Polish Journal of Microbiology
2009, Vol. 58, No 2, 99–104

ORIGINAL PAPER

Expression of Human Serum Albumin – L7/L12 (*Brucella abortus* Ribosomal Protein) Fusion Protein in *Saccharomyces cerevisiae*

IRAJ PAKZAD[1], ABBAS REZAEE[2], MOHAMMAD EMANEINI[3], AHMAD ZAVARAN HOSSEINI[2], BAHMAN TABBARAEE[4] and MOROVAT TAHERIKALANI[1]*

[1] Department of Microbiology, School of Medicine, Ilam University of Medical Sciences, Ilam, Iran
[2] Department of Bacteriology, Medical Sciences Faculty, Tarbiat Modares University
[3] Department of Microbiology, School of Medicine, Tehran University of Medical Sciences
[4] Department of Bacterium Vaccine, Pasteur Institute of Iran

Received 24 August 2008, revised 3 April 2009, accepted 5 April 2009

Abstract

Brucella abortus is a facultative intracellular gram-negative bacterial pathogen that causes abortion in pregnant cattle and undulant fever in humans. The immunogenic *B. abortus* ribosomal protein L7/L12 is a promising candidate antigen for the development of subunit vaccines against brucellosis. It has already been expressed in several bacteria and has been used as DNA vaccine. In order to construct yeast expressing vector for the tHSA-L7/L12 fusion protein, the *l7/l12* ribosomal gene was amplified by PCR. The expression plasmid pYtHSA-L7/L12 was constructed by inserting the L7/L12 gene into the pYHSA5 shuttle vector (containing inulinase signal sequence, HSA gene and Gal10 promoter). The recombinant vector was transformed into *S. cerevisiae* and was then induced by galactose. The secreted recombinant fusion protein was detected in supernatant by SDS-PAGE and confirmed by western blot analysis using anti-HSA and anti-L7/L12 antibodies. Fusion protein was purified by affinity chromatography and its amount was approximately 500 µg /liter.

Key words: *Brucella abortus*, gene expression in *Saccharomyces cerevisiae*, human serum albumin, L7/L12

Introduction

Brucella abortus is a facultative intracellular gram-negative bacterium that infects humans and domestic animals. Brucellosis is an important zoonotic disease that causes abortion in cattle, and undulant fever in humans. Brucellosis is considered a major health problem in most the countries all over the world (Moreno *et al*., 2002).

To control brucellosis in human, it is necessary to control it in animal first. At present, live attenuated *B. abortus* strain S19 is used to immunize animals; however, the vaccine has the three following major disadvantages. First, S19 can mainly cause abortion when administered to pregnant cattle; second, S19 is pathogenic for human; third, the vaccine induces antibodies in vaccinated cattle, a false manifestation that may be mistaken for field infection diagnosis. Therefore, developing a more effective and safer vaccine is necessary to control the disease (Schurig *et al*., 2002).

Protection against *B. abortus* is considered to be depended on cell mediate immunity. The *B. abortus* L7/L12 ribosomal protein has been identified as a major component in cellular immunity response (Ko and Splitter, 2003; Cloeckaert *et al*., Oliveira and Splitter, 1994; 1996). Lipopolysaccharide (LPS) of *Brucella* is an important component in humeral immunity to brucellosis (Huang *et al*., 1999).

A noninfectious vaccine to humans but effective in stimulating a broad protective immune response is needed to control brucellosis. To develop this type of *Brucella* vaccine, several research groups are pursuing different strategies, including development of subunit vaccines (Oliveira and Splitter, 1996), utilization of bacterial vectors (Onate *et al*., 1999), and overexpression of protective homologous antigen (Vemulapalli *et al*., 2000).

