Recombinant Protein and DNA Vaccine Construct of *Brucella abortus* L7/L12 Gene Elicits Immune Response

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ABSTRACT

Both cell mediated and humoral immune responses are required for recovery from *Brucella abortus* infection. In the development of vaccines capable of providing immunity against brucellosis, L7/L12 50S ribosomal protein has been demonstrated to be one of the protective immunogens of *Brucella abortus.* The recombinant *Brucella abortus* L7/L12 ribosomal protein previously produced in the *E. coli* system was purified by affinity chromatography. The DNA vaccine (pVL7/L12) construct was purified in bulk by endotoxin free reagents. The present study was designed to evaluate cell mediated immune response and humoral immune response after the intramuscular vaccination of mice with DNA vaccine alone (Group 1), recombinant protein alone (Group 2) and DNA vaccine followed by recombinant protein boosting (Group 3). The protective efficacy of the said vaccines also evaluated. Animals vaccinated with pVL7/L12 (DNA vaccine) alone did not develop L7/L12 specific antibodies at least until 60 days of vaccination. But recombinant protein induced a strong antibody titer (1:32000) after 60 days of post immunization which exhibited a dominance of immunoglobulin IgG2a over IgG1. L7/L12 DNA vaccine elicited a T-cell proliferation response and also induced the production of IFN-γ. The lymphocyte proliferation and IFN- γ production was significantly higher ($P<0.001$) in Group 3 than the other groups. Group 3 also induced strong IgG2a response. The DNA vaccine followed by protein boosting induced a strong significant level of protection in mice against challenge with *B. abortus* 544. The level of protection in Group 3 was significantly higher than the DNA vaccine or recombinant protein alone. Altogether, the data generated in the present study suggested that L7/L12 DNA vaccine followed by recombinant protein boosting is a good candidate for use in future studies of vaccination against brucellosis.

Keywords: *Brucella abortus*, DNA vaccine, Immune response, Immunoglobulin, Recombinant protein.

Introduction

Brucella abortus S19 a smooth, avirulent strain is used to vaccinate cattle. Although the S19 vaccine gives considerable protection to cattle, it is pathogenic to humans and it is not effective in controlling the infection of udder, through which milk used for human consumption is contaminated with the bacterium. Further, the vaccine strain, cross reacts with the serological tests used to diagnose clinically infected cases. This is a major drawback in any control program. In the given scenario various researchers are in the process of developing safe and efficacious vaccines against brucellosis. Subunit nucleic acid vaccine strategies which negate many of the demerits of traditional vaccines give us an opportunity to find a suitable solution to control the disease. Many immunogenic proteins like Omp 28, Omp 31, Cu-Zn SOD, L7/L12 have been tried as recombinant subunit,

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nucleic acid, synthetic peptide vaccines.L7/L12 50S ribosomal protein is a promising candidate because it has given good results in mice models. Further, Brucellin INRA (sLPS free cytosolic extract from rough *B. melitensis* B115) which is a purified protein used in skin test to screen positive cases of brucellosis fails to elicit a delayed type hyperimmune (DTH) response, when the L7/L12 fraction is removed from the purified protein [1]. L7/L12 ribosomal protein participates in bacterial protein synthesis by binding elongation factor G and TU. Like any other ribosomal protein this 12 kDa protein encoded by a 375 bp gene is also conserved across many genera of bacteria like *E. coli,* Mycobacteria etc. The exact antigenic motif recognized by the host immune system has not yet been elucidated. L7/L12 with its helix turn helix DNA binding motif makes it a suitable candidate to screen for putative T cell epitopes [2]. Any vaccine against brucellosis should give a predominantly Th1 mediated immune response to clear the infection. IFN-γ (interferon) and TNF-α have been implicated as key cytokines needed to confer protection against the disease. Keeping all this in mind the present work was undertaken with the objectives of analyzing cellular immune response and humoral immune response against L7/L12 recombinant protein and its DNA vaccine construct in laboratory animal and to study the protective efficacy of recombinant protein and its DNA vaccine construct against challenge in laboratory animal.

Material and methods

Bacterial cultures

Brucella abortus S19 and *Brucella abortus* 544 were obtained from Brucella Referral Lab., Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar, India.

Experimental animals

Apparently healthy female Swiss albino mice (4 to 6 weeks age) were obtained from the Laboratory Animal Research Section, IVRI, Izatnagar, India. The animals were kept under conventional housing condition and provided feed and water *ad lib.*

Immunoconjugates

Anti-mouse IgG (H+L) HRPO (horse radish peroxidase) conjugate, anti-mouse IgG (Fab) HRPO and anti-mouse (IgG1-IgG2a) conjugate were used for ELISA and immunoblotting.

Gamma-Interferon EIA kit

Sandwich ELISA kit was used to estimate mouse Gamma-Interferon level.

Enzymes

Restriction endonucleases, Taq DNA polymerase, Proteinase-K, RNase-A were used in this study.

Oligonucleotide primers

Oligonucleotide primers were designed from published sequence of *B. abortus*. The primers BA1 (Forward) and BA2 (Reverse) both had linear sequences at their 5' ends.

