African Journal of Microbiology Research Vol. 6(4), pp. 802-808, 30 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1119
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Construction of a novel recombinant vector as *Brucella melitensis vacB* gene knockout candidate

Rahmatollah Yazdani*, Abbas Doosti and Payam Ghasemi Dehkordi

Biotechnology Research Center, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran.

Accepted 18 November, 2011

Brucella melitensis **is a Gram negative coccobacillus bacterium from the Brucellaceae family. Brucellosis is an infectious zoonotic disease affecting most species of domestic animals, but sheep and goats, particularly milking breeds, are the most susceptible. Cattle may occasionally be affected and the disease may appear in pigs.** ***vacB*** **gene is one important gene of** ***B. melitensis*** **that encoded a protein (RNase R) and in** ***B. melitensis*** **and** ***Brucella abortus*** **has no impact on bacterial virulence. The aim of the present study was to construct a novel recombinant vector as** ***B. melitensis vacB*** **gene knockouts candidate.** ***B. melitensis*** **were collected from Microbiology laboratory of Islamic Azad University of Shahrekord Branch and cultured into** ***Brucella*** **agar. Genomic deoxyribonucleic acid (DNA) was extracted and the polymerase chain reaction (PCR) were performed using designed primers for amplification of upstream and downstream regions of** ***vacB*** **gene of** ***B. melitensis*** **and** ***kan*** **gene of pET-28 vector. Then, amplified fragments were cloned using T/A cloning technique and the construction was transformed into competent** ***Escherichia coli*** **Top10F' strain in LB media. The final construction was confirmed by digestion with** ***Xho*****I,** ***Kpn*****I,** ***Xba*****I, and** ***BamH*****I restriction enzymes. PCR amplified products for flanking regions of** ***vacB*** **gene and** ***kan*** **gene on 1% agarose gel revealed 609, 595, and 870 bp, fragments, respectively. The results showed that** ***vacB*** **gene and** ***kan*** **gene were cloned in** ***E. coli*** **successfully. Sub-cloning of all fragment into pET-32 vector were done successfully and pET-32-Up-Kan-Down recombinant vector was generated. The findings of this study showed that the designed novel recombinant construct (pET-32-Up-Kan-Down recombinant plasmid) is useful for genetic engineering and for manipulate of** ***vacB*** **gene from** ***B. melitensis*****.**

Key words: Virulence-associated gene (*vac*B), *kan* gene, *Brucella melitensis*, the polymerase chain reaction (PCR) and cloning.

INTRODUCTION

Brucella is coccobacilli or short rods 0.6 to 1.5 µm long by 0.5 to 0.7 µm in width. They are arranged singly and less frequently in pairs or small groups (Moreno and Moriyón, 2002; Wu et al., 2006). The morphology of *Brucella* is fairly constant except in old cultures, where pleomorphic non-encapsulated, and non-motile. They do not form spores, pili, or flagella (Corbel, 1997). *Brucella* members are aerobic, but some strains require an atmosphere containing 5 to 10% carbon dioxide (CO_2) added for growth, especially on primary isolation (Hallez et al., 2007). The optimum pH for growth varies from 6.6 to 7.4, and culture media should be adequately buffered near pH 6.8 for optimum growth. The optimum growth temperature is 36 to 38°C, but most strains can growth between 20 and 40°C (González et al., 2008). *Brucella* requires biotin, thiamin and nicotinamide. The growth is improved by serum or blood, but haemin (V-factor) and nicotinamide-adenine dinucleotide (X-factor) are not required. The growth of most *Brucella* strains is inhibited

*****Corresponding author. E-mail: biologyshki@yahoo.com. Tel: +98-381-3361001. Fax: +98-381-3361001.

Abbreviations: ***vacB*** **gene,** virulence-associated gene; ***B. melitensis*****,** *Brucella melitensis*; ***kan*** **gene,** kanamycin resistance gene.

on media containing bile salts, tellurite or selenite (Ferrero et al., 2009).

Brucella abortus (*B. abortus*) is responsible for bovine brucellosis and in other primary hosts such as moose, elk, and bison. Secondary hosts of *B. abortus* include goats, sheep, pigs, and humans (Hoffmann et al., 1990; Guzman-Verri et al., 2001). *Brucella melitensis* (*B. melitensis*) is the most virulent *Brucella* spp. and infects mainly goats, although humans are a secondary host. Other species of *Brucella* include *Brucella suis*, *Brucella ovis*, and *Brucella canis* (Edmonds et al., 2002).

B. melitensis is aerobic, Gram-negative, facultative intracellular pathogens that are the causative agents of brucellosis in both humans and animals (DelVecchio et al., 2002). Originally named “Micrococcus melitensis”, *B. melitensis* was discovered by Lieutenant Colonel David Bruce in 1887 from British soldiers residing in Malta. *B. melitensis* is a world-wide disease which remains most problematic in developing countries. Although considered to be eradicated in the United States, *B. melitensis* is a continuing cause for concern because of its status as a potential agent of biological warfare (Moreno and Moriyon, 2002).