The *B. abortus* L7/L12 ribosomal protein has been identified as an immunodominant antigen (Oliveira and Splitter, 1994). The recombinant L7/L12 protein

* Corresponding author: M. Taherikalani, Department of Microbiology, School of Medicine, Ilam University of Medical Sciences, Banganjab, I.R. of Iran, Ilam; phone: (+98) 841-223-5747; fax: (+98) 841-222-7136; **e-mail: taherikalani@gmail.com**

and the plasmid encoding the L7/L12 gene have demonstrated to be able to elicit strong CMI and engender protection from *Brucella* infection in mice; however, the protective effect is much lower than that of the live attenuated *B. abortus* vaccine S19 (Moreno *et al.*, 2002; Oliveira and Splitter, 1996 and 1994). Researchers have also observed the protective role of other types of L7/L12 based-vaccines utilizing different vectors such as vaccinia virus and *Lactococcus lactis*, nevertheless the protection level is far less than that of the currently used live *B. abortus* vaccine (Baloglu *et al.*, 2005; Pontes *et al.*, 2003, Ribeiro *et al.*, 2002). These results suggest that vaccines based on L7/L12 alone could not induce enough protection, regardless of the type of L7/L12 vaccine used. However, other evidence shows that polyvalent vaccines, including protein vaccines and DNA vaccines, can engender more effective protection than univalent vaccines in some cases (Johnston and Bystryn, 2005; Schirmbeck *et al.*, 2002). Thus, polyvalent vaccines combining L7/L12 with other immunogenic antigen(s) such as lipopolysaccharide (LPS) of *Brucella*, which is an important component in humeral immunity to brucellosis, will be a strategy to offer higher protection levels for *Brucella* infection (Huang *et al.*, 1999), One of the best subunit vaccine is a combination, a complex or a conjugation of L7/L12 with LPS.

Considering affinity of LPS for human serum albumin (HSA) (Fujiwara and Amisaki, 2008), in this research in order to develop a complex vaccine, we fused *l7*/*l12* gene to *hsa* and constructed a shuttle vector pYHSA5-L7/L12 (containing, inulinase signal sequence of *Kluyveromyces marxianus*, human serum albumin cDNA, *l7*/*l12* gene, Gal10 promoter), and expressed in *S. cerevisiae*.

Experimental

Materials and Methods

Strains, plasmids and media. *B. abortus* S19 was provided by Dr. B.Tabbaraee (Pasteur Institute of Iran) and was grown on Brucella broth (Difco, USA) at 37°C for 72 h. *S. cerevisiae* was the yeast strain used as the host for expression of fusion protein (truncated HSA-L7/L12) 2805(Matα pep4::His3 prb-Δ1.6R can1 his3-20 ura3-52). The yeast was cultured in a rotary incubator (200 rpm) at 30°C in SC-Ura, where the uracil deficient complete synthetic medium contained 2% glucose (Difco, USA). *Escherichia coli* DH5α (Stratagene, USA) (*f*-gyr A96 Nlar, recA1Thi-1 hsdR17 r-k m+k) was used for cloning, sequencing and maintaining various DNA fragments. The medium Luria Bertani (LB) (QUELAB, UK) was used for culturing *E. coli*. Shuttle vector pYHSA5 contained inulinase signal sequence (*Kluyveromyces marxianus*), human serum albumin cDNA, Gal10 promoter, and ura3 selective marker (Kang *et al*., 2000). The yeast transformation was carried out by the modified lithium acetate method (Hill *et al*., 1991; Rezaee *et al*., 2005).

Amplification of the *B. abortus l7*/*l12* ribosomal gene by PCR. According to L7/L12 nucleotide sequence (Gene Bank accession Number L19101), and nucleotide sequence of pYHSA5 shuttle vector, primers were synthesized by MWG Company (Germany). These primers create two XbaI site at 5' and 3' ends of the gene. *B. abortus* S19 DNA chromosomal was extracted (Cheng *et al*., 2006) and *l7*/*l12* gene was amplified by pfu polymerase (Fermentase) at 2.5 mM $MgSO_4$ (Fermentase), 0.5 ρmol primer/1λ reaction, annealing temperature 55°C in thermal cycler (FGEN02TD Techneh, UK). The PCR product was purified by PCR product Gel extraction kit (Roche) and confirmed by EcoRI and HindIII. The PCR product was digested with XbaI and ligated to pYHSA5 plasmid. Direction of *l7*/*l12* gene in pYtHSA-L7/L12 was determined by enzyme digestion along with sequencing. PYtHSA5-l7/L12 plasmid and PCR product were sequenced by MWG Company (Germany).

