Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Differential Stimulatory Activities of Smooth and Rough *Brucella abortus* Lipopolysaccharide in Murine Macrophages

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ARTICLE HISTORY

Received: September 10, 2011
Revised: October 20, 2011
Accepted: January 12, 2012

Key words:
Brucella abortus
Cell stimulation
Lysozyme
Nitric oxide
Reactive oxygen species

ABSTRACT

Brucella abortus lipopolysaccharide (LPS) was isolated and purified from rough (RB51) and smooth (S2308) strains of *Brucella*. The LPS preparations were used to treat murine (RAW 264.7) macrophages in order to study their differential effects. Treated macrophages were tested by lysozyme release test (LRT), nitroblue tetrazolium test (NBT) and nitric oxide (NO) assay, respectively. Rough *Brucella* LPS induced significantly higher levels of lysozyme release, oxidative stress, and nitric oxide in murine macrophages than smooth *Brucella* LPS or combined LPS (rough + smooth LPS). These responses were dose-dependent. Macrophages treated with rough LPS were more *Brucella*cidal than those treated with smooth LPS. The minimal stimulation of murine macrophages by *Brucella* smooth LPS may provide basis for less active immune responses against smooth strains.

To Cite This Article: Akhtar R, YO He, CB Larson, ZI Chaudhary and MUD Ahmad, 2012. Differential stimulatory activities of smooth and rough *Brucella abortus* lipopolysaccharide in murine macrophages. Pak Vet J, 32(3): 339-344.

INTRODUCTION

Brucellosis is a zoonotic disease transmitted mainly by oral, respiratory, cutaneous, ocular and sexual routes. The etiological agent of brucellosis is a non-motile, non-spore forming and facultative intracellular bacterium of the genus *Brucella*. Ten *Brucella* species examined exhibit variation in their host specificities and pathogenicity. The frequency of brucellosis varies from country to country (Gul and Khan, 2007), but is higher in agrarian countries including the Middle East and South West Asia (Abubakar *et al*., 2012).

The outer membrane of *Brucella* cell wall contains a component called lipopolysaccharide (LPS) (Munir *et al*., 2010). *Brucella* LPS is a non-classical endotoxin that plays a pivotal role in host–*Brucella* interactions and various aspects of *Brucella* pathogenesis such as phagolysosome fusion, cytokine secretion, apoptosis and phagocytosis modifications. *Brucella* LPS is considered one of the macrophages stimulator other than cytokines, interferon-gamma (IFN-γ) and tumor necrosis factor (TNF-α), which either act independently or in combination to elicit macrophages activation. An evidence (Goldstein *et al*., 1992) suggests that amongst all the macrophages stimulators, *Brucella* LPS is of particular interest because it exhibits minimal endotoxic activity (10,000 times less toxic than *E. coli* LPS and 1000 times less toxic than *Salmonella typhimurium* LPS). This property makes *Brucella* LPS attractive for future usage in immune cells stimulation and as an adjuvant in future *Brucella* vaccines.

Most previous studies of *Brucella* LPS have emphasized extraction procedures, biological properties, anti-LPS antibodies detection and immunogenic mimicking of LPS epitopes however, the precise role of LPS in induction of anti-*Brucella* immunity is still unresolved. Therefore, to better understand the differential immunological role of *Brucella* smooth and rough LPS, it is crucial to study their differential stimulatory activities in treated macrophages. In this study, we used rough and smooth LPS preparations to study their synergistic or antagonistic effects.

MATERIALS AND METHODS

LPS extraction and lyophilization: Lipopolysaccharide from *Brucella* rough (RB51) and smooth (S2308) strains was extracted using a phenol extraction method (Bhattacharjee *et al*., 2002). The crude LPS were purified using the method of Lee and Tsai (1999).