The primary host of *B. melitensis* is the caprine host as well as the ovine host, although the pathogen can be problematic in cattle, camels, and humans as secondary hosts (Nielson and Duncan, 1990). In female goats and sheep the primary symptom is abortion, but larger doses of the pathogen cause mastitis and joint problems. In male goats, orchitis is uncommon, and if observed it is generally unilateral. In male sheep, however, the disease is often asymptomatic, although orchitis and hygromas are a rare observation (Alton, 1990).

B. melitensis infection in sheep appears to occur endemically in the Mediterranean region, especially along its northern and eastern shores, stretching through Central Asia as far south as the Arabian peninsula and as far east as Mongolia (Rezaei-Sadaghiani et al., 1996; Wu et al., 2006). Parts of Latin America are also seriously affected, especially Mexico, Peru and northern Argentina. The disease is prevalent on the Southern and Eastern edges of Mediterranean basin, particularly in Tunisia, Libya, Egypt, Syria, and in the Arabian Peninsula and Iran. The disease also occurs in Africa and India. However, North America (except Mexico) is believed to be free, as are Northern Europe (except for sporadic incursions from the south), Southeast Asia, Australia and New Zealand (Elzer et al., 2002). In Iran, the causative organism of brucellosis was isolated from human blood culture in 1932, bovine fetus in 1944, and sheep and goats milk in 1950 (Samar et al., 1996; Khadjeh et al., 1999). Human brucellosis is also common in many parts of north, central and South America, particularly in Mexico, Brazil, Colombia and Peru. New foci of human brucellosis have emerged particularly in central Asia (Pappas et al., 2006).

Of the three different biovars of *B. melitensis* biovar 3 predominates almost exclusively in Mediterranean countries and Middle East, while biovar 1 seems to predominate in Latin America (Sauret and Vilissova, 2002). The biovars 1 and 2 have also been reported in some southern European countries. However, the precise recognition of biovar 3, especially its differentiation from biovar 2 appears sometimes equivocal (Schurig et al., 2002).

In Iran, *B. melitensis* was first isolated from a sheep in Isfahan in 1950 and subsequently its biovar 1 was sporadically isolated in different regions of the country from sheep and goats as well as cattle, camel, sheep-dogs, and human being (Khadjeh et al., 1999). Meanwhile, *B. melitensis* biovars 2 and 3 are of considerable importance and have been frequently isolated from sheep, goats and human beings (Zowghi and Ebadi, 1985; Akbarmehr, 2011). *B. melitensis* biovar 1 is responsible for the disease in regions of Isfahan, Khorasan, Guilan (north), Khoozestan (south), Yazd (central) and Kermanshah (west), whereas in Tehran and Azarbaijan, biovars 1, 2 and 3 are the responsible ones. Surprisingly, *B. suis*, *Brucella neotoma*, *B. ovis* and *B. canis* were not isolated in Iran (Zowghi and Ebadi, 1988; Zowghi et al., 1996).

Virulence-associated gene (*vacB*) is one of important gene of *B. melitensis* and exhibited an exoribonuclease (RNase R) activity. The *vacB* gene encoded a protein (RNase R) of 798 amino acid residues with a molecular mass of 90.5 kDa. The *vac*B mutant also exhibited reduced motility, and these growth and motility phenotype defects were restored after complementation of the *vacB* mutant (Erova et al., 2008). *vacB* was first defined as a virulence factor, but on the other hand, *vacB* is known to be an exoribonuclease RNase R involved in mRNA posttranscriptional processing, ribosome rescue, and rRNA methylation (van Buul and Knippenberg, 1985; Tobe et al., 1992; Cheng et al., 1998; Cheng and Deutscher, 2003). The process of mRNA decay is integral to the posttranscriptional control of gene expression, and mRNA turnover is a means of coordinating this process, first through integration with control of transcription and export and translation of mRNAs and second through enabling mRNAs involved in similar processes to decay at similar rates (Wilusz and Wilusz, 2004; Cheng and Deutscher, 2005).

Detection of *Brucella* species and bacterial infection is important. The serological and bacteriological methods are not sensitive enough to differentiate bacterial isolates and strains (Morata et al., 1999). These techniques involve bacterial restriction endonuclease analysis, monoclonal antibodies, several different antigen capture enzyme-linked immunosorbent assays (ELISA), pulsed field gel electrophoresis (PFGE) (Navarro et al., 2004; Navarro et al., 2006). Molecular study such as polymerase chain reaction (PCR) assays has many advantages over other methods for the detection of *Brucella* species. The PCR technique itself is fairly easy

to perform, sensitive, specific, rapid, affordable, and data can be acquired in less than one day for detection of *Brucella* species. Other advantages include the targeting of *Brucella*-specific deoxyribonucleic acid (DNA) and the independence of antibody-based testing, which largely reduce the potential for cross-reaction-based false positive results (Queipo-Ortuño et al., 1997; Navarro et al., 2004).

