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Expression, Purification and Characterization of the Mixed Total -OMP- CagA from *Brucella abortus* and *Helicobacter pylori* as Vaccine Candidate

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***Brucella* can causes brucellosis in humans and animals, the Most significance of this bacterium is due to its use in biologic wars and bioterrorism. Vaccination strategy has played a high role in decreasing *Brucella* infections in many countries of the world. So, this study aimed to investigate the expression, purification and characterization of the mixed Total -OMP- CagA from *Brucella abortus* and *H. pylori* as vaccine candidate. After bioinformatics designing by suitable softwares, OMPs proteins of *B. abortus* extracted, and recombinant protein CagA that cloned in Pet28a, previously, transformed to the *E. coli* BL21 as expression host. Then recombinant protein purified by nickel column. Then, CagA and OMPs proteins confirmed with SDS-PAGE and western blotting. In this study construct of Total OMP-CagA was synthesized. OMPs proteins with sizes 25-27 and 36-38 kDa were extracted. Protein 32 KDa of CagA with concentration 700μg/ml expressed and purified, successfully. In this study, the recombinant protein CagA of *H. pylori* successfully expressed and total OMPs of *B. abortus* were successfully extracted and combined together to construct a vaccine candidate. But, complementary studies are required to evaluate the immunological features of mixed OMPs- CagA as novel and efficient vaccine candidate against *H. pylori*.**

Keywords: *Brucella. abortus*, OMP- CagA, Vaccine candidate.

Brucella can causes brucellosis in humans and animals[1]. At now there are six *Brucella* species, of which four species are pathogenic to humans making brucellosis a zoonotic disease[2] which yearly more than half a million people are affected[3]. The Most significance of this bacterium is due to its use in biologic wars and bioterrorism[2]. Vaccination strategy has played a high role in decreasing *Brucella* infections in many countries of the world, so, vaccine is a preeminent factor in control of the disease[4]. In spite of animal brucellosis vaccines are commercially accessible but there is no safe and effective human vaccine for *Brucella* species, for this reason different studies on *Brucella* have recently been focused for development of safe and effective human vaccines against *Brucella* infections[5]. However, it still remains a mystery in the 21st century[6]. In recent years *Brucella* has caused disease in new locations

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and also has observed as re-emerging in some areas, has been shown to cause changes in the epidemiology of this disease and result in increased the role of *Brucella* in travel-related disease. It seems that in the near future will not reduce the disease burden, because there is no effective vaccine for humans and prevention of disease depend on achievement in intricate and expensive projects which done in animals for its control and eradication[3]. OMPs are among the most important structural components of this bacterium and act as virulence factors to induce immune responses. Because *Brucella* is lack of flagella and capsule, OMPs providing the most important role in the pathogenesis of this bacterium[7]. Because these proteins create pore-like structures are essential to export any protein of this bacterium and the most immune responses are formed against the cell wall structure components. Regarding that *Brucella* is an intracellular bacterium, cellular immune responses against this group of proteins of cell wall structure components are involved in creating an effective response and protection[8]. In scientists opinion, *H. Pylori* acts as an important cause of cancer in humans and type I carcinogen[9]. Reports showed that the *H. pylori* infection occurs in more than half of the world population[10]. In total, *H. pylori* is responsible for approximately 75% of total gastric cancers and 63.4% of the entire stomach cancers worldwide[10]. Almost 70% of all strains of *H. pylori* have CagA factor[11], and more than 90% of the strains that have been isolated from patients with duodenal ulcers and cancer are CagA +. This protein is one of the most important immunogenic factors of this bacterium that causes production of specific antibodies, stimulating the *specific* CD4+ T cell responses in the gastric mucosa, increasing the risk of cancer, the development of atrophic gastritis, ulcer formation and delayed recovery, the induction of the expression of oncogenes and mutations in genes P53, atherosclerosis, ischemic heart disease, coronary heart disease and increased production of PGE2 and ROS(12). Given the high prevalence of brucellosis and lack of suitable vaccines with a very high efficiency, this study aimed to investigate the expression, purification and characterization of the mixed Total -OMP- CagA from *Brucella abortus* and *H. pylori* as vaccine candidate.