Characterization of *Brucella* LPS: Purified LPS samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, 15% resolving gel (2.4 g urea, 1.25 mL resolving gel buffer, 5 mL acrylamide solution, 2 mL distilled water, 15µL 10% ammonium persulfate, 5µL TEMED) and 4.9% stacking gel (1.25 mL stacking gel buffer, 0.8 mL acrylamide solution, 2.9 mL distilled water, 50 µL of ammonium persulfate solution, 5µL TEMED) were used. LPS samples (50µL) were solubilized with equal amount of Laemmli sample buffer (Cat # 161-0737, Bio-Rad, Hercules, USA) at 100°C for five minutes. The wells were loaded with appropriate volumes of sample. Electrophoresis was carried out at 80 V and was terminated when the dye front reached the bottom of gel. Bio-Rad Precision Plus Protein Dual Color Standards (Cat #161-0374, Bio-Rad, Hercules, USA) was used as a standard for determination of molecular weights. The gel was fixed in a solution containing 10% glacial acetic acid, 2.5% glycerol, 40% methanol and 47.5% distilled water for 20 min. This step was followed by silver staining (Cat # 161-0448, 161-0462, 161-0463, 161-0464, Bio-Rad, Hercules, USA).

Cell culture: RAW 264.7 murine macrophages obtained from the American Type Culture Collection (ATCC# CRL-2278, Rockville, USA) were cultured, and maintained as described by (Baldwin and Parent, 2002).

Lysozyme induction in LPS-treated macrophages: Lysozyme release assay was performed by using a Petri plate method. Agarose gel 1% containing 0.5 mg/mL of dried *Micrococcus lysodeikticus* cells (Cat #LS008736, Worthington, USA) were suspended in a 0.1M phosphate citrate buffer pH 5.8. Twenty five milliliters of agarose were poured into each plate. After solidification holes of 35 mm in diameter were punched into the gel. Murine macrophages with a starting concentration of $2.5x10^5$ /mL were cultured at 37°C for 48 hrs in Dulbecco's Modified Eagle Medium (DMEM) (Cat#12430, Invitrogen GIBCO, Carlsbad, USA) in 24 well plates. Varying concentrations (0.02. 0.2, 2, 20, 200 µg/mL) of each *Brucella* LPS preparation (rough LPS, smooth LPS, and combination of rough and smooth LPSs (equal amounts) were respectively added into each well. The samples were incubated at 37°C for two hours with shaking. The incubation mixtures were centrifuged at 4°C at 3,000 x *g* for 10 min. The supernatants were stored at -70°C until assayed. Suitable aliquots of each supernatant 25 µL were loaded into the wells in the agarose plate. Each sample was assayed in triplicate. Commercially prepared egg white lysozyme (Cat# L-6876, Sigma, St. Louis, USA) of concentration ranging from 0.02 to 20 µg/mL were used as a standard curve. The plates were incubated at 37°C for 24 hrs. Each plate was scanned (HP Scanjet G4050) and radius of cleared zone around each well was measured with a ruler and the amount of lysozyme release calculated from the standard curve. The background (DMEM) value was subtracted from each calculated value (Rasool *et al*., 1992).

Reactive oxygen species (ROS) induction in LPS-treated macrophages: Murine macrophages were cultured in DMEM in 24 well plates at a starting concentration of $2.5X10^5$ /mL in 200 µL of 0.1% NBT in 0.15 M NaCl. Appropriate cell concentrations were added to each well and the plates incubated at 37°C for 60 min. Following incubation with varying concentrations of LPS (same range as in lysozyme assay) were added to each well and the samples were incubated again at 37°C for 30 min. Reactions were stopped by adding equal volume (500 µL) of 0.1N HCl with subsequent centrifugation at 4°C at 800 x *g* for 15 min. The pellets were dried at 37°C in the dark. Dioxane (1mL) was added to each pellet and incubated at 85°C for 20 min followed by centrifugation as described above. The optical density of the clarified supernatant was determined at 580 nm. *E. coli* LPS and purified superoxide dismutase (SOD) were used as positive and negative controls respectively (Yang *et al*., 2011).

Reactive nitrogen intermediates (RNI) induction in LPS-treated macrophages: Murine macrophages were cultured in 96 well plates at a concentration of $2.5X10^5$ cells /mL (2 mL each well) treated with varying concentrations of LPS (described above) and were incubated for 12 hrs. The LPS-treated macrophages were centrifuged and the resulting supernatants were mixed with an equal volume of Griess reagent (one part 0.1% naphthylethylenediamine dihydrochloride and one part 1% sulfanilamide contained in 5% phosphoric acid) in new plates. After 10 min at 25°C, the color change was determined at $OD_{540.}$ Each experiment was performed in triplicate. A standard curve was generated using increasing concentrations of sodium nitrite (0.3125 to 20 µg/mL) contained s in DMEM. Positive controls were run using macrophages pretreated with *E. coli* LPS. The NO production by iNOS was inhibited by L-NMMA for confirmation. The absorbance values of the standards, controls, and test samples was converted to ng/mL of nitrite by comparison with absorbance of sodium nitrite standards within a linear curve fit (Waters *et al*., 2002).