Gene cloning or molecular cloning is the act of making copies, or clones, of a single gene. Once a gene is identified, clones can be used in many areas of biomedical and industrial research. Genetic engineering is the process of cloning genes into new organisms, or altering the DNA sequence to change the protein product. Bacterial plasmids used in gene cloning naturally contain genes that encode some form of antibiotic resistance (Sambrook and Russell, 2001). Cloning of *vacB* gene of *B. melitensis* and other genes from *Brucella* species and destroy these genes by construction of a novel recombinant vector should yield protective recombinants vaccine in near future.

Kanamycin is inactivated by bacterial aminophosphotransferases (APHs). The APHs inactivate kanamycin by transferring the γ-phosphate of ATP to the hydroxyl group in the 3' position of the pseudosaccharide. The kanamycin resistance gene (*kan* gene) gene codes for kanamycin resistance. Several versions of this gene exist, with varying crossover resistance to other antibiotics such as neomycin or gentamycin. The *kan* gene is provided with a bacterial promoter and, after transformation of the vector, is therefore expressed in *Escherichia coli* cells.

Recent advances in molecular biology and genetic engineering have made gene replacement and construction of a novel recombinant vector and it is useful for directed mutagenesis and creates a new mutant strain of bacteria. The purpose of the present study was to construct a novel recombinant vector that carries a kanamycin resistance gene replacement in flanking regions of VacB gene from *B. melitensis*.

MATERIALS AND METHODS

Bacterial collection and culture conditions

Smooth virulent *B. melitensis* was maintained as frozen glycerol stocks were collected from Microbiology laboratory of Islamic Azad University of Shahrekord Branch and cultured into *Brucella* agar (Becton Dickinson. Microbiological Systems, Franklin Lakes, NJ, USA) and incubated for 18 h with shaking (200 rpm) at 37°C with 5% CO_2 until the log growth phase (OD600 = 0.9) was reached.

Strains, plasmids construction, and media

T-Vector using TOPO T/A cloning kit (Invitrogen, San Diego, CA) with *E. coli* strain Top10F' (*in vitrogen*, the netherlands) were used for cloning and maintenance of DNA fragment. Bacterial cultures were grown at 37°C in Luria-Bertani (LB) broth and LB agar plates.

Extraction of genomic DNA from *B. melitensis*

Bacterial DNA was extracted from colonies of bacteria using DNP™ Kit (CinnaGen, Iran) according to the manufacturer's instructions. The quality of extracted DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm according to the method described by Sambrook and Russell (2001).

Amplification of *kanamycin* gene, and flanking regions of VacB gene

Oligonucleotide primers were designed for amplification of flanking regions of VacB gene of *B. melitensis* according to the published sequence. The sequences of these primers were B-upV-F: 5'-CTCGAGGCAGCCACAGCCCAATCG-3' and B-upV-R: 5'-GGATCCCACCAGATCGCCGCTTTC-3' (accession number: AF174645) contain XhoI and BamHI restriction sites and B-dowV-F: 5'-GGTACCACCCTGCGTGAGTTCCTGC-3' and B-dowV-R: 5'-TCTAGAAGACGAAAGCCCCTGCC-3'(accession number: AF174645) contain KpnI and XbaI restriction sites were used for amplification of upstream and downstream region of VacB gene, respectively. Furthermore, Kan-F: 5'-GGATCCACAAGGGGTGTTATGAGC-3' and Kan-R: 5'-GGTACCTCAAATATGTATCCGCTC-3' primers contain BamHI and KpnI restriction sites were designed for amplification of kan gene and pET-28 vector were used as template.

Three sets of PCR programs were performed separately in high volume for amplification of kan gene and upstream and downstream regions of *vacB* gene in a gradient palm cycler (Corbett Research, Australia). Amplification programs were carried out in 50 µl total reaction volumes, each containing 1 µg of template DNA, 1 µM of each primer, 5 µM of 10X PCR buffer, 2 mM $MgCl_2$, 200 mM dNTPs, and 1 unit of Taq DNA polymerase (Fermentas, Germany). For the optimal amplification of flanking regions of VacB gene and kan gene, an initial denaturation step of 94°C was held for 5 min. Each reaction was then subjected to denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min over 32 cycles. Lastly, a prolonged extension phase was programmed for 5 min at 72°C and amplified samples were holed at 4°C.

