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Live micro-encapsulated *Brucella abortus* vaccine strains offer enhanced protection and sustained immune response in BALB/c mice

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Abstract

Two sustained release vaccine delivery formulations, micro-encapsulated live *Brucella abortus* vaccine strains S19 and RB51 in alginate microspheres, were analyzed for entrapment efficacy and *in-vitro* release kinetic studies.

The level of protection offered to female BALB/c mice after sub-cutaneous (S/C) immunization with both encapsulated vaccine formulations 15 days after intra-peritoneal (I/P) challenge on 30th days post immunization (DPI) with wild type *B. abortus* 544 strain was significantly higher (P<0.01) compared to non-encapsulated live versions. In addition, the protection offered by the encapsulated RB51 formulation was superior (P<0.01) compared to encapsulated S19.The mean number of colony forming units (log_{10} CFU) persisting in spleen in all four experimental groups of immunized mice at 15 day post challenge (DPC) did not differ significantly.

Subtle differences in the antibody isotype and cytokine response pattern were observed during the pre and the post challenge stage in different groups of mice immunized with encapsulated S19, encapsulated RB51, non-encapsulated S19 and RB51. Compared to non-encapsulated version and saline inoculated controls, the enhanced protection exhibited by micro-encapsulated vaccines was reflected in significantly different (P <0.01) IgG1, IgG2b, IgG3 titers in mice immunized with encapsulated RB51, but not with encapsulated S19 at 15 DPC; however, encapsulated S19 immunized group showed significantly different (P<0.01) IgG2a titers. Although, both the mice groups immunized with encapsulated S19 and RB51 elicited significantly higher (P<0.01) IFN-γ response compared to S19 and RB51 non-encapsulates and controls at 15 DPC, the difference (P<0.05) in IL-2 response could be observed in encapsulated RB51 immunized group but not in encapsulated S19. The salient features of pre-challenge immune response in mice immunized with encapsulated S19 smooth strain were characterized by significantly elevated IgG1, IgG2a, IgG2b, IgG3 titers (P<0.05; P<0.05; P<0.01; P<0.01 respectively) at 30 DPI, also, IgG2a (P<0.05) and IgG2b (P<0.01) titers differed

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significantly as early as 7 DPI, accompanied by elevated IL-2 ($P<0.05$) at 21 DPI; while the encapsulated RB51 rough strain elicited enhanced IgG1 ($P<0.05$, $P<0.05$ and $P<0.01$) at 14,21 and 30 DPI and IgG2b ($P<0.01$) response at 30 DPI.

Overall comparison indicated that RB51 micro-encapsulated vaccine formulation is probably a potential candidate as it offered the best level of protection upon challenge and elicited most appropriate immune response.

Keywords: *Brucella abortus,* microencapsulation, vaccine, cytokines, antibody.

Introduction

Bovine brucellosis is mostly caused by a Gram negative intra-cellular pathogen *Brucella abortus* and is one of the major causes of infertility and abortion in cattle and buffaloes (1, 2). The disease causes significant economic loss to the dairy industry, and it has been estimated that the losses accounted for cattle and buffaloes in India was approximately US$ 3.25 billion (3, 4). The pathogen is excreted in milk, uterine, vaginal discharge and semen from infected animals, and humans can acquire infection by aerosol, direct contact through skin abrasions or consumption of unpasteurized milk (5, 6).

Bovine brucellosis is generally controlled by test and slaughter strategy or by mass calf-hood vaccination of females, restriction in the movement of animals and adoption of biosecurity measures (7). Two live attenuated *B. abortus* vaccines S19 and RB51 has been successfully used in mass immunization program in different countries for control of bovine brucellosis (8,9,10). However, the S19 vaccine produces residual virulence in some proportion of immunized animals, and excretes the vaccine strain in the environment, infecting humans (11). The efficacy of protection due to immunization with S19 vaccine varies from 65-70%, but the duration of protection beyond 7 years has not been documented (12,13). Vaccination using S19 strain interferes with standard diagnostic serological tests (14) since anti O-lipopolysaccharide (O-LPS) antibodies are detected in case of natural infection and also due to immunization. This interference is absent following immunization with RB51 vaccine since it lacks the O-LPS. However, RB51 is a rifampicin resistant strain, and exposure of humans to this strain from vaccinated animals or during vaccination has been cited; this observation is significant as it complicates therapeutic regimen with antibiotics in exposed humans (15). In order to address and improve the current limits of immunogenicity, duration and safety offered by the two *B. abortus* S19 and RB51 vaccines, sustained-release delivery vehicles carrying transposon and deletion marked attenuated mutants of *vbjR Brucella meltensis* (16) and *B. abortus* (17) has been used for testing the efficacy of these candidates in mice. These studies in mouse models had showed enhanced efficacy of immunogenicity, protection and safety. The present study was aimed at testing whether the protective and immunogenic efficacy of *Brucella abortus* S19 and RB51 vaccine strains cross linked by Poly-L-lysine and encapsulated in alginate microspheres were superior to non-encapsulated controls in a BALB/c mouse model.

Materials and Methods

Mice : 6 to 8 week old female BALB/c mice were obtained from Small Animal Testing (SAT) unit, Indian Immunologicals Limited, Hyderabad and acclimatized for 2 weeks before start of the experiment. All experimental procedure and animals care were done as per the guideline of Institutional Animal Ethical (IAEC) Committee (Approval No. IIL-R & D SA06/2010).

