

Full Length Research Paper

Evaluation of the currently used polymerase chain reaction assays for molecular detection of *Brucella* species

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The diagnosis of brucellosis is the corner stone in any control and eradication program. Therefore, the main objective of the present study was to apply more advanced techniques for rapid and accurate diagnosis of brucellosis that can overcome the draw backs of the traditional diagnostic techniques. Different polymerase chain reaction (PCR) assays were applied in the present study, either singly or in a multiplex format, that enable to detect and differentiate most of *Brucella* species. The PCR assay detection limit was evaluated in a preliminary study. The obtained results recommend the PCR assay as a valuable, rapid, very specific, highly sensitive and safe laboratory diagnostic test that can be used not only for detection of *Brucella* antigen either in culture or in clinical samples but also in differentiating most of the virulent and vaccinal strains.

Key words: *Brucella* species, polymerase chain reaction (PCR) assays, diagnosis, vaccinal strains, *Brucella* antigen.

INTRODUCTION

Brucellosis is a widespread infectious disease of animals and humans. The infection is widely distributed in Egypt and Mediterranean countries (Sayour et al., 1970; Hamdy, 1992). The disease in animals is manifested by reproductive failure, which includes abortion, birth of unthrifty calves and retained placenta in female animals. Lesions in *Brucella* infected males are largely confined to genital organs including testicles, seminal vesicles and epididymes (Morgan and Mackinnon, 1979). The disease can also lead to a highly diverse illness in humans (Probert et al., 2004).

The gold standard diagnostic technique continues to be based on isolation of suspicious bacterial colonies from host tissues, milk or vaginal exudates, followed by bacteriological characterization (Alton et al., 1975). This process has serious drawbacks, as it is time consuming,

complicated, laborious, need highly skilled personal, besides the zoonotic nature of most *Brucella* species which is potentially hazardous, moreover, the results are not always definitive (Bricker, 2002). On the other hand, there is no single serological test of choice for diagnosis and control of brucellosis (Morgan, 1967; Ibrahim et al., 1996). Frania (1985) concluded that serological tests proved to be either too sensitive giving false positive results, or too specific giving false negative results, beside misdiagnosis due to cross reactivity of other gram negative bacteria such as *Yersinia enterocolitica* with smooth *Brucella* species.

However, quick and accurate identification of vaccine strains would be required in many circumstances especially when the fate of the vaccine has to be determined after vaccination, in epidemiological studies or in differentiation among vaccinal strains and wild-type field strains.

Numerous PCR based assays have been developed and evaluated for the identification of *Brucella* species to improve the diagnostic capabilities ranging from general

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Table 1. PCR primers used for single and multiplex PCR.

Gene	Primer ¹	Primer sequence ²	Amplicon size (bp)
<i>TEM</i>	F	TTAACTGGCGAACTACTTAC	247
	R	GTCTATTTTCGTTTCATCCATA	
<i>SHV</i>	F	AGGATTGACTGCCTTTTTFG	393
	R	ATTTGCTGATTTTCGCTCG	
<i>CMY-2</i>	F	GACAGCCTCTTTCTCCACA	1,000
	R	TGGACACGAAGGCTACGTA	
<i>CTXM-1</i> group	F	TTAGGAARTGTGCCGCTGYA	688
	R	CGATATCGTTGGTGGTRCCAT	
<i>CTXM-2</i> group	F	CGTTAACGGCACGATGAC	404
	R	CGATATCGTTGGTGGTRCCAT	
<i>CTXM-9</i> group	F	TCAAGCCTGCCGATCTGGT	561
	R	TGATTCTCGCCGCTGAAG	
<i>CTXM-8/25</i> group	F	AACRCRCAGACGCTCTAC	326
	R	TCGAGCCGGAASGTGTYAT	

¹F = forward; R = reverse.

