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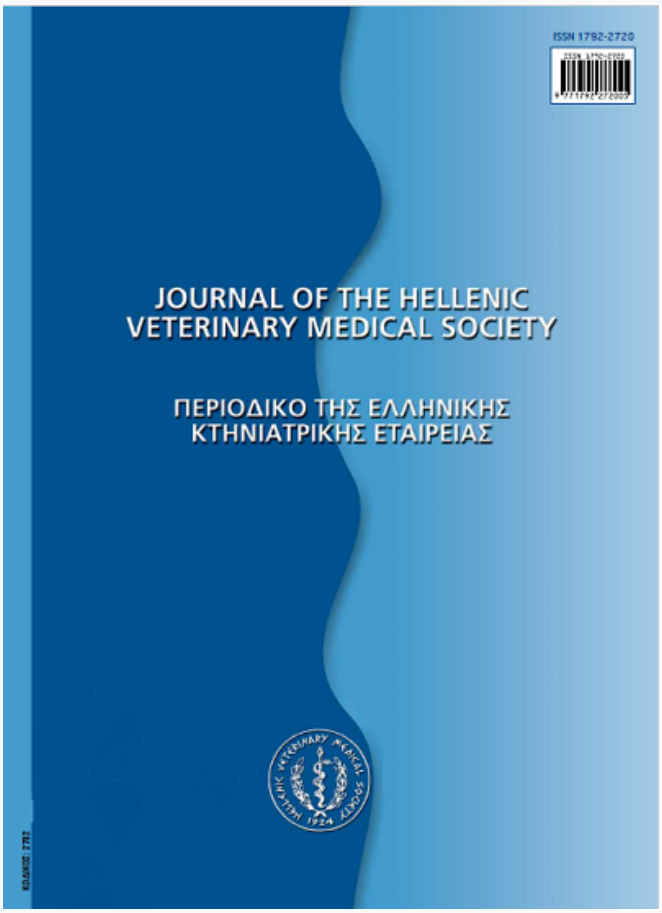

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Site-directed mutagenesis in *Brucella abortus* S19 by overlap extension PCR-based procedure

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ABSTRACT. Introduction of a site-directed mutation can be effective method to evaluate properties of various genes. Brucellosis is one of the most common zoonotic infectious diseases, which causes great economic losses. Thus, determination of pathogenicity factors in the genus *Brucella* can lead to the control of this health problem. Due to the importance of site-directed mutations in identification of genomic structure, overlap extension polymerase chain reaction (PCR) has been introduced as an improved technique for the removal and replacement of gene targets. In this study, three DNA fragments were amplified and combined using a two-step PCR with specific primers. The resulting fusion PCR product, obtained without any change in the nucleotide sequence, was cloned in a specific position in the pBluescript II SK (-) plasmid using restriction enzymes. Finally, the construct was transferred into cells of *Brucella abortus* S19 by electroporation and replaced the target gene (*wbkA*) in the genome of the bacterium. PCR analysis was performed on kanamycin-resistant colonies to provide genetic evidence that the *B. abortus wbkA* gene was interrupted by the kanamycin cassette. The results of this study show that the optimized modified technique, splicing by overlap extension PCR, is effective in creating mutations in the bacterial genome and can easily be used in *Brucella* spp.

Keywords: *Brucella abortus* S19, Mutation, Overlap extension polymerase chain reaction

