



Patho-Physiological Response of LPS Defective *Brucella abortus* S19 Δ *per* in Experimentally Infected Mice

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ABSTRACT

Brucellosis is an anthroponotic infectious disease in which infected animals pose a threat to humans. This disease has considerable social, economic and international trade importance. Despite past and current efforts to eradicate brucellosis, a large number of new human cases are reported annually worldwide. In this study, patho-physiological response to *Brucella abortus* S19 Δ *per* infection in BALB/c mice was assessed in comparison to its parent strain, *B. abortus* S19 and virulent strain, *B. abortus* 544. Immunohistochemical analysis confirmed the presence of bacteria in liver and spleen. Comparatively lower serum Aspartate aminotransferase (AST) level and observation of less number of microgranulomas in liver indicated that *B. abortus* S19 Δ *per* was less infectious and failed to cause active infection unlike S19 and 544 strain. S19 Δ *per*, thus could be a safer vaccine candidate as an alternate to the S19 vaccine strain. Hematological studies indicated clinical manifestation of thrombocytopenia in different *Brucella* infected mice including S19 Δ *per*. Therefore, measurement of platelets count and serum AST level may offer as reliable indicators of brucellosis in clinical cases.

Keywords: Brucellosis, perosamine synthetase, lipopolysaccharides, thrombocytopenia, aspartate aminotransferase, microgranuloma

Brucellosis is a global zoonotic disease having potential to cause great economic losses in farm animals and health hazards among human beings. In India, bovine brucellosis incurred losses of 3.4 billion USD per annum (about 20,000 crores INR) (Singh *et al.*, 2015). Infected animals are the source of human brucellosis that affects a sizeable number of people in the world (Moreno and Moriyon, 2006). Therefore, control of animal brucellosis is an essential step to combat infection in human (Radostits *et al.*, 2007). Vaccination is an economic and the only way to control animal brucellosis as stamping out is not a choice in the Indian scenario. The *B. abortus* S19 vaccine (S19) is used for animal vaccination in India and many other countries. Among other drawbacks, S19 strain possesses

residual virulence causing abortion in when used in adult animals. In our previous study, a perosamine synthetase deletion (*per*) mutant of *B. abortus* S19 (S19 Δ *per*) has been developed which showed high immunogenicity. S19 Δ *per* exhibits intermediate rough phenotype with truncated lipopolysaccharide (LPS) (Lalsiamthara, *et al.*, 2015).

Mouse remains the most widely used experimental animal model for *in-vivo* study of *Brucella* infection (Silva *et al.*, 2011). Brucellosis affects the vital organ such as liver, lung, kidney, heart and spleen, leading to their damage or impairment of their functions according to the stage of infection that is reflected in changing the complete blood counts (CBC) and serum biochemistry profile of infected

animals (Al-Majali, 2005). Various cultural, serological and molecular methods are employed for the diagnosis of brucellosis. The “Gold standard” for the diagnosis of brucellosis is isolation and characterization of the organisms from the infected individual but it is arduous, time-consuming and hazardous to laboratory workers.

The present study was carried out to assess the health status of BALB/c mouse after infection with S19 Δ *per*, vaccine strain S19 and virulent *B. abortus* 544 strain (S544). Hematological and serum biochemical parameters along with the histological examination of vital organs were performed to evaluate the extent of infection caused by S19 Δ *per*.

MATERIALS AND METHODS

Ethics statement and animals

Normal, healthy, female BALB/c mice (4-6 weeks) were housed in the institute experimental animal house facility and the animals received water and food *ad lib*. Animal experiments were performed with the approval and following the guidelines of the Institute Animal Ethics Committee (IAEC). *Brucella* organisms were handled and processed in accordance to guidelines provided by Institute Biosafety Committee (IBSC), IVRI.

Mouse inoculation

Brucella abortus strain 544 (S544) was obtained from *Brucella* referral laboratory, Division of Veterinary Public Health, IVRI, Izatnagar.

Table 1: Bacterial inoculation of BALB/c mice with different *Brucella abortus* strains, S19 Δ *per*, S19 and S544 through intraperitoneal (i.p) route

Sl. No.	Experimental groups	No. of BALB/c mice	Dose and route
1	<i>B. abortus</i> S19 Δ <i>per</i>	6	5×10^5 CFU, i.p.
2	<i>B. abortus</i> S19	6	5×10^5 CFU, i.p.
3	<i>B. abortus</i> strain 544	6	5×10^5 CFU, i.p.
4	PBS	6	200 μ l, i.p.

