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Author: Farhad Safarpoor Dehkordi
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Incidence study of *Brucella abortus* and *Brucella melitensis* in Bovine and Buffalo semen samples by Real-Time PCR assay in Iran

Farhad Safarpoor Dehkordi [1)*]

[1)] Young Researchers Club, Faculty of Veterinary Medcine, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran

*Corresponding author: Farhad Safarpoor Dehkordi, Young Researchers Club, Faculty of Veterinary Medcine, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran
Tel.: 98 9365819491, Fax: 98 3813381892, E-mail: Dr.Farhads@yahoo.com

Running Title: Brucellosis in large ruminants

ABSTRACT. Advances in molecular sciences allow us to make extensive use of equipment in order to meet needs. In this present study to overcome the shortcomings of culture and serological methods for detection and identification of *Brucella abortus* and *Brucella melitensis* in bovine and buffalo semen samples, a conventional and real-time PCR assays were used. All samples were collected and transferred to laboratory, genomic DNA was extracted and the molecular methods by specific primers were performed. In total 241 (23.86%) bovine and 261 (26.48%) buffalo semen samples were positive for

presence of *Brucella* species by culture method. After single step PCR it was recognized that 255 (25.24%) bovine and 271 (28.28%) buffalo semen samples were positive for incidence of *Brucella* species by conventional PCR. After using of real-time PCR, it was recognized that 156 (15.44%) and 63 (6.23%) bovine and 188 (19.62%) and 58 (6.05%) of buffalo semen samples were infected by *Brucella abortus* and *Brucella melitensis*, respectively. Beside, the results of real-time PCR showed that 3.56% and 2.27% of bovine and 2.6% and 1.87% of buffalo semen samples were infected with unknown species and both species, respectively. The sensitivity and specificity of conventional PCR were obtained 100% and 98.17% in bovine and 100% and 98.56% in buffalo semen samples. The CT values had significant differences ($p<0.05$) between the incidences of both *Brucella abortus* and *Brucella melitensis* in bovine with buffalo semen samples.

KEY WORDS: *Brucella abortus*, *Brucella melitensis*, Real-time PCR, Semen samples, Iran

INTRODUCTION

Brucellosis is a worldwide highly contagious zoonotic bacterial disease of human and many species of animals that caused by gram-negative, aerobic and facultative intracellular bacterium of the genus *Brucella*. Disease 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 [3]. *Brucella* species (*Brucella* spp.) are classically classified into 6 main species including *Brucella abortus* (*B. abortus*), *B. melitensis*, *B. suis*, *B. canis*, *B. ovis* and *B. neotomae* [15] and among these species, *B. abortus* and *B. melitensis* caused an abortion in ruminants [28].

In some countries especially in Europe and Asia (including Iran) where animals like camelid and buffaloes are kept in close contact with infected

sheep, goat and cattles, infections and abortions can also be caused by *B. melitensis* and *B. abortus* [40,18,42].

In Iran, *B. abortus* was first isolated from a bovine fetus in 1944 [11] and *B. melitensis* was first isolated from a sheep in Isfahan province in 1952 [21] and then brucellosis has been reported from various parts of Iran. In the majority of cases of brucellosis in Iran, *B. abortus* and *B. melitensis* are the main pathogens. *Brucella* varies in the frequency with which they infect particular host species. Thus *B. abortus* infects cattle, but sometimes can be transmitted to many other hosts and *B. melitensis* primarily infects sheep and goats, but can be transmitted to other species. Artificial insemination is used to induce fertility in livestock in Iran and other sites of the world. Therefore, quality and hygiene of semen samples should be considered. There are various methods for monitoring the semen health and quality and according to high economic losses of brucellosis; accurate, safe and sensitive diagnostic methods play a vital role in control and eradication of brucellosis in human and animals. There are various assays for diagnosis of brucellosis such as culture, serological and molecular methods. Culture method requires a living host and is both time consuming and hazardous [34]. The diagnosis of brucellosis by serological responses, which can be unspecific due to cross-reaction or subsensitive reactions in samples from areas with a low or subclinical prevalence of brucellosis [5]. 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 [34].

