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A PCR method with internal control for detection of Brucella spp. from bovine abortion samples

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SUMMARY

Brucella spp. are important infectious agents in bovine abortions worldwide. The bacteriological culture of *Brucella* spp. is fastidious and time consuming procedure as a classical laboratory method. *Brucella* spp. can be detected by using different molecular techniques. The aim of this study is to develop a PCR technique with an internal control for detection of *Brucella* spp. from bovine abortion samples. For this purpose, the sensitivities of three different primer pairs (BgF/BgR, B4/B5 and JP-R/JP-F) were compared. Bovine 12S gene specific primer pairs (12SM-FW/12SBT-REV2) were used as an internal control. The sensitivity of BgF/BgR primers was found higher than the other primer sets. A PCR assay was developed by combining BgF/BgR primer sets and primers for bovine 12S. This protocol was tested and validated by using abomasal contents of two Brucella-positive and eighteen Brucella-negative clinical samples. In conclusion, the developed PCR method with an internal positive control has a potential for use in direct detection and identification of the *Brucella* spp. from bovine abortion samples.

Keywords: *Brucella* spp., PCR, Internal Control

RESUMÉ

Développement d'une méthode PCR avec contrôle interne pour détection de *Brucella spp.* à partir de prélèvements d'avortements bovins

Brucella spp. est un agent d'avortement important chez les bovins. La culture bactériologique de *Brucella* spp. est fastidieuse, la lecture est tardive et ce, malgré qu'elle soit considérée comme la méthode classique. *Brucella* spp. peut être détecté par l'utilisation de différentes techniques moléculaires. Le but de cette étude est de développer une technique PCR avec un contrôle interne pour la détection de *Brucella* spp. à partir des prélèvements réalisés sur les avortons bovins. Pour cette raison, la sensibilité de trois paires d'amorces (BgF/BgR, B4/B5 and JP-R/JP-F) a été comparée. Le paire d'amorce spécifique pour le gène 12S (12SM-FW/12SBT-REV2) a été utilisé comme un contrôle interne. La sensibilité d'amorces de BgF/BgR s'est révélée plus élevée que celle des autres amorces. Un test PCR a été développé par combinaison d'amorces BgF/BgR avec celles de 12S. Ce protocole a été testé par utilisation de deux prélèvements positifs et dix huit échantillons négatifs de contenu abomasal prélevés sur avortons.
A la lumière de ces résultats, il s'avère que la méthode PCR développée avec un contrôle interne positif peut être utilisée pour la détection et l'identification de *Brucella* spp. à partir de prélèvements d'avortements bovins.

Mots clés : *Brucella* spp., PCR, Contrôle Interne

Introduction

Brucella spp. is one of the common infectious agents of abortion in cows and it has a zoonotic potential (25). Brucellosis is common in many parts of the world, especially in the Mediterranean countries (12). Serological and culture methods are used frequently in the diagnosis of brucellosis. However, these methods have disadvantages such as false-negative results, time-consuming and pathogenic transmission to laboratory employees (19, 26). In addition, culturing of *Brucella* requires a biosafety level-3 (BSL-3) laboratory, which is not readily available in many cases. Because of isolation problems, significance of molecular based detection techniques are increasing. PCR techniques for diagnosis of *Brucella* spp. are used successfully both from different clinical samples and pure cultures (4, 14, 15). In several studies the PCR was compared with culture methods and it was found that PCR was more sensitive than culture (7, 11, 18). However, DNA preparations may affect sensitivity of analyses and it is not uncommon to encounter with false negative results due to inhibitory substances in clinical materials (11). Besides, host DNA can interfere with PCR detection of *Brucella* DNA. Therefore, presumptive negative samples for Brucella should be confirmed by comparison to the internal DNA controls (e.g., host DNA specific primers or by adding known amount *Brucella* DNA) (20, 22). It is also advisable that the clinical samples may be diluted before use as template in PCR for decreasing the inhibitory substances that may be present (27). However, dilution process would also inadvertently decrease the amount of target organisms. Therefore, the use of optimal parameters including the primer sets is critical for the performance of PCR with high sensitivity and specificity. Additionally, the European Standardization Committee proposed the usage of internal amplification control in PCR (2).

The aim of this study was to develop and to validate a PCR protocol with internal control for the detection of *Brucella* species from bovine abortion samples.

