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Research paper

# Attenuated *Salmonella* secreting *Brucella* protective antigens confer dual-faceted protection against brucellosis and salmonellosis in a mouse model

Amal Senevirathne, Chamith Hewawaduge, John Hwa Lee*

*College of Veterinary Medicine, Chonbuk National University, Iksan Campus, 54596, Iksan, Republic of Korea*

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ABSTRACT

We demonstrated the use of attenuated *Salmonella* strains secreting *Brucella* antigens SodC, Omp19, BLS, and PrpA as live vaccine candidates against *Brucella abortus* infection and presented their cross-protection against *Salmonella* infections using a BALB/c mice model. Here, a single immunization with each individual strain was capable of establishing significantly high ($p < 0.05$) *Brucella*-specific systemic immunoglobulin (Ig)G and secretory IgA (sIgA) responses compared to control mice. Upon stimulation of the splenocytes harvested from immunized mice with the respective antigens SodC, Omp19, BLS, and PrpA, significant increases in splenocyte proliferative responses against all four antigens versus PBS and vector controls were observed ($p < 0.05$). Additionally, interferon-γ and interleukin-4 secretion clearly demonstrated an uplift of these cytokines in all four strains upon immunization compared to the control groups. However, a significantly high response was noted in the mice groups immunized with *Salmonella* secreting SodC and Omp19 only. Upon virulent *Brucella abortus* 544 challenge, all four antigens presented a significantly high protection index (PI) in the spleen, as follows: 0.85 for SodC; 0.96 for Omp19; 0.6 for BLS; and 0.66 for PrpA. In contrast, in the liver, the same antigens resulted in PI values of 1.37, 1.14, 1.12, and 1.81, respectively. Immunological profiling of immunized mice against *Salmonella*-specific immune responses also showed significant elicitation of both humoral and cell-mediated immune responses as measured by IgG, sIgA, splenocyte proliferation, and cytokine induction. In addition, full protection against virulent *Salmonella* challenge was shown with no mortality in immunized mice, whereas 100% (8/8) mortality was observed in control mice over a two-week post-*Salmonella* challenge. In conclusion, we show that the live attenuated *Salmonella* delivering *Brucella* protective antigens may efficiently confer dual protection against both brucellosis and salmonellosis in immunized mice.

## 1. Introduction

Brucellosis is a disease caused by a group of intracellular Gram-negative bacteria of the genus *Brucella*. It has previously been recognized as a class B bioterror agent by the United States Centers for Disease Control and Prevention (Dabral et al., 2014). It infects both humans and animals by way of ingestion; inhalation; or contact exposure, where already-infected organisms can act as a reservoir of infection (Yang et al., 2013). The disease rarely results in death in humans; however, its outcomes remain severely debilitating. It also poses a significant economic loss in domesticated animals due to the loss of progeny, a reduction in milk yield, and infertility (Xavier et al., 2009). The lack of a safe vaccine for both animal and human brucellosis remains a significant hurdle to overcome. As a result, many scientific research investigations have been launched in search of alternative vaccine strategies for the conventional live forms of *Brucella* vaccines such as *Brucella abortus* S19 and *Brucella melitensis* Rev-1, which preserve considerable residual virulence (Yang et al., 2013). Recent studies have also demonstrated the ability of attenuated *Salmonella* strains to deliver various heterologous antigens in order to induce protective immune responses against various infections (Kim et al., 2018; Lalsiamthara and Lee, 2017; Osorio et al., 2009). Considering the *Salmonella* pathogenesis, an anti-*Brucella* vaccine could be developed using recombinant *Salmonella* strains, which express and secrete *Brucella* protective antigens in order to deliver protection against *Brucella abortus* infection. As an intracellular pathogen, *Salmonella* can be employed to deliver protective *Brucella* antigens directly into macrophages for efficient antigen presentation (Gog et al., 2012). Additional advantages such as an intrinsic adjuvant effect (Chaudhari and Lee, 2013), sufficiency of a single inoculation for a lasting immunity (Lalsiamthara and Lee, 2017), ability to multiply and present an amplitude of antigens, and the active penetration of natural barriers that protect subunit

* Corresponding author.
*E-mail address:* johnhlee@jbnu.ac.kr (J.H. Lee).

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antigens from degradation by host digestive enzymes also can be expected in conjunction with *Salmonella* as an antigen delivery platform.