BA1

5'- CGC CGT CGA CAT GGC TGA TCT CGC AAA GATC - 3'

BA2

5'- GCC CCT GCA GTC TAG ATT ACT TGA GTT CAA CCT TGGC-3'

Vectors and host cells

Ins T/A easy vector and DH5α *E. coli* culture were used in this study.

Plasmid isolation kit

Endotoxin free plasmid Maxi kit was used for bulk DNA vaccine production.

Molecular weight marker

Medium range protein molecular weight marker; Pre-stained molecular weight marker and 100 bp DNA ladder were used.

Recombinant clones

pPROC L7/L12 for prokaryotic expression and pVL7/L12 for DNA vaccine were obtained from IVRI, India.

Preparation of competent cells

E. coli stain DH5α was made competent by $CaCl_2$ method. *E. coli* strain DH5α was grown for 2 h in shaking incubator at 37°C in 10 ml test tube in LB broth without ampicillin till the OD of culture 0.2 - 0.35 was attained. One ml culture was taken in each 1.5 ml eppendorf tube and pelleted at 6000 rpm for 10 min. The pellet was used for subsequent steps. 200 µl of 0.1M $CaCl_2$ was added in each tube and mixed properly. Further it was centrifuged at 6000 rpm for 10 min at 4°C. The pellet was recovered after discarding the supernatant. The pellet was re-suspended in 200 µl 0.1M $CaCl_2$ and kept in ice for 45 min. Each aliquot of cells was used for transformation.

Transformation of PPROL7/L12 and pVL7/L12

2 µl of each recombinant plasmid was mixed with 200 µl of competent cell aliquot and kept in ice for 1 h. Positive and negative controls were always kept. Heat shock to the cells was given at 42°C for 90 sec and cells were then immediately transferred back to ice for 5 min. About 600 µl of LB medium was added to the transformed cells and incubated at 37°C for 45 min. The cells were then plated out into the LB agar containing ampicillin for pPROL7/L12 and kanamycin for pVL7/L12. The plates were incubated at 37°C overnight. After incubation the plates were observed for presence of colonies and stored at 4°C.

Screening of recombinant clones

Few colonies were picked at random and incubated in 3 ml LB broth containing ampicillin or kanamycin. The broths were incubated overnight at 37°C under constant shaking. The plasmids were extracted following alkali lysis method [3].

Screening by PCR

Plasmid DNA from transformed bacteria was used as template for amplification of L7/L12 gene. The PCR mixture consisted of 50 ng of template, 30 pM of each primers BA1 and BA2, 200 µM of each dNTPs, 5 µl of 10X PCR buffer and 2.5 units of Taq DNA polymerase. The volume of the reaction was made up to 50 µl with distilled water. PCR was performed using a DNA thermal cycler with the following conditions: 5 min. at 94°C followed by 30 cycles consisting of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, finally an extension of 10 min at 72°C. Around 10 µl of amplified product was then analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

Screening by restriction endonuclease digestion

Plasmid isolated from transformed bacteria was subjected to double digestion with restriction enzymes. Approximately 1.0 µg of plasmid was digested with 1 unit of restriction endonucleases, *BamH*1 and *Xba*1 in reaction volume of 20 µl. Digested plasmid was electrophoresed on 1.0% agarose gel stained with ethidium bromide.

Sequencing of transformed clones

After screening by PCR and RE digestion, the recombinant clones were to confirm the correct reading frame and sequence changes, if any.

Isolation of plasmid DNA from pVL7/L12 for the production of DNA vaccine

DNA vaccine grade recombinant pVL7/L12 having complete gene in right orientation was prepared using standard kit following the manufacturer's protocol.

Quantification of plasmid DNA concentration

The concentration and purity of DNA was analyzed by UV spectrophotometer. The purity of plasmid DNA was checked spectrophotometrically by considering the ratio of OD260/OD280.

Production of recombinant protein from pPROC L7/L12 in bulk

Induction of expression

Overnight culture of the recombinant clone was inoculated in 500 ml of LB broth containing ampicillin (100 µg/ml) and grown at 37°C with vigorous shaking. The culture was induced with IPTG to final concentration of 1 mM when the OD at 600 nm reached 0.5. After 5 h the culture was washed thrice with PBS and re-suspended in 25 ml PBS. Then the culture was stored at -20°C till further use.

SDS-PAGE analysis of the protein

SDS-PAGE analysis was carried out as described by Laemmli [4] in a vertical mini-gel electrophoresis apparatus using 15% resolving gel having a stacking gel of 5% concentration. Electrophoresis was carried out at a constant current of 18 mA, until the tracking dye reached the bottom of the gel. The gel was taken out from the plates and stained with 0.25% (W/V) Coomassie brilliant blue R-250 prepared in methanol, glacial acetic acid and water (45:10:45) and destained

with destaining solution (20 parts methanol : 10 parts glacial acetic acid and 70 parts distilled water).

Purification of recombinant L7/L12 protein

Polyhistidine tagged fusion protein was purified under denaturing conditions by metal chelate affinity chromatography with certain modifications introduced in the purification parameter.50 µl of induced culture was re-suspended in 4 ml of lysis buffer (buffer B) containing imidazole (20 mM), 1M NaCl, glycerol (20%) and Tritonx 100 (25%). It was held at room temperature for 1 h with intermittent mixing. The cell debris was removed by centrifugation at 10,000 rpm for 20 min. The supernatant was incubated with 1 ml of Ni-NTA resin for 40 min and packed into column. Thereafter column was washed thoroughly with wash buffer (buffer C). The fusion protein was eluted in 0.5 ml x 4X with the elution buffer (buffer E). Five elutes were collected. Elutes were checked by SDS-PAGE as described earlier. Elutes were dialyzed against PBS to remove urea.

