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Immunological Evaluation of Fc Receptor Mediated Antigen Delivery of *Brucella melitensis* Recombinant Outer Membrane Protein 28 (Romp 28) in Mice

Ramya Kalaivanan^{1*}, Sankar Palanisamy¹, Tapas Kumar Goswami², Pallab Chaudhury² and G. C. Ram²

¹Veterinary College and Research Institute, Namakkal, Tamil Nadu Veterinary and Animal Sciences University, Chennai, India

²Indian Veterinary Research Institute, Izatnagar Bareilly, India

*Corresponding author

ABSTRACT

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The present study was aimed to prepare a conjugate of Fc fragment of mice IgG with rOMP 28 of *Brucella melitensis* and investigate its ability to influence the kinetics of humoral and cellular immune response in comparison to its unconjugated counterpart and the rOMP 28 alone in the mice model. Among all the groups, the group of mice immunized with rOmp28 alone induced highest level of humoral immune response and the vaccine strain group(S19) conferred highest level of cellular response which was evidenced by higher nitric oxide concentration and the clearance of virulent *B. abortus* 544 from the spleen upon challenge infection. The conjugated group conferred low level of humoral response compared to the rOMP28 alone which may be due to the involvement of inhibitory FcγIIR in the regulation of immune responses, soluble forms of FcγR inhibiting antigen presentation and irreversible loss of Fc receptors on the macrophage plasma membrane. The S19 strain of *B. abortus*, a live vaccine conferred high level of protection when challenged with virulent *B. abortus* 544 which indicate whole cell organism composed of various immunogenic components might have been involved in inducing protection.

Introduction

Brucellosis is an economically significant disease-causing abortion and infertility in infected animals and undulant fever in humans. The disease is caused by Gram-negative, nonmotile, non-spore-forming,

partially acid fast, strict aerobe, some strains require carbon dioxide, especially on primary isolation, intracellular coccobacilli or short rods and are oxidase, catalase, nitrate reductase and urease positive (Young *et al.*, 1985). *B. abortus*, *B. melitensis*, *B. suis* and *B. canis* preferentially infects cattle, sheep and

goats, pigs and dogs respectively. All the above species infect humans with *B. melitensis* being the most common. *Brucella abortus* causes abortion and infertility in cattle. Efforts have been made to control this important disease through vaccination with live attenuated *B. abortus* strain 19 and killed strain 45/20. Although these vaccines have been found effective against brucellosis, they have some disadvantages, such as the ability to cause disease in humans, the possibility of causing abortion when administered to pregnant cattle and the diagnostic difficulty of distinguishing field infections from vaccinated animals (Baldi *et al.*, 1996). A vaccine that will be noninfectious and effective in stimulating a broad protective immune response is needed to control brucellosis. With the advent of genetic engineering techniques, it is now possible to generate non-infectious vaccines like subunit and DNA vaccines for economically important disease like brucellosis. One such approach was the production of recombinant proteins from zoonotically important *Brucella* sp. and its use as vaccine candidate.

Brucella OMPs are divided based on their molecular mass into group 1 antigens with a molecular mass of 94 kDa, group 2 antigens of approximately 41–43 kDa and group 3 antigens of 30 kDa. Omp28 and Omp25 are both group 3 antigens and are distinct from each other based on molecular mass (Santos *et al.*, 1984). The 28 kDa OMP (OMP28) of *B. melitensis* belongs to group III antigens. OMP28, also been termed as BP26 and CP28, is an immune dominant antigen localized in the periplasm and this protein has been a target molecule for detection of anti-*Brucella* antibodies (Lindler *et al.*, 1996; Debarh *et al.*, 1996; Cloeckert *et al.*, 2001).

Effective immune response to a disease-causing agent is highly essential to get rid of the infection. Even though available vaccines

are competent to induce desired immune response, glitches during the differential diagnosis of infected and vaccinated animal prompt us to seek alternative strategies. The current study was undertaken for the targeted delivery of the weakly immunogenic subunit vaccine to be conjugated with Fc portion of mice immunoglobulin to immune effector cells like B cells, NK cells, Neutrophils, macrophages etc., through the Fc gamma receptor (FcγR) they possess (Qiu *et al.*, 1990). FcR again acts as a trigger molecule for inflammatory, allergic, cytolytic, endocytic, and phagocytic activities of immune cells (Fridman *et al.*, 1992). So FcR responsible for receptor mediated endocytosis can be targeted to enhance the antigen uptake. Fc receptors responsible for various isotypes of immunoglobulin are located on different types of cells involved in immune response. In this way it is possible to achieve enhanced immune response even with very less available antigen by artificially augmenting antigen uptake by the cells possessing surface receptors. These receptors were also effectively used by the immunologists to target the vaccines to a particular cell to induce high level of immune response without any adjuvant.

In the present study, recombinant outer membrane protein 28 (rOMP 28) of *Brucella melitensis* was used to study the efficacy of receptor mediated antigen delivery system. The *E. coli* expressed purified rOMP 28 protein was conjugated with Fc portion of mouse IgG using glutaraldehyde as a crosslinker and the kinetics of immune response was studied in the mice model.

Materials and Methods

Experimental animals

Apparently healthy Swiss albino mice of 4-6 weeks age were obtained from the Laboratory

Animal Resource Section, Indian Veterinary Research Institute, Izatnagar. The animals were housed in cages and provided feed and water *ad libitum*. The guidelines of Institutional animal ethics committee were followed for maintenance, handling and care of animals.

Biologicals

Brucella abortus 544 virulent strain and *Brucella abortus* S-19 live vaccine strain was obtained from the *Brucella* Referral Lab., Division of Veterinary Public Health, IVRI, Izatnagar. Anti-mouse IgG and its isotypes (Total IgG, IgG1, IgG2a and IgG2b) HRPO conjugates (Sigma) were used for the assay of immune globulins.

Separation of serum

Blood was collected from the mice and kept at room temperature undisturbed for 2 hours and overnight at 4°C. The clotted blood was subjected to centrifugation at 2000 rpm for 10 minutes at 4°C. Separated serum was collected and pooled together and stored at -20°C for immunoglobulin (IgG) precipitation.

Ammonium sulphate precipitation of Immuno globulins

Saturated ammonium sulphate solution was prepared in double distilled water and kept in incubator for overnight, clarified by filtration using 0.22 µm filter and pH was adjusted to 7.4. Four ml of saturated ammonium sulphate solution was added drop wise to about 6ml of serum with continuous stirring in order to achieve a final concentration of 40 % (v/v) saturation. The solution was left undisturbed overnight at 4°C and centrifuged at 2500 rpm for 10 minutes. The supernatant was discarded and the precipitate thus obtained was redissolved in distilled water to its original volume and reprecipitated with

saturated ammonium sulphate solution to final concentration of 40% (v/v). The precipitate containing immune globulins (IgG) was collected again by centrifugation and dissolved in Phosphate Buffer Saline (PBS pH 7.4).