Furthermore, a *Salmonella*-vectored *Brucella* subunit vaccine may provide protection against salmonellosis in immunized animals. Some investigations have reported that multiple infections along with salmonellosis can result in increased disease outcomes in infected animals (Borewicz et al., 2015; El-Seedy et al., 2016; Sharma, 2014). The existence of such an enhanced pathogenesis may be due to an immunomodulatory effect of *Salmonella*, which suppresses or delays host immune responses (Ibarra and Steele-Mortimer, 2009), facilitating the systemic infection of multiple species. Under acute conditions, salmonellosis can also lead to abortions and deaths (Coburn et al., 2007), causing a productivity loss in animal industries. Therefore, a vaccine candidate able to be effective against both *Brucella* and *Salmonella* infections among farm animals could be particularly advantageous for managing these productivity-related diseases via a single immunization effort, thus saving both time and resources.

The attenuated *Salmonella* Typhimurium strain used in the current study, JOL912, is a genetically engineered *ΔlonΔcpxRΔasd* triple mutant with a smooth phenotype (Hur and Lee, 2011). JOL912 was transformed with an "*asd*"-complemented plasmid vector designated as pJHL65 harboring *Brucella*-protective immunogens (Supplementary Fig. 1), Cu–Zn superoxide dismutase (SodC) (Muñoz-Montesino et al., 2004), outer membrane protein 19 (Omp19) (Pasquevich et al., 2011), lumazine synthase (BLS) (Zhao et al., 2009), and proline racemase subunit A (PrpA) (Lalsiamthara and Lee, 2017). Each individual strain was designed to constitutively secrete these antigens for the gradual development of a degree of protective immunity against *Brucella*. In the present study, we immunized BALB/c mice with each of the attenuated *Salmonella* strains, delivering the *Brucella* antigens SodC, Omp19, BLS and PrpA, and investigated the development of cellular and humoral immune responses against each of the *Brucella* antigens. We also investigated *Salmonella*-specific immunity and revealed that the immunization of mice with the *Salmonella* strains delivering *Brucella* antigens can effectively confer simultaneous protection against brucellosis and salmonellosis in immunized mice.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in the supplementary Table 1. Attenuated *Salmonella* and *Escherichia coli* strains were routinely grown at 37 °C in Luria-Bertani (LB) broth with or without appropriate antibiotics, unless otherwise indicated. The *Brucella abortus* 544 challenge strain was routinely grown in *Brucella* medium (BD, Sparks, MD, USA) in an aerobic or 5% $CO_2$ atmosphere.

### 2.2. Construction of attenuated Salmonella mutant delivering Brucella antigens

Construction of *asd* complemented pJHL65 plasmid constitutively expressing *Brucella* immunogens were described in previous report (Lalsiamthara and Lee, 2017). Briefly, *Brucella* genes were cloned in-frame, downstream to the beta-lactamase secretory signal sequence (*bla* ss) in order to externalize respective antigens out of the bacterium. The resultant plasmids were denoted as pJHL65::*sodC*, pJHL65::*omp19*, pJHL65::*bls* and pJHL65::*prpA*. The recombinant plasmids were then transformed into an *asd* mutant auxotrophic *Salmonella* mutant strain JOL912. Resultant strains were designated as JOL1874, JOL1875, JOL1876 and JOL1877, respectively. The presence of respective gene in JOL912 was confirmed by colony PCR. Secretion of *Brucella* antigens by the attenuated *Salmonella* strain were confirmed by Western blot analysis utilizing polyclonal antibodies produced in rabbits against the each individual *Brucella* antigen (Supplementary Fig. 2).

Each open reading frame, *sodC*, *omp19*, *bls* and *prpA* were cloned into pET28a + vector (Novagen, Madison, WI, USA). Recombinant plasmids were then transformed into *E. coli* BL21 DE strains (Novagen, Madison Wim USA) for overexpression and purification (Hajam and Lee, 2017). The expressed proteins were purified using Ni-NTA (Qiagen, Valencia, CA, USA) affinity column chromatography (Poly-Prep, Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. Purified proteins were quantified by a Bradford assay (Bradford, 1976), filtered, and stored at −20 °C until further use.