²Bold indicates N = T or C; R = A or G

identification of the genus *Brucella* (Genus-Specific PCR assay), that is designed to explicate a single unique genetic locus that was highly conserved in *Brucella* (e.g. 43 KDa omp, BCSP31, IS6501/711 or 16SrRNA genes) (Fekete et al., 1990; Baily et al., 1992; Herman and De Ridder, 1992; Halling et al., 1993; Romero et al., 1995a; Da Costa et al., 1996; Casanas et al., 2001). The differential identification of *Brucella* species (differential PCR-based assay), that depends on strain locus specific multiplexing (e.g. AMOS-PCR based on IS711, PCR-RFLP or RAPD-PCR), (Bricker and Halling, 1994; Bricker and Halling, 1995; Sifuentes et al., 1997; Tcherneva et al., 2000; Adone et al., 2001; Redkar et al., 2001; Probert et al., 2004; Ocampo-Sosa et al., 2005). Differential PCR based assays are particularly useful for epidemiological trace back or for species-specific eradication programs (Bricker, 2002). PCR assay has been shown to be a valuable rapid and sensitive technique in many national and international publications (Husseinen, 1993; Gabal et al., 1994; Amin et al., 1995; 2001; Hamdy and Amin, 2002; Richtzenhain et al., 2002; Tantillo et al., 2003; El-Faki et al., 2005b; Garin-Bastuji et al., 2006; Gupta et al., 2006a; Leary et al., 2006).

MATERIALS AND METHODS

Brucella strains

Six *Brucella* reference and vaccinal strains (*Brucella abortus* strain 544, *B. abortus* strain 19, *B. abortus* strain RB51, *B. abortus* strain 2308, *Brucella melitensis* strain M16 and *B. melitensis* Rev-1) were used in this study. One *B. melitensis* biovar- 3 field isolate was also used. All strains were kindly obtained from the Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt.

Clinical samples

A total of 30 samples were obtained and used in the current study.

7 of them from aborted animals suspected to have Brucellosis. 2 samples of foetal fluids collected from aborting cows; 4 samples of internal organs (lung, liver, kidney and heart) were collected from aborted foetal. 1 sample of stomach content was collected from aborted fetus and another 23 tissue samples were collected from serologically positive cows.

DNA amplification by PCR assays

Different PCR assays were performed in the present study. The amplification was performed in a programmable heating block, (Primus Thermal Cycler, MWG Biotech, Germany). To ensure reproducibility, each PCR was performed at least twice, the primers used and the concentration of PCR reaction components as well as the amplification conditions employed in different PCR assays (Bricker and Halling, 1995).

PCR conditions (25 µl reaction volume). All solutions should be thawed and kept cool, gently mixed and brief centrifuged, added in a thin walled PCR tube on ice. The following PCR components were added in each PCR tube: 2X PCR master mix 12.5 µl, DNA template 150 to 200 ng, each primer 0.5 µM and nuclease free water up to 25 µl. Amplification conditions, after gently mixing and brief centrifugation of each PCR tube, all tubes were placed in the thermal cycler and amplification program was initial denaturation at 95°C for 4 min, 35 cycles: denaturation step 95°C for 1.20 min, annealing step at 55.5°C for 2 min and extension step at 72°C for 2 min. The final extension at 72°C for 10 min. The amplified product was resolved using 1.5% agarose gel electrophoresis that is stained with ethidium bromide and photographed by photo documentation system (UVP, USA) (Sambrook et al., 1989).

Different sets of primers were synthesized by Fermentas, AB.Gene (MWG, oligosynthesis- Germany). Different PCR assays used (single or multiplex assays). The first to fourth PCR assays were shown in Table 1. The first PCR assay was used for detection of genus *Brucella*, the second PCR assay was used for detection of *B. abortus* (biovar 1, 2 and 4), the third PCR assay was used for differentiation of *B. abortus* S19 from other brucellae, the fourth PCR assay was used for differentiation of *B. abortus* strain RB51/2308 and the fifth PCR assay was carried out using primer sets (B4, B5, Ba-SP and IS711-SP) for the detection of *Brucella* species and differentiation of *B. abortus* species from *B. melitensis*. The sixth PCR assay was carried out using primer sets (B4, B5, Eri1, and Eri2) for differentiation of *B. abortus* S19 from other *Brucella*

