

Brucella Phage Lysate Bacterin Induces Elevated TLRs and Cytokines Response in Murine Model

Ankita Jain¹, Mayank Rawat³, Soumendu Chakaravarti², Abhishek¹,
Vinod Chaturvedi², Losa Chesti¹, Ankush K. Niranjani¹,
Rajat Varshney¹ and Bablu Kumar^{2*}

¹Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Bareilly - 243 122, India.

²Division of Biological Products, Indian Veterinary Research Institute, Bareilly - 243 122, India.

³Division of Biological Standardization, Indian Veterinary Research Institute, Bareilly - 243122, India.

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TLRs sense pathogen associated molecular patterns of invading microbes which initiate intracellular signaling cascades and lead to production of pro-inflammatory cytokines which in turn help in bridging innate and adaptive immune response. TLRs and cytokines response of plain and alum adsorbed lysate of *Brucella abortus* strain 19 in mice model were evaluated by relative expression of the transcript by real time PCR. Three different groups of mice immunized with plain lysate bacterin, alum adjuvanted lysate, standard *B. abortus* strain 19 vaccine respectively and an unimmunized control group were used in present study. The results showed higher expression of TLR2, TLR4, TLR9, IL-4, IL-12 and INF- α transcripts in spleen of all the immunized mice groups as compared to unimmunized control groups. The relative higher transcripts expression of TLRs and cytokines in alum adjuvanted lysate as comparison to plain lysate bacterin suggest better immuno-protective response of alum adjuvanted phage lysate bacterin against the brucellosis.

Keywords: Brucella phage lysate, murine, TLRs, Cytokines.

Brucellosis is a major bacterial zoonoses worldwide (Manish *et al.*, 2013) caused by *Brucella* species. *B. abortus* causes spontaneous abortion in cattle, lead to major economical losses in dairy and chronic infection in humans (Moriyón *et al.*, 2004; Pappas *et al.*, 2005). Vaccination plays a central role in bovine brucellosis control programs and has been successfully used worldwide for decades to control the disease (Olsen and Stoffregen, 2005). The development of an

efficacious and safe vaccine for brucellosis has been a challenge for scientists for many years. Despite enormous advances and the development of *B. abortus* S19 (Graves, 1943) and RB51 (Schurig *et al.*, 2002; Stern *et al.*, 1971) vaccines, the search for improved vaccines has never ended. Although the available vaccines are effective in controlling brucellosis, they have numerous drawbacks like abortifacient potential for pregnant animals (Smith and Ficht, 1990), residual virulence ability for human (Spink *et al.*, 1962), the complex manufacturing process etc. Apart from that, when S19 or RB51 preparations are used in animals, the interference of vaccine induced antibody response with

* To whom all correspondence should be addressed.
E-mail: babbacteriol@gmail.com

conventional serological tests (Stevens *et al.*, 1994; Stevens *et al.*, 1995; Schurig *et al.*, 2002) also creates problems during the surveillance programs. In past, inactivated *Brucella* vaccines have also been tried with limited success (Plommet *et al.*, 1970). The major drawback of chemically killed vaccines include high production cost, poor protection, residual virulence, batch to batch variation, denaturation of conformational epitopes, unaccepted local reaction, serological problems (Moriyón *et al.*, 2004).

Continuous efforts by various researchers have been made in past to develop alternate effective and safe vaccine candidate against brucellosis. Till date there is no licensed human vaccine against brucellosis. Several new generation vaccines like recombinant vaccine (Al-Mariri *et al.*, 2001; Oliveira *et al.*, 1996), DNA vaccines (Munoz *et al.*, 2004), genetically modified vaccines, vectored vaccines (Ruecker and Guzmán, 2012) have been tried with varying degree of success but none showed the results equivalent to the Brucella S19 vaccine (Schurig *et al.*, 2002).

