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RESEARCH ARTICLE

GENERATION AND CHARACTERIZATION OF A RECOMBINANT CHIMERIC PROTEIN (rGB) AND ITS POTENTIAL ROLE AS A SUBUNIT VACCINE AGAINST BRUCELLOSIS

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ABSTRACT

Brucellosis is one of the most common zoonotic diseases worldwide, caused by the infection of *Brucella* spp. Despite of extensive research, till now no licensed vaccines are available against *Brucella* infection for human application. In the present study, a recombinant chimeric protein rGB contriving immunologically active regions of Gapdh and bvrS from *B. abortus* S19 was constructed, cloned and expressed in *E. coli* host cell. Group of mice immunized with purified rGB protein elicited both Th1 and Th2 mediated immunity as indicated by the higher secretion of IgG2a and IgG1 antibody isotypes in the sera. This observation was further supported by the elevated cytokine profiles of IFN-γ and IL-2 reflecting Th1 and IL- 4 as an indicator of Th2 response upon stimulation of splenocytes with purified rGB antigen. The *in-vitro* lymphocyte proliferation assay resulted in a higher splenic lymphocyte response in immunized mice upon antigen stimulation in comparison with control mice implicating the development of elevated immune responses. Macrophage monolayer supplemented with anti-rGB polysera illustrated efficient protection (>85% survival, $P < 0.001$) against challenge of *B. abortus* strain 544. These findings demonstrate the potential efficacy of fusion protein rGB as a candidate subunit vaccine and showed the development of memory response and functional role of antibodies in protection against *Brucella* infections.

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INTRODUCTION

Brucellosis is one of the world's major zoonoses that still is of veterinarian, public health and economic concern in many parts of the world. Although, brucellosis in livestock and transmission of infection to the human population has been significantly decreased following the instigation of effective vaccination-based control and prevention programmes in parts of the world, it remains an uncontrolled problem in regions of high endemicity such as the Mediterranean, Middle East, Africa, Latin America and parts of Asia including India (Mantur *et al*., 2007; Corbel, 2006; Renukaradhya *et al*., 2002). Attenuated, live *Brucella* strains such as *B. abortus* S19, *B. abortus* RB51 and *B. melitensis* Rev.1 are being used as vaccines to control brucellosis in domestic animals. There is no licensed vaccine for prevention of human brucellosis. The development of new-generation vaccine systems to prevent brucellosis is a pressing need to combat this category B bioweapon agent.

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Multiple studies from our group and others have conclusively demonstrated that chimeric proteins have enhanced immune efficiency in activating humoral and cellular immune responses (Fernandes *et al*., 1995; Singh *et al*., 2014). Thus, bivalent vaccine combining immunogenic antigens of *Brucella* will be a strategy to offer higher protection levels for *Brucella* infections. In *Brucella,* the two component regulatory system (BvrS/BvrR) is a membrane bound homodimeric protein which is predicted to be essential for sensing the phagosomal environment and changing from an extracellular to intracellular life style (Guzman-Verri *et al*., 2002; Lo´pez-Goni *et al*., 2002). This system acts as a cascade of protein phosphorylation which alters membrane protein expression and allows *Brucella* to bind and penetrate through the lysosomal membrane into the host cells (Baldwin and Goenka, 2006). So far, the BvrS is the best characterized two component system associated with the virulence of *Brucella*. Earlier studies on BvrS insertional mutants showed reduced multiplication and invasiveness in macrophages and HeLa cells *in vitro*. Also, *Brucella* defective in the BvrS system rendered minimal persistence in mice spleens, suggesting the inability to inhibit lysosomal fusion, thereby eliminated by the host cell immune