The dissolved precipitate was dialyzed against PBS (pH 7.4) in dialysis tubing having a molecular weight cut off 12 kDa with several changes of PBS until free from ammonium sulphate. The presence of ammonium sulphate in the immunoglobulin precipitate was detected by Nessler's reagent.

Purification of immunoglobulin G using protein- A agarose

About 1ml of Protein A agarose (Sigma) was taken in a syringe column and allowed to settle. Once the column has settled, it was washed with 20 column volumes of Buffer A as per the manufacturer's instruction. The immunoglobulin dialyzed against PBS was then applied to the column and the column was washed with 10 column volumes of buffer A.

The bound IgG was then eluted using 3 column volumes of buffer B pH 4.5. The eluates were neutralized by collecting in 0.1M NaOH to prevent denaturation. The different fractions of the eluates were checked for the purity in 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) described by Laemmli (1970). The eluates with pure immune globulins were chosen and pooled for digestion.

Fragmentation of mouse immunoglobulin G with preactivated papain

The purified IgG was subjected to fragmentation with preactivated papain (Parham *et al.*, 1982). Four mg of papain was dissolved in 2ml of 0.1M acetate buffer, pH

5.5 containing 0.003M EDTA and 50mM cysteine hydrochloride. This solution of papain was incubated at 37°C for 30 min for preactivation. Following incubation, the papain solution was dialyzed against 0.1M acetate buffer with 0.003M EDTA for 6 hrs in order to remove the cysteine. Papain was preactivated just before starting the digestion. Papain concentration was determined spectrophotometrically against 0.1M acetate buffer with 0.003M EDTA as blank.

Six mg of purified mouse IgG was equilibrated by dialysis against 0.1M acetate buffer, pH 5.5 with 0.003M EDTA for overnight. To about 6 µg of mouse IgG, 300 µg of preactivated papain was added and incubated in a water bath for 12 hrs at 37°C in 0.1M acetate buffer with 0.003M EDTA.

After 12hrs of incubation, the digestion reaction was stopped by addition of iodoacetamide to achieve a final concentration of 30mM. The digested protein samples were dialyzed against PBS (pH 7.4) to remove the acetate buffer. The digested products were analyzed by SDS PAGE (12.5%) in the absence of beta mercaptoethanol.

Purification of Fc fragment in protein A agarose

The digested products were applied on the column containing Protein A agarose and washed with 10 column volumes of buffer A. Then the bound Fc fragment was eluted with buffer B (pH 4.5). The eluates were collected and pH was brought to neutral with 0.1N NaOH immediately to prevent denaturation. The purity of the Protein A agarose purified Fc fragment was confirmed in SDS PAGE (12.5%) under non reducing condition. The concentration of the Fc fragment was determined spectrophotometrically against buffer B as blank.

Purification of recombinant outer membrane protein 28 of *Brucella melitensis*

The *E. coli* cell having the gene for outer membrane protein 28 of *Brucella melitensis* in *pPROC* expression vector was obtained from Dr. Pallab Chaudhuri, Division of Bacteriology was grown in LB medium till the OD values reached 0.5 at 600 nm. The *E. coli* cells were then induced with 1 mM IPTG and allowed to grow further for 6 h at 37°C. The *E. coli* cells were harvested and the *rOMP* 28 was purified using Ni-NTA agarose according to the instructions of the manufacturer (Qiagen, USA) and analyzed on SDSPAGE. The concentration of the protein was determined as per the described the method (Lowry *et al.*, 1951) as well as spectrophotometrically at 280nm.

Conjugation of *rOMP* 28 with IgG Fc using glutaraldehyde

Conjugation was done following the protocol described by Baron and Baltimore (1982). Approximately 2.5 mg Fc fragment was added to 2.5 mg of *rOMP* 28. The Fc fragment and *rOMP* 28 were dialyzed against PBS-pH 7.4 before use. About 0.5 ml of glutaraldehyde solution (20 mM) was added immediately to the mixture drop wise, mixing gently with a magnetic stirrer for one hour at room temperature. After one hour, once the mixture turns milky, the reaction was stopped by adding 100µl of glycine solution (10 mM final concentration) to inactivate the unreacted glutaraldehyde. Conjugated protein was dialyzed against 4 to 5 liters of PBS, pH 7.4 for 48 hours at 4°C to remove the glutaraldehyde and glycine from conjugate solution. The conjugation was confirmed by resolving in 12.5% SDS PAGE.

The protein concentration of the conjugate was determined spectrophotometrically at 280nm against PBS as blank.

Preparation of glutaraldehyde treated OMP 28

About 3 mg of purified rOMP 28 dialyzed against PBS (pH 7.4) was treated with 20 mM glutaraldehyde for one hour and the reaction was stopped with 10mM glycine. Glutaraldehyde treated rOMP 28 was dialyzed against 4 litres of PBS (pH 7.4) for 48 hrs.

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

The *B. abortus* S19 and *B. abortus* 544 were sub cultured separately on tryptose phosphate agar slant. *B. abortus* S19 slants were incubated at 37°C for 48 to 72 h. However, *B. abortus* 544 slant cultures were incubated at 37°C under 5% CO₂ tension for 72 to 96 h. The viable bacterial count was determined as per the described method (Alton *et al.*, 1975). Briefly, serial ten-fold dilution (10⁻¹ to 10⁻¹⁰) of the bacterial suspensions (*B. abortus* S19 and *B. abortus* 544 harvested from slants) were made in tryptose phosphate broth. About 20 µl from each dilution starting from 10⁻⁵ to 10⁻¹⁰ was plated on tryptose phosphate agar in triplicate. The plates were incubated at 37°C for 48-72 h for *B. abortus* S19 and 72-96 h at 37°C in 5% CO₂ tension for *B. abortus* 544. Plates showing 30-300 CFU were selected for colony count. The mean of all the plates were multiplied by dilution factor and viable bacteria were counted as colony forming unit (cfu)/ml. Then 1x10⁵ cfu/ml of *B. abortus* S19 was used for intra peritoneal immunization and 1x10⁵ cfu/ml of *B. abortus* 544 were used for challenge study.

Immunization of mice

A total of 60 Swiss albino mice of either sex were randomly distributed into six groups and caged separately. All the groups were

immunized on day 0, 14 and 21 as shown in the Table 1 except the *Brucella abortus* S19 which was given as a single dose.

Assessment of humoral immune response

Indirect ELISA

To assess the humoral immune responses blood was collected from the immunized and control groups on day 0, 7, 14, 21 and 28 days post vaccination. The immunoassay plates (Maxisorp, Nunc, Denmark) were coated with purified rOMP28 protein at a concentration of 100 ng per well, diluted in 0.1M bicarbonate buffer (pH 9.0) and incubated overnight at 4°C. The wells were emptied and washed five times with phosphate buffer saline-Tween20 (PBST) and blocked with PBST containing 5% BSA. Immunoassay plates were charged with sera at a dilution of 1:100 and incubated at 37°C for 1 h and washed five times with PBST. The plates were incubated with respective HRPO conjugates for 1 h at 37°C. After washing with PBST, substrate solution containing Ortho-phenyl diamine (OPD) and H₂O₂ were added. Color development was stopped by adding 2M H₂SO₄ after incubating the plates for 10 minutes in dark at room temperature. Absorbance/Optical density (OD) values were recorded at 490 nm wavelength in an ELISA reader.