1 2 3 4 5 6 7 8 9 10 11 12



Figure 1. Detection limits of *B. abortus* RB51 DNA by PCR using B4, B5 primer set. Lane 1: 100 bp marker; lanes 2 to 10: Different concentrations from 1.7×10^{10} to 1.7×10^1 CFU/ml and lane 12: Negative control.

species. The seventh PCR assay was carried out using primer sets (B4, B5, IS711-SP, Ba-sp and RB51/ 2308 primer) for differentiation of *B. abortus* strain RB51 from other *B. abortus* species. While the eighth PCR assay was carried out using primer sets (B4, B5, Ba-SP, IS711-SP, Eri1, and Eri2) for differentiation of *B. abortus* strain 19 from *B. abortus* strain RB51. Moreover, the ninth PCR assay was carried out for detection of *B. melitensis* using primer sets (B4, B5, Bm-SP, IS711-SP, Eri1 and Eri2). Bm- SP 5' AAA TCG CGT CCT TGC TGG TCT GA 3', IS711- SP- 5' TCG CGA TCA CTT AAG GGC CTT CAT 3' (Bricker and Halling, 1994).

The tenth PCR assay was carried out using primer sets (B4, B5, Ba-SP, Bm-SP, IS711-SP, RB51/ 2308 primer, Eri1 and Eri2) for differentiation and discrimination between all tested *Brucella* species.

Application of PCR on field samples

Thirty field samples were tested using different PCR assays. The first PCR assay using primer set (B4 and B5), which indicate the presence or absence of *Brucella* organism. The second assay was multiplex PCR using primer sets (B4, B5, IS711-SP, Bm-SP, Eri1, and Eri2), for detection and differentiation of *B. melitensis*. Another PCR assay using primer sets (Bm-SP, IS711-SP, Eri1 and Eri2) were applied on the tissue samples collected from the serologically positive cattle.

RESULTS AND DISCUSSION

As with any disease, control of brucellosis would benefit from improvements in diagnostic methods, because of the disadvantages of the traditional diagnostic techniques (Bricker, 2002). In the current study advanced techniques for direct detection and differentiation of different *Brucella* species were performed. Different PCR assays were applied either singly or in a multiplex format in order to investigate the adaptation of PCR amplification assay as rapid, simple, highly sensitive, very specific technique for the detection and differentiation of *Brucella* species either following culture or directly in field samples.

In preliminary study, the relative sensitivity of the PCR assay was determined. The detection threshold of PCR,

using the B4 and B5 primer set, was (1.7×10^2 CFU/ml), Figure 1. While using RB 51/2308 primer set was (1.7×10^3 CFU/ml), Figure 2. Such results suggest the PCR amplification assay as a highly sensitive technique. Even only a few DNA molecules can be amplified as in samples with low titer of pathogen or highly contaminated with other micro-organisms. These results agree with the previously reported data by (Cetinkaya et al., 1999; Cortez et al., 2001; Richtzenhain et al., 2002). The first PCR assay (PCR assay 1) was reported by Baily et al. (1992). Such assay was designated to amplify genus-specific target DNA sequence. The primer set used for amplification was specific for the gene encoding a 31 KDa *Brucella* outer membrane protein (BCSP31); the gene encodes an antigenic, periplasmic protein of unknown function. It is conserved in all species and biovars of *Brucella* examined. The results indicated that the product was successfully amplified from all the examined *Brucella* reference, vaccinal and field isolates strain (Figure 3). The results indicated the PCR as a valuable tool for rapid diagnosis of brucellosis. These results are in agreement with the conclusions previously reported by (Gabal et al., 1994; DaCosta et al., 1996; Ibrahim et al., 1996; Radwan and Ibrahim, 2000; Casanas et al., 2001).