system (Cristina Viadas *et al*., 2010). Interestingly, the glyceraldehyde-3-phosphate-dehydrogenase (Gapdh) which is a key enzyme involved in glycolytic pathway has been associated with pathogenesis and reported as an immunogenic molecule in several infectious disease models such as Candidiasis, Lyme disease and Chagas' diseases (Harper *et al*., 2003). Gapdh is known to be a putative vaccine candidate against *Schistosoma japonicum* and *S. mansoni* infections (Argiro *et al*., 2000; Waine *et al*., 1993). Gapdh is a prominent T and B cell reactive protein in *Brucella* and advised to be a suitable target antigen for immunoprotective approaches against this bacterium (Fugier *et al*., 2009). The present strategy envisages the use of chimeric gene approach to obtain the fusion protein that contains truncated portion of immunodominant Gapdh fused with conserved region of BvrS from *B. abortus* S19. Mice immunized with recombinant Glyceraldehyde-3-phosphate-dehydrogenase fused with *Brucella* virulence related sensory antigen (rGB) was evaluated for its antigenicity, immunogenicity and the protective efficacy against *Brucella* infections was also investigated *in vitro* using macrophage killing assay.

MATERIAL AND METHODS

Bacterial strains and media

The *B. abortus* S19 was used to isolate *gapdh* and *bvrS* genes and pathogenic strains like *B. abortus* 544 and *B. melitensis* 16M were used in *in vitro* assays (Table 1). The bacterial strains were routinely grown on tryptic soy broth (TSB) or *Brucella* broth plated on *Brucella* agar and incubated at 37 °C for 48 h -72 h. The *E. coli* BL21DE3 was propagated in LB broth/agar with appropriate antibiotics and maintained overnight at 37 °C. The media used in this study were procured from Hi-Media labs, Mumbai, India. All Cultures were maintained in 15 % glycerol stocks at -80 °C.

Amplification of *B. abortus gapdh* and *bvrS* genes

The nucleotide sequences corresponding 1 to 166 and 1 to 193 amino acids in *B. abortus gapdh a*nd *bvrS* genes, respectively were PCR amplified using forward and reverse primers flanked with *Bam*HI and *Kpn*I restriction sites, subsequently for the directional cloning in pRSETC vector. In another set of reaction, the reverse and forward primers of *gapdh* and *bvrS* genes, respectively, flanked with nucleotide sequences coding for glycine linker residues to splice both amplicons by SOE PCR. The PCR was carried out using respective primers in Eppendorf master cycler gradient (Hamburg, Germany). Each 20µL reaction contained 10 pM of primers, 100 µM of dNTPs, and 2.0 unit of *pfu* DNA polymerase, 1× *pfu* PCR buffer (with 25 mM $MgSO_4$) with 100 ng of template DNA. PCR was taken through 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 60 s and extension at 72 °C for 90 s. The DNA was denatured for 5 min in the beginning and finally extended for 10 min at 72 °C. PCR products were analyzed in 1.2% agarose (Lonza). PCR amplified products of each gene were purified with the aid of PCR purification kit (Sigma, India).

Construction of a chimeric gene

The *gapdh* and *bvrS* PCR amplicons harboring overlapping sequences were mixed in equal concentration and spliced together by overlap extension PCR to synthesize the chimeric gene *rGB*. Briefly, the SOE PCR was performed in a 30 µl reaction mix comprising 200 ng each of purified *gapdh* and *bvrS* DNA amplicons, 20 pM of forward and reverse primers of *gapdh* and *bvrS* genes respectively, 1× *pfu* PCR buffer (with 25 mM $MgSO_4$), 2U *pfu* polymerase and 100 µM dNTP mix. The PCR reaction mix was taken through 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 1.30 min and extension at 72 °C for 2.30 min. The SOE PCR products were examined by agarose gel electrophoresis and the amplicons (excised from the gel) were purified using GenElute -gel extraction kit (Sigma, India).