Media and culture condition for expression. YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose) was used for cultivation of the host and yeast transformation. Yeast Nitrogen Base without uracil (Difco) was used for selecting the yeast transformant and seed culture (Rezaee *et al*., 2001). For induction of tHSA-L7/L12 expression, the yeast transformants were grown in shake-flasks containing YPDG media (1% yeast extract, 2% Bactopeptone, and 1% glucose at varying concentration of Galactose). The shake-flask cultures were performed at 30°C and 150rpm (Kang *et al*., 2000).

Immunoblotting. Western blot analysis of culture supernatant of yeast cells was carried out using polyclonal antibodies raised against human serum albumin and anti-L7/L12 ribosomal protein antibodies (a donation from Dr. Philippe Langella, France)

Protein purification. First, the supernatant of induced cells was collected by centrifugation (1300×g, 3 min) containing phenylmethylsulphonyl fluoride (PMSF, Merck), then the expressed protein from the supernatant was precipitated by 60% ammonium sulfate (Merck); second, it was dialyzed against water, and last it was purified by affinity chromatography column CN-Br activated sepharose CL-4B (Biogen) and anti-HSA antibody.

Results

Construction of pYtHSA-L7/L12 shuttle vector. PCR was used successfully to amplify a gene fragment encoding the sequence of L7/L12. PCR product has about 400 bp on 1% agarose gel electrophoresis

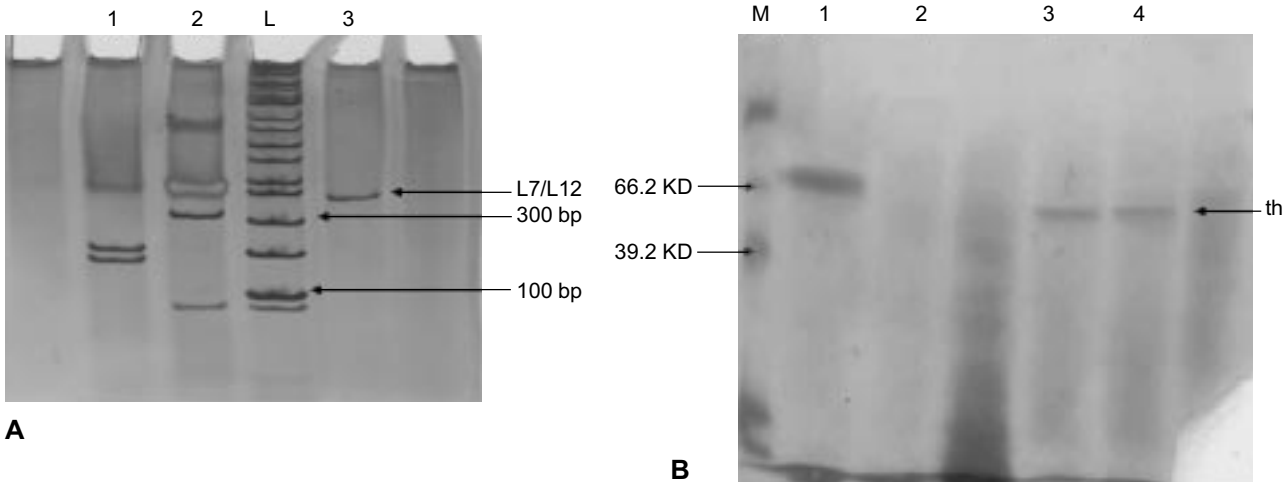

Fig. 1. (A) – Electrophoresis of PCR product on 1% agarose gel (lane 3), PCR product digestion by EcoRI (lane 1) and HindIII (lane 2); (B) – silver-stained SDS-PAGE of culture supernatant from induced and non induced cells, M-protein molecular weight marker, HSA recombinant was produced by *S.cerevisiae* with pYHSA5 (lane 1), (lane 2) – strain 2805 of *S. cerevisiae* as negative control, the culture supernatants at 0 h (lane 2), 24 h and 36 h (lanes 3, 4). The arrows indicate the fusion protein after 24 h and 36 h of growth.

