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Original Article

Development of New Modified Simple Polymerase Chain Reaction and Real-time Polymerase Chain Reaction for the Identification of Iranian *Brucella abortus* Strains

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ABSTRACT

Brucellosis is primarily a worldwide zoonotic disease caused by *Brucella* species. The genus *Brucella* contains highly infectious species that are classified as biological threat agents. In this regard, the identification of *Brucella* can be a time-consuming and labor-intensive process posing a real risk of laboratory-acquired infection to the laboratory staff. This study aimed to present a novel conventional and real-time polymerase chain reaction (PCR) assay for the identification of *Brucella abortus* strains. Regarding this, two primers (bru ab2) were designed based on the unique loci encoding autotransporter-associated beta strand repeat-containing protein (ID:YP00113760). A total of 56 *Brucella* strains (e.g., reference, vaccinal, and field isolates) and *Yersinia enterocolitica*, as a non-*Brucella* isolate, were evaluated in conventional and real-time PCR systems. The results of the study indicated that 0.4 ng and 400 FG of genomic DNA of *B. abortus* strains can be detected by conventional and real-time PCR, respectively. The primers, bru ab2, were suitable for both PCR methods. Both methods were specific for the detection of all strains of the bacterium; however, real-time PCR assay was 1000-fold more sensitive than the conventional PCR method. Therefore, this new detection system could be a suitable selective modified method for the accurate identification of all *B. abortus* strains.

Keywords: *Brucella abortus*, Identification, PCR, Real-time PCR

Développement d'une Nouvelle Réaction en Chaîne de Polymérase Simple Modifiée et d'une Réaction en Chaîne de Polymérase en Temps Réel pour l'identification des Souches Iraniennes de *Brucella abortus*

Résumé: La brucellose est principalement une maladie zoonotique dans le monde entier causée par l'espèce *Brucella.* Le genre *Brucella* contient des espèces hautement infectieuses classées comme agents de menace biologique. À cet égard, l'identification de *Brucella* peut être un processus long et exigeant en main-d'œuvre, présentant un risque réel d'infection acquise en laboratoire pour le personnel de laboratoire. Cette étude visait à présenter un nouveau test de réaction en chaîne par polymérase (PCR) classique et en temps réel pour l'identification des souches de *Brucella abortus*. À cet égard, deux amorces (bru ab2) ont été conçues sur la base des locus uniques codant pour une protéine contenant une répétition de brin bêta associée à un autotransporteur (ID: YP00113760). Un total de 56 souches de Brucella (par exemple, des isolats de référence, vaccinal et de terrain) et Yersinia enterocolitica, en tant qu'isolât non-Brucella, ont été évalués dans des systèmes de PCR conventionnels et en temps réel. Les résultats de l'étude ont indiqué que l'ADN génomique des souches de *B. abortus* à 0,4 ng et 400 FG peut être détecté par PCR conventionnelle et en temps réel, respectivement. Les amorces, bru ab2, convenaient aux deux méthodes de PCR. Les deux méthodes étaient spécifiques à la détection de toutes les souches de la bactérie; cependant, le test de PCR en temps réel était 1000 fois plus sensible que le

modifiée sélective et appropriée pour l'identification précise de toutes les souches de *B. abortus*.
Mots-clés: *Brucella abortus*, Identification, PCR, PCR en temps réel