Cloning and expression of *rGB* chimeric gene

The *rGB* PCR product harboring *Bam*HI and *Kpn*I restriction sites at 5' & 3' ends and the pRSETC vector were digested with corresponding restriction enzymes, purified by gel extraction and ligated together (16 °C overnight) to form pRSETC-GB recombinant vector. The *E. coli* BL21DE3 transformed with recombinant vector pRSETC-rGB and selected on LB agar supplemented with ampicillin. Recombinant clones contriving pRSETC-rGB were further screened by PCR using T7 primers. PCR positive *E. coli* BL21DE3- pRSETC-rGB clones were inoculated in LB broth supplemented with ampicillin and the late log phase cultures (O.D. at 600 nm: 0.6-0.8) were induced with 1mM IPTG. Cells were pelleted after 5 h of induction and examined by SDS PAGE. The recombinant protein was purified from 500 mL broth culture of *E. coli* BL21DE3- pRSETC-GB clone under denatured conditions by immobilized metal affinity chromatography using Ni-NTA slurry (Qiagen, Germany) according to the manufacturer's protocol. The recombinant purified protein was dialyzed against Phosphate Buffer Saline (PBS) (pH 7.4) for 4 h at 4 °C and purity of eluted proteins was ascertained by SDS-PAGE and quantified by Bradford method. The concentration of the protein determined to be 1.6 mg/ml.

Immunization schedule

Six week old female BALB/c mice were immunized with 50 µg of recombinant protein at an interval of 14 days (0, 14, 28 and 42 days). The first immunization was with Freund's complete adjuvant (FCA) and subsequent doses were provided with incomplete Freund's adjuvant (IFA) intramuscularly in hind legs.

Serum antibody titre/ Measurement of antibody response

Sera was obtained from the immunized mice biweekly and stored at -20 °C as separate aliquots. The antibody titer in sera of immunized mice was measured by indirect ELISA. Briefly, microtiter plates were coated with 100 µl of rGB protein (10 $\mu g\ ml^{-1}$) in carbonate-bicarbonate buffer and the unbound sites were blocked with skimmed milk powder (5% w/v) in PBS. Serially diluted test sera was added to the wells and maintained at 37 °C for 2 h, followed by similar incubation with Horse Radish Peroxidase (HRPO) labeled polyvalent Ig antibody. Each well was washed thrice with PBST (PBS containing 0.05% Tween 20) after incubation, the plate was developed

with substrate O-phenylenediamine dihydrochloride (OPD) in citrate phosphate buffer (pH 4.5 ± 0.2) in presence of H_2O_2 4 µl ml^{-1} concentration. Antibody titer was measured in ELISA plate reader (Infinite M200 pro; Tecan, Grodig, Austria) at O.D. 470 nm. BALB/c mice immunized with rGB chimeric protein developed a high titre (1:64000) circulating immunoglobulins post 42 days of immunization.

Western blot analysis

The whole cell extracts of *B. abortus* along with the purified rGB protein were transferred onto 0.45 µm pore size PVDF (Polyvinylidenedifluoride) membrane (Millipore, USA) by the method described by Towbin *et al*. (1979) using 25 mM Tris hydrocloride-192 mM glycine buffer (pH 8.3), containing 20% methanol at a constant voltage of 70 volts for 70 min in a transblot cell (BioRad mini protein, USA). The free sites were saturated by incubating in 5% skimmed milk powder in PBS, overnight at 4 °C. Membrane was washed with PBS to remove unbound milk protein then incubated with 1:1000 dilution of anti-rGB mouse antiserum. Membranes were washed thrice with PBST (Phosphate Buffered Saline with Tween 20) and further incubated with goat anti-mouse IgG-HRP conjugate (1:2000 dilution in PBS) (Sigma chemicals, USA) for 1 h at 37 °C. Chromogenic reaction was observed by incubating with Diaminobenzidine (DAB)-H_2O_2 substrate. The Polysera rose against the rGB chimeric protein specifically detected the native proteins of Gapdh and bvrS in whole cell extracts of *B. abortus* S19 and also in rGB protein.

In-vitro lymphocyte proliferation

Spleen was collected aseptically from euthanized experimental mice and homogenized in Dulbecco's modified Eagle's medium (DMEM) to recover the splenocytes. The splenocytes were pelleted by centrifugation at 800×g for 5 min at 37 °C and the erythrocytes were lysed by incubation with RBC lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate and 127 µM EDTA) at 37 °C for 3-5 min followed by two washes in sterile PBS. The splenocytes in DMEM media (1×10^5 cells ml^{-1}) was distributed in 96 well culture plates and incubated at 37 °C with 5% CO_2. A gradient concentration of purified rGB protein (5, 10, 15, 20, 25, 30 and 35 µg) was added in lymphocyte suspension and incubated at 37 °C (5% CO_2) for 72 h. Concanavalin A (con A) at 10 µg was used as a positive control/ mitogen. Proliferation of lymphocytes were assessed by incubating with 50 µg of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reagent in dark for 1 h at 37 °C. Reaction was developed with 200 µl of DMSO (Dimethyl Sulfoxide) and observed at 570 nm in micro-titer plate reader (Infinite M200 pro; Tecan, Grodig, Austria). All the supplements and media were procured from Sigma, India.

