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Comparative Study of Lipopolysaccharide and Killed Vaccines of *Brucella suis* Field Isolate in Mice

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A B S T R A C T

In the present study, we developed a killed and LPS based *Brucella suis* vaccine and evaluated the humoral and cell mediated immune response in mice. *Brucella suis* isolated from field and developed as vaccine. Lipopolysaccharides from *B. suis* were administered to the nude mice, in addition to killed vaccine from that species whereas mineral oil was used as an adjuvant. The vaccines from *B. suis* is considered as smooth strain with smooth dissociation. Serum was collected from clotted blood samples of day 0, 7, 14 and 21 from the mice (control, adjuvant, BM-killed, BM-LPS vaccine, BS-killed and BS-LPS groups). In BS-killed and BS-LPS treated groups, the immune response was very significant in BS-LPS group alone. Though, the immune response was lower in BS-LPS vaccine group (p value = 0.0075) in comparison to BS-killed vaccine group (p value = 0.0009), 14 days post vaccination. There is a significant spike on the immune response of the BS-LPS group (p value < 0.0001), 21 days post vaccination. In *B. suis* vaccinated groups, the TNFα, IFNγ, IL4, IL10 and IL12 expressions were up-regulated in BS-LPS treated animals with a p value of 0.0198, 0.0142, 0.0195, 0.0384 and 0.03 respectively.

Introduction

Brucellosis is one of the world's most widespread contagious zoonotic diseases which has been reported in almost all species of animals and brucellosis is an economically important disease in productive animals worldwide. To control the brucellosis so many vaccines were developed. Recently it was found that bacterial lipopolysaccharide as a potential candidate for vaccine development. The lipopolysaccharide (LPS) phenotype of *Brucella* species is either smooth or rough if they possess or lack the surface exposed O-polysaccharides (O-PS) chain respectively.

Lipid A, fatty acids, a core region, and a polysaccharide O-side chain were the components of smooth strains LPS of

Brucella spp. The O-PS plays a major role in virulence associated with smooth LPS (SLPS) in that mutant smooth strains fail to survive in macrophages. *Brucella* S-LPS are refractive to the actions of polycationic molecules and it show that smooth strains of *Brucella* can resist the cationic bactericidal peptides of the phagocytes. S-LPS have also been found to confer antiphagocytic properties upon *Brucella* and are unable to activate the alternative pathway of the complement cascade. Yu *et al.,* (2005) reported that polysaccharide (from bacterial capsule or LPS) protein conjugates are usually immunogens in mice, rabbits and humans. In the present study, we developed a killed and LPS based *Brucella suis* vaccine and evaluated the humoral and cell mediated immune response in mice.

Materials and Methods

Brucella reference culture

Reference strains of *Brucella abortus S19*, *Brucella melitensis* and *Brucella suis* were obtained from Indian Veterinary Research Institute (I.V.R.I), Izatnagar and used as positive control. The slant was stored in 2-8^{o}C.

Bacterial isolation

Brucella suis was isolated from swine with history of abortion from different farms. Blood (68), milk (6), vaginal swab (168) and aborted foetus (1) were collected. Isolation of *Brucella spp.* was done according to the procedure detailed in Bergey's Manual of Systemic Bacteriology (Bergey *et al.,* 1984; OIE, 2009).

The aborted materials were enriched with *Brucella* broth at 37^{o} C for three days. The three days old enriched suspension were directly streaked on the *Brucella* selective medium with *Brucella* selective supplement which was prepared as prescribed by the manufacturer.

Biochemical tests such as H_2S production, urease activity, growth in different concentration of basic fuchsin, thionin and safranin O to find the biovar (OIE, 2009). DNA was extracted from colonies for bruce ladder polymerase chain reaction (PCR) (Lopez goni *et al.,* 2008).

Extraction of LPS

LPS extraction was carried out as per the protocol described by Westphal and Jann, (1965) with few modifications.

Limulus amebocyte lysate assay

The potency of LPS samples were determined by the limulus amebocyte assay gel clot method (LONZA, Walkersville, USA) which had a sensitivity of 0.06 endotoxin units per millilitre (UE/ml), according to the protocol of Friberg, (1987).