Analysis of PCR products

The amplified products were detected in 1% agarose gel electrophoresis. The electrode buffer was TBE (Tris-base 10.8 g 89 mM, Boric acid 5.5 g 2 mM, EDTA (pH 8.0) 4 ml of 0.5 M EDTA (pH 8.0), combine all components in sufficient H_2O and stir to dissolve). Aliquots of 10 µl of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. The 1 kb DNA ladder (Fermentas, Germany) was used as a molecular weight marker to determine the length of the amplified fragments. After electrophoresis, the gel was stained with ethidium bromide and examined under UV light and photographed were obtained in UVIdoc gel documentation systems (UK).

Cloning of *vacB* gene and plasmid construction

PCR products purified using gel extraction kit (Bioneer Co., South-Korea) after gel electrophoresis according to manufacture's protocol. All PCR products were cloned into T-vector using TOPO T/A cloning kit (*in vitrogen*, USA) and the recombinant vectors were

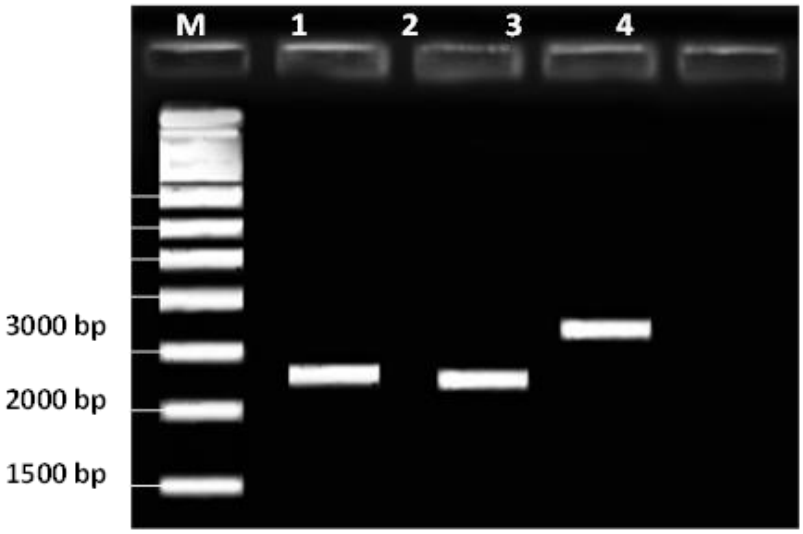

Figure 1. Agarose gel electrophoresis of PCR amplified products of flanking regions of *vacB* gene and *kan* gene (Line M is 1 kb DNA marker (Fermentas, Germany), lines 1 and 2 are upstream and downstream regions of *vacB* gene, respectively, line 3 is *kan* gene, and line 4 is PCR negative control sample without bacterial DNA).

transformed under heat shock (42°C) and calcium chloride for 90s into *E. coli* TOP10F' competent cells. Then, competent cells cultured in LB media (Merck Co., Germany). The presence of *kan* gene and flanking regions of *vacB* gene was confirmed by restriction enzyme analysis.

Sub-cloning of the *VacB* and *kan* genes

The Up-VacB fragment was removed from the TOPO vector by *Xho*I-*BamH*I double digestion and subcloned in *Xho*I-*BamH*I linearized pET-32 to get pET-32-up. Then, *kan* fragment was released from the TOPO vector by *BamH*I-*Kpn*I double digestion and subcloned into *BamH*I-*Kpn*I linearized pET-32-Up producing pET-32-Up-Kan. And final TOPO-Down double digested using *Kpn*I-*Xba*I and down fragment of *vacB* subcloned into *Kpn*I-*Xba*I linearized pET-32-up-kan to producing pET-32-Up-Kan-Down recombinant vector. The final construct was confirmed by double digestion by *Xho*I-*Xba*I and PCR using B-upV-F and B-dowV-R primers.

RESULTS AND DISCUSSION

Gene amplification

DNA was successfully extracted and PCR amplified products for flanking regions of *VacB* and *kan* genes on 1% agarose gel revealed 609 bp, 595 bp and 870 bp, fragments, respectively (Figure 1).

Verification of colonies that contain recombinant vector

The upstream and downstream regions of *vacB* gene of *B. melitensis* and *kan* gene were cloned with T/A cloning technique in T-vector successfully. Chemical competent cells of *E. coli* Top10F' strain were transformed with pET-32-Up-Kan-Down recombinant plasmid. Flanking regions of *vacB* gene and *kan* gene had restriction point of *Xho*I, *BamH*I, *Kpn*I, and *Xba*I were inserted in polyclonal site (PCS) in pET-32.

TOP10F' competent cells were used for transformation and cultured in LB media containing ampicyline. Plasmid purification and *Kpn*I, *Xba*I, *Xho*I and *BamH*I restriction endonuclease digestion of pET-32-Up-Kan-Down recombinant plasmid, confirmed the correction of upstream and downstream regions of *vacB* gene of *B. melitensis* and *kan* gene cloning. Figure 2 shows recombinant plasmids after digestion. A 7974 bp large fragment is related to pET-32 vector (5900 bp) and 609, 595 and 870 fragments are upstream and downstream regions of *vacB* gene and *kan* gene bands, respectively.