INTRODUCTION

Brucella is a Gram-negative, intracellular, and non-motile bacterium causing brucellosis, one of the most important zoonotic diseases threatening public health by infecting many organs and soft tissues, including mammary glands. This infection frequently results in abortion, low milk production, and fetal mortality in animals (Davis and Troy, 2005; Troy et al., 2005; Tiller et al., 2010). The *Brucella* genus has six classical species, including *B. abortus* (cattle), *B. suis* (pigs), *B. melitensis* (goats and sheep), *B. canis* (dogs), *B. ovis* (rams), and *B. neotomae* (desert rats). In addition, four newly described species are *B. ceti*, *B. pinnipedialis* (marine mammals), *B. microti* (common vole), and *B. inopinata* (associated with a human infection and recognized as the newest member) (Ficht, 2010). Although many efforts have been made to control this disease in many countries, there still remain regions where the infection persists in domestic animals and may consequently be transmitted to humans (Dean et al., 2012). *Brucella abortus*, *B. melitensis*, *B. suis*, *B. canis*, and strains isolated from marine mammals are the species causing human brucellosis (Sohn et al., 2003; Ullah et al., 2014). The disease is usually transmitted from infected animals to humans through direct contact with animal or the consumption of their products, mostly dairy products (Pappas et al., 2005). Brucellosis in humans is characterized by undulant fever, malaise, and myalgia in acute phase, as well as late adverse effects on various organs and tissues as a chronic disease. In animals, the disease may cause genital infections, abortion, and fetal mortality (Probert et al., 2004). In Iran, brucellosis is an endemic disease, and the most important species of *Brucella* are *B. melitensis* and *B. abortus* (Najafi et al., 2011). The laboratory detection of Brucella is largely based on bacteriological methods, such as the culture (e.g., blood, milk, and genital excretions) (Molavi et al., 2014). On the other hand, serological methods are indirect laboratory methods applied for the diagnosis of human and animal brucellosis, based on immune response to antigenic epitopes, such as lipopolysaccharide (Adone and Pasquali, 2013). These processes are time-consuming, and cross-reactions may decrease sensitivity and specificity (O'Grady et al., 2014). Moreover, they are accompanied by a real risk of laboratory-acquired infections (Probert et al., 2004). The determination and biotyping of *Brucella* need laboratory level 3, and are mostly performed in reference laboratory (Smirnova et al., 2013). In addition, some of *Brucella* species have subspecies (biovar), for example *B. abortus* (biovar 7), *B. melitensis* (biovar 3), and *B. suis* (biovar 5). Molecular methods, such as PCR, are helpful techniques for the identification and typing of *Brucella*. Conventional and real-time PCR as the common methods in this field are able to detect few amount of genomic DNA. *Brucella* species display a high degree of DNA homology based on DNA-DNA hybridization studies (T et al., 2012). Therefore, the molecular discrimination of all biovars of a species from other species requires the identification of unique loci in genome. Several genomic targets, such as *glk*, *bcsp31*, *per*, *IS711, alkB/IS711,* and *BMEI1162/IS711*, have been used for the identification of *Brucella* at genus, species, and biovars levels (Gopaul et al., 2014). Therefore, this research aimed to investigate a suitable conserve genomic position as a unique and similar region among all biovars of *B. abortus* for the discrimination of the bacterium (all biotypes) from other *Brucella* species by conventional and real-time PCR.

MATERIAL AND METHODS

Bacterial strains and DNA extraction. Totally, 56 *Brucella* strains, consisting of *B. abortus*, *B. melitensis*, *B. suis*, *B. neotoma*, reference, vaccinal, and field strains, were prepared. Thereafter, field strains were collected from the infected cows in provinces with high infection prevalence in Iran. Milk samples were collected and cultured in *Brucella* Agar containing modified selective supplements for primary isolation. Subsequently, the samples were incubated at 37 ºC for 5 days. Biovars of *Brucella* colonies were confirmed by CO_2 requirement, dye sensitivity in thionin, basic fuchsin, growth characteristics in streptomycin, lysis with Tbilisi phage, and agglutination with monospecific A and M antisera. Yersinia enterocolitica 0:9 was used as non-*Brucella* strain (Table 1). Pure *Brucella* strains were diluted in 200 µl phosphate buffered saline. High Pure PCR Template Preparation Kit was used for DNA extraction (Roche cat. no. 11796828001). Concentration of extracted DNA was determined using the Nano Drop spectrophotometer (Nano-Drop ND-1000. Wilmington, DE 19810 USA).