Determination of antigen specific antibodies by western blotting

The production of antigen specific antibodies in sera of the animals immunized with various antigen preparations were determined by Western blot analysis. The purified rOMP 28 (2.5µg) was electrophoretically resolved in SDS PAGE (12.5%) and the resolved proteins were electrophoretically transferred to polyvinyl difluoride (PVDF).

Semi dry blotting steps were followed as described by Towbin *et al.*, (1979).

Assessment of cell mediated immune response

Lymphocyte transformation test (LTT)

Spleens from two immunized mice from each group were taken after 30 and 40 days of first inoculation after sacrifice, splenocytes were collected by repeated perfusion with sterile PBS using insulin syringe. The splenocytes were isolated using Histopaque (1077). To 1.5 ml of histopaque, 3 ml of splenocyte suspension was overlaid and centrifuged at 1600 rpm for 40 min. The interface containing lymphocytes was collected in a fresh tube and two washings were done with sterile PBS and finally resuspended in RPMI 1640 growth medium. The blastogenic response of splenocytes was assessed by MTT colorimetric method as described by Mosmann (1983).

Briefly, 100µl of splenocyte cell suspension (2×10^6 cells/ml) was added to 96-well flat bottom tissue culture plate (Griener). Then 100µl of RPMI-1640 growth medium containing either Con A (for positive control) @ 20µg/ml or antigen (rOMP 28) @ 2µg/ml was added in triplicate wells. For negative control, 100 µl of growth medium without antigen or mitogen was added in triplicate wells. The plate was sealed properly with adhesive tape and incubated at 37°C in a humidified chamber with 5% CO₂. After 96 hours of incubation, 20µl of MTT solution (5 mg/ml in PBS) was added to each well and further incubated at 37°C for 4 hr. The plates were then centrifuged at 1,500 rpm for 10 min and 100µl of culture supernatant was discarded from each well. Finally, 150µl of dimethyl sulfoxide (DMSO) was added to each well and mixed thoroughly avoiding air bubbles. OD measured at 550nm.

Stimulation index (SI) was calculated using the following formula:

$$SI_{(MTT)} = \frac{\text{Mean OD of stimulated culture}}{\text{Mean OD of unstimulated culture}}$$

Nitric oxide production assay

Splenocytes from the immunized groups were isolated as mentioned in the lymphocyte proliferation assay. In this assay, the RPMI 1640 medium (without phenol red) was supplemented with 5 mM L- arginine. One ml of cell suspension containing 1×10^6 cells was plated in triplicate in 96 well plates. The splenocytes from different groups were activated with rOMP28@10µg/ml. The plates were incubated at 37°C and 5% CO₂ for 2 days. Culture supernatants were collected from all the wells at 24, 30 and 40 hrs intervals. The collected supernatants were stored at -20°C until NO estimation. For nitric oxide (NO) estimation, different concentrations of NaNO₂ (Sodium nitrite) was used for preparing standard curve.

In a 96 well ELISA plate, to 50µl of the cell culture supernatant or standard, 50 µl of Griess reagent were added and incubated at 37°C for 30 min. Absorbance was measured at 550nm. The NO level in the sample was extrapolated using the standard curve (NaNO₂ concentration vs O.D at 550 nm).

Protection study by challenge infection

All the groups of mice were challenged 45 days post primary immunization with 1×10^5 CFU of *Brucella abortus* Strain 54 in 200µl of sterile PBS intraperitoneally. Four weeks after challenge, three mice from each group were sacrificed for splenic clearance assay. Spleen from each mouse was removed, homogenized in 2 ml of TPB (Tryptose Phosphate Broth).

A tenfold serial dilution was made in TPB and 20µl suspension was plated on Tryptose phosphate agar. *B. abortus* 544 colonies were counted after incubation for 72-96 h at 37°C under 5% CO₂. The difference of log organism burdens in spleen of PBS control (unimmunized) mice and the vaccinated groups was considered as protection in terms of splenic clearance.

Statistical analysis

The results of ELISA and lymphocyte proliferation assay are expressed as mean ± standard error (SE). In protection studies, log values of total no. of Brucella per spleen of mice were calculated and protection was assessed by subtracting the log colony forming unit of immunize mice from those of PBS control. One-way analysis of variance was used to test the statistical significance by SPSS 11.0. *P* value less than 0.01 was considered statistically significant as it showed significantly high level of protection as compared to PBS control group.

Results and Discussion

Precipitation and determination of concentration of mouse immunoglobulin

The serum precipitated with saturated ammonium sulphate solution revealed two thick bands, one about 55kDa and other about 25kDa indicating high concentration of immunoglobulin with some other protein bands in 12.5% SDS PAGE. The concentration of the precipitated immunoglobulin was determined by spectrophotometer as 2mg/ml.

Isolation and purification of mouse immunoglobulin G using Protein A agarose

The purity of the Protein A agarose purified IgG was analyzed for purity in 12.5%

SDSPAGE (Fig. 1) and also in 10% native PAGE. The fractions were free from any contaminant proteins and contained only IgG of 150 kDa (Fig. 2). The concentration of the purified immunoglobulin was determined spectrophotometrically as 1.25mg/ml against buffer B as blank.

Digestion of mouse IgG with preactivated papain

The concentration of the preactivated papain was determined spectrophotometrically as 1.13mg/ml and used for digestion. The mouse IgG after digestion with the activated papain was checked in SDSPAGE (12.5%) under non reducing conditions and the gel revealed three detectable bands representing 110 kDa undigested IgG, 50 kDa Fab and 27 kDa Fc fragment which confirmed the digestion steps (Fig. 3).

Purification of Fc fragment in protein A agarose

The eluted fractions were checked for the presence of Fc fragment in 12.5% non-reducing SDS PAGE revealed single pure band of about 27 kDa representing Fc fragment (Fig. 4). The concentration of protein A agarose purified Fc fragment was determined spectrophotometrically as 400 µg/ml of fraction at 280 nm against buffer Bas blank.

Purification of recombinant outer membrane protein 28 (rOMP 28) of *brucella melitensis*

The eluted fractions of Ni-NTA column were checked in 12.5% SDS PAGE and 28 kDa rOMP was noticed in different eluates. The eluates containing high concentration of rOMP 28 were pooled and dialyzed (Fig. 5). The concentration of the purified rOMP28 after pooling was determined as 1mg/ml by

Lowry method as well as by UV spectrophotometry.

Confirmation of conjugate formation

The IgG Fc and rOMP 28 conjugate formation was confirmed in 12.5% SDS-PAGE which revealed one band in the range of 50 and 55 kDa and another band in the range of 25 and 30 kDa which is suggestive of unconjugated Fc and/or rOMP28 proteins (Fig. 6). The concentration of the conjugate was determined as 310 µg/ml in UV spectrophotometer.