Estimation of concentration of recombinant protein

Concentration of recombinant protein, L7/L12 was estimated by protein-estimation kit according to the protocol originally described by Lowry *et al.* [5] taking BSA as a standard.

Preparation of *B. abortus* 544 for challenge study and *B. abortus* S19 for vaccination

Brucella abortus 544 and *B. abortus* S19 were subcultured separately on Tryptose phosphate agar on Tubes as slant culture and incubated at 37°C for 48 h. *B. abortus* 544 culture was incubated under 5% CO_2 tension. The growths were harvested with tryptose phosphate broth by gentle shaking.

Viable count was determined as per method described by Alton *et al*. [6] with some modifications. Tryptose phosphate agar was used for the enumeration of the organisms. Serial 10-fold dilution of the suspension was made up to 10^{-10} in tryptose phosphate broth. From the dilution 10^{-1} to 10^{-10}, 10 µl was taken and plating was done on tryptose phosphate agar using three replica for each dilution. The plates were incubated at 37°C for 48-72 h. For *B. abortus* 544 plates were incubated under 5% CO_2 tension. Plates showing 30-300 cfu were selected for colony count. The mean of all the plates was multiplied by dilution factor. This was taken on the number of viable *Brucella* in the suspension.

Preparation of sonicated antigen from Brucella

Sonicated antigen was prepared following the method described by Zoha and Carmichael [7] with slight modification. The antigen was prepared from live *Brucella abortus* S19. The organisms were first heat killed at 65°C for 2 h, and then it as harvested in PBS. After centrifugation at 10,000 *g* for 20 min, cell sediment was washed again in PBS. Equal volume of sediment and PBS was mixed and sonicated for 30 cycles for 1 min each at amplitude of 30. The sonicated cell suspension was centrifuged at 15,000 *g* for 1 h. The supernatant was taken as sonicated antigen extract. Concentration of this sonicated antigen was estimated by protein-estimation kit as originally described by Lowry *et al.* [5].

Experimental vaccination trial in mice

A total of 75 number of six week old female Swiss albino mice were housed in cage and fed *ad lib*. Mice were allowed to acclimatize for 1 week before vaccination. Five vaccine groups of 15 mice each were immunized by deep intramuscular route with various preparations as given below:

Group 1

50 µg (a total volume of 300 µl in PBS) of L7/L12 DNA vaccine (PVL7/L12) was injected to each mouse. Two boosters were given with same concentration of DNA vaccine on day 15 and day 30.

Group 2

30 µg (a total volume of 150 µl in PBS) of recombinant L7/L12 protein was emulsified with 150 µl of Freund's incomplete adjuvant was injected to each mouse. 2 Booster were given with same concentration of protein on day 15 and day 30.

Group 3

50 µg (a total volume of 300 µl in PBS) of pVL7/L12 was injected to each mice. Two boosters were given with 30 µg of recombinant L7/L12 protein to each mice at day 15 and day 30.

Group 4

50 µg (a total volume of 300 µl in PBS) of pVax vector was injected to each mice as vector control. Boostering were done by same concentration of vector on day 15 and day 30.

Group 5

300 µl of PBS to each mouse.

Study on humoral immune response

Serum samples were collected from the mice on day 0, day 7, day 14, day 30, day 45 and day 60.

Detection of antibody by ELISA from collected serum samples

The presence of serum immunoglobulin G (IgG), IgG1 and IgG2a isotypes with specificity to L7/L12 was determined by indirect ELISA. The purified recombinant L7/L12 was diluted to 500 ng/ml in carbonate buffer (pH 9.6) and used to coat the wells of polystyrene plates (100µl/well). The plates were incubated at 4°C overnight. Next day plates were washed thrice with PBS-T (phosphate-buffered saline containing 0.1% Tween-20). Blocking was done with 1% BSA in PBST and incubated for 1 h at 37°C. Plates were washed thrice with PBS-T. Sera samples collected at different days along with negative control in the dilution of 1:200 were put in 100 µl quantity per wall. The plates were incubated at 37°C for 60 min and washed thrice as before. Anti-mouse conjugates were added (100 µl/well) at an appropriate dilution. The plates were again incubated at 37°C for 60 min and washed thrice as before. Finally 100 µl of freshly prepared substrate solution was added to each well. The plates were incubated for 15 min in the dark and the reaction was stopped by addition of 100 µl of 0.18 M H_2SO_4 per well. Absorbance was measured at 492 nm.

