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Comparative experimental study of *Brucella melitensis* and its lipopolysaccharide in mouse model infected via subcutaneous route of exposure

Abdinasir Yusuf Osman[a,b,*], Abdul Aziz Saharee[b], Faez Firdaus Jesse[b], Arifah Abdul Kadir[c]

[a] Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Pengkalan Chepa, 16100 Kota Bharu, Kelantan, Malaysia
[b] Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[c] Department of Preclinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

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ABSTRACT

Brucella melitensis is a major zoonotic pathogen in which lipopolysaccharide (LPS) is believed to play a major role in the diseases pathogenesis. To study the immunopathophysiological aspects, we established a mouse model experimentally infected with whole cell of *B. melitensis* and its lipopolysaccharide via subcutaneous route of exposure. Eighty four mice, BALB/c, both sexes with equal gender distribution and 6-8 weeks-old were randomly assigned into 3 groups. Group 1 (n = 36) were subcutaneoulsy inoculated with 0.4 mL 10^9 of *B. melitensis* while group 2 (n = 36) were subcutaneously challenged with 0.4 mL 10^9 of LPS. Group 3 (n = 12) was challenged subcuatneously with phosphate buffered saline and served as a control group. Animals were observed for clinical signs, haematological and histopathological analysis for a period of 24 days post-inoculation. Our results revealed that *B. melitensis* infected group demonstrated significant clinical signs and histopathological evidence than LPS infected group. However, both infected groups showed elevated levels of interleukins (IL-1β & IL6), antibody levels (IgM & IgG) as early as 3 days post-infection with predominance in LPS infected group. For hormone analysis, low levels of progesterone, estradiol and testosterone were observed in both *B. melitensis* and LPS challenged groups throughout the study period. Moreover, in *B. melitensis* infected groups, the organism was re-isolated from the organs and tissues of gastrointestinal, respiratory and reproductive systems; thereby confirming the possible transmission of the disease dynamics. Moreover, LPS stimulated significantly the innate and acquired immune system without significant systemic dysfunction suggesting the potentiality of the protective properties of this component as an alternative vaccine for brucellosis infection. This report is the first detailed investigation comparing the infection progression and host responses in relation to the immunopathophysiological aspects in mouse model after subcutaneous inoculation with *B. melitensis* and its lipopolysaccharide.

1. Introduction

Brucella melitensis is a major zoonotic pathogen with significant economic implications in animal industry worldwide [1,2]. The pathogen affects a wide range of mammalian hosts that include domestic herds and wild life species with special predominance in small ruminants [3,4]. The disease is manifested as fertility related issues involving abortion in pregnant females during trimester, retained placenta, weak offspring and metritis [5]. Rams experience orchitis, epididymitis and polyarthritis [6,7]. *Brucella melitensis* has long been believed to be the most virulent pathogen among the zoonotic species of the genus *Brucella* causing devastating epidemics with high morbidity and mortality in Mediterranean Basin, Middle East, Africa, Latin America and central Asia [8–10]. Infections are believed to occur by ingestion, inhalation or direct contact of the organism with broken skin or mucous membranes as well as venereal transmission and latent infection of neonates [6]. Higher incidence of *B. melitensis* is associated with environmental and management factors which include moist, humid conditions, high animal population density, extensive free grazing system and poor husbandry practice [2]. *Brucella melitensis* along with its LPS, a componenet that plays a major role of the pathogenesis, is generally believed to enter hosts through the subcutaneous, respiratory and/or oral routes leading to septicaemia in animals [11,12]. In the process of entering the host through the respiratory tract, both *B.*

* Corresponding author. Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Pengkalan Chepa, 16100 Kota Bharu, Kelantan, Malaysia.
E-mail address: abdinasir@umk.edu.my (A.Y. Osman).

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melitensis and LPS are believed to stimulate the humoral and cell mediated immune response [13]. Subcutaneously administered antigens have shown to elicit mucosal immune response in distant site such as respiratory, reproductive and urinary tracts [14,15]. In animals, the microflora along with the pH of stomach may provide adverse effects to antigens administered orally; hence, there is a need to clarify alternatively whether or not subcutaneously administered *B. melitensis* and its LPS establishe the infection and trigger the necessary humoral, cellular immune response in the host. Therefore, the aim of present study was to investigate the clinical signs, histopathological changes, cytokine and antibody immune response as well as the sex-related hormonal changes in mice experimentally infected with *B. melitensis* and its LPS via subcutaneous route of exposure.

2. Materials and methods

2.1. Ethical statement

The study was independently reviewed and approved by an ethical board of the Faculty of Veterinary Medicine, Universiti Putra Malaysia with reference no. UPM/IACUC/AUP-R049/2015.