The AMOS PCR assay (Bricker and Halling, 1994), for identification of *Brucella* species, was based on the existence of IS711 copies specific for each of the *Brucella* species, that exploiting the multi-copy element IS711 (Halling et al., 1993), which is also known as IS6501 (Bettach et al., 1993). As it is much easier and more valuable to identify an isolate as S19, RB51 or any other *Brucella* strain, the assay was modified (Bricker and Halling, 1995), by the introduction of new primers (Eri1, Eri2 and RB51/2308 primer) that can detect and differentiate *B. abortus* S19 vaccine strain and *B. abortus* strain RB51 and/ or its parent strain 2308. The test was previously evaluated and successfully applied as a diagnostic tool (Fekete et al., 1992; Leal-Klevezas et al., 1995;

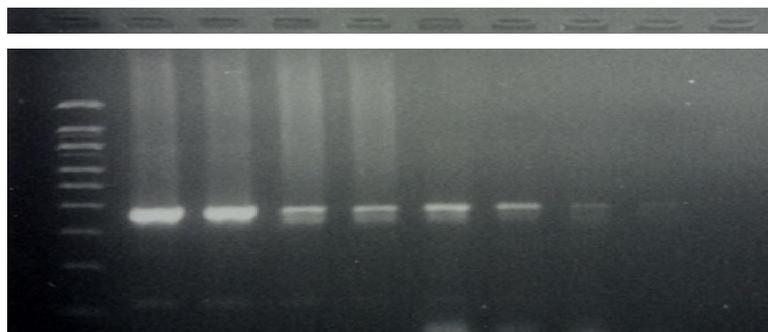


Figure 2. Detection limits of *B. abortus* RB51 DNA by PCR using IS711-SP, RB51/ 2308 primer set. Lane 1: 100bp marker; lanes 2 to 11: Different concentrations from 1.7×10^{10} to 1.7×10^1 CFU/ml and lane 10: Negative control. PCR assays on DNA extracted from *Brucella* reference and vaccinal strains as well as field isolate.



Figure 3. PCR amplification of *B. species*-DNA from *Brucella* vaccinal and reference strains using the primer set (B4, B5). Lane 1: 100 bp molecular weight marker; lane 2: *B. melitensis* 16 M; lane 3: *B. abortus* strain 2308; lane 4: *B. abortus* strain 19; lane 5: *B. abortus* strain RB51; lane 6: *B. abortus* strain 544; lane 7: *B. melitensis* Rev.1 and lane 8: Negative control.

Romero et al., 1995a; 1995b; Ewalt and Bricker, 2000; Adon et al., 2001), applied with modified version (Redkar et al., 2001; Ewalt and Bricker, 2003; Ocampo-Sosa et al., 2005). Amplification of both 731 and 178 bp indicates that the strain is *B. melitensis*. On the other hand, amplification of both 498 and 178 bp indicates that, the strain is *B. abortus* (biotype 1, 2 or 4) while amplification of only the 498 bp indicates *B. abortus* strain 19. Failure to amplify either the 731 bp or the 498 bp and amplification of only the 178 bp indicate that the strain is any *Brucella* species other than the above mentioned species and biotypes (Bricker and Halling, 1995). In this study, each primer set in the AMOS cocktail was tested alone in a single test (PCR assays 2, 3 and 4), Figures 4, 5 and 6.

There were some previous trials to discriminate among the species of the genus *Brucella* using the wide possibilities of PCR techniques (Fekete et al., 1992b;

Bricker and Halling, 1994,1995; DaCosta et al., 1996; Ouahrani-Bettach et al., 1996; Techerneva et al., 1996, 2000; Sifuentes et al., 1997; Redkar et al., 2001; Ocampo-Sosa, 2005; Ferrao-Beck et al., 2006; Garcia-Yoldi et al., 2006), who obtained similar results to that obtained in the present study.