The S19 vaccine strain was obtained from the Division of Biological Products, IVRI. *Brucella* organisms were

grown on tryptose soya agar (TSA) at 37 °C for 48 hours, suspended in phosphate buffer saline solution, for enumeration of colony-forming-units (CFU). BALB/c mice were randomly segregated into 4 groups. Six mice in each of the three treatment groups were inoculated intraperitoneally (i.p.) with a suspension containing 5×10^5 CFU of S19 Δ *per*, S19 and S544, respectively (Table 1).

Blood collection

Blood was collected from retro-orbital sinus of each mouse on 0 day and 15 days post infection (dpi). Serum was separated by centrifugation at 3000 rpm for 10 min at 4°C in a bench top centrifuge (Eppendorf) and stored at -20 °C till used.

Necropsy

All mice were euthanized humanely immediately after blood collection at 15 dpi. Liver, lung, heart, kidney and spleen were fixed in 10% neutral buffered formalin and processed for histopathology. Tissues were embedded in paraffin, sectioned, and mounted on glass slides, and then stained with hematoxylin and eosin (Luna, 1968).

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded tissue sections of liver and spleen were mounted on poly-L-Lysine (Sigma) pre-coated slides followed by deparaffinization in xylene, rehydration in graded ethanol, and quenching of endoperoxidase activity using 3% H₂O₂ in methanol. Antigen retrieval was done in heat mediated antigen retrieval solution according to manufacturer’s instructions (Vector Labs, USA). To block non-specific sites, 2.5% normal non-immune goat serum (Vector Labs, USA) was used. The sections were incubated with rabbit anti-*Brucella* serum as primary antibodies followed by goat anti-rabbit IgG (whole molecule)-Peroxidase conjugate (Sigma, USA) and developed using DAB (3,3'-diaminobenzidine tetrahydrochloride) enhanced liquid substrate System (Sigma, USA). Immunostained sections were counterstained with Mayer’s hematoxylin (Sigma, USA) and mounted with a tissue mounting medium (CC/ Mount™, Sigma, USA).

Haematology

For DLC (differential leukocyte counts), an EDTA coated blood on clean grease free slide was fixed with methanol and stained with Giemsa stain.

The Complete blood count (CBC) was conducted using ABX Micros ESV60 haem-analyzer (ABX Vet Package, HORIBA ABX Diagnostic, France) immediately after blood collection (Table 2).

Serological tests

Sera samples collected on day 0 and 15 dpi were analyzed for anti-*Brucella* antibodies using Rose Bengal coloured antigen obtained from the Division of Biological products, IVRI. Rose Bengal Plat agglutination Test (RBPT) assay was performed using a volume of 30 μ l of serum and an equal volume of RBPT antigen on a glass slide. Formation of agglutination is indicative of positive reactivity.

Serum biochemistry profile

Serum clinical chemistry profile for albumin, globulin, blood-urea-nitrogen (BUN), cholesterol, creatinine, total protein, triglyceride, glucose, alanine aminotransferase

(ALT) and aspartate aminotransferase (AST) was determined by an automated analyzer (Vet Test 8008, IDEXX Laboratories, USA) using IDEXX Vet Test slides.

Statistical analysis

It was performed using IBM SPSS version 20.0 program. The difference in mean values among various experimental groups were compared using one-way ANOVA with Tukey's HSD test and considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Hematology

The results of hematological parameters of *Brucella* inoculated BALB/c mice are presented in Table 2. Thrombocytopenia was observed in all *Brucella* inoculated groups that was significantly more pronounced in S19 Δ per infected mice. The S19 Δ per infected mice showed lymphocytopenia that was significantly more evident in strain 544 infected group. There was a significant difference in the number of lymphocytes and platelets among different infected groups. Neutrophilia was also observed in all infected mice.

