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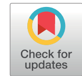
Study of serum bactericidal and splenic activity of Total-OMP- CagA combination from *Brucella abortus* and *Helicobacter pylori* in BALB/c mouse model



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ABSTRACT

Background: *Brucella* is a Gram-negative and facultative intracellular organism that causes brucellosis, a common zoonotic disease. Over 500,000 people are annually affected by brucellosis. *Brucella* is highly infectious through inhalation route; for this reason it is used for biological warfare aims. This study aimed to study the serum bactericidal and splenic activity of Total-OMP-r CagA immunogens from *Brucella abortus* and *Helicobacter pylori* in a BALB/c mouse model.
Methods: Immunization of BALB/c mice was performed with immunogenic proteins three times subcutaneously (S.C.) at 14-day intervals. The protective effects of two component vaccines with CpG adjuvant were evaluated after mice were challenged with *H. pylori* ss1 and *Brucella abortus strain 544*. The specific IgG1 and IgG2a antibodies in sera were assessed using ELISA test. For measuring the antigen-specific IL-4, IL-12 and IFN-γ responses in sera of immunized mice after challenge, RT-PCR technique was applied. Twenty days after the challenge, mice were killed then gastric, splenic and serum samples were assessed and bacterial colony count was measured based on the pour plate count agar.
Results: The results indicated that rCagA + OMP decreased bacterial colonization in these tissues, and significant difference was observed between test and control groups (p value$^{<}$0.001).
Conclusion: Our results showed that the combination vaccine was effective against an oral exposure and the bacterial burden in the spleen, serum and gastric tissues were reduced in mice immunized with the Total- OMP-CagA.

1. Introduction

Brucellosis is a widespread zoonotic disease mainly transmitted through cattle, sheep, goats, pigs and camels or direct contact with blood, placenta, fetuses or uterine secretions, consumption of contaminated raw animal products [1]. Over 500,000 people are annually contaminated with infections are caused by this bacterium [2]. *Brucella* spp., is highly infectious through aerosol route, for this reason, it as an attractive pathogen to be used as a potential agent for biological warfare purposes [3]. Brucellosis there is around the world [4]. The prevalence of brucellosis is growing due to the international tourism and migration [5]. Brucellosis has a high frequency of morbidity both in humans and animals; and this organism has a high health and economic burden especially in developing countries [6]. Methods of prevention are health education to decrease occupational and food-borne risks, and pasteurization of dairy products. However, final prevention of human infection depended on the elimination of the infection among domestic animals. This purpose can probably be obtained by an appropriate

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immunization of animals as well as elimination of infected animals [7]. Recently there are many vaccines for application in humans and animals, but none of those are quite safe and their efficacy isn't 100% [8]. Outer membrane proteins (OMPs) of *Brucella* are virulence factors of this organism and play a significant role in pathogenesis of *Brucella*, nowadays are raised as one of the important vaccine candidates [9]. The role of *H. pylori* in gastric cancer has been verified and many factors have been recognized in association to the pathogenicity of this bacterium, the *cagA* gene has been detected in about 80%–100% of *H. pylori* strains isolated from people with gastric cancer [10]. Therefore, due to the lack of safe and more effective vaccine against *Brucella*, this study aimed to evaluate the serum bactericidal and splenic activity of Total-OMP- rCagA combination from *B. abortus* and *H. pylori* in BALB/c Mouse model.

2. Material and methods

2.1. Ethics statement

All experimental protocols with animals were carried out in firm consistent with international ethical standards for animal experimentation (Helsinki Declaration and its amendments, Amsterdam Protocol of welfare and animal protection and National Institutes of Health, USANIH, and guidelines).

2.2. Mice and bacterial strains

Six-to eight-week-old female BALB/c mice were purchased from the Pasteur institute, Tehran. OMP proteins were prepared of *B. abortus S1* and for challenging were used of *B. abortus* 544 biovar 1. S19 strain was provided from Razi Institute and other strains were prepared of Pasteur Institute, Tehran.