The use of lytic phages to inactivate bacteria and the application of the phage lysate as a vaccine antigen is an alternate approach to develop candidate vaccine against bacterial disease but it has been poorly explored. Recently the protective ability of phage lysate bacterins against brucellosis (Jain *et al.*, 2015) and Salmonellosis (Verma *et al.*, 2012) has been evaluated by various workers. The exact mechanism for protective ability of this candidate vaccine has been poorly understood. It is hypothesized that due to specific lysis of bacteria by bacteriophage presents large number of unaltered conformational epitopes which can stimulate both innate and adaptive immune response to elicit robust immune response in the body. Amongst various antigens LPS, lipoprotein, Outer membrane proteins, DNA are the major antigens supposed to be present in phage lysate preparation which are considered as important PAMPs to be recognized by innate immune system of defense.

In the recent years, there has much emphasis on improvement of vaccine utilizing better adjuvant targeting innate immune response to enhance both humoral and cell-mediated immunity. The profound capacity of TLRs mediated

immunity can be exploited to augment the antigen specific immune response for many of the vaccine antigens. Emerging evidences indicate that engagement of multiple TLRs with their respective agonists can result in cross-talk between them. In an attempt to understand mechanism of immune response elicited by phage lysate in present study we are reporting the TLR mediated immune response of plain and alum adjuvanted phage lysate of *B. abortus* strain 19 in mice.

MATERIALS AND METHODS

Mice

Apparently healthy female Swiss albino mice (4 to 6 week age, about 20 gm) were obtained from the Laboratory Animal Research (LAR) section unit, IVRI, Izatnagar. The animals were kept under conventional housing condition and provided feed and water *ad lib*. All animal studies have been conducted as approved by the Ethics Committee of the Indian Veterinary Research Institute, Izatnagar.

Bacterial strain

Culture of *B. abortus* S19 maintained in Brucella Laboratory, Biological product division, Indian Veterinary Research Institute (IVRI), Izatnagar was used in the study. The purity and identity of culture were tested by morphological, cultural and biochemical examination.

Brucellaphage

A phage showing consistent lytic activity against *B. abortus* S19 was isolated in the Department of Veterinary Microbiology, Veterinary College, Guru Angad Dev Veterinary and Animal Science University, Ludhiana, Punjab, India and used for the present studies.

Generation of phage lysate

Conditions for generation of stable phage lysate of Brucella S19 were optimized in our laboratory system as per Jain *et al.*, 2015 with slight modification. For comparative evaluation of TLR response of phage lysate and the standard Brucella S19 vaccine, a pool of equal volume of 3 lysate batches was used as the final test preparation. Safety test was conducted by injecting 0.5 ml of test preparation through intramuscular, subcutaneous, intravenous or intraperitoneal route into 5 mice kept in 4 separate groups. The animals were observed for any untoward reaction or

mortality till 21st day of inoculation.

Characterization of phage lysate

Biochemical composition of each batch of lysate was determined by estimating total protein (Lowry, 1940) and carbohydrate (concentration Dubois *et al.*, 1956) concentration. The phage count was determined by using serial dilution method.

Experimental population and immunization

4-5 week old healthy swiss albino female mice obtained from Laboratory Animal Research (LAR) unit IVRI Izatnagar were randomly distributed in to four experimental groups consisting of 6 animals each. Each mouse of group I and II were injected with plain phage lysate and alum adjuvanated phage lysate respectively in a dose volume of 0.1 ml through subcutaneous route. Mice of group III received Brucella S19 vaccine (1×10^5 cfu/mice: subcutaneously) and kept as positive control. Mice of group IV kept as negative control, received buffered Brucella saline (BSS).

Collection of sample

Two randomly selected mice from each group were sacrificed on 3, 7 and 14th day post immunization (DPI). Spleen tissue was collected aseptically, washed three times with PBS and stored in RNA Later (Sigma Aldrich, USA) at -20°C for RNA isolation.