Therefore in order to facilitate these problems, and in spite of the high degrees of genetic similarity of *Brucella* spp., several conventional and real-

time PCR assays which are easier, faster, safer, more convenient and accurate than traditional methods have been developed [7, 45, 12].

So the two-fold purposes of this current study were to introducing the real-time PCR for detection and identification of *B. melitensis* and *B. abortus* and determination of the incidences rate of them in bovine and buffalo semen samples in Iran.

MATERIALS AND METHODS

Samples

From January to May 2011, a total of 1,010, bovine and 958 buffalo semen samples were collected randomly from 683 commercial herds in various parts of Iran. The animals which their semen samples were collected for this study were clinically healthy and the 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 semen samples were kept at –20 °C until processing. Each semen sample was divided to two parts and one part was cultured and from another part genomic DNA was extracted.

Brucella culture method

Bacterial isolation trial from samples were made on blood agar base (Oxoid) supplemented with 5% defibrinated sheep erythrocytes and antibiotics (Vancomycin, Nalidixic acid, bacitracin, nystatin and cyclohexamide at the

doose recommended in OIE manual 2000). Cultures were incubated for 10 days with 5% CO_2 at 37°C. Bacterial isolated were identified according to the conventional procedures [39].

DNA extraction

From each animal, 10 ml of semen were collected with using artificial vagina. For *Brucella* DNA detection, the assay that was introduced by Consuelo Vanegas et al., (2009) was used [50]. Purification of DNA was achieved using a genomic DNA purification kit (Fermentas, GmbH, Germany) and the total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell [46].

Conventional PCR

In this present study, the conventional PCR assay designed by Leal-Klevezas et al., (1995) was used [25]. This PCR to screen the *Brucella* spp. detected DNA sequence of the gene coding the outer membrane protein (omp-2) reported for *Brucella* in GenBank database located at NCBI [25]. The forward primer sequence is 5'-GCGCTCAGGCTGCCGACGCAA-3', and the reverse primer sequence is 5'-ACCAGCCATTGCGGTCGGTA-3'.

The PCR reaction was performed in a total volume of 25 µl containing 10 µl DNA concentrated in 2 µl of DNA sample, 0.5 mM $MgCl_2$, 0.2 mM dNTPmix, 0.8 µM 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, 65 °C for 40 sec, 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 (Master Cycler Gradiant, Eppendrof, Germany). A negative control (sterile water), and a positive control DNA from *B. abortus* strain S19 (S19 vaccine strain) (Razi Institute, Iran), were included in each amplification run.

Gel electrophoresis

The PCR-amplified products (OMP2: 113 bp) were examined by electrophoresis (120 V/208 mA) in a 1.5% agarose gel, stained with a solution of ethidium bromide (0.004 μg/ml) and examined under UV illumination. The *Brucella* DNA extracted from *B. abortus* S19 vaccine strain was used as positive control.

Real-Time PCR

The real-time PCR for species segregation were based on unique genetic loci of *B. melitensis* and *B. abortus*. The primer set (that was designed by the author) consisted of BMEII0466 (5′-TCGCATCGGCAGTTTCAA/ CCAGCTTTTGGCCTTTTCC-3′) (112 bp) with the Cy5-CCTCGGCATGGCCCGCAA-BHQ-2 (5′Fluorophore→3′Quencher) internal probe for *B. abortus* and BruAb2_0168 (5′-GCACACTCACCTTCCACAACAA/CCCCGTTCTGCACCAGACT-3′) (222 bp) with the FAM-TGGAACGACCTTTGCAGGCGAGATC-BHQ-1 internal probe for *B. melitensis*. In this study the starting quantity of DNA from each serial dilution is plotted as a function of threshold cycle (CT) values to obtain a standard curve. In the other hand the CT is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

A typical 25 µl reaction contained: 12.5 µl TaqMan® Universal PCR Master Mix (foodproof® *Brucella* Detection Kit), a 300 nM concentration of each forward and reverse primer (BioNeer Corporation, South Korea), a 200 nM concentration of the probe (BioNeer Corporation, Republic of Korea), and 2.5 ng of sample DNA. TaqMan Master Mix Real time PCRs reactions were carried out using a RotorGene 6000 instrument (Corbett Research, Australia). The reaction mixture was initially incubated for 10 min at 95 °C. Amplification was performed for 45 cycles of denaturation at 95 °C for 20 s, annealing and extension at 62 °C for 1 min. In this reaction the foodproof® *Brucella* Internal Control (White cap) and foodproof® *Brucella* Control Template (Purple cap) were used as an internal and positive control, respectively.