2.3. Extraction of OMPs B. abortus strain S19

Broth cultures containing bacteria were centrifuged for 4 min at 6000 rpm and the sedimentation resolved in 10 mm Tris buffer solution. 2.1 mm PMSF, lysozyme (10 mg per gram of bacteria) and 1 mm EDTA were added to the bacterium culture and incubated overnight at 37 °C. Sarcosinate with a concentration of 1% was added and incubated for 2 h at 37 °C. Sonication was performed 30 times, each time for a second, with 10-s intervals, with the power of 20 kHz Mgcl2 (0.001M) was added in order to inhibit EDTA. RNase and DNase were added at 300 μg/g of dry weight of bacteria and incubated 2 h at 37 °C. Centrifugation was done at 5000 g for 30 min at 4 °C for 30 min. The upper solution was removed and centrifuged at 40000 g for 30 min at 4 °C (36).

2.4. Induction of recombinant vector with IPTG

E. coli BL21 containing *pET-28a/cagA* was dedicated by Dr. Esmaeili (Associated Professor of BMSU). *E. coli* cells harboring expression vector *pET-28a/cagA* were grown in LB medium supplemented with kanamycin (30 μg/ml) and chloramphenicol (34 μg/ml) at 37 °C to an OD 600 = 0.6–0.8 for induction, IPTG (Sigma, USA) was added to a final concentration of 1 mM and for growth of bacteria were incubated at 37 °C for 4 h. The bacterial cells were harvested and suspended in lysis buffer (Nacl 0.5 M, PMSF 1 mM, EDTA 10 mM, 1%(v/v), Triton X-100, 20 mM Tris- HCl, pH: 7.5) then was frozen, thawed and sonicated on ice in the presence of PMSF 1 mM (Sigma). Recombinant CagA proteins were centrifuged at 14000 g for 20 min and were collected in the supernatant phase.

2.5. Immunizations and experimental studies

Four groups of mice (N = 12/groups) were immunized three times in interval 14 days (0, 14, 28) subcutaneously with final volume 0.1 mL (S.C). Immunizations were done with rCagA (10 μg) + CpG (15 μg) [Group 1], OMP (10 μg) +CpG (15 μg) [Group 2], rCagA(5 μg) +OMP(10 μg) + CpG (15 μg) [Group 3], PBS (0.1 Ml) [Group 4]. Sera were collected 14 days after each immunization. ELISA was used to measuring the serum IgG1, total IgG and IgG2a antibodies specific to *H. pylori* antigens. Fourteen days after last immunization, mice were challenged with 5×10^4 CFU of *H. pylori SS1* and *B. abortus strain 544*. Antigen specific IL-4 and IFNγ responses were measured using ELISA kit in spleen of immunized mice before and after challenge and also, IL-10, IL-12 and TGFβ after challenge. The mice were killed and used to assay naive splenocyte responses to stimulating desired antigens. Spleens were sterility collected from mice. Spleens were removed and ground through a screen mesh. The lysis of RBC in splenocyte samples were performed by "ACK (Ammonium-Chloride-Potassium) lysis buffer". Cell suspensions of mice were washed, centrifuged and solution filtered with 0.45 μm filter. Finally, lymphocytes were then resuspend at 5×10^6 cells/ml in complete RPMI with 10% FCS [11]. Also gastric and splenic tissues of mice were separated and placed in a plate, Then 1 ml of sterile saline was added to the plate and with the bottom of the syringe was crushed the spleen, Then from serum and the liquid inside plate, the dilution plates 1/10, 1/100 were prepared and cultured on *Brucella* and blood agar media enriched with fetal calf serum that is required for the growth of *H. pylori*. In temperature 37 °C with 10% CO2 for *H. pylor*i and 5% CO2 for *Brucella* were incubated for 3–5 days. Then the numbers of grown colonies in each group according to their dilutions were calculated [12].

2.6. Study of serum and splenic bactericidal effects

Mice spleen was isolated and their fragments cultured on Blood and Brucella agar media. Serial dilution prepared and bacterial colonies were counted as CFU based on pour plate count agar. Sera samples were cultured on *Brucella* agar then bacteria count was carried out. *H. pylori* cultures were performed on Brucella agar enriched with 5% defibrinated sheep blood, FCS (Fetal calf serum) and incubated in microaerophilic conditions.

