

Evaluation of immunogenicity and protective efficacy of a liposome containing *Brucella abortus* S19 outer membrane protein in BALB/c mice

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Summary

The use of liposome as an adjuvant and a vaccine carrier has been cited previously in the literature. It has also been shown to be effective in enhancing the immunogenicity of vaccine candidates. BALB/c mice immunized subcutaneously with outer membrane protein (OMP) of *Brucella abortus* S19 vaccine strain entrapped in a commercial cationic liposome (S19-OMP-liposome) for vaccine delivery, showed enhanced protection ($P<0.05$) compared to groups of mice inoculated with S19 OMP alone, S19 live *B. abortus* vaccine and liposome alone, when challenged intra-peritoneally with virulent *B. abortus* strain 544 at 30 days post-immunization (DPI). The S19-OMP-liposome preparation was found to be safer compared to the live *B. abortus* S19 vaccine at 15 days post challenge (DPC), as evidenced by the significant difference in spleen weight between S19-OMP-liposome, S19 OMP and S19 live as well as the liposome control groups ($P<0.01$). Antibody isotype response profiles of the experimental groups indicated that the immune response was Th1 cell mediated. The protective advantage conferred to mice immunized with S19-OMP entrapped in liposome over those immunized with the live *B. abortus* S19 version, could probably be related to the significantly different response of IgG2b at 30 DPI ($P<0.01$), IgG2a ($P<0.01$), IgG2b ($P<0.01$) and IgG3 ($P<0.05$) at the DPC stages, respectively.

Key words: *Brucella*, Liposome, Mice

Introduction

Brucella abortus is the causative agent of bovine brucellosis. It causes spontaneous abortion, placentitis and infertility in pregnant cattle, hence resulting in considerable economic loss for the dairy industry (Xavier *et al.*, 2009). It also causes infection in humans which lead to persistent undulant fever. Controlling bovine brucellosis is mainly achieved by mass immunization with live attenuated *B. abortus* smooth strain S19 (Graves, 1943) or the rough mutant strain RB51 (Crasta *et al.*, 2008), but it does not confer absolute protection to cattle (Alton *et al.*, 1984). It may revert to pathogenic form (Corner and Alton, 1981) and show residual virulence in natural hosts. The vaccine strains are also pathogenic to humans as they are excreted through milk or other secretions (Nicoletti, 1989).

Attempts have been made earlier to use purified antigens from the cell membrane of *B. abortus* to immunize cattle. Purified sub unit vaccines are immunogenic and safe since the possibility of reversal to virulence is eliminated. The immunogenicity of the outer membrane protein (OMP) can be improved by

entrapping the OMP within liposomes that have the potential to improve the efficacy of delivery to the target antigen processing cells (APC) (Onurdag *et al.*, 2008).

In earlier studies, the multi-lamellar molecular structure of bio-degradable cationic liposomes with an appropriate formulation of various antigens derived from *F. tularensis* and *B. melitensis* have shown versatile adjuvant property, safety, enhancement of prophylactic efficacy and the ability to elicit a strong immune response in *in vitro* as well *in vivo* models (Kulikov *et al.*, 1985; Wong *et al.*, 1992; Gladerio *et al.*, 1995; Vitas *et al.*, 1995; Vitas *et al.*, 1996; Ireland *et al.*, 2010). The ability of cationic liposomes to deliver liposome bound antigens in sustained and concentrated forms directly to macrophages for their further processing to APC has been cited as a reason for the better clearance of infection (Alving *et al.*, 1986; Richard *et al.*, 1998).

In this study, the immunogenic and protective efficacy of the outer membrane protein of *B. abortus* strain S19 (S19-OMP) entrapped in cationic liposome as a vaccine delivery system is evaluated by immunization and challenge experiments in a BALB/c mouse model.