RNA isolation and cDNA synthesis

Spleen tissues were processed for total RNA isolation using RiboZolTM (Amresco, USA) as per the manufacturer's protocol and the complementary DNA (cDNA) was synthesized from total RNA using RevertaidTM First Strand cDNA Synthesis Kit (Thermo Scientific, USA), following manufacturer's instructions. 1 µg of total RNA was treated with 1 µl of DNaseI (Fermentas USA) prior to reverse transcription to remove any DNA contamination from extracted RNA. The extracted RNA was assessed for quantity and purity using nanodrop Spectrophotometer (nanodrop Ltd, UK) and stored at -80°C until further use. The reverse transcription was performed using M-MuLVReverse Transcriptase (Thermo scientific, USA). Briefly, 1 µg of total RNA was mixed with 1 µl of random hexamer primers (Thermo scientific, USA) in a 0.2 ml PCR tube and incubated at 65°C for 5 min to remove any secondary structure within the RNA. Then the tubes were snap chilled on ice which was followed by addition of 4 µl of 5x RT-buffer, 1.5 µl of 10Mm dNTP mix, 1 µl of reverse

transcriptase enzyme, 1 µl of RiboLock RNase inhibitor in a final 20 µl volume of reaction. The contents were mixed properly and incubated at 42°C for 1h in a thermo cyclor for reverse transcription to occur. Finally the reactions were terminated by heating at 90°C for 2 min to inactivate M-MuLVRT enzyme. The cDNA prepared was stored at -80°C for further use.

Real time quantification

The differential mRNA Expression levels of TLR2, TLR4, TLR9, IL-4, INF- γ and IL-12 genes were analyzed by real time PCR using the Maxima SYBR Green (Thermo Scientific, USA) on ABI 7900 real time PCR system (Applied Biosystem, USA) using published gene specific primers (Table 1). β -Actin was used as the reference gene. Expression levels of the target genes were calculated relative to the expression of the β -Actin gene and expressed as n-fold increase or decrease relative to the control. Real time RT-PCR was conducted in a final volume of 10 µl containing 5 µl SYBR green PCR master mix (Thermo scientific, USA), 1 µl cDNA as the PCR template and 1 µl (5 pmol) forward and reverse primers. Real time RT-PCR was performed using the following cycling parameters: 1 cycle at 95°C for 5 min followed by 40 cycles of 95°C for 15s and annealing at temperature as shown in table 1 for 30s, and a dissociation stage of 95°C for 15s, 60°C for 30s, 95°C for 15s and 60°C for 15s. The change in the gene expression was calculated as $2^{-\Delta\Delta Ct}$ and the value indicated in an n-fold difference relative to the calibrator.

Statistical analysis

The relative expression of target gene (TLR) were evaluated by analysis of variance (ANOVA) followed by Duncan's post hoc test in IBM SPSS statistics 20 software. Result are expressed as Mean \pm SEM. The level of significance was set at $P < 0.05$.

RESULTS

Production and characterization of Lysate: Lysate batches produced through 3 separate cycles did not show any significant variation with respect to protein and polysaccharide contents. Carbohydrate and protein concentration of the pooled lysate were found to be 360 µg and 62 µg/ml respectively.

Safety result: Test preparation did not produce any untoward effect in mice till 21st DPI.

The relative expression of TLRs gene

Expression level of TLR2 gene was significantly ($P < 0.05$) up-regulated in all the immunized group at all the time interval under study as compared to unimmunized control group (Fig. 1). Expression of TLR2 was highest in all the immunized group at 3rd DPI which gradually declined up to day 14th. However in alum adjuvanted Phage lysate group after attaining peak on 3rd DPI, declined on 7th DPI and increased again on 14th DPI. TLR2 expression was invariably higher in mice immunized with Brucella S19 vaccine at almost all day interval. The relative expression of TLR4 transcripts were significantly ($P < 0.05$) up-

regulated in all the immunized group at all the time intervals under study and peaked at 3rd DPI in all the groups and gradually declined thereafter up to 14th DPI as compared to unimmunized mice group (Fig. 2). But alum adjuvanted phage lysate group showed almost equivalent relative expression level of TLR4 as compared to Brucella S19 on 7th DPI and 14th DPI. Similar trends were observed in expression level of TLR9 gene (Fig. 3)