Western blot analysis

Serum samples of different days in different dilutions were assayed by Western blot. Western blotting was carried out according to Towbin *et al*. [8] with some modifications. Purified r-L7/L12 fusion protein, sonicated Brucella antigen and prestained marker were loaded on 15% SDS-PAGE and electro-blotted on to nitrocellulse paper (NCP) at 2 mAmp/cm2 constant current for an hour, the transfer was carried out in semidry electro blot apparatus. After the transfer the nitrocellulose paper was blocked by 5% skim milk powder for overnight. After completion of blocking, the NCP was washed thoroughly followed by cutting. Strips were incubated with appropriate dilutions of different serum at 37°C for 1 h. After the reaction primary antibody, NCP was washed three times with PBS-T and incubated with antimouse IgG-HRPO conjugate for 1 h. Color development was carried out with substrate DAB/$NiCl_2$/H_2O_2 visualization solution. After the optimum color was developed, the NCP was washed with excess water to stop the reaction.

Detection of cell mediated immune response

Splenocyte cultures and lymphocyte proliferation assay [9]. Mice were sacrificed at various time intervals of post immunization for collection of splenocytes to detect the *in vitro* CMI response. After one week, 3 weeks and 6 weeks of post immunization mice were sacrificed and their spleens were removed under aseptic conditions.

Several perfusions were made in spleen by using 1 ml tuberculin syringe having fine needle. Then the cells were slowly washed out by PBS-D solution. About 3 ml of washed cells was aseptically layered over 3 ml Histopaque in 15 ml plastic centrifuge tubes. The tubes were centrifuged at 1400 rpm for 40 min at 4°C. The interface ring rich in mononuclear cells were collected carefully and washed twice in sterile PBS-D at 1200 rpm for 10 min each time. The cells were then re-suspended in RPMI 1640 and medium washed again. Finally the cells were suspended in RPMI 1640. Viability of cells was ascertained by trypan blue dye exclusion method and cell concentration was adjusted to achieve a cell count of 4 x 10^6 cells/ml. Splenocytes were cultured at 37°C with 5% CO_2 in a 96-well flat-bottom plate at a concentration of 4 x 10^5 viable cells/well in the presence of 0.4 µg sonicated *B. abortus* S19 proteins, 0.08 µg of purified L7/L12, 0.25 µg of Concavalin A per well, or no additives (non-stimulated control) according to Onate *et al*. [10]. Phenol red free RPMI-1640 medium supplemented with 2 mM L glutamine, 10% heat inactivated fetal calf serum and 50 µl of penicillin-streptomycin was used for culturing the splenocytes. After 96 h, 20 µl of MTT solution (5 mg/ml in PBS) was added to each well and further incubated for 4 hrs and the plates were then centrifuged at 1500 rpm for 10 min. 100 µl of culture supernatant was discarded from each well. Finally, 150 µl of dimethylsulfoxide (DMSO) was added to each well and mixed thoroughly avoiding any foam formation in order to dissolve formazan crystals. The intensity of colour development was measured by taking absorbance (OD) at wavelength of 570 nm with the reference wavelength of 630 nm in microplate reader.

Stimulation index (SI) was calculated using the following formula

OD of stimulated culture =

$$\frac{\text{Mean SI (MTT)}}{\text{Mean OD of non-stimulated culture}}$$

Detection of Gamma-Interferon level in immunized mice

For detection of IFN-γ, culture supernatants of spleen cells were collected after 48 h of antigen stimulation and tested for the presence of IFN-γ by antigen capture ELISA using Opt EIA Set Mouse IFN-γ. All assays were performed in triplicate.

The concentration of IFN-γ in the culture supernatants was calculated for each experimental group using a linear-regression equation obtained from the absorbance values of the standards.

Protection experiments

A total of 72 no. six weeks old female Swiss albino mice were housed into six groups. Mice were allowed to acclimatize for 1 week before vaccination. 12 mice were taken for each group as given below.

Immunization with different antigens

Group 1: DNA vaccine (pVL7/L12)

Group 2: Recombinant protein vaccine (pPROL7/L12)

Group 3: DNA vaccine followed by protein boosting

Group 4: *B. abortus* S19 vaccine (positive control group)

Group 5: PBS (control group)

Group 6: PVax1 vector (Vector control group)

Vaccination schedule, doses and routes of immunization:

DNA vaccine and pVax1 vector were given through intramuscular route at dose rate of 50 µg per animal. Two boosters immunization were performed at day 15 and day 30 of primary immunization. Recombinant protein vaccine was given through intramuscular route at a dose of 30 µg per animal. Two boosters immunization were performed at day 15 and day 30 of primary immunization. One group of animals was given DNA vaccine and two boosters immunization were performed with recombinant protein vaccine. *B. abortus* S19 was given through intra-peritoneal route at dose rate 5 x 10^4 cfu (colony forming unit) per animal. No booster immunization was performed in this group. PBS control group was received only phosphate buffer saline (0.2 ml) by intramuscular route.

Challenge of animals by pathogenic Brucella

Thirty days after booster immunization all the mice were challenged with virulent *Brucella abortus* spp. Animals were challenged with 5 x 10^4 live cells of *B. abortus* 544 strain by intraperitoneal route. Four weeks after challenge, the mice were sacrificed for splenic clearance assay. Spleens were removed from the mice aseptically, homogenized in 2 ml of TPB (Tryptose phosphate broth). 10-fold serial dilutions were made in TPB and plated in triplicate. *B. abortus* 544 colonies were counted after 3 days of incubation at 37°C with 5-10% CO_2.

Statistical analyses

The data for lymphocyte proliferation, detection of IFN-γ and splenic clearance were analyzed by one way analysis of variance and mean effect by using Duncan multiple range test [11].s L7/L12 gene.