Materials and Methods

Mice

Six to eight-week-old female BAB/c mice were used in this study, all provided by the Small Animal Testing (SAT) Unit, Indian Immunologicals Limited, Hyderabad. The mice were caged in biosafety level 3 (BSL 3) facilities and cared for 1 week before the start of the experiments. All experiments were approved by the Institutional Ethical Committee (IAEC) and the Committee for the Purpose of Control of Experiment of Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Government of India, and were conducted according to the standard operating procedures (SOP) and guidelines of IAEC/CPCSEA.

Bacterial culture

Bulk culture of the *B. abortus* S19 strain, obtained from the National Dairy Development Board, Anand, was grown in an aerated stirred-tank bioreactor using soya casein digest medium (BD, USA) and used for extraction of the outer membrane protein. Mice were immunized with 1.1×10^5 colony forming units (CFU) of *B. abortus* S19 vaccine (Bruvax, Indian Immunologicals Limited, Hyderabad, India). After re-constitution and dose adjustment, 0.1 ml *B. abortus* wild type strain 544 (ATCC, USA) was used for mice challenge experiments.

Extraction and purification of *Brucella abortus* S19OMP complex

Extraction of outer membrane protein complex was carried out according to the method described earlier by Verstrete *et al.* (1982). Downstream processing, such as cell physical disruption, purification and efficacy of extraction by electro-immunoblot transfer assay and S19-OMP complex estimation was performed as described previously by Mythili *et al.* (2012).

Formulation of S19-OMP-liposome

The cationic liposome 1,2, dioleoyl-3-dimethylammonium-propane (DODAP/1,2, dioleoyl-sn-glycero-3-phospho-ethanolamine (DOPE) (50:50 mol/mol) was obtained commercially (Formumax®, USA) and used for the entrapment of the S19-OMP complex obtained as described above. The formulation was prepared fresh before immunization by manual mixing of DODAP/DOPE with S19-OMP at the recommended ratio of 1:20 (50 µL of DODAP/DOPE with 950 µL of S19-OMP) by repeated inversion (20-40 times) of the mixture in a microcentrifuge tube at room temperature, so as to obtain a concentration of 50 µg of S19-OMP/mice/dose. The process resulted in the binding of the OMP extracted from *B. abortus* S19 to the cationic liposome DODAP/DOPE.

Immunization of mice

Each group of six BALB/c mice was immunized subcutaneously with a 50 µg formulation of liposome-encapsulated *B. abortus* S19 OMP (S19-OMP) as

described above as well as OMP alone once at day 0 of immunization. Six mice were simultaneously immunized with 1.1×10^5 CFU live attenuated *B. abortus* S19 once on day 0 of the immunization. Those injected with liposome alone acted as positive and negative controls on day 0 of the immunization.

Bleeding and mice challenge experiments

Serum samples from mice were collected for the antibody assay from the infra-orbital sinus using the capillary tube insertion method after proper restraining and before immunization on day 0 and days 7, 14, 21, 30 and 45 post-immunization. Mice were challenged intraperitoneally with 2.2×10^5 CFU/mice of wild type *B. abortus* strain 544 irrespective of the treatment group, day 30 post-immunization.

Protective efficacy in mice

On day 15 post-challenge, mice were euthanized by CO₂ asphyxiation. The spleen of each mouse from each group was collected aseptically to determine the bacterial load of wild type *B. abortus* challenge strain (OIE, 2009). The spleen was homogenized using mortar and pestle and re-suspended in PBS (pH = 6.4) in volumes 10 times its weight. Ten-fold serial dilutions of suspension from each spleen were plated on tryptic soya agar (TSA) to determine the load of the *B. abortus* strain 544. Spleens from control groups were similarly processed and samples from mice immunized with *B. abortus* S19 were plated on potato infusion agar (PIA). The spleen from mice immunized with liposome alone as negative control was plated on TSA to determine the bacterial load of the *B. abortus* 544 challenged. TSA plates were incubated at 37°C for 5-7 days with 5% CO₂ to recover the wild type challenge strain. PIA plates were incubated at 37°C for the same period in air, without CO₂ atmosphere. CFU enumeration from each plate was done manually. The CFU was expressed after the arithmetic values were subjected to logarithmic transformation ($\log y = x/\log x$; where $x = \text{CFU/ml}$). The protection expressed as log₁₀ CFU values were compared for each group to determine the protective efficacy of each treatment.