(Fig. 1, A; lane 3). For preliminary confirmation, the fragment was subjected to digestion by EcoRI and HindIII. Digestion with EcoRI yielded 165 and 210 bp fragments, while digestion with HindIII yielded 75 and 300 bp fragments at the expected positions (Fig. 1, A, lanes 1, 2). Ligation of PCR product in pYHSA5 was confirmed by XbaI digestion. The sequencing of PCR product fragment revealed complete homology at the nucleotide level to *l7/l12* gene in NCBI.

Extracellular production of fusion protein tHSA-L7/L12 in *S. cerevisiae*. The pYtHSA-L7/L12 shuttle vector has signal sequence, thus, the product was expected to secrete into the extracellular. Culture supernatant from the induced and noninduced cells was done by electrophoresis on SDS-PAGE and stained by silver nitrate staining (Fig. 1, B). *S. cerevisiae* strain 2805 was used as a negative control. Optimum condition of culture for the high level expression of fusion protein was glucose 1%, and galactose 2% at 24, 36 h. (Fig. 1, B). In samples from induced culture supernatant a band of molecular mass ~52 KD was present (Fig. 1, B). Considering the length of two fused genes this weight was expected.

Western blotting of fusion protein. Anti-HSA and anti-L7/L12 antibody from rabbit was used for blotting (Fig. 2). The result show that the fusion protein could be detected by both the antibodies. This indicated that molecular structure of fusion protein

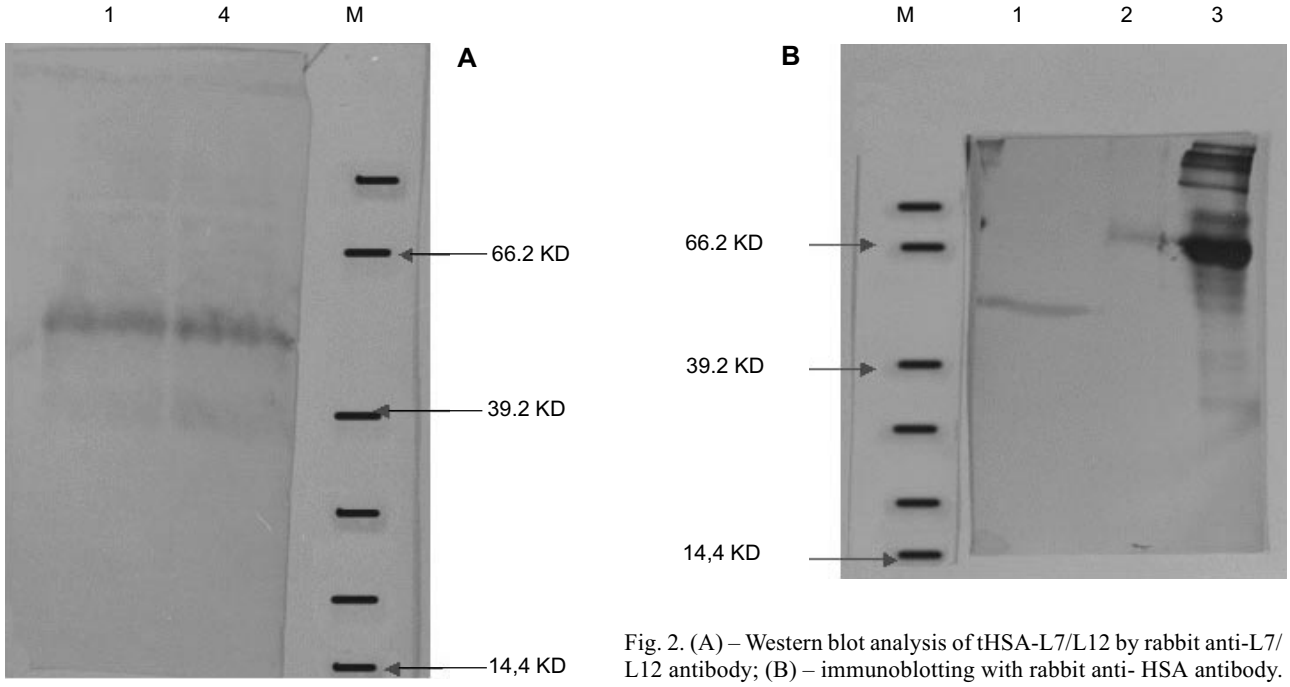

Fig. 2. (A) – Western blot analysis of tHSA-L7/L12 by rabbit anti-L7/L12 antibody; (B) – immunoblotting with rabbit anti- HSA antibody.