Sequencing

In order to confirm the PCR results, sequencing method was used. For this reason, PCR products of some positive samples were purified with High pure PCR product purification kit (Roche Applied Science, Germany) according to manufacturer's recommendations. Single DNA strands were sequenced with ABI 3730 XL device and Sanger sequencing method (Macrogen, Korea). Result of the sequence of each gene was aligned with the gene sequences recorded in the GenBank database located at NCBI.

Statistical analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA), ANOVA test analysis were performed and

differences were considered significant at values of $p<0.05$. In this study distribution of CT values was compared between bovine and buffalo semen samples using ANOVA (Analysis of Variance) test.

RESULTS

Culture

After culturing, 241 out of 1,010 (23.86%) bovine and 261 out of 958 (26.48%) buffalo semen samples were positive for presence of *Brucella* spp. in Iranian bovine and buffalo herds (Table 1).

Conventional PCR

The presence of *Brucella* DNA was detected by single-step PCR from 526 semen samples out of 1,968 animals (26.72%) (Figure 1). The incidence of *Brucella* spp. in bovine and buffalo semen samples were 25.24% and 28.28% in Iran (Table 2). According to the table 2, Sistan Va Balochestan province had a highest incidence of *Brucella* spp. in bovine (29.88%) and buffalo (36.08%) while Hormozgan province had a lowest incidence of *Brucella* spp. in bovine (17.27%) and buffalo (20.86%) in Iran.

We evaluated the relative sensitivity and specificity of conventional PCR compared with culture method. the sensitivity and specificity of conventional PCR assay were obtained 100% and 98.17% and 100% and 98.56% for detection of *Brucella* spp. in bovine and buffalo semen samples, respectively (Table 3). Therefore this study showed the high sensitivity and specificity of PCR method.

188 *Real-Time PCR*

189 After real-time PCR, it was recognized that from a total 1,010, bovine semen
190 samples, 156 (15.44%), 63 (6.23%) and 36 (3.56) and from a total 958
191 buffalo semen samples, 188 (19.62%), 58 (6.05%) and 25 (2.6%) were
192 positive for *B. abortus*, *B. melitensis* and unknown species, respectively
193 (Table 2). In addition, after using of BMEII0466 and BruAb2-0168 gene
194 specific primers, it was recognized that 23 (2.27%) and 18 (1.87%) of bovine
195 and buffalo semen samples were infected with both bacteria (*B. abortus* and
196 *B. melitensis*) (Table 2).

197 *Statistical analysis and evaluation of CT values*

198 Statistical analysis showed significant differences ($p<0.01$) between *B.*
199 *abortus* and *B. melitensis* detected in bovine and buffaloes semen samples
200 and $p<0.05$ between presences of *Brucella* spp. in bovine with buffalo semen
201 samples. In this study after curve analysis the CT value less than 43 was
202 indicating as positive (Figure 2). The CT values which obtained from the
203 real-time PCR had significant differences ($p<0.01$) between the incidence of
204 both species of bacteria in bovine and buffalo semen samples and $p<0.05$
205 between the incidences of both *B. melitensis* and *B. abortus* in bovine with
206 buffalo semen samples. Our results showed that the CT values, representing
207 amount of *Brucella* DNA, in buffalo was higher than bovine for both
208 *Brucella* spp..
209

210 These results indicated that the incidence of *B. abortus* and *B. melitensis* in
211 bovine semen samples were entirely higher than buffalo. This study showed

212 that the incidence of this bacterium is high in semen samples of Iranian
213 bovine and buffalo herds.