### 2.3. Immunization and challenge

All animal experiments involving the use of specific pathogens free BALB/c mice were approved by the Chonbuk National University Animal Ethics Committee (CBNU2015-00085) in accordance with the guidelines of the Korean Council on Animal Care and Korean Animal Protection Law, 2007; Article 13. Five weeks old female BALB/c mice (N = 60) were randomly divided into 6 groups (n = 10) and once immunized either with PBS, JOL1874, JOL1875, JOL1876, JOL1877 and JOL912 *Salmonella* vector only via the intraperitoneal route. Each vaccine strain was prepared by diluting exponentially growing *Salmonella* strain at $1x10^7$ CFU/ml in 100 µl of PBS. The *Salmonella* Typhimurium vector control was administered at $1x10^7$ CFU/ml in PBS. Pre-immunized serum and intestinal wash samples were collected two days before the immunization. Thereafter, serum and intestinal wash samples were collected every two weeks according to previously described method (Lalsiamthara and Lee, 2017). After two weeks of immunization, 5 mice from each group were euthanized and splenocytes were aseptically collected for cytokine and splenocyte proliferation assays. At four weeks of immunization, remaining mice were challenged with $2x10^5$ CFU/mice using a virulent *Brucella abortus* strain 544. After two weeks, challenged mice were sacrificed and *Brucella* load in spleen and liver were enumerated as per the previously described procedure (Lalsiamthara and Lee, 2017) using whole spleens and livers of immunized mice. The protection index resulted in each organ was also calculated for each group as previously described (Lalsiamthara and Lee, 2017). In order to investigate the protection against *Salmonella* infection, two separate mice groups (n = 8) were either immunized with PBS or *Salmonella* strain delivering SodC antigen at $1x10^7$ CFU/ml in 100 µl. Thirty days after the immunization, mice were intraperitoneally challenged with a virulent *Salmonella* strains JOL401 at $1x10^6$ CFU/mice concentration. Immunological profiling was done simultaneously with *Brucella* immunization experiment. The virulence-related mortality was recorded daily basis. Dead mice were collected and the *Salmonella* load in spleen was determined. Two weeks after the challenge, remaining mice were sacrificed, splenic *Salmonella* load was determined in whole spleen samples according to the same aforementioned procedure and cultured on brilliant green agar (BGA) plates (BD, Sparks, MD, USA) for enumeration.

### 2.4. Antigen-specific IgG and secretory IgA responses

Indirect ELISA was used to assess SodC, Omp19, BLS and PrpA specific IgG and secretory IgA (sIgA) responses in the sera and intestinal wash samples according to a previously described method (Won et al., 2017). *Salmonella*-specific IgG and sIgA responses were also measured in immunized mice. Recombinant *Brucella* protein antigens and *Salmonella* outer membrane proteins were used as the coating antigen at 300 ng/well and 250 ng/well, respectively for the determination of *Brucella* and *Salmonella*-specific antibody responses. To assess the antibody response in PBS control mice, purified SodC was used for coating at 300 ng/well concentration.

### 2.5. Antigen-specific splenocyte proliferation

Antigen-specific cell-mediated immune responses (CMI) were evaluated in splenocyte proliferation assay. Two weeks post immunization,

splenocytes were harvested from immunized mice groups, using 5 mice per each group. Cells were adjusted to $1x10^5$ cells/well in 96 well plates and stimulated with specific *Brucella* immunizing antigens or with *Salmonella* outer membrane proteins 10 μg/ml. The PBS, vector controls vaccinated mice splenocytes were stimulated with SodC purified protein. Cell proliferative responses were measured by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] based assay as described previously (Denizot and Lang, 1986).

### 2.6. Cytokine responses

Splenocytes from immunized mice were aseptically harvested using 5 mice per each group. Splenocytes $1x10^6$ cells/ml was stimulated with 200 ng of respective *Brucella* antigen or *Salmonella* outer membrane proteins for 24 h. Total RNA was isolated by RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Complementary DNA was prepared from 1 μg of RNA using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to manufacturer's instructions and stored at −20 °C till use. Real-time PCR assay (qRT-PCR) for gene expression studies was performed with the ABI applied biosystems using Power SYBR Green PCR Master Mix (#4367659, Applied Biosystems, USA) as described previously (Hajam and Lee, 2017). The relative amounts of IFN-γ and IL-4 cytokine mRNA present (normalized with GAPDH) was determined by 2-ΔΔCT method (Pfaffl, 2001) and expressed as log10 fold induction.

### 2.7. Statistical analysis

The statistical significance between immunized and non-immunized test subjects was analyzed using GraphPad Prism 6.00 program (San Diego, CA, USA.) One way analysis of variance (ANOVA) with Tukey`s multiple comparison test was conducted to determine the statistical difference among test group where the *P* value $< 0.05$ were considered significant.