Table 2: Hematological parameters (mean \pm SE) of BALB/c mice at day 15th post-inoculation

Sl. No.	Blood parameters	<i>B. abortus</i> strain 544	<i>B. abortus</i> S19	<i>B. abortus</i> S19 Δ per	Healthy (PBS)
		(n=6)	(n=6)	(n=6)	(n=6)
1	RBC ($\times 10^6/\mu$ l)	8.266 \pm 0.409 ^a	8.621 \pm 0.739 ^a	9.220 \pm 0.125 ^a	9.185 \pm 0.295 ^a
2	Hb (g/dl)	14.783 \pm 0.681 ^a	15.150 \pm 1.173 ^a	16.133 \pm 0.156 ^a	16.750 \pm 0.240 ^a
3	PCV (%)	40.350 \pm 2.103 ^a	42.016 \pm 3.916 ^a	44.833 \pm 0.537 ^a	47.350 \pm 0.689 ^a
4	MCV (fl)	49.000 \pm 0.447 ^a	48.666 \pm 0.760 ^a	48.500 \pm 0.223 ^a	51.833 \pm 0.792 ^b
5	MCH (pg)	17.900 \pm 0.313 ^a	17.683 \pm 0.231 ^a	17.483 \pm 0.107 ^a	18.300 \pm 0.628 ^a
6	MCHC (g/dl)	36.683 \pm 0.494 ^a	36.400 \pm 0.679 ^a	35.983 \pm 0.270 ^a	35.316 \pm 0.748 ^a
7	Platelets ($\times 10^3/\mu$ l)	845.333 \pm 28.842 ^a	816.666 \pm 15.79 ^{ab}	729.833 \pm 21.097 ^b	980.166 \pm 27.065 ^c
8	WBC ($\times 10^3/\mu$ l)	16.650 \pm 2.293 ^a	15.066 \pm 1.151 ^a	15.033 \pm 1.334 ^a	14.900 \pm 0.970 ^a
9	Neutrophils (%)	44.333 \pm 1.926 ^a	43.833 \pm 1.904 ^a	36.333 \pm 3.480 ^a	16.833 \pm 0.9457 ^b
10	Eosinophils (%)	0.000 \pm 0.0000 ^a	0.0000 \pm 0.0000 ^a	0.166 \pm 0.166 ^a	0.166 \pm 0.166 ^a
11	Basophils (%)	0.000 \pm 0.000 ^a	0.333 \pm 0.210 ^a	1.166 \pm 0.477 ^a	0.333 \pm 0.333 ^a
12	Lymphocytes (%)	50.333 \pm 1.282 ^a	52.666 \pm 2.275 ^{ac}	60.500 \pm 3.422 ^c	78.500 \pm 1.522 ^b
13	Monocytes (%)	5.333 \pm 1.744 ^a	3.166 \pm 1.514 ^a	1.833 \pm 0.307 ^a	4.166 \pm 1.681 ^a

Note: Abbreviations: RBC (Red blood cells), Hb (Hemoglobin), PCV (Packed cell volume), MCV (Mean corpuscular volume, MCH (Mean corpuscular hemoglobin), MCHC (Mean corpuscular hemoglobin concentration); Values represent mean \pm SE. The mean values were compared between groups by one-way ANOVA followed by Tukey's HSD (honestly significant difference)-multiple comparison test. Means sharing the similar superscripts in the same row are not significantly different from each other ($P < 0.05$).

Table 3: Serum biochemical parameters (mean ± SE) of BALB/c mice at day 15th post-inoculation with 5×10^5 CFU S19 Δ *per*, S19 and S544

Sl. No.	Serum biochemical parameters	<i>B. abortus</i> strain 544 (n=6)	<i>B. abortus</i> S19 (n=6)	<i>B. abortus</i> S19 Δ <i>per</i> (n=6)	Healthy (PBS) (n=6)
1	BUN (mg/dl)	23.000 ± 1.290 ^a	12.750 ± 1.5478 ^b	12.250 ± 0.946 ^b	11.250 ± 1.108 ^b
2	Creatinine (mg/dl)	0.375 ± 0.025 ^{ab}	0.400 ± 0.040 ^b	0.275 ± 0.0250 ^{ac}	0.250 ± 0.0287 ^c
3	Cholesterol (mg/dl)	117.250 ± 10.483 ^a	74.750 ± 7.110 ^b	76.750 ± 4.607 ^b	95.500 ± 7.005 ^{ab}
4	AST (U/L)	368.500 ± 7.984 ^a	273.250 ± 7.318 ^b	214.500 ± 8.026 ^c	186.750 ± 2.286 ^d
5	ALT (U/L)	57.250 ± 3.966 ^{ab}	71.500 ± 7.762 ^a	52.750 ± 5.618 ^{ab}	44.500 ± 6.409 ^b
6	Glucose (mg/dl)	133.500 ± 10.935 ^a	106.750 ± 15.991 ^a	119.250 ± 18.445 ^a	124.000 ± 8.784 ^a
7	TP (g/dl)	6.775 ± 0.311 ^a	5.5750 ± 0.0478 ^b	6.300 ± 0.158 ^{ab}	6.425 ± 0.332 ^{ab}
8	TRIG (mg/dl)	100.750 ± 6.896 ^a	108.500 ± 6.383 ^a	95.750 ± 0.478 ^a	96.000 ± 5.802 ^a
9	Albumin (g/dl)	2.400 ± 0.147 ^b	1.900 ± 0.040 ^a	2.250 ± 0.028 ^{ab}	2.425 ± 0.125 ^b
10	Globulin (g/dl)	4.375±0.179 ^a	3.675±0.025 ^b	4.075±0.165 ^{ab}	3.975±0.197 ^{ab}