In the PCR assays (2, 3 and 4), each primer in the AMOS cocktail alone was tested in a single test; the encouraging result was promising to evaluate the possibility of multiplexing through different steps (PCR assays 5, 6, 7, 8 and 9). In PCR assay (5) a multiplex PCR assay was employed using primer sets (B4, B5, Ba-SP and IS711-SP) for detection of *Brucella* species with identification of *B. abortus* species from *B. melitensis*, 223 and 498 bp bands were successfully revealed from *B. abortus* only, while only one band of 223 bp obtained from *B. melitensis* (Figure 7). In the next PCR assay (PCR assay 6) B4, B5, Eri1 and Eri2 primer sets were



Figure 4. PCR amplification of *B. abortus* (biovar 1, 2 and 4) using the primer set (Ba-SP, IS711-SP). Lane 1: 100 bp molecular weight marker; lane 2: *B. abortus* strain 2308; lane 3: *B. abortus* S19; lane 4: *B. abortus* strain RB51; lane 5: *B. abortus* strain 544; lane 6: *B. melitensis* 16M strain; lane 7: *B. melitensis* Rev.1 and lane 8: Negative control.

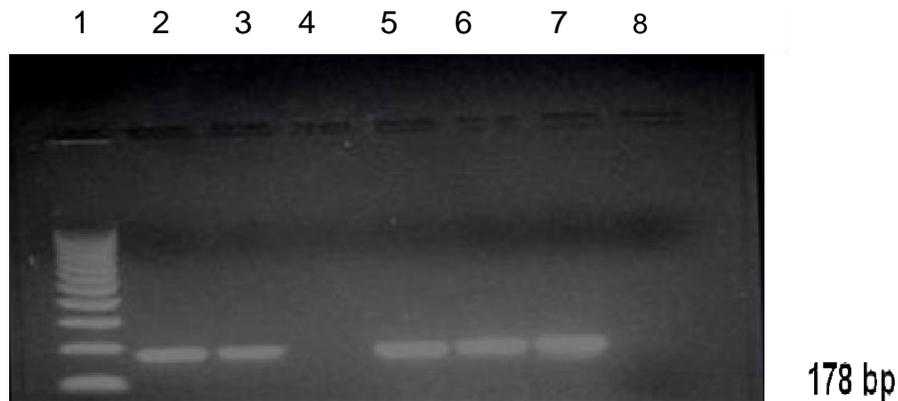


Figure 5. PCR amplification of *B. species*-DNA from *Brucella* vaccinal and reference strains using the primer set (Eri1, Eri2). Lane 1: 100 bp molecular weight marker; lane 2: *B. abortus* strain 544; lane 3: *B. abortus* RB51; lane 4: *B. abortus* S19; lane 5: *B. melitensis* 16M; lane 6: *B. melitensis* Rev.1; lane 7: *B. abortus* strain 2308 and lane 8: Negative control.



Figure 6. PCR amplification of *B. species*-DNA from *Brucella* vaccinal and reference strains using the primer set (RB51/ 2308 primer, IS711-SP). Lane 1: 100 bp molecular weight marker; lane 2: *B. abortus* RB51; lane 3: *B. abortus* strain 2308; lane 4: *B. abortus* S19; lane 5: *B. abortus* strain 544 and lane 6: Negative control.

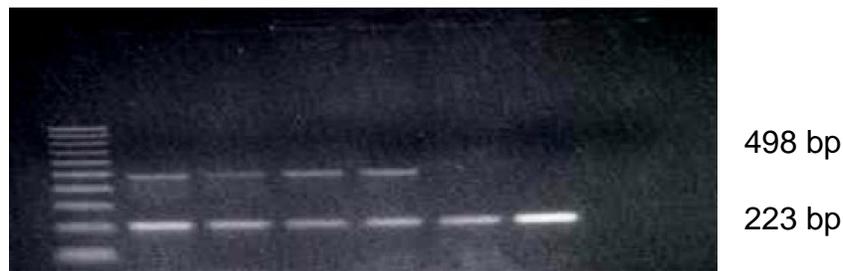


Figure 7. PCR amplification of *B. species*-DNA from *Brucella* vaccinal and reference strains using the primer sets (B4, B5, Ba-SP and IS711-SP). Lane 1: 100 bp molecular weight marker; lane 2: *B. abortus* RB51; lane 3: *B. abortus* strain 2308; lane 4: *B. abortus* S19; lane 5: *B. abortus* strain 544; lane 6: *B. melitensis* 16M; lane 7: *B. melitensis* strain Rev.1 and lane 8: Negative control.