## 3. Results

### 3.1. JOL912-derived strains expressed Brucella antigens

The construction of attenuated *Salmonella* mutant strains expressing and secreting *Brucella* antigens has been previously described (Lalsiamthara and Lee, 2017). Insertion of each gene in the host plasmid vector pJHL65 was confirmed by colony PCR using specific primer sets. The vector pJHL65 provides the *bla* signal sequence upstream of each protein antigen to facilitate efficient protein secretion. The secretion efficacy of each protein antigen was confirmed by Western blot analysis (Supplementary Fig. 2).

### 3.2. Brucella antigen–specific systemic IgG and IgA antibody responses

To investigate the contribution of each antigen component in the elicitation of *Brucella* specific humoral immunity, the serum IgG (Fig. 1A) and intestinal sIgA (Fig. 1B) antibody responses were evaluated in immunized mice. Single intraperitoneal immunizations with JOL1874 (pJHL65::*sodC*), JOL1875 (pJHL65::*omp19*), JOL1876 (pJHL65::*bls*), and JOL1877 (pJHL65::*prpA*), respectively, were conducted. The antibody responses were then compared against the test controls, PBS, and vector-only strain. All immunized groups with individual attenuated *Salmonella* strains showed a significant ($p < 0.05$) increase in IgG and sIgA antibody levels compared to the control group by the fourth week postimmunization. Of particular significance, the IgG antibody response prompted by JOL1874 (pJHL65::*sodC*), JOL1875 (pJHL65::*omp19*), and JOL1876 (pJHL65::*bls*) at two weeks postimmunization was significantly higher than the response in the PBS control group. Specifically, at two weeks postimmunization, the highest IgG response was elicited by JOL1875 (pJHL65::*omp19*) among all four

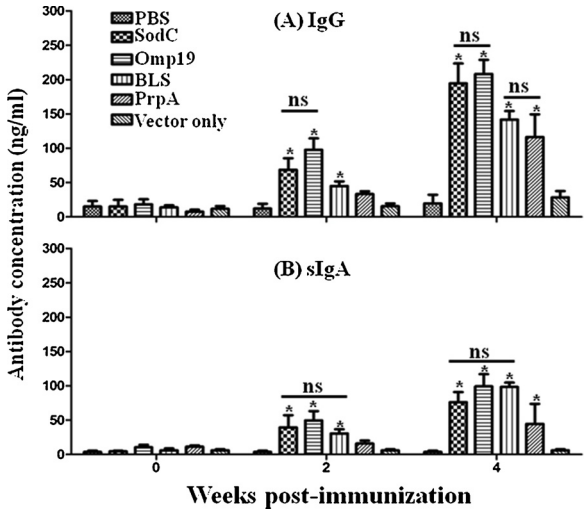


**Fig. 1.** Systemic IgG and mucosal IgA antibody responses in mice immunized intraperitoneally with PBS, *Salmonella* delivering *Brucella* antigens SodC, Omp19, BLS, PrpA and vector only strains. (A) Serum IgG antibody responses (B) Intestinal IgA antibody responses. Both IgG and IgA responses were measured in indirect ELISA using purified *Brucella* antigen proteins. Each data points represent mean ± standard deviation (SD) of five mice per group. *p < 0.05. ns; non-significant.

attenuated *Salmonella* strains. The sIgA response derived by all *Salmonella* strains also showed a significantly high increase ($p < 0.05$) at four weeks postimmunization versus that in the PBS and vector controls. Except for the immunization with JOL1877 (pJHL65::*prpA*), all strains resulted in a significantly high sIgA response as early as two weeks postimmunization. Successful elicitation of both IgG and sIgA responses suggest that *Salmonella* delivery vectors have effectively delivered heterologous antigens into the immunized mice.

### 3.3. Splenocyte proliferative response

To investigate the CMI responses in immunized mice, an MTT-based splenocyte proliferation test was conducted. Splenocytes were harvested from each mouse group (n = 5) at 14 days postimmunization and stimulated with the respective purified antigens—that is, the same that were delivered by the attenuated *Salmonella* strains. The PBS and vector controls were stimulated with SodC purified protein. To assess the *Salmonella*-specific CMI responses, splenocytes were stimulated with purified *Salmonella* outer membrane proteins. All immunized mice with *Salmonella* vaccine strains showed a significantly ($p < 0.05$) high splenocyte proliferative response in comparison with the PBS and vector controls (Fig. 2). In addition, co-establishment of a significantly high *Salmonella*-specific CMI response was also evident in immunized mice.