Material and Methods

For the detection of *Brucella* spp. three different primer pairs were used. Reference *Brucella (B.) abortus* S19 strain

was used as positive control. Also, a set of primer pair was used as internal control to detect bovine 12S gene (Table I). A total of 20 bovine aborted foetus abomasal contents were used as clinical samples for validating the developed technique. These samples were collected from the naturally infected cases in different farms in Hatay Province, Turkey. For using as template in the PCR assays, nucleic acids were isolated one milliliter of from each sample by phenol-chloroform extraction method (21).

Bacterial DNA was extracted from control organism and DNA concentration was measured to 30 ng/µl (Nano drop Spectrophotometer, ND 1000, USA) to be used as the template in PCR. Then, 10-fold serial dilutions of stock DNA solution was prepared and used as template from 3 ng/µl to 30 f/µl concentrations (21). Sensitivities of primer pairs for *Brucella* spp. were compared. Individual PCR assays with each pair of primer were performed according to the references as shown in Table I. The most sensitive Brucella primer pair was then combined with the bovine specific primer pair for developing the PCR assay with an internal control for detection of *Brucella* spp. directly from abomasal contents of aborted foetus as clinical samples. Extracted DNA from clinical samples was used to try developed technique. The multiplex PCR assay was performed according to the recommendations of Henegariu et al. (10).

The PCR amplification mixture was carried out in a final volume of 25 µl. The mixture was consisted of 2 µl of extracted DNA template, 1,5 U of *Taq* DNA polymerase (Vivantis Technologies), 2,5 µl of 10x PCR buffer (10X ViBuffer A, without $MgCl_2$), 3 mM $MgCl_2$, 200 µM each of dNTPs (Vivantis Technologies), and 20 pmole of each primer.

After an initial denaturation at 95 °C for 3 min, the PCR protocol was: 60 s of template denaturation at 94 °C, 60 s of primer annealing at 54 °C, and 90 s of primer extension at 72 °C (total of 35 cycles), with a final extension at 72 °C for 5 min. The samples were analyzed by electrophoresis in a 1,5% agarose gel, stained with ethidium bromide (0.5 mg/mL), and DNA bands were visualized under UV light.

Results

Three *Brucella*-specific primer sets (BgF/BgR, B4/B5 and JP-R/JP-F) were first evaluated for detection of pure *Brucella* genomic DNA in PCR; the highest sensitivity was achieved with the BgR/BgF primer pair (300 fg was the lowest concentration of genomic DNA that gave a positive reaction). It was followed by B4/B5 primer set (detection limit was 3 pg DNA), and the lowest sensitivity was found with JP-F/JP-R primers (detection limit, 30 pg DNA). While BgR/BgF primers (208bp amplification product) and B4/B5 primers (223 bp amplification product) didn't amplify any nonspecific products, in PCR with JP-F/JP-R primers (193 bp amplification product) nonspecific amplification products were seen between 800 and 900 bp. Specific bands for the primer sets are shown in Fig 1.

PCR	Primer name	Sequences of primers	Length of amplicon	Reference
Set 1	B4	5'- TGGCTCGGTTGCCAATATCAA- 3'	223bp	4
	B5	5'- CGCGCTTGCCTTTCAGGTCTG -3'		
Set 2	JP-R	5'- ACC AGC CAT TGC GGT CGG TA -3'	193bp	15
	JP-F	5'- GCG CTC AGG CTG CCG ACG CAA -3'		
Set 3	BgF	5'-CAATCTCGGAACTGGCCATCTCGAACGGTAT-3'	208bp	14
	BgR	5'-ATGTTATAGATGAGGTCGTCCGGCTGCTTGG-3'		
Internal control	12SM-FW	5'-CTAGAGGAGCCTGTTCTATAATCGATAA-3'	346bp	16
	12SBT-REV2	5'-AAATAGGGTTAGATGCACTGAATCCAT-3'		

Table I: The properties of primer pairs used in this study.