Evaluation of humoral immune response

Indirect ELISA

The humoral immune response against rOMP 28 specific IgG isotypes was measured by indirect ELISA. The level of the different subclasses of antibodies in the test serum at dilution of 1:100 was expressed as absorbance (OD₄₉₂) of the color complex developed in the assay (indirect ELISA).

Isotype profile

It is known that different vaccination approaches and the route of antigen delivery can affect the antibody isotype and T helper cell type in an immune response. IgG2a is produced as a consequence of Th1 subset lymphocyte activation, whereas Th2 subset lymphocyte activation enhances IgG1 and suppresses IgG2a (Abbas *et al.*, 1996; Mossman and Coffman 1989).

The total IgG antibody level started increasing significantly from day 7 post immunization till day 21 post immunization in all the groups except those groups which received PBS and *S19 strain*. On day 7 and 14 post immunization, animals from group 5 showed highest responses which were

maintained till day 21, followed by group 2, and 4. Significant increase in the group 3 animals could be observed on day 21 after the administration of first booster dose on day 14. However, the IgG antibody response started decreasing on day 28 post immunization. Kinetics of antibody response revealed that mice immunized with rOMP 28 conjugated with Fc portion showed less significant difference in antibody production in comparison to other immunized groups. The vaccine strain immunized mice formed less significant IgG antibodies against rOMP 28 compared to all other groups (Fig. 7).

IgG1 response

The IgG1 antibody response against rOMP 28 were found to be highly significant on day 14, 21 and 28 in between groups on day 7. The antigen specific IgG1 antibody levels were increasing from day 7 to day 28. The highest response was found in animals from group 3 on 28 days post immunization followed by group 6 and 2. The vaccine strain immunized mice produced less significant IgG1 antibodies against rOMP 28 compared amongst all other groups (Fig. 8).

IgG2a response

The IgG2a antibody response against rOMP28 were found to be increasing right from day 7 till 28 days post immunization in all the groups except group 7. The highest response was observed on group 5 followed by group 2, 4 and 3. The vaccine strain immunized mice produced less significant IgG2a antibodies against rOMP 28 compared amongst all other groups (Fig. 9).

IgG2b response

The IgG2b antibody response against rOMP 28 was not found to be significant ($P < 0.101$) on day 7 post immunization but were highly

significant on all other days ($P < 0.000$) between groups. Significant increase in the level of IgG2b antibody was observed till day 21 post immunization whereas, a mixed type of response was observed among groups on day 28. The vaccine strain immunized mice produced less significant IgG2a antibodies against *rOMP 28* compared amongst all other groups (Fig. 10).

Determination of antigen specific antibodies by western blotting

To confirm the presence of specific antibodies against *rOMP 28*, the sera from different group of animals were analyzed by western blotting. All the group of animals reacted almost similarly to *rOMP 28* except the PBS which has not reacted at all. The S19 group of animals showed less reactivity to *rOMP 28* when compared to other groups (Fig. 11).

Evaluation of cell mediated immune response

Lymphocyte proliferation assay

The stimulation indices of the stimulated splenocytes ranged between 0.87 ± 0.01 to 1.5 ± 0.00 . The blastogenic responses were found to be higher on day 30 post immunization which was reduced after 40 days post immunization. On day 30 group 2 mice exhibited highest blastogenic response which is followed by group 5, 4, 3, 6 and control. Proliferation of splenocytes after second week of last immunization was reduced. Splenocytes from all groups of mice including PBS control showed similar proliferative responses upon stimulation with Con A (Fig. 12).

Nitric oxide estimation

The macrophage function in terms of production of nitric oxide was assessed by

estimating the level of nitrite in the splenocyte culture supernatant collected after 96 hrs of incubation which was stimulated with *rOMP 28* on days 30 and 45 of primary immunization. The nitrite concentration ranged from $5 \mu\text{M}$ to $22 \mu\text{M}$. Among all the groups, group 6 (vaccine strain S19) showed highest level of nitrite concentration on day 30 as well as on day 45 post immunization. The remaining groups also showed variable levels of nitrite levels on different days (Fig. 13).

Protection Study

Protection assay in the form of bacterial clearance was carried out after 30 days of giving challenge infection with virulent *B. abortus 544* to the vaccinated and control mice. Challenge dose was fixed as 1×10^5 organisms per animal intra peritoneally. The level of infection/clearance in each group was evaluated by determining the colony forming unit (CFU) of bacteria in the spleen. Three mice from each group were sacrificed to determine the protection level. The results indicated group 6, conferred highest protection with 1.05 log unit of protection followed by group 5 with 0.04 log unit of protection. However, the groups immunized with *rOMP 28* and conjugate vaccine conferred very less protection (Table 2 and Fig. 14).

Discussion

Brucellosis is an economically important zoonotic disease caused by a facultative intracellular bacterium that causes abortion and infertility in cattle and undulant fever in humans. (Young *et al.*, 1985) Because of its serious economic and medical consequences, efforts have been made to control this important disease through vaccination with live attenuated *B. abortus* strain 19 and killed strain 45/20 in cattle. Although these vaccines have been found effective against brucellosis,

they do have some disadvantages, such as the ability to cause disease in humans, the possibility of causing abortion when administered to pregnant cattle, and the diagnostic difficulty of distinguishing field infections from vaccinated animals (Baldi *et al.*, 1996). A vaccine that will be noninfectious and effective in stimulating a broad protective immune response is needed to control brucellosis. With the advent of genetic engineering techniques, it is now possible to generate noninfectious vaccines like subunit and DNA vaccines for economically important disease like brucellosis. Brucella protein named CP28, BP26, or Omp28 has been identified as an immunodominant antigen in infected cattle, sheep, goats, and humans (Gupta *et al.*, 2010). In this study, the recombinant outer membrane protein 28 (*rOMP* 28) has been investigated as vaccine candidate.

The state of immunity against a particular microorganism should be established in the host in order to get rid of infection. Several factors play role in inducing an effective immune response viz, molecular weight, number of epitopes, conformation, degradability, the way in which the immune cells recognize the antigen etc., and many of these components fail to induce a desirable protective immune response by itself. In such cases, it needs to be adjuvanted or targeted to the immune cells capable of inducing a protective response. One such approach is investigated in this research through Fc receptor mediated antigen delivery to induce potent immunity by targeting the antigen *rOMP* 28 subunit vaccine to immune effector cells.

Receptors for Fc portion of IgG (Fc γ R) play an essential role in antibody-dependent immune responses (Ravetch and Kinett, 1991). These Fc receptors can recognize the immune complex made of IgG and an antigen

and are involved in receptor mediated phagocytosis and able to present antigens to T cells, secretion of reactive oxygen intermediates and lysosomal hydroxylases and ultimately mediates antibody dependent cell mediated cytotoxicity (ADCC). The dendritic cells possessing FcRs are involved in cross presentation. These receptors transmit either activating or inhibitory signals *via* immune receptor tyrosine-based activation motifs (ITAMs) or immune receptor tyrosine-based inhibitory motifs (ITIMs), respectively (Ravetch and Lanier 2000) provide a link between humoral and cell mediated response by targeting immune complexes to effector cells.