### 3.4. Cytokine responses

The cytokine response of immunization was evaluated in post-immunized splenocytes harvested from each mouse group. Splenocytes from *Salmonella* vaccine strains were stimulated with the respective antigens delivered by each strain using purified proteins, while the PBS and vector control groups were stimulated with purified sodC proteins. Both Th1 (IFN- γ) (Fig. 3A) and Th2 (IL-4) (Fig. 3B)–type cytokine responses were evaluated by qRT-PCR assay at the transcription level. A significantly high *Brucella*-specific IFN-γ response was observed only with the JOL1874 (pJHL65::*sodC*) and JOL1875 (pJHL65::*omp19*) strains versus the control groups. The *Brucella*-specific IL-4 induction was noted to be significantly high in all four *Salmonella* strains delivering *Brucella* antigens upon stimulation.

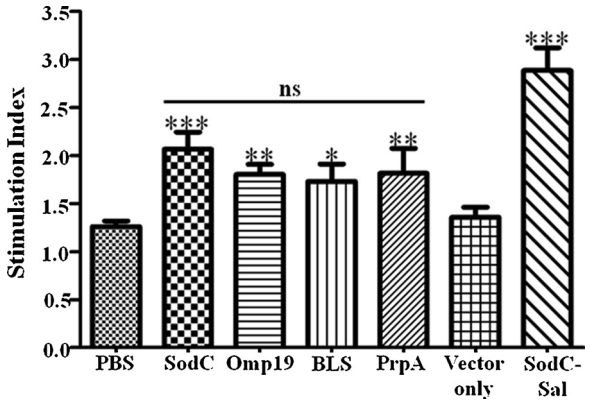


**Fig. 2.** Splenocyte proliferative response in mice vaccinated intraperitoneally with PBS, *Salmonella* delivering *Brucella* antigens SodC, Omp19, BLS, PrpA and vector only strain. Splenocytes ($1 \times 10^5$/ml) isolated from immunized mice were stimulated with their respective antigens or *Salmonella* specific protein (10 μg/ml) for 72 h and proliferative responses were assayed in MTT based assay. Each data point indicates mean stimulation index ± standard deviation (SD) of five mice per group. *$p < 0.01$. **$p < 0.001$, ***$p < 0.0001$. ns; non-significant. SodC-Sal; mice immunized with *Salmonella* delivering SodC and stimulated with *Salmonella* outer membrane proteins.

### 3.5. Protective efficacy against Brucella abortus infection

To determine the protective efficacy of *Salmonella*-delivered *Brucella* antigens, immunized and control mice were intraperitoneally challenged with the *Brucella abortus* strain 544 variant at four weeks after immunization. Two weeks after the challenge, the mice were euthanized and the bacterial load was determined in whole spleen and liver samples. Ultimately, all four attenuated *Salmonella* strains presented significantly ($p < 0.05$) low numbers of challenged *Brucella* in both the spleen (Fig. 4A) and liver (Fig. 4B) samples as compared with the PBS and vector control groups. The highest PI in the spleen was seen in mice immunized with SodC and Omp19, with PI values of 0.85 ± 0.08 and 0.96 ± 0.23, respectively, whereas in the liver, PrpA obtained the highest PI (1.81 ± 0.31) among all of the mouse groups (Supplementary Table 2).

### 3.6. Immune responses and protective efficacy against Salmonella infection

To investigate the protection against *Salmonella* infection derived by immunization with attenuated *Salmonella* strains, a separate mouse group was immunized with JOL1874. The development of anti-*Salmonella* IgG (Fig. 5A) and sIgA (Fig. 5B) antibody responses was then assessed in serum and intestinal wash samples. Of note, both IgG and sIgA levels were significantly ($p < 0.05$) higher than in the PBS control group at two weeks postimmunization. Nearly a two-fold increase in antibody titers was observed at four weeks after immunization. In order to assess the protective efficacy against *Salmonella* infection, eight mice from each immunized and nonimmunized group were intraperitoneally challenged with a wild-type virulent *Salmonella* Typhimurium strain, JOL401. The protection efficacy in immunized mice was 100% with zero mortality, whereas the control PBS group experienced 100% mortality (8/8) (Fig. 5C) over a two-week period post-challenge. Further, examination of splenic *Salmonella* recovery revealed a complete clearance from *Salmonella* infection after two weeks of post-challenge in immunized mice (Fig. 5D).