214 **DISCUSSION**

215 Our study showed that the semen samples of infected bovine and buffaloes
216 maybe played an important role in distribution of brucellosis in Iran.
217 Unfortunately, despite the high incidence of this bacterium in bovine and
218 buffalo semen samples, researches about detection of *Brucella* in semen
219 samples were very low. In addition, in majority of cases the semen samples
220 are not well controlled for presence of *Brucella* and other pathogens.
221 Besides, if laboratories want to control them, the traditional diagnostic
222 methods like an Enzyme Linked Immuno Sorbent Assay (ELISA) are
223 commonly used. Diagnosis of brucellosis by ELISA method is not
224 recommended because it can be unspecific and subsensitive due to cross-
225 reaction with other pathogens including *Yersinia enterocolitica*, *Salmonella*
226 genus, *Escherichia coli* O:157 and other *Brucella* spp. [9,10, 35]. Therefore,
227 requires of PCR as an accurate, safe, sensitive, fast and specific assay for
228 detection and differentiation of *Brucella* spp. is so essential in these cases.
229 Despite this, the real-time PCR assay has some advantages compared to the
230 conventional PCR; it is an important diagnostic tool yielding reliable and
231 reproducible results, does not require post-PCR analysis (gel electrophoresis,
232 hybridization), and the risk of cross contamination is limited than
233 conventional method but the real-time PCR is more expensive than
234 conventional PCR. Many studies have shown that the conventional method
235 for detecting *Brucella* spp. is technically time-consuming and labor-intensive
236 than real-time PCR assay [5,53]. In addition to above, the real-time PCR
237 assay used in this study allows precise detection of two *Brucella* spp. (*B.*
238 *abortus* and *B. melitensis*) and can simplify the procedure by testing

presumptive *Brucella* genome taken directly from semen samples. According to some features the six different species of *Brucella* were recognized, all of these species show high degrees of genetic similarity. Therefore, conventional PCR technique, most often, is not able to precise distinction between *Brucella* spp.. In addition to real-time PCR, multiplex-PCR assay can be used for simultaneous detection and identification of these two pathogens but studies showed that the high sensitivity and specificity of real-time PCR against multiplex PCR [54]. In addition multiplex PCR need to post-PCR analysis like gel electrophoresis and this can make it tedious and time consuming.

Since, PCR has been developed for the detection of *Brucella* in a wide variety of clinical samples such as semen [24], blood [38], milk [43], aborted fetuses [8] and lymphoid tissue [17] and in all of these studies PCR has been introduced as an accurate and sensitive assay for detection of *Brucella* spp.

The high incidence of brucellosis in semen samples of unspecific host (buffalo) of this present study maybe showed that these animals have been maintained in close association with infected sheep and cattles. In addition, this high prevalence of brucellosis in animals in Sistan Va Balochestan province probably represents low veterinary facilities of this province, geographical and climate conditions and finally importation of infected livestock from the neighboring countries like Pakistan and Afghanistan.

To our knowledge, the volume of semen used was also a critical factor affecting the amplification reaction in our experiment. Although the sensitivity of PCR detection would increase on increasing the volume of semen due to the increase in the number of organisms present, it would also increase the inhibitory factors present in the semen. So it was essential to

compromise on the volume of semen used such that there was no PCR inhibition. This volume would also be largely dependent on the method of DNA extraction followed [52] but in total there are many factors that maybe affect the incidence and epidemiology of brucellosis such as processing milk and milk products, socioeconomic status, climatic conditions, social customs, food habits, husbandry practices and environment hygiene [26].

Previous study showed that Iran, Saudi Arabia, Jordan, Syria and Oman had the highest incidence of brucellosis among the countries of the Near East region [41]. Studies about brucellosis from various parts of Iran [44, 36] and various species such as sheep [57], goat [1], cattle [55], camel [23], dog [31], buffalo [36], human [22] and horse [48] indicated that brucellosis is one of the most important endemic zoonotic diseases in Iran. Brucellosis causes the great economic losses in Iran. A previous report from Iran indicated that the prevalence rate of brucellosis was 37/100,000 in human, 340/10,000 in sheep and goat and 56/10,000 in cattle in East of Iran during 2002-2006 [6].