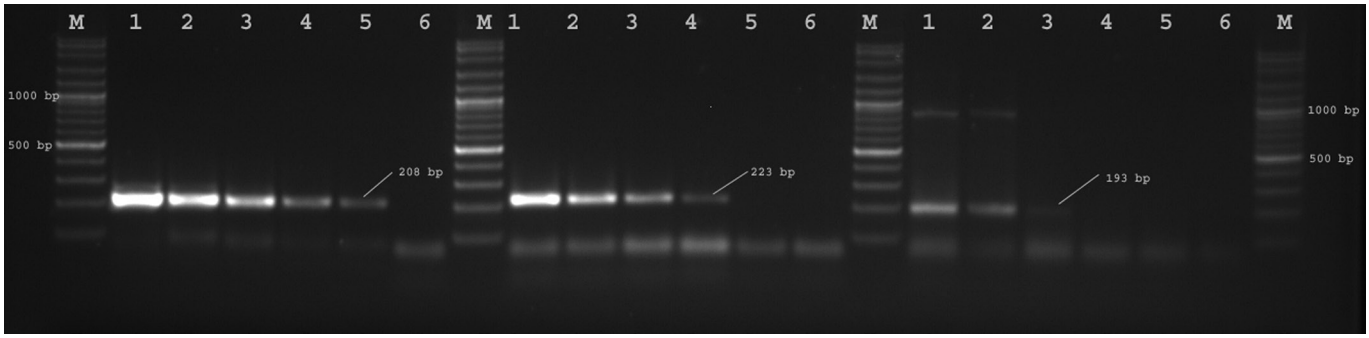

Figure 1: Amplification products of individual PCR assay with primers for *Brucella* spp. as tested on pure genomic DNA. Lane M:VC 100bp Plus DNA Ladder (Vivantis Technologies), Lanes 1-6:Products of BgF/BgR primers (208 bp), Lanes 7-12: Products of JP-R/JP-F primers (193 bp), Lines 13-18: Products of B4 /B5 primers (223 bp).

In aborted fetal samples, cattle 12S rRNA gene was detected and cattle specimens were verified by using specific primers (Fig 2).

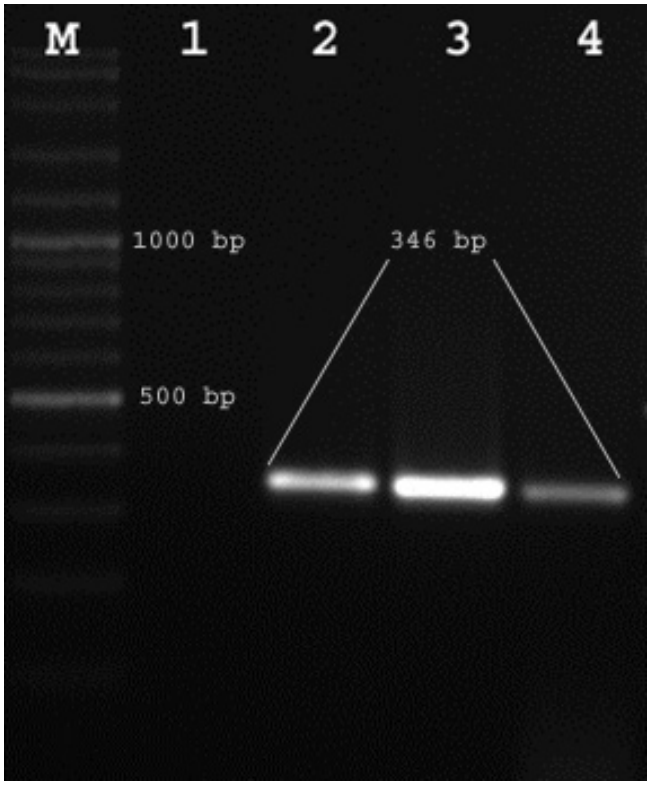

Figure 2: The amplification products of 12SM-FW and 12SBT-REV2 primers specific for Bovine 12S gene. Lane M: VC 100bp Plus DNA Ladder (Vivantis Technologies).Lane 1: Negative control, Lanes 2-4: PCR positive abomasal content samples (346 bp).

In the PCR analyses of clinical specimens, two (2) of all samples (20) were found positive for Brucella with BgR/BgF primers (208bp amplification product). Also, the developed PCR assay has been tested on clinical samples that were known to be positive (n=2) and negative (n=18) fetal bovine samples for *Brucella* spp. Specific amplification products for Brucella spp. (208 bp) and host specific internal control (346 bp) of developed PCR assay with internal control were (multiplex PCR) shown in Fig 3.

Discussion

Brucella spp. is one of the most important infectious abortion agents and causes economic losses in cattle farming. Due to the disadvantages of culture and serological methods, use of molecular methods is increasing in practice day by day. In this study, three different primer pairs were compared for diagnosis of *Brucella* spp. The most sensitive pair was combined with bovine specific primer pair and a PCR assay was developed for *Brucella* spp. with internal control. The developed technique was validated by using positive and negative clinical samples.