MATERIALS AND METHODS

Synthetic Primers, Cloning and Construction of Recombinant Plasmid

In our study was used from cloned cagA gene which had been prepared in our previous work. But will be explained about it, briefly. At first cagA sequence obtained from NCBI, then the single primer design performed by Primer 3 and Gene runner softwares for *H. pylori* 26695 cagA gene target fragment. At this stage, Bioinformatics Studies in silico with softwares related with each section was performed. To understand the spatial structure of proteins which their primer sequences were (F: 52 - *aaggatccactaacgaaaccattgacca* -32 and R: 52 - aagagctcactccctcaactctaacatt-32 which allowed amplifying fragment with length of 841bp) had been designed by software, and Protein Modeling performed. Two enzymes cutting site *Bam* HI and *Sac* I inserted into the 52 and *Sac* I 32 ends, respectively. The DNA construct was cloned in the *E. Coli* DH5a vector and *E.coli* strain Top10 was transformed. Transformed *E.coli* was selected on LB agar plate containing 100 mg/ml of kanamycin. PCR, enzyme digestion and sequence determination were used for confirmation of transformed colonies. Then, the *cagA gene* was *cloned into pET28a to form recombinant expression vector*[13].

Expression of recombinant protein CagA in *E.coli* BL21b strain

In the next stage, pET/cagA transformed to the E.coli BL21 strain as expression host, subsequently proper transformation of selected colony was confirmed by PCR, enzyme digestion and sequencing. Transformed cells were cultured in 5 ml of LB (Luria bertani) broth containing 100 mg/ml kanamycin and shaken on shaker incubator at 37°C until OD reached 0.4-0.6 at 600 nm. Then, 2 ml of growing bacterium was laboring for inoculation of 500 ml of LB broth containing 100 mg/ml kanamycin and was shaken at 37°C until cell density in the OD 600 nm reached 0.4-0.6. Protein expression that was induced by IPTG (Isopropyl â-D-1-thiogalactopyranoside) in different concentrations which include 0.2, 0.5 and 1 mM, were submitted for shaking on the shaker incubator. In order to determine the best time and temperature, and induced cells were incubated at 4, 18, 28 and 37°C for various times: 4, 8, 16 and 24 h. Then

collected cells were sonicated 3 times for 2 min and 2 min interval was allowed among cycles. Cells were pelleted by centrifugation at 14,000xg for 15 min at 4°C.

***Brucella* strains**

In this study *Brucella* OMPs antigens were used from *Brucella abortus* strain S19**,** *this strain* prepared *as* lyophilized *form* Pasteur *Institute* of Karaj, Iran.

OMPs extracted from *B. abortus* S19

Medium containing the bacterium centrifuged for 4 minutes at 6000 rpm and the resulted precipitant dissolved in 10 mM Tris buffer, PMSF with concentration of 1 mM, lysozyme (10 mg per one gram of bacterium) and EDTA with concentration 1 mM added to the bacterium mix and incubated overnight at 37 °*C*. After that sarcosine added at a concentration of 1% and put 2 hours at 37 °*C*. Sonication 30 times, each time for one second, 10-second intervals, with power 20 kHz was done. Mgcl2 added (with Molarity 0.001) to inhibit the EDTA. Then RNase and DNase added with the amount of 300 micrograms per gram of dry weight of bacterium and incubated 2 h at 37 °*C*. Then compound centrifuged at 5000 g for 30 minutes and the temperature of 4 °*C*. In the next step, supernatant removed and centrifuged for 30 min at 40000g and 4 °*C*. Finally, the supernatant was taken and centrifuged for 30 min at 40000g and 4 °*C*. This supernatant contained the bacterial outer membrane proteins that were stored until use at -20 °*C*.

Confirmation of extracted outer membrane proteins (OMPs) and recombinant protein CagA by SDS-PAGE and western blotting Finally, to check that the recombinant protein CagA was in the supernatant (periplasmic space) or pellet (inclusion bodies) and for confirmation of extracted outer membrane proteins SDS-PAGE was performed. Separation of protein was performed on 12% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE) (14). Pellet and supernatant of sonicated cells were suspended in sample buffer and heated at 95°C for 7 min; then, 10 µl of each sample was runned on SDS-PAGE gel. Staining of protein bands carried out by Coomassie Brilliant Blue R250 and bands size were assessed by protein marker (Thermo scientific). In western blotting technique, the proteins which separated by SDS-PAGE gel were transferred to PVDF membrane (Amersham) and immunobloting was done using anti-poly histidine-peroxidase monoclonal antibody (Sigma-aldrich). Based on manufacturer's recommendations, finally, protein bands were revealed by Western Blot Chemiluminescent kit (takapouzist). It is noted that the antibody dilution which was used in this experiment was 1/2000**.**

Purification of recombinant fusion protein from *E. coli* lysate

Due to the existence of 6 His-tag at the C-terminus of proteins, Ni-NTA column was applied to purify recombinant protein CagA. 10 ml of denaturing lysis buffer, potassium phosphate buffer, 150 mM NaCl and 10% of glycerol were used to resuspend cell pellet and supernatant. Then the sonication was performed, the lysates were loaded on the Ni-NTA column and washing steps were carried out by imidazole (15 mM), and protein CagA was eluted with imidazole (500 mM). Subsequently dialysis by PBS buffer was performed. The purity of recombinant protein was assessed by SDS-PAGE and Western blotting. Protein concentrations were determined by Bicinchoninic Acid Protein assay Kit (Parstoos).

Measurement of protein concentration by the Bradford method

Bradford method is a rapid and sensitive method for measuring a protein concentration. In this method, Comas blue (CBB) attached to the protein. Therefore, on wavelength 595 nm, protein CagA-dye complex was measured.

RESULTS

The results of SDS PAGE and western blotting

The figure 1 and 2 are related to the SDS-PAGE of the recombinant protein CagA, also for confirmation of this protein the western blot method with Anti-His tag was used. In figure 3 and 4 OMPs proteins show the bands with sizes 25-27 and 36-38 kDa. The concentration of recombinant protein CagA using Bradford method was 700µg/ml.

DISCUSSION

Brucellosis is the most common zoonotic disease between human and animal, as every year more than half a million people are infected with it.

For this reason the eradication of this bacterium is very important(15). *Brucella* outer membrane proteins (OMPs) play an important role in stimulating the host immune system and are as key components in producing subunit vaccine candidates in humans and animals(16). (11). In the prevention against brucellosis, the vaccination have more important role. Currently, for prevention of brucellosis in cattle and sheep used from two types of *Brucella* live attenuated vaccines, which include ; *B. abortus* strains S19 and RB51 and Rev-1 strain of *B. melitensis(17). H. pylori* CagA is one of the most immunogenic proteins that stimulate the production of specific antibodies and specific lymphocytes TCD4 +, which can be used to enhance the immunogenicity of antigens, polysaccharides and cell-mediated immune stimulation(11). Regarding to the importance of this bacterium and its role in gastric cancer and related diseases, the urgent need to eradicate this bacterium will be felt. So the different subunits of the bacterium such as UreB, HspA, FlaA, FlaB, CagA, VacA, HpaA have been used as vaccine candidates(18). Due to *Brucella* is intracellular bacterium, a vaccine is valuable that can stimulate humoral and cellular immune system together. The live vaccine *B. abortus* S19 activate humoral immunity and one of its drawbacks is antibody production after vaccination that always problems resulted from it is raised(19). Live vaccine *B. abortus* RB51 stimulate cellular immune system and immunogenicity capability of it has documented, but the its efficacy is only 40%(20). According to