Immunoassay

The levels of immunoglobulin antibody isotypes against anti-*Brucella* outer membrane protein were assayed by indirect ELISA. The well of the microtiter plate (Nunc, Denmark) was coated with 100 ng of OMP/100 µL/well in carbonate-bicarbonate buffer (pH = 9.6). The optimal concentration of antigen and serum dilution was determined by checker-board titration utilizing pre- and post-vaccinated serum. IgG antibody isotype IgG1, IgG2a, IgG2b and IgG3 against OMP was determined using a monoclonal isotype antibody kit (SIGMA, USA) with a 1:1000 dilution in 2% skimmed milk as the blocking buffer and incubated for 1 h at 37°C. Plates were washed 4-5 times with PBS-T before each subsequent step. 100 µL of recombinant protein A/G peroxidase (Thermo Scientific, USA) with a

1:20,000 dilution in skimmed milk were used as secondary conjugate after washing with PBS-T. 100 μ L of Peroxidase substrate in citrate buffer (Tetra-methylbenzidine with H_2O_2) were added in each well after 1 h of incubation at 37°C and washed with PBS-T. The reaction was stopped with 1.25 M H_2SO_4 . After 10 min of color development and absorbance, optical density was measured at 450 nm (SYNERGY ST, BioTek, USA). The antibody titers were expressed as the reciprocal of 2 logarithmic transformations with the Mean \pm SD of the pre-vaccinate sera with a 1:50 dilution as a cut off value for each antibody isotype.

Statistical analysis

The intensity of infection and protective efficacy of each group at 15 DPC were expressed as the mean log CFU \pm SD. The values were analyzed statistically by Student's t-tests, followed by an analysis of variance (ANOVA) with a Tukey's Honestly Significant Difference (HSD) post-test for all groups. The specific isotype antibodies were expressed as reciprocal of log 2 end-point dilutions and analyzed by ANOVAs for statistical difference followed by Tukey's HSD tests (Snedcor and Cochran, 1980).

Results

Antibody response

The antibody isotype response to IgG1 in mice immunized with S19-OMP-liposome and live *B. abortus* S19 vaccine differed significantly ($P < 0.01$) for mice inoculated with liposome alone as a negative control group at day 30 post immunization (DPI) and day 15 post challenge (DPC) (Fig. 1a). However, the mean IgG1 titers of mice groups immunized with S19-OMP-liposome were not statistically different from those vaccinated with live *B. abortus* S19 vaccine (Table 1); neither the IgG1 titers of mice group immunized S19-OMP-liposome differed statistically from those immunized with S19-OMP (Table 2).

The mean levels of IgG2a were significantly different ($P < 0.01$) for mice immunized with S19-OMP-liposome, those receiving OMP alone and those vaccinated with live S19 on DPI 30 and DPC 15 compared with the liposome inoculated controls (Fig. 1b). However, at DPC 15, the mean IgG2a titers in groups immunized with S19-OMP-liposome differed significantly ($P < 0.01$) from those immunized with S19 live vaccine (Fig. 1b, Table 1); as well as those immunized with S19-OMP alone ($P < 0.05$) (Fig. 1b, Table 2). In addition, the mean levels in mice immunized with S19-OMP differed statistically ($P < 0.05$) from those given the S19 live vaccine (Fig. 1b).

