

Full Article

Amplification, cloning and expression of *Brucella melitensis* bp26 gene (OMP28) isolated from Markazi province (Iran) and purification of Bp26 Protein

Hosseini¹, S.D., Azizpour^{*2}, M., Akbari³, N., Basiri³, H., Behrozikhah⁴, A.M., Eskandari¹, S.

1. Department of Pathobiology, Razi Vaccine & Serum Research Institute, Karaj, Iran

2. Department of Microbiology, Science and Research branch, Islamic Azad University, Arak, Iran

3. Department of Microbiology, Islamic Azad University, Arak Branch, Arak, Iran

4. Department of Brucella vaccine, Razi Vaccine & Serum Research Institute, Karaj, Iran

Received 02 Oct 2012; accepted 15 May 2013

ABSTRACT

Brucellosis is a debilitating disease that imposes costs on both economy and society. It is shown that although the vaccine can prevent abortion, it does not provide complete protection against infection. In Iran, *Brucella melitensis* is a common causative agent for brucellosis and BP26 protein of this bacterium having a good antigenicity and an important vaccine candidate. In this study *B. melitensis* bp26 gene was cloned first in to PTZ57R/T vector and accessed on the PET28a vector and sequenced. Recombinant vector transformed and expressed in to *E. coli* BL21 (DE3) and then recombinant protein was purified with Ni-NTA column of chromatography against His tag. Obtained rOmp28 could be used as a research experimental tool to find its potential as a detection kit and vaccine candidate.

Keywords: *Brucella melitensis*, bp26 gene, OMP28, brucellosis, cloning, expression

INTRODUCTION

Brucellosis is a worldwide zoonosis that caused by *Brucella* spp. and the most important zoonotic disease in terms of social and economic impacts (Godfrid *et al* 2010, Abbas & Aldeewan 2009). This bacterium is Gram-negative coccobacilli, facultative intracellular that can involve many organs and tissues (Chaudhuri *et al* 2010, Thavaselvam *et al* 2010). In spite of the attempts to control the disease, brucellosis remains endemic among domestic animals and humans (Abbas

& Aldeewan 2009). *B. melitensis* is the most frequently isolated species from all domestic ruminants (Álvarez *et al* 2010, Cloeckaert *et al* 2002) and is the major cause of human brucellosis worldwide (Mikeljon *et al* 2010, Cloeckaert *et al* 2002). In Iran, Brucellosis is an endemic disease and incidence of the disease in this country is 34 per 100 000 per year (Najafi *et al* 2011). *B. melitensis* was first isolated from a sheep in Isfahan province in 1952 (Kaveh 1952) and then brucellosis has been reported from various species such as sheep (Zowghi *et al* 2008), goat (Akbarmehr & Ghiyamirad 2011), cattle (Zowghi & Ebadi 1985), camel (Khadjeh

* Author for correspondence. Email: maryam.azizpour@gmail.com

et al 1999), dog (Mosallanejad et al 2009), buffalo (Nowroozi-Asl et al 2007), human (Kazemi et al 2008) and horse (Tahamtan et al 2010) from different parts of Iran. In spite of being pursued for more than one century, a definitive diagnostic technique is not available yet (Padilla Poester et al 2009). The diagnosis of brucellosis is usually performed by a combination of methods. As the sensitivity of the bacterial culture is very low and depends on many factors such as disease stage, culture medium and availability of viable bacteria in the samples (Yagupsky 1999, Mantur & Mangalgi 2004), serodiagnosis is carried out by detection antibody generated against brucella immunogenetic proteins. Furthermore, it is time consuming and not routinely performed (Ghodasara et al 2010). Serological tests which, although rapid, often lead to false positive reaction (Wen-xing et al 2011). Vaccination may be the most economical means of controlling the brucellosis (Cassataro et al 2007b). The attenuated strains such as *B. melitensis* Rev 1, *B. abortus* S19 and RB51 are used to control brucellosis in domestic animals. These currently available live vaccines present serious side effects, which restrict their use as ideal vaccines. The most crucial of these drawbacks is that the live vaccines, whilst attenuated, remain virulent for human and cattle (Vahedi et al 2011, Mantur & Amarnath 2008). In addition, the vaccination is not fully efficacious and interferes with the diagnosis of field infection. The identification of *Brucella* spp. protective antigens is essential for the development of future subcellular vaccines, which avoid the drawbacks of live attenuated vaccines. The outer membrane proteins (OMPs) of *Brucella* spp. were initially identified in the early 1980s (Dubray & Bezar 1980) and have been extensively characterized as potential immunogenic and protective antigens (Cloekaert et al 1995; Cassataro et al 2007a). The major OMPs were classified according to their apparent molecular mass (Cloekaert et al 1992). Three major OMPs, Omp25 (25-27 kDa), Omp2b (36-38 kDa) and Omp31 (31-34 kDa) have been identified (Dubray & Bezar 1980, Verstrete et al 1982, Cloekaert et al

