*, and *B. melitensis*. *J Clin Microbiol* 2004; 42(3): 1290-3. [http://dx.doi.org/10.1128/JCM.42.3.1290-1293.2004] [PMID: 15004098]