

Protection against *Brucella abortus* 544 Strain Infection in BALB/c Mice by Subcutaneous Administration of Multicomponent Vaccine of rCagA Conjugated with LPS + CpG

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Brucellosis is one of the commonest zoonotic disease that is spread throughout world. In this study, we tested subcutaneous vaccines against brucellosis with formulation lipopolysaccharide (LPS) of *Brucella abortus* S19 strain Conjugated with the *Helicobacter pylori*'s recombinant CagA protein and with CpG in BALB/c mouse model. BALB/c mice were immunized with different formulations three times subcutaneous at 14-day intervals. The protective effects of two component vaccines plus CpG Adjuvant were assessed after *Brucella abortus* 544 challenge in different studies. The specific IgG antibodies in serum were studied by ELISA, and Antigen specific IL-4, IL-10 and IFN- γ responses were measured in spleen of immunized mice after challenge using ELISA test. Clearance of *B. abortus* carried according standard protocol. *B. abortus*-specific IgG1 and IgG2a isotopes were measured from sera before and post challenge. In this study the IgG2a/IgG1 ratio in the mice were <1 . Analysis of lymphocyte proliferation of mice showed that rCagA with concentration 1 μ g increase lymphocyte proliferation excellent compared to control group. CpG oligodeoxy nucleotides is proper to induce Th1-biased immune responses. Immunization of mice with rCagA-LPS + CpG induced a strong local and systemic Th1 immune response. Although both immunization groups LPS + CpG and rCagA-LPS + CpG had increased numbers of IFN- γ secreting splenocytes ($P<0.05$). There was no significant difference in IFN- γ production between the rCagA conjugated with LPS + CpG and others groups. Conversely, the rCagA conjugated with LPS + CpG group had a increase in more IL-4 than IFN- γ and serum IgG1 titers higher than IgG2a ($P<0.05$). Naïve splenocytes produced high levels of IL-10 after challenge with rCagA conjugated with LPS + CpG that indicate LPS in order prevention of inflammation by induced Th1/Th2 balance response. Vaccine current formulations enable decrease colonization and bacterial load in mice spleens after complete vaccination according immunization schedule. Vaccinations induce Th1 and Th2 immune responses. In this study, mice were protected from infection with *B. abortus* 544 strain showed 1-fold reduction in the number of *B. abortus* in the spleen compared to non immunized mice. In conclusion, vaccination with current formulation in animal model was safe and was able to induce appropriate responses and also decrease *B. abortus* 544 strain colonization but suggest that rCagA combined with other Components of brucella.

Key words: Brucella, LPS, CagA protein, vaccine, challenge.

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Brucellosis is the commonest zoonotic disease in the world, especially around the Mediterranean Sea, South America and Middle East, and more than 500,000 new cases of Brucellosis are reported yearly. However that figure

underestimates the magnitude of the problem moreover, Brucellosis have many considerable economic and social importance^{1,2}. Humans can be infected by inhalation of aerosol, contact with infected animals, or by ingestion of contaminated animal products. This disease causes high various clinical manifestations in humans, and any organ can be affected that include encephalitis, meningitis, spondylitis, orchitis, prostatitis, arthritis and endocarditis^{1,3}. Although brucellosis in domestic animals almost has been controlled in most developed countries but it is an important public and animal health problem in the developing countries¹.

The cytotoxin-associated gene A (CagA) is part of the cag pathogenicity island (cagPAI), which is found in disease-causing strains of *H. pylori*⁴. This bacterium express surface protein with a Molecular weight of 120-128 KD with name CagA, and A dominant characteristic of this protein is high antigenicity⁵.

Development of an effective vaccine against brucellosis for human use is necessary specially due to the possibility application it as biowarfare agent. The vaccines that so far studied included administration of live, attenuated *Brucella mutants* or injection of killed whole cells, antigenic fractions of cell lysates, or recombinant protein antigens administered with adjuvants⁶. The researchs indicate that Cellular immune responses to play an important role in eradication or containment of intracellular infections such as brucellae^{7,8,9}. Systemic specific anti LPS antibodies, have also been shown to be protective¹⁰. At present, there is no vaccine licensed for use against brucellosis in humans.