Measurement of cytokine production in splenocytes *in vitro*

Immunized and non-immunized mice were euthanized and single cell suspensions of splenocytes were prepared and adjusted to the concentration of 1×10^5 cells/ml complete medium (Himedia, India) supplemented with 10% fetal calf serum. Cytokine analysis of T-lymphocytes were carried out in 24 well plates in presence of rGB protein for 72 h. Following incubation, culture supernatant were harvested and assayed immediately. These supernatants were screened for secreted IFN-γ, IL-2, IL-4 and IL-5 using mouse IL enzyme linked immunosorbent assay kit (Sigma, India).The levels of different secreted cytokines were evaluated in triplicate from culture supernatants of splenic T cells isolated from control and rGB protein exposed mice using a ELISA plate reader (Tecan, Grodig, Austria). The statistical significance of differences in the selected cytokine levels were determined by the Turkey's T test and the obtained results were determined using Student's test, statistical significance of differences in the cytokine level were calculated by comparing the cytokine production of T cells from rGB exposed mice to that of control mice.

Macrophage killing assay

Two hour before infection, the freshly grown culture of *B. abortus* 544 was transferred into 37 °C shaker incubator. RAW 264.7 cells were exposed with bacteria at multiplicity of infection (MOI) of 10 in presence of gradient concentration of anti-rGB polysera and control cells were treated with naïve mouse polysera. After 72 h of infection at 37 °C, the percentage viable macrophage cells in each treatment were measured by MTT assay.

Statistical analysis

All the experiments were repeated twice with similar conditions. Results were presented as the mean value ± standard error (SE) and their graphs were created with the help of Graph Pad Prism6 software. Statistical difference between various treatment groups were analyzed by uni-variate (One way Anova). Statistical differences were assumed with p value ($p < 0.05$, $p < 0.01$ and $p < 0.001$).

RESULTS

Construction, cloning, expression and purification of recombinant chimeric protein-rGB

Gene sequences corresponding to 1 to 166 and 1 to 193 amino acids in *B. abortus Gapdh* and *bvrS* genes, respectively were PCR amplified (supplementary data, Figure). The chimeric gene was found to have no deletions or point mutations when checked by sequencing.

Table 1. List of bacterial strains used in the study

Strains	Sources
Escherichia coli DH5α	Invitrogen, Bangalore, Karnataka, India
Escherichia coli BL21 (DE3)	Invitrogen, Bangalore, Karnataka, India
Brucella abortus S19	DRDE, Gwalior, India
Brucella abortus S99	DFRL, Karnataka, India
Brucella abortus 544	DFRL, Karnataka, India
*Brucella melitensis*16M	DFRL, Karnataka, India

This gene was successfully inserted into pRSETC vector by restriction digestion and ligation mediated cloning and transformed into *E. coli* BL21DE3 host cells. PCR-positive clones were induced with 1mM IPTG for 5 h at 37 °C, and the expression of recombinant protein was detected in 12% SDS-

PAGE gel stained with Coomassie blue (Fig. 1). The relative size of the protein was in agreement with the predicted size of 42 kDa. Expressed protein was found to be concentrated as inclusion bodies, and the purification was carried out in denaturing conditions using immobilized metal affinity chromatography. Purified rGB protein was refolded by PBS mediated dialysis at 4 °C.