Results

Screening of recombinant clones (pPROL7/L12 and pVL7/L12)

Screening by PCR

Plasmid DNA from transformed bacteria was used as a template for PCR reaction and BA1 and BA2 used as forward and reverse primers. A single band of 375 bp was found in agarose gel electrophoresis of the amplified product. It was same as desired size of the gene.

Screening by Restriction endonuclease digestion

Plasmid DNA isolated from transformed bacteria was subjected to double digestion by *BamH*1 and Xba1. On agarose gel electrophoresis of the digested product the band size was same as PCR product.

Sequencing of transformed clones

The recombinant clones were sequenced. Sequences of both the clones were same and correct reading frame. The sequences were analyzed in PROTEAN programme of laser gene software. The predicted protein structure and amino acid sequence was same as desired L7/L12 protein.

Isolation of vaccine grade endotoxin free plasmid DNA and quantification

Plasmid DNA was isolated in bulk by using standard kit. Plasmid was checked in agarose gel electrophoresis. Further, the plasmid DNA was also checked for purity and concentration spectrophotometrically by recording OD at 260 and 280 nm. The purified DNA had the yield of around 400 µg from 250 ml culture. The ratio of OD260/OD280 was 1.8, indicating DNA preparations to be pure and free from RNA and protein contaminations.

Production and quantification of recombinant protein from pPRO: L7/L12 in bulk

The culture was induced with IPTG when OD at 600 nm reached 0.5. After 5 h induction the culture was pelleted and washed with PBS. Then the culture was lysed by Buffer 'B' (pH 8.0). The lysate was checked in SDS-PAGE. A distinct band was observed and that band was absent in non-induced culture. After checking polyhistidine tagged fusion protein was purified under denaturing conditions by metal chelated affinity chromatography (as per manufacturer's protocol), with some modification introduced in the purification parameter. Different elutes were checked in SDS-PAGE. No non-specific band was found in the purified protein when analyzed in SDS-PAGE. Pure distinct single band at 22 kDa position was observed. Then elutes were subjected to dialysis against PBS. Then the concentration of the purified dialyzed protein was estimated by protein-estimation kit. The concentration of the protein was 0.5 µg/µl). The purified protein had the yield of around 4.5 mg from 500 ml of culture. Hence the recombinant clone had given high level of expression.

Humoral immune response studies in mice

Detection of antibody by ELISA from collected serum samples

The presence of serum immunoglobulin G (IgG), IgG1, and IgG2a isotypes with specificity to recombinant L7/L12 protein was determined by indirect ELISA. The level of anti L7/L12 antibodies were measured in pooled serum from the DNA vaccine alone (Group 1), recombinant protein alone (Group 2), DNA vaccine followed by protein boosting (Group 3), Vector control (Group 4) and PBS control (Group 5). Animals inoculated with DNA vaccine showed very low levels of anti L7/L12 antibody. Recombinant protein vaccine group showed a sharp increase in IgG level at 21 days of immunization and it was increased up to 45 days immunization and maintained up to 60 days. But there was a sharp increase in serum IgG level after 30 days of immunization in case of Group 3 and it was maintained at least up to 60 days of immunization. None of the animals inoculated with pVAX vector and PBS control showed anti-L7/L12 antibodies. Serum of Groups 2 and 3 were pre-dominantly IgG2a. High level of IgG2a was detected after 21 days of immunization in case of Group 2 and 30 days of post immunization in case of Group 3. But after 45 days of immunization the level of IgG2a was higher in Group 3 than Group 2 and it was maintained at 60 days of post immunization. A rise in serum IgG1 level was observed at 21 days in case of Group 2 but then it declined gradually. But in case of Group 3 the IgG1 level was very less.

In a repeat experiment the titration of the sera samples of recombinant protein vaccinated mice were done in ELISA. Brucella positive serum also has been titrated. After 30 days of post immunization the titer was 1:32000 and maintained up to 60 days of post immunization. But the titer declined at 90 days post-immunization. Only 1:16000 serum titer was detected after 90 days of immunization. Brucella positive serum also showed high titer (1:32000).

Western blot analysis

In western blot analysis recombinant L7/L12 protein and *Brucella* sonicated antigen reacted to Group 2 serum. After 14 days of immunization of Group 2 and after 30 days of immunization in case of Group 3 serum in a dilution of 1:1000 strongly reacted to *Brucella* antigen. *Brucella* positive serum in a dilution of 1:1000 strongly reacted to L7/L12 recombinant protein. Serum collected from Group 2 at 90 days of post immunization strongly reacted to L7/L12 recombinant protein in 1:1000 dilution. None of the sera collected from DNA vaccine alone (Group 1) reacted in western blot. Also serum from the vector (pVax) control and from the PBS control group did not give any reaction in western blot.