Phage Typing. The fields and reference isolates of *Brucella* were grouped into species and biovars as the following by phage typing as a golden standard test (Filippov et al., 2013):

1. Biovar 1 of abortus 544 as standard biovar and 20 field isolated samples of *Brucella abortus*

2. Biovar 1 of melitensis 16M as standard biovar and 30 field isolated samples *of Brucella melitensis*

Primer designing. Genomic comparison among *Brucella* species and biovars was performed to achieve a suitable specific primer. Bioinformatics evaluation of genomic polymorphism and determination of the specificity of the primers were compared with the data of the GenBank database using the Basic Local Alignment Search Tool. Primers were designed using Oligo software (version 5). The sequences of Ab2 forward and reverse were TGAGCGGTGACAGCCAA GG and GCCAGAACCCAGCTTCAGA, respectively.

DNA amplification by conventional polymerase chain reaction. The master mixture prepared for DNA amplification by conventional PCR contained 1.5 U of *Taq* DNA polymerase (Promega Biotec), 10 pmol of each primer, 40 ng template DNA, 1 mM $MgCl_2$, and 2.5 mM dNTP in a volume of 25 µl. The amplification was performed in the Bio-Rad My Cycler Thermal cycler. The thermal program was included initial denaturation for 4 min at 95 °C, denaturation for 30 sec at 94 °C, annealing for 45 sec at 55 °C, extension for 1 min at 72 °C, and final extension for 10 min at 72 °C. Thereafter, the gradient PCR method was used to find optimum annealing temperature at 55-65 °C. Furthermore, two different concentrations of *Taq* DNA polymerase (0.5 U and 1 U) were utilized in each reaction. Subsequently, Agarose gel (1.8%) was prepared and electrophoresed. Eventually, post staining was performed by ethidium bromide to visualize PCR products on ultraviolet illumination.

DNA amplification by real-time polymerase chain reaction. Each reaction mixture in real-time PCR contained 10 µl 2X buffer, 5 pmol of each primer, and 4 ng template DNA in a total volume of 20 µl. Amplification was performed in applied biosystem 7500 Real-time PCR. Thermal condition entailed the activation of polymerase (95 °C for 2 min), followed by 40 cycles of 15 sec at 95 °C, 1 min at 55 °C, and 1 min at 65 °C. Double-stranded PCR product fragment was measured at 6 °C. In the optimization stage, a thermal range of 65-68 °C for 40 and 50 sec was used in the extension step. In addition, two-step thermocycling was examined for 15 sec at 95 °C and 40 sec at 68 °C. The data were collected during the detection of fluorescence signal affected by the binding of SYBR green to amplicon. Agarose gel (1.8%) was used and electrophoresed to confirm the real-time fluorescence data.

Specificity and Sensitivity. Specificity was determined by using several *B. abortus* strains and biovars, as well as other *Brucella* species and non-*Brucella* strains (Table 1). To discern the sensitivity,

Table 1. Species and biovars used in the study

Species and biovar	Strain	Number	Source	Geography position
B. abortus 1	544	1	Reference	Weybridge- England
B. abortus 1	S99	1	Reference	Weybridge- England
B. abortus 1	S19	1	Vaccinal	RVSRI – IRAN
B. abortus 1	RB 51	1	Vaccinal	RVSRI – IRAN
B. abortus 2	-	1	Field isolate	IRAN
B. abortus 3	-	15	Field isolate	IRAN
B. abortus 5	-	1	Field isolate	IRAN
B. abortus 7(9)	-	1	Field isolate	IRAN
B. melitensis	16 M	1	Reference Field isolate	Weybridge- England
B. melitensis	-	30		IRAN
B. suis	1330	1	Reference	Weybridge- England
B. neotoma	5k33	1	Reference	Weybridge- England
Yersinia enterocolitica	O:9	1	Field isolate	IRAN