Determination of intracellular survival of *Brucella* in murine macrophages: Suitable aliquots of RAW 264.7 macrophages containing $2.5x10^5$/mL cells were placed in 24 well tissue culture plates (Cat# 3047, Becton Dickinson, Franklin Lakes, NJ, USA). The macrophages were stimulated with rough, smooth or a mixture of *Brucella* LPS (200 µg/mL each) as described previously s and incubated at 37°C in 5% CO_2 for 24 hrs. The macrophages were challenged with *Brucella* strain RB51 at a multiplicity of infection (MOI) of 100 for one hour. The medium was aspirated and the cells were rinsed three times with phosphate buffered saline. One milliliter DMEM containing 50 µg/mL gentamycin was then aliquoted into each well. At the end of chase periods of one, six and 24 hrs, the host cells were lysed with one milliliter per well of 0.1% Triton X-100. Trypticase soy agar (TSA, Difco, MI, USA) plates were inoculated in triplicate with 50 µL of each lysate using a 1:10 serial dilution and evaluated for cfu. Since rough strains grow slower, the cfu were counted after 4-5 days as described previously (Riley and Robertson, 1984).

Statistical analysis: Statistical analysis was made by student's *t* test for two-group comparison. A $P<0.05$ was considered to be statistically significant.

RESULTS

SDS-PAGE revealed that rough LPS had a banded pattern whereas smooth LPS presented a smeared pattern (Fig 1). The apparent molecular weight of each sample was calculated from its respective Rf values using a standard constructed with commercial molecular marker mixture. *Brucella* smooth LPS showed one band of approximately 81.2 kDa, while the *Brucella* rough LPS samples showed a total of s three bands. The first band approximated 95.4 kDa while the second band approximated 72.4 kDa. A third or last band at bottom of *Brucella* rough LPS approximated 70.7 kDa (Table 1a and 1b).

Each *Brucella* LPS preparation (rough, smooth and combined) stimulated RAW 264.7 macrophages differently and induced differing levels of lysozyme, ROS and NO. Rough LPS from RB51 strain evoked elevated lysozyme production in murine macrophages compared to *Brucella* smooth and combined (rough + smooth 1:1) LPS preparations. Approximately twice the amount of lysozyme was induced with *Brucella* rough LPS compare to *Brucella* smooth or combined LPSs ($P<0.05$). In contrast, the amount of lysozyme induced by *Brucella* combined LPS preparation was marginally higher than smooth LPS (Fig 2 and 3).

The present study shows in murine macrophages highest ROS induction by *Brucella* rough LPS, lower inductions by *Brucella* combined LPSs and lowest induction with *Brucella* smooth LPS ($P<0.05$) (Fig 4). It is interesting to note a similar pattern of increased stimulation by rough *Brucella* LPS was observed in terms of nitric oxide induction from the murine macrophages ($P<0.05$) while *Brucella* smooth LPS induced a lower levels of NO than that induced by the combined LPS preparation (Fig 5). The results of the cfu assay confirmed the increased stimulation of murine macrophages by *Brucella* rough LPS. At one hour post-infection when murine macrophages infected with *Brucella* were pre-treated with rough LPS, few viable *Brucella* were found (1.7×10^4 from murine macrophages). After six to 24 hours post infection, no bacteria were retrieved from macrophages, suggesting that a majority of the bacteria were phagocytosed and killed. Indirectly these results suggest that substantial activation of macrophages has occurred. In contrast, at one hour post infection, macrophages stimulated with smooth *Brucella* LPS retained viable *Brucella* cells (1.3×10^5 in murine macrophages). After six hours post-infection 9.5×10^4 *Brucella* cells survived and at 24 hrs post-infection, 5.5×10^4 viable *Brucella* were found. In contrast, pre-treatment of murine macrophages with a combination of smooth and rough *Brucella* LPS (1:1) resulted in a decreased number of viable bacteria (6.6×10^4, 2.9×10^4 and 1.8×10^4 cells at one, six and twenty four hours post-infection, respectively) as compared to the pre-treatment with *Brucella* smooth LPS. Of each of the three LPS treatments, rough LPS stimulated macrophages contained the least number of live *Brucella* after infection. A slight decrease in viable *Brucella* occurred during the first hour after which time the number remained stable (Fig 6).