Bacterial Strain : Freeze dried vials of *Brucella abortus* strain S19 obtained from USDA and RB51 from Virginia Tech, USA were respectively grown on Potato Infusion Agar (BD, USA) and Tryptic Soya Agar slant (Difco,USA) with 5% serum and dextrose (20% w/v) with Rifampicin at a concentration of 20 µg/ml for 4-5 days .Bacteria were harvested from the surface into phosphate buffer saline (PBS) with pH 6.4 .The cultures were pelleted by centrifugation at 4000

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rpm for 20 minutes , re-suspended in PBS 6.4 and washed thrice before getting a final suspension .The viable count of organisms was determined by serial dilution plating method. The culture was finally re-suspended at a final concentration of 6.3 x 10^7 CFU / ml for S19 and 4.7 x 10^7 CFU / ml for RB51.

Preparation and characterization of B.abortus S19 and RB51 microsphere: Microspheres of *B.abortus* S19 and RB51 loaded with 6.3 x 10^7 CFU / ml for S19 and 4.7 x 10^7 CFU / ml respectively were prepared by method previously described (17) with minor modification where the encapsulates were prepared by employing Homogenizer (Polytron) at 2000 rpm for 10 minutes .The culture was permanently cross-linked with Poly-L-lysine and final covering of alginate matrix. Prior to permanent cross-linking with Poly-L-lysine, 1 ml of microspheres suspensions were treated with depolymerizing solution (Tri sodium-citrate 50mM, 0.455mM NaCl and 10mM 3-Morpholinopropane-1-sulfonic acid (MOPS). After treatment, cultures were re-suspended in 1 ml of Brucella broth (BD, USA) and incubated at 37 ° C for 24 hours with 5% CO_2 for RB51 and without CO_2 for S19. After incubation, serially diluted culture was plated on specified plate for S19 and RB51 to determine the post-encapsulation viable count of both organisms. The entrapment efficacy of both the strains were calculated by dividing post-encapsulation count of bacteria by pre-encapsulation count and expressed in per cent. The presence of bacteria encapsulated, morphology and size were determined by direct microscopy (OLYMPUS, Model No. BX 50, Japan) at 100X magnification under oil immersion. Three optical fields were observed and mean diameter of microsphere was determined.

In-vitro bacterial release from the microencapsulate : One ml. of encapsulated culture containing 1.1 x 10^9 CFU/ml for S19 and 2.3 X 10^9 CFU/ml of RB51 were re-suspended in 9 ml. Brucella broth and incubated at 37 ºC for 24 hours. After 24 hours, the culture is spinned at low rpm (1500 -2000) for 5 minutes and the microencapsulates were re-suspended in 10 ml of fresh Brucella broth. One ml of broth culture post spinning was taken for each strain and plated onto Potato infusion agar (PIA) plate for S19 and in 5% Serum dextrose agar (SDA) with Rifampicin for RB51 respectively. Plates were incubated at 37 ºC with and without CO_2 for 3-4 days for RB51 and S19 respectively.. The colonies of the bacteria were counted from each plate and expressed as CFU/ml in terms of number of release of bacteria versus time. The above procedure was repeated till no further release was seen.

Antigen preparation : *B.abortus* S19 and RB51 were grown on PIA and SDA slant with rifampicin respectively and incubated for 4-5 days at 37 ° C .Confluent growth of bacterial lawn were harvested in PBS (pH 6.4) from each slant separately and checked for purity of the culture. The cultures were pelleted by centrifugation at 2000 rpm for 10 minutes in centrifuge tube. The pelleted culture was washed thrice with PBS buffer .The culture was finally re-suspended in PBS (pH 6.4) at fixed volume and equal amount of acetone (50 % v/v) were added to culture. The acetone mixed culture was kept for stirring for 24 hours to kill the bacteria. After 24 hours, 100 µl of killed culture were plated and checked for the viability. The killed culture was confirmed by negative growth on the plate. The protein content of acetone killed antigen were assessed by Bichinconic Acid Method (BCA Method) and used for evaluation of anti-Brucella specific antibody in mice (18).

Immunization of mice : Thirty 6-to 8 weeks' old female BALB/c mice were randomly divided in 5 groups of 6 mice each. Two groups of mice were immunized with a single dose of 0.1ml microencapsulated *B.abortus* S19 and RB51 containing a final count of 1.3 x 10^5 CFU and 2.7 X 10^5 CFU by sub-cutaneous route respectively as per the OIE protocol (19) . Two groups of positive control mice received non-encapsulated live *B.abortus* S19 1.1 x 10^5 CFU and 1.9 x 10^5

CFU for RB51. One group of negative control mice was injected with MOPS buffer.

Bleeding and challenge of mice : Mice were bled by inserting capillary tube in the infra-orbital sinus on day 0 and on 7,14,21,30 and 45 days post-immunization. All mice irrespective of treatment were intra-peritoneally challenged on day 30 with 2.2 x 10^5 CFU of wild type *B.abortus* strain 544.

Protection efficacy : Mice in each group were euthanized by CO_2 asphyxiation on 15 days post-challenge (DPC). Spleen from each individual mouse was collected aseptically. The size and weight of each spleen was determined to observe the presence or absence of spleenomegaly. Spleen was homogenized using sterile mortar and pestle and re-suspended in PBS with volume in ml equal to 10 times the weight of spleen. Ten fold serial dilution of spleen were prepared and 200 µl of suspension in duplicate were plated on PIA plate for S19 and SDA with rifampicin plate for RB51.The suspension was simultaneously plated on Tryptic Soya Agar (TSA) plate in duplicate to determine the bacterial load of *B.abortus* 544 strain in spleen. SDA and TSA plate were incubated at 37 ° C for 4-5 days at 5% CO_2 and PIA plate without CO_2 .The colonies of each Brucella strain were counted and the values were expressed after logarithmic transformation. The efficacy of micro-encapsulated with non-encapsulated live *B.abortus* vaccine and unvaccinated control group were determined by comparing the $\log_{10}$ CFU protection value of treatment group with non-vaccinated group (19).