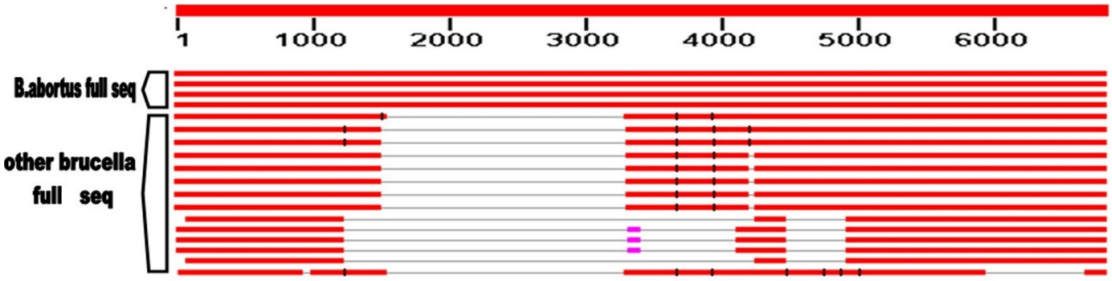

Figure 1. Genomic comparison of 18 complete genomes of Brucella species

well-purified *B. abortus* 544 genome, as a standard strain, was diluted in 10-fold serial dilutions, and each dilution was used as template DNA in conventional and real-time PCR. The lowest amount of detectable DNA was considered as the limit of detection.

RESULTS

Bioinformatics evaluation of single nucleotide polymorphism (i.e., deletion and insertion of genome) was not helpful to cover all biovars of *B. abortus*. Therefore, a specific genomic region was found in chromosome 2 GI: 376270715. In this sequence, position genomic deletion was performed in all *Brucella* species, except for *B. abortus*, which can be used for the identification of *B. abortus* among other species (Figure 1). This deletion was carried out in genomic position encoding autotransporter-associated beta strand repeat-containing protein ID: YP 005113760. The results indicated a distinct, sharp, and specific band in the position of 215 bp without any non-specific bands. Gradient PCR method indicated that the optimum annealing temperature was 58 °C (Figure 2).

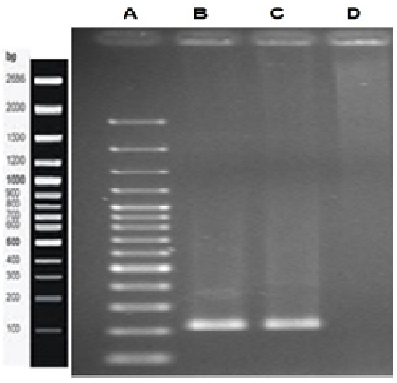

Figure 2. Conventional polymerase chain reaction product (215bp) of Brucella abortus 544 (genomic10-fold serial dilutions) visualized in agarose gel 2%; A) 100 bp DNA ladder, B) 4 ng DNA template, C) 0.4 ng DNA template, D) 0.04 ng DNA template.

Within the three steps of real-time PCR, there was no non-specific melting curve. In addition, no specific

reliable melting curve was observed at 85±0.03 with denaturation for 15 sec at 95 °C and annealing-extension at 68 °C in the two-step thermocycling (Figure 3).

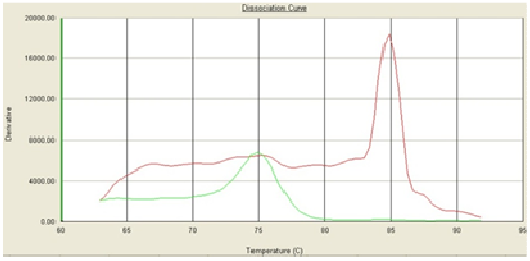

Figure 3. Melting curve of *Brucella abortus* 544.

Therefore, in real-time PCR assay, all *Brucella* strains had distinct melting curve at 85±0.03 °C. Figure 3 displays the amplicon-specific signals with a melting temperature of 85±0.05 °C and other *Brucella* and non-*Brucella* strains without any fluorescent signals. Conventional PCR was able to detect 0.4 ng genomic DNA; however, this amount was 400 FG in real-time PCR (Figure 4).