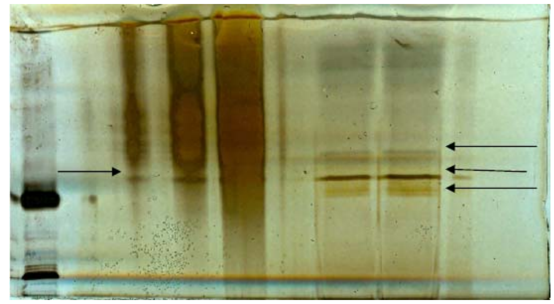

Fig 1: Silver stained SDS-PAGE profiles of smooth and rough *Brucella* LPS preparations. Lanes 1-3 contain smooth LPS 5µL, 15µL, and 25µL, respectively. Lanes 4-5 contain 5µL and 7µl rough LPS respectively. The "M" lane contains molecular weight markers.

A

B

C

Fig 2: Visualization of lysozyme released based using agarose plate assay. The LPS samples are (A) rough RB51 LPS, (B) smooth S2308, and (C) combined S2308+RB51 LPSs. The concentration of each sample was 200µg/mL in each well.

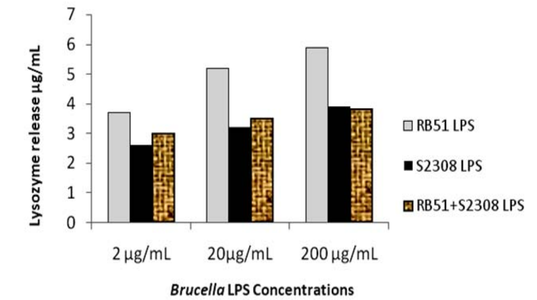

Fig 3: Differential induction of lysozyme in murine macrophages treated with *Brucella* LPS preparations. A higher level of lysozyme was induced by rough (RB51) LPS than smooth (S2308) or combined (RB51 + S2308) LPSs.

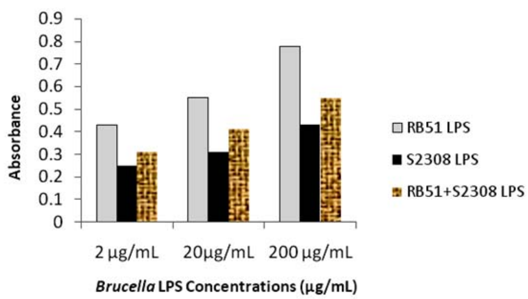

Fig 4: Differential induction of reactive oxygen species (ROS) in murine macrophages treated with *Brucella* LPS preparations. A higher level of NBT reduction observed with rough (RB51) LPS than smooth (S2308) or combined (RB51 + S2308) LPS. NBT= Nitroblue tetrazolium

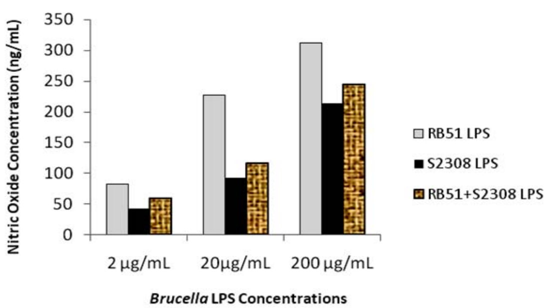

Fig 5: Differential induction of nitric oxide in murine macrophages treated with *Brucella* LPS preparations. A higher level of nitric oxide induced with rough (RB51) LPS than smooth (S2308) or combined (RB51 + S2308) LPS.

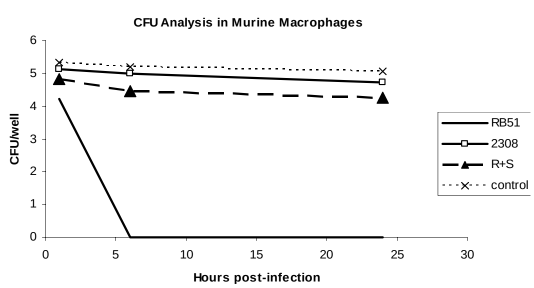

Fig 6: Kinetics of *Brucella* survival inside RAW 264.7 macrophages pre-treated with rough, smooth, or combined *Brucella* LPSs. Macrophages were infected with RB51 at a MOI of 100.