Experimental design

The main objective of the study is to develop LPS and killed vaccine from local isolates of *B.melitensis* and *B.suis* which needs mice model for vaccine efficacy studies. BALB/c mice are routinely used for various immunological studies. Hence in the present study to rule out humoral and cell mediated response this strain of mice was selected.

Immunization procedure

Four separate groups of six male BALB/c mice were injected through intramuscular in this experiment with LPS and killed vaccines which were prepared from local isolates of *B. melitensis* and *B. suis* (10 µg LPS in polysaccharide content), in 0.2 ml of 0.9 per

cent NaCl and killed vaccines prepared from *B. melitensis* and *B. suis* (1.2-1.5X10^9) organisms inactivated in 0.1 per cent formalin with mineral oil as adjuvant. Two separate groups of six male BALB/c mice were kept as control animal injected with adjuvant and 0.9 per cent NaCl as per Sharifat *et al.,* (2009) with few modification.

Blood collection

The animals were bled prior to immunization subsequently on 7, 14 and 21 days post immunization. Blood samples were collected from mice via retro orbital route in 5 per cent EDTA tubes and serum was collected for cell mediated and humoral immunity. The animals were sacrificed on 21st day collected for assessment of cytokine expressions.

Assessment of humoral immunity by Enzyme-linked immunosorbent assay

Specific antibody molecules produced against the killed and LPS of *B.suis* were demonstrated by enzyme-linked immunosorbent assay (ELISA). Assessment of mouse *Brucella* antibody assay was carried out as per manufacturer guideline Mouse Brucella Antibody IgG ELISA kit (Bioassay Technology, China).

Assessment of cell mediated immunity by cytokine expression

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood by density gradient centrifugation. PBMCs were collected after centrifugation on Histopaque®-1077 (Sigma Aldrich, France) for mice. PBMC culture was done as per Puech *et al.,* (2015). RNA isolation was done as per Chomczynski and Mackey (1995). c-DNA synthesis were done. q-RT PCR (CFX96 Touch, Biorad, Inc.,) was done with primers designed for cytokines such as tumour necrosis factor- α, interferon –ϒ, interleukin 4, interleukin 10 and interleukin 12.

Statistical analysis

Two way ANOVA was done to study the humoral and cell mediated immunity post vaccination with *Brucella suis* killed and LPS vaccine by Graph Pad prism 6 Software.

Results and Discussion

Isolation of *Brucella suis* was done and confirmed by biochemical and bruce ladder the results were shown in Figure 1. Assessment of antibody level post vaccination with *B.suis* killed and LPS vaccines by Mouse antibody *Brucella* IgG ELISA and analysed statistically by two way ANOVA method. There was no significant difference between the control, adjuvant on 0, 7, 14 and 21 days of post vaccination whereas there was significant difference in *B. suis* killed with a P value of 0.0009 and 0.0075 on 14 and 21 days of post vaccination. There was highly significant difference in 21 days of *B. suis* LPS post vaccination with a P-value of < 0.0001 shown in table 3, q-RTPCR was carried out as per the protocol, the expression of different genes such as TNF-α, Interferon-ϒ, IL4, IL10 and IL12 for different groups showed in Table 3 and Figure 3 and were analysed Two way ANOVA tests statistically using Graph Pad Prism software (Table 1).

In the present study, lipopolysaccharide from *B.suis* was administered to the nude mice, in addition to killed vaccine from that species whereas mineral oil was used as an adjuvant. The vaccines from *B. suis* is considered as smooth strain with smooth dissociation. Serum was collected from clotted blood samples of day 0, 7, 14 and 21 from the mice (control, adjuvant, BM-killed, BM-LPS vaccine, BS-killed and BS-LPS groups). In

BS-killed and BS-LPS treated groups, the immune response was very significant in BS-LPS group alone. Though, the immune response was lower in BS-LPS vaccine group (p value = 0.0075) in comparison to BS-killed vaccine group (p value = 0.0009), 14 days post vaccination. There is a significant spike on the immune response of the BS-LPS group (p value < 0.0001), 21 days post vaccination. This also suggests that the LPS initiate a significant immune response, but lately. BS-LPS vaccinated group exhibit significant immune response only after 14 days of vaccination. This suggests that the LPS though previously reported not to possess significant in triggering an innate immune response, thus initiate adaptive immune response in mice (Fig. 2 and Table 1–4).