Similarly, the mean IgG2b response in mice immunized with S19-OMP-liposome, S19-OMP alone, and the S19 live vaccine were significantly different ($P < 0.01$) from those inoculated with liposome controls at DPI and DPC stages (Fig. 1c). The salient response was significantly heightened IgG2b levels ($P < 0.01$) in mice immunized with S19-OMP-liposome and S19-OMP compared to those immunized with S19 live vaccine

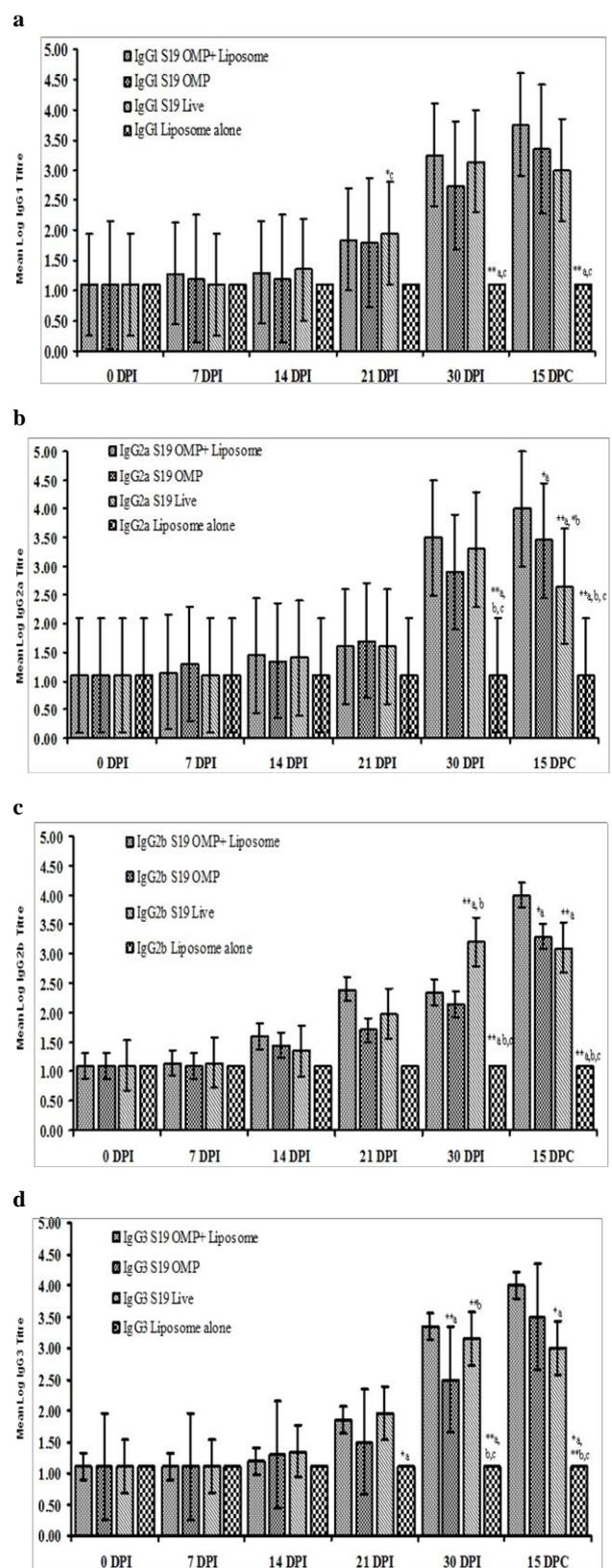


Fig. 1: Mean log 2 isotype antibody titer of mice immunized with *B. abortus* S19 Liposomed – OMP (a), OMP alone (b) and S19 live vaccinated mice (c) with liposome alone (d) as negative control on DPI 0, 7, 14, 21, and 30 and DPC 15. Figs. 1a-d: Response of IgG1, IgG2a, IgG2b and IgG3 differ significantly between groups, days and between days and groups ($P < 0.01$)

($P < 0.01$) at DPI 30. Most significantly, the mean IgG2b levels of mice immunized with the S19-OMP-liposome and those with the live *B. abortus* S19 vaccine were statistically different at 15 DPC ($P < 0.01$) (Table 1).