Investigations revealed that CpG DNA mixed to antigens is efficient adjuvant for vaccine-induced Th1-cell responses in mice¹³. *Brucella* periplasmic binding protein P39 used with CpG as an adjuvant has been shown to elicit a Th1-type immune response and to provide protection to immunized mice challenged with virulent *B. abortus* strain 544¹².

We developed a vaccine composed of purified *B. abortus* LPS as a conjugate with the *H. pylori*'s recombinant CagA (rCagA) protein and with CpG. In the present study we studied the protective efficacies of purified LPS and LPS- rCagA and CpG vaccine delivered by subcutaneous (s.c.)

immunization of mice against intraperitoneally (IP) challenge with virulent *B. abortus* strain 544.

MATERIALS AND METHODS

Extraction and characterization of *B. abortus* LPS

Bacterial colonies were collected and sonicated, then extraction of *B. abortus* S19 strains LPS, were carried by Westphal method. Sonicated bacterial solutions was added with same volume of hot phenol-water (9:1, V:V) and then samples were shaken for 30 min at 65-70 rpm. After centrifugation at 3500rpm/30min in 4° C aqueous phases were collected (this step were repeated 3 times). All collected phenolic phases dialyzed against distilled water for 48 h for elimination of phenol also PH=7.4. The LPS extracts were concentrated to 1/5 of the early volume and then digested with use of RNaseH and DNase I (Sigma) enzymes with final concentration of 50 µg /mL at 37° C at 4h. The digested extract were washed in boiling-water for 15 min and then placed at 4° C overnight. The obtained supernatants were centrifugated at 3000 rpm/min for 30 min then were dialyzed against distilled water for 48-72 h. By centrifugation at 5000 rpm/min for 30 min precipitates were collected, then resuspended in distilled water to remove residual alcohol and dialyzed against distilled water for 48 h. The LPS extracts were centrifuged at 100 000 g for 2 h and pellets were dialyzed in distilled water then were lyophilized¹³.

Production of *H. pylori*'s recombinant CagA protein (rCagA)

Single primer pair was used to amplify *H. pylori* cagA gene target fragment based on Gene Bank. The primers had a *Bam* HI site incorporated into the 5' end and a *Sac* I site at the 3' end and their sequences as follows: F: 5'-aaggatccactaacg-aaaccattgacca-3 and R: 5'-aagagctcactcc-tcaacttaacatt-3' that enable amplify fragment to length of nearly 841 bp.

Escherichia coli DH₅± and *E.coli* BL21 were applied as cloning and expression prokaryotic hosts. The pJET1.2 (Fermentas) and pET28a (novagen) were applied for cloning and expression of target ORF. A primer pair was designed to amplify the terminal 841 bp of coding region of cagA.

Recombinant CagA proteins were

centrifuged in 14000 g for 20 min and were collected in supernatant phase. Purified recombinant CagA were also subjected to western blot analysis¹⁴.

Mice

6 to 8 weeks-old male BALB/c mice (obtained from Baqiyatallah University, Iran) were adapted and randomly distributed into experimental groups. Mice were kept in conventional animal facilities and received water and food, in accordance with pertinent US Federal regulations and policies.

Bacterial strains

B. abortus strain 544 (virulent strains) and *B. abortus* S19 (vaccinal strain) were cultured in Brucella Broth (Merck) supplemented and incubated as described^{15,16}.

Lymphocyte proliferation test

The mice were immunized three times with

10 µg of rCagA, LPS, LPS-rCagA conjugate and CpG, in 100µl sterile PBS with 14 days interval, three times by S.C. immunization. 2 weeks after last immunization, spleens were removed and suspended in cold PBS (4°C) under sterile condition from immunized and non-immunized mice; RBCs were lysed using NH₄Cl buffer. Cell suspension was prepared in complete RPMI 1640 (Gibco) and adjusted to 5 × 10⁶ cells per milliliters. 100µl of cell suspension was added to each well of flat bottom 96 well plates and reCagA at concentration of 1µg/ml was added to each one and as negative controls some wells were not added antigen. Volume of all wells adjusted to 200 µl and as positive we used Phytohemagglutinin (PHA) at final concentration of 5µg/ml. After incubation for 72 h at 37°C in 5% CO₂ humid incubator, cell proliferation was measured by using 3(4,5-dimethylthiazol-2-yl)-2,5-