Table.1 List of *Brucella* specific primers used for PCR study

Primer	Gene target	Sequence (5'-3')	Amplicon size (bp)	Reference
BMEI0998F	Glycosyltransferase, gene wboA	ATC CTA TTG CCC CGA TAA GG	1682	*Lopez Goni et al.*, 2008
BMEI0997R		GCT TCG CAT TTT CAC TGT AGC		
BMEI0535F	Immunodominant antigen, gene bp26	GCG CAT TCT TCG GTT ATG AA	450	
BMEI0536R		CGC AGG CGA AAA CAG CTA TAA		
BMEII0843F	Outer membrane protein, gene omp31	TTT ACA CAG GCA ATC CAG CA	1071	
BMEII0844R		GCG TCC AGT TGT TGT TGA TG		
BMEI1436F	Polysaccharide deacetylase	ACG CAG ACG ACC TTC GGT AT	794	
BMEI1435R		TTT ATC CAT CGC CCT GTC AC		
BMEII0428R	Erythritol catabolism, gene eryC (Derythrulose-1- phosphate dehydrogenase)	GCC GCT ATT ATG TGG ACT GG	587	
BMEII0428R		AAT GAC TTC ACG GTC GTT CG		
BR0953F	ABC transporter binding protein	GGA ACA CTA CGC CAC CTT GT	272	
BR0953R		GAT GGA GCA AAC GCT GAA G		
BMEI0752F	Ribosomal protein S12, gene rpsL	CAG GCA AAC CCT CAG AAG C	218	
BMEI0752R		GAT GTG GTA ACG CAC ACC AA		
BMEII0987F	Transcriptional regulator, CRP family	CGC AGA CAG TGA CCA TCA AA	152	
BMEII0987R		GTA TTC AGC CCC CGT TAC CT		

(F) = Forward primer; (R) = Reverse primer

Table.2 Experimental design and number of animals

Treatment / Replicate	Blank control	Adjuvant	LPS *B. melitensis*	LPS – *B. suis*	Killed vaccine *B. melitensis*	Killed vaccine - *B. suis*	Total
R1	2	2	2	2	2	2	12
R2	2	2	2	2	2	2	12
R3	2	2	2	2	2	2	12
Total	**6**	**6**	**6**	**6**	**6**	**6**	**36**

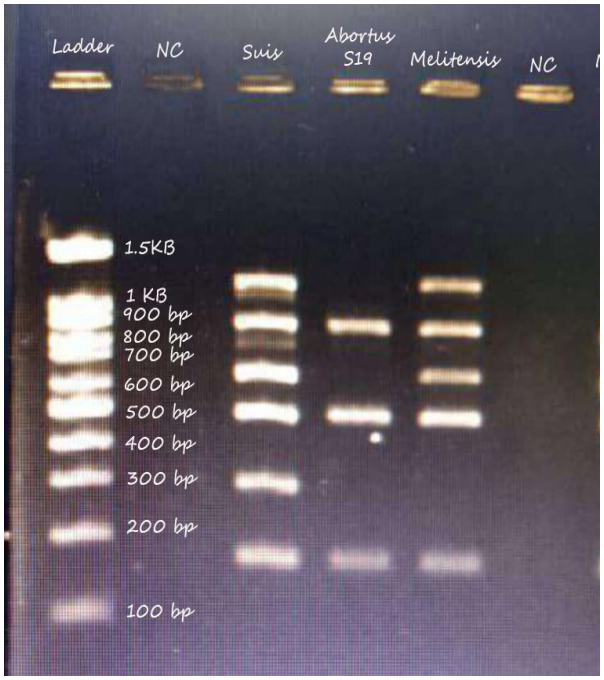
Table.3 List of Cytokine specific primers used for Cell mediated immunity study in *Mus musculus*

