



Immune response strategies of *Brucella melitensis* and their antigens in rats

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Abstract

Brucella melitensis is an intracellular bacterium and is the main brucella species that cause abortion and placenta retention in sheep and goats. It has many mechanisms to evade the immune response. The current study aimed to investigate *Brucella melitensis* strategies for producing immune responses in rats after challenging the bacterium. For this purpose, live and killed *Brucella melitensis* REV1 strain was given to rats subcutaneously, and immunological markers like TLR2, TLR4, IFN- γ , and anti-brucella antibodies were determined. The results showed that the level of immunological markers like TLR2 and TLR4 did not significantly increase in rat groups inoculated with live *Brucella melitensis*, while it increased in the rats' groups vaccinated with the sonicated *Brucella melitensis*; also, the results showed an increase in the level of IFN- γ and anti-brucella antibody titers in all animal groups. The study concluded that the inoculation with killed bacteria and REV1 could protect the animals against challenging doses, as seen when the groups were inoculated with challenge dose of the bacterium.

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Introduction

Brucella is gram negative intracellular zoonotic bacteria, that causes many diseases to human and animals (1). *Brucella melitensis* is the main *Brucella* species that caused abortion and placenta retention in sheep and goats (2). Live attenuated and killed vaccines were used for the protection of animals and humans. REV1 vaccine which is prepared from live attenuated *Brucella melitensis* strain is the main vaccine types used for sheep and goat's protective against brucellosis (3). The protective effects of the REV1 vaccine are due to the stimulation of T- helper and T-cytotoxic with their mediators (3,4). Both cellular and humoral immune responses were activated after *Brucella* infection or vaccination with a live and killed vaccine (5). Innate immunity plays an important role in host protection against *Brucella* by activation of neutrophil and NK cells. Both macrophages and dendritic cells play an important role as

antigens presenting cells which consider the key to immune responses (6,7). Toll-like receptors (TLRs) recognized pathogen associated molecular patterns (PAMPs), which lead to the activation of dendritic cells and produce proinflammatory cytokines (8). They are 13 types of TLR in rats. Bacterial compounds such as LPS, flagella and lipopeptide will be recognized by TLR2, TLR4, TLR5, and TLR9 (9). Both TLR2 and TLR4 can recognized bacterial components and are considered triggers to adaptive immune response. *Brucella* can evade immune system mechanism by many pathways this activity prevents both TLR2 (outer membrane proteins) and TLR4 (lipopolysaccharide) from activation (10,11). Cellular immune responses against *Brucella* and other intracellular pathogen involve activation of CD4 and CD8 T-cells, host Protection occurs by Th1 mediated, interferon- γ (IFN- γ) which is an important cytokine of Th1 that stimulate of macrophages (12). The Aim of the study was to investigate the ability of live and

killed *Brucella melitensis* and REV1 to produce immune response and to protect the animal from infection.

Materials and methods

Ethical approve

Experiments were performed on laboratory animals after obtaining the approval of the Scientific Committee at the College of Veterinary Medicine, University of Tikrit.

Pathogenic *Brucella melitensis* isolates

The pathogenic *Brucella melitensis* isolates were isolated from aborted goats, bacterial isolation was conducted by using of Trypton soya broth (Himedia, India) as transport media and brucella basal agar (Biolive, Italy) was used and 5% of sterile horse blood was added to the agar and one ampule of Brucella selective supplement (Himedia, India) was added for each 500 ml of media for the isolation of *Brucella melitensis*, each sample inoculated in two agar plates, one inoculated aerobically and other inoculated un aerobically, gram stain and biochemical tests were applied according to Alton *et al.* (13). The isolated consider as *Brucella melitensis* if grow aerobically and un aerobically as pearl white colonies, appear as gram negative coccobacilli, positive to oxidase, catalase and urease test (13).

Vaccine isolates

Brucevac, Jovac, Jordan which contain 5×10^8 CFU/ml viable live attenuated *Brucella melitensis* Rev1 strain, according to results of LD₅₀.

Whole Sonicated *Brucella melitensis*

Prepared by using of ultra-sonicator (Karl Klob, Germany) at 12 Peak with 2 minutes intervals between them, for 30 minutes in a cold environment (ice) according to Mitove *et al.* (14).

Experimental animals

Adult male rats at age of three months and weight 150-180 grams were housed in the animal house, Tikrit University, College of Veterinary Medicine.

Experimental animals grouping

four groups, 8 rats in each group. First group, were inoculated subcutaneously with pathogenic *Brucella melitensis* in a dose of 0.25 ml from bacterial suspension of concentration 10^5 CFU/ml. Second group, were inoculated subcutaneously with REV1 in a dose of 10^5 CFU/ml. Third group, were inoculated with 2 milligrams of Whole Sonicated *Brucella melitensis* (after dissolving with phosphate buffer saline). Fourth group, negative control

inoculated with 1ml of phosphate buffer saline. After 21 days, 2-3 ml of blood were collected and serum separated to determine of immunomarkers such as TLR2, TLR4 and IFN γ and antibodies titer.

Immunological marker

TLR4 determined in serum by using of Rat toll-like receptor 4 (TLR4) Sandwich ELISA Kit (CUSABIO- USA) the results calculated by OD and standard curve method for evaluation. TLR2 determined in serum by using of Rat Toll Like Receptor 2 (TLR-2) Sandwich ELISA Kit- (MYBIOSOURCE- USA) the results calculated by OD and standard curve method for evaluation. Rat IFN-gamma quantizing Sandwich ELISA kit (BIO TECH- USA) the results calculated by OD and standard curve method for evaluation.