Determine of cytokine response in -vivo : Mice were bled and serum were obtained from individual mouse of each group on day 0,7,14,21 and day 30 post-immunization and 15 day post-challenge for determination of cytokine level. Quantification of different cytokine (IL-2, IL-6, and IFN-γ) was determined by ELISA (eBioscience, Germany) as per the manufacturer's instruction.

Determination of anti-Brucella specific antibody : To determine the anti-Brucella specific antibody response, mice were bled and serum was collected on day 0, 7,14,21,30 days post immunization (DPI) and on 15 DPC. Sera were used for measuring IgG isotypes. Acetone killed whole cell *B.abortus* S19 and RB51 antigen were used to coat 96 well ELISA plate (Nunc, Denmark) at a concentration of 100ng /100µl / well. Optimal concentration of antigen and serum dilution were determined by checker board titration employing pre immunized and vaccinated sera and the method was qualified for inter-personal and inter-day variability in triplicate by Bland-Altman Plot using MedCalc Software (Data not shown). After overnight incubation at 4 °C, plates were washed 4 to 5 times with PBS and 0.1% Tween-20 (Sigma, Germany) and blocked with 2 % skimmed milk (BD, USA) and kept for 1 hour at 37 ° C. After wash, serum was added at initial dilution of 1:50 in same buffer and serially diluted further two-fold to determine the end-point titer. After addition of serum, plates were incubated at 37 ° C for 1 hour. After washing with PBS-T to remove unbound antibody, mouse monoclonal IgG isotype antibody (SIGMA, Germany) were added at 1: 1000 dilution for each plate in 2% skimmed milk and incubated at 37 ° C for 1 hour. 100 µl of purified recombinant protein A/G peroxidase (ThermoScientific, USA) at 1:20,000 dilutions in 2% skimmed milk was added after washing with PBS-T. After incubation and washing, added 100µl of peroxidase substrate (1 Tablet of Tetramethyl Benzidine dissolved in 10ml of citrate buffer with 3µl of H_2O_2) was added in each well and kept for 10 minutes at room temperature in dark. The reaction was stopped by 100µl of 1.25 M H_2SO_4 and read the absorbance value at 450nm (Synergy ST, BioTek). The Mean OD value $\pm$3 Standard Deviation of pre-vaccinated mouse sera at 1:50 dilution were taken as cut off value for each isotype.

Statistical analysis : The *in vitro* release of bacteria from micro-encapsule was expressed as $\log_{10}$ CFU plotted on Y axis versus time in days on X axis. The difference in spleen weight, intensity of infection and protective efficacy of

vaccination (bacterial clearance from spleen) of each group of 6 mice at 15 DPC was expressed as mean $\log_{10}$ CFU± Standard Deviation was analyzed by Student's *t* test, and the significance of differences between the groups were determined by analysis of variance (ANOVA) followed by a Tukey's honestly significant difference (HSD) post-test comparing all groups to one another. The cytokine production *in vivo* was expressed as mean cytokine concentration ±Standard Deviation of each group of 6 mice. The anti-Brucella specific isotype antibodies were expressed as the reciprocal of $\log_2$ end point dilution ±Standard Deviation. The significance of differences between groups for both anti-*Brucella* specific antibodies and cytokines were analyzed by employing ANOVA followed by Tukey's HSD. For ANOVA and Tukey's HSD, P value of <0.05 were considered statistically significant (17, 20).

Results

Microsphere preparation and characterization : Microscopic studies revealed that the preparations from live *B. abortus* S19 and RB51 strains derived from cultures of Batch I and II were of uniform spherical shape ranging from 100 to 200 µm in size, with dotted centers suggestive of proper encapsulation (Fig. 1a and 1b). The viability of micro-encapsulated S19 and RB51 preparations after dissolution of capsules did not show significant batch to batch variation (Table 1). The *in vitro* release kinetics study on microsphere preparations incorporating *B.abortus* S19 and RB51 indicated the initial burst and continued sustained release till 24 days and 27 days for S19 and RB51, respectively (Fig. 2).

Protection efficacy : At 15 DPC (45 DPI) mice from all immunized groups demonstrated a statistically significant decrease in bacterial load in spleen as compared to MOPS inoculated naive mice control, with a 1.40-log reduction (P<0.01) from non-encapsulated S19, 1.99-log reduction (p<0.01) from encapsulated S19, a 3.22-log reduction (p<0.01) from non-encapsulated RB51 and a 3.89-log reduction (p<0.01) from encapsulated RB51 relative to naïve mice (Table 2). Both groups of mice immunized with encapsulated S19 and RB51 showed significantly higher level of protection upon challenge compared to non-encapsulated S19 and RB51 (P<0.01) (Table 2). Moreover, the level of protection offered by encapsulated RB51 (higher by 1.9-log) was significantly higher than that