2002). Previously, CP28, BP26 or Omp28 from *Brucella* has been identified independently by three research groups as an immunodominant antigen in infected cattle, sheep, goats, and humans (Wen-xing et al 2011, Gupta et al 2010, Thavaselvam et al 2010). These characteristics support Omp28 as a promising subunit for detection kit and vaccine candidate against brucellosis. To achieve a new vaccine and diagnostic tools, specially to produce a recombinant vaccine and antigen, initial practical steps including isolation, extraction and purification of the vaccine candidates are necessary. So, in this study the *B. melitensis* bp26 gene was selected and expressed in *E. coli* BL21(DE3) as a host.

MATERIALS AND METHODS

Bacterial strain. *B. melitensis* strain 8594 was procured from the Karaj Razi vaccine and serum Research institute.

PCR amplification. The genomic DNA was purified from the bacterial culture according to the phenol/chloroform protocol (Matrone et al 2009). The purity and quantity of the purified genome was estimated using a spectrophotometer. Then, a primer pair was designed based on the known sequence of omp28 gene in the gene bank for amplification of *B. melitensis* bp26 gene, including 5'-AGGATCCATG AACACTCGTGCTAG-3' (forward) and 5'-AAGCTTC TTGATTTCAAAAACGAC-3' as reverse. The PCR was conducted in a final volume of 25 µl as shown in a table 1 and programmed at a denaturation 94 °C for 1 min, annealing 60 °C for 1 min, and polymerization 72 °C for 1 min for 34 cycles with Extra polymerization in 72 °C for 10 min. The PCR product consisting 748 bp was checked using agarose gel electrophoresis and then purified using a Fermentas Silica Bead DNA Gel Extraction Kit.

Cloning and transformation. The expected gene was cloned into PTZ57R/T vector and transformed in to *E. coli* DH5α competent cells and then the transformation mixture was spread on Luria-Bertani (LB) agarose culture medium containing isopropyl-

beta-D-thiogalactoside (IPTG), X-gal and ampicillin (50 µg/ml). The transformed colonies were screened desired gene performing colony PCR. After verification, the recombinant plasmid was extracted and digested with two restriction enzymes (BamHI and HindIII Fermentas Fast Digestion Enzyme). The bp26 gene was ligated into pET28a vector and then transformed in to *E. coli* BL21 (DE3).

Induction of expression and purification of recombinant protein. One recombinant colony inoculates in to 10 ml LB containing kanamycin and grew at 37°C overnight. One millilitre of the culture inoculated in to 60 ml fresh LB medium and grown at 37°C overnight with vigorous shaking. After reaching OD600 to 0.4-0.6, IPTG (1.0 mM) was added. All induced cell as well as noninduced cell were pelleted at 4,000 x g for 15 min and stored at -20°C for next uses. The pellets lysed in sample buffer and the suspension were boiled for 10 min and analysed on 10% SDS-PAGE. Protein Purification of the cell lysate was carried out using a Qiagen Ni-NTA column of chromatography against His tag with a denaturation purification protocol as specified by the manufacturer. The purified protein was analysed on SDS-PAGE.