## 4. Discussion

The scope of the present study was to investigate the contribution of each individual attenuated *Salmonella* strain in delivering *Brucella* antigens in the development of immunity against *Brucella* while co-establishing *Salmonella*-specific protection in BALB/c mice model. We have demonstrated here the existence of a significant protection against both the *Brucella abortus* and *Salmonella* virulent challenges in mice via lowering systemic pathogen establishment by evaluating challenged bacterial loads in spleen and liver samples of immunized mice. The reality of most farming industries is that animals can experience multiple infections that remain undiagnosed and which can cause huge economic losses. This is true especially in developing countries where the overall level of hygiene can be low. In such a context, multispecies protection provided in a single vaccine may be very practical in disease management. In this study, we evaluated the dual-faceted protection that can be achieved by *Salmonella* vectors secreting the highly conserved *Brucella* protective antigens SodC, Omp19, BLS, and PrpA against brucellosis as well as *Salmonella* vector–derived protection against salmonellosis in mice. Further, for the first time, we have investigated the contribution of *Salmonella*-delivered antigen components in the development of humoral and cell-mediated immunity and the subsequent protection against virulent challenge with each pathogen.

The secretion of each *Brucella* antigen delivered by an attenuated *Salmonella* strain under the *bla* secretory signal was confirmed by Western blot analysis. The expressed protein remained immunologically active, as evidenced by the elicitation of antigen-specific humoral and cellular immune responses. The humoral and CMI responses provoked by each antigen candidate showed a correlation with the subsequent protection resulting against the virulent *Brucella* challenge. All four antigens significantly induced both the IgG and sIgA production and antigen-specific CMI responses in immunized groups as

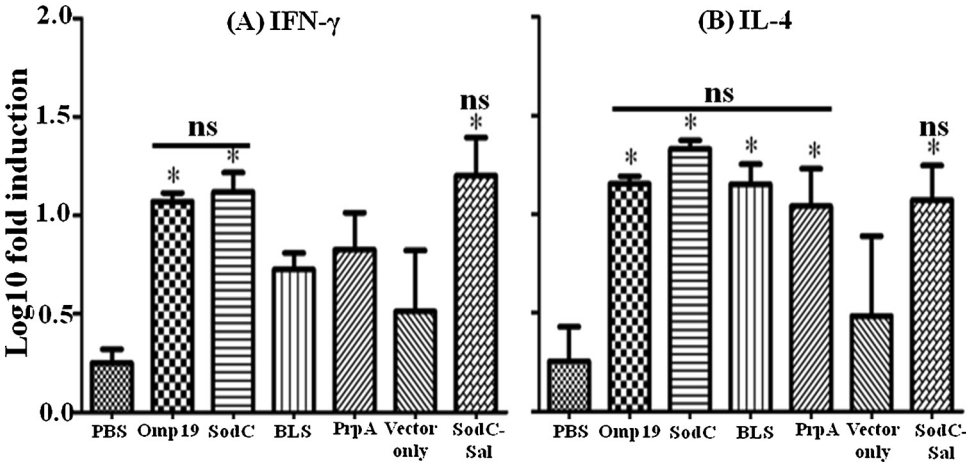


**Fig. 3.** Cytokine responses in mice immunized intraperitoneally with PBS, *Salmonella* delivering *Brucella* antigens SodC, Omp19, BLS, PrpA and vector only strains. Splenocytes ($1 \times 10^6$/ml) isolated from immunized mice were stimulated with their respective antigens or *Salmonella* protein for 24 h and then analyzed for induction of IL-4 and IFN-γ mRNA transcription levels by qRT-PCR assay. Results are expressed as relative fold change in mRNA transcription levels of stimulated splenocytes from immunized mice compared to the *Salmonella* vector control group. Gene expressions were normalized to Beta actin and mRNA levels of naive stimulated cells were used as the calibrator. Data presented are mean ± SD of five mice per group. *$p < 0.05$. ns; non-significant. SodC-Sal; mice immunized with *Salmonella* delivering SodC and stimulated with *Salmonella* outer membrane proteins.