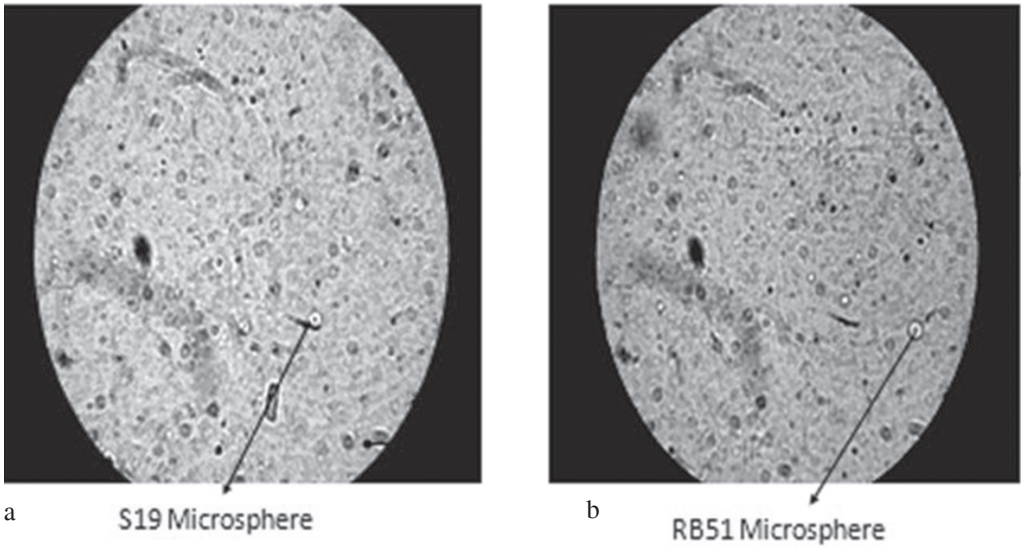

Fig. 1. Microscopic evidence of microencapsulated *B.abortus* S19 (1a) and RB51 (1b) after permanent cross-linking with Poly-L-lysine (100 x).

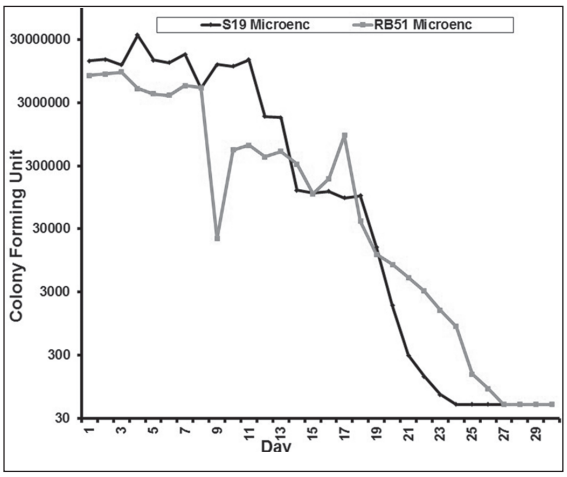

Fig. 2. Kinetics of live bacteria released from microsphere. 1ml of microencapsulates were suspended in 9 ml of Brucella broth and incubated at 37 ° C for 30 days. 1 ml of aliquot was withdrawn from each strain and plated on specific media plate to determine the release of bacteria from microspheres on each day.

offered by encapsulated S19 (P<0.01) (Table 2). However, the mean log10 CFU in spleen of vaccine strains in all groups of immunized mice at 15 DPC did not differ significantly (Table 2). Statistical difference was not observed in the mean spleen weight in all groups of immunized mice at 15 DPC (data not shown).

Comparison of immune response in mice after immunization and challenge: ***Encapsulated live B. abortus S19 and RB51 versus non-encapsulated live S19 and RB51 :*** The IgG1 isotype response in mice immunized with non-encapsulated live RB51 differed (P< 0.01) from MOPS inoculated controls at 7 DPI but in those immunized with encapsulated RB51 the levels differed (P< 0.01) at 7, 14, 21 and 30 DPI. The IgG1 response of mice immunized with encapsulated RB51 and non-encapsulated formats differed at 7 DPI (P< 0.05) and at 14, 21, 30 DPI (P<0.01). Mice groups immunized with non-encapsulated and encapsulated S19 showed IgG1 levels that differed (P<0.01) from MOPS inoculated controls at 30 DPI (Fig.3a). In addition IgG1 response in mice immunized with encapsulated S19 differed (P<0.05) from those inoculated non-encapsulated form at 30 DPI. The levels of IgG1 elicited in mice immunized with RB51 encapsulated form also differed from those immunized with S19 encapsulated form (P<0.05) at 7 and 14 DPI. It was observed that mice immunized with live non-encapsulated S19 and RB51 had significantly different (P<0.01) IgG1 levels with respect to the MOPS inoculated controls at 15 DPC. Further, at 15 DPC the IgG1 levels of mice immunized with encapsulated RB51 were significantly different (P<0.01) from non-encapsulated live RB51.

The IgG2a levels were significantly higher in mice immunized with non-encapsulated RB51 as well as encapsulated RB51 compared to MOPS inoculated controls (P<0.01) at 7,14,21,30

Table 1. Entrapment efficacy of Brucella *abortus* live vaccine with alginate matrix in two different set of preparations.

Batch I : *In –Vitro* Kinetics				
	Pre-Count (X)	**Post –Count (Y)**	**Y/ X x 100**	**%**
S19 RB51	1.9 X 10^9 CFU / ml 3.7 X 10^9 CFU / ml	1.1 X 10^9 CFU / ml 2.3 x10^9 CFU / ml	0.5789 0.6216	57.89 62.16
Batch II : Vaccine Batch For Mice Immunization				
S19 RB51	6.3 x 10^7 CFU / ml 4.7 x10^7 CFU / ml	4.1 x10^7 CFU / ml 2.6 x10^7 CFU / ml	0.6507 0.5531	65.07 55.31

Table 2. Enhanced protection in female BALB/c mice immunized sub-cutaneously with encapsulated *B.abortus* S19 and RB51 compared to non-encapsulated and MOPS control groups followed by intra-peritoneal challenge with 544 strain. Difference in log protection values were compared between experimental and control group(e). Mice immunized with S19 micro-encapsulated form (a); RB51 micro-encapsulated form (b); S19 non-encapsulated form (c); RB51 non-encapsulated form (d) and MPOS buffer inoculated control groupe; S** significant P<0.01 by Student 't' test.