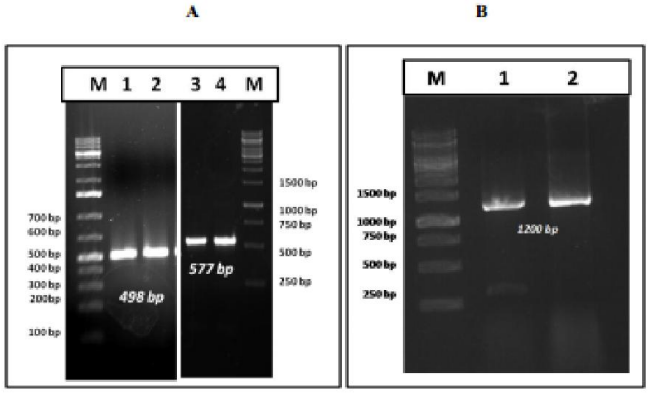

Figure A. PCR amplification of Gapdh, BvrS and chimeric gene GB genes: (A) Lanc M-100 base pair DNA marker; Lane 1&2- PCR amplified gapdh gene: Lane 3&4-PCR amplified BvrS gene; (B) Lane M- 1 Kb DNA marker; lanc 1,2-PCR amplified chimeric GB gene

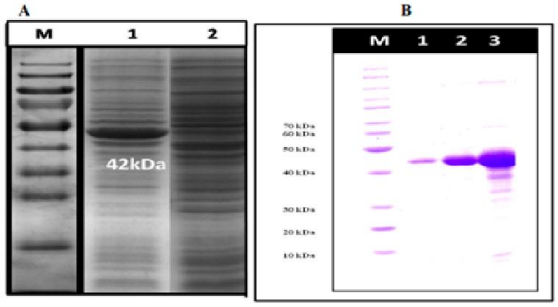

Figure 1: Expression of rGB protein: (A) Lane M- Prestained protein ladder; Lane-1 positive clone: Lane-2 Uninduced control. **(B) Purification of rGB protein:** Lane M- Unstained protein ladder; Lane-1,2 & 3: Elutions of Purified r-GB protein.

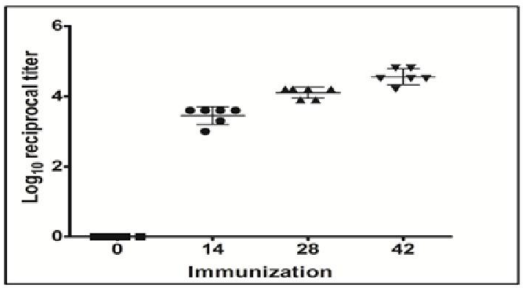

Figure 2: The immunogenicity of protein was assessed by indirect ELISA method: Antibody titer was checked in serum samples collected from immunized BALB/c mice at different time intervals of immunization.

Humoral immune responses

Sera collected at different time point of immunization were screened for the development of anti-rGB antibody titer in the immunized and sham immunized group of mice. A significant increase in rGB specific antibody titer were observed since first booster immunization and there was progressive increase (1:64000) until the final immunization at 42nd day. This signifies elevated level of humoral immune response in mice. The sham immunized group of mice found with no specific antibody titer (Fig. 2). As IgG2a/IgG2b and IgG1 antibody isotypes were known to be the markers of Th1 and Th2 immune responses, respectively. The quantification of these antibody isotypes primarily indicated the T-cell mediated immunity. The chimeric protein was able to elicit both Th1 and Th2 mediated immune response as revealed by higher secretion of IgG1/ IgG3 and IgG2a/ IgG2b antibodies in the immunized mice (Fig. 3). The level of antibody absorbance was measured at 470 nm.

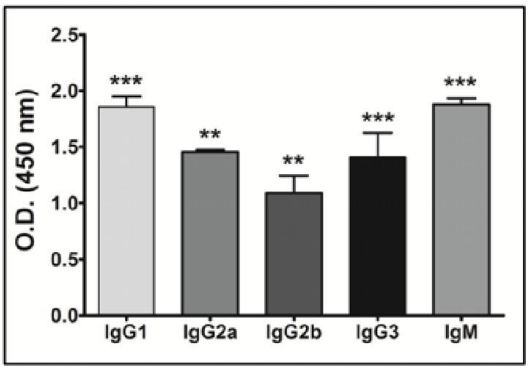

Figure 3: Antibody isotype profiling of rGB immunized mice: The titers of anti- rGB specific IgM, IgG1, IgG2a, IgG2b and IgG3 were presented as a log10 reciprocal of the summative titer. IgG isotypes revealed the immune response biased towards the Th1 and Th2 type in the rGB immunized mice.