B. melitensis is facultative intracellular bacteria that survive and replicate in both phagocytic and non-phagocytic cells (DelVecchio et al., 2002). *B. melitensis* is the most virulent strain in human infection, with only 1 to10 organisms needed for infection and with undulant fever as the primary symptom (Mantur et al., 2007). Brucellosis in humans is a disease that has the potential to affect several systems with symptoms ranging from mild to severe. Transmission is normally from infected animals. Incubation periods may vary from weeks to several months before symptoms fully develop (Franco et al., 2007). Infected individuals may experience undulating fever, fatigue, and headaches, as well as joint and back pain. These symptoms may have long-term or chronic effects in some patients. More serious symptoms are

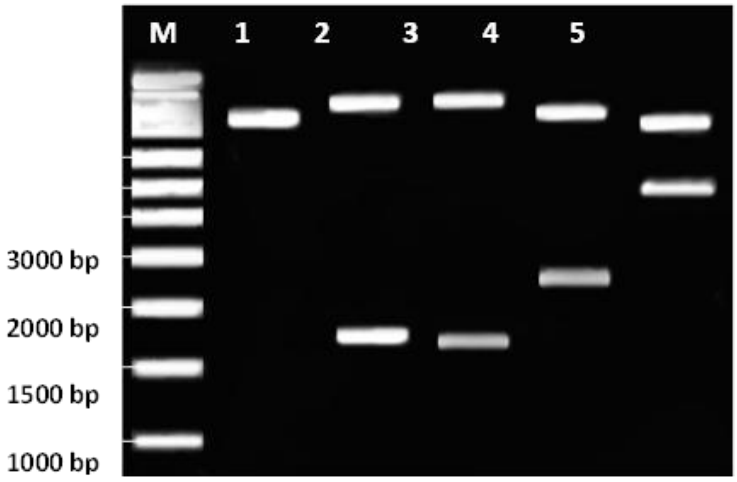

Figure 2. Analysis of digested pET-32-Up-Kan-Down recombinant plasmid using *Xho*I, *BamH*I, *Kpn*I and *Xba*I restriction endonuclease enzymes (Line M is 1 kb DNA ladder (Fermentas, Germany), line 1 is pET-32 vector without inserted sequence, lines 2, 3 and 4 are digested pET-32-Up-Kan-Down recombinant vector to fragments of upstream and downstream regions of *vacB* gene, and *kan* gene respectively, and line 5 is pET-32-Up-Kan-Down recombinant vector using *Xho*I and *Xba*I restriction enzymes to Up-Kan-Down recombinant fragment).

observed in cases where the bacterium has migrated to the central nervous system or endocardium, in which case meningitis, endocarditis and psychoneurosis can occur (Pappas et al., 2006; Franco et al., 2007). Although brucellosis is not typically considered a fatal disease, human cases left untreated can result in mortality (Park et al., 2007; Franco et al., 2007). It is believed that many cases of human brucellosis remain undiagnosed and thus unreported. This may be due to the similarity of initial symptoms to those of influenza (Chain et al., 2005).

There is no human vaccine for brucellosis and the *B. melitensis* Rev.1 vaccine for animals is infectious to humans (Moriyon et al., 2004). This vaccine, although effective, is potentially abortigenic and produces anti-LPS antibodies that can interfere with diagnostic tests (González et al., 2008). Vaccines developed from strains of *B. abortus* are not effective in providing protection against infection with *B. melitensis* (Davis and Elzer, 2002; Schurig et al., 2002).

B. melitensis is highly contagious and can be easily disseminated with devastating effects to public and agricultural health. *B. melitensis*, as well as *B. suis* and *B. abortus*, have been deemed potential bioterrorism agents by the United States Centers for disease control and prevention (Edmonds et al., 2002).

Brucellosis is a disease of major economic and zoonotic importance, a strategy for its control in small ruminants is essential in endemic areas. The initial aim of the strategy selected will be the reduction of infection in the animal population to such a level that the impact of the disease on human health as well as on animal health and production will be minimized (Davis and Elzer, 2002). Subsequent steps can include eradication from a region by test and slaughter and, following successful eradication, measures to prevent reintroduction of the disease. Control of a zoonosis is a general term that embraces all the measures designed to reduce the incidence and prevalence of a disease in a defined animal population (Nijskens et al., 2008).