Vaccine Group	Recovery of challenge strain *B.abortus* 544 (Log 10 CFU)	Recovery of vaccine strain *B.abortus* (Log 10 CFU)	Protection indices
Microencapsulated Live RB51	1.87	2.68	3.89**a,b,e
Non-encapsulated Live RB51	2.54	1.95	3.22**b,d,e
Microencapsulated Live S19	3.77	3.83	1.99**a,e
Non-encapsulated Live S19	4.36	3.23	1.40**a,c,e
Unvaccinated MOPS Buffer control	5.76	-	

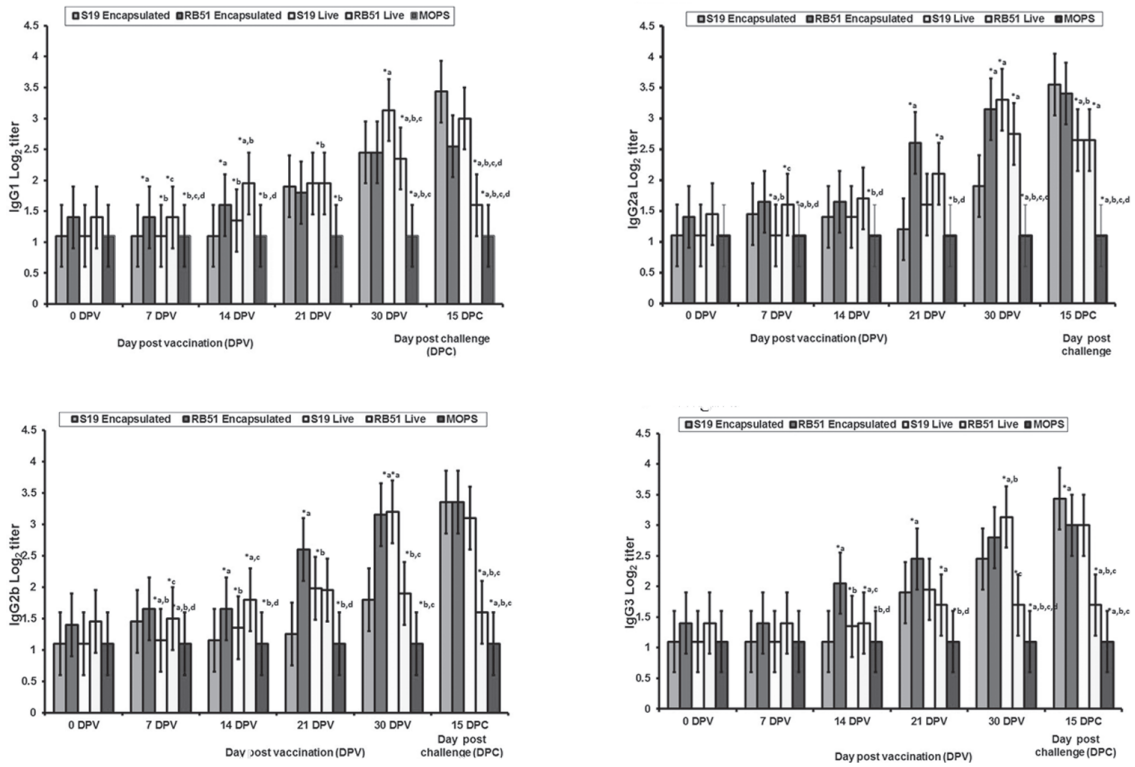

Fig.3. Anti-brucella isotype specific antibody response in BALB / c mice immunized with live microencapsulated *B.abortus* S19(a) and RB51(b) and live non-encapsulated S19(c) and RB51(d) with MOPS control group (e) on days 0,7,14, 21,30 and 15 day post-challenge ; Fig 3a , 3b ,3c and 3d : Response of IgG1 , IgG2a ,IgG2b and IgG3 differ significantly between group , days and day and group ($p < 0.001$)

DPI and also at 15 DPC (Fig.3b). Similarly, in mice groups immunized with non-encapsulated S19 the IgG2a differed from MOPs inoculated controls at 7 DPI ($P<0.05$) and 30 DPI ($P<0.01$), whereas in mice immunized with encapsulated S19 differed significantly from MOPS control at 7 DPI ($P<0.05$) and 30 DPI ($P<0.01$) respectively. The IgG2a levels were higher in mice immunized with S19 encapsulated format compared non-encapsulated group at 7DPI ($P<0.05$), 30 DPI ($P<0.01$) and at 15 DPC ($P<0.01$). The IgG2a were significantly elevated ($P<0.01$) in mice immunized with S19 encapsulated version compared to those immunized with RB51 encapsulated group at 30 DPI.

The IgG2b levels were significantly elevated in mice immunized with non-encapsulated S19 ($P<0.05$ at 30 DPI and 15 DPC), non-encapsulated RB51 ($P<0.05$ at 7, 14 and 21 DPI), encapsulated S19 ($P<0.01$ at 30 DPI and 15 DPC) and encapsulated RB51 ($P<0.01$ at 7, 14, 21 DPI and 15 DPC) compared to MOPs inoculated controls (Fig.3c). Significantly different IgG2b levels were observed in mice immunized with S19 encapsulated version compared to live non-encapsulated S19 ($P<0.01$) at 7 and 30 DPI, whereas in groups of mice immunized with encapsulated RB51 the levels differed ($P<0.01$) at 21 DPI and 15 DPC. Further mice immunized with encapsulated RB51 had significantly different IgG2b levels ($P<0.01$) compared to the group immunized with encapsulated S19 at 14, 21 and 30 DPI.