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INTRODUCTION

Gram-negative coccobacilli belonging to the genus *Brucella* are facultative intracellular bacteria infecting many species of animals and occasionally humans (Poester et al., 2013). The deletion of virulence genes is one of the ways to reduce their virulence. Site-directed mutagenesis with in a bacterial chromosome is a preferred method for studying a particular region of DNA (Datsenko and Wanner, 2000; Kahl-McDonagh and Ficht, 2006; Soler-Lloréns, 2014). Mutagenesis by the overlap extension polymerase chain reaction (OE-PCR) has become a standard method for creating mutations, including specific point mutations, insertions, or deletions within a particular DNA sequence of interest (Horton et al., 1990; Chou et al., 2004). Initial PCR generates overlapping gene segments, which are used as templates for another round of PCR to create a full-length product. Adjacent primers generate overlapping, complementary 3′ ends on the intermediate segments and introduce nucleotide substitutions, insertions, or deletions for site-directed mutagenesis or for gene splicing by changing nucleotides found at the junction of adjoining gene segments (Horton et al., 1990; Horton 1995). The overlapping strands of these intermediate products hybridize at the 3′ region in a subsequent PCR and are extended to generate a full-length product amplified using flanking primers that include restriction enzyme sites for inserting the product into an expression vector for cloning purposes (Bryksin et al., 2010).

In *B. abortus*, a *wbkA*-homologous gene was predicted to be absolutely required for the O-side chain production. The WbkA protein can interact with WboA to elongate the *Brucella* LPS O-sidechain by α-1,2 and α-1,3 links (Monreal et al., 2003). According to one of the known problem of *B. abortus* S19, the presence of smooth LPS interferes with the discrimination between infected and vaccinated animals, disruption of the gene in *B. abortus* S19 can be useful approach to obtain an unable strain to induce antibodies that interfere with the diagnosis (Ugalde et al., 2003).

In this study, we applied overlapping extension PCR strategy to insert linear DNA fragments into a specific location in the bacterial genome. PCR products were designed to carry insertion sequences flanked by 500 base pairs (bp) of homology to the target site on the chromosome. This application was used to disrupt the gene (*wbkA*) encoding a glycosyltransferase in *Brucella abortus* S19.

MATERIALS AND METHODS

Primary PCR. The primers used for PCR are listed in Table 1. The upstream and downstream segments of the *wbkA* gene were amplified from *B.abortus* S19 genomic DNA using primers 1 and 2 and 3 and 4, respectively. Primers 5 and 6 were designed to amplify the kanamycin resistance (KanR) cassette from the pET-28 vector in parallel PCR reaction. All primers were designed using Primer Express software v.3.0 (Applied Biosystems), (Takapozist, Iran). PCR was conducted in a 50 μL volume containing 10 ng of total genomic DNA or 0.1 ng of plasmid DNA, 1.5 U of *Pfu* polymerase (Fermentas,

Table 1. Sequences of the primers. The underlined segments in primers 2 and 3 are complementary to KanR cassette of the pET-28 vector. The underlined segments in primers 1 and 4 are XhoI and XbaI cleavage sites, respectively.

Primers	Sequence	Restriction Enzyme
1. Up – F	5 ‘ - CGCTCGAGAATCGACTGGAGGCTGTACAAG 3‘	*Xho I*
2. Up – R	5 ‘ - GTTTCCCGTTGAATATGGCTCATTCCTTCTATGAAGCTAATTGTTTGATC3‘	
3. Down – F	5 ‘ - CATTTGATGCTCGATGAGTTTTTCTAAATTACTTGCGAATATCGGTC3‘	
4. Down – R	5 ‘ - GCTCTAGACTCCTTTGTAATCGCTCATCTG 3‘	*Xba I*
5. Kan – F	5 ‘ - ATGAGCCATATTCAACGGGAAAC 3‘	
6. Kan – R	5 ‘ -TTAGAAAAACTCATCGAGCATCAAATG 3‘	