Detection of cell mediated immune response

Lymphocyte proliferation assay

Lymphocyte proliferation assay by MTT was conducted at 3 different time intervals after immunization. After first week, third week and sixth week of post immunization, mice were sacrificed and their spleens were removed under aseptic conditions. Splenocytes were separated by the method described

earlier. Splenocytes were cultured with different stimulus and stimulation indices were measured by MTT colorometric assay. Splenocytes from none of the mice immunized with DNA vaccine (Group 1), recombinant protein (Group 2) had an increased proliferative response to recombinant L7/L12 and sonicated antigen after 1 week of immunization. The splenocytes from the three experimental groups and two control groups had very similar proliferative response to the mitogen ConA throughout the study. After 3rd week of immunization splenocytes from Group 1, Groups 2 and 3 showed significantly higher proliferation ($P<0.001$) than the control groups. When stimulated by recombinant protein antigen, the Groups 1 and 2 showed stimulation indices 1.256±0.0197 and 1.147±0.0165 respectively. Splenocytes when stimulated by sonicated antigen the stimulation indices of Groups 1 and 2 were 1.230±0.0197 and 1.194±0.0162 respectively. But in comparison to others the Group 3 showed highest stimulation index (1.277±0.0170) when stimulated with *Brucella* sonicated antigen. It was significantly higher ($P<0.001$) than the control groups. The control group did not show increased stimulation indices in any case, either stimulated by recombinant protein or *Brucella* sonicated antigen. Hence, Group 3 (DNA vaccine followed by protein boosting) showed highest proliferative response and Group 2 (Recombinant protein group showed least increased proliferative response when they are stimulated by sonicated *Brucella* antigen. Proliferation level increased significantly in Groups 1 and 3 after 6th week of immunization. Group 3 showed highest proliferation. Group 3 splenocytes when stimulated by sonicated antigen the mean value of stimulation indices was 1.609±0.0129. The value was significantly higher ($P<0.001$) than the control group. The mean stimulation index of Group 3 splenocytes in case of recombinant protein stimulation was 1.524±0.0179. That was also significantly higher ($P<0.001$) than that of control value. When Groups 1 and 2 splenocytes were stimulated by sonicated antigen the mean values of stimulation indices were 1.362±0.0283 and 1.270±0.0169 respectively. In case of recombinant protein stimulation the mean values were 1.340±0.0306 and 1.215±0.0192 respectively. The highest proliferation was found in Group 3. Group 2 showed least increased proliferation in comparison to Groups 1 and 3. Significant difference also found in between Groups 1 and 3. Hence DNA vaccine followed by protein boosting gave best proliferative response in comparison to DNA vaccine alone or recombinant protein alone.

Interferon-Gamma assay

IFN-γ assay by Opt EIA kit was conducted at 3 different time intervals after immunization. It was done simultaneously with lymphocyte proliferation assay. Splenocytes were cultured with different stimulus and then supernatants were collected and assayed by antigen capture ELISA. The concentration of IFN-γ in the culture supernatants was calculated for each experimental group using a linear regression equation obtained from the absorbance values of the standards. DNA vaccine (Group 1) and Recombinant protein (Group 2) did not show increased level of IFN-γ concentration after 1 week of immunization. After 3rd week of immunization, the entire vaccinated group showed increased level of IFN-γ. In comparison to other the Group 3 showed highest level of IFN-γ when stimulated with sonicated antigen. IFN-γ level was significantly higher ($P<0.001$) in Groups 1 and 3 after 6th week of immunization. Group 3 showed highest level of IFN-γ when stimulated by sonicated antigen. None of the control mice showed increased level of IFN-γ after stimulation.

Efficacy of vaccine in generating protective immunity against *B. abortus* 544

Protection experiments were carried out by challenging vaccinated and control mice by intraperitoneal injection of virulent *B. abortus* 544 and the level of infection was evaluated by determining the CFU in their spleens. The results indicate that Group 1, Groups 2 and 3 showed significantly higher ($P<0.05$) protection compared to the non-immunized control group. To compare the extent to which mice could be protected, Group 1 induced 1.204 log protection, Group 2 induced 1.289 log protection, Group 3 induced 1.427 log protection and the S19 strain induced 2.662 log protection. No significant difference was found between groups injected with pVax vector and PBS. DNA vaccine followed by protein boosting showed higher degree of protection against Brucella infection in comparison to DNA vaccine and recombinant protein vaccine alone.

Table 1: Mean OD values of L7/L12 specific IgG response of different experimental groups of mice in ELISA

S.No.	Experimental groups	0 day	7 day	14 day	21 day	30 day	45 day	60 day
1.	DNA vaccine	0.254	0.243	0.276	0.319	0.304	0.439	0.424
2.	Recombinant protein	0.235	0.602	0.672	1.652	2.187	2.540	2.469
3.	DNA vaccine followed by protein boostering	0.206	0.255	0.247	0.88	2.548	3.190	3.304
4.	Vector (pVax control)	0.178	0.219	0.220	0.295	0.417	0.381	0.337
5.	**PBS control**	**0.193**	**0.205**	**0.237**	**0.251**	**0.280**	**0.264**	**0.259**

Each value represents the OD of pooled serum samples of five mice under same group

Table 2:Mean OD vales of L7/L12 specific immunoglobulin isotype (IgG2a) antibody response of mice vaccinated with recombinant L7/L12 protein alone and DNA vaccine followed by protein boosting group by ELISA

S.No.	Group No.	Experimental groups	0 day	7 day	14 day	21 day	30 day	45 day	60 day
1.	2	Recombinant protein	0.144	0.415	0.473	1.279	1.382	1.856	**1.861**
2..	**3**	**DNA vaccine followed by protein boostering**	**0.189**	**0.220**	**0.239**	**0.756**	**1.250**	**3.251**	**3.268**