Cytokine expression profile

There was significant ($P < 0.05$) increase in IL-4 expression at 3rd, 7th and 14th DPI (Fig. 4) and peaked on 7th DPI in Brucella S19 and plain lysate group but on 14th DPI in alum adjuvanted group. The expression of IFN- γ (Fig. 5) peaked on 3rd DPI in all the immunized group and declined up to 14th

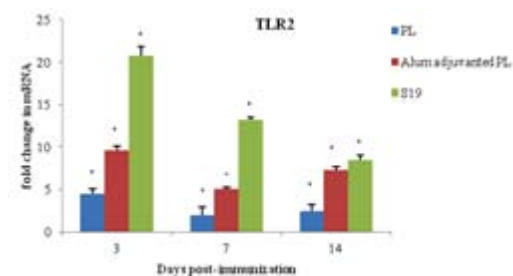


Fig. 1. Relative expression profile of TLR2 in *Brucella* phage lysate (PL) bacterin, alum adjuvanted phage lysate and *Brucella* S19 immunized mice. The data are expressed as mean \pm SDE.

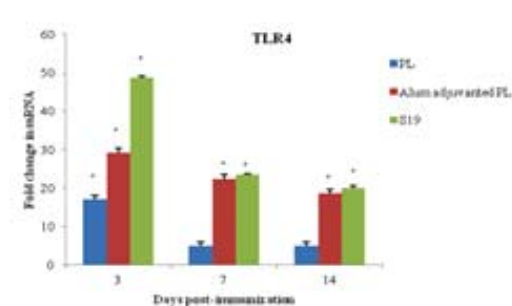


Fig. 2. Relative expression profile of TLR4 in *Brucella* phage lysate (PL) bacterin, alum adjuvanted phage lysate and *Brucella* S19 immunized mice. The data are expressed as mean \pm SDE.

Table 1.

Gene	Primer	Annealing (°C)	Primer sequence	Reference
TLR2	Forward	54	5'-AAGAGGAAGCCCAAGAAAGC-3'	Renshaw <i>et al.</i> , 2002
	Reverse		5'-CGATGGAATCGATGATGTTG-3'	Renshaw <i>et al.</i> , 2002
TLR4	Forward	54	5'-ACCTGGCTGGTTTACACGTC-3'	Renshaw <i>et al.</i> , 2002
	Reverse		5'-CTGCCAGAGACATTGCAGAA-3'	Renshaw <i>et al.</i> , 2002
TLR9	Forward	54	5'-ACTGAGCACCCCTGCTTCTA-3'	Renshaw <i>et al.</i> , 2002
	Reverse		5'-AGATTAGTCAGCGGCAGGAA-3'	Renshaw <i>et al.</i> , 2002
IL12	Forward	54	5'-TTGCTGGTGTCTCCAATCATG-3'	Chan <i>et al.</i> , 2012
	Reverse		5'-GTCACAGGTGAGGTTCACTGTTTC-3'	Chan <i>et al.</i> , 2012
INF γ	Forward	54	5'-GCTTTGCAGCTCTTCCTCATG-3'	Chan <i>et al.</i> , 2012
	Reverse		5'-CTTCCACATCTATGCCACTTGAG-3'	Chan <i>et al.</i> , 2012
IL4	Forward	54	5'-GAGACTCTTTCGGGCTTTTCG-3'	Chan <i>et al.</i> , 2012
	Reverse		5'-CAGGAAGTCTTTCAGTGATGTGG-3'	Chan <i>et al.</i> , 2012
β -actin	Forward	60	5'-CAATAGTGATGACCTGGCCGT-3'	Overbergh <i>et al.</i> 1999
	Reverse		5'-AGAGGGAAATCGTGCGTGAC-3'	Overbergh <i>et al.</i> 1999