The prevalence of *B. abortus* observed in bovine in this study (15.44%) is higher than Egypt (5.44%) [45] and Ethiopia (4.9%) [29] but our results are lower than Punjab (20.67%) [4], India (18.81%) [20], Sokoto State (25.25%) [19] and Kenya (77.5%) [33]. The incidence of *B. abortus* observed in buffalo in this present study (19.62%) is higher than Egypt (0.3%) [41] and Punjab (India) (13.4%) [12] but is lower than Africa (30%) [51]. To our knowledge, there is no prevalence report of brucellosis caused by *B. melitensis* in bovine and buffalo being and this present study is the first prevalence report of *B. melitensis* observed in bovine and buffalo semen samples in the world.

The results of our 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*. To our knowledge, buffaloes maybe are not the primary hosts of *Brucella*, but they can be infected with both *B. abortus* and *B. melitensis*. Consequently, the prevalence of brucellosis in buffaloes is dependent to infection rate of primary hosts being in contact with them. In the other hand, spread of brucellosis in buffaloes depends on the *Brucella* spp. prevalent in other animals sharing their habitat and on the husbandry methods of the different species. Studies indicated that the multivariable logistic regression model on both individual and herd levels revealed large herds and contact with small ruminants as risk factors for prevalence of brucellosis [2].

Despite the advances made in surveillance and control, the prevalence of brucellosis is increasing in many developing countries due to various sanitary, socioeconomic, and political factors [37]. In extensive management system the prevalence of brucellosis among various species of animal is low [30].

The results of this present study showed that the bovine and buffalo can be the important reservoir for transmission of this zoonotic disease to human in Iran. This present study shows that molecular methods such as conventional and real-time PCR are accurate, trustful and rapid assays for detection and identification of *B. abortus* and *B. melitensis* in bovine and buffalo semen samples but the real-time PCR assay is better. To our knowledge, 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 that was 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 be used by and by.

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513 **Table 1.** Isolation of *Brucella* spp. from semen samples by culture method.

Provinces	No. samples		Culture (%)	
	Bovine	Buffalo	Bovine	Buffalo
Khozestan	388	210	100 (25.77)	67 (31.9)
Boshehr	131	300	27 (20.61)	76 (25.33)
Hormozgan	220	254	36 (16.36)	50 (19.68)
Sistan Va Balochestan	271	194	78 (28.78)	68 (35.05)
Total	1,010	958	241 (23.86)	261 (26.48)

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515 **Table 2.** Incidences of *B. melitensis*, *B. abortus* and *Brucella* spp. in bovine
516 and buffalo semen samples by evaluation of conventional and Real-Time
517 PCR methods in Iran.

Provinces	**No. samples**		**Conventional PCR (%)**		**Novel real-time PCR (%)**							
					Bovine				**Buffalo**			
	Bovine	**Buffalo**	**Bovine**	**Buffalo**	***B. abortus***	***B. melitensis***	**Unknown**	**Both bacteria**	***B. abortus***	***B. melitensis***	**Unknown**	**Both bacteria**
Khozestan	**388**	**210**	**108**	**70**	**70**	**18**	**20**	**9**	**52**	**10**	**8**	**4**

			(27.83)	(33.3)	(18.04)	(4.63)	(5.15)	(2.06)	(24.76)	(4.76)	(3.8)	(1.9)
Boshehr	**131**	**300**	**28 (21.37)**	**78 (26)**	**17 (12.97)**	**7 (5.34)**	**4 (3.05)**	**2 (1.52)**	**55 (18.33)**	**18 (6)**	**5 (1.66)**	**5 (1.66)**
Hormozgan	**220**	**254**	**38 (17.27)**	**53 (20.86)**	**23 (10.45)**	**12 (5.45)**	**3 (1.36)**	**5 (2.27)**	**38 (14.96)**	**10 (3.93)**	**5 (1.7)**	**3 (1.18)**
Sistan Va Balochestan	**271**	**194**	**81 (29.88)**	**70 (36.08)**	**46 (16.97)**	**26 (9.59)**	**9 (3.32)**	**8 (2.95)**	**43 (22.16)**	**20 (10.3)**	**7 (3.6)**	**6 (3.09)**
Total	**1,010**	**958**	**255 (25.24)**	**271 (28.28)**	**156 (15.44)**	**63 (6.23)**	**36 (3.56)**	**23 (2.27)**	**188 (19.62)**	**58 (6.05)**	**25 (2.6)**	**18 (1.87)**

Table 3. Evaluation of sensitivity and specificity of conventional PCR for detection of *Brucella* spp. in bovine (A) and buffalo (B).