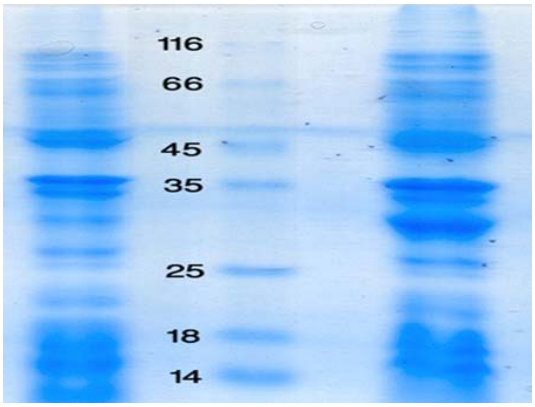

Fig. 1. Expressed CagA recombinant protein on (SDS-PAGE 12% w / v) stained with Coomassie Brilliant *Blue G-250*. Wells T1, T2, T3, T4 are related to the bacterial pellet induced by IPTG, 4 h after induction. Well T0: non-induced bacterial pellet

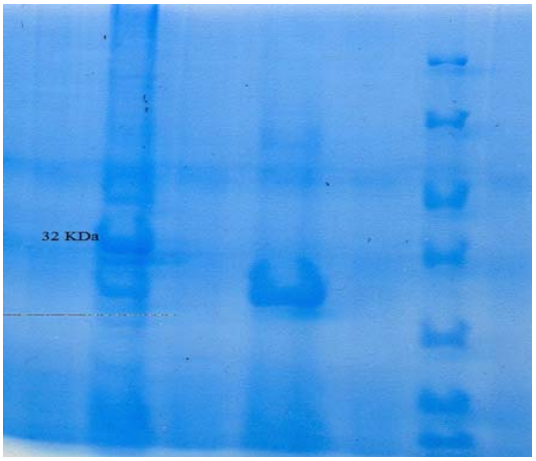

Fig. 2. Purified recombinant protein using a nickel column, right well is related to the protein marker, middle well: purified protein 32 KDa of CagA, left well; before protein purification

Fig. 3. Western blotting image of *Brucella* OMPs proteins in compared to the protein marker

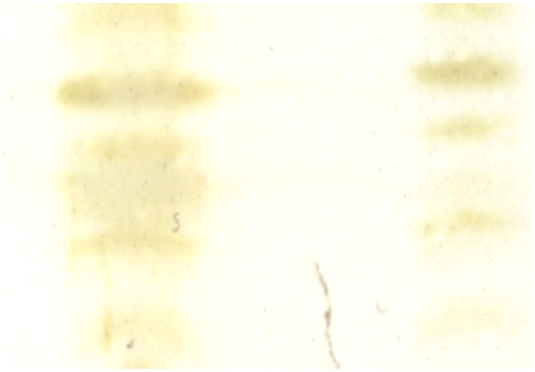

Fig. 4. Western blotting image of *Brucella* OMPs proteins

the OMPs proteins to activation of both arms of the cellular and humoral immune system and have antigenic and strong Immunogenetics properties has combined with strong immunogenic protein CagA will increase the immunogenicity (humoral and cellular) against both *Brucella* and *H. pylori*. *Brucella* OMPs combined with strong immunogenic protein rCagA to develop a vaccine with high efficacy and immunogenicity is needed to overcome on present problems and preparation of a vaccine with better immunity**.** In this study, 841 bp of 52 cagA gene was used to design primers. One of our aims of choosing this fragment was selecting an area first would have present in all strains which possess this gene, secondly, is the lack of diversity and antigenic changes and EPI motif, thirdly, has motif stimulating humoral and cellular immune responses(21). In studies that have been done, the complete recombinant protein CagA and its C terminal have used for immunization, but for the first time in present study has applied the N-terminal region of it for immunization(22). Regarding that the selected 32-kDa fragment was lack of EPYIA motif, in result was lack of a variable and mutagen region. Using bioinformatics software showed that this fragment is capable of stimulating humoral and cellular system. Researchers used from this bacterium OMPs as recombinant and natural form and DNA vaccine encoding OMPs for the immunization of animals alone or in combination with other antigens, but none had successful clearance(23, 24). Unlike other studies, here we used of total OMP accompanying with recombinant protein CagA to overcome on this problem.

CONCLUSION

In this study, the recombinant protein CagA of *H. pylori* successfully expressed and total OMPs of *B. abortus* were successfully extracted and combined together to construct a vaccine candidate. But, complementary studies are required to evaluate the immunological features of mixed OMPs- CagA as novel and efficient vaccine candidate against *H. pylori*.

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