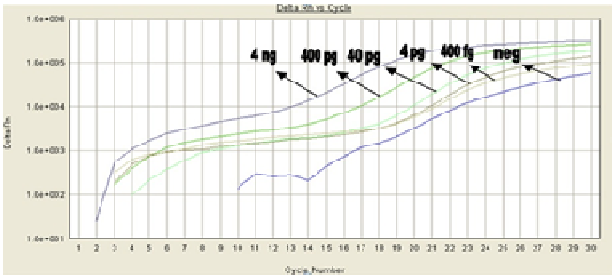

Figure 4. Real-time polymerase chain reaction amplification curves of Brucella abortus 544 (genomic10-fold serial dilutions).

Sensitivity and specificity of *bru ab*2 primers were evaluated in several *B. abortus* strains, involving standard, vaccinal, antigenic, and field isolates, as well as in non-*B. abortus* strain, including other *Brucella* species and non-*Brucella* strain. All strains and biovars of *B. abortus* were detected by conventional PCR assays based on the presence of 215 bp fragment that was the same in all strains of *B. abortus* without any cross-reaction with other examined species (Figure 5).

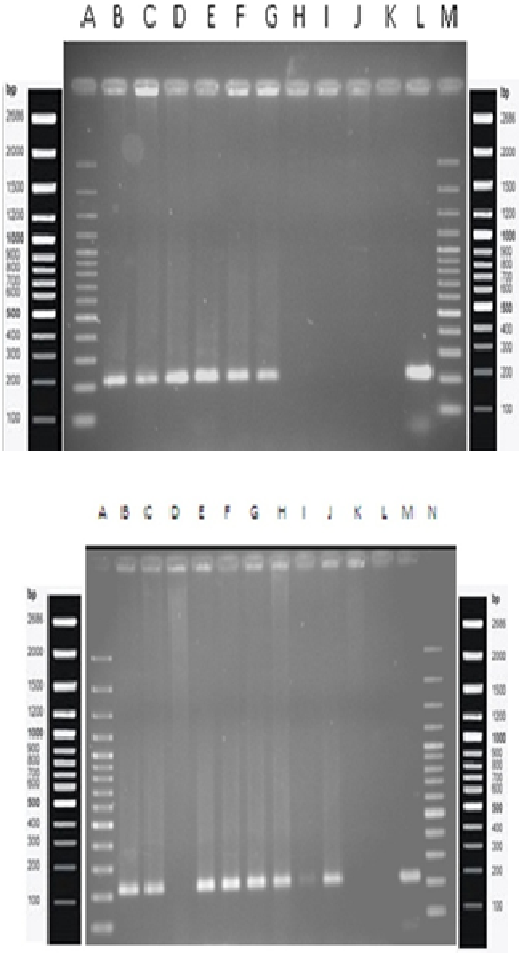

Figure 5. Polymerase chain reaction product (215 bp) visualized by ethidium bromide staining on a 1.8% agarose gel; **Above**: **A**) 100 bp DNA ladder, **B**) *B. abortus* biovar (bv) 1, **C**) *B. abortus* bv 2, D) *B. abortus* bv 3, **E**) *B. abortus* bv 4 (field strain), **F**) *B. abortus* bv 5, **G**) *B. abortus* bv 7-9, **H**) *B. melitensis* 16M, **I**) *B. suis* 1330, J: *B. neotoma* 5k33, **K**) Yersinia enterocolitica O:9, **L**) *B. abortus* 544, **M**) 100 bp DNA ladder; **Below: A**) 100 bp DNA ladder, **B**) *B. abortus* S99, **C**) *B. abortus* Rb51, **D**) *B. melitensis* 16M, **E**) *B. abortus* S19, **F, G, H, I, and J**) *B. abortus* bv 3 (field strains), **K**) *B. suis* 1330, **L**) *B. neotoma* 5k33, **M**) *B. abortus* 544, **N**) 100 bp DNA ladder

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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