**Received: 26.4.2006** **Accepted: 12.9.2006**

*Original Article*

# Detection of Brucella abortus by alkB and IS711 based primers

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## Abstract

**BACKGROUND**: Brucellosis is a zoonotic disease, which involves both animals and human. Although the conventional methods have been widely used for its laboratory diagnosis, the PCR techniques have proved to be useful due to specificity, sensitivity and the rapidness. Various target sequences of brucella bacterium such as OMP2, 16s RNA and IS711 have been used for the primer designing. All primer sets have shown different sensitivities and specificities. In present investigation, PCR protocol and primer designated based on IS711 and a fragment of chromosomal DNA all were optimized with standard genome and clinical samples.

**METHODS**: Numerous tissue samples (liver, kidney, lymph node, and uterus) were prepared and were cultured by the bacteriological standard methods along with the serology positive human samples. PCR protocol was optimized and the primer's sensitivity and the specificity were checked using pure genome of *B. abortus*. All samples were tested by the standard bacteriological methods. The samples were then subject to PCR amplification and the PCR product was confirmed using the RFLP technique.

**RESULTS**: The culture results indicated a poor sensitivity as it was previously reported. The PCR product 157 bp was observed on the agarose gel indicating that significant number of clinical samples (human brucellosis cases) were positive by PCR but not by the culture method. Although *B. abortus* DNA was detected in all the culture positive veterinary specimens, some cross-reactions with close related bacteria were observed that might influence the interpretation of the results.

**CONCLUSIONS**: The sensitivity of the present PCR protocol was significantly higher when alk B and IS711 based primers were used in compare to each of the alkB and IS711 based primers alone. More research will be needed to improve the specificity and sensitivity of the PCR protocol before recommending for routine laboratory works.

**KEY WORDS:** Brucella, PCR, Brucellosis.

JRMS 2007; 12(2): 62-67

Brucellosis continues to be of great health concern and economic importance in many countries including Iran. The disease is caused by aerobic gram-negative bacteria of the genus Brucella. Among the genus, B. abortus and B. melitensis are the leading cause of brucellosis in livestock. They are also the most important causal agent of brucellosis in humans [1]. Infections in animals that are caused by Brucella spp. frequently result in abortions and diminished levels of milk production. From clinical point of view, Brucella abortus is one of the most prevalent Brucella spp [2]. Once the acute period of the disease is over, animals may show little or no disease symptoms. The brucella cells can chronically be located in the

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supramammary lymphatic nodes and the mammary glands of nearly 80% of the infected animals. Thus, in healthy animals, the pathogen continues to hide in the body fluids [3]. People my consume milk in the form of raw milk and cheese; therefore the pathogen could be easily acquired by the consumers. Brucellosis may be transmitted to healthy individuals through the gastrointestinal route. Some occupational factors may play a major role in disease transmission to humans. The role of the infected aerosols in Brucella dissemination has also been well documented [4]. Therefore, there is no doubt that the control of the disease in animals will have an immediate effect on the incidence of brucellosis in humans.

The current diagnosis of brucellosis is based on the serological and the microbiological tests. It is well known that the serological methods are not always sensitive and/or specific [5]. The follow up of the treatment procedures of the chronic or the recurrent cases of brucellosis in some human cases generally relies on the serological findings that have not yet reached satisfactory results [6]. Moreover, they have repeatedly been reported to cross-react to antigens other than those from Brucella spp [7, 8]. Microbiological isolation and identification are the most reliable methods of diagnosis for brucellosis. Those procedures are not always successful; they are cumbersome, and represent a great risk of infection for laboratory technicians. Molecular detection methods have been widely used for Brucella diagnosis in the last decade [9]. PCR technique provides a promising option for the diagnosis of brucellosis. It is a potentially useful method which has been used alone or in combination with the labeled probes for the detection of Brucella species from isolated bacteria [10] or highly contaminated aborted tissues [11]. Different target sequences have been selected for Brucella DNA amplification [12, 13]. The most frequent genes which have been used for replication are omp2, 16s RNA, IS711, utilizing erythritol gene, and protective gene [14]. Because of the high sensitivity and the species specificity of the IS711/alkb based primers, we selected this protocol to evaluate its sufficiency after optimization and testing some clinical samples that we had obtained from clinically ill individuals.

## Methods

Reference Brucella abortus strains including S19, S544 and S103 were kindly donated by the research laboratories at Tarbiat Modares University and Tehran University of Medical Sciences, and they were preserved at -20°C. Different tissue specimens including breast tissue, liver, lymph node, kidney, and uterus of the slaughtered diseased bovine were prepared from the city's slaughterhouse. The clinical samples of the serologically positive patients with brucellosis were prepared from Baqyatollah hospital. The bacterial strains used as negative controls were selected either because of their close relationship with the genus of Brucella or because of their reported cross-reactivity in serological tests. All the samples were first cultured according to the standard bacteriological methods. For Microbiological analysis of human samples, three ml of the dispense blood containing heparin or sodium citrate (as an anticoagulant) was inoculated onto biphasic medium as described elsewhere [14]. The blood cultures were incubated in the vertical position at 37°C. Aliquots were plated onto the standard Brucella agar weekly during a 3-week period. The inoculated plates were incubated at 37°C under aerobic and vellobiotic conditions. The suspected Brucella colonies were identified by conventional biochemical and serological methods.