Gene	5’ - 3’ Forward	3’- 5’ Reverse	Amplicon size
INF-γ	GTGATTGCGGGGTTGTATCT	CACATTCGAGTGCTGTCTGG	197
TNF-α	CAAACCACCAAGTGGAGGAG	GTGGGTGAGGAGCACGTAGT	179
IL4	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG	162
IL10	AGTCACCAACCTGTCCCTTG	GAACAGGCCACAGTTCCATT	177
IL12	TCAACCCCCAGCTAGTTGTC	TGTTCTTCGTTGCTGTGAGG	177
ACTB	TGTTACCAACTGGGACGACA	GGGGTGTTGAAGGTCTCAAA	165

Table.4 Assessment of antibody level post vaccination with *B.suis* killed and LPS vaccines vaccine by Mouse antibody *Brucella* IgG ELISA

	Control			**Adjuvant**			***Brucella suis* killed**			***Brucella suis* LPS**		
Day 0	0.471	0.374	0.576	0.473	0.513	0.497	0.379	0.478	0.374	0.398	0.615	0.497
Day 7	0.483	0.578	0.387	0.672	0.471	0.523	0.674	0.554	0.435	0.639	0.597	0.734
Day 14	0.491	0.427	0	0.702	0.698	0.691	0.884	0.774	0.829	0.697	0.713	0.749
Day 21	0.478	0.524	0.476	0.701	0.699	0.712	0.724	0.784	0.713	1.731	1.385	1.234

Fig.1 Bruce ladder-PCR for confirmation of *Brucella suis*



Lane 1- 100bp ladder, Lane 2 negative control, Lane 3 *Brucella suis*
Lane 4 *Brucella abortus* S19, Lane 4 *Brucella melitensis*, Lane 5 Negative control

Fig.2 Assessment of immune response post vaccination with *B.suis* killed and LPS vaccine by Mouse antibody *Brucella* IgG ELISA

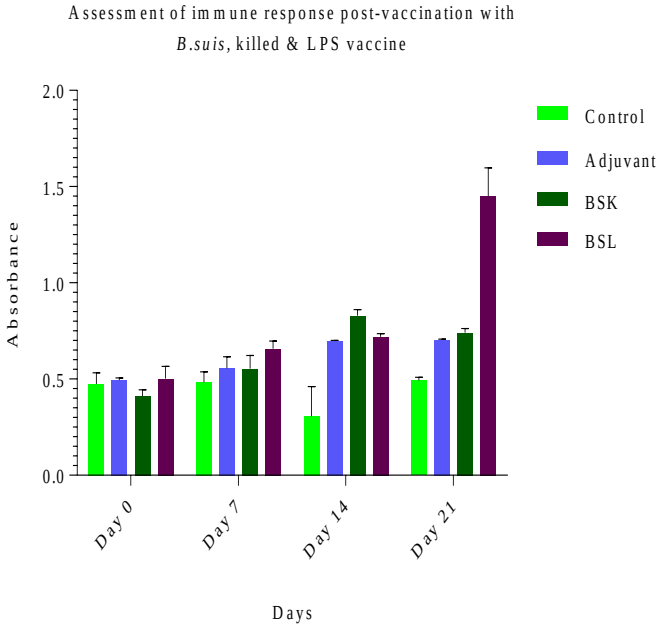
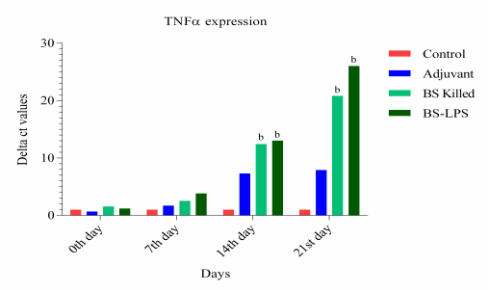
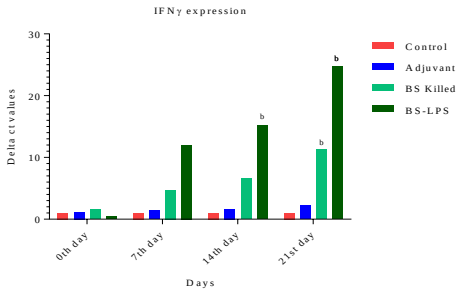
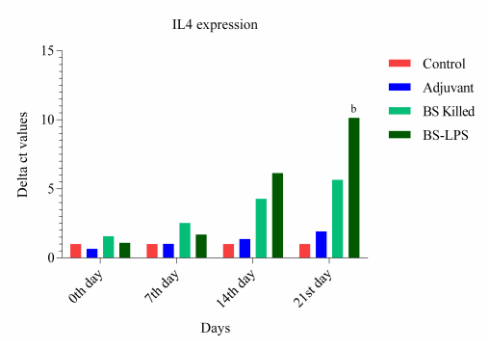
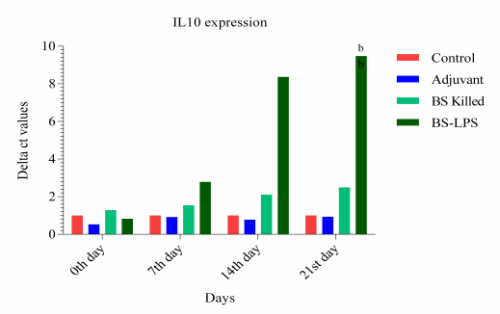


