Journal of Animal and Veterinary Advances 10 (23): 3085-3089, 2011
ISSN: 1680-5593

Immunologic Evaluation of Surface 18 kDa Protein of *Brucella abortus* S19 Cojugated with Detoxified Lps as an Antigen Useful for Human Brucellosis Vaccine in BALB/c Mice Model

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Abstract: Available vaccines against *Brucella* species are live attenuated Brucella strains with some disadvantages. So as to design an improved vaccine to be used in animals and humans, the researchers aim to engineer a subunit brucellosis vaccine. Immunologic evaluation of Outer Membrane Proteins (OMPs) of Brucella cells has a key role in development of prevention and diagnosis programs. Particularly, the researchers are interested in the 18 kDa outer membrane lipoprotein of *Brucella abortus* (Omp19) as it is most exposed to the host immune system and is anticipated to be capable of eliciting immune response. As the immunodominant antigen of smooth Brucellae is LPS, the principle agent of serodiagnosis of the infection, the researchers used detoxified Lps which loses fatty acid chain and includes compelet polysacharid fragment. The aim of this study is to produce a serological assessment of recombinant Omp19 (rOmp19) cojugated with detoxified Lps in a way to prevent brucellosis. *Brucella omp19* gene was cloned in pET28a (+) and expressed in *E. coli* BL21 (DE3) by 1 mM IPTG. Recombinant protein was purified using nickel resin and confirmed by western blot analysis using polyclonal antibodies and conjugated with detoxified Lps which was applied to detect antibodies in BALB/c mice brucellosis cases' serum samples via western-blotting. Overall these results indicate that rOmp19 conjugated with detoxified Lps would be a useful candidate for a subunit vaccine against human and animal brucellosis.

Key words: Brucellosis, *Brucella abortus* rOmp19, purification, detoxified LPs, vaccine, Iran

INTRODUCTION

Brucellae are facultative intracellular pathogen which cause worldwide brucellosis, the most important zoonoses that affect domestic animals and humans (Emslie and Nel, 2002). Animal infection causes abortion and reduces fertility in livestock which leads to significant economic losses all over the world (Chimana *et al*., 2010). Human brucellosis is known to have nonspecific manifestations such as undulant fever, osteomyelitis and arthritis (Buzgan *et al*., 2009). Brucellosis is typically acquired after consuming contaminated foods, especially unpasteurized dairy product or after occupational contact with infected animals (Franco *et al*., 2007). Thus, prevention of infected cases with improvement of new Brucella vaccines are challenging researchers from the standpoint of control and eradication. Generally, three vaccine strains (*Brucella abortus* S19 and RB51 strains and *Brucella melitensis* Rev1) are applied for the control of brucellosis in livestock (Davis and Elzer, 2002). The most important problems associated with the use of these vaccine strains are the residual virulence of these strains for humans and the development of agglutinating antibodies in animals vaccinated as adults which are indistinguishable from those elicited by natural infection. Also there is an ever being problem with immunoglobulines to Brucella LPS which cross react with that of certain Gram negative bacteria (Andrew *et al*., 1973; Erasmus and Bergh, 1985; Schurig *et al*., 1991). Efficient treatment of humans depends on elongated antibiotic therapy so, control of human brucellosis is dependent on control of animal infection and food products safety (Skalsky *et al*., 2008). With no available safe human vaccine, despite suggestion, the identification of Brucella antigens and eliciting immune responses would be of great importance to the development of subunit vaccines or diagnostic tests that avoid the disadvantages of currently used ones. OMPs are surface proteins acting as protective antigens. OMPs categorize according to apparent molecular mass, 88-94, 36-38 and 25-27 kDa. They are resistant to nonionic

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detergents and EDTA (Cloeckaert *et al*., 2002). In addition, there are several minor OMPs with low molecular weight in *B. abortus* (Omp19, Omp16.5 and Omp10) (Bowden *et al*., 2000; Tibor *et al*., 2002). Here, the researchers evaluated the immunologic feature of recombinant Omp19 conjugated with detoxified Lps as a vaccine against Brucellosis in BALB/c mice model. Omp19 or 18 kDa immonoreactive protein is a useful antigen for diagnosis of brucellosis (De Mot *et al*., 1996). This OMP has structural features of bacterial lipoprotein present in all 8 *Brucella* species and their biovars. The detection of antibodies to Omp19 of *Brucella abortus* is useful for the diagnosis of human and animal brucellosis. Because of the high immunogenicity of Omp19, it is potentially useful for designing brucellosis vaccines.