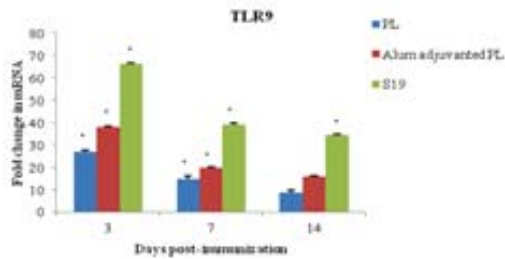


Fig. 3. Relative expression profile of TLR9 in Brucella phage lysate (PL) bacterin, alum adjuvanted phage lysate and Brucella S19 immunized mice. The data are expressed as mean±SDE

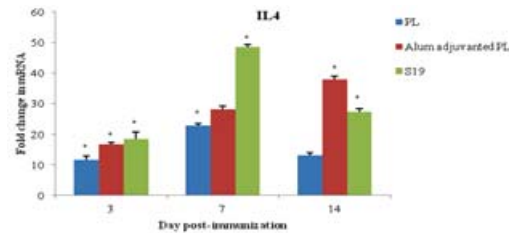


Fig. 4. Relative expression profile of IL4 in Brucella phage lysate (PL) bacterin, alum adjuvanted phage lysate and Brucella S19 immunized mice. The data are expressed as mean±SDE

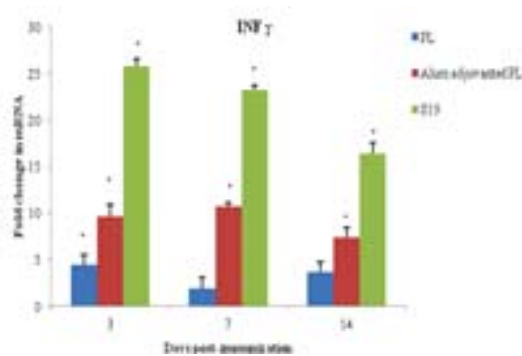


Fig. 5. Relative expression profile of INF³ in Brucella phage lysate (PL) bacterin, alum adjuvanted phage lysate and Brucella S19 immunized mice. The data are expressed as mean±SDE

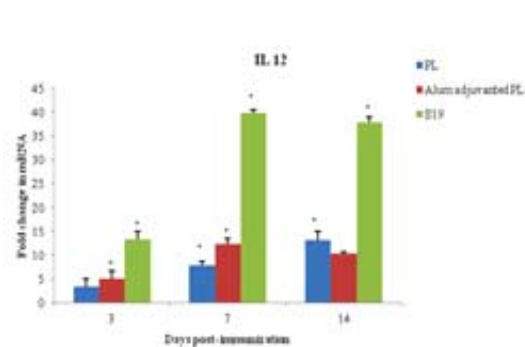


Fig. 6. Relative expression profile of IL12 in Brucella phage lysate (PL) bacterin, alum adjuvanted phage lysate and Brucella S19 immunized mice. The data are expressed as mean±SDE

day. In case of IL-12, significantly higher ($P < 0.01$) expression was observed at all the time interval studied after immunization (Fig. 6). IL-12 expression level peaked on 7th DPI at all immunized group and persisted up to 14th DPI. However in group immunized with standard Brucella S19 vaccine the level of expression of almost all genes were significantly higher than lysate group.

DISCUSSION

In this study, we have evaluated the expression of toll like receptor and cytokine transcripts in spleen of mice model against phage lysate bacterin of Brucella S19 to understand the immuno-protective mechanism of Phage lysate bacterin. Phage lysate which contains varied type of microbial components is expected to stimulate

innate immune response which can guide towards better adaptive immune response in the body. The most common PAMPs of bacteria such as lipopolysaccharide (LPS), lipoproteins and unmethylated CpG are recognized by TLR4, TLR2 and TLR9 respectively (Kawai and Akira, 2007). TLR signaling induced production of several pro-inflammatory cytokines (Kawai and Akira, 2006). But no such systemic studies have been conducted in this regard. So in an effort to understand mechanism of protection in the present report, we are reporting TLRs and cytokines mediated immune responses of phage lysate bacterin of *B. abortus* S19 in mice model via evaluation of TLR2, TLR4, TLR9, IL-4 IL-12 and INF-3. Such kind of study will be helpful in understanding the mechanism of immune protection of phage lysate bacterin. As per previous reports,

acute phase of brucellosis start from the 3rd day to the 2nd-3rd week which is marked by rapid increase the bacterial load in target organs, inflammation of the spleen and lymph node (Hort *et al.*, 2003). Phage lysate bacterin contains several unaltered conformational epitopes which recognized by TLRs found on the surface and endosomal membrane of different types of immune cells (macrophage, DC, neutrophil etc.). Among all the TLRs, TLR4, TLR2 and TLR9 play important role in controlling Brucella infection (Oliveira *et al.*, 2008). Up-regulation of TLRs and cytokines transcripts were observed in immunized mice groups in comparison to unimmunized group in present study.

In our study TLR9, IL-12 and INF- α expression were significantly up-regulated at every interval which indicate bacterial CpG motif of phage lysate bacterin act as TLR9 agonist and reaffirm the previous observations that activation of intracellularly located TLR9 with bacterial CpG motif lead to the production of IL12, which drive the Th1 immune response (Oliveira *et al.*, 2011) and increases the immunogenicity of peptide, DNA, tumor cells or DC based vaccines (van Duin *et al.*, 2006). TLR9 expression was higher in mice group immunized with Brucella S19 in compared to plain and alum adjuvanted phage lysate group at 3rd, 7th and 14th DPI which indicate that CMI response is relatively higher in Brucella S19 in comparison to plain and alum adjuvanted phage lysate bacterin. However the CMI response of phage lysate bacterin was sufficient enough to protect intracellular infection of *B. abortus* in mice as reported by Jain *et al.*, 2015. The relative higher expression of TLR9 in live Brucella S19 vaccine group may be due to mimicking of natural way of releasing and sensing of bacterial CpG motif by TLR9 in effective manner and higher antigen load.

In our study, TLR2, TLR4 and IL-12 expression were also significantly up-regulated in all immunizing groups as compared to negative control at every interval which suggest that LPS as a TLR4 agonist and lipoprotein as TLR2 agonist of Phage lysate bacterin augment the immunoprotective mechanism of phage lysate bacterin. Our findings were in accordance with previous reports, TLR2 recognize *B. abortus* lipoprotein omp19 and omp16 induce the expression of IL-12 and other pro inflammatory cytokines (Barrionuevo *et al.*,

2008). TLR4 activation strongly enhances Th1 type cellular and humoral immune responses and typically induces robust IL-12 production (Ulrich *et al.*, 1995 and Evans *et al.*, 2003). Although TLR2 and TLR4 expression were found to be higher in mice immunized with Brucella S19 in compared to alum adjuvanted phage lysate at 3rd DPI however TLR2 expression was almost similar in both group at 14th DPI and TLR4 expression was almost similar in both group at 7th and 14th DPI, which suggest that alum adjuvanted phage lysate bacterin may be alternative approach to combat the brucella infection at very low antigenic biomass while live Brucella S19 vaccine require high antigenic biomass. Expression of IL4 transcript significantly up-regulate in all the immunized groups in comparison to unimmunized group. In Brucella S19 group, the maximum IL-4 expression was observed at 7th DPI which reduced at 14th DPI. IL-4 expression was significantly higher in alum adjuvanted phage lysate group than Brucella S19 group and plain phage lysate at 14th DPI which reaffirm the previous observations that alum adjuvant drive Th2 immune response preferentially and help in early protection.

CONCLUSION

On the basis of present observation, we found that alum adjuvanted phage lysate bacterin produce sufficient expression level of TLRs and cytokines which in turn suggest alum adjuvanted phage lysate bacterin in comparison to plain lysate produce sufficient immune-protection in form of Th1, Th2, cell mediated and humoral immune response.

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