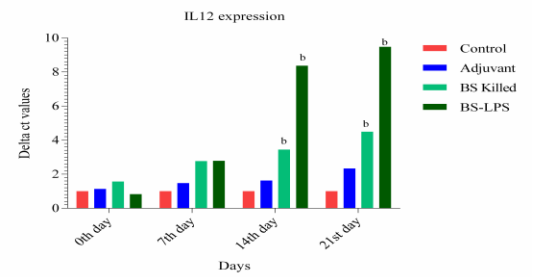
Fig.3 Relative Quantification of cytokine m-RNA expression levels post vaccination with *B. suis* killed and LPS vaccine by q-RTPCR











In *B. suis* vaccinated groups, the TNFα, IFNγ, IL4, IL10 and IL12 expressions were upregulated in BS-LPS treated animals with a p value of 0.0198, 0.0142, 0.0195, 0.0384 and 0.03 respectively. In BS-killed vaccine groups, though the expression was elevated, it was not as significant as noticed in the BS-LPS treated group.

Generally, initial response of *Brucella* spp. is to infect the neutrophils followed by infection of the macrophages, the cells of innate immune response. Also, *Brucella* can infect both phagocytic and non-phagocytic cells, in vitro and in vivo. *Brucella* consists of lipopolysaccharides which are less virulent and a dose of more than 10-fold is required to generate an immune response, in vivo. As a mechanism of primary immune response, the entering brucella is engulfed by macrophages. This key bactericidal response is primarily initiated by two cytokines, namely, gamma interferon (IFNγ) and tumor necrosis factor (TNFα), specifically, the response of CD4+, CD8+, and γδ T cells mediated production of IFNγ activate the bactericidal response within macrophages and minimize the favorable conditions for intracellular survival of *Brucella*. In addition, IL4, IL10 and IL12 also

play major role against *Brucella* infection. Generally, during brucella infection, IL4 expression levels are found to be lower. Also, in vaccinated or unvaccinated conditions, IL4 level did not show a significant change as previously reported. This also implies that IL4 does not contribute more towards the immune response mechanisms during Brucella infection (Pasquali *et al.*, 2001). It was previously reported that IL10 expression during vaccination levels were found to be higher and does not downregulate the expression of IFN γ. This implies that the role of IL-10 in immune response is limited to offset the Th1 cytokines production rather an exaggerated proinflammatory response (Pasquali *et al.*, 2001). Further, IL12, a cytokine which plays crucial role in activating interferon producing NK and T helper cells leads to antibacterial response mechanism (Zhan and Cheers, 1995; Siadat *et al.*, 2015).

In the present study, IL4 expression was found to be elevated in contrast to what has been already reported. Also, there is evidence suggesting elevated expression of IL-10 is detrimental to brucellosis. However, regulation of cytokines collectively plays a significant role in immune response mechanisms when compared to a single cytokine. Also, it has been reported that brucella-LPS as poorly endotoxic and does not trigger considerable innate immune response which is in contrast to the present study (Moreno *et al.*, 1981; Lapaque *et al.*, 2005, 5).

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