RESULTS

The concentration of *B. melitensis* genomic DNA was 803 µl/ng which was used as a template for bp26 gene amplification. So, the size of PCR product subjected on 1% agarose gel has accordance with the part of bp26 gene size (~747) in gene bank (Figure 1). The transformed DH5α colonies (with recombinant PTZ57R/T vector) were confirmed and selected for having bp26 gene using colony PCR method and the product was analysed on 1% agarose gel (Figure 2). The recombinant PET28a having bp26 gene transformed in to the BL21 (DE3) and confirmed by colony PCR method as shown in Figure 3. The recombinant PET28a sequenced and results showed that Iranian *B. melitensis* bp26 gene was completely identical to *B. melitensis* bp26 gene sequence in Gene bank. Then results (Figure 4) suggested 1 mM IPTG

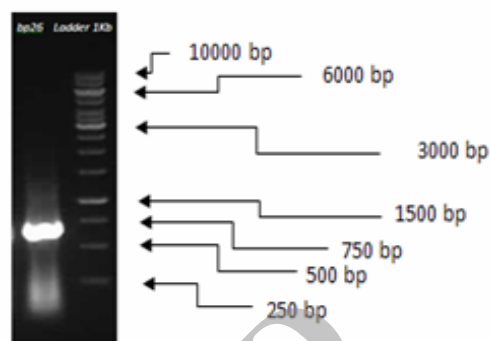


Figure 1. The part of bp26 gene size which amplified on 1% agarose gel: Lane 1; bp26 gene, Lane 2; Fermentas 1 Kb DNA Ladder.

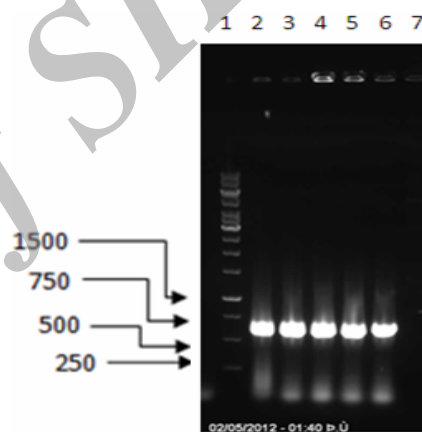


Figure 2. colony PCR from random selected DH5α white colonies on 1% agarose gel: Lane 1; Fermentas 1 Kb DNA Ladder, Lane 2,3,4 and 5; positive colonies, Lane 6,7 and 8; negative colonies.

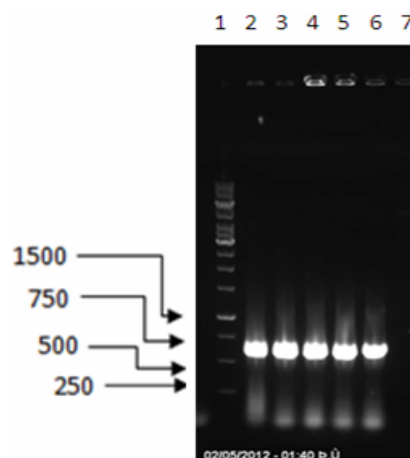


Figure 3. colony PCR from 6 random selected colonies on 1% agarose gel: Lane 1; Fermentas 1 Kb DNA Ladder, Lane 2, 3, 4, 5 and 6; positive colonies.

and 3 hr. of induction under shaking condition as optimized for expression (Figure 4).

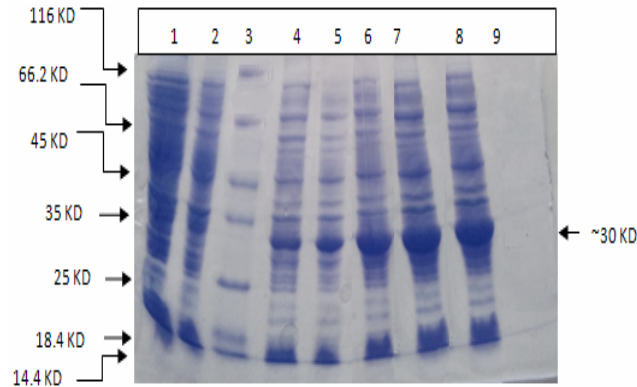


Figure 4. bp26 gene expression on SDS-PAGE: Lane 1; protein expression in transformed BL21(DE3) with PET28a vector before adding IPTG, Lane 2; transformed BL21 (DE3) with PET28a vector 1 hour after adding IPTG, Lane 3; Fermentas protein Ladder, Lane 4; transformed BL21 (DE3) with recombinant PET28a vector before adding IPTG, Lane 5; 1 hour after adding IPTG, Lane 6; 2 hour after adding IPTG, Lane 7; 3 hour after adding IPTG, Lane 8; 5 hour after adding IPTG, Lane 9; supernatant