MATERIALS AND METHODS

B. abortus S19 was cultured on Brucella agar. *Escherichia coli* $DH_5\alpha$ and *E. coli* BL21 (DE3) were applied as cloning and expression prokaryotic hosts, respectively. The pJET1.2 (Fermentas) and pET28a (+) (Novagen) were applied for cloning and expression of target ORF, respectively. A primer pair was designed to amplify the whole coding region of Omp19 (ACCESSION: U35742) consisting of FB18F 5'-AACGGATCCATGGG AATTTCAAAAGCAAGTCTGCTC-3' and FB18R 5'-AA AAAGCTTTCAGCCCAACAGCGTCACGGCCTGC-3' including restriction sequences of BamHI and HindIII, respectively. Complete omp19 ORF was amplified with a high fidelity enzyme PrimSTAR® HS DNA polymerase. *Brucella abortus omp19* gene was amplified with PrimSTAR® HS DNA polymerase, cloned in pJET1.2. The pJET1.2 bearing the gene coding for *B. abortus* Omp19 was double digested with BamHI and HindIII and verified by sequencing and aligned with reference sequence. The corresponding gene was sub-cloned in pET28a (+). Recombinant pET28a (+)-omp19 vectors were transformed into *E. coli* BL21 (DE3). Expression of recombinant Omp19 was induced by adding 1 mM IPTG in OD_{600} 0.6. Samples of 3 h of incubation at 37°C were collected. Crude cells were lysed by adding sample buffer and boiling in 95°C water bath. Sample lysates were subjected to SDS-PAGE in mini gels (BioRad) with 15% polyacrylamid resolving gel of followed by Coomassie Brilliant Blue G-250 staining. Cell pellet of 2 L of 4 h inducted culture collected and was purified according to the universal protocol by QIAGEN manual (QIAexpressionist™). Briefly, cell pellet of 4 h induced BL21 harboring pET28-omp19 were resuspended in a lysis buffer (Buffer A) containing PMSF as antiprotease and sonicated. Cell debris were separated by centrifugation, NI-NTA resin (QIAGEN) were added to the supernatant and shacked for 1 h. Afterwards resins were collected on a cartridge and washed subsequently. Recombinant protein was eluted with 250 mM imidazole and elution reagent removed by dialysis against phosphate buffered saline. Purified recombinant Omp19 and crude induced BL21 cell lysate were separated on 15% polyacrylamide gel distinctly and electro-transferred to PVDF sheet using Bio Rad transblott system. Sheet was blocked by bovine serum albumin. The protein was probed by polyclonal rabbit antibodies to *Brucella abortus* outer membrane protein preparations. HRP conjugated anti-rabbit IgG applied to the sheet as the secondary antibody. Sheets were finally developed with diamino benzidine. Purified recombinant Omp19 were also subjected to western blot analysis and probed by 1:3000 dilutions of 10 serum samples from brucellosis patients. These were treated with HRP-anti-human IgG and developed as described above. The 20 μg of purified rOmp19 conjugated with detoxified Lps and combined with ferund's adjuvant used to immunize BALB/c mice.

Antigen was injected subcutaneously as a primary followed by two boosters with 2 weeks of intervals. Splenic lymphocytes were removed and cultured. Lymphocytic cultures were stimulated with 10 μg mL^{-1} of rOmp19.