Antibody titer

Anti-*Brucella melitensis* antibodies titer was determined by Tube agglutination test by use of kit of *Brucella melitensis* antigen (Febrile Serodiagnostic - LINEAR CHEMICALS- SPAIN).

Challenge dose

After 30 days of the first treatment, all animal groups were inoculated subcutaneously with 1ml of 10^6 CFU/ml pathogenic *Brucella melitensis*, then the animals were observed for 14 days and any clinical sings or death were recorded.

Statistical analyses

Conducted by version 9.1 of the SPSS program. one-way Variance Analysis (ANOVA) were used, least significant difference (LSD) test to detect the differences among means. $P < 0.05$.

Results

The result of the ELSA test shows significant increase in levels of TLR2 and TLR4 in the group Vaccinated Whole Sonicated *Brucella melitensis* in compare with other groups as in table 1. Also, the current study showed a significant increase in IFN- γ and antibody titer in all treated groups in comparison with the control group. The result of challenge dose in rats showed that high ability of REV1 vaccine and previous infection in protection of rats against recurrent infection with *Brucella melitensis* as appeared in group 1 and 2, since only 12.5% of animals dead in case of group 2 (inoculated with live bacteria), while no dead were seen in group 1 (REV1 group).

Table 1: TLR2, TLR4, IFN- γ and antibodies titer in the studied groups

Animal groups	TLR2	TLR4	Gamma interferon	Antibody titer
Group 1	22 \pm 2.76b	101 \pm 3.1b	680 \pm 39.1d	400 (640-160) d
Group 2	22 \pm 3.5b	98 \pm 2.7b	530 \pm 24.7c	260 (320-160) b
Group 3	29 \pm 1.9c	118 \pm 2.9c	470 \pm 38.7b	320 (640-160) c
Group 4	20 \pm 2.51a	94.2 \pm 7.1a	300 \pm 21.3a	0

Difference letters refer to significant difference in $P \leq 0.05$.

Discussion

Many studies mentioned that bacteria become resistant to many commonly used antibiotics (15,16); therefore, it is important to protect animals from infection through vaccination. Brucellosis is an endemic disease in many countries of the Middle East including Iraq, despite the high attempts to control the disease in animals using vaccination programs (17,18). In the current study, a local bacterial isolate was used in order to induce an immune response in laboratory animals in addition to the REV1 vaccine. The result showed significant increase in levels of TLR2 and TLR4 in group Vaccinated Whole Sonicated *Brucella melitensis* when compared with other groups. These results agreed with Solanki *et al.* (19). Detection of Brucella by Toll-like receptors (TLRs) is an essential activation step in innate immune response, *Brucella* escaped from innate immunity mechanisms, by using hydrophobicity phenomena of *Brucella* cell envelope, these phenomena delayed or reduced inflammatory response by minimizing pattern recognition receptors (such as TLR) stimulation of the host (20,21). TLR2 and TLR4 are involved in DC activation and maturation and the absence of TLR2 and 4 significantly reduced the expression of the maturation markers in DC (22). The current study noted that TLR2 and TLR4 increase only in the group that is immunized with whole Sonicated *Brucella melitensis* which lose their ability to escape from innate immunity mechanisms. The lower antigenicity of *Brucella* smooth lipopolysaccharides (such as *Brucella melitensis*) to induce NOS-2 and COX-2 leads to lower activation ability of monocyte chemoattractant protein-1 (MCP-1) and macrophage-inflammatory protein that's lead to reduce the production of pro-inflammatory cytokines (23,24). The highly protective ability of REV1 in comparison with Sonicated antigen due to the ability of the REV1 vaccine to stimulate both humeral and cellular immune responses (25). The presence of one animal out of 8 animals in the control group not dying, may be due to individual variation in the ability of immune response of animals.

Conclusion

Brucella melitensis has ability to escape from innate immune response, so that TLR2 and TLR4 levels not

increase, live and killed *Brucella melitensis* caused an increase in antibody and IFN- γ levels and protect experimental animal when challenged with pathogenic *Brucella melitensis*.

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Conflict of interest

The authors declare that there is no conflict of interest

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استراتيجيات الاستجابة المناعية للبروسيللا المالطية ومستضاداتها في الجرذان

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الخلاصة

تعد البروسيللا المالطية من بكتيريا الداخل خلوية وهي النوع الرئيسي من البروسيللا التي تسبب الإجهاض واحتباس المشيمة في الأغنام والماعز. تمتلك العديد من الآليات للتهرب من الاستجابة المناعية. هدفت الدراسة الحالية إلى التحقيق في استراتيجيات البروسيللا المالطية لإنتاج استجابة مناعية في الجرذان بعد التحدي مع بكتيريا البروسيللا المالطية. لهذا الغرض تم إعطاء البروسيللا المالطية عنرة ريف ١ الحية والمقتولة لمجاميع الفئران تحت الجلد، تم قياس الواسمات المناعية مثل المستقبلات الشبيهة بالنول ٢ و ٤ والإنترفيرون كاما والأجسام المضادة للبروسيللا لدى هذه المجاميع. أظهرت النتائج أن مستوى الواسمات المناعية مثل المستقبلات الشبيهة بالنول ٢ و ٤ لم يرتفع في مجموعات الجرذان الملقحة ببروسيللا المالطية الحية، بينما زادت في مجموعات الجرذان التي تم تلقيحها بالبكتيريا المعرصة للأمواج فوق صوتية، كما أظهرت النتائج زيادة في مستوى الإنترفيرون كاما ومضاد البروسيللا في جميع مجموعات الحيوانات. توصلت الدراسة إلى أن التلقيح بالبكتيريا المقتولة لعنرة ريف ١ يمكن أن يحمي الحيوان من العدوى كما واضح عند تلقيح المجموعات بجرعة التحدي.