Human brucellosis is diagnosed using serological testing and by culturing bacteria from blood, lymph, or cerebrospinal fluid (Moriyon et al., 2004). Disadvantages of this method include the slow growth of *Brucella* in culture and a potentially low number of colony forming units (CFU) present in clinical samples due to the stage of infection or to the use of antibiotics prior to sample collection (Moriyon et al., 2004; Franco et al., 2007). The Rose Bengal test is also useful for human diagnosis, as well as specially designed ELISAs (Orduna et al., 2000). The tube agglutination test was the first test used for diagnosis of brucellosis in humans and was later adapted for use in animals. In this test, sera is diluted and added to a tube containing a standard quantity of killed *Brucella* cells. The occurrence of clearing and agglutination following incubation is considered a positive result (Mert et al., 2003). Wright's serum agglutination test, which measures the titer of anti-*Brucella* antibodies, and Huddleson's slide agglutination test, in which serum agglutination can be rapidly detected, is successful diagnostic tools for brucellosis (Mert et al., 2003). Studies surrounding the use of molecular-based diagnosis using the PCR have also been explored with varied results. It is also crucial to obtain a detailed case history of any travels to endemic countries or ingestion of any untreated

animal products imported from endemic countries (Hong et al., 2005; Kattar et al., 2007). *B. melitensis vacB* gene that encodes an exoribonuclease RNase R involved in bacterial virulence. In present study we constructed a novel recombinant vector carries a kanamycin resistance gene replacement in upstream and downstream region of *vacB* gene of *B. melitensis*. A majority of the studies concerned with exoribonucleases indicate their prominent role in quality control of rRNA, cell cycle-regulated degradation of tmRNA (small stable RNA), and mRNA decay, which is a significant determinant of gene expression (Cheng and Deutscher, 2003; 2005). Since publication of the report regarding the role of the *vacB* gene in the pathogenesis of serotypes of *Shigella* spp. and enteroinvasive *E. coli* strains only recently did studies emerge that indicated a potential contribution of RNases, in particular PNPase, in regulating bacterial virulence (Clements et al., 2002; Rosenzweig et al., 2005; Ygberg et al., 2006). However, the role of RNase R in bacterial virulence is not fully explored. Interestingly, the *vacB* gene of *B. melitensis* and *B. abortus* has no impact on bacterial virulence (Miyoshi et al., 2007).

Chao-Yu et al. 2003 constructed of a recombinant vector based on AAV that carried human endothelial nitric-oxide synthase gene. Their study showed pSNAV-eNOS was successfully constructed with the ability to express human endothelial nitric oxide synthase (eNOS mRNA in cultured mammalian cells (Chao-Yu et al., 2003). The method used in their study somewhat same to the present research.

The recombinant bacteria have become the useful tool in various aspects on basic knowledge and applied science. In our research, we have constructed the novel recombinant plasmid carries a kanamycin resistance gene replacement in upstream and downstream region of *vacB* gene of *B. melitensis* by modifying the native indigenous plasmid of *E. coli*. The new recombinant construct (pET-32-Up-Kan-Down recombinant plasmid) in this study was useful for genetics engineering and manipulate of *vacB* gene and induce a new phenotype in *B. melitensis* and reduce the amplification of bacterial cells via inserting wild type gene with recombinant gene in future.

ACKNOWLEDGEMENTS

The authors would like to express their deep sense of gratitude and sincere thanks to the staff of the Biotechnology Research Center of Islamic Azad University of Shahrekord Branch in southwest Iran.

REFERENCES

Akbarmehr J (2011). The prevalence of *Brucella abortus* and *Brucella melitensis* in local cheese produced in Sarab city, Iran and its public health implication. Afr. J. Microbiol. Res., 5(12): 1500-1503.

Alton G (1990). From: Animal Brucellosis. CRC Press, Boca Raton.

Chain PS, Comerci DJ, Tolmasky ME, Larimer FW, Malfatti SA, Vergez LM, Aguero F, Land ML, Ugalde RA, Garcia E (2005). Whole-genome analyses of speciation events in pathogenic brucellae. Infect. Immun., 73: 8353-8361.

Cheng ZF, Deutscher MP (2003). Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. Proc. Natl. Acad. Sci. USA, 100: 6388-6393.

Cheng ZF, Deutscher MP (2005). An important role for RNase R in mRNA decay. Mol. Cell., 17: 313-318.

Cheng ZF, Zuo Y, Li Z, Rudd KE, Deutscher MP (1998). The *vacB* gene required for virulence in *Shigella flexneri* and *Escherichia coli* encodes the exoribonuclease RNase R. J. Biol. Chem., 273: 14077-14080.

Chen-Xia H, Chao-Yu M, Ji-Hua Y, Hao-Ming C, Da-Ru L, Ding-Feng S, Jing-Lun X (2003). Construction of a recombinant vector based on AAV carrying human endothelial nitric-oxide synthase gene. Acta Pharmacol. Sin., 24(7): 637-640.