RESULTS

Omp19 of *Brucella abortus* was successfully expressed in the PET28a (+) vector as inclusion bodies in *E. coli* BL21 (DE3) cells. *E. coli* BL21 containing the plasmid encoding the 18 kDa protein were grown and cultured and induced by IPTG 1 mM to express recombinant protein. Cloning and expression of rOmp19 make it available as a potential candidate for design of specific chemotherapeutic agents in further investigations. The inclusion bodies were solubilised in 8 M urea (Fig. 1) and the protein was refolded by dialysis

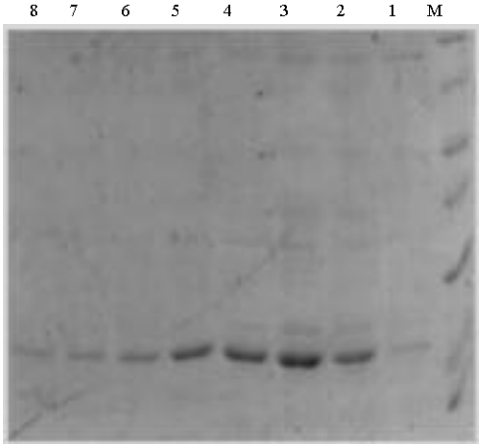

Fig. 1: rOmp19 was purified using Ni-NTA resin

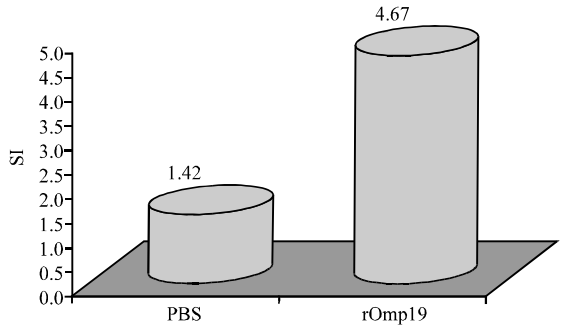

Fig. 2: Lymphocytic cultures were stimulated with 10 μg mL^{-1} of rOmp19. Lymphocyte proliferation response to the protein antigen was assessed via MTT test

against PBS. SDS-PAGE analysis of induced culture samples compared to non-induced one and non-transformed *E. coli* BL21 (DE3) shows the expression of a protein of approximately 20 kDa. This was consistent with the expected protein of 17.53 kDa plus pET28a (+) added amino acids. The expression reaches the maximum rate in 2 h after induction and remains unchanged in the next 2 h. Amidation method was used to conjugate the 18 kDa protein of *Brucella abortus* S19 with detoxified Lps and conjugated molecules purified from non one. The 20 μg of mentioned protein combined with ferund's adjuvant used to immunize BALB/c mice cultured. Lymphocytes were stimulated with 10 μg mL^{-1} of rOmp19 (Fig. 2). Lymphocyte proliferation response to the antigen was evaluated by MTT test and measure up to that of non-immunized mice lymphocytic cultures. Polyclonal antiserum successfully recognized rOmp19 in crude cell lysate. Purified recombinant Omp19 and the conjugated or combination forms successfully recognized by rabbit polyclonal anti serum and sera from 10 microbiologically confirmed hospitalized patients. This point out that epitops of rOmp19 is at least partially similar to the native component. These results obviously indicate that the conjugated form of rOmp19 with d-Lps shows the most protection rather than the other forms of the antigen.