IgG3 levels were significantly elevated ($P < 0.01$) in mice immunized with the S19-OMP-liposome, S19-OMP, and the S19 vaccine compared to liposome inoculated control mice at the DPI 30 and DPC 15 stages (Fig. 1d). Also, at 30 DPI, the mean IgG3 levels in mice inoculated with S19-OMP-liposome were statistically different ($P < 0.01$) from those vaccinated with S19-OMP alone (Fig. 1d). Similarly, the IgG3 levels in mice immunized with S19-OMP alone were different from those that received the live S19 vaccine ($P < 0.01$) at DPI 30 (Fig. 1d). Importantly, the mean IgG3 titers of mice immunized with S19-OMP-liposome were significantly elevated ($P < 0.05$) compared to those inoculated with the live S19 vaccine at the DPC stage (Fig. 1d).

Protection efficacy and safety

The level of protection, i.e. protection index, expressed as mean log₁₀ CFU, was estimated by immunizing mice with S19-OMP-liposome, S19-OMP and S19 live vaccines, respectively, and challenged with virulent *B. abortus* 544 culture on day 30 post-immunization. The most striking feature was the significantly enhanced level of protection by an order of magnitude of 0.84 logs (protective index = 2.36) conferred by the immunization of mice with S19-OMP-liposome compared to those immunized with live S19 vaccine (protective index = 1.52, $P < 0.05$) (Table 3). The efficacy of the protection enhancement (greater by 1.32 log) caused by the delivery of the antigen S19-OMP entrapped in cationic liposome formulation was observed by comparing the difference in the level of protection achieved by immunization with S19-OMP alone ($P < 0.05$) (Table 3). However, the level of protection

Table 1: Suggested immune response mechanism that empowered mice immunized with liposome entrapped S19-OMP protective advantage over the group immunized with *B. abortus* live S19 vaccine

Vaccine formulation/antibody isotype immune response	DPI stages	15 DPC stage
Antibody isotype		
IgG1		
S19-OMP entrapped in liposome	NS	NS
Live <i>B. abortus</i> S19	NS	NS
IgG2a		
S19-OMP entrapped in liposome	NS	**
Live <i>B. abortus</i> S19	NS	NS
IgG2b		
S19-OMP entrapped in liposome	(30 DPI)**	**
Live <i>B. abortus</i> S19	NS	NS
IgG3		
S19-OMP entrapped in liposome	NS	*
Live <i>B. abortus</i> S19	NS	NS

* Significant $P < 0.05$, and ** Significant $P < 0.01$ by Tukey's HSD. Protective advantage to mice conferred by immunization with S19-OMP entrapped in liposome vaccine compared to live *B. abortus* S19 version could probably be due to significantly different response of IgG2b at DPI 30 and that of IgG2a, IgG2b and IgG3 at DPC stages. NS: Statistically not significant

Table 2: Suggested immune response mechanism that empowered mice immunized with liposome entrapped S19-OMP protective advantage over the group immunized with S19-OMP alone

Vaccine formulation/isotype antibody response	DPI stages	15 DPC stage
Antibody isotype response		
IgG1		
S19-OMP entrapped in liposome	NS	NS
S19-OMP alone	NS	NS
IgG2a		
S19-OMP entrapped in liposome	NS	*
S19-OMP alone	NS	NS
IgG2b		
S19-OMP entrapped in liposome	NS	*
S19-OMP alone	NS	NS
IgG3		
S19-OMP entrapped in liposome	(30 DPI)**	NS
S19-OMP alone	NS	NS

* Significant $P < 0.05$, and ** Significant $P < 0.01$ Tukey's HSD. Protective advantage to mice conferred by immunization with S19-OMP entrapped in liposome vaccine compared to S19-OMP alone could probably be due to the significantly different response of IgG3 at 30 DPI and IgG2a and IgG2b at the DPC stages. NS: Statistically not significant

Table 3: Protective indices of *Brucella abortus* S19 OMP with liposome (a), S19 OMP alone (b), and S19 live attenuated vaccine (c) with liposome alone (d) as control in mice