The IgG3 levels were significantly elevated in mice immunized with non-encapsulated S19 ($P<0.01$ at 15 DPC), encapsulated S19 ($P<0.01$ at 15 DPC), non-encapsulated RB51 ($P<0.01$ at 14, 21 and 30 DPI) and encapsulated RB51 ($P<0.01$ at 14 and 30 DPI, and at 15 DPC, $P<0.05$ at 21 DPI) compared to MOPs controls. Compared to non-encapsulated S19 and RB51, IgG3 was significantly elevated ($P<0.01$) in mice immunized with encapsulated S19 at 30 DPI and those immunized with encapsulated RB51 at 15 DPC. Moreover, IgG3 found to differ significantly in mice immunized with encapsulated S19 compared to those immunized with RB51 at 14 DPI ($P<0.01$) and 21 DPI ($P<0.05$) (Fig.3d).

The IL-2 levels were significantly different ($P<0.05$) in mice immunized with encapsulated S19 compared groups of mice immunized with non-encapsulated S19 at 21 DPI, and MOPS controls at 21 DPI ($P<0.05$) and at 15 DPC ($P<0.01$). In addition the IL-2 level were statistically different in mice immunized with non-encapsulated S19 and RB51 compared to MOPS at 15 DPC ($P<0.01$). Also, IL-2 was significantly different ($P<0.05$) in mice immunized with encapsulated RB51 from non-encapsulated RB51 and MOPs control mice groups at 15 DPC (Fig.4a). The IFN-γ levels in mice immunized with non-encapsulated S19 differed ($P<0.01$) from their MOPS control at 15 DPC. Both groups of mice immunized with S19 and RB51 encapsulated forms showed significantly elevated IFN-γ levels ($P<0.01$) compared to non-encapsulated S19 and RB51 as well as to the MOPS control at 15 DPC (Fig.4b). However the IL-6 levels did not differ significantly among groups after immunization or post-challenge (Fig.4c).

Discussion

Microspheres prepared from live *B. abortus* S19 and RB51 strains ranged from 100-200 µm. Particle size is an important factor for the effective uptake of the immunogen by antigen processing cells/ macrophages (21). Natural and synthetic micro-particles of similar size ranging from 10 to 300 µm has been used to encapsulate Influenza A, HBsAg , *B. abortus* and *B. melitensis* antigens (14, 21, 22), and while the micro-encapsulated Influenza A and HBsAg immunogens were reported to elicit appropriate immune response (21), the micro-encapsulated *B. abortus* and *B. melitensis* exhibited improved protection (16,17). Although the bacterial viability post-encapsulation did not differ statistically between strains and batches in the current study, the values were lower (mean 60%) as compared to previous report (17). The reason for lower viability may probably because of the employment of a

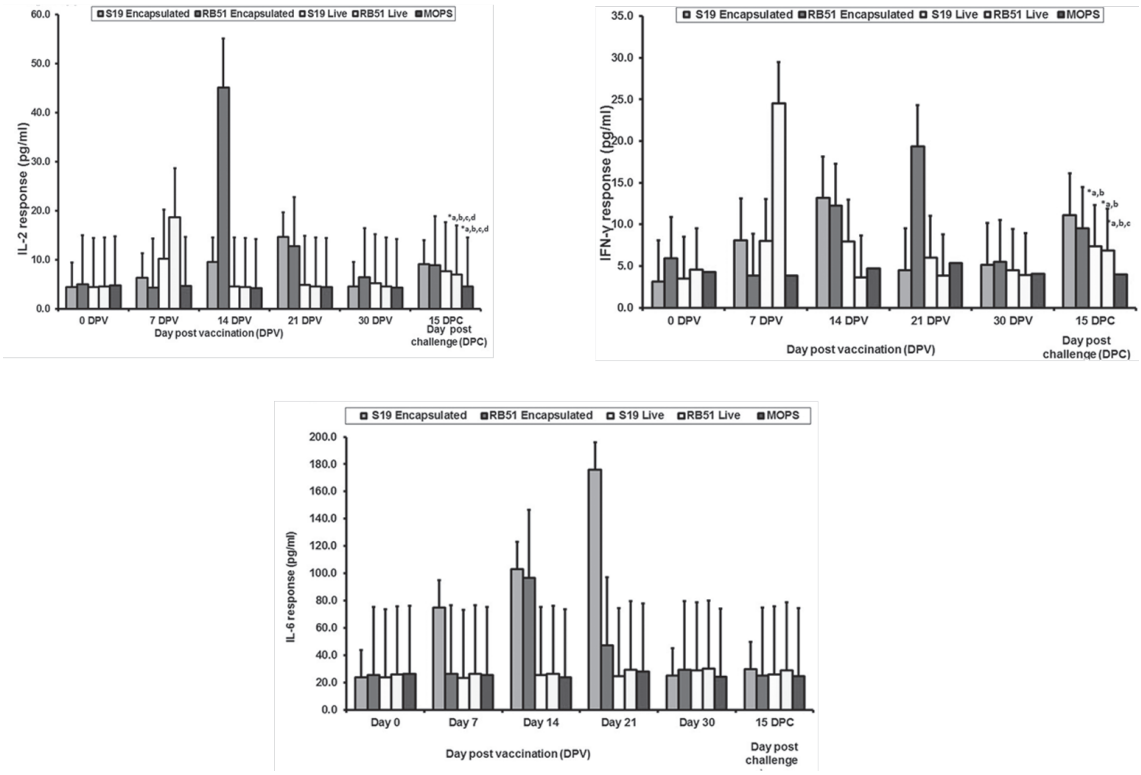

Fig. 4. Comparative quantification of cytokine in serum samples of female BALB / c immunized with live encapsulated *B.abortus* S19(a) and RB51(b) vaccines with non-encapsulated live S19(c) and RB51(d) and MOPS control group(e) on day 0 , 7,14,21, 30 and 15 day post-challenge with *B.abortus* 544 ; Fig 4a and 4b : Response of IL-2 and IFN-γ differ significantly between day and group by ANOVA ($p<0.001$ and $p< 0.015$ respectively).IL-6 does not differ significantly between days and group (4c)