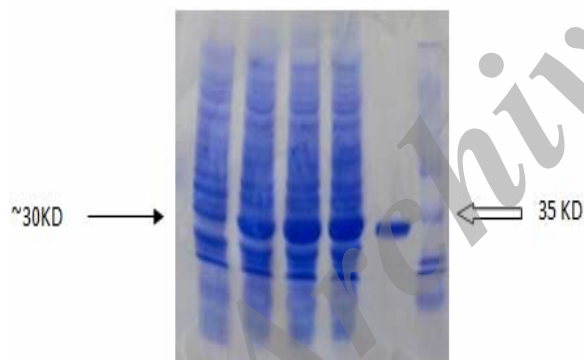


Figure 5. rBP26 protein expression on SDS-PAGE: Lane 1; transformed BL21 (DE3) with recombinant PET28a vector before adding IPTG, Lane 2; 1.5 hour after adding IPTG, Lane 3; 2 hour after adding IPTG, Lane 4; 5 hour after adding IPTG, Lane 5; purified protein, Lane 6; Fermentas protein Ladder.

DISCUSSION

In the absence of an effective isolation procedure, serological tests, such as ELISA methods and agglutination tests are relied on for the clinical diagnosis of brucellosis. Several studies have compared the tests available for the diagnosis of brucellosis

(Farazi & Hosseini 2012). As, cross-reaction with other bacteria can be occurred in the serological tests (Varshochi *et al* 2010, Vrioni *et al* 2007) So, since 1996 scientists attempted to segregate and express *Brucella* spp OMPs to get the recombinant protein antigens. Previous studies demonstrated that BP26 protein can be immunodominant antigen in infected cattle, sheep, goats and humans (Wen-xing *et al* 2011, Gupta *et al* 2010, Thavaselvam *et al* 2010). The use of rBP26 protein instead of the protein extracted from *Brucella* spp. for the diagnosis of brucellosis has at least two advantages: (a) preparation of the rBP26 protein is less time consuming and has a high yield, and (b) the high risk incurred by laboratory personnel when manipulating pathogenic *Brucella* spp. is avoided (Wen-xing *et al* 2011, Gupta *et al* 2010, Thavaselvam *et al* 2010). In the study, high level expression of 6xHis Taged protein Bp26 has been carried out by using PET vector based on T7 promoter transcription-translation system in *E.coli*. The cloning of the bp26 gene in the PET28a expression system led to the expression of a protein with size of approximately 30 kDa that was similar to other studies published in pubmed (Thavaselvam *et al* 2010). The nucleotide sequence of the achieved gene also confirmed the integrity of the cloning in comparison of the Gene Bank data base (data not shown). In spite of many previous studies that shown expression protein in a soluble form (Gupta *et al* 2010, Thavaselvam *et al* 2010) but, in the study the expressed protein was insoluble so that purification was done under denature condition. On cloning in this system, 6His tag was also coexpressed with the complete Omp28 protein so that makes easy to achieve purification of the protein using a Ni-NTA agarose columns. The results suggested that an IPTG concentration of 1 mM and 3 h of incubation under shaking conditions was optimum for expression of the protein. Hence, the rOmp28 was purified successfully and could be used as a antigen in diagnostic tools and a vaccine candidate for future studies. Recombinant BP26 protein production from *B. melitensis* isolated in

Markazi province using PET28a vector is possible and available for future studies.

Acknowledgment

The authors thank Dr Behrozikhah, the head of Brucella Department of Razi vaccine and serum Research institute, Karaj, for providing the Markazi province *B.melitensis* isolate and all of persons in Razi vaccine and serum Research institute, Arak Branch, for their constant support, guidance and inspiration.

References

- Abbas, B. A. & Aldeewan, A. B. (2009). Occurrence and epidemiology of *Brucella* SPP. In raw milk samples at Basrah province IRAQ. *Bulgarian Journal of Veterinary Medicine* 12: 136-142
- Akbarmehr, J. & Ghiyamirad, M. (2011). Serological survey of brucellosis in livestock animals in Sarab City (East Azarbayjan province, Iran). *African Journal of Microbiology Research* 5: 1220-1223.
- Álvarez, J., Luis, S.J., Garcia, N., Serrat, C., Pérez-Sancho, M., Gonzalez, S. et al. (2010). Management of an outbreak of brucellosis due to *B. melitensis* in dairy cattle in Spain. *Research in Veterinary Science* doi:10.1016/j.rvsc.
- Cassataro, J., Pasquevich, K.A., Estein, S.M., Laplagne, D.A., Velikovskiy, C.A., de la Barrera, S., Bowden, R., Fossati, C.A., Giambartolomei, G.H. & Goldbaum, F.A. (2007b). A recombinant subunit vaccine based on the insertion of 27 amino acids from Omp31 to the N-terminus of BLS induced a similar degree of protection against *B. ovis* than Rev.1 vaccination. *Vaccine* 25: 4437-4446.
- Cassataro, J., Pasquevich, K.A., Estein, S.M., Laplagne, D.A., Velikovskiy, C.A., de la Barrera, S., Bowden, R., Fossati, C.A., Giambartolomei, G.H. & Goldbaum, F.A. (2007a). A DNA vaccine coding for the chimera BLSOmp31 induced a better degree of protection against *B. ovis* and a similar degree of protection against *B. melitensis* than Rev.1 vaccination. *Vaccine* 25: 5958-5967.
- Cloekaert, A., Vizcaino, N., Paquet, J.Y., Bowden, R.A. & Elzer, P.H. (2002). Major outer membrane proteins of *Brucella* spp: past, present and future. *Elsevier Veterinary microbiology* 90: 229-247.
- Cloekaert, A., Zygmunt, M.S., de Wergifosse, P., Dubray, G. & Limet, J.N. (1992). Demonstration of peptidoglycan-associated *Brucella* outer-membrane proteins by use of monoclonal antibodies. *Journal of General Microbiology* 138: 1543-1550.
- Cloekaert, C., Jacques, I., Limet, J.N. & Dubray, G. (1995). Immunogenic properties of *Brucella melitensis* cell-wall fractions in BALB/c mice. *Journal of Medical Microbiology* 42: 200-208.
- Dubray, G. & Bezar, G. (1980). Isolation of three *Brucella abortus* cell-wall antigens protective in murine experimental brucellosis. *Annales de Recherches Veterinaires* 11: 367-373.
- Farazi, A.A. & Hoseini, S.D. (2012). Diagnostic validity of the conventional brucellosis serological tests in. *Arak Medical University Journal* 14(6, Supplement 3): 71-77
- Godfroid, J., Nielsen, K. & Saegerman, C. (2010). Diagnosis of Brucellosis in Livestock and Wildlife. *Croatian Medical Journal* 51: 296-305.
- Gupta, V.K., Kumari, R., Vohra, J., Singh, S.V. & Vihan, V.S. (2010). Comparative evaluation of recombinant BP26 protein for serological diagnosis of *Brucella melitensis* infection in goats. *Small Ruminant Research* 93: 119-125
- Gupta, V.K., Kumari, R., Vohra, J., Singh, S.V. & Vihan, V.S. (2007). Serological diagnostic potential of recombinant outer membrane protein (Omp31) from *Brucella melitensis* in goat and sheep brucellosis. *Small Ruminant Research* 70: 260-266.
- Kaveh, M. (1952). Brucellosis in general. *Journal of the Faculty of Veterinary Medicine* 1: 15-66.
- Kazemi, B., YousefiNamin, S.A., Dowlatshahi, M., Bandepour, M., Kafilzadeh, F., Gachkar, L., Mahmoudinejad, F., Samarghandi, A. & Mardani, M. (2008). Detection of *Brucella* by peripheral blood PCR and comparison with culture and serological methods in suspected cases. *Iranian Journal of Public Health* 37: 96-102.
- Khadjeh, G., Zowghi, E. & Zarif-fard, M.R. (1999). Incidence of brucellosis in one-humped camels of Boushehr, Iran. *Archives of Razi Institute* 50: 83-86.
- Mantur, B.G. & Amarnath, S.K. (2008). Brucellosis in India. *Journal of Biosciences* 33: 539-547
- Mantur, B. G. & Mangalgi, S. S. (2004). Evaluation of conventional Castaneda and lysis centrifugation blood culture techniques for diagnosis of human brucellosis. *Journal of Clinical Microbiology* 42: 4327-4328
- Matrone, M., Keid, L.B., Rocha, V.C.M., Vejarano, M.P., Ikuta, C.Y., Rodriguez, C.A.R., Ferreira, F., Dias, R.A. & Ferreira Neto, J.S. (2009). Evaluation of DNA extraction protocols for *Brucella abortus* PCR detection in aborted fetuses or calves born from cows experimentally infected with strain 2308. *Brazilian Journal of Microbiology* 40: 480-489.