2.7. RT- PCR

Primers were designed for detection of cytokines of IFNγ, IL-12, IL-4 and *B.actin* gene. Firstly, the whole sequence of the relevant genes was searched from NCBI site, and primer design was performed by proper online softwares. Forward and Reverse primers were blasted in the Primer-BLAST section of NCBI BLAST Gene Bank [13]. In the next step, primer pairs were studied by Oligo analyzer software. After this initial stage and after examination and confirmation of primers, they were evaluated in silico PCR amplification online software. After designing of primers were ordered to the Pishgam Company for synthesis, after their receiving, the primers were provided in eppendorf tubes lyophilized. Deionized distilled water was used for diluting primers and they were prepared with the appropriate concentrations. To prepare stock, the primers were frizzed at −20 °C. Primer sequences used were as follows; F IL-12: 5′ GGAAGCACGGCAGCAGAATA 3'; R-IL-12: 5′ AACTTGAGG GAGAAGTAGGAATGG 3′(282 bp); F IFN-γ: 5′ ACTGGCAAAAGGATGG TGAC 3'; R IFN-γ: 5′ TGAGCTCATTGAATGCTTGG 3′ (Amplicon 237 bp); F IL-4: 5′ GCCTGCTTTTCACATGAGGT 3'; R IL-4; 5′ AAATATGCG AAGCACCTTGG 3′(Amplicon 250 bp); F B-actin: AGCCATGTACGTAG CCATCC; R B-actin; CTCTCAGCTGTGGTGGTGAA (228 bp). After preparing a stock solution, working solution 10 p.m from primers was prepared. RT-PCR technique was applied to studying of gene expression.

2.8. Assay of IgG1 and IgG2α titer

IgG1 and IgG2α responses were measured in sera of immunized

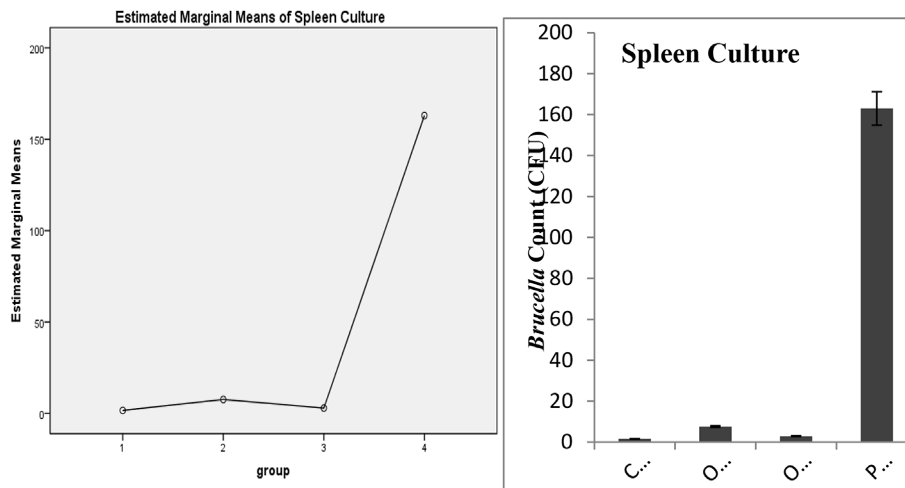

Fig. 1. The results of splenic clearance of *Brucella* in 4 mice groups. Results indicated that clearance of Brucella decreased in group rCagA + TN-OMP + CpG.

mice after challenge using ELISA test.

2.9. Statistical analysis

For comparison of responses between intra and inter groups of mice and multiple groups, respectively, ANOVA and Tukey tests were used. Two-way analysis of variance and LSD, using SPSS V13 statistical software, compared the levels of proliferative responses to antigens. The levels of proliferative responses to antigens were performed by two-way analysis of variance and LSD. Values less than 0.05 were considered as a statistically significant.

3. Results

3.1. The results of the clearance of bacteria in spleen

Mice spleens isolated and their fragments were cultured on proper media. Colonies of *Brucella* were counted based on CFU (Fig. 1). The statistical analysis also showed the significant differences between test groups compared to the control groups (p value$^<$0.01).

3.2. Serum clearance of Brucella

Sera samples were cultured on *Brucella* agar and then bacteria count were carried out based on CFU (Fig. 2). The statistical results of the analysis of spleen clearance show the significant differences between groups compared to the control groups (p value$^<$0.01).

3.3. Immunization reduced H. pylori colonization in mice gastric tissue

As was shown in Fig. 3, after challenge, mice were killed and gastric samples were assessed and bacteria were counted based on CFU. The results indicated that rCagA + OMP decreased bacterial colonization; a significant difference was seen between test and control groups (p value$^<$0.001).

3.4. Clearance of Brucella and H. pylori in gastric, splenic and blood tissues in mice

Clearance of *Brucella* and *H. pylori* was evaluated in serum, splenic and gastric tissues of all mouse groups. Results indicated a higher clearance of *Brucella* and *H. pylori* in the rCagA + TN-OMP + CpG group compared with other groups (Fig. 4). Brucella count in blood and spleen tissues decreased in all but the control group.

3.5. Results of expression of cytokines with RT-PCR technique

RT-PCR protocol was performed according to the material and method section. For observation of results, the 1% agarose gel was used (Fig. 5). As shown in Fig. 4, RT-PCR analysis before and after treatment revealed that the gene expression of the IFNγ, IL-12 and IL-4 were significantly expressed in the presence of vaccine formulation while these genes had higher expression in encountering of bacteria with immunogens. Expression of B-actin housekeeping gene in test and control groups remained relatively constant.

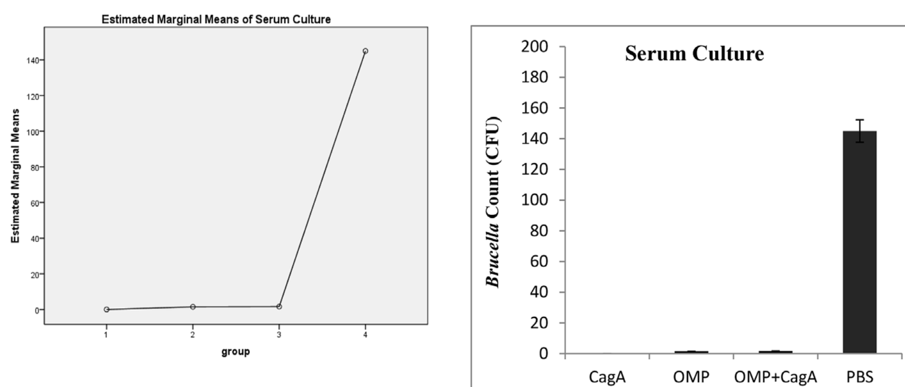

Fig. 2. The results of serum clearance of *Brucella* in 4 mice groups. Results indicated that clearance of Brucella decreased in group rCagA + TN-OMP + CpG.

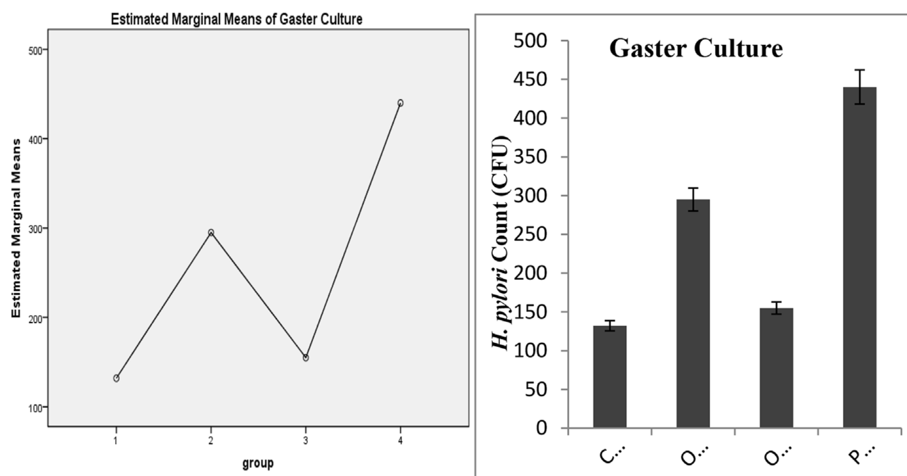

Fig. 3. The results of gastric clearance of *H. pylori* in 4 mice groups. Results indicated that clearance of *H. pylori* decreased in group rCagA + TN-OMP + CpG.