Serum was used as a source of immunoglobulin since it harvests high concentration and considering the ease of collection. Serum pooled from 40 mice yielded large quantities of immunoglobulin as evidenced spectrophotometrically. Since the study requires only IgG, Protein A agarose (Sigma) was used for the isolation of pure IgG. The Protein A bound the IgG isotype alone and leaving all other isotypes in the elute. The purified IgG fractions obtained from eluting the Protein A agarose revealed only two bands at 55 kDa and another around 25 kDa indicating the heavy and light chains of IgG in SDS-PAGE which was in accordance with Ey *et al.*, (1978) and Singh *et al.*, (2008).

The purified immunoglobulin was used to obtain Fc fragment by subjecting to fragmentation with preactivated papain. Digestion of the mouse IgG yielded undigested IgG, Fab and Fc fragments (110,55 and 27 kDa) along with one more band which represents papain (24kDa) in native PAGE (Singh *et al.*, 2008; Coleman and Mahler 2003). The Fc fragment purified in the Protein A agarose column revealed a single band of Fc fragment of about 27 kDa

demonstrating the purity of the preparation. The efficiency of conjugation assured in SDS PAGE revealing a high molecular weight band around 55 kDa enable us to proceed for immunization.

The present study was conceptualized with the hypothesis that receptor mediated antigen delivery will trigger the immune response against *rOMP* 28. The humoral immune response kinetics was studied after immunization in all the groups up to 28 days post immunization. The IgG response was found to be on increasing till day 21 and started decreasing on day 28 indicative of the recombinant protein's ability to induce humoral immune response which further increased upon booster immunization. The total IgG response throughout the study period remained highest in the group immunized with *rOMP* 28 alone and *Fc+rOMP* 28 (unconjugated) suggesting that unaltered proteins could be a better antigen than conjugated proteins. The lesser total IgG response in conjugate group throughout the study in comparison to *rOMP* 28 alone group could be attributed to the irreversible loss of *Fc* receptors from the macrophage plasma membrane (Mellman *et al.*, 1983) or can be ascribed to the alterations in the immune conjugate due to conjugation which might have resulted in a generation of different/novel immunogenic peptides to induce the immune response.

The IgG1 profile of the immunized groups showed increasing trends from day 7 and further increase could be appreciated after the two boosters on day 14 and 21 with the highest response on day 28. However, on day 28 the conjugate group showed highest level IgG1 response suggestive of activation of Th2 subset of lymphocytes (Abbas *et al.*, 1996; Mossman and Coffman, 1983). The magnitude of IgG2a response indicative of Th1 subset of lymphocyte activation was similar to that of total IgG response with

unaltered *rOMP* 28 showing the maximum antibody response. The antibody response of the vaccine strain (group 6) mice to the *rOMP* 28 was the least when compared to the other groups in indirect ELISA as well as in western blotting. The current live attenuated vaccines against animal brucellosis induce high titer antibodies against the O polysaccharide of the LPS. Furthermore, the immune gold labeling of *OMP* 28 on the 16M smooth strain of *B. melitensis* detected by electron microscope was specific but at a low level, suggesting that *OMP* 28 is not highly exposed on the surface of these cells. The *OMP* 28 of *B. melitensis* was not recognized by sera obtained from *B. abortus* 2308 infected cattle or swine naturally infected with *B. suis* (Blasco and Diaz 1993). The vaccine strain *B. abortus* S19 developed from virulent *B. abortus* strain may have a different type of *OMP* due to spontaneous mutation which was not detected by the *rOMP* 28 of *Brucella abortus* 544 strain (Lindler *et al.*, 1996).

In a study to evaluate BP26/ *OMP*28 in serological diagnosis of *B. melitensis* infection in sheep, the absorbance values in indirect ELISA did not reach those observed in indirect ELISA using purified O polysaccharide as an antigen. The antibody response to BP26/*OMP* 28 was delayed and much weaker than to O polysaccharide. Hence, the weak response to *OMP* 28 suggests that it can be used to differentiate infected and vaccinated animals (Cloeckert *et al.*, 2001)

Cell mediated immunity plays an important role in the protection against intracellular organisms like *Brucella*. The cell mediated immunity against *rOMP*28 in mice evaluated by *in vitro* stimulation of lymphocytes with *rOMP* 28 induced highest proliferation in group 2 which received *rOMP* 28 alone on 30 days post immunization.

Table.1 Immunization schedule in mice

Groups	Inoculum	Dose/mice	Route of Immunization
1.	PBS	0.1ml	S/C
2.	rOMP28 alone	50 µg	S/C
3.	Conjugate of rOMP28 with Fc fragment	100µg	S/C
4.	Glutaraldehyde treated rOMP 28	50µg	S/C
5.	Fc + rOMP 28	50 µg + 50µg	S/C
6.	<i>Brucella abortus</i> S19 (Vaccine strain)	1x10 ⁵ CFU	I/P

Table.2 Table representing CFU of *B.abortus* 544 in spleen after challenge in different immunized groups

Experimental Groups	No. of <i>B.abortus</i> 544 in spleen	Log ₁₀ CFU of <i>B.abortus</i> in spleen Mean ± SE	Log unit of protection
I	3.87x10 ⁵	5.55 ± 0.09	0
II	1.23x10 ⁶	6.08 ± 0.05	-0.50
III	4.37x10 ⁵	5.6 ± 0.02	-0.05
IV	3.87x10 ⁵	5.58 ± 0.04	0
V	3.5x10 ⁵	5.53 ± 0.04	0.04
VI	3.37x10 ⁴	4.51 ± 0.07	1.05

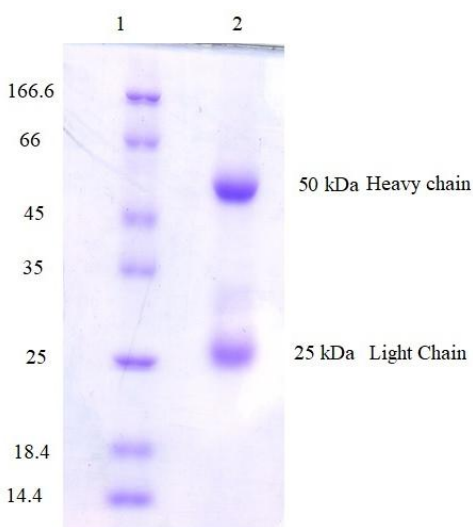


Fig. 1 Protein A purified mouse IgG in 12.5% SDS PAGE

Lane 1: Protein Molecular Weight Marker
Lane 2: Purified IgG fragments

Fig.1 Protein A purified Mice IgG in 12.5 % SDS PAGE

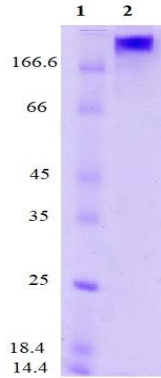


Fig.2 10% Native PAGE showing Protein A purified mouse IgG
Lane 1: Protein Molecular weight marker (kDa)
Lane 2: Protein A purified Mouse IgG (150 kDa)