The DNA extraction was performed by the extraction techniques that were previously described [15]. The PCR components including taq polymerase, dNTPs and PCR reagents were provided from internal sources (Sina gene LTD). Different gram-negative and gram-positive bacteria included S. aureus, group B streptococcus, Klebsiella, E-coli, and Proteus. The Enterobacteria and Pseudomonas were collected from clinical laboratories and were used for specificity tests. Two distinct sequence targets of alkB and IS711 gene were used for

primer designing. The primers designed from those regions of Brucella genome had previously shown good sensitivity and specificity [6]. The 157 fragments were amplified with BAF/BAR that were derived from alkB genetic elements and IS711 gene [9].

BAF) 5'- CCATTGAAGTCTGGCGAGC-3'

BAR) 5'- CGATGCGAGAAAACATTGACCG-3'

All the amplifications were performed in a total volume of µl contained 0.5 µM of each primer, 200 mM dNTPs, 2.5 U taq DNA polymerase, 5 mM $MgCl_2$, 10 X PCR buffered (KCl 500 mM, Tris-HCl 200 mM, and $MgCl_2$ 50 mM). The extract sample (DNA) was first diluted with 20 µl of double distilled water and 2 µl were added to the PCR mixture. Amplification using primers BAF/BAR consisted of an initial denaturation for the PCR profile was set as follows: 1 min of template denaturation at 94°C, 1 min of primer annealing at 54°C and 1 min of primer extension at 72°C for total of 35 cycles [10]. The PCR products were confirmed by RFLP technique using specific endonuclease enzyme.

## Results

The PCR protocol optimization was preformed with the purified extract genomic DNA of standard B. abortus. The agarose gel of the sensitivity and specify tests are shown in figures 1 and 2. The results of PCR, serology test and culture of clinical samples have been compared (table 1). The sequential dilutions of purified genomic DNA from reference B. abortus cultures showed a clear amplification with as little as $10^{-4}$ of template of reaction mixture (figure 1). The results indicated that both the PCR and the culture could diagnose the B. abortus in the clinical specimens of suspected patients. When bovine specimens were examined by the two methods, the PCR technique showed a much higher sensitivity (table 2). The DNA of B. abortus was detected in all the 10 samples but only 5 samples were positive by the standard bacteriological methods. The PCR products of 157 bp were confirmed by RFLP methods (figure 3). The results of the confirmation test by the restriction enzyme analysis indicated that the PCR products were cut in two pieces exactly at the specific site of sequence (124 and 33 bp) as expected.

**Table 1.** Comparison of PCR results of clinical samples with serology and culture.

| Samples | Culture | Serology | | | PCR |
|---|---|---|---|---|---|
| | | Wright | Coombs-Wright | 2ME | |
| 1 | + | + | ND | + | + |
| 2 | + | + | ND | + | + |
| 3 | + | + | ND | + | + |
| 4 | + | + | + | + | + |
| 5 | + | + | ND | + | + |

**ND** = Not Done

**Table 2.** Comparison of PCR results of bovine samples with serology and culture.

| Samples | Results | | |
|---|---|---|---|
| | PCR | Culture | Serology |
| Lymph node | + | - | + |
| Kidney | + | + | + |
| Uterus | + | + | + |
| Breast | + | - | + |
| Placenta | + | + | + |
| Lymph node | + | + | + |
| Liver | + | - | + |
| Kidney | + | + | + |
| Lymph node | + | - | + |
| Liver | + | - | + |


**Figure 1.** Sensitivity test of primers.
Lane 1: $10^{-1}$ purified Brucella genome,
lane 2: $10^{-2}$ purified Brucella genome,
lane 3: $10^{-3}$ purified Brucella genome,
lane 4: DNA ladder, lane 5: $10^{-4}$ purified Brucella genome, lane 6: negative control.