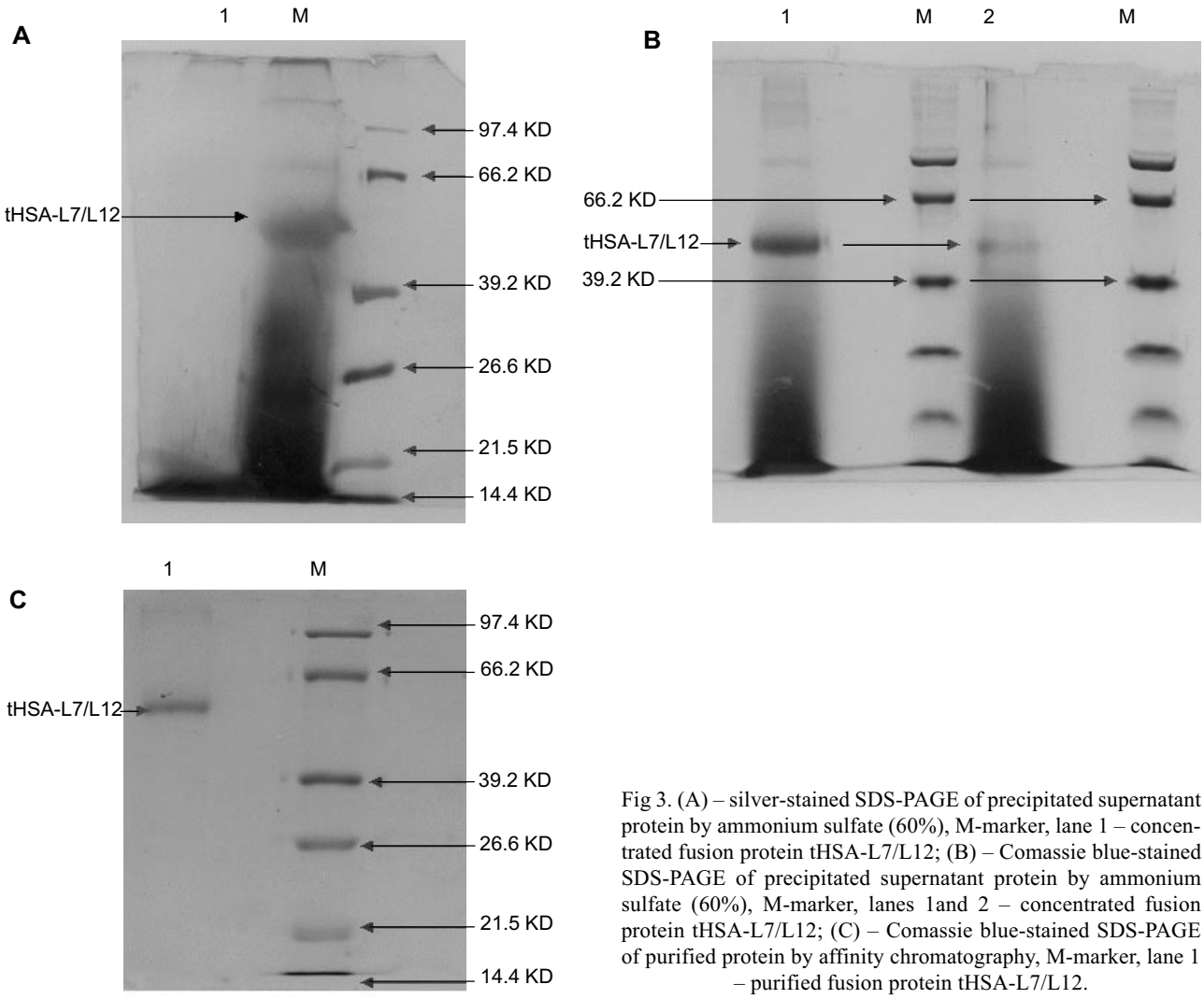

Fig 3. (A) – silver-stained SDS-PAGE of precipitated supernatant protein by ammonium sulfate (60%), M-marker, lane 1 – concentrated fusion protein tHSA-L7/L12; (B) – Comassie blue-stained SDS-PAGE of precipitated supernatant protein by ammonium sulfate (60%), M-marker, lanes 1and 2 – concentrated fusion protein tHSA-L7/L12; (C) – Comassie blue-stained SDS-PAGE of purified protein by affinity chromatography, M-marker, lane 1 – purified fusion protein tHSA-L7/L12.

was constructed in a form that that the two protein fragments were identifiable.

Extracellular protein purification. After precipitation of supernatant protein by ammonium sulfate (60%) and overnight dialyzing against water and purification by affinity chromatography, (Fig. 3) the amount of the purified fusion protein was demonstrated to be 0.5 mg/liter.

Discussion

To develop a new generation of vaccines to prevent brucellosis, it is vital to overcome the three main drawbacks of the currently used live *Brucella* vaccines, *i.e.*, causing abortion in pregnant animals, pathogenicity for humans, and inducing antibodies that interfere with the diagnosis of field infection in vaccinated animals (Cheville *et al*., 1993; Corner and Alton, 1981; Moriyon *et al*., 2004). Improvements in the methods for gene cloning and protein purification have led to the use of purified recombinant proteins as cellular vaccines in experimental trials (Oliveira and Splitter, 1996).

So far, a variety of protein and non-protein antigens of *Brucella* or a combination of them has been introduced as a vaccine (Vizcaino *et al*., 1996; Huang *et al*., 1999; Kang *et al*., 2000). Considering the point that immunity against brucellosis needs induction of both the two immune responses specially cellular immunity, as well as the fact that most the antigens of *Brucella structure* don't enjoy the ability to induce protective response on their own (Cloeckaert *et al*., 2002), it seems that an effective subunit vaccine will be considered to be a combination of several antigens (Ko and Splitter, 2003; Cloeckaert *et al*., 2002).