2.2. Animals and experimental procedure

Eighty four clinically healthy mice of approximately 6–8 weeks of age were divided into three groups with equal gender distribution of 36 animals each in group 1 (*B.melitensis*) and group 2 (LPS). The animals in group 3 (Control) consisted of 12 mice with equal gender distribution. Animals were kept in individual cages, fed on pellets and drinking water was given *ad libitum*. Access to veterinary care was available at all times and the animal wellbeing was assessed regularly. Briefly, animals in *B. melitensis* group, single inoculum of 0.4 mL containing 10^9 of *B. melitensis* was subcutaneously challenged while animals in LPS group, a single dose of 0.4 mL containing LPS extracted from *B. melitensis* was subcutaneously inoculated. Animals in control group, however, a single dose of 0.4 mL phosphate buffered saline (PBS) of pH7 was used subcutaneously. All mice were observed at 12 h, 48 h, and 72 h and then at 7 days interval for clinical signs, histopathological evaluation, and blood analysis for antibody, cytokine and steroid hormone levels for up to 24 days post-infection.

2.3. Synchronization

Prior to the experiment, all females in each group were synchronized as previously described by Ref. [16].

2.4. Bacterial strain and inoculum preparation

A stock culture of *B. melitensis*, an epidemic strain that previously isolated from an outbreak in Malaysia, was used to prepare the inoculums. The inoculum was prepared according to the procedures described previously by Refs. [5,17].

2.5. LPS extraction from B. melitensis

The lipopolysaccharide (LPS) of *B. melitensis* was extracted using Intron Biotechnology® LPS Extraction Kit. All the guidelines and instructions of the kit were followed as described by Ref. [18].

2.6. Route of exposure

2.6.1. Subcutaneous inoculation

Challenges were conducted by inserting the appropriate needle into subcutaneous tissues on the plantar surface of the left hind footpad and 0.4 mL of the suitable suspension of *B. melitensis*, LPS or PBS were injected slowly in the groups 1–3, respectively. This mode of infection for the purpose of quality control as it often represents a rapid, inexpensive, and simple method of parenteral substance administration. Substances administered subcutaneously often are absorbed at a slower rate compared with other parenteral routes, providing a sustained effect.

2.7. Clinical observation

All mice were observed at 12 h, 48 h, 72 h and then at 7 days interval for clinical signs for up to 24 days post-infection. The clinical signs were scored based on the scoring system described earlier for experimental infections [19] as follows: appetite (mild, moderate and severe) 1–3; eye discharge (mild, moderate and excessive) 1–3; fur condition (mild, moderate and severe) 1–3. A total clinical score was calculated for each observational time for each mouse from which mean clinical score was derived and then mean total clinical score for each group at each observational time was subsequently derived.

2.8. Histopathological examination

Representative samples that included liver, spleen, lungs, heart, kidneys, jejunum, ileum, cecum, proximal and distal portions of the colon, testes, epididymis, ovary, uterine, brain, and pituitary gland, were examined. All tissue samples were preserved in neutral buffered 10% formalin except brain and pituitary gland tissue in which 40% of formalin is used. For histologic analysis, tissue sections were processed in an automatic tissue processor (Leica TP 1020, Germany) embedded in paraffin wax, sectioned with rotatory microtome (Leica Jung Multicut 2045, Germany) at 4 μM thick and stained with Harris' hemotoxylin and eosin (H&E). The stained sectioned were observed through photomicroscope (Nikon Eclipse 50i, Japan) and the histologic lesions recorded and overall grading for severity was assigned. No significant lesion = o; mild lesion = 1; moderate lesion = 2 and severe lesion = 3.

2.9. Bacteriology

Representative samples of the different organs collected aseptically from the whole cell treated group were subjected for cultural evaluation. The cultures were incubated at 37 °C and examined for appearance of typical colonies of *B. melitensis*. These samples were subjected for further confirmation using Polymerase Chain Reaction (PCR) technique [20].

2.10. Cytokine and antibody quantification

Blood was collected from mice of various groups and centrifuged for 10 min at 800 μg at 4 °C. The sera were then frozen and stored at −20 °C until further examination. The serum levels of interleukins (IL-1ß & IL-6) and antibody levels (IgM & IgG) were measured using a Mouse Elisa kit, 96T (QAYEE) according to the manufacturer's guidelines with all steps carried out at room temperature.

2.11. Hormone analysis

Beckman Coulter Immunotech Radioimmunoassay kits were used for the *invitro* determination of progesterone and estradiol concentration in the sera of the experimental units. The extraction of the sex-related hormones was performed according to procedures published earlier by the same authors [17].

2.12. Statistical analysis

All data were analyzed statistically as well as biologically. Evaluation of the effects was performed using the following model: $\gamma = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$ where ε = residual error. Significantly