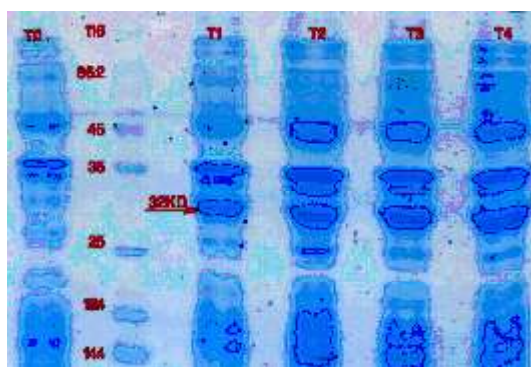


Fig. 1. Coomassie blue staining for recombinant *E. coli* BL21 (DE3) expressed *H. pylori* recombinant CagA (T1, T2, T3 and T4) and non induced *E. coli* BL21 (T0)

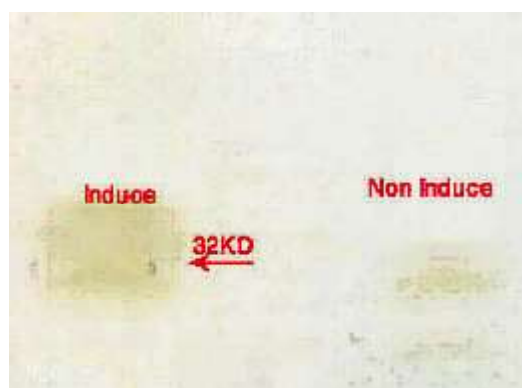


Fig. 2. Western blot analyses for induced *E. coli* BL21 (DE3) expressed *H. pylori* recombinant CagA

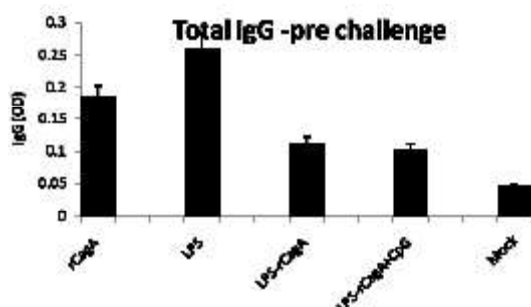


Fig. 3. Total IgG response against specific antigens pre challenge. Sera from four mice per group collected 2 weeks after the last immunization were assayed individually by ELISA.

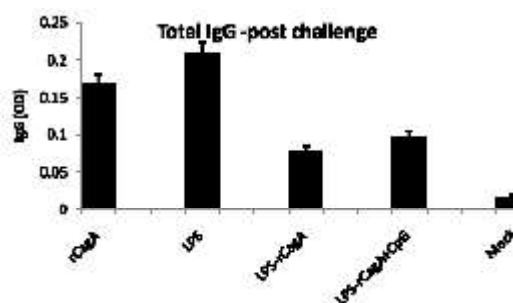


Fig. 4. Total IgG response against specific antigens post challenge. Sera from four mice per group collected 4 weeks after challenge were assayed individually by ELISA

diphenyltetrazolium bromide; thiazolyl-blue (MTT) dye assay. Briefly, 20 μ l MTT was added to each well and plates were further incubated at 37 °C for 4 h. Following incubation, the plates were centrifuged at 300 g for 10 min and then supernatant was aspirated carefully and formazan crystals were solubilized by adding 100 μ l dimethyl sulfoxide into each well. The absorbance of each well was then determined at a wavelength of 540 nm. Stimulation Index (SI) was calculated according to formula: SI= OD of the wells stimulated with antigen / OD of the wells containing only the cells without antigen stimulation^{17,18}.

Immunization and challenge of mice

Groups of male BALB/c mice were

immunized S.C. with rCagA, purified *B. abortus* LPS, LPS-rCagA conjugate, LPS-rCagA conjugate + CpG vaccine. Control mice were immunized with sterile phosphate-buffered saline (PBS) (0.01 M sodium phosphate, 0.14M sodium chloride; pH 7.5) subcutaneously. The procedure used for s.c immunization, mice was given 10-30 μ g vaccine in 200 μ l sterile PBS under the right hind thigh, using a 1ml syringe fitted with a 25 gauge needle. Three doses of vaccine were given 2 weeks apart. 10-30 μ l of vaccine containing 10 μ g LPS or 10 μ g LPS and 10 μ g rCagA for the LPS-rCagA conjugated vaccine or 10 μ g LPS, 10 μ g rCagA and 10 μ g CpG for the conjugated LPS-rCagA + CpG vaccine in sterile PBS was administered. Blood was collected