Fig.2 Protein A purified mouse IgG in 10% native PAGE

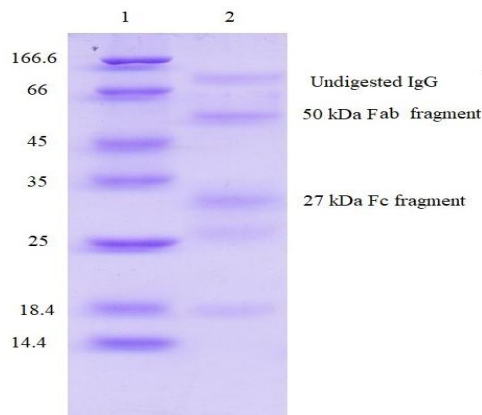


Fig.3 Papain digested mice IgG in 12.5% Native PAGE
Lane 1: Protein molecular weight marker
Lane 2: Papain digested fragment of IgG(50kDa Fab fragment and 27 kDa Fc fragment)

Fig.3 Papain digested mice IgG in 12.5% Native PAGE

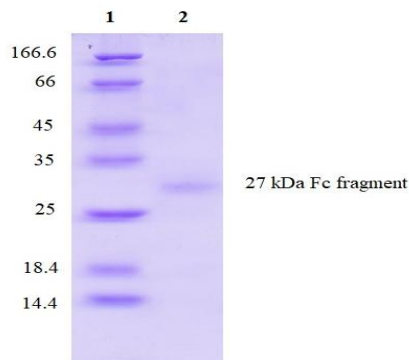


Fig.4 Protein A purified Fc fragment of mouse IgG
Lane1: Protein Molecular Weight Marker
Lane2: Purified Fc fragment of IgG (27 kDa)

Fig.4 Protein A purified Fc fragment of IgG

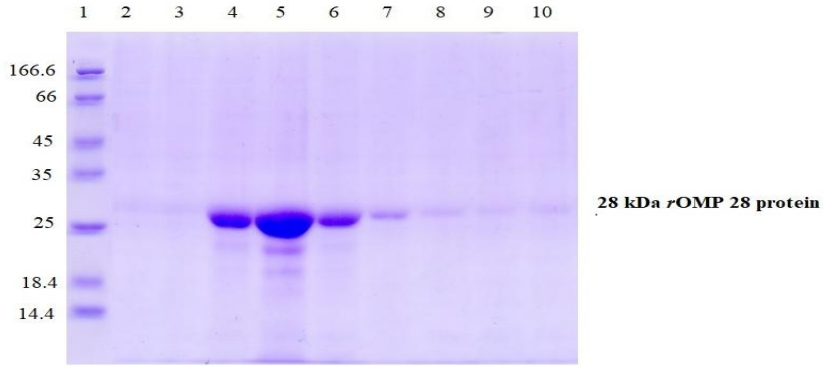


Fig.5 12.5% SDS PAGE analysis of expressed *rOMP 28* protein
Lane1: Protein Molecular Weight Marker
Lane 2-10: Fractions of *rOMP 28* eluted from Ni-NTA affinity column

Fig.5 12.5% SDS PAGE analysis of expressed *rOMP 28* protein

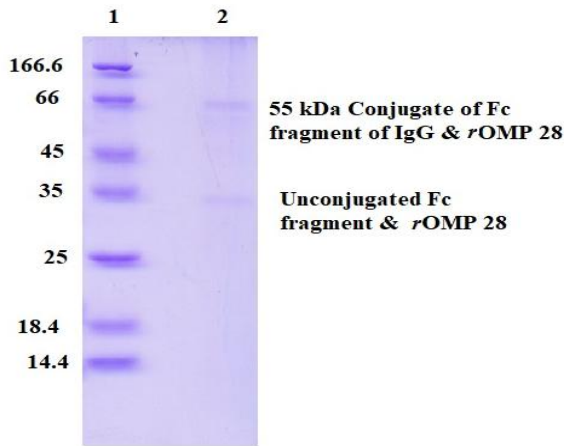


Fig.6 Conjugate prepared from Fc fragment of IgG and *rOMP 28*
Lane 1: Protein Molecular Weight Marker
Lane 2: Conjugate of Fc fragment of IgG and *rOMP 28*(55 kDa)