Clements MO, Eriksson S, Thompson A, Lucchini S, Hinton JC, Normark S, Rhen M (2002). Polynucleotide phosphorylase is a global regulator of virulence and persistency in *Salmonella enterica*. Proc. Natl. Acad. Sci. USA. 99: 8784-8789.

Corbel MJ (1997). Recent Advances in brucellosis. J. Med. Microbiol., 46: 101-103.

Davis DS, Elzer PH (2002). *Brucella* vaccines in wildlife. Vet. Microbiol., 90(1-4): 533-544.

DelVecchio VG, Kapatral V, Elzer P, Patra G, Mujer CV (2002). The genome of *Brucella melitensis*. Vet. Microbiol., 90(1-4): 587-592.

Edmonds MD, Cloeckaert A, Elzer PH (2002). *Brucella* species lacking the major outer membrane protein Omp25 are attenuated in mice and protect against *Brucella melitensis* and *Brucella ovis*. Vet. Microbiol., 88(3): 205-221.

Elzer PH, Hagius SD, Davis DS, DelVecchio VG, Enright FM (2002). Characterization of the caprine model for ruminant brucellosis. Vet. Microbiol., 90(1-4): 425-431.

Erova T, Kosykh VGE, Fadl AA, Sha J, Horneman AJ, Chopra AK (2008). Cold shock exoribonuclease R (VacB) is involved in *Aeromonas hydrophila* pathogenesis. J. Bacteriol., 190(10): 3467-3474.

Ferrero MC, Fossati CA, Baldi PC (2009). Smooth *Brucella* strains invade and replicate in human lung epithelial cells without inducing cell death. Microb. Infect., 11(4): 476-483.

Franco MP, Mulder M, Gilman RH, Smits HL (2007). Human Brucellosis. Lancet Infect. Dis., 7: 775-786.

González D, Grilló MJ, De Miguel MJ, Ali T, Arce-Gorvel V, Delrue RM, Conde-Alvarez R, Muñoz P, López-Goñi I, Iriarte M, Marín CM, Weintraub A, Widmalm G, Zygmunt M, Letesson JJ, Gorvel JP, Blasco JM, Moriyón I (2008). Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. Public Library of Science One. 3(7): 2760.

Guzman-Verri C, Chaves-Olarte E, von Eichel-Streiber C, López-Goñi I, Thelestam M, Arvidson S, Gorvel JP, Moreno E (2001). GTPases of the Rho subfamily are required for *Brucella abortus* internalization in nonprofessional phagocytes: direct activation of Cdc42. J. Biol. Chem., 276(48): 44435-44443.

Hallez R, Letesson JJ, Vandenhaute J, De Bolle X (2007). Gateway-based destination vectors for functional analyses of bacterial ORFeomes: Application to the Min system in *Brucella abortus*. Appl. Environ. Microbiol., 73(4): 1375-1379.

Hoffmann EM, Shapiro SJ, Nicoletti P (1990). Evaluation of serologic and cellular immune responses of cattle to a nonlipopolysaccharide antigen from *Brucella abortus*. Am. J. Vet. Res., 51(2): 216-221.

Hong SJ, Tran QA, Keiler KC (2005). Cell cycle-regulated degradation of tmRNA is controlled by RNase R and SmpB. Mol. Microbiol., 57: 565-575.

Kattar MM, Zalloua PA, Araj GF, Samaha-Kfoury J, Shbaklo H, Kanj SS, Khalife S, Deeb M (2007). Development and evaluation of real-time polymerase chain reaction assays on whole blood and paraffin-embedded tissues for rapid diagnosis of human brucellosis. Diagn. Microbiol. Infect. Dis., 59(1): 23-32.

Khadjeh G, Zowghi E, Zarif-fard MR (1999). Incidence of brucellosis in one-humped camels of Boshehhr, Iran. Arch. Inst. Razi., 50: 83-86.