A

	Culture positive	Culture negative	Total
Conventional PCR positive	241*[a]	14[c]	255
Conventional PCR negative	-[b]	755**[d]	755
Total	241[a+b]	769[c+d]	1,010

*Sensitivity $= \frac{a}{a+b} = 100\%$

523 **Specificity: $\frac{d}{d+c}$ = 98.17%

524 B

	Culture positive	Culture negative	Total
Conventional PCR positive	261*[a]	10[c]	271
Conventional PCR negative	-[b]	687**[d]	687
Total	261[a+b]	697[c+d]	958

525 *Sensitivity = $\frac{a}{a+b}$ = 100%

526 **Specificity: $\frac{d}{d+c}$ = 98.56%

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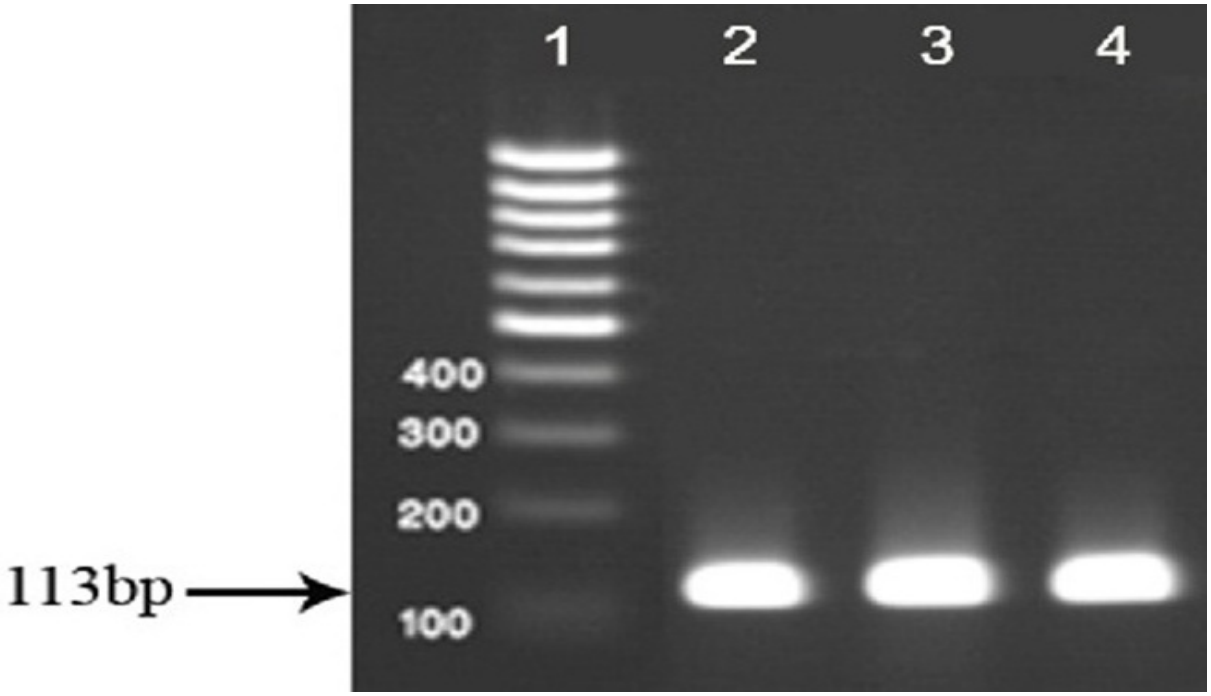

Fig. 1. Conventional PCR for detection of *Brucella* spp. lane 1 is 100 bp ladder, lanes 2 and 3 are positive samples for bovine and buffalo semen and lane 4 is positive control.