DISCUSSION

Brucellosis is a worldwide distributed zoonotic disease which is endemic in most regions of the developing countries (Glynn and Lynn, 2008). Diagnosis of the disease is mainly according to the isolation of the pathogen from the blood samples which is time consuming because of slow growing of Brucellae (Giorgio *et al*., 2003). A common alternative is serological diagnosis in which mainly antibodies to LPS of the bacterium is recognized and has the deficiency of low specificity by cross-reacting with some gram negative species (Al-Attas *et al*., 2000). Prevention of the disease is only by animal vaccination. Live attenuated vaccines (*Brucella abortus* S19 and RB51 strains and *Brucella melitensis* Rev1) are licensed to prevent animal brucellosis. They gives short term immunity and interfers with serological tests (Shumilov *et al*., 2010). There is no immunization strategy for human. Although, the major antigen of smooth Brucellae is lipopolysaccharide (Gonzalez *et al*., 2008) the OMPs of *Brucella* species have shown broadly immunogenic properties in previous studies. They categorize to major Omps (Cloeckaert *et al*., 2002) and the minors, (Omp19, Omp16.5 and Omp10) according to molecular mass (Tibor *et al*., 1996). Researches on the protective ability of minor OMPs continues. Omp19 is an immunoreactive outer membrane lipoprotein of Brucellae and previously showed that it is recognized with serum antibodies of brucellosis patients (Hemmen *et al*., 1995). The researchers first produced the recombinant Omp19 in common host *E. coli* BL21 successfully. The results indicate that the recombinant Omp19, expressed in *E. coli* host retained its epitopes after purification at least partially that can interact with serum antibodies. Mice were immunized with 20 μg of recombinant protein combined with ferund's adjuvant subcutaneously at days 0 and two boosters with 2 weeks of intervals. The first group of BALB/c mice was injected with rOmp19 showed 1.35 log unit protection. Significantly different from PBS-immunized mice and S19 immunized mice as control group. In previous studies, Karina and the colleagues illustrated almost the same protection about 1.38 against *B. abortus* 544 in BALB/c mice immunized with L-Omp19+IFA (Pasquevich *et al*., 2009). The most important brucella antigen is Lps (Cardoso *et al*., 2006) with key role in immunogenisity but it is not usable directly and should be detoxified. On the other hand, they have T-cell independent immunity and are not able to cause immune memory. So, it's need the polysacharid structure is commbinated or conjugated with a carrior protein. With the same thought, Apurba Bhattacharjee and the colleagues showed that intranasal immunization with *B. melitensis* LPS as a noncovalent complex with *Neisseria meningitidis* group B outer membrane protein (LPS-GBOMP) subunit vaccine significantly protects mice against intranasal challenge with virulent *B. melitensis* (Bhattacharjee *et al*., 2002). So, the researchers immunized the second BALB/c mice group with rOmp19+detoxified LPs that showed 1.69 log unit protection. Winter and the colleagues had compared the difference of smooth and rough Lps, they vaccinated mice with a complex of porin and smooth lipopolysaccharide (porin-S-LPS) extracted from virulent *Brucella abortus* 2308 presented considerable protection, equal to that reached by vaccination with living attenuated *B. abortus* 19, on the contrary the vaccination

Table 1: Log protection unit of (Porin-S-LPS) and (Porin-R-LPS) extracted from virulent *Brucella abortus* 2308

Groups	Log unit protection	Clearance (%)
Porn+SLps	1.76	25
Porn+RLps	1.33	19

Table 2: Comparison of (Porin-S-LPS) and (Porin-R-LPS) extracted from virulent *Brucella abortus* 2308 as brucellosis vaccine with different forms of Omp19. The vaccine made up of long chains of OPS showed more effectiveness to short one. They both indicate less protection in comparison to conjugated form of rOmp19

Groups	Log unit protection	Clearance (%)
rOmp19	1.30	26
rOmp19+dLps	1.69	38
rOmp19-dLps	2.90	44

Table 3: Protection against *B. abortus* 554 in BALB/c mice immunized with L-Omp16 or L-Omp19 protein in IFA

	Protection against *B. abortus* 544 with the indicated vaccine dose			
	30 μg		10 μg	
Vaccine	Log_{10} CFU of bacteria[a]	Protection (U)	Log_{10} CFU of bacteria[a]	Protection (U)
H38+IFA	2.88±0.17[b]	2.02	ND	ND
B. abortus S19	ND	ND	2.90±0.21[b]	2.13
L-Omp19+IFA	3.12±0.16[b]	1.78	3.59±0.36[b, d]	1.44
L-Omp19+IFA	3.18±0.12[b]	1.72	3.65±0.63[b, d]	1.38
PBS+IFA	4.90±0.40[c]	0	5.03±0.38[d]	0