Fig.6 Conjugate of Fc fragment of IgG and *rOMP 28*

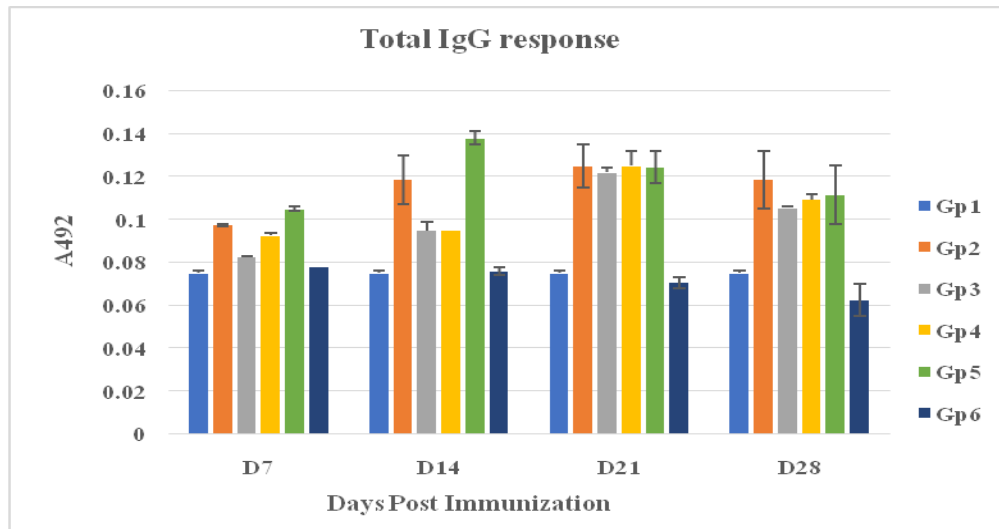


Fig.7 Total IgG response to immunization with *rOMP 28* in different groups

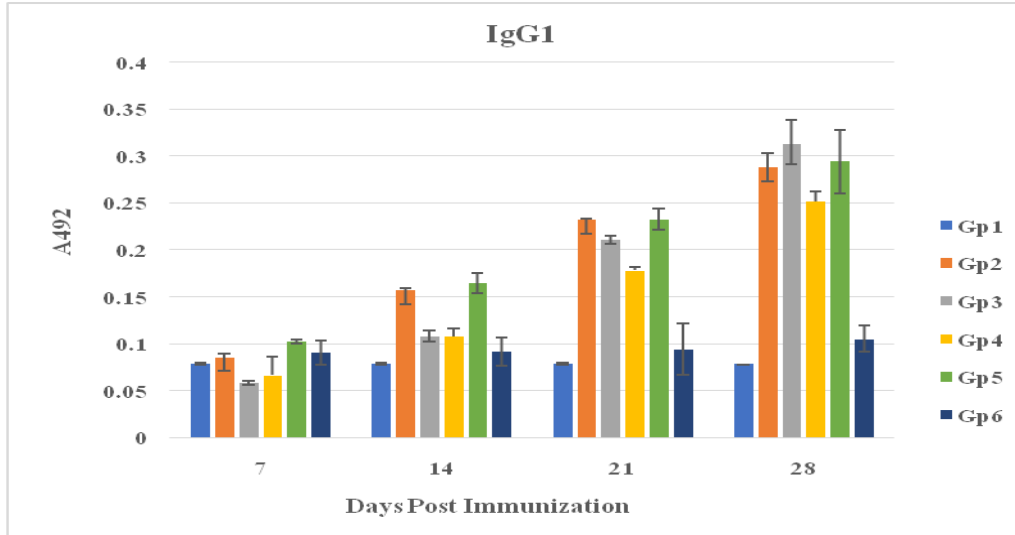


Fig.8 IgG1 response to immunization with *r*OMP 28 in different groups

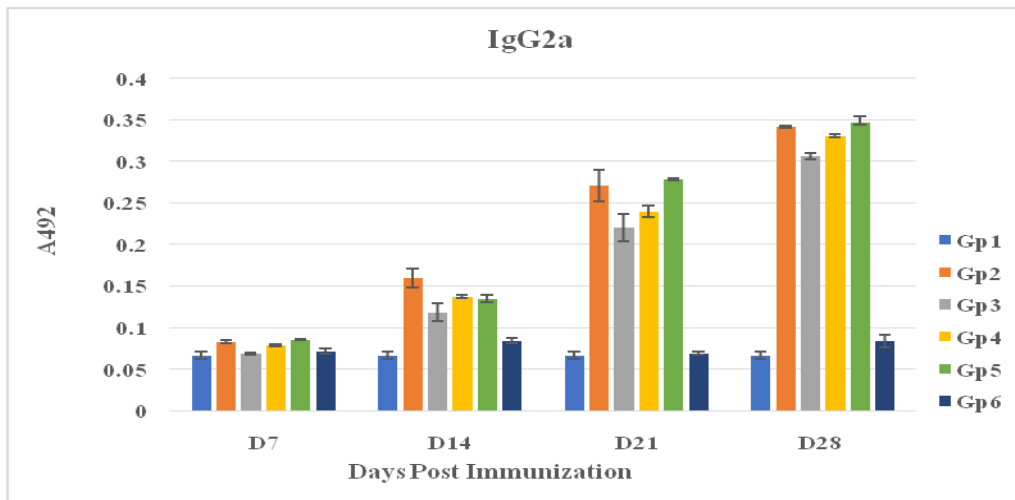


Fig.9 IgG2a response to immunization with *r*OMP 28 in different groups

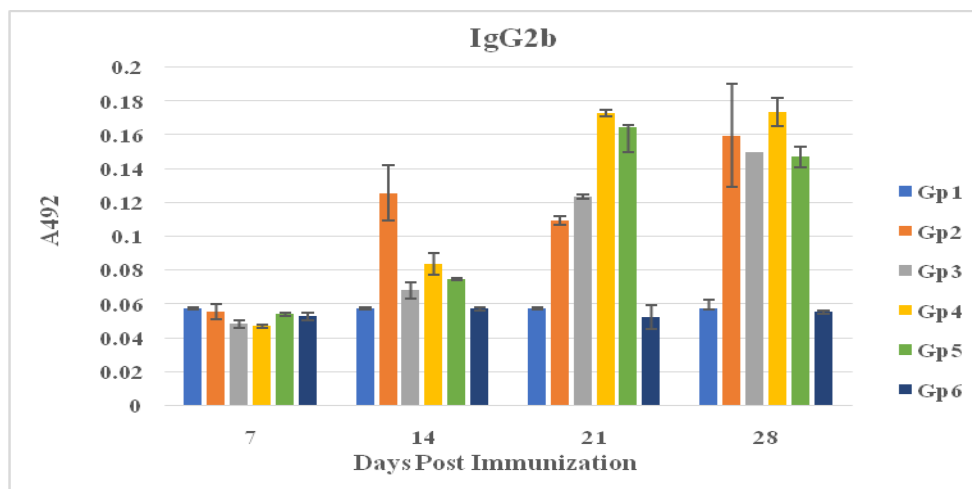


Fig.10 IgG2b response to immunization with *r*OMP 28 in different groups

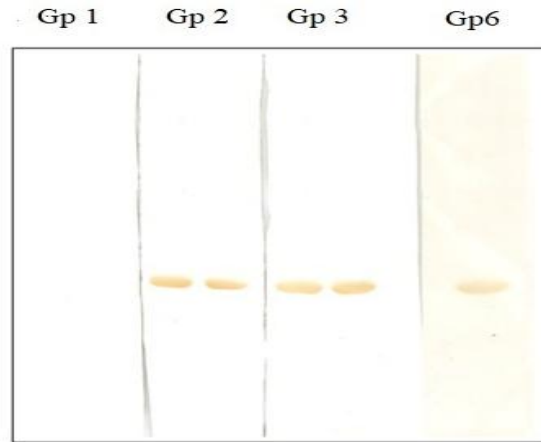


Fig. 11 Western blot analysis of Group 1,2,3 & 6 against rOMP 28

Fig.11 Western blot analysis of immunized groups against rOMP 28

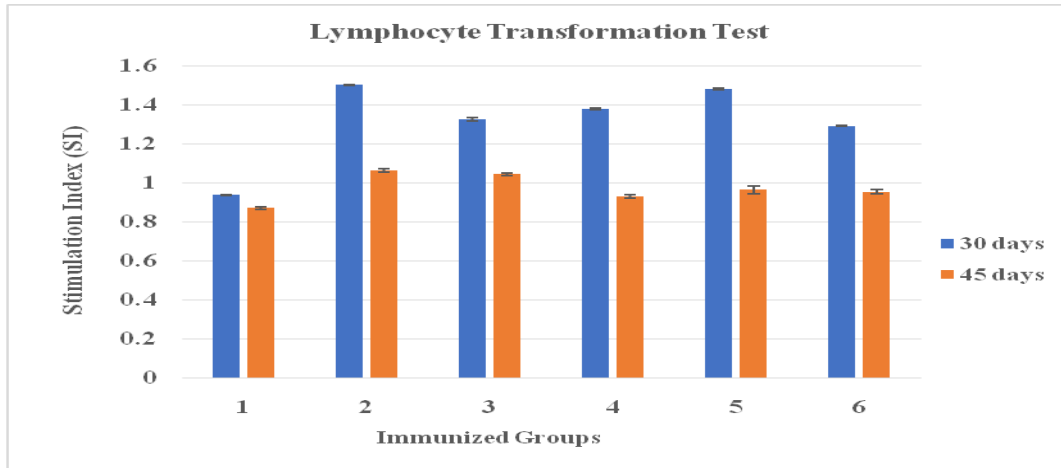


Fig.12 Lymphocyte transformation assay of mice immunized with rOMP 28

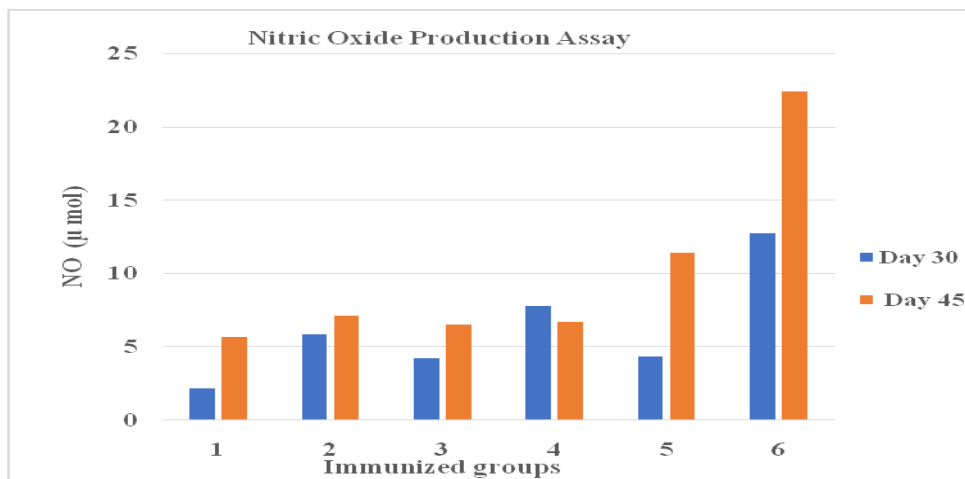


Fig.13 Nitric oxide production in 96hrs culture supernatant ($\mu\text{mol}/2 \times 10^6$ cells) on different days from the splenocytes of immunized mice

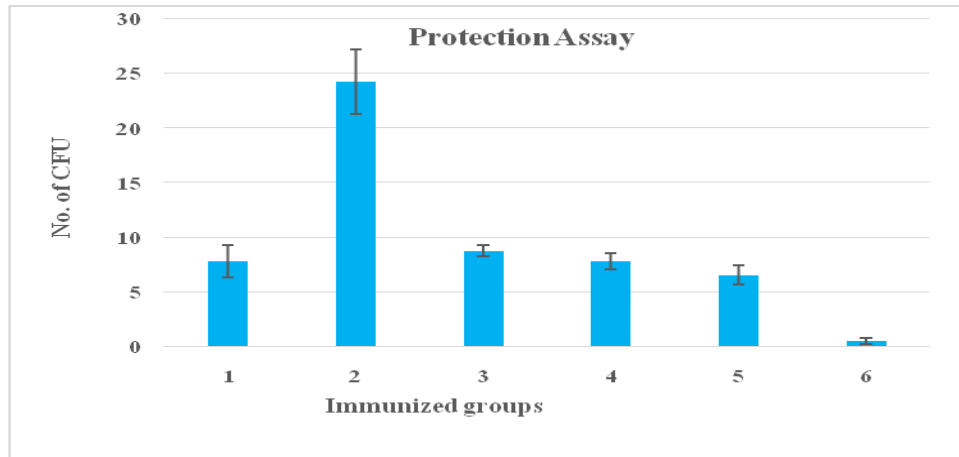


Fig.14 Diagram representing CFU of *B.abortus* 544 in spleen after challenge in different immunized groups

However, 45 days after immunization, the proliferation started decreasing in all the groups suggestive of weak cell mediated immunity induced by subunit vaccine *rOMP* 28. Nitric oxide synthesized by the inducible nitric oxide synthase of macrophages is a predominant microbicidal agent. The nitric oxide production was highest in the S19 group both on 30- and 45-days post immunization which may be the reason for the highest level of protection conferred by S19 group after challenge. The low level of nitric oxide in rest of the groups in the present study clearly emphasizes its role in conferring protection against the intracellular bacteria like *Brucella*. The present study discloses though the antibody response in indirect ELISA in the *B. abortus*S19 group is lowest among all the groups using *rOMP* 28 as antigen, the protection conferred by the S19 vaccine was found to be highest.

In conclusion, *Brucella* being an intracellular pathogen needs to be cleared out by the cell mediated immune system and innate immune cells like macrophages. Hence, it may be inferred as the *rOMP* 28 which constitutes a little part of the organism alone may not be able to provide a full protection against the infection. This was evident in the results of protection study after challenge.