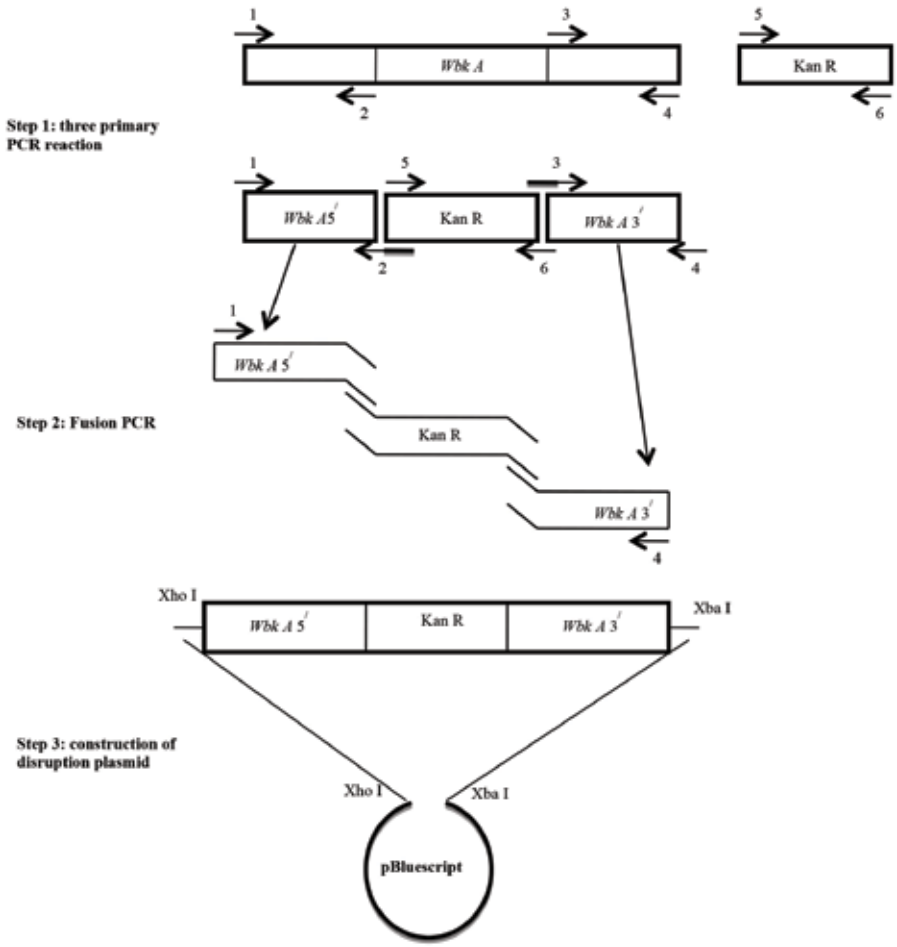
Latvia), 5 µL of 10x *Pfu* polymerase buffer, 10 mM deoxynucleotide triphosphates, and 0.5 µM each primer. A DNA engine thermal cycler (Techne, UK) was used with the following amplification conditions: 95 °C for 4 minutes; 35 cycles of 95 °C for 1 min, 55 °C, 53 °C or 52 °C for 1 min for the upstream, downstream or KanR cassette reaction, respectively, and 72 °C for 2 min; and 72 °C for 10 min for final extension. PCR products were visualized on a 1% agarose gel containing Tris borate EDTA buffer, and the linear DNA was detected using an ultraviolet transilluminator. Each PCR product was purified by the Gel Extraction Kit (MBST, Iran).

Fusion PCR. According to Figure 1, the upstream and downstream segments of the *wbkA* gene amplification primers (2 and 3) have 5´- tails complementary to the KanR cassette. Equimolar (1 ng) concentrations of the three segments amplified in the primary PCR reactions and primers 1 and 4 were used for fusion PCR. The PCR conditions were the same as in the primary PCR, except that the annealing temperature was increased to 60 °C and the extension time was changed to 3 min. PCR products were purified as in the previous step.

Cloning. The purified fusion PCR product and the pBlueScript vector were digested with the *XbaI* and *XhoI* restriction enzymes (Fermentas) at 15 °C for 16 h. Ligation was performed using T4 DNA ligase (Fermentas) at 22 °C for 90 min.