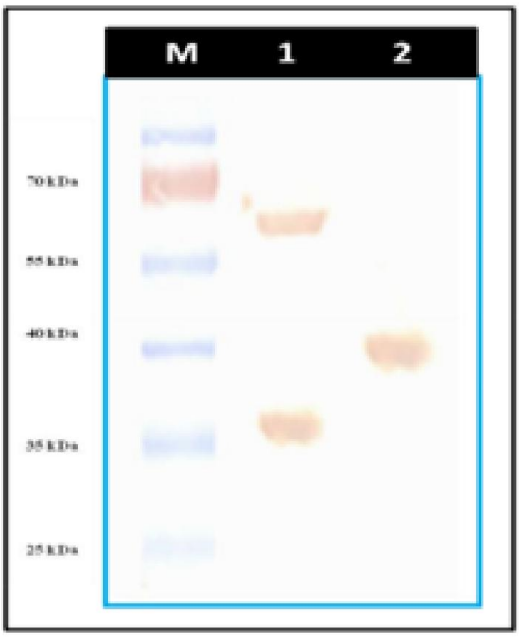

Figure 4: Western blot analysis with the anti-rGB polysera against whole cell lysates of *B. abortus* S19 and rGB protein: Lane M- Prestained protein ladder; Lane 1- whole cell lysates of *B. abortus* S19; Lane 2- rGB chimeric protein.

Immunoreactivity of anti-rGB polysera

Anti- rGB polysera used as probe in Western blot analysis showed strong reactivity with the purified recombinant protein

as well as against whole cell extracts of *B. abortus* S19. Whole cell extracts were further resolved by SDS-PAGE and transferred onto nitrocellulose membrane. The resulting Western blot analysis showed specific binding of anti-rGB polysera at 36 kDa (gapdh) and 66 kDa (bvrS) individual proteins from *B. abortus* S19 among whole cell extracts (Fig. 4). The reactivity of anti-rGB polysera with rGB protein as well as with the native *B. abortus* strain in Western blot assay at the precise molecular weight inferred the antigenic similarity of the recombinant protein with that of the native proteins.

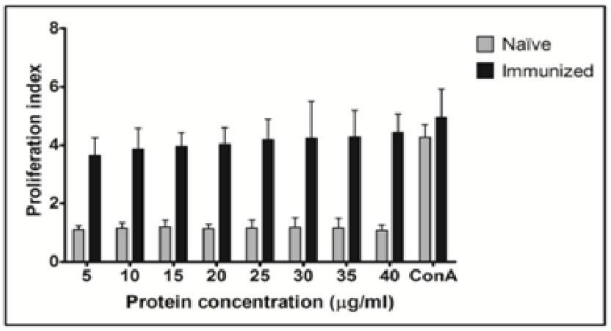

Figure 5. Proliferation of crude spleen lymphocytes extracted form rGB-immunized mice exposed with a gradient concentration of rGB purified protein incubated at 37^{0}C for 72 h: Concanavalin-A (Con-A) was considered as positive control in the experiment

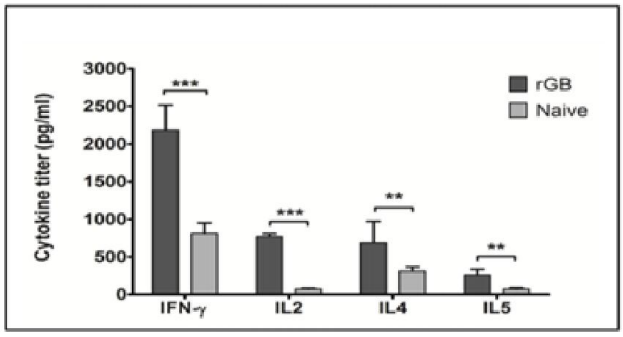

Figure 6. Cytokine profile analysis of the rGB immunized mice: Higher secretion of IFN-γ was measured from the group of immunized mice, Equal concentration of IL-2 as well as IL-4 also recored