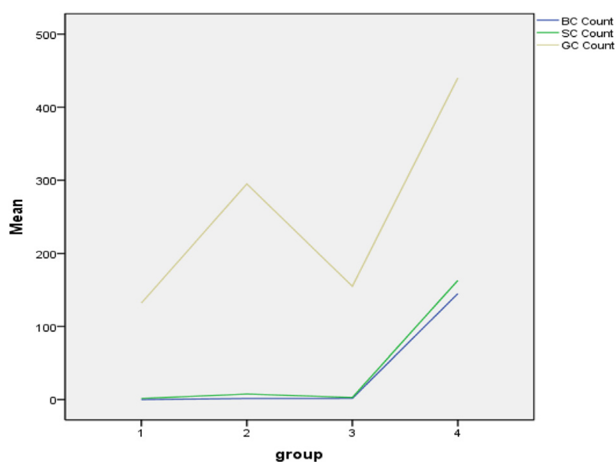

Fig. 4. The total results of clearance of *Brucella* and *H. pylori* in serum, splenic and gastric tissues of all mice groups. Gc: Gastric count, Sc: spleen count, Bc: blood (serum) count. Results indicated that clearance of *Brucella* and *H. pylori* decreased more than other groups in group rCagA + TN-OMP + CpG.

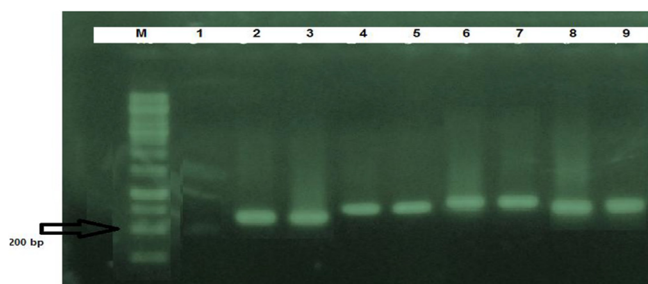

Fig. 5. Results of cytokine expression patterns with RT-PCR. Lane M. Marker 100 bp, lane 2 gene expression of IFNγ before immunization with TN-OMP + rCagA + CpG combination, lanes 2 shows gene expression of IFNγ post immunization with mentioned combination, lanes 3is correspond with gene expression of IL-10 post immunization with desired combination, lanes 4 and 5 present gene expression of IL-4 after immunization with TN-OMP + rCagA + CpG combination, lanes 6 and 7 show gene expression of IL-12 after immunization with mentioned combination, lane 8 is related with gene expression of B-actin Housekeeping gene before immunization and lane 9 is associated with expression of B-actin Housekeeping gene post immunization with the vaccine formulation.

3.6. Results of IgG2α and IgG1 to TN-OMP + rCagA

Specific IgG1 and IgG2a isotypes were measured in sera after the challenge. In this study the IgG2a/IgG1 ratio in the mice immunized with r*CagA* and TN-OMP CpG was > 1, indicating a Th1 type response that is important for clearance (Fig. 6). In other groups IgG2a/IgG1 ratio was < 1 that indicated a humoral immune response.