Table 3: Mean OD values of L7/L12 specific IgG1 antibody response of mice vaccinated with recombinant L7/L12 protein alone and DNA vaccine followed by protein boosting group by ELISA

S.No.	Group No.	Experimental groups	0 day	7 day	14 day	21 day	30 day	45 day	60 day
1.	2	Recombinant protein	0.248	0.453	0.635	1.203	1.165	0.921	1.865
2..	**3**	**DNA vaccine followed by protein boostering**	**0.178**	**0.243**	**0.204**	**0.458**	**0.621**	**0.542**	**0.460**

Table 4: Mean OD values of L7/L12 specific IgG response of different dilution in different days interval of post immunization with recombinant L7/L12 protein and Brucella infected serum

Dilution	Brucella positive	14 day	21 day	30 day	45 day	60 day	90 day	0 day
1000	1.3895	0.3285	0.775	1.5415	0.639	1.7145	1.5785	0.089
2000	1.096	0.181	0.4065	1.172	1.208	1.4565	0.998	0.07
4000	0.7395	0.096	0.2175	0.8395	0.855	1.158	0.6725	0.047
8000	0.4335	0.0855	0.122	0.521	0.519	0.7945	0.40	0.05
16000	0.25	0.066	0.076	0.284	0.301	0.4925	0.2325	0.046
32000	**0.1445**	**0.0545**	**0.057**	**0.16**	**0.172**	**0.296**	**0.125**	**0.0525**

Table 5:Detection of titer at different day intervals of post immunization with recombinant L7/L12 protein and Brucella infected serum

Days	Titer
Brucella positive	32000
14 days	2000
21 days	8000
30 days	32000
45 days	32000
60 days	32000
90 days	16000
0 day	**<1000**

Table 6: Antigenic specific blastogenic response of splenocytes of mice at 1 week of post vaccination as measured by MTT colorimetric assay

Experimental groups	Number of animals	Stimulation index±SE	
		Recombinant protein antigen	Sonicated antigen
DNA vaccine	3	1.07±0.0125[a]	1.058±0.0130[a]
Recombinant protein	3	0.955±0.0098[a]	1.039±0.0122[a]
DNA vaccine followed by protein boostering	3	1.030±0.0106[a]	0.932±0.00216[a]
Vector (pVax control)	3	0.961±0.0272[a]	0.998±0.0085[a]
PBS control	**3**	**0.973±0.0201[a]**	**0.856±0.0104**

[a]Denotes no significant different between the groups

Table 7:Lymphocyte proliferative response by MTT at 3rd week of post-immunization in mice

Experimental groups	Number of animals	Stimulation index±SE	
		Recombinant protein antigen	Sonicated antigen
DNA vaccine	4	1.256±0.0197[a]	1.230±0.0197[ab]
Recombinant protein	4	1.147±0.0165[b]	1.194±0.0162[b]
DNA vaccine followed by protein boostering	4	1.242±0.0164[a]	1.277±0.0170[a]
Vector (pVax control)	4	1.089±0.0175[c]	1.020±0.0197[c]
PBS control	**4**	**0.893±0.0185[d]**	**1.018±0.0167[c]**

[a,b,c,d]Means bearing different superscripts in a column differ significantly (P<0.001)

Table 8: Lymphocyte proliferative response by MTT at 6th week of post-immunization in mice

Experimental groups	Number of animals	Stimulation index±SE	
		Recombinant protein antigen	Sonicated antigen
DNA vaccine	4	1.340±0.0306[b]	1.362±0.0283[b]
Recombinant protein	4	1.215±0.0192[c]	1.270±0.0169[c]
DNA vaccine followed by protein boostering	4	1.524±0.0179[a]	1.609±0.0129[a]
Vector (pVax control)	4	1.013±0.013[d]	1.058±0.0165[d]
PBS control	**4**	**1.044±0.0314[d]**	**1.036±0.0350[d]**

[a,b,c,d]Means bearing different superscripts in a column differ significantly (P<0.001)

Table 9: IFN-γ levels measured by sandwich ELISA at 1 week of post-immunization in mice

Experimental groups	Number of animals	Concentration of IFN-γ (ng/ml)±SE	
		Stimulated by recombinant L7/L12 protein	Stimulated by sonicated Brucella protein
DNA vaccine	3	0.318±0.0514[a]	0.302±0.0730[b]
Recombinant protein	3	0.290±0.0322[a]	0.317±0.0477[c]
DNA vaccine followed by protein boostering	3	0.311±0.0656[a]	0.325±0.080[a]
Vector (pVax control)	3	0.294±0.0246[a]	0.285±0.033[d]
PBS control	**3**	**0.288±0.0315[a]**	**0.291±0.048[d]**

[a]No significant different between the groups

Table 10:IFN-γ levels measured by sandwich ELISA at 3 weeks of post immunization in mice