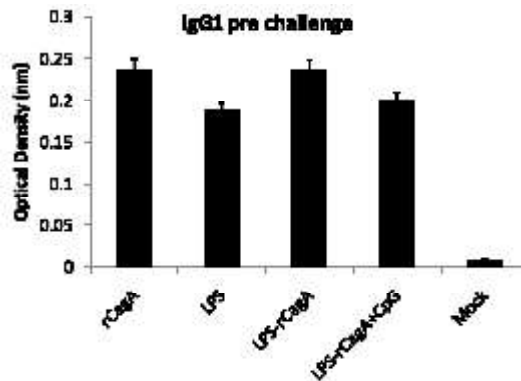


Fig . 5. IgG1 response against specific antigens pre challenge. Sera from four mice per group collected 2 weeks after the last immunization were assayed individually by ELISA

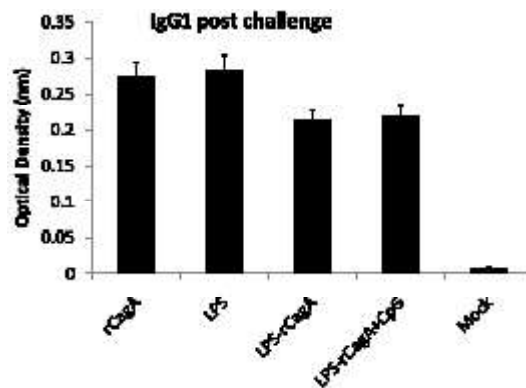


Fig . 6. IgG1 response against specific antigens post challenge. Sera from four mice per group collected 4 weeks after challenge were assayed individually by ELISA

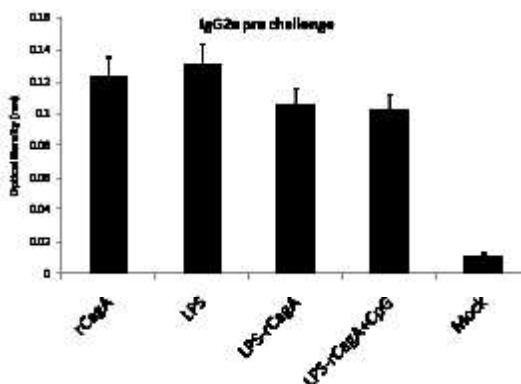


Fig. 7. IgG2a response against specific antigens pre challenge. Sera from four mice per group collected 2 weeks after the last immunization were assayed individually by ELISA

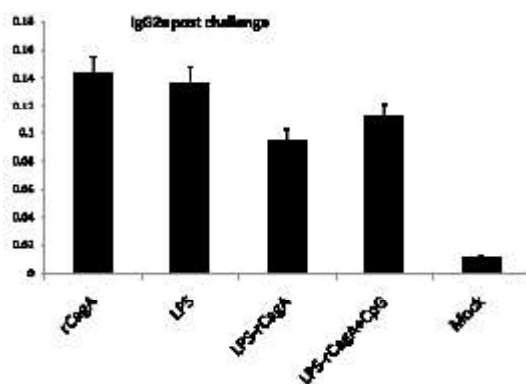


Fig . 8. IgG2a response against specific antigens post challenge. Sera from four mice per group collected 4 weeks after challenge were assayed individually by ELISA

from six euthanized mice in each group 2 weeks after the third dose of vaccine sera were collected and stored at -20°C until they were analyzed for Serum IgG1, IgG total and IgG2 \pm antibodies specific to LPS of *B. abortus* by an enzyme-linked immunosorbent assay (ELISA).

Groups of immunized mice (six mice in each group) were challenged intraperitoneally 2 weeks after the third dose of vaccine with 10^4 colony-forming unit (CFU) of *B. abortus* strain 544 suspended in 200 μl PBS as described previously¹⁹. Blood and spleens were aseptically collected from anesthetized mice 4 weeks post challenge. Antigen specific IL-4, IL-10 and IFN- γ responses were measured in spleen of immunized mice before and post challenge using ELISA test. The numbers of *Brucella* CFU in spleen were determined by dilution and culture on brucella agar²⁰.