On the other hand, since the live vaccine is composed of lot many components like OMP, LPS, etc. these fractions of the organism might have a role in conferring protective immune response.

Overall, the lower immune response of both humoral and cell mediated in the conjugate group can be attributed to following factors. First of all, two types of Fc γ Rs are expressed by the immune cells that are activating which include Fc γ RI and Fc γ RIII and the inhibitory Fc γ RII. There are reports to support this view that there is an augmented humoral response and anaphylactic responses by Fc γ RII deficient mice (Toshiyuki *et al.*, 1996; Nimmerjahn and Ravetch, 2005). The uptake of antigens via distinct extracellular and intracellular Fc γ Rs influences antigen presentation, and determines whether the antigen is degraded or presented and the type of epitopes that are presented (Guilliams *et al.*, 2014). There is also evidence that soluble forms of Fc γ Rs are released by the Langerhan cells of mice to inhibit Fc γ R mediated antigen presentation (Esposito- Farese *et al.*, 1995). Apart from the Fc γ R mediated antigen uptake, the fate of the Fc receptors after endocytosis was different. Studies suggests that receptor mediated phagocytosis led to the selective and irreversible removal of Fc receptors (>50%)

from macrophage plasma membrane (Mellman *et al.*, 1983). It is also notable that due to the high circulating levels of IgG in mouse serum (~6 mg/mL), it is expected that the binding sites of high-affinity IgG receptors are constitutively saturated by IgG *in vivo*, at least on circulating cells. The ability of FcRs to induce biological functions following interaction with immunoglobulins (i.e. activation, inhibition, internalization, routing, transport) depend on the molecular state of the ligands, whether monomeric, present in an immune complex, or opsonizing cells, viruses, or bacteria. Moreover, binding of monomeric IgG by mouse FcγR I and FcγR IV has not been reported to lead to biological functions whereas multimeric immune globulins, however, trigger activating FcRs to mediate intracellular signaling leading to a diversity of cellular responses including phagocytosis, cell differentiation, cell migration, degranulation, secretion of mediators, antibody-dependent cellular cytotoxicity (Bruhns and Johnson, 2015).

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