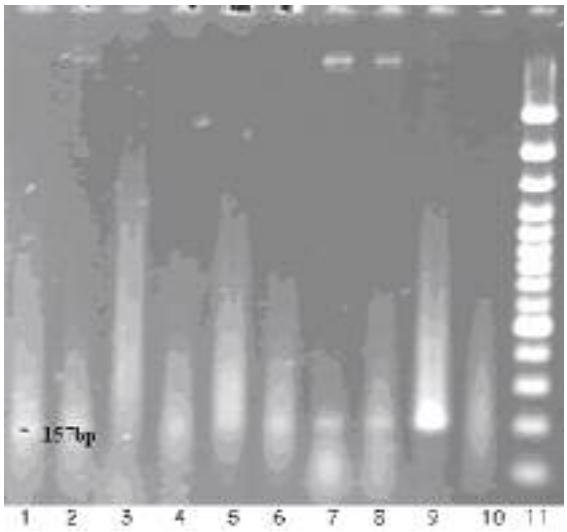


**Figure 2.** Specificity test of primers.
Lane 1: E. coli, lane 2: Pseudomonas,
lane 3: Shigella, lane 4: Proteus,
lane 5: group B Streptococcus,
lane 6: S. aureus, lane 7: Klebsiella,
lane 8: Enterobacter, lane 9: positive control,
lane 10: negative control, lane 11: DNA ladder.

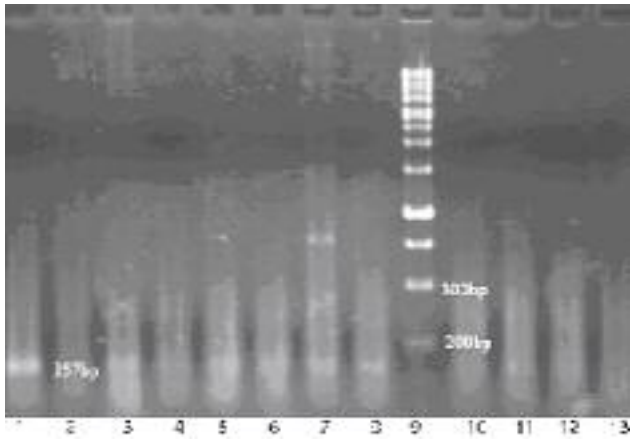


**Figure 3.** Typical electrophoresis profile of amplified B. abortus DNA. Lane 1: positive control, lane 2: lymph node, lane 3: liver,
lane 4: uterus, lane 5: breast, lane 6: uterus,
lane 7: lymph node, lane 8; kidney,
lane 9: DNA ladder, lane 10: liver,
lane 11: lymph node, lane 12: liver,
lane 13: negative control.

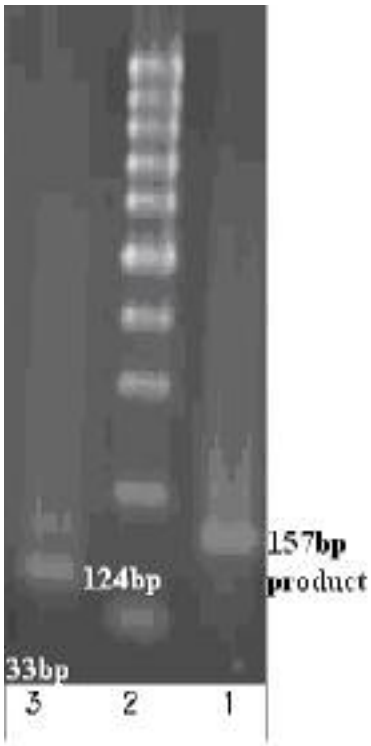


**Figure 4.** Restriction enzyme analysis of PCR products.

## Discussion

Although brucellosis has been largely eradicated from some parts of the world, it is a continuing and apparently increasing problem in many parts of the world (including The Middle East) both through its economic impact on livestock and as a cause of human disease. In Kuwait, where it has been well studied, the annual incidence has risen from 1.2 in 1976 to 69/100,000 in 1985 [16]. The human's Brucellosis is annually being reported worldwide [3]. Six species of Brucella are recognized. The three of them including B. abortus, B. melitensis, and B. suis are the significant causes of zoonotic human diseases. The six Brucella species form a closely related group (with the DNA homologies) were estimated to include more than 90% of this strain, while ribosomal RNA studies suggest that they have no close relatives amongst other pathogenic bacteria [17]. Because of its potential to detect very small numbers of organisms, the polymerase chain reaction has been applied for the diagnosis of a number of infectious agents, particularly viruses and bacteria that had previously caused diagnostic difficulties. The publication by Baily et al on the nucleotide sequences of a B. abortus alkB gene and IS711 as a diagnosis approach, provided the basis for the application of this approach to brucellosis [9]. Different reports indicated that when both the alkB sequence and the IS711 are used for the primer designing, the test sensitivity would be higher compared with the primer sets from each of the sequences alone. This argument is also confirmed by the present data (unpublished observation). Our results indicate that the mentioned primer set have satisfactory sensitivity and specificity. Although most of the PCR protocols and primer optimization for brucella detection have been achieved by purified DNA extracted from the standard strains, we used 15 clinical and veterinary samples for the evaluation of optimized protocol. In order to confirm the identities of the amplified fragments, restriction analysis were carried out on the basis of the restriction map for the alkB gene.

### Acknowledgment

This research was supported by the grant number 344-5/5-312, from Baqyatallah Medical Sciences University.

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