Experimental groups	Number of animals	Concentration of IFN-γ (ng/ml)±SE	
		Stimulated by recombinant L7/L12 protein	Stimulated by recombinant L7/L12 protein
DNA vaccine	4	1.872±0.121[a]	2.196±0.246[b]
Recombinant protein	4	1.256±0.0347[b]	1.488±0.0406[b]
DNA vaccine followed by protein boostering	4	1.760±0.260[a]	2.320±0.177[a]
Vector (pVax control)	4	0.302±0.0164[c]	0.290±0.0248[c]
PBS control	**4**	**0.289±0.0217[c]**	**0.311±0.0309**

[a,b,c,d]Means bearing different superscripts in a column differ significantly (P<0.001)

Table 11: IFN-γ levels measured by sandwich ELISA at 6 weeks of post-immunization in mice

Experimental groups	Number of animals	Concentration of IFN-γ (ng/ml)±SE	
		Stimulated by recombinant L7/L12 protein	Stimulated by recombinant L7/L12 protein
DNA vaccine	4	2.85±0.178[a]	3.165±0.192[b]
Recombinant protein	4	1.547±0.135[b]	1.79±0.141[c]
DNA vaccine followed by protein boostering	4	3.217±0.191[a]	4.66±0.287[a]
Vector (pVax control)	4	0.310±0.0583[c]	0.323±0.0626[d]
PBS control	**4**	**0.296±0.0335[c]**	**0.308±0.0219[d]**

[a,b,c,d]Means bearing different superscripts in a column differ significantly (P<0.001)

Table 12:Protection conferred against *Brucella abortus* 544 in mice after immunization with recombinant protein and DNA vaccine after 30 days post challenge

Vaccine	Log_{10} CFU of *B. abortus* 544 in spleen (Mean±SE)	Log_{10} units of protection
DNA vaccine	4.228±0.165	1.204[b]
Recombinant protein	4.143±0.058	1.289[c]
DNA vaccine followed by protein boostering	4.005±0.207	1.427[a]
Vector (pVax control)	2.81±0.471	2.622[d]
PBS control	5.432±0.123	-
pVax	**5.344±0.208**	**0.088**

[a,b,c,d]Means bearing different superscripts in a column differ significantly (P<0.05) as compared to control values

Discussion

In the present study protection experiments were carried out by challenging vaccinated and control mice by intra-peritoneal infection of virulent *B. abortus* S44, and the level of infection was evaluated by determining the cfu in their spleens. Mice which were immunized with rL7/L12 protein induced 1.289 log protection and mice vaccinated with S19, induced 2.622 log protection. These results were significantly higher (P<0.05) than that of vector control and PBS control. Among the recombinant antigens that have been tested previously so far, HtrA, GroEL, GroES, UvrA and YajC induced cellular and humoral immune responses in mice, but only the L7/L12 [12,13] elicited some level of protection. So there is a agreement between the present work and previous studies. *E. coli* expressing the Cu/Zn SOD also conferred a significant level of protection (1.77 log units) [13]. Onate *et al.* [13] also reported that the complete Cu/Zn SOD protein could induce a protective immune response in mice. But that report was contradictory with the results of Tabatabai and Pugh [14] who were unable to induce a protective immune response in mice using a combination of adjuvants and purified recombinant Cu/Zn SOD protein as the vaccine antigen, although some level of protection was induced with synthetic peptides of the protein and adjuvant.DNA vaccination is a relatively novel and powerful method of immunization DNA vaccination is a powerful method of immunization that induces strong cellular immune responses to a wide range of pathogens in many animal models for different diseases [15]. In the present study DNA vaccination induced 1.204 log protection that was lower than that of recombinant protein vaccination alone and DNA vaccine followed by protein boosting but significantly higher (P<0.001) than that of pVax vector control and PBS control. Based on the results obtained with DNA vaccine against other pathogenic intracellular bacteria, many studies were being developed using immunizations that code for proteins with immunogenic properties for brucellosis. The results of those experiments showed that those vaccines induced an immune response and some level of protection against challenge with pathogenic strain of *B. abortus* [10,16]. Previous reports demonstrated that intramuscular inoculation with a DNA vaccine that coded for L7/L12 elicited a strong protective response [17]. As described previously, viable *B. abortus* RB51 conferred over 2 log units of protection [14]. Munoz-Montesino *et al.* [18] concluded that intra-spleen delivery of a DNA vaccine containing the Cu/Zn SOD was able to generate a protective immunity but intramuscular administration of that vaccine in the same doses and under the same conditions as the subcutaneous inoculation did not lead to a significant protective immune response [10]. Those results demonstrated a higher efficacy of the subcutaneous route of administration and they were in agreement with the results obtained by Cano *et al.* [19] and Maloy *et al.* [20].

Conclusion

In the present study, the lower antibody response after DNA vaccine immunization could be due to the low level of antigen processing by exogenous pathway. Group 2 and Group 3 showed high level of antibody response up to 60 days of post immunization. Increased level of IgG2a over IgG1 indicated that the experimental vaccine given a Th1 type of response. Maximum splenocyte proliferative response and IFN-γ level has been observed in case of DNA vaccine followed by protein boosting. Recombinant protein potentiated the effect of L7/L12 DNA vaccine to direct the immune response towards Th1 type. DNA vaccine followed by protein boosting gave higher level of protection than DNA or protein alone. To overcome silencing of the genes, in order to generate DNA vaccine that expresses for extended periods of time, protein boosting is necessary. S19 vaccination has given highest protection level than that of other vaccinated groups in the present study.

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