Outer membrane antigens, specifically LPS are exposed to direct contact with host immune system; therefore, antigens will be a main part of the probable subunit vaccine (Jacques *et al.*, 1991; Jacques, 1996; Cloeckaert *et al*., 2002). Recently, researchers have noticed the stimulating ability of cellular immune by cytoplasm proteins, specifically L7/L12. They recognize it as the most important protein of this bacteria

for stimulating cellular immunity. Immunity of L7/L12 recombinant protein has been demonstrated by researchers (Oliveira and Splitter, 1994 and 1995; Schurig *et al*., 2002). They recommend that a probable suitable subunit vaccine must contain a combination or a complex or a conjugation of LPS and L7/L12.

Considering the fact that polyvalent vaccines can induce a more intensive immune response than the univalent vaccine in some cases (Johnston and Bystryn. 2005; Schirmbeck *et al*., 2002), besides the idea that bacterial LPS has affinity for bonding with HSA (Fujiwara and Amisaki, 2008), we come to the concept of utilizing fusion protein advantages by producing a fusion protein, and bacterial LPS bonding with L7/L12 protein through the possible simplest method. Up to now, due to limitations such as glycosylations, only a few tendencies have expressed prokaryotic proteins in *S. cerevisiae*. However, there are some examples, such as RTEM-β-lactamases, *Staphylococcus aureus* nuclease A, TSST-1 and Shiga-like toxin I A-chain that are expressed in *S. cerevisiae* (Pines and London, 1991; Pines *et al*., 1988).

In this our study, tHSA-L7/L12 fusion protein was expressed. In order to construct pYtHSA-L7/L12 vector, first multiplied *l7*/*l12* gene was verified by enzyme digestion, then it was confirmed by sequencing. The sequence was exactly the same as that of *l7*/*l12* gene in NCBI gene bank.