Lymphocyte isolation

The mice were sacrificed and used to assay naive splenocyte responses to antigen stimulation. Spleens were sterilely collected from

all mice at the time sacrifice. Spleens were removed and ground through a screen mesh. Red Blood Cells (RBCs) in splenocyte samples were lysed with ACK (Ammonium-Chloride-Potassium) lysis buffer. Cell suspensions from all mice in each group were washed, centrifuged and passed through a 0.45 μm filter. All lymphocytes were then resuspended at 5×10^6 cells/ml in complete RPMI with 10% FCS^{17,18}.

Study of *B. abortus* infection in spleen

One to three (1/3) of spleen tissue in each mice were taken and were analyzed for a quantitative of *B. abortus* infection. Mice spleens were placed in 200 μl of brucella broth, homogenized using a sterile ground –glass pounder. The serial dilutions of 0.1, 0.01, 0.001 and 0.0001 from spleen cultured on brucella agar in a 10% CO_2 incubator for 5-7 days. *B. abortus* detection carried on gram staining, Urease, catalase and oxidase²¹.

Cytokine responses

Spleens cell suspensions from immunized or control mice were prepared in complete medium and plated at 250000 /well in 24-well flat-bottom

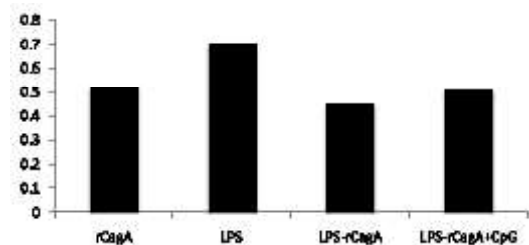


Fig. 9. IgG2a/IgG1 ratio pre challenge

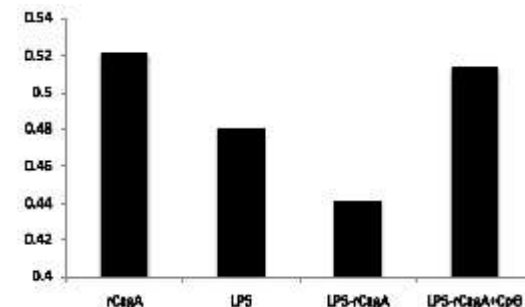


Fig. 10. IgG2a/IgG1 ratio post challenge

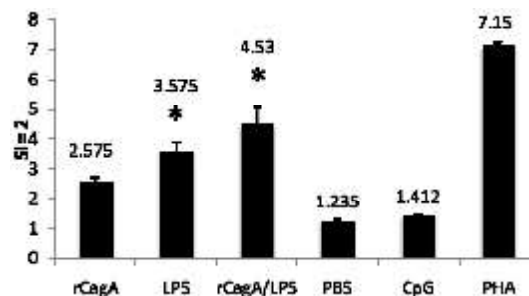


Fig. 11. Proliferation of splenocytes from BALB/c mice immunized with the recombinant CagA, LPS, LPS-rCagA conjugate, CpG and PHA



Fig. 12. IFN- γ response against specific antigens pre challenge

plates (Nunc; Nunc, Roskilde, Denmark). Cells were stimulated in vitro at 37°C in 5% CO₂ with rCagA (5 µg/ml), or complete medium alone. Supernatants were taken after 72 h of culture and stored at -70° C until assayed for cytokine production. IFN-γ, IL-10 and IL-4 in culture supernatants were measured by sandwich ELISA using paired cytokine specific Antibodies according to the manufacturer's instructions (PharMingen, San Diego, CA),²¹.

Statistical analysis

The CFU data were normalized by log

transformation and evaluated by ANOVA followed by Dunnett's post hoc test. Differences between CFU from some experimental groups were also evaluated by Student's *t*-test. The cellular and humoral responses were compared using the non-parametric Mann–Whitney *U*-test.