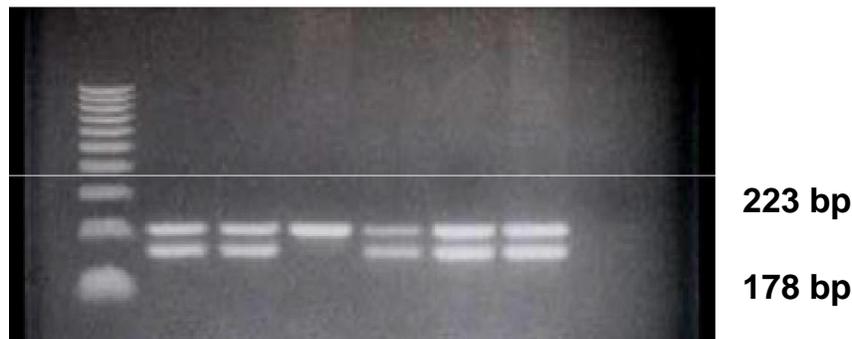


Figure 8. PCR amplification of *B. species*-DNA from *Brucella* vaccinal and reference strains using the primer sets (B4, B5, Eri1, and Eri2). Lane 1: 100 bp marker; lane 2: *B. abortus* strain 2308; lane 3: *B. abortus* strain RB51; lane 4: *B. abortus* S19; lane 5: *B. abortus* strain 544; lane 6: *B. melitensis* strain 16M; lane 7: *B. melitensis* strain Rev.1 and lane 8: Negative control.

used for differentiation of *B. abortus* strain 19 from other *Brucella* species as expected 2 bands of 223 and 178 bp were obtained from all *Brucella* strains except strain 19 show only one band of 223 bp (Figure 8). While in PCR assay (7) another multiplex primer sets were used (B4, B5, IS711-SP, Ba-SP and RB51/2308 primer) were used for differentiation of *B. abortus* strain RB51 and its parent strain 2308 from other *B. abortus* strains by revealing 3 different bands at 223, 364 and 498 bp from strain RB51/2308, while 2 bands obtained only (223 and 498 bp) from other *B. abortus* strains (Figure 9). An important mPCR (PCR 8) was used for differentiation of *B. abortus* S19 from *B. abortus* strain RB51 (which are the most common vaccine strains) among other brucellae (Figure 10). In PCR assay (9) a mPCR assay using primer sets (B4, B5, Bm-SP, IS711-SP, Eri1 and Eri2) for differentiation of *B. melitensis* from other *Brucellae*, 3 expected products of 731, 223 and 178 bp were obtained from different *B. melitensis* strains (Figure 11).

After some trials of PCR optimization it was possible to apply the multiplexing PCR (PCR assay 10) on any suspected *Brucella* isolate to confirm whether it is a wild type *Brucella* field strain or strain 19 or RB51 vaccine strain in a single test (Figure 12). The earlier mentioned results are in agreement with those previously obtained by (Bricker and Halling, 1994; Ewalt and Bricker, 2000; Ocampo-Sosa et al., 2005; Garcia-Yoldi et al., 2006).

In the present study, a 223 bp amplicon was obtained from 4 tissues samples out of 7 as shown in (Figure 13) using PCR assay (1) indicating presence of *Brucella* species, and a 731, 223 and 178 bp amplicons were obtained from the same samples using PCR assay (9) indicating that all the identified samples were *B. melitensis* as shown in Figure 14.

Another 23 tissue samples were obtained from serologically positive animals. On applying PCR assay (using primer sets B4, B5, Bm-Sp and IS711-SP), 21 out of 23 samples were positive giving amplified products at



Figure 9. PCR amplification of *B. species*-DNA from *Brucella* vaccinal and reference strains using the primer sets (B4, B5, IS711-SP, RB51/ 2308 and Ba-SP). Lane 1: 100 bp marker; lane 2: *B. abortus* strain 2308; lane 3: *B. abortus* strain RB51; lane 4: *B. abortus* S19; lane 5: *B. abortus* strain 544 and lane 6: Negative control.