Escherichia coli DH5α competent cells were used for transformation with pBlueScript containing the

Figure 1. Schematic illustration of OE-PCR and construction of the gene disruption pBlueScript plasmid.



insert. Transformed bacteria were cultured on Luria Bertani agar containing 50 μg/mL of ampicillin, isopropyl ß-D-1-thiogalactopyranoside (IPTG, 0.1 M), and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal, 20 mg/mL), and selected colonies were analyzed for the presence of the construct by restriction enzyme digestion. Sequencing of the constructed plasmid was performed with T3 promoter specific and M13 forward and reverse primers (Gene Fanavaran, Iran).

Electroporation. Electroporation was used to introduce the construct into *B. abortus* S19. *Brucella* cells were grown for 20 h in Trypticase soy broth (TSB) at 37 °C with vigorous shaking, then pelleted, and washed three times with an equal volume of sterile cold water. The cells were suspended in 80 μL of cold 10% glycerol. In cuvettes, 10 μg of plasmid DNA was added to 40 μL of the electrocompetent bacteria, which were then electroporated in a BioRad gene Pulser at 2.5 kV, 5 ms and 25 μF. One milliliter of TSB was added and the mix was incubated at 37 ◦C with shaking for 6 h. The electroporation mix was then plated on *Brucella* agar (BA) containing 30 μg/mL of kanamycin. The plates were incubated at 37 ◦C for four days (Miyoshi et al., 2007).

RESULTS

Splicing by overlap extension PCR. OE-PCR was initially employed for fusion of three DNA fragments. The six PCR primers (1 and 2, 3 and 4 and 5 and 6) were used in the first round of PCR to amplify three fragments of 512, 531, and 816 bp, respectively. The primers were designed so that flanking primers 1 and 4 had unique restriction sites for *XhoI* and *XbaI*, respectively. In order to avoid unwanted incorporations of new mutations, *Pfu* polymerase was used as the PCR enzyme instead of *Taq* DNA polymerase. In the fusion PCR, the three fragments were allowed to join to generate a full length (1,846 bp) fragment using primers 1 and 4 (Figure 2A).

Cloning, screening and sequencing. The resulting cassette was introduced into the pBlueScript vector using *XhoI* and *XbaI* restriction enzyme sites and expressed in *E. coli* DH5α. The expression of the recombinant plasmid was detected by white-blue screening on IPTG, X-gal, and ampicillin medium. The plasmid DNA from white colonies was digested with *XhoI* and *XbaI* (Figure 2B). The sequencing results were confirmed by comparison with the original sequences using the Basic Local Alignment Search Tool (BLAST) resources.

Δ*wbkA B. abortus* S19 mutant construction. Electroporation performed with the survival rate between 25 - 50%. The stable gene replacement candidates were isolated by positive selection on BA plates containing 30 μg/mL of kanamycin. To confirm that the *B. abortus wbkA* gene was interrupted by the kanamycin cassette, PCR analysis was performed on genomic DNA from kanamycin-resistant colonies of the *B. abortus* S19 Δ*wbkA* strain. The specific primer sequences for the *wbkA* gene, described above (1 and 4), were used for PCR amplification (Figure 2C).

DISCUSSION

B. abortus is the causative agent of human and animal brucellosis. Isolation, identification, and characterization of new antigens and virulence factors are the goals of many researchers (Nikolich et al., 2010; Xavier et al., 2010). The aim of this short study was to devise an efficient strategy to introduce site-directed mutations in a bacterial genome, using *B. abortus* S19 as a model organism. Site-directed mutagenesis is used widely to create desired changes in genes (Higuchi et al., 1988; Monreal et al., 2003). Various site-directed mutagenesis protocols, both PCR and non PCR-based, have been described (Allen et al., 1998; Ugalde et al., 2003). Mutagenesis by overlap extension has been previously described as a method for site-directed mutagenesis to create base substitutions, insertions, and/or deletions (Horton, 1995; Bryksin and Matsumura, 2010).