RESULTS

Construction of the recombinant *E. coli* BL21 (DE3) strains and their expressions of *H. pylori* recombinant CagA

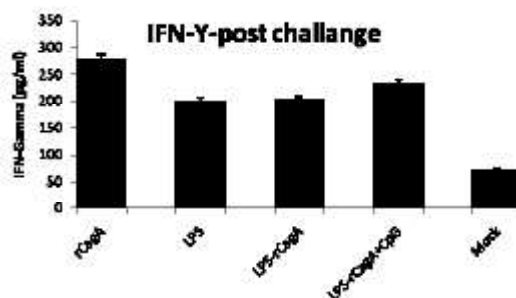


Fig . 13. IFN-γ response against specific antigens post challenge

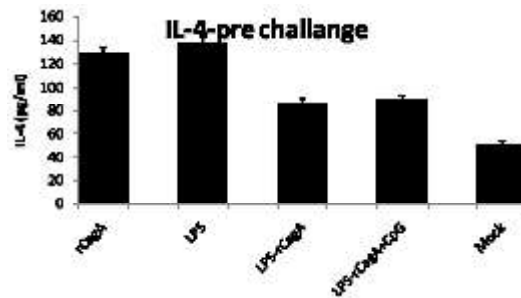


Fig . 14. IL-4 response against specific antigens pre challenge

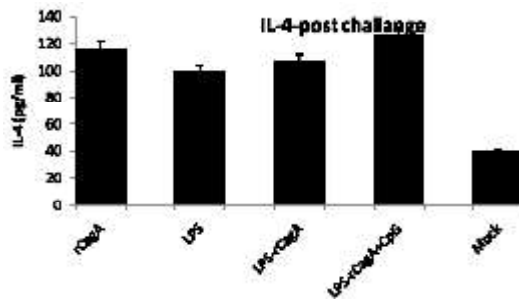


Fig . 15. IL-4 response against specific antigens post challenge

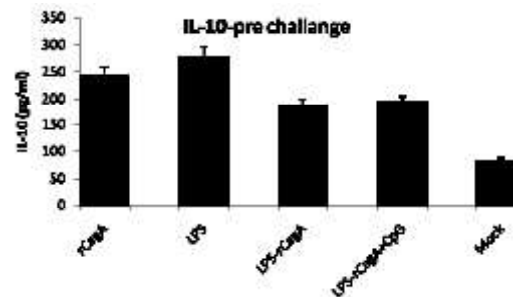


Fig . 16. IL-10 response against specific antigens pre challenge

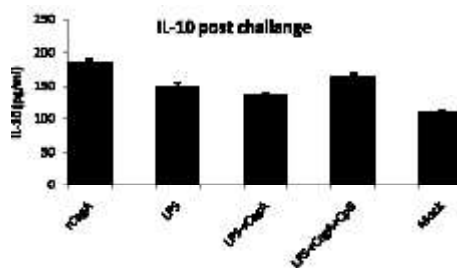


Fig . 17. IL-10 response against specific antigens post challenge

The *H. pylori* rCagA gene was subcloned into pET28a, then recombinant plasmid pET28a - rCagA was successfully electroporated into *Escherichia coli* (*E. coli*) BL21 (DE3) to form recombinant *E. coli* BL21 (DE3) pET28a - rCagA. The expressed *H. pylori* rCagA was visualized by Coomassie blue staining (Fig. 1) and Western blot analyses (Fig. 2). It turned out that the *H. pylori* rCagA protein was expressed by recombinant *E. coli* BL21 (DE3) pET28a - rCagA strains (band 1), while *E. coli* BL21 (DE3) pET28a alone did not express any detectable *H. pylori* rCagA (band 3).

Immune response in mice

Antibodies response

Indirect ELISA demonstrated LPS stimulated a mixture of Total IgG, IgG1 and IgG2a two weeks after the last vaccination. Total IgG response against a specific antigen was higher in mice group vaccinated with LPS than LPS-rCagA conjugate and LPS-rCagA conjugate + CpG on pre and post challenged mice (Fig 3 and 4).

All groups (Except LPS-rCagA conjugate with LPS-rCagA + CpG groupe) have significantly difference from Total IgG antibody titers ($P < 0.05$).

Immunization with LPS elicited a strong specific IgG response, but decreased after challenge with *B. abortus* strain 544 (Fig. 4). Immunization with LPS-rCagA conjugate and LPS-rCagA + CpG induced lower levels of antibodies.

The anti- LPS IgG subtype titers of mouse sera after vaccination are shown (Fig. 5,6,7,8).

The anti-LPS IgG1 titers have significantly difference between all groups ($P < 0.05$). Also there was significant difference in anti-LPS IgG2a antibodies ($P < 0.05$).