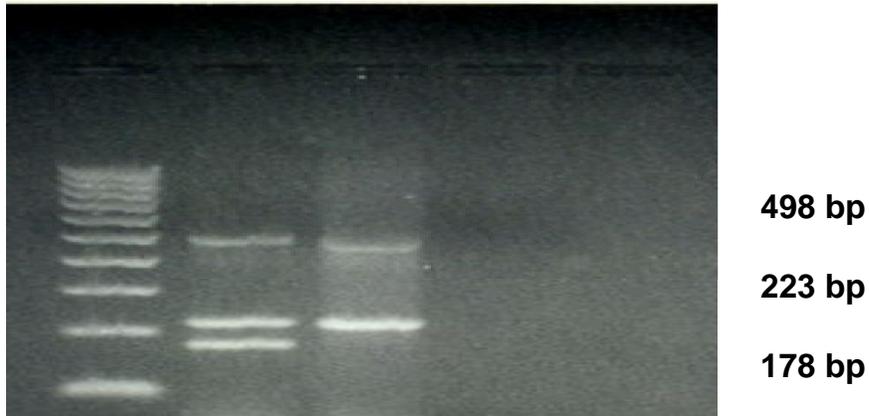


Figure 10. PCR amplification of *B. species*-DNA from *Brucella* vaccinal and reference strains using the primer sets (B4, B5, Ba- SP, IS711- SP, Eri1, and Eri2). Lane 1: 100 bp marker; lane 2: *B. abortus* strain RB51; lane 3: *B. abortus* S19 and lane 4: negative control.

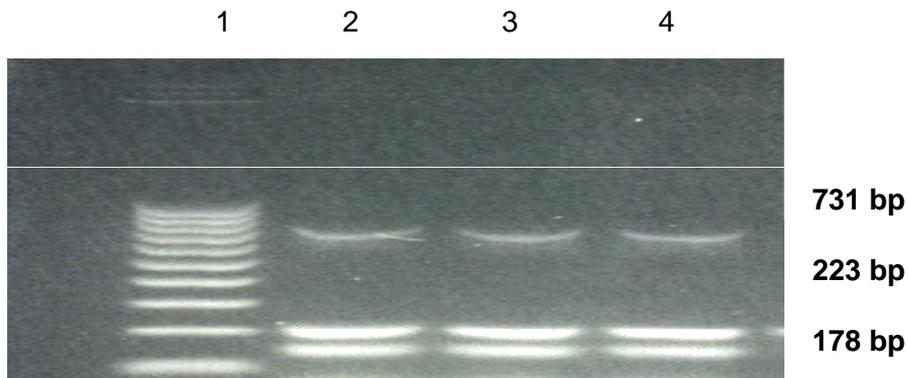


Figure 11. PCR amplification of *B. species*-DNA from *Brucella* vaccinal and reference strains using the primer sets (B4, B5, Bm-SP, IS711-SP, Eri1 and Eri2). Lane 1: 100 bp marker; lane 2: *B. melitensis* 16M; lane 3: *B. melitensis* strain Rev.1 and lane 4: Field isolate (*B. melitensis* biovar 3).