The constructed strain containing pYtHSA5-L7/L12 secreted tHSA-L7/L12 fusion protein into the supernatant. The Bradford result of supernatant and purified protein showed (data not shown) that the amount of the production compared with the HSA expressed by the same vector was low. This fusion protein was confirmed by HSA and L7/L12 specific antibodies. This shows that precision of designing fusion protein gene and its expression in *S. cerevisiae* has retained antigenic characteristics. In addition, the finding shows that the nature of this fusion protein can be appropriated for immunological evaluation.

Conclusion. Regarding tHSA_L7/L12 fusion protein properties, this protein can be used with *Brucella* LPS as a new subunit vaccine. However, immunization assay requires more investigations with this fusion protein and LPS. It seems necessary to accomplish more studies to determine an established definition of the fusion protein and its combination as an give the immune response and a protection provider against *B. abortus* infection.

Acknowledgments

We are grateful to Kang who kindly gave us the plasmid pYHSA5; we also thank Philippe langella for kindly giving us the anti-L7/L12 antibodies. We would also like to thank Mr. Shohanizad for the edition of this manuscript.

Literature

Rezaee MA., A. Rezaee, S.M. Moazzeni, Y. Yasuda, K. Tochikubo, S.N. Pirayeh and M. Arzanlou. 2005. Expression of *Escherichia coli* heat-labile enterotoxin B subunit (LTB) in *Saccharomyces cerevisiae*. *J. Microbiol*. 43: 354–360.

Baloglu S., S.M. Boyle, R. Vemulapalli, N. Sriranganathan, G. G. Schurig and T.E. Toth. 2005. Immune responses of mice to vaccinia virus recombinants expressing either *Listeria monocytogenes* partial listeriolysin or *Brucella abortus* ribosomal L7/L12 protein. *Vet. Microbiol.* 109: 11–17.

Cheng HR. and N. Jiang. 2006. Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnol. Lett.* 28: 55–59.

Cheville N.F., M.G. Stevens, A.E. Jensen, F.M. Tatum and S.M. Halling. 1993. Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of *Brucella abortus*. *Am. J. Vet. Res.* 54: 1591–1597.

Cloeckaert A., N. Vizcaino, J.Y. Paquet, R. Bowden and P.H. Elzer. 2002. Major outer membrane proteins of *Brucella* spp: past, present and futur. *Vet. Microbiol*. 90: 229–247.

Corner L.A. and G.G. Alton. 1981. Persistence of *Brucella abortus* strain 19 infection in adult cattle vaccinated with reduced doses. *Res. Vet. Sci.* 31: 342–344.

Fujiwara S. and T. Amisaki. 2008. Identification of high affinity fatty acid binding sites on human serum albumin by MM-PBSA method. *Biophys J.* 94: 95–103.

Hill J., K.A. Donald, D.E. Griffiths and G. Donald. 1991. DMSO enhanced whole cell yeast transformation. *Nucleic Acids Res.* 19: 57–91.

Huang L., A.M. Krieg, N. Eller and D.E. Scott. 1999. Induction and regulation of Th1-inducing cytokines by bacterial DNA, lipopolysaccharid, and heat-inactived bacteria. *Infect. Immun*. 67: 6257–6263.

Johnston D. and J.C. Bystryn. 2005. Heterogeneous antibody response to polyvalent melanoma vaccines in syngeneic mice. *Cancer Immunol. Immunother.* 54: 345–350.

Jacques I., V. Olivier-Bernardin and G. Dubray. 1991. Induction of antibody and protective responses in mice by brucella O-polysaccharide-BSA conjugate. *Vaccine* 9: 896–900.

Jacques M. 1996. Role of lipooligosaccharides and lipopolysaccharide in bacterial adherence. *Trends. Microbiol*. 4: 408–410

Kang HA., E.S. Choi, W.K. Hong, J.Y. Kim, S.M. Ko, J.H. Sohn and S.K. Rhee. 2000. Proteolytic stability of recombinant human serum albumin secreted in the yeast *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 53: 575–582.