Compared with results of LPS-rCagA conjugate vaccine, The CpG that adjuvanted to

LPS-rCagA conjugate, failed to induced a significant IgG respons .

As shown in Fig. 5 and Fig. 7, LPS - immunization elicited high levels of IgG1 as well as IgG2a antibodies on pre challenged mice.

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Proliferative responses to purified antigens.

Figure 11 shows the pattern of the proliferative response of splenocytes. Splenocytes from animals immunized with the recombinant CagA protein proliferated in response to the specific antigen. All animals immunized responded to the polyclonal stimulant PHA as a positive proliferation control.

There was significant difference between rCagA with LPS-rCagA conjugate ($p = 0.001$), LPS with LPS-rCagA conjugate ($p = 0.046$), CpG, LPS and LPS-rCagA conjugate with PBS ($P < 0.05$) and all groups with PHA ($p = 0$).

Cellular immune response

To further investigate the cellular immune

Table 1. Protection against *B. abortus* strain 544 compared with mice injected with PBS (control)

Group	Vaccine	Log10 units of brucella in spleen
1	rCagA	10
2	LPS	20
3	LPS-rCagA conjugate	12
4	LPS-rCagA conjugate + CpG	12
5	PBS	150

response induced by the vaccines, the cytokines (IFN-g, IL-4 and IL-10) production were analyzed.

Sandwich ELISA assays were used to determine IFN-g, IL-4 and IL-10 levels in the supernatants of the splenocytes cells and the antigens (2 weeks post-vaccination and 4 weeks post-challenge) (Fig. 12,13,14,15,16,17).

Statistic analysis of IFN-g titers showed significant difference between all groups with Mock ($p=0$). But there was no significant difference between other groups. Also analysis of IL-10 titers have Similar results.

In the other hand statistic analysis for IL-4 significant difference between rCagA with LPS-rCagA conjugate and LPS-rCagA+ CpG groups ($p=0.001$). why?

Statistic analysis between IFN-g and IL-4 titers showed significant difference on all groups ($p=0$).

Statistic analysis between IFN-g and IL-10 titers showed significant difference on all groups ($p=0$).

Statistic analysis between IL-10 and IL-4 titers showed no significant difference on all groups.

Protection assay

To analyse the vaccine efficacy of rCagA, LPS, LPS-rCagA conjugate and LPS-rCagA conjugate + CpG against virulent *B. abortus* strain 544 challenge infection, an *in vivo* protection study in BALB/c mice was performed. In this experiment, protection was defined by subtracting mean counts to the vaccinated group from the mean of the mice receiving PBS. The vaccine efficacy was calculated as the decimal logarithm (\log_{10}) of protection. Table 1 shows that the protection decimal logarithms in mice immunized with rCagA, LPS, LPS-rCagA conjugate and LPS-rCagA conjugate + CpG were 4 weeks after the challenge, respectively.

DISCUSSION

Brucella is facultative intracellular pathogen that preferentially infect macrophages²². Prevention of brucellosis is only by animal vaccination. Live attenuated vaccines by *B. abortus* S19 and RB51 strains and *B. melitensis* Rev1, are licensed to prevent animal brucellosis. They give short term immunity²³. There is no immunization strategy for human thus development of an

effective subunit vaccine against brucellosis is necessary. *B. abortus* LPS has been shown to be 10,000-fold less pyrogenic than *E. coli* LPS in eliciting fever in rabbits and 268-fold less potent in killing sensitized mice²⁴.

Adjuvant increases the efficacy of vaccines by increasing immune response to an antigen²⁵. CpG DNA is a djuvant that induce B cell and T cell responses²⁶.

Brucella is a bacterium virulence genes that modify phagocytosis, phagolysosome fusion, cytokine secretion, and apoptosis^{27,28,29}. IFN-g has a central role in the pathogenesis of brucellosis by activating macrophages, producing reactive oxygen species and nitrogen intermediates; by inducing apoptosis, enhancing cell differentiation and cytokine production; by converting IgG to IgG2a; and by increasing the expression of antigen-presenting molecules³⁰. So strong cellular immune responses with associated high levels of IFN-g are believed to be important for long-term protection against intracellular pathogens such as *Brucella*^{31,32}. Moreover, the nitric oxide (NO)-mediated killing possibly plays a role in *Brucella* killing³³. The antigens of *brucella* induce specific B cell responses to secrete and both types of Th cells induce the secretion of IgM and IgG3³⁴. The meningococcal outer membrane protein (GBOMP) is also known to increase expression of costimulatory molecules on murine B cells and to enhance antibody responses to polysaccharide antigens³⁵.