[a]The content of bacteria in spleens is represented as the mean log CFU = Standard deviation/group (n = 5 mice group^{-1}). ND, Not Done; [b]Significantly different from PBS-immunized mice ($p<0.01$, estimated by Dunnett's test); [c]Significantly different from H38-immunized mice ($p<0.01$, estimated by Dunnett's test); [d]Significantly different from S19-immunized mice ($p<0.01$, estimated by Dunnett's test)

Table 4: L-Omp19 log protection unit

Gruop	Log unit protection	Clearance (%)
L-Omp19	1.38	27

Table 5: L-Omp19 and rOmp19 indicate almost the same protection. The combination form show more protection than L-Omp19 and the native form but the conjugation form show the most protection

Groups	Log unit protection	Clearance (%)
rOmp19	1.35	26
rOmp19+dLps	1.69	38
rOmp19-dLps	2.90	44

Table 6: Protection against *B. abortus* 554 in BALB/c mice immunized with L-Omp16, U-Omp16, L-Omp19 or U-Omp19 protein with IFA

Vaccine (dose (μg))	Adjuvant	Log_{10} CFU of *B. abortus* 544[a]	Protection (U)
B. abortus S19	None	2.90±1.18[b]	2.18
L-Omp 16 (10)	IFA	3.58±0.39[b, c]	1.50
U-Omp 16 (10)	IFA	3.11±0.23[b]	1.97
L-Omp 19 (10)	IFA	3.65±0.64[b, d]	1.43
U-Omp 19 (10)	IFA	3.23±0.65[b]	1.85
PBS	IFA	5.08±0.43[d]	0.00

[a]The content of bacteria in spleens is represented as the mean log CFU± Standard deviation/group (n = 8 mice group^{-1}); [b]Significantly different from PBS-immunized mice ($p<0.01$, estimated by Dunnett's test); [c]Significantly different from S19-immunized mice ($p<0.05$, estimated by Dunnett's test); [d]Significantly different from S19-immunized mice ($p<0.01$, estimated by Dunnett's test)

with porin complexed with rough LPS (Porin-R-LPS) from a rough mutant of strain 2308 provided no protection.

Table 7: U-Omp19 and L-Omp19 log protection unit

Groups	Log unit protection	Clearance (%)
U-Omp19	1.85	36
L-Omp19	1.43	28

Table 8: Although, un lipided form of Omp19 showed more protection to lipided and native forms but it's less than combination form and rOmp19-dLps shows the most protection to the all

Groups	Log unit protection	Clearance (%)
rOmp19	1.35	26
rOmp19+dLps	1.69	38
rOmp19-dLps	2.90	44

Antibodies specific for porin or R-LPS were found in minor quantities in vaccinated mice. They resulted that the O-polysaccharide will form an necessary component of an efficient subcellular vaccine against *B. abortus* and that O-polysaccharide specific antibodies has a main function in protective immunity in brucellosis (Winter *et al*., 1988) and the recent test confirmed this. When we immunized the last mice group with conjugated form of rOmp19-Lps, the most protection (2.9) was observed (Table 1-8 coparatively). Comparison of conjugation and combination forms of protein with d-Lps confirm the positive effectiveness of conjugation on protective immunity.

CONCLUSION

Recognition of antibody to the conjugated form of recombinant protein in serum samples of infected cases suggests the stimulation of immune response to this protein during brucellosis. The researchers have engineered a sub-unit or conjugated vaccine for human brucellosis.

ACKNOWLEDGEMENTS

The researchers would like to thank the colleagues in the Department of Microbiology, Faculty of Sciences, Azad University-Karaj branch and Bacteriology Department of Tarbiat Modares University. The researchers are also grateful to Amir Hossein Samadi Kafil for his comments on the manuscript. This study was the M.Sc thesis.

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