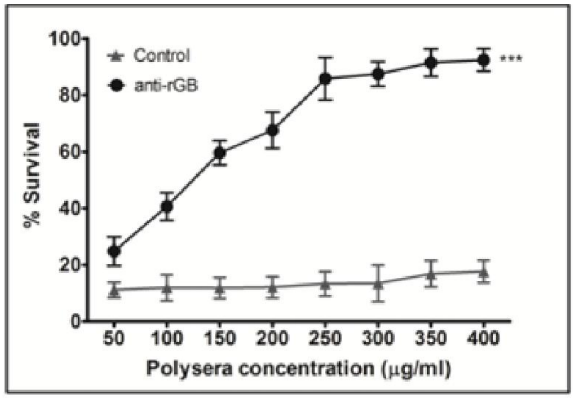

Figure 7: Macrophage monolayer supplemented with anti-rGB polysera was exposed to the challenge of *B. abortus* 544: Significant survival rate (>85%) was achieved with the immunized polysera supplementation and naïve mice polysera used as control were observed with no protection.

In-vitro proliferation of lymphocyte from rGB immunized mice

The capability of rGB antigen to proliferate T lymphocytes was assessed by the in-vitro proliferation of lymphocyte upon exposure with a gradient concentration of purified rGB protein. Splenocytes obtained from immunized group of mice were observed with significant increase in proliferation index (P.I.) with highest value (P.I. 5.28) at 30 µg/ml protein concentration ($p < 0.001$). In contrast, the sham immunized mice splenocytes were observed with no significant increase in proliferation index. The positive control, ConA (10 µg/ml) showed proliferative index of 9.0 and 8.56 in immunized and sham immunized mice group, respectively (Fig. 5). The rGB showed progressive proliferation of primed splenic lymphocytes up to 30 µg ml^{-1} concentration of chimeric protein.

Cytokine profile analysis of the rGB immunized mice

The high proliferation of lymphocytes observed in the splenocytes of rGB immunized mice prompted us to assess the levels of Th1 and Th2 mediated cytokines expressed in the T and B- cell population. The profile of cytokine secretion in culture supernatants of spleen cells from immunized mice and control were evaluated by ELISA. The rGB immunized mice significantly stimulated the production of IFN-γ, IL-2, IL-4 and IL-5 but not from non immunized animals (Fig. 6). This indicates the chimera when used as immunogen significantly produced mixed type of Th1 and Th2 immune responses. Con A induced the production of IFN- γ, IL-2, IL-4 and IL-5 in all groups (data not shown).

In-vitro protective efficacy of anti-rGB antibodies against infection of *B. abortus* 544

The protective efficacy of anti-rGB antibodies were analyzed by *in-vitro* challenging of macrophage cell lines with *B. abortus* 544. Anti-rGB polysera was found to elicit significantly higher protection of more than 85% survival ($P < 0.001$) with 250 µg/ml supplementation of polysera against *Brucella* mediated intracellular killing of macrophage cell line. Further increase in polysera concentration between 300 to 400 µg/ml produced statistically insignificant results ($P < 0.001$). Whereas polysera from the naïve mice were observed with considerably lower protective efficacy (Fig.7).

DISCUSSION

In modern medicine, vaccination or immunotherapy has either virtually eradicated or dramatically reduced the incidence of diseases such as polio, tetanus, tuberculosis, chicken pox, measles, hepatitis etc. The approach using vaccinations has exploited the ability of the immune system to prevent infectious diseases. Stimulation of cell mediated immune response as well as adequate levels of antibodies and their appropriate isotypes to confer protection against the infectious agents is critically dependant on the strong immunogen and continuous presence of antigen in the immunized animals. The vaccines available against brucellosis are live attenuated strains, causing a number of serious side effects and threat of revert into their active pathogenic form (Pappas *et al*., 2005).