Apurba K. Bhattacharjee *et al.* demonstrated that the serum anti-LPS antibody response persists at a high plateau for at least 16 weeks after the second dose of vaccine and is still at one-fourth of its peak level at the 25-week mark⁶. This prolonged, high-titer response may partially reflect the unusual prolonged persistence of *Brucella* LPS on the surface of antigen-presenting cells³⁶, but it may also reflect a contribution of the adjuvant protein component of the vaccine. The adjuvant protein increases expression of co stimulatory molecules on murine B cells and enhances antibody responses to polysaccharide antigens³⁷. The pattern of antibody subclass expressed by mice immunized with the adjuvant protein -LPS is also different from the pattern evoked by immunization of mice with *B. abortus* LPS or infection with *B. abortus*^{38,39,40}.

Interestingly, Kurtz and Berman found that immunization with *B. abortus* LPS containing 5 to 6% protein by weight led to production of IgG1 antibodies directed against the protein component and IgG3 antibodies directed against the LPS component, while immunization with *B. abortus* LPS containing <1% protein evoked primarily IgG3 directed against LPS determinants³⁹. Caroff et al. have shown that the *B. abortus* structure consists of an unbranched linear homopolymer of 1,2-linked 4,6-dideoxy-4-formamido- α -D-mannopyranosyl residues⁴¹. Al-Mariri et al. shown that the decimal logarithms of the protection of P39 protein with the CpG adjuvant vaccine were 1.28 and 1.6, 4 and 8 weeks post-infection respectively¹².

The present studies also demonstrate that subcutaneously immunization with LPS-rCagA significantly protects mice from dissemination of infection to the spleen when animals are challenged intraperitoneally with *B. abortus* strain 544. The degree of protection against dissemination after intraperitoneally challenge in the present study was similar to that previously reported with a live, attenuated vaccine administered i.p.⁴².

Vaccine modifications to elicit antibodies of different subclasses or greater avidity or addition of protein antigens to enhance cell mediated immune responses may enhance efficacy and lead to inhibition of local, as well as disseminated, infection.

There have been a number of studies to show the protective effect of brucella subunit vaccines in animal models. Numerous studies have demonstrated that immunization of mice with killed smooth strains of *B. abortus* or *B. melitensis* reduces the number of *B. abortus* CFU in the spleen or liver when mice are challenged intravenously or intraperitoneally (i.p.)^{43,44,45}.

Brucella O-polysaccharide- specific monoclonal antibodies were shown to provide protection against challenge with *B. melitensis* and *B. abortus* smooth strains^{46,47}. The protection demonstrated in the studies described above was defined as a 1- to 2-log reduction in the intensity of infection of spleens and livers after intravenous or intraperitoneal challenge of mice. CpG oligonucleotides contain unmethylated CG motifs similar to those found in bacterial DNA that trigger Toll-like receptor 9 (TLR9) in the vertebrate immune system⁴⁸.

We developed a vaccine composed of purified *B. abortus* lipopolysaccharide (LPS) as a conjugate with the *Helicobacter pylori*'s recombinant CagA protein and with CpG. *Helicobacter pylori*'s recombinant CagA protein has been shown to enhance the immunogenicity of peptide and polysaccharide vaccines. We have shown that immunization of mice with this LPS-rCagA vaccine elicited both humoral and cellular responses in mice¹⁴. We define protection as complete clearance of bacteria from the organs studied.

In the present study we studied the protective efficacies of purified LPS and LPS-rCagA and CpG vaccine delivered by subcutaneous (s.c.) immunization of mice against intraperitoneally (IP) challenge with virulent *B. abortus* strain 544. In this study 32CagA fragment of N-terminal was selected and In this study the IgG1/IgG2a ratio in the mice was <1 and also IFN- γ promoted indicating a Th1 type response. The balance between Th1 / Th2 responses is essential for *H. pylori* clearance. Th2 responses are needed for sufficient antibody production. However, Th1 responses are seen to occur more often in natural cases of *Brucella* infections. Multi component rCagA-LPS + CpG reduced 1.2 log bacteria load.

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