homogenizer for carrying out the encapsulation instead of a specific encapsulator as cited in a previous study (17). Homogenizers generate high stress on cells due to shear force, the encapsulator on the other hand produce minimal stress that helps maintain viability of preparations. Similarly, the release of S19 and RB51 from microencapsulated preparations as observed by *in vitro* release kinetic studies lasted for 24 and 27 days, respectively, compared to a previous report of 36 days in case of a microencapsulated mutant *B. melitensis* (17). Various physicochemical factors may affect the release, including the concentration, surface charge, ligand composition and hydrophobicity (21, 22).

Micro-encapsulated live S19 and RB51 provided enhanced protection in mice by order of magnitude of 0.59-log and 0.67-log respectively compared to non-encapsulated forms when a protocol of sub-cutaneous immunization, challenge at 30 DPI and recovery of challenge strain *B. abortus* 544 at 15 DPC was followed. The efficacy of protection in the current study was similar to a study reported previously (17) where mice immunized intra-peritoneally with live *B. abortus* S19 vjbR::kan mutant encapsulated in alginate microsphere exhibited

superior efficacy (0.8-log) that was statistically different ($P<0.05$) than its non-encapsulated format, following intra-peritoneal challenge with *B. abortus* 2308 at 32 weeks post-immunization and evaluation of the load of *B. abortus* 2308 in spleen at 1 week post-challenge. While in a separate previous study mice immunized intra-peritoneally with encapsulated *B. melitensis* 16M vjbR::Tn5 mutant and challenged intra-peritoneally at 9 weeks post-immunization with *B. melitensis* 16M strain showed much higher level of enhanced protection of the order of 1.84-log compared to non-encapsulates controls (16). Improved efficacy of vaccines by microencapsulation of antigens employing biodegradable polymers have been reported (14, 23, and 24). The degree of efficacy may be affected by the choice of microspheres (natural/ synthetic), physiochemical character of the antigen such as the composition of the outer membrane (25), the nature of lipo-polysaccharide (smooth and rough) (26) as well as the immunization and challenge protocol adopted for the model for testing the efficacy. The most salient finding in the current study was the exhibition of superior protection offered by the encapsulated live *B. abortus* RB51 rough strain by an order of magnitude of 1.9-log compared to encapsulated S19. Probable reasons for enhanced efficacy may be ascribed to the facts such as structural and functional differences in outer membrane in rough *Brucella* strains (25). Further it has been shown that the nature and degree of attachment, mode of entry into a cell (in non-opsonized conditions) and intra-cellular trafficking (in human monocytes, vero cells and macrophages) of rough and smooth strains of *Brucella* are quite distinct (26, 27, 28), the rough strains also induced higher amounts of pro and anti-inflammatory cytokines from monocytes (28).

The characteristics of antibody isotype and cell mediated immune response appeared to be influenced by the choice of the vaccine strain and whether it was used as an encapsulated or a non-encapsulated version for immunization. The probable mechanisms that could have conferred the group of mice a protective advantage by immunization with micro-encapsulated S19 and RB51 compared to the un-encapsulated versions are summarized (Table 3). Similarly, the probable mechanism that conferred the mice group immunized with micro-encapsulated RB51 over those immunized with micro-encapsulated S19 is summarized (Table 4). Thus it appears that antibody isotype levels differs between the encapsulated versions in the post-immunization stage alone, however, no such differences in IL-2 and IFN-γ were observed between the two encapsulated groups either at the post-immunization and post challenge stage.

The significantly different levels of IgG2a, IL-2 and IFN-γ in mice immunized with encapsulated S19 compared to the non-encapsulated group at the DPC stage was probably related to better protection by an order of magnitude of 0.59 logs. While better protection by an order of magnitude of 0.67 logs provided by immunization of mice with encapsulated RB51 version compared to non-encapsulated group may have been due to significantly different levels IgG1, IgG2b, IgG3, IL-2 and IFN-γ at the DPC stage. Therefore it appears that production of significantly higher levels of IgG2a, IL-2 and IFN-γ (S19) or significantly higher levels of IgG1, IgG2b, IL-2 and IFN-γ (RB51) in encapsulated groups compared to the non-encapsulated versions at the DPC stage was due to more efficient recall and recruitment of these specific immune memory cells. This observation probably conferred the protective advantage to mice groups immunized with encapsulated versions over those immunized with live *B. abortus* vaccines. Improved protection observed in mice immunized with encapsulated S19 versions is probably also linked to significantly different levels of IgG2a, IgG2b, IgG3 and IL-2 in the DPI stage. Since together in tandem, they may have been contributory factors that could have possibly facilitated more efficient priming of the immune system, leading to more efficient recruitment of memory cells, later at the DPC stage. But unlike

Table 3. NS: Statistically not significant; S*: Significant P< 0.05; and S** significant P<0.01 by Tukey's Honesty Significant Difference. Suggested immune response mechanisms that empowered mice immunized with micro-encapsulated vaccine a protective advantage over the group immunized with non-encapsulated form. Probable mechanisms: Protective advantage to mice conferred by immunization with micro-encapsulated S19 compared to the non-encapsulated form could probably be due to significantly different response of IgG2a, IL-2 and IFN-γ at the DPC stage and that of IgG2a, IgG2b, IgG3 and IL-2 at the DPI stages. Together in tandem this characteristic immune response at the DPI stages probably facilitated more efficient priming of immune cells leading to efficient recruitment immune memory cells later at the DPC stage. Similarly, protective advantage to mice conferred by immunization with micro-encapsulated RB51 compared to the non-encapsulated form could probably be due to significantly different response of IgG1, IgG2b, IgG3, IL-2 and IFN-γ at the DPC stage and that of IgG1 and IgG2a at the DPI stages.