4. Discussion

One of the major challenges associated with vaccines against brucellosis is to increase their effectiveness and safety. The live attenuated vaccines for brucellosis are the S19 and Rev 1 types which are mostly used in animals and have relatively good safety [14]. However, the most important concern for these vaccines is their virulence in humans [15]. OMPs of *Brucella* play a very important role in activating the host immune system and have been introduced as a successful combination vaccine candidate in humans and animals [16]. On the other hand, in previous studies, recombinant OMP 31, 19, 16, 25 and 18 were used as immunogenic antigens but none of them showed a successful clearance [17,18]. For this reason, we used from total OMP of *Brucella* in this study. Combination of OMP with the robust immunogenic protein rCagA will raise the immunogenicity (humoral and cellular) against *Brucella* and *H. pylori* together. Various factors of *H. pylori* have been introduced as vaccine candidates but none has shown proper efficacy against this bacterium, and the effectiveness of these vaccines are limited [19]. Possibly, the appropriate immunity against *H. pylori*

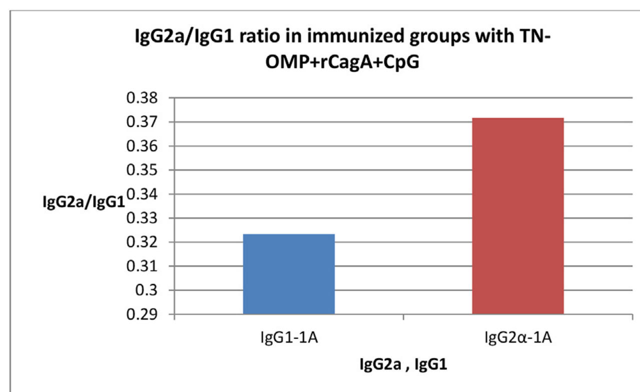

Fig. 6. Results of assessment of IgG2α/IgG1 in group TN-OMP + rCagA + CpG post challenge. This Figure indicates IgG2a/IgG1 ratio in group TN-OMP + rCagA + CpG is > 1 that suggests a cellular immune response.

obtained by combining the different virulence factors with various activities in pathogenesis of the infection [20]. The CagA and OMP are among the most important virulence factors and the major protective antigens, and they have been applied in vaccination experiments to inhibit *H. pylori* and *Brucella* infection in mouse model [21], in present study we used from *Brucella* total OMP as a basic antigen factor accompanied by *H. pylori* rCagA. The study conducted by Rossi et al. showed that the use of a combination (CagA, VacA, and NAP) can considerably reduce the colonization rate of *H. pylori* in gastric tissue [22]. In line with above study, our study revealed that the combination (CagA-OMP) decreases the numbers of bacteria in gastric tissue after challenge with *H. pylori*. Again, a study carried out by Esmaeili et al. emphasized that the combination (CagA-LPS) dramatically reduced the colonization of *H. pylori* in gastric mucus, which is due to the strong Th1 response, 6-fold reduction in the numbers of *H. pylori* in the gastric tissue compared to non-immunized mice has been observed in protected mice of infection with *H. pylori* [23].

In another study by Siadat et al. an LPS was conjugated with an OMP (outer membrane vesicles of meningococci), results revealed that the LPS of this bacterium is an important virulent factor, which is able to escape from the immune system by creating a balance between Th1 and Th2. They concluded that this conjugate is capable of Th1 stimulation and Th2 inhibition. They also showed that immunization with *Brucella* LPS alone may result in a meaningful induction of IFN-γ compared with OMV + LPS and FA + LPS groups ($p > 0.5$), although titers IL-4 and IL-10 slightly increased, whereas in the conjugated vaccine, the values of IL-4 and IL-10 were greater [24,25]. In a study by Stein et al., in 2009, it was shown that BLS Omp31 (*Brucella spp*. Omp31) induced a protective IFN-γ and IgG response against *B. ovis* in male sheep [26]. rCagA 32 KD is a hydrophilic, surface-exposed antigen that induces cellular and humoral immunity responses. The *H. pylori* serotype O2 is unique, because does not express Lewis antigen but also induces a strong Th1 immune response which aids in the protection or clearance against *H. pylori* infection [27,28]. Conjugation of these proteins with rCagA as an adjuvant which is a strong immunogenic protein will increase the immune response against TN-OMP [29].