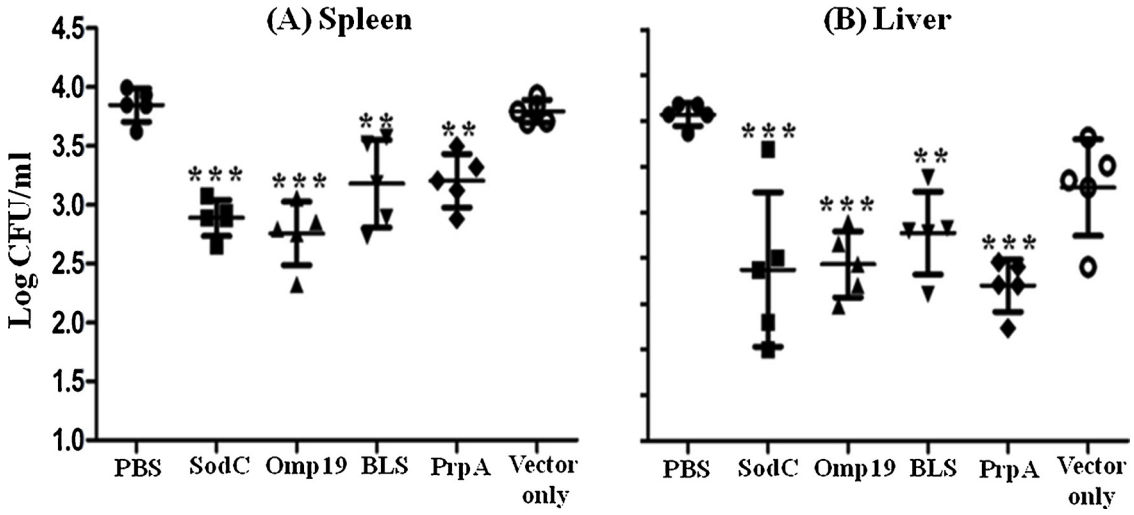


**Fig. 4.** Post challenge *Brucella* recovery in spleens and livers of mice immunized intraperitoneally with PBS, *Salmonella* delivering *Brucella* antigens SodC, Omp19, BLS, PrpA and vector only strains. All mice were challenged with $1 \times 10^5$ CFU/mice with a virulent *B. abortus* strain 544 after 30 days of immunization. Fourteen days after the challenge, mice were euthanized and challenged *Brucella* was recovered from the spleen. The data was expressed as the mean protective index standard deviation (SD) of five mice per group.**$p < 0.001$, ***$p < 0.0001$.

compared with in the PBS and vector control groups. These responses clearly demonstrate the successful engagement of each antigen component in anti-*Brucella* adaptive immunity. Furthermore, the CMI responses indirectly indicated that each antigen component is capable of stimulating T-cell subpopulation, which ultimately modulates whole protective immune responses via the engagement of both CD4 and CD8 T-cell populations (Ko and Splitter, 2003). Particularly CD4-derived IFN-γ–mediated type I immunity is considered an essential component in protective efforts against *Brucella* infection (Yang et al., 2013). Investigation into immune-modulatory cytokine profiles in immunized mice groups has demonstrated efficient engagement of both Th1- and Th2-type immune responses as evidenced by both the expression of IFN-γ and IL-4, respectively. Prominent IL-4 responses were observed in all four attenuated *Salmonella* strains delivering *Brucella*. However, *Brucella*-specific IFN-γ responses were significantly high only in the *Salmonella* strains secreting the SodC and Omp19 antigens. The IFN-γ responses promoted in the BLS- and PrpA-vaccinated groups were high; however, these groups did not present a significant difference as compared with that in the PBS control group. The highest IFN-γ responses were derived by the *Salmonella* strains JOL1874 (pJHL65::*sodC*) and