- Mikeljon, P.N., Warren, R.L., Lindler, L. E. & Izadjoo, M.J. (2010). Attenuation of defined *Brucella melitensis* wboA mutants. *Vaccine* 28S: F12–F16.
- Najafi, N., Ghassemian, R., Davoody, A.R. and Tayebi, A. (2010). An unusual complication of a common endemic disease: clinical and laboratory aspects of patients with *brucella* epididymoorchitis in the north of Iran. *BMC Research Notes* 4:286
- Nowroozi-Asl, A., Oliaei, A. & Poormahmood-Shalgahian, M. (2007). A serological survey of *Brucella* spp. in water buffalo in Khoozestan province, Iran. *Italian Journal of Animal Science* 6: 825-827.
- Padilla, P.F., Nielsen, K., Ernesto, S.L. & Ling, Y.W. (2010). Diagnosis of Brucellosis. *The Open Veterinary Science Journal* 4: 46-60.
- Seleem, M.N., Stephen, M.B. & Sriranganathan, N. (2010). Brucellosis: A re-emerging zoonosis. *Veterinary Microbiology* 140: 392–398.
- Tahamtan, Y., Namavari, M.M., Mohammadi, G. & MoazeniJula, G. (2010). Prevalence of brucellosis in horse north-east of Iran. *Journal of Equine Veterinary Science* 30: 376-378.
- Thavaselvam, D., Kumar, A., Tiwari, S., Mishra, M. & Prakash, A. (2010). Cloning and expression of the immunoreactive *Brucella melitensis* 28 kDa outer-membrane protein (Omp28) encoding gene and evaluation of the potential of Omp28 for clinical diagnosis of brucellosis. *Journal of Medical Microbiology* 59: 421–428.
- Vahedi, F., Talebi, A. F., Ghorbani, E., Behroozikhah, A. M., Shahriari Ahmadi, F. & Mahmoudi, M. (2011). Isolation, cloning and expression of the *Brucella melitensis* Omp31 gene. *Iranian Journal of Veterinary Research, Shiraz University* Vol. 12, No. 2: 156-157.
- Varshochi, M., Majidi, J., Amini, M., Ghabili, K. & Mohammadali, M.shoji. False positive seroreactivity to brucellosis in tuberculosis patients: a prevalence study. *International Journal of General Medicine* 2011; 4: 207–210.
- Verstrete, D.R., Creasy, M.T., Caveney, N.T., Baldwin, C.L., Blab, M.W. & Winter, A.J. (1982). Outer membrane proteins of *Brucella abortus*: isolation and characterization. *Infection and Immunity* 35: 979-989.
- Vrioni, G., Kouvardas, S., Pagarliota, A., Charalampaki, N., Tarpatzi, A. & Zerva, L. (2007). Serologic diagnosis of human brucellosis using Wright, Rose Bengal, *Brucella* capt and Elisa tests in a nonendemic area in Greece. 17th European Congress of Clinical Microbiology and Infectious Diseases ICC, Munich, Germany, 31 Mar - 04 Apr 2007.
- Yagupsky, P. (1999). Detection of *Brucellae* in Blood Cultures. *Clinical Microbiology* 37(11): 3437–3442.
- Young, E. J. (1995). An overview of human Brucellosis. *Clinical Infectious Disease* 21:283-287.
- Wen-xing, Liu., Sen, Hu., Zu-jian, Qiao., Wei-ye, Chen., Lin-tao, Liu., Fang-kun, Wang., Rong-hong, Hua., Zhi-gao, Bu. & Xiang-rui, Li. (2011). Expression, purification, and improved antigenic specificity of a truncated recombinant bp26 protein of *Brucella melitensis* M5-90: a potential antigen for differential serodiagnosis of brucellosis in sheep and goats. *International Union of Biochemistry and Molecular Biology* 58: 32–38.
- Zowghi, E. and Ebadi, A. (1985). Naturally occurring *Brucella melitensis* infection in cattle in Iran. *Revue scientifique et technique* 4: 811-814.
- Zowghi, E., Ebadi, A. & Yarahmadi, M. 2008. Isolation and identification of *Brucella* organisms in Iran. *Iranian Journal of Clinical Infectious Diseases* 3: 185-188.