Original OE- PCR, known as OE-PCR, was employed to precisely and quickly splice two fragments without the use of restriction enzymes (Horton et al., 1990; Horton, 1995; Higuchi et al., 1988) and to produce linear DNA with long flanking homology regions to the genome (Bikard et al., 2013). In the two-step mutation process used in this study, the KanR cassette was first inserted between upstream and downstream regions of the *wbkA* gene by homologous recombination. Then, this construct was cloned

using restriction enzymes (*Xba-I* and *Xho-I*) into the pBlueScript plasmid. The recombinant plasmid was introduced into *B. abortus* S19 cells by electroporation. Selection for KanR colonies was the initial criterion for identifying potential positive clones, which were confirmed by colony PCR. Although OE-PCR is relatively straightforward, efficient, and reliable, it is not recommended as the first step of PCR-mediated mutation (Denamur and Matic, 2006; Luo et al., 2013).

CONCLUSION

This protocol provides an easy method for correction and deletion of nucleotides, and for single point mutations, which could be accomplished by two rounds of PCR. In this study, the method described was used on a fastidious bacterium (*B. abortus*), and it can be extended to other bacterial species for genome analysis.

ACKNOWLEDGMENTS

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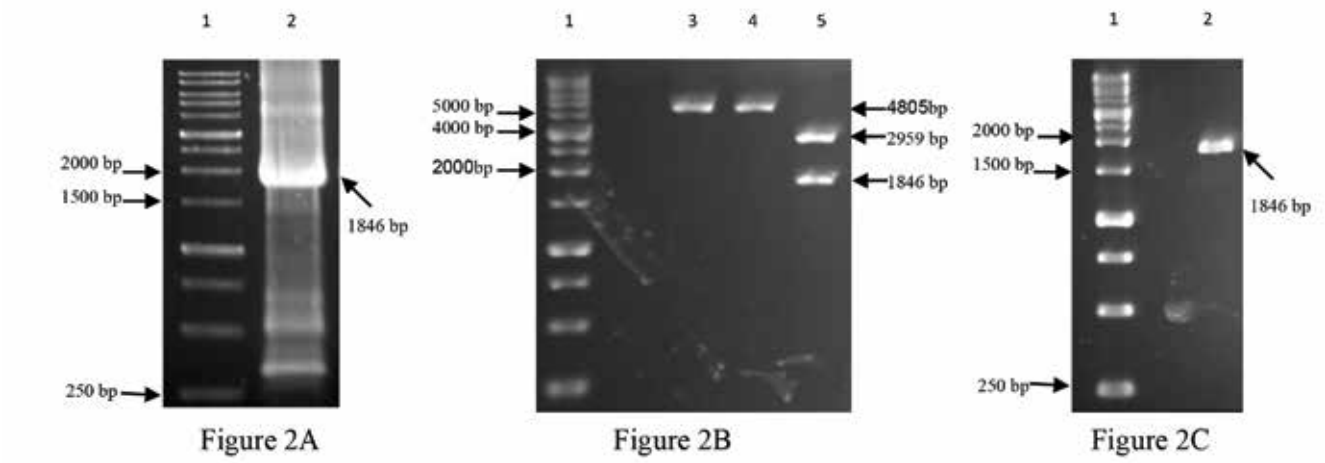
• The authors declare that there is no conflict of interests.

Figure 2. Results of PCR reactions and digestion with restriction enzymes (1% agarose gels).



A: Lane 1, 1 kb DNA marker; Lane 2, the fusion PCR product.

B: Lane 1, 1 kb DNA marker; Lanes 3 and 4, pBlueScript containing the construct, digested with Xba-I and Xho-I, respectively; Lane 5, pBlueScript containing the construct, double digested with Xba-I and Xho-I; pBlueScript (2,959 bp) and the construct (1,846 bp) are separated.

C: Lane 1, 1 kb DNA marker; Lane 2, the PCR product obtained with primers 1 and 4 using extracted DNA from electroporated bacteria and grown on rich medium with the antibiotic kanamycin.

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