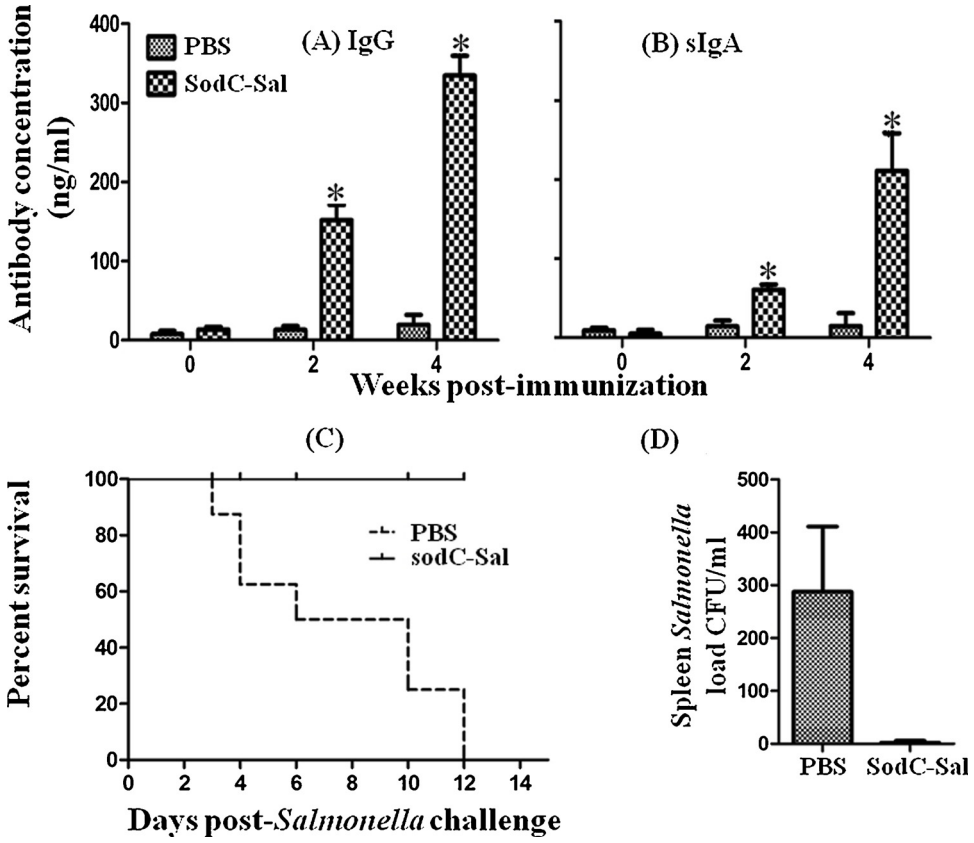


**Fig. 5.** *Salmonella* specific systemic IgG and mucosal IgA antibody responses in mice immunized intraperitoneally with PBS or *Salmonella* delivering *Brucella* antigen SodC and protective efficacy. (A) Serum IgG antibody responses (B) Intestinal sIgA antibody responses. Both IgG and IgA responses were measured in indirect ELISA using purified *Salmonella*-specific protein. Each data points represent mean ± standard deviation (SD) of four mice per group. *$p < 0.05$. ns; non-significant. To determine the protective efficacy, mice immunized with either PBS or *Salmonella* delivering *Brucella* antigen were challenged at 30 days post immunization with a virulent *Salmonella* strain. (C) Survival rate of immunized and PBS control groups. (D) Bacterial load in mice post-challenge. Each data points represent mean ± standard deviation (SD) of eight mice per group.

JOL1875 (pJHL65::*omp19*), followed by JOL1876 (pJHL65::*bls*) and JOL1877 (pJHL65::*prpA*), showing the relative importance of the SodC and Omp19 antigens in the vaccine formulation. However, considering the PI of each vaccine strain in the obtained spleen and liver samples, the findings suggest that the PrpA antigen also plays a crucial role in reducing the bacterial burden in liver tissues. Thus, *Salmonella*-delivered SodC, Omp19, and PrpA can be recognized as a major contributor to vaccine efficacy.

Further, immunogenic profiling of *Salmonella*-specific systemic IgG, intestinal sIgA, and cell-mediated and cytokine responses also suggested the successful establishment of protective immunity against *Salmonella* infection. Being an intracellular pathogen, *Salmonella* too showed the clear engagement of both type I and type II immune responses, as demarcated by IFN-γ and IL-4 cytokine responses. Importantly, this attenuated *Salmonella* strain provided a full degree of protection in the immunized mice groups with zero mortality, resulting in almost complete splenic *Salmonella* clearance at two weeks post-virulent challenge, while the mice treated with PBS experienced 100% mortality within two weeks post-challenge.

In conclusion, we have demonstrated in the present research that an attenuated *Salmonella* strain delivering *Brucella* antigens can elicit *Brucella*- and *Salmonella*-specific humoral and cell-mediated immune responses, offering a two-faceted protection approach against *Brucella abortus* and *Salmonella* infections in a mouse model. Further experiments may be required to investigate the protective efficacy of attenuated *Salmonella* immunization in other farm animals such as cattle and goats, which more typically serve as natural hosts for *Brucella* infection in farming environments.

## Conflict of interest

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

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