Note: Abbreviations : BUN (Blood urea nitrogen), ALT (Alanine aminotransferase), AST (Aspartate aminotranferase), TP (Total protein), TRIG (Triglycerides); Values represent mean ± SE. The mean values were compared between groups by one-way ANOVA followed Tukey’s HSD (honestly significant difference)-multiple comparison test. Statistical significant difference in mean values between groups are indicated by different superscript letters at p<0.05.

Serum biochemical parameters

The analyzed results of various serum biochemical parameters are presented in Table 3.

In comparison with healthy animals, aspartate aminotransferase (AST) level was found considerably elevated all *Brucella* infected groups. However, the level of AST was considerably lower in the S19 Δ *per* group than S544 and S19 infected mice. In addition, the level of blood urea nitrogen (BUN) was observed significantly lower in the S19 Δ *per* group than S544 infected mice only. The difference in the concentration of other parameters such as albumin, cholesterol, creatinine, Triglycerides (TRIG), Total protein (TP), ALT, glucose and globulin was found insignificant in the serum of S19 Δ *per* group.

Histopathological findings

Liver

Microscopically, the liver of S544 infected BALB/c mice showed extensive infiltration of mononuclear cells around the blood vessels and also in the focal areas in the parenchyma along with macrophages forming microgranuloma like structures. In few animals, the focal areas of hepatic degeneration and necrosis were also seen. The presence of few neutrophils at isolated areas with MNCs

was also found in some cases. Similarly, the liver of *B. abortus* S19 vaccinated BALB/c mice showed minute foci of infiltration in the parenchyma which was characterized by the presence of MNCs and few neutrophils. The liver of mice immunized with S19 showed comparatively less inflammatory changes as compared to the mice infected with S544. Among different experimental group, liver of mice inoculated with S19 Δ *per* showed the least number of foci of microgranuloma formation and infiltration of MNCs (Fig. 1).

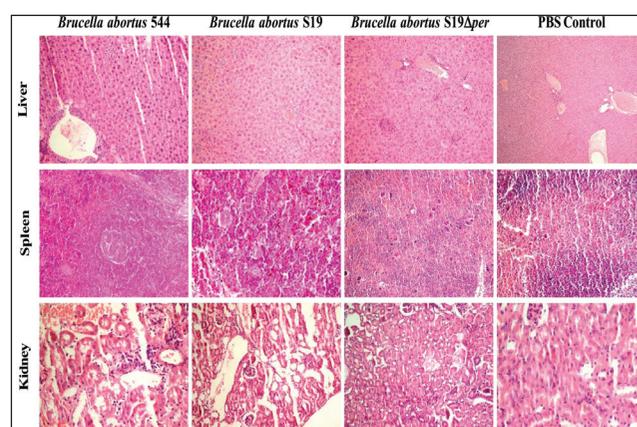


Fig. 1: Histopathology of visceral organs of BALB/c mice at day 15th post-inoculation with 5×10^5 CFU of virulent *B. abortus* strain 544, *B. abortus* S19 and *B. abortus* S19 Δ *per*

Spleen

Spleen of BALB/c mice infected with strain S544 and S19 revealed non follicular proliferation of the lymphocytes along with macrophages indicating highly reactive spleen. The red pulp was sparsely distributed which was occupied by the lymphocytes in mice spleen. Focal areas also showed the presence of the lympholysis in some cases. Similarly, the spleen of the S19 Δ *per* inoculated mice also revealed reactive changes characterized by variable sized lymphocytes (Fig. 1).