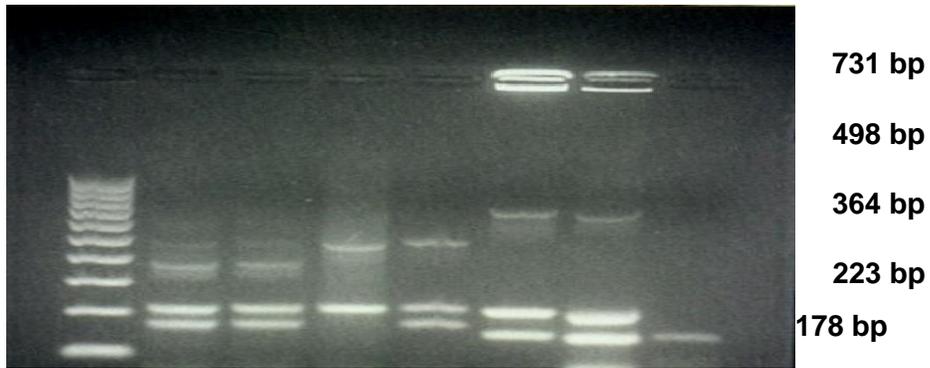


Figure 12. PCR amplification of *B. species*-(B4, B5, Ba-SP, Bm-SP, IS711-SP, RB51/ 2308 primer, Eri1 and Eri2). Lane 1: 100 bp marker; lane 2: *B. abortus* strain 2308; lane 3: *B. abortus* strain RB51; lane 4: *B. abortus* S19; lane 5: *B. abortus* strain 544; lane 6: *B. melitensis* strain 16 M; lane 7: *B. melitensis* strain Rev.1 and lane 8: negative control.

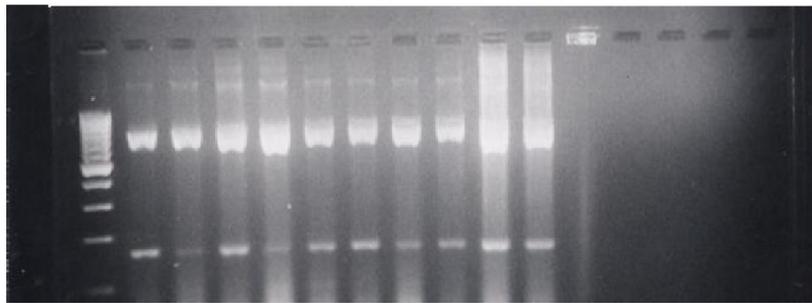


Figure 13. PCR amplification of *Brucella* species DNA from aborted bovine fetus samples using (B4, B5) primer set. Lane 1: 100 bp marker; lane 3 to 5: Positive field samples; lane 6 to 7: Negative field samples; lane 2: positive control and lane 8: Negative control.

1 2 3 4 5 6 7 8



Figure 14. PCR amplification of *Brucella* species DNA from aborted bovine fetus samples using (B4, B5, Bm- SP, IS711-SP, eri1 and eri2) primer sets. Lane 1: 100 bp marker; lane 3 to 5: Positive field samples; lane 6 to 7: Negative field samples; lane 2: positive control and lane 8: Negative control.



731 bp

178 bp

Figure 15. PCR amplification of *Brucella* species DNA from different tissue samples using (Bm-SP, IS711-SP, Eri1 and Eri2) primer sets. Lane 1: 100 bp marker; lane 2 to 11: Positive field samples; lane 2: positive control and lane 10: Negative control.

224 and 731 bp (Figure 14). These results indicating that all positive samples identified as *Brucella* species were *B. melitensis* (Figure 15). Similar results were previously reported (Amin et al., 1995; Hamdy and Amin, 2002; Rijpens et al., 1996; Gupta et al., 2006a; 2006b) also succeeded to detect Brucella DNA in field samples.

Two out of 23 samples were PCR negative but seropositive animals could be explained by many factors; such as the PCR inhibitors which could be the cause of amplification failure. Although protocol for DNA extraction was used to eliminate inhibitors, persistence of such inhibitors in some samples could be the reason of some false negative PCR (Manterola et al., 2003). Another factor of false negative PCR is the number of *Brucella* organisms below the detection threshold, degradation of target DNA in the samples and inefficient DNA extraction (Romero et al., 1995b).

In conclusion, PCR based assays have been proved to be an important alternative rapid technique that overcome problems and disadvantages of currently used traditional methods. The PCR is very specific and highly sensitive technique that can be used not only for detection of Brucella antigen either in culture or in clinical samples but also in differentiating most of virulent and vaccine strains. The possibility of using the PCR technique to detect the DNA of dead bacteria and even in samples highly contaminated with other micro-organisms could increase the rate of detecting infected animals. A great attention should be paid for the optimal reaction condition. The assay was optimized to allow maximum sensitivity even in the multiplex format. The multiplex format of the assay reduces the reagent cost and save time required to perform testing for brucellosis.

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