Ko J. and Splitter A.G. 2003. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and human. *Clin. Microbiol. Rev*. 16: 65–78.

Kurar E. and G.A. Splitter. 1997. Nucleic acid vaccination of *Brucella abortus* ribosomal L7/L12 gene elicits immune response. *Vaccine* 15: 1851–1857.

Moreno E., I. Moriyon. and A. Cloeckaert. 2002. *Brucella evolution* and taxonomy. *Vet. Microbiol*. 90: 209–227.

Moriyon I., M.J. Grillo, D. Monreal, D. Gonzalez, C. Marin, I. Lopez-Goni, R.C. Mainar-Jaime, E. Moreno and J.M. Blasco. 2004. Rough vaccines in animal brucellosis: structural and genetic basis and present status. *Vet. Res.* 35: 1–38.

Oliveira S. C. and G.A. Splitter. 1994. Subcloning and expression of the *Brucella abortus* L7/L12 ribosomal gene and T-lymphocyte recognition of the recombinant protein. *Infect. Immun.* 62: 5201–5204.

Oliveira S. C. and G.A. Splitter. 1995. CD8_ type 1 CD44hi CD45 RBlo T lymphocytes control intracellular *Brucella abortus* infection as demonstrated in major histocompatibility complex class I- and class II-deficient mice. *Eur. J. Immunol.* 25: 2551–2557.

Oliveira S.C. and G.A. Splitter. 1996. Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against *Brucella abortus* infection. *Vaccine* 14: 959–962

Oliveira S.C., Y. Zhu and G.A. Splitter. 1994. Recombinant L7/L12 ribosomal protein and gamma-irradiated *Brucella abortus* induce a T-helper 1 subset response from murine CD4_ T cells. *Immunology* 83:659–664.

Onate A.A., R. Vemulapalli, E. Andrews, G.G. Schurig, S. Boyle and H. Folch. 1999. Vaccination with live *Escherichia coli* expressing *Brucella abortus* Cu/Zn superoxide dismutase protects mice against virulent *B. abortus*. *Infect. Immun.* 67: 986–988.

Pines, O., and A. London. 1991. Expression and secretion of *Staphylococcus nuclease* A in yeast: effects of amino terminal sequences. *J. Gen. Microbiol*. 137: 771–778.

Pines, O., C.A. Lunn, and M. Inouye. 1988. Defective *E. coli* signal peptidase function in yeast. *Mol. Microbiol*. 2: 209–217.

Pontes, D. S., F. A. Dorella, L. A. Ribeiro, A. Miyoshi, Y. Le Loir, A. Gruss, S. C. Oliveira, P. Langella, and V. Azevedo. 2003. Induction of partial protection in mice after oral administration of *Lactococcus lactis* producing *Brucella abortus* L7/L12 antigen. *J. Drug Target.* 11: 489–493.

Ribeiro L.A., V. Azevedo, Y. Le Loir, S.C. Oliveira, Y. Dieye, J.C. Piard, A. Gruss and P. Langella. 2002. Production and targeting of the *Brucella abortus* antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. *Appl. Environ. Microbiol.* 68: 910–916.

Schirmbeck R., M. Kwissa, N. Fissolo, S. Elkholy, P. Riedl and J. Reimann. 2002. Priming polyvalent immunity by DNA vaccines expressing chimeric antigens with a stress protein-capturing, viral J-domain. *FASEB J.* 16:1108–1110

Schurig G.G., J.M. Corbel and N. Sriranganathan. 2002. Brucellosis vaccine: past, present and future. *Vet. Microbiol.* 90: 479–496.

Vemulapalli R., Y. He, S. Cravero, N. Sriranganathan, S.M. Boyle and G.G. Schurig. 2000. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. *Infect.Immun.* 68:3286–3289.

Vizcaino N., A. Cloeckaert, G. Dubray and M.S. Zygmunt. 1996. Cloning nucleotide sequence and expression of the gene coding for a ribosome releasing factor homologous protein of *Brucella melitensis*. *Infect. Immun*. 64: 4834–4837