Mentioned studies with our study present the fundamental role of rCagA and related combinations in inducing of immunity and protection against *H. pylori and Brucella*. The significantly low values of IL-4 and IL-10 compared with IFN-γ in all groups indicated this formulation induced Th1 response, which can be considered as an important factor in the efficiency of the *Brucella* vaccine. After immunization in the animal model, the value of IFN-γ in relation to IL-4 and IL-10 were expressed in all groups, indicating an appropriate cellular immune response through TH1 and the secretion of IFN-γ. The results of our study showed that the TN-OMP along with rCagA protein is a successful candidate for immunization.

IgG2a titers predominated over IgG1 titers during immunization with antigens TN-OMP along with rCagA protein. The induction of a specific IgG2a subclass during an immune response is also affected by the Th1/Th2 balance. The simultaneous use of TN-OMP and rCagA along with CpG has a synergistic effects and promotes a Th1 immune response (IFN-γ, IL-12, IgG2a), which aids in the protection and clearance of the *H. pylori* infection. Clearance was associated with high levels of IFN-γ and IgG2a. The balance between Th1/Th2 responses is essential for the clearance of *H. pylori*. Th2 responses are needed for sufficient antibody production. However, Th1 responses are seen to occur more often in natural cases of *H. pylori and Brucella* infections.

Formation of antigen-antibody complexes facilitates neutralization and opsonization of the pathogen, which can also lead to the neutralization of the CagA toxin. Because OMP induces humoral and cellular immunity, we selected it for immunization accompanied with rCagA. The results demonstrated that a combination of OMP + rCagA induced a higher cellular and humeral immune response than any of them alone.

Due to the adverse side effects of attenuated vaccines against *Brucella*, it is necessary to explore alternative vaccination options. Subunit vaccines are safer, so we can use appropriate antigens for immunization. The search for a suitable vaccine for use in humans is required, because there is also biological warfare. Researchers have so far investigated LPS, r OMP and OMP as conjugated or accompanied by other proteins, but in this study we select a 32 KD fragment of CagA without EPIYA motif combined TN-OMP.

Nowadays, Brucellosis is a global problem. With the exceptions of a small number of countries that have eradicated Brucellosis or have no incidence of it, most countries are affected. In the latest report from WHO, only 17 countries in the world have no incidence of Brucellosis. In the Eastern Mediterranean and Middle East, all countries were affected and Iran is one of the endemic regions with a high incidence of both animal and human Brucellosis. Statistically, the economic losses due to the animal and human Brucellosis have been estimated to be very high in Iran. The lack of an effective human vaccine and the adverse side effects of animal vaccines are reasons why this disease has not yet been eradicated on a global scale. Appropriate immune against *Brucella* are based on the cellular immune responses. In other words, enhancing the killing power of macrophages depends on the secretion of cytokines like IFN-γ. Therefore, it is essential to select an appropriate antigen capable of inducing cellular responses to eradicate this bacterium from the body tissues.

Given that rCagA is a hydrophilic protein, it enhanced the immune response. In other studies, an OMP with a weaker immune response has commonly been used. Therefore, in this study we propose the use of TN-OMP without significant conformational alterations as an immunization candidate, due to the fact that total proteins are exposed and are capable of inducing a potent immune response. Furthermore, a TN-OMP with hydrophilic protein can play a more effective role in enhancing humoral and cellular responses.

The present study showed that the total outer membrane protein that is naturally produced has a conformational structure with suitable epitopes that stimulate cellular and humoral immunity. Conjugating the antigenic epitopes may also be changed and reduce the immune response.

Hence, the literature shows that rCagA and *Brucella* OMP can be suitable candidates for vaccines. Due to the changes and diversity in the C-terminal segment of CagA gene in various isolations of *H. Pylori*, in this study we used a protected N-terminal segment with antigenic and immunogenic properties, to induce an appropriate immune response. Because this segment is protected and has suitable folding properties, it can induce a better immune response.

5. Conclusion

Our results showed that the vaccine combination was effective against an oral exposure and the bacterial burden in the spleen, serum, and gastric tissues were noticeably reduced in mice received immunogenes (Total- OMP-CagA).

Conflicts of interest

None declared.

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