Mantur BG, Amarnath SK, Shinde RS (2007). Review of clinical and laboratory features of human brucellosis. Indian J. Med. Microbiol., 25: 188-202.
Mert A, Ozaras R, Tabak F, Bilir M, Yilmaz M, Kurt C (2003). The sensitivity and specificity of *Brucella* agglutination tests. Diagn. Microbiol. Infect. Dis., 46: 241-243.
Miyoshi A, Rosinha GM, Camargo IL, Trant CM, Cardoso FC, Azevedo V, Oliveira SC (2007). The role of the *vacB* gene in the pathogenesis of *Brucella abortus*. Microb. Infect., 9: 375-381.
Morata P, Queiopo-Ortuno MI, Reguera JM, Garcia-Ordonez MA, Pichardo C, De Dios Colmenero J (1999). Posttreatment Follow-Up of Brucellosis by PCR Assay. J. Clin. Microbiol., 37(12): 4163-4166.
Moreno E, Moriyón I (2002). *Brucella melitensis*: A nasty bug with hidden credentials for virulence. Proc. Natl. Acad. Sci., 99(1): 1-3.
Moriyon I, Grillo MJ, Monreal D, González D, Marin C, Lopez-Goni I, Mainar-Jaime RC, Moreno E, Blasco JM (2004). Rough vaccines in animal brucellosis: Structural and genetic basis and present status. Vet. Res., 35(1): 1-38.
Navarro E, Casao MA, Solera J (2004). Diagnosis of human brucellosis using PCR. Expert Rev. Mol. Diagn., 4(1): 115-123.
Navarro E, Segura JC, Castano MJ, Solera J (2006). Use of Real-Time quantitative Polymerase Chain Reactoin to monitor the evolution of *Brucella melitensis* DNA load during therapy and post-therapy follow-up in patients with brucellosis. Clin. Infect. Dis., 42: 1266-1273.
Nielson K, Duncan JR (1990). Animal Brucellosis. CRC Press, Inc., Boca Raton.
Nijskens C, Copin R, De Bolle X, Letesson JJ (2008). Intracellular rescuing of a *B. melitensis* 16M *virB* mutant by co-infection with a wild type strain. Microbiol. Pathol., 45(2): 134-141.
Orduna A, Almaraz A, Prado A, Gutierrez MP, Garcia-Pascual A, Duenas A, Cuervo M, Abad R, Hernández B, Lorenzo B, Bratos MA, Torres AR (2000). Evaluation of an immunocapture-agglutination test (Brucellacapt) for serodiagnosis of human brucellosis. J. Clin. Microbiol., 38(11): 4000-4005.
Pappas G, Christou L, Akritidis N, Tsianos EV (2006). Quinolones for brucellosis: treating old diseases with new drugs. Clin. Microbiol. Infect., 12(9): 823-825.
Park KW, Kim DM, Park CY, Kim HL, Jang SJ, Choi YS, Park MY, Song HJ, Lee SH (2007). Fatal systemic infection with multifocal liver and lung nodules caused by *Brucella abortus*. Am. J. Trop. Med. Hyg., 77(6): 1120-1123.
Queipo-Ortuño MI, Morata P, Ocón P, Manchado P, Colmenero JD (1997). Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. J. Clin. Microbiol., 35(11): 2927-2930.
Rezaei-Sadaghiani R, Zowghi E, Marhemati-Khamene B, Mahpeikar HA (1996). *Brucella melitensis* infection in sheep-dogs in Iran. Arch. Inst. Razi., 46/47: 1-7.
Rosenzweig JA, Weltman G, Plano GV, Schesser K (2005). Modulation of Yersinia type three secretion system by the S1 domain of polynucleotide phosphorylase. J. Biol. Chem., 280: 156-163.
Samar G, Aminian N, Vahdani F, Zowghi E (1996). A survey on human brucellosis in Iran. Arch. Inst. Razi., 46/47: 17-26.
Sambrook J, Russell DW (2001). Molecular cloning: A laboratory manual. 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Sauret JM, Vilissova N (2002). Human Brucellosis. J. Am. Board Fam. Med., 15(5): 401-406.
Schurig GG, Sriranganathan N, Corbel MJ (2002). Brucellosis vaccines: past, present and future. Vet. Microbiol., 90(1-4): 479-496.
Tobe T, Sasakawa C, Okada N, Honma Y, Yoshikawa M (1992). vacB, a novel chromosomal gene required for expression of virulence genes on the large plasmid of *Shigella flexneri*. J. Bacteriol., 174: 6359-6367.
van Buul CP, van Knippenberg PH (1985). Nucleotide sequence of the *ksgA* gene of *Escherichia coli*: comparison of methyltransferases effecting dimethylation of adenosine in rRNA. Gene., 38: 65-72.
Wilusz CJ, Wilusz J (2004). Bringing the role of mRNA decay in the control of gene expression into focus. Trends Genet., 20: 491-497.
Wu Q, Pei J, Turse C, Ficht TA (2006). Mariner mutagenesis of *Brucella melitensis* reveals genes with previously uncharacterized roles in virulence and survival. BMC Microbiol., 6: 102.
Ygberg SE, Clements MO, Rytkonen A, Thompson A, Holden DW, Hinton JC, Rhen M (2006). Polynucleotide phosphorylase negatively controls spv virulence gene expression in *Salmonella enterica*. Infect. Immun., 74: 1243-1254.
Zowghi E, Ebadi A (1985). Naturally occurring *Brucella melitensis* infection in cattle in Iran. Rev. Sci. Tech. O.I.E., 4(4): 811-814.
Zowghi E, Ebadi A (1988). Brucellosis in camels in Iran. Rev. Sci. Tech. O.I.E., 7(2): 383-386.
Zowghi E, Hedayeti AH, Ebadi A, Yarahmadi M (1996). *Brucella* infection causing abortion in human beings. Arch. Inst. Razi., 46/47: 35-39.