Kidney

Few focal foci of MNCs infiltration were found in the renal parenchyma and around blood vessels in the S544 infected mice. Some cases also revealed mild degenerative changes in the tubular epithelium. Similar changes were noticed in the S19 and S19 Δ *per* inoculated groups but the number of foci was less. (Fig. 1)

The presence of *Brucella* in infected liver and spleen was also confirmed by immunohistochemistry (Fig. 2).

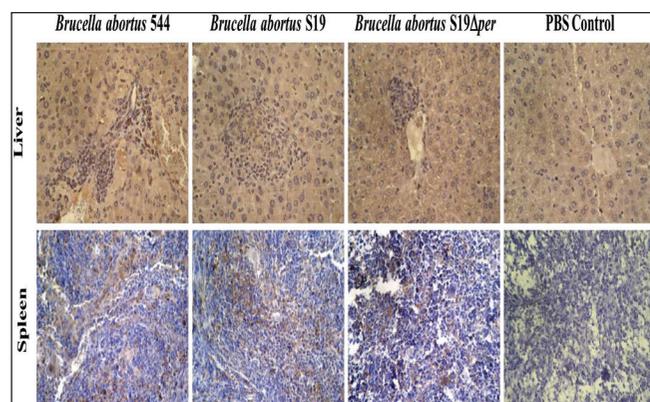


Fig. 2: Immunohistochemical detection of *Brucella* antigen (Golden brown deposition) in liver and spleen of BALB/c mice at day 15th post-inoculation with 5×10^5 CFU of virulent *B. abortus* strain 544, *B. abortus* S19 and *B. abortus* S19 Δ *per*. Staining of uninfected (control) tissue produced no signal. IHC \times DAB \times 400

Mouse remains the most suitable experimental animal to investigate *Brucella* infection *in-vivo*. The results of vaccine testing and infection studies depend upon breed, sex, age, genetic makeup and physiological status of mice, in addition to the virulence, dosage and route of inoculation of *Brucella* strain. Clinicopathological observations and

clinical chemistry profile are commonly evaluated in mice as a disease model for biomedical research (Hall, 1992; Irausquin, 1992; Kanno, 1992). To overcome the flaws of the currently used *B. abortus* S19 vaccine, an equally immunogenic LPS defective mutant of S19 (S19 Δ *per*) was generated (Lalsiamthara *et al.*, 2015). The authors reported that S19 Δ *per* produces intermediate rough colonies due to truncated LPS. Further, it was shown that S19 Δ *per* conferred solid immunity against virulent challenge with S544 in a murine model. The present study was carried out to evaluate the safety aspects of S19 Δ *per in-vivo* in the murine model. To determine the extent of infection, vital organs of BALB/c mice infected with S19 Δ *per*, S19 vaccine and virulent 544 strain, were collected at 15th dpi.

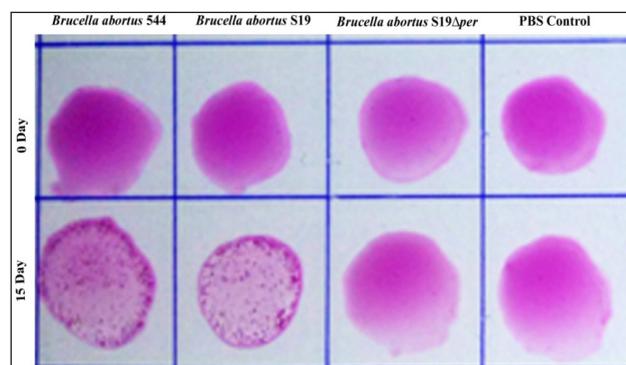


Fig. 3: RBPT on mice sera collected after 15 day on inoculation. BALB/c mice were inoculated with 5×10^5 CFU of virulent *B. abortus* strain 544, *B. abortus* S19 and *B. abortus* S19 Δ *per*. At day 15th post inoculation, S544 and S19 mice strongly reacted with RBPT characterized by the formation of agglutination; S19 Δ *per* immunized mice showed RBPT negative results

Extensive microgranulomatous lesions in liver, depletion of red pulp in spleen, MNCs infiltration forming foci in renal parenchyma were characteristics of S544 infection in mice. Pugh *et al.* (1989) confirmed the depletion of splenic lymphoid tissue in *B. abortus* 2308 infected BALB/c as well as in other strains of mice. Histological findings in liver confirm that *B. abortus* produces granulomatous lesions and acute inflammatory responses in mice (Riglar and Cheers, 1980; Cheers, 1984; Ko *et al.*, 2002a; Ko *et al.*, 2002b). Liver is the first organ exhibiting microgranuloma as an important pathological lesion (Grillo *et al.*, 2012). Granulomas consisted of histiocytic cells mixed with monocytes, lymphoid cells and granulocytes, several of which having ingested *Brucella* antigen (Yoneyama and Ichida, 2005). Granuloma formation localizes the

pathogens which are then easily engulfed by the phagocytic cells. *Brucella* starts multiplying in the cytoplasm resulting transformation of phagocytic cells into epithelioid cells. As expected, golden brown deposition as a positive-IHC signal confirmed the presence of *Brucella* inside granulomas and also support IHC specificity (Fig. 2). The presence of comparatively less number of microgranuloma in the liver and less number of foci in kidney depicts less infectious nature of S19 Δ *per*. This property makes S19 Δ *per* a more safe vaccine candidate than its parent strain, S19. The attenuated biological activity of S19 Δ *per* is attributed to its truncated lipopolysaccharide (LPS) structure.

Lympholysis, as we observed in *Brucella* inoculated mice spleen was associated with temporally decreased percentage of T and B cells in the spleen (Araya *et al.*, 1989). From circulation, monocytes migrate into tissue and mature into macrophage and dendritic cells. Thus the population of macrophages, that were initially present in small number, rapidly increase as result of the immune or inflammatory cascade. The infiltrated macrophages phagocytosed brucellae facilitated by the deposition of C3b following antibody-independent activation of complement components (Corbeil *et al.*, 1988). The influx of phagocytic cells generates exuberant inflammatory and immune responses leading to the elimination of organisms.

Hematological analysis of S544, S19 and S19 Δ *per* inoculated mice on 15th dpi revealed lymphopenia, thrombocytopenia, and neutrophilia. Immune-mediated thrombocytopenia was one of the hematological complications in a case of acute human brucellosis (Hayrettin *et al.*, 1998; Gurkan *et al.*, 2003; Tunccan *et al.*, 2014). The presence of pancytopenia with severe thrombocytopenia in acute brucellosis can imitate several primary hematological diseases (Yilmaz *et al.*, 2007; Erduran *et al.*, 2010). The exact cause of thrombocytopenia in brucellosis is still obscure but it may be due to hypersplenism, disseminated intravascular coagulation, hemophagocytosis, bone marrow suppression, immune destruction of platelets and adherence of thrombocyte to damaged vascular surfaces (Yilmaz *et al.*, 2007; Erduran *et al.*, 2010).

Altered serum enzyme activities are the trusted indicators of pathological changes in different tissues and may be used as a valuable diagnostic tool in domestic animals. Serum enzyme activities are the indicators of pathological

changes and not a specific disease. The purpose of studying serum biochemical parameters was to evaluate potential changes in serum enzymes activities and protein fractions in sera of brucellosis-infected mice, which would help in elucidating the pathophysiology of *Brucella* infection.

In the present study, an increase in AST level was observed in the serum of all *Brucella* infected mice which are in line with the findings of El-Boshy *et al.* (2009) in camels, Nath *et al.* (2014) and Elazab (2015) in *Brucella* infected cows. Measuring AST level in serum is valuable for detecting liver dysfunction in several types of its disorder, diseases and hepatic injury. In comparison with healthy mice, the AST level in *B. abortus* S19 Δ *per* was higher, but the AST level was significantly lower than S544 and S19 inoculated mice clearly indicate the less deleterious effect of S19 Δ *per* on the liver functions which was also reflected in less number of microgranulomas as a pathological lesion (Fig. 1).

CONCLUSION

S19 Δ *per* with truncated LPS showed a lower potential to establish infection in mice. This property of attenuation would make S19 Δ *per* a more safe vaccine candidate as an alternative to the S19 vaccine. Measuring serum AST level and platelets count may be a complementary assay for diagnosis of brucellosis.

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Conflicts of interest: None

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