Table 1a: Rf Values and Log Molecular Weight of Marker Bands.

Molecular weight of marker band	Log molecular weight	Rf Value
250	2.39	0.17
100	2.0	0.49
75	1.8	0.61
50	1.69	0.76
37	1.56	0.86
25	1.39	0.89
20	1.30	0.93
15	1.17	0.95
10	1.0	0.98

Table 1b: Rf Values and Log Molecular Weight of Sample Bands.

Sample Bands	Molecular weight of marker band	Log molecular weight	Rf Value
Silver stained gel			
Lanes 1-3: Smooth LPS band	81.2	1.91	0.59
Lanes 4-5: Rough LPS band 1	95.4	1.98	0.53
Lanes 4-5: Rough LPS band 2	72.4	1.86	0.58
Lanes 4-5: Rough LPS band 3	70.7	1.85	0.67

DISCUSSION

The characterization of both *Brucella* rough and smooth LPSs revealed the structural differences between two. *Brucella* smooth LPS presented a smeared pattern that could be due multiplicity of its high molecular weight forms as compared to *Brucella* rough LPS/or possibly the involvement of associated proteins.

The characterization of *Brucella* rough and smooth LPSs was followed by the assessment of their relevant stimulatory activities separately and in combination. The stimulatory activity of each of the three types of *Brucella* LPS was measured in terms of lysozyme, ROS and RNI production in murine RAW 264.7 macrophages. Lysozymes are secretory enzymes of macrophages and their production increases on cell stimulation (Osman *et al*., 2010). This perspective of *Brucella* killing, macrophages stimulation would be beneficial since lysozyme has ability to break *Brucella* cell wall. In this study lysozyme release assay was used as a parameter to measure macrophage stimulation. There could be many reasons for enhanced induction of lysozyme by *Brucella* rough LPS as compared to *Brucella* smooth and combined LPSs. One possibility is the absence of the LPS O-chain. *Brucella* smooth strains have a complete LPS (all three domains including O-chain, polysaccharide and lipid A), while the attenuated rough *Brucella* strains lack a O-chain (Martin-Martin *et al*., 2011). The increase in macrophagic stimulation could be due to naked membrane determinants that may in turn be attributed to the absence of O-chain in rough *Brucella* LPS (Rittig *et al*., 2003).

On other hand, *Brucella* smooth LPS did not induce significant level of lysozyme in murine macrophages. This could be explained by the fact that *Brucella* smooth LPS is able to enhance cAMP production and subsequently inhibit phagosome-lysosome fusion that may be responsible for decreased lysozyme induction in murine macrophages. The observation that there is a reduction in induction of lysozyme also explains prolonged survival of *Brucella* smooth strains inside the phagocytes (Martirosyan *et al*., 2011). Lysozyme induction in cells stimulated with combined *Brucella* LPSs was lower than that observed with *Brucella* rough LPS, but greater than that observed with *Brucella* smooth

LPS. This response shows the intermediatory action of these combined LPSs or it may be concluded that individually rough LPS has more stimulatory activity that is decreased by the use of a combination of both rough and smooth LPS preparation. These results suggest an antagonistic effect of rough and smooth LPS on each other.

Macrophages when stimulated increase their utilization of oxygen (respiratory burst) and convert oxygen to reactive oxygen species (ROS). ROS damage the fatty acid side chains contained in *Brucella* cell wall. For defense against such molecules *Brucella* produced the enzymes (catalase, superoxide dismutase and peroxidase) that directly detoxify ROS and decrease its production. It is possible that rough *Brucella* LPS produces lower amounts of catalase and superoxide dismutase (SOD) than smooth LPS needed for the neutralization of ROS. This hypothesis is supported by the observations of Latimer *et al*. (1992) who found that *Brucella* smooth strain S2308 expressed elevated levels of catalase and SOD activity due to the presence of gene encoding for the Cu/Zn superoxide dismutase (SOD) in this strain that is mutated (deleted) in rough strain. Moreover, the differential NADPH oxidase and myeloperoxidase systems of *Brucella* rough and smooth strains may be responsible for their differential ROS production as the smooth *Brucella* strains inhibit “metabolic burst” accompanying phagocytosis by interference with myloperoxidase (Steele *et al*., 2010). Our results also suggest that *Brucella* LPSs (rough and smooth) and their respective strains behave in the same manner.