Vaccine group (n=6 in each group)	Recovery of challenge strain <i>B. abortus</i> 544 (Log 10 CFU)	Recovery of vaccine strain <i>B. abortus</i> (Log 10 CFU)	Protection indices (Log 10)	Mean spleen weight (g) at 15 DPC
S19-OMP-liposome	3.55	-	2.36 ^{a,b,c}	0.165 ^{a,c,d}
S19-OMP	4.87	-	1.04 ^{a,c}	0.145 ^{a,c,d}
Live <i>B. abortus</i> S19	4.39	3.23	1.52 ^{a,b}	0.255 ^{a,b}
Liposome inoculated control	5.91	-	-	0.312 ^{a,b}

* Significant P<0.05, Tukey's Honesty significant difference

(1.04 log) offered to mice by immunization with the S19-OMP formulation was significantly lower (lesser by 0.48 log) compared to the group immunized with the live S19 vaccine (1.52 log) (P<0.05) (Table 3).

Reduction in splenomegaly, as measured by the decrease in mean spleen weight in mice groups vaccinated with live S19 strain did not differ statistically from the control group inoculated with liposome alone (Table 3). In contrast, the reduction in mean spleen weight in the group immunized with S19-OMP-liposome and S19-OMP (P<0.05) was significantly different compared to the liposome inoculated controls (Table 3). Interestingly, the S19-OMP-liposome formulation was found safer than the S19 live vaccine since the mean spleen weight of mice immunized with S19-OMP-liposome was significantly lower compared to those immunized with the live S19 vaccine at 15 DPC (P<0.05) (Table 3).

Discussion

Citing Allison and Gregoriadis (1974), Shek and Sabiston (1981) explored the role of liposome associated protein antigens, and pointed to the initial finding regarding the immunization of animals with diphtheria toxoid entrapped in liposome which results in enhanced antibody production. With particular reference to *Brucella*, it has been shown that the administration of Hernandez-Caselles *et al.* (1989) encapsulated in cationic liposomes eliminates *B. melitensis* from the liver and spleen of infected mice. However, the anionic formulation cleared the infection from the spleen less effectively (Hernández-Caselles *et al.*, 1989). The enhanced efficacy of the potentiation of humoral immune responses to lipopolysaccharide and o-polysaccharide antigens of *B. abortus* in mice using liposome as a vaccine carrier has also been demonstrated by Wong *et al.* (1992). Later, it was shown by Vitas *et al.* (1996) that liposomal gentamicin had protective effects against systemic acute experimental murine brucellosis due to *B. abortus*. The role of recombinant outer membrane proteins (rOMP) such as rOMP25, rOMP28 and rL7/L12 as sub-unit vaccine candidates has been also studied, and it has been concluded that rOMP candidates are efficacious in mouse models, if immunization and challenge experiments are employed (Mallick *et al.*, 2007; Kaushik *et al.*, 2010; Goel and Bhatnagar, 2012; Lim *et al.*, 2012).

Liposome encapsulated rL7/L12 protein (Mallick *et*

al., 2007) and rOMP25 (Goel *et al.*, 2013) vaccine candidates have been recently tested in BALB/c mouse models. In both studies the candidates were shown to be able to elicit enhanced humoral and cell mediated immune responses compared to the live S19 vaccine and control groups. In addition, when BALB/c mice were primed by sub-cutaneous immunization with a 50 µg dose of rL7/L12 entrapped in liposomes that employed egg-phosphatidylcholine and cholesterol in a 2:1 ratio and boosted by the same route with a 30 µg dose on days 21 and 28 after priming, they induced better systemic clearance of the virulent challenge strain *B. abortus* 544 compared to the live *B. abortus* S19 vaccinated controls at DPC 15 (Mallick *et al.*, 2007). However, BALB/c mice were able to clear the organism, *B. abortus* 544 challenge strain, from their spleen only when they were primed and boosted with the subcutaneous administration of 50 µg of rOMP25 entrapped in phosphatidyl-ethanolamine (PE) and phosphatidylcholine (PC) (in 8:2 ratio) 15 days after priming compared to groups of mice immunized with the live S19 vaccine (Goel *et al.*, 2013). The protective efficacies resulting from the immunization of BALB/c mice with 50 µg rOMP25 in PE-PC liposome without a booster were not statistically different from the S19 vaccinated group (Goel *et al.*, 2013). In contrast to the reports cited above, we used a commercial cationic liposome, wherein the DODAP and DOPE mixture was supplied in a 50:50 (mol/mol) ratio to entrap purified total OMP extracted from *B. abortus* S19. Immunization of BALB/c mice with this formulation in a 50 µg subcutaneous dose regimen without a booster was able to confer significantly higher protection (log 0.84) compared to the mice immunized with the live S19 vaccine at DPC 15. The present finding indicates our formulation was much more efficacious compared to the previous report (Goel *et al.*, 2013). Also, by adopting a single prime immunization strategy in the current study, the comparative efficacy of protection in the S19-OMP-liposome immunized BALB/c mice with respect to the S19-OMP show far better results at DPC 15 (log 2.36 versus log 1.04) compared to a previous study by Mallick *et al.* (2007). the comparative protection indices of mice immunized with liposome encapsulated rL7/L12 protein and rL7/L12 protein in IFA were shown as log 1.61 and log 0.066 units at DPC 30, respectively.

In the studies cited above (Mallick *et al.*, 2007; Goel *et al.*, 2013) liposome formulations demonstrated a predominant IgG2a antibody isotype immune response,

nevertheless, cell mediated immunity indicated that the nature of the response could be influenced by the nature of the formulation. For instance, while immunization with rOMP25 in the PE-PC formulation induced both Th1 and Th2 cellular responses, the rL7/L12 in the PC-cholesterol formulation induced a Th1 response, despite the fact that most egg-based PC-cholesterol formulations are usually known to stimulate Th2 cells. It appears, therefore, that liposome formulations are distinctly unique in their ability to elicit a very specific and distinct immune response. The uniqueness of a particular liposome formulation in the elicitation of a specific response was observed in our study. Since single immunization with a 50 µg subcutaneous dose with S19-OMP entrapped in DODAP/DOPE liposomes at the DPI 30 and the DPC 15 stages was able to induce significantly elevated IgG2b responses compared to IgG1 responses, as well as significantly higher IgG2a and IgG3 response at the DPC 15, a bias is indicated towards Th1 based cellular immunity. All formulations employed in this study have shown significantly enhanced levels in the mean antibody titers of all isotypes (IgG1, IgG2a, IgG2b and IgG3) compared to the group immunized with liposome alone at the DPI and DPC stages indicating the involvement of Th1 and Th2 cells in the immune response. During the post-challenge period, the IgG1 response did not differ significantly, nevertheless, the IgG2a response was significantly elevated along with IgG2b and IgG3 in the S19-OMP-liposome immunized group in contrast to mice immunized with the live S19 vaccine, indicating the role of Th1 cells in conferring better protection. Earlier studies that used *Brucella* antigens such as rOMP28 as an immunizing antigen have shown strong support for the switching Th1/Th2 mechanism of *Brucella* immunity in mice as evidenced by a 20 fold increase in IgG2a compared to IgG1. This indicates a predominant role of the Th1 directed cellular immunity at the post-challenge stage (Lim *et al.*, 2012). It has been shown earlier that mice infected with *B. abortus* S19 and 2308 strains induce intense, protracted polyclonal IgG2a and IgG3 responses compared to IgG1 and IgM isotypes between weeks 4-8 post-infection. Furthermore, as suggested earlier, the polyclonal IgG2a and IgG3 response is mediated via IFN- γ by *B. abortus* primarily in a T cell independent fashion during the first week of infection, and from the T cells thereafter (Elzer *et al.*, 1994). Current studies on the S19-OMP-liposome in mice exhibit an immune response mechanism similar to that suggested above.

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Conflict of interest

The authors do not have any conflict of interest.

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