There are different reports about sensitivity of primers for PCR detection of *Brucella* spp. Navarro et al. (20), reported that B4/B5 primers were more sensitive than JP-R/JP-F primers, and they also showed that human genomic DNA decreased the PCR efficiency in *Brucella* spp. detection. Similarly, Mitka et al. (18) and Baddour and Alkhalifa (3) reported similar result in human blood samples. Also, Ghodasara et al. (6) indicated that the most sensitive primer pair was B4/B5 primers in PCR detection of *Brucella* spp. in animal clinical samples.

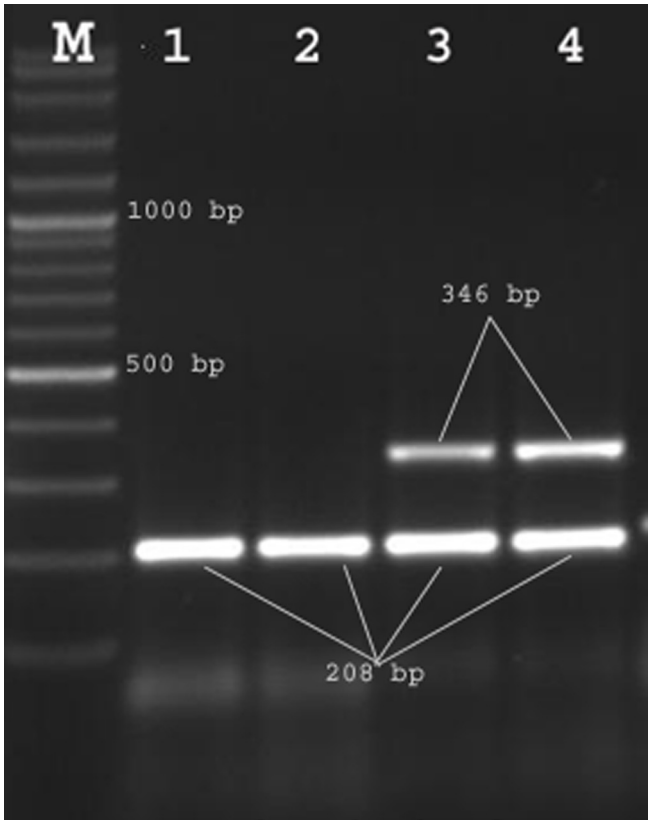

Figure 3: Simplex and multiplex PCR assay amplification product for Brucella spp.in the positive clinical specimens. Lane M:VC 100bp Plus DNA Ladder (Firma), Lanes 1-2: Products of simplex PCR for Brucella spp. with BgF/BgR primers (208 bp), Lanes 3-4: Products of duplex PCR for Brucella spp. with BgF/BgR primers (208 bp) and for bovine gene 12SM-FW and 12SBT-REV2 primers (346 bp) .

In this study, BGF/BGR primer pair, reported by Kumar et al. (14), was found the most sensitive primers for detection of *Brucella* species. In a previous report, B4/B5 primer pair was found more sensitive than JPF/JPR primer pair, similar to our findings in this study. Moreover, BGF/BGR primer pair which was found to be the most sensitive primers for *Brucella* spp. was combined with the bovine-specific primer pair 12SM-FW and 12SBT-REV2. This multiples PCR protocol has been tested with the clinical samples, and the result were the same as the simplex PCR results.

The use of the internal amplification control is effective for the detection of the false negative results caused by inhibitors or mistakes in the various stages of PCR analysis. There are many strategies for usage of internal control. For this reason, a bacterial (17) or viral (23) DNA can be added to the mix which doesn't exist naturally in specimen, or synthetic DNA or plasmids (1,8) can be added to the PCR mix. Using host

DNA specific primers are also useful for checking the PCR procedure failures from sampling stage to amplification (9).

Abortions cause significant economic losses in cattle farming. The economic losses in an abortion case is $700 in USA (24), 480 $ in UK (13) and 538 $ in Turkey (5). Accurate, reliable and rapid diagnosis of the causative agent is important for prevention and helps reduce the economic losses. In this study, *Brucella* specific primers combined with bovine DNA specific primers effectively. As conclusion, the developed PCR method with internal positive control has a potential use for the detection and identification of *Brucella* spp. directly from cattle abortion cases such as aborted foetuses, milk and vaginal swap samples. For further studies, applying this method with ruminant gene specific primers can increase its further use.

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