Increased macrophage stimulation by *Brucella* rough LPS was verified by increased nitric oxide (NO) production. This is one of the most important mediators of immune cells that exhibit potent anti-*Brucella* activity, which inhibits cellular respiration of *Brucella*. Nitric oxide synthase has three isoforms and iNOS is responsible for high output of NO production. The present studies revealed increased production of nitric oxide by *Brucella* rough LPS treated murine as compared to *Brucella* smooth and combined LPSs. It may be due to differential iNOS expression by smooth and rough LPS of *Brucella.* These results parallel those of Serafino *et al*. (2007) who found that NO production was higher in macrophages infected with rough RB51 strain as compared to smooth S2308 or S19 strains. Our results are also consistent with the findings of Gangtsetse *et al*. (2003) who extracted smooth LPS from *B. melitensis* and found that it did not induce eminent production of NO in RAW264.7 macrophages. Decreased induction of NO by smooth *Brucella* LPS in murine macrophages may be due to interaction of superoxide with RNI that may produce products other than nitrite.

Enhanced stimulatory activity of *Brucella* rough LPS demonstrated by increased production of lysozyme, ROS and nitric oxide was positively affirmed by a lower number of viable *Brucella* surviving in murine macrophages treated with *Brucella* rough, smooth and combined (rough + smooth 1:1) LPS. The cfu results indicate higher anti-*Brucella* activity of murine macrophages treated with *Brucella* rough LPS than smooth or combined LPS preparations s when subsequently challenged with *Brucella* rough strain (RB51). These findings are supported by the previous studies of Vassalos *et al*. (2009) who reported that *Brucella* rough strains undergo rapid internalization and ultimately increased killing as compared to *Brucella* smooth LPS. The lower stimulatory response by smooth *Brucella* LPS observed in present study may be due to the presence of differing stimulatory pathways for smooth and rough *Brucella* LPS pathways or the non-activation of P38 and ERK1/2MAP kinases pathways during macrophage infection with *Brucella* smooth strain (Pei *et al*., 2008). Chen and He (2009) suggested that prevention of macrophages apoptosis by *Brucella* smooth S2308 strains may be responsible for prolong survival of these strains inside macrophages. Rough RB51 strain may promote apoptosis and necrotic cell death and be along with host cells. However, our results are not in agreement to an independent study employing human monocytes (Rittig *et al*., 2003). These authors found that *Brucella* smooth and rough LPS preparations reduced the number of intracellular viable bacteria to a similar extent and the kinetics remained the same. The differences in these studies are probably attributable to species differences in cell stimulation.

In contrast to most of the previous studies that have compared cell stimulation with whole *Brucella* rough and smooth strains, our experiments have focused on analysis of the different roles in cellular metabolism of *Brucella* smooth and rough LPS preparations. Our results assist in elucidating the role of *Brucella* LPS as a legend in macrophage stimulation and intracellular pathogenesis of smooth versus rough strains. Lower levels of macrophages stimulation may be a key factor in the survival of *Brucella* smooth strains as compared to rough strains in macrophages. It is tempting to speculate that lower level of stimulation by smooth LPS may also contribute to *Brucella* virulence and resistance.

Conclusions: This study provides an experimentally supported explanation why *Brucella* rough, smooth and combined LPS preparations exhibit different properties and stimulated murine macrophages in different ways. These experiments show s greater potency of *Brucella* rough LPS in enhancing lysozyme, reactive oxygen species and nitric oxide production as compared to smooth LPS and the combination of both smooth and rough LPSs. This observation may be a key factor in revealing the survival of different *Brucella* stains within macrophages and as such may support the link between macrophages activation and *Brucella* killing. Since activated macrophages successfully deal with intracellular *Brucella* it is possible that LPS-mediated activation of macrophages may prevent infection by stimulating macrophages and other immune cells, increasing phagocytosis and ultimately host defense. The use of a combined *Brucella* LPS preparation had no dramatic effect on immune cell stimulation and its use as a mean of producing a successful multiple LPS vaccine may not produce fruitful results.

Acknowledgments: This project was supported by Higher Education Commission Pakistan.

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