Therefore, a variety of approaches are being pursued to develop a safe vaccine against human brucellosis. Large numbers of subunit vaccines are being explored as promising, safe and efficacious molecules for protection of at-risk workers, general populations living in endemic areas and military personnel in the event of a bioterrorism scenario. Various types of protective antigens have been characterized for their role in protective immunity against brucellosis, of these, metabolic and regulatory associated gene targets are drawing attentions as they offer conserved nature in different species of *Brucella* and capable of inducing both T and B cell protective epitopes. Nucleoside diphosphate kinase (Ndk), Glucokinase (Glk) and Glyceraldehyde 3 phosphate dehydrogenase (Gapdh) are some of the proteins well established as protective molecules for brucellosis (Hop *et al*., 2015). One of the features of Gapdh is that it is present on the cell surface of several prokaryotes including *Brucella*, where it is involved in *Brucella* containing vacuole formation (BCV) thereby contributing to evade the host immune system and multiplication of *Brucella* in the invading host [Roy *et al*., 2006]. Previous studies conducted by several laboratories, indicated that antigenically conserved proteins with Gapdh activity have potential as a target for vaccines in several microorganisms [Argiro *et al*., 2000; Bolton *et al*., 2004; Fontaine *et al*., 2002; Gil-Navarro *et al*., 1997; Rosinha *et al*., 2002]. Despite its role in the expression of several membrane proteins, the BvrS is a major virulence factor associated with *Brucella* and the mutants in BvrS component are easily eliminated by the host immune system *in vivo* (Sachse *et al*., 2002). Thus, we believe that the *B. abortus* Gapdh and BvrS proteins can be a good vaccine candidate against brucellosis. To test the potential of Gapdh and the BvrS as components of a chimeric vaccine against this bacterium, we cloned and constructed a chimera composed of immunodominant Gapdh and the conserved BvrS proteins using flexible Glycine linkers so as to retain the nativity of individual domains in the fusion construct [Trinh, R. 2004]. Here we show that mice immunized with the chimera rGB developed both humoral and cell mediated immune response.

Protective antigen's efficacy can depend on the T and B lymphocytic responses and the degree of protection offered in the immunized host. Assessment of rGB immunized mice splenocytes for T-cell proliferation exerted substantial level of lymphocyte stimulation at the minimal protein concentration ($p< 0.001$). Immunity against *Brucella* relies on both Th1 and Th2 mediated immune responses which is characterized based on the production of IgG2a and IgG1 isotypes respectively (Hop *et al*., 2016). The antibody isotypes measured in the rGB immunized mice sera produced the profile of both IgG1 and IgG2a isotype in the significant manner preceded by the chimeric protein. We observed significant rise in the levels of IL-4 (Fig 3B), an anti-inflammatory cytokine that enhances B cell survival, proliferation and antibody production. Interestingly, the rGB immunized mice splenocytes elicited a significant IFN-γ response, a cytokine critical for the activation of macrophages and a requisite for controlling *Brucella* infections (Baldwin *et al*., 2006). It is therefore likely that prolonged elevated levels of IFN-γ in mice vaccinated with rGB contributed to the enhancement of cellular immunity. The presence of IL-2 and IL-4 further suggests Th2 and Th1-biased immune responses engendered by the chimeric protein. This observation suggests that the symmetry in cytokine production levels could be important in providing a more efficacious immune response to brucellosis. Previous reports have clearly indicated that r-Gapdh alone induced a Th1-type of immune response in mice (Gracia *et al*., 2002). The nature of rGB-induced complete cell mediated immune response is unclear at present. However, it is possible that an auxiliary virulence function of the BvrS may be responsible for the robust immune response directed towards it. These studies may shed further light on the immunological aspects of both the genes (*Gapdh* and *BvrS*) which are known to be involved in the *Brucella* stealthy strategy to establish successful infections in the host cells. Protective levels of rGB protein were analyzed by *in vitro* challenging of macrophage cells (RAW 264.7) with *B. abortus* 544 and found higher protection against *Brucella* mediated intracellular killing of macrophages. Taken together, the data suggests that rGB protein as a potential candidate subunit vaccine for brucellosis. BvrS and/or Gapdh may also represent promising subunit vaccine candidates when used alone and/or in combination with other known protective antigens. In this regard, we plan to evaluate such antigen cocktails in the near future, using heterologous species of *Brucella* in mice and in other host species.

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