A. Suggested immune response mechanisms that empowered mice immunized with micro-encapsulated S19 vaccine a protective advantage

Immune response	**DPI stages**	**15 DPC stage**
Antibody isotype		
IgG1	NS	S*
IgG2a	(7 DPI S*) (30 DPI S**)	S**
IgG2b	(7 and 30 DPI) S**	NS
IgG3	(30 DPI) S**	NS
Cytokine response		
IL-2	(21 DPI)S*	S*
IFN-γ	NS	S**

B. Suggested immune response mechanisms that empowered mice immunized with micro-encapsulated RB51 vaccine a protective advantage

Immune response	**DPI stages**	**15 DPC stage**
Antibody response		
IgG1	(14 and 21 DPI)S* (30 DPI S**)	S*
IgG2a	NS	NS
IgG2b	(21 DPI) S* (30 DPI)S**	S**
IgG3	NS	S**
Cytokine response		
IL-2	NS	S*
IFN-γ	NS	S**

the above observation, significantly elevated levels of IgG1 and IgG2a in case of mice immunized with encapsulated version of rough strain *B. abortus* RB51, at the DPI stage, were sufficient enough to render improved priming; that in turn, could have contributed to better recall of memory T cells at the DPC stage.

Similarly, the superior protective efficacy of encapsulated versions of RB51 compared to S19 could probably be linked to significantly higher levels of IgG1 (early stage - 7 DPI) and IgG2b (middle and late stage - 14, 21, 30 DPI) at the DPI stage in mice immunized with encapsulated RB51, but not significantly enhanced IgG2a (late stage -21 and 30 DPI) and IgG3 (mid and late

Table 4. NS: Statistically not significant; S*: Significant P< 0.05; and S**: Significant P<0.01 Tukeys Honesty Significant Difference. Suggested immune response mechanisms that empowered mice immunized with micro-encapsulated RB51 vaccine a protective advantage over the group immunized with S19 encapsulated form. Probable mechanisms: Protective advantage to mice conferred by immunization with micro-encapsulated RB51 compared to the micro-encapsulated S19 form could probably be due to significantly different response of IgG1 at 7 DPI and IgG2b at 14, 21 and 30 DPI, but not significantly elevated IgG2a at 21 and 30 DPI and IgG3 at 14 and 21 DPI as noticed in mice immunized with micro-encapsulated S19.

Micro-encapsulated vaccine form / Immune response	**DPI stages**	**15 DPC stage**
Antibody response		
IgG1		
S19	NS	NS
RB51	(7 and 14 DPI) S*	NS
IgG2a		
S19	(21 and 30 DPI) S*	NS
RB51	NS	NS
IgG2b		
S19	NS	NS
RB51	(14, 21 and 30 DPI) S*	NS
IgG3		
S19	(14 and 21 DPI) S*	NS
RB51	NS	NS
Cytokine response		
IL-2		
S19	NS	NS
RB51	NS	NS
IFN-γ		
S19	NS	NS
RB51	NS	NS

stage -14 and 21 DPI) levels; as noticed in mice immunized with encapsulated S19 compared to encapsulated RB51. This evidence is supported by the observation that the levels of antibody isotypes were statistically non-significant when both encapsulated versions were compared at the DPC stage. Neither, the levels of IL-2 and IFN-γ differed statistically between the groups of mice immunized with encapsulated S19 and RB51 either at the DPI or the DPC stage. These observations emphasize that most probably priming of the immune cell subsets secreting IgG1 and IgG2b at the DPI stage confers the encapsulated rough *B. abortus* RB51 version with a significant protective advantage (>1.9 log) over the encapsulated smooth *B. abortus* S19 version.

The above repertoire of immune response events described above while comparing encapsulated with non-encapsulated groups probably suggested that for both encapsulated versions, in the DPC stage, the cellular immunity was due to both the Th1 and the Th2 types, and these responses were significantly more

pronounced than the non-encapsulated versions, leading to better protection in encapsulated groups. However, during the DPI stages, in the S19 encapsulated group the Th1 driven immune response was more pronounced than controls. This was distinct from that noticed in the RB51 encapsulated group where cells of both Th1 and Th2 lineage were recruited for eliciting a more pronounced immune response than the controls. The most important observation that emerged from this study was that the protective advantage offered by immunization of BALB/c mice with encapsulated RB51 over those with encapsulated S19 seemed to be linked to cellular events that were distinct in character and was noticed in the DPI stage alone. The Th1 driven response alone was more pronounced in the S19 encapsulated group than in the RB51 encapsulated group, as evidenced by recruitment of B cell subsets secreting significantly elevated levels IgG2a and IgG3, in contrast, in RB51 encapsulated mice both the Th1 and Th2 driven responses were prominent compared to the S19 encapsulated group, as evidenced by recruitment of a different subset of B cells secreting significantly elevated levels of IgG2b and IgG1. This observation may have been responsible for conferring the mice immunized with rough encapsulated RB51 the protective advantage over those immunized with smooth encapsulated S19, since no other significantly different Th1 or Th2 driven activity was noticed among the two groups in terms of antibody isotype or cytokine response (IL-2, IFN-γ) either at DPI or DPC stages. The essential involvement Th1 and Th2 cells in the immunity to *Brucella* have been previously described (14, 17, 29, 30, 31 32,). Our current observations are in agreement to these previous reports.

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