Brucella melitensis Rev.1 live attenuated Vaccine and its DNA induced IFN-γ and anti-ds DNA antibodies production in rats

Nawal Utba* **, Raghad Qays**
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Abstract:
Brucellosis is one of the five common bacterial zoonoses in the world caused by organisms belonging to the genus *Brucella.* Immune recognition of bacterial infection may contribute to cytokine, as well as antibody production that are characteristic of innate and adaptive responses. In this study, the presence of attenuated live *Brucella melitenses* Rev1 bacteria or its DNA induced the immune system to produce IFN-γ and anti-ds DNA antibody. In respect to IFN-γ released, the *B. melitensis* Rev 1 attenuated live vaccine was able to stimulate the immune system more than the DNA (P≤0.05). Such finding could be attributed to the whole attenuated bacteria that have immunogenic factors other than the DNA like cell wall component and outer membrane. On the other hand, the *B. meliensis* Rev 1 DNA activated the B cell to secret anti-ds DNA antibodies significantly higher (P≤ 0.05) than live attenuated vaccine, and the level of antibodies was increased to parallel the concentration increases of injected DNA.

Keywords: *Brucella melitensis* Rev 1, IFN-γ, anti-ds DNA antibodies.

اللقاح المضعف لبكتريا*Brucella melitensis* Rev 1 و الدنا المستخلص منها يحفز انتاج الانترفيرون – كاما والاضداد للحمض النووي في الجرذان

نوال عتبه* **، رغد قيس**
قسم علوم الحياة، كليه العلوم، جامعه بغداد، بغداد، العراق.

الخلاصه.
تعدالحمى المالطيه واحدة من خمسةامراض بكتيرية شيوعا في العالم وهي ذات منشاحيواني والتي تسببها الكائنات التي تنتمي إلى جنس البروسيلا . ويمكن ان يساهم التعرف المناعي للاصابه البكتيرية في انتاج الحركيات الخلويه والاضداد عن طريق تحفيز الاستجابة المناعيه الفطرية والمكتسبه. وفي هذه الدراسة حقنت البكتريا المضعفه*B. melitensesf* Rev1 و الحمض النووي لتلك البكتريا في الجرذان وحفز جهازها المناعي لإنتاج الإنترفيرون كاما والاضداد للحمض النووي. فيما يتعلق الإنترفيرون كاما، كان اللقاح المضعف ل *Brucella melitensis* Rev 1 قادر على تحفيز الجهاز المناعي معنوي لانتاج الانترفيرون كاماأكثر من الحمض النووي (الدنا) للبكتريا (P ≤ 0.05). ويمكن أن يعزى هذا الاستنتاج لان البكتيرياالكامله المضعفه لديهاعوامل أخرى بالاضافه الى الحمض النووي مثل مكونات جدار الخلية و الغشاء الخارجي وهذه يمكن ان تحفز الاستجابه المناعيه . من ناحية أخرى، فإن الحمض النووي للبكتريا المضعفه *B. meliensis* Rev 1

*Email:anooutba2014@Yahoo.com

نشط الخلايا البائية لانتاج الاضداد للحمض النووي أعلى معنويا ($P \leq 0.05$) بكثير من اللقاح المضعف ، وازداد مستوى الاضداد مع زيادة تركيز الحمض النووي للبكتريا المحقون في الجرذان.

Introduction:

Brucellosis is a zoonotic disease that is caused by *Brucella* species with more than 500,000 new cases reported annually. Four species; *Brucella melitensis*, *Brucella abortus*, *Brucella suis* and *Brucella canis* are currently known to be pathogenic to humans. In animals, brucellosis can have a huge economic impact, since infection can lead to abortions, stillbirths, and loss of fertility in livestock [1]. *Brucella* spp. are Gram-negative coccobacilli, aerobic, urease positive, non-motile bacteria, which cause brucellosis in humans and in a variety of animal species [2]. Brucellosis in humans is a debilitating disease characterized by fever, sweats, and aches. In approximately 5% of cases it can be fatal when complications, usually endocarditis arise. The illness can last a number of weeks, and even with antibiotic treatment, relapses can occur [3].

Brucellosis is common in developing countries and areas without effective animal disease control policies. In these countries, the microorganisms are usually transmitted through ingestion, inhalation, or direct skin contact. Unpasteurized milk is a common source of infection, as is inhalation from carcasses among abattoir workers [4]. The Middle East has traditionally been considered as an endemic area. Data were recently made available for the incidence of human brucellosis in Iraq, underlining the huge endemicity of the disease in this region, despite ongoing attempts to control both animal and human disease [5]. Iraq is one of the countries with an annual incidence of 8-50 cases/10^5 population [6]. There are several live attenuated vaccines licensed for use in animals. Of these, the most widely used are *B. melitensis* Rev.1and *B. abortus* S19 or RB51. These vaccines are unsuitable for use in humans since they are insufficiently attenuated and still cause disease [7].

Brucella melitensis Rev 1 is a vaccine effective against the brucellosis of sheep and goat caused by *B. melitensis*; the commonest source of human infection. However, Rev 1 carries a smooth lipopolysaccharide with an O-polysaccharide that elicits antibodies response in vaccinated animal [8]. This study aimed to compare between the effectiveness of *B. melitensis Rev*.1 a live attenuated Vaccine and *Brucella melitensis Rev*.1 DNA in inducing the immune system to produce IFN-γ and Anti-ds DNA antibodies.

Materials and methods:

Aborvac-R Lamb™ *Brucella melitensis Rev*.1 a live attenuated Vaccine from Vetal company (Turkey) was used in this study. Freeze-dried vaccine was diluted with vaccine diluents according to the manufacturer instructions with concentration of 1-3 × 10^9 bacteria/ml.

DNA Extraction:

Brucella melitensis Rev.1 DNA were extracted and purified from Aborvac-R Lamb *B. melitensis* Rev.1 vaccines using a Wizard genomic DNA purification kit (Promega Corporation) according to the manufacturer instructions. The quantity and quality of DNA were determined by evaluation of the ratio at OD_{280} to OD_{260}. The DNA concentration also was determined by reading the OD at 260 nm [9].

Laboratory animals:

Swiss white male rats aged 6-8 weeks were the laboratory animals in this work. They had free access to water and food. The animals were randomly distributed into three groups; A, B, and C.

Injection protocol:

Group A injected with 0.1 ml of two different concentrations (four rats for each concentration) of *B. melitencesis* Rev.1 attenuated vaccine; 0.05 × 10^9 bacteria/ml and 0.1 × 10^9 bacteria/ml designated as groups A1 and A2, respectively. In regard to group B, *B. melitencesis* Rev.1 DNA was dissolved in Tris-EDTA buffer at two different concentrations; 9.13µg/ml and 45.6µg/ml. Each concentration was injected subcutaneously in four rats; hence each subgroup was labeled as B1 and B2, respectively. Group C was injected with 0.1 ml of TE buffer; therefore it was considered as control group. Thereafter, sera were collected from all animal groups after one day and 14 days of injection.

Rat IFN-γ and Anti-ds DNA antibodies level estimation:

Rat sera were tested for the concentration of anti-ds DNA Abs and IFN-γ following the manufacturer instructions of EIAab Rat Anti-ds DNA (China) and Rat IFN-γ immunoassy kits (R&D system, UK), respectively. These immunoassay kits allow *in vitro* quantitative determination of rat anti-ds DNA Ab concentrations and IFN-γ concentrations in serum. Each assay was performed in duplicate.

Statistical analysis:

Data are presented as mean ± standard deviation. T- Test was employed for data analysis using Microsoft EXCELL 2007 application. Differences were considered significant when $P \leq 0.05$.

Results and discussion

In this study, the attenuated live bacterial vaccine and its DNA stimulated rat immune system to produce IFN-γ in serum table 1 and anti-ds DNA antibodies table 2.

Table 1-IFN-γ concentrations in rats group after injected with *Brucella melitensis* Rev.1 live attenuated Vaccine and *Brucella melitensis* Rev.1DNA.

Rat groups	Mean ± SD pg/ml After one day	Mean ± SD pg/ml After 14 days	P value
Group **A1** injected with 0.05×10^9 bacteria/ml	32.92± 5.64	482± 55.86	P=0.0036
Group **A2** injected with 0.1×10^9 bacteria/ml	41.31± 11.17	732.33± 144.53	P=0.01
Group **B1** injected with 9.13µg/ml bacterial DNA	397.98± 85.2	474.88± 37.39	P=0.181
Group **B2** injected with 45.6 µg/ml bacterial DNA	411.55± 124.8	527.56± 57	P=0.177
Group **C** injected with 0.1 ml TE buffer	18.82 ±0.57	17.85 ± 3.2	P=0.276

SD= standard deviation**.** $P \leq 0.05$ between A, B, C groups.

The concentration of IFN-γ between one day and 14 days were highly significance increased in groups A1 and A2 while in groups B1, B2 and C were not significant which mean that time effected on IFN-γ concentration in group A may be in this time the bacterial cell was destroy and be more immunogenic**.** Otherwise, the IFN-γ concentrations were significantly higher in rats injected with vaccine (groups A1 and A2) and bacterial DNA (groups B1and B2) than control group (group C) after one day and 14 days of injection. Also the concentration of INF-γ in each groups A1and A2 increased significantly than both group B1 and B2 after one day. However, IFN-γ concentrations are significantly increased in-group A2 than B1 and non-significant between A1 with B1, A1 with B2 and A2 with B2 after 14 days.

We can conclude that the attenuated live vaccine was able to stimulate the immune system more than the DNA. Such finding could be attributed to the whole attenuated bacteria have immunogenic factors other than the DNA like cell wall component and outer membrane. However, longer period (14 days vs. 1 day) stimulated of the immune system to recognize the bacteria infection or its derivatives and released the cytokines. Tavakoli *et al*. [10] mentioned that the spleenocytes stimulated by *B. melitensis*, and *B. melitensis* strain Rev1, as attenuated live vaccine DNAs, induced significant quantities of IFN-γ on day 5 in comparison to control; nevertheless, IFN-γ increased insignificantly after one day. Yamamoto *et al*. [11] reported that bacterial DNA could induce murine NK cells to produce IFN- γ and attributed this effect to palindromic sequences present in bacterial DNA.

On the other hand, the Anti-ds DNA antibodies concentrations were significant differences ($P \leq 0.05$) between one day than 14 days table 2; in group A2 significantly higher and in group B2 significantly lower. Furthermore, there were significant differences ($P \leq 0.05$) between A1, A2, and B1, B2 groups than group C after one day and 14 days which means that the presence of attenuated live *B. melitenses* Rev1 bacteria or its DNA induced the immune system to produce Anti-ds DNA Ab. Moreover, between groups we found that there were significant differences ($P \leq 0.05$) in Anti-ds DNA Ab concentration of A1 than A2, B1and B2 after one day, between A1than B2 after 14days, between A2 than B1and B2 after one day, between A2 than B2 after 14 days and between B1 than B2 after one day and 14 days.

Table 2-Anti-ds DNA antibodies concentration in rats group after injected with two different concentrations of *B. melitensis* Rev.1 live attenuated Vaccine and *B. melitensis* Rev.1DNA.

Rat groups	Mean ± SD After one day IU/ml	Mean ± SD After 14 days IU/ml	P value
Group **A1** injected with 0.05×10^9 bacteria/ml	3.145 ± 0.0919	7.805± 2.538	P= 0.060
Group **A2** injected with 0.1×10^9 bacteria/ml	4.695± 0.38	9.48± 1.8	P= 0.035
Group **B1** injected with 9.13µg/ml bacterial DNA	19.06± 2.24	13.59± 2.54	P= 0.075
Group **B2** injected with 45.6 µg/ml bacterial DNA	34.325± 2.70	25.37± 1.04	P=0.022
Group **C** injected with 0.1 ml TE buffer	1.69± 0.26	1.53±0 .014	P= 0.24

SD= standard deviation**.** P≤ 0.05 between A, B, C groups.

The result indicates that *B. meliensis* DNA activated the B cell to secreted antibodies, and the amount of Antibodies increased with the increases concentration of injected DNA. Also, it induced the production of antibodies more than the vaccine. Our result was agreed with Al-Mathkhury et *al*, [12] findings that the rats' immune system was stimulated to produce anti-DNA antibodies after intraperitoneally injection with bacterial DNA and not agreed with the results obtained by Deng and Tarkowski [13] as they stated that serum levels of antibodies specific for ds-DNA and ss-DNA were low in *Staphylococcus aureus* bacterial DNA injected mice, and in comparison with the autoantibody levels of control mice, showed no difference.

The recognition of bacteria as nonself agents by mammalian cells is key in mounting an innate response to control infection. Several bacterial antigens, known as pathogen-associated molecular patterns (PAMPs), are sensed as "nonself" molecules by host immune cells, using receptors of the innate immune system. PAMPs are, for the majority, cell-wall molecules. Some PAMPs are found in both Gram-negative and Gram-positive bacteria, (lipopeptides, peptidoglycan, flagellin, and bacterial DNA). Others are specific either for Gram-negative bacteria (LPS), Gram-positive bacteria (lipoteichoic acid), or mycobacteria (lipoarabinomannan) [14].

As intracellular organisms, protection against *Brucella* infection requires cell-mediated immunity, which includes $CD4^+$ and $CD8^+$ T lymphocytes, Th1-type cytokines such as IFN-γ, Tumor necrosis factor- alfa (TNF-α), activated macrophages and dendritic cells. Therefore, host control of infection requires a set of cells and factors which together promote a complex response against *Brucella*. $CD8^+$ T cells have the predominant role for optimal protection against *Brucella* infection. This protection can be performed by a type 1 cytokine profile production, mainly IFN-γ, and lysis of *Brucella*-infected macrophages. Lysis of these macrophages releases the bacteria to the extracellular milieu enabling uptake by other activated macrophages in an IFN-γ-rich microenvironment. These cells presents augmented anti-brucellae mechanisms and are able to destruct the pathogen, inhibiting *Brucella* spread [15].

Intensive interest is being directed towards the use of bacterial derivatives which promote Th1-like responses. DNA is an essential macromolecule whose immunologic properties vary with sequence heterogeneity. While mammalian DNA is immunologically inert, bacterial DNA has potent immunological properties. It appears to function as one of the "danger signals" to trigger innate immunity against infection as well as triggering a specific adaptive immune response [10].

Experimental evidence has demonstrated that toll- like receptor-9 (TLR9) mediates CpG-ODN immunostimulatory activity in murine and human immune cells [16]. A number of studies in mammalian cells concerning the *in vitro* and *in vivo* immunostimulatory effects of CpG-ODN have previously been reported. These studies have shown that bacterial DNA and synthetic CpG-ODN stimulate variety of cells including B lymphocytes, natural killer cells, macrophages and dendritic cells which result in production of cytokines, including IFN-γ, IFN-α, TNF-α, IL-1, IL-6, IL-12 and IL-18 [17].

Bacterial infection stimulates the host to mount a rapid inflammatory response. A 6-base DNA motif consisting of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines was

shown to contribute to this response by inducing polygonal B-cell activation. This stimulatory motif is 20 times more common in the DNA of bacteria than higher vertebrates. The same stimulatory motif induces the rapid and coordinated secretion of interleukin-6 (IL-6), IL-12, and IFN- γ but not IL-2, IL-3, IL-4, IL-5, or IL-10 *in vivo* and *in vitro*. Stimulatory CpG DNA motifs induced B, T, and natural killer cells to secrete cytokine more effectively than did lipopolysaccharide. Thus, immune recognition of bacterial DNA may contribute to the cytokine, as well as the antibody production characteristic of an innate inflammatory response [17].

There is a strong evidence suggests that anti-ds DNA Ab of the IgG isotype are able to shuttle nuclei acid fragments through the plasma membrane causing activation and secretion of inflammatory cytokines [18].

Since the presence of attenuated live *B. melitenses* Rev1 bacteria or its DNA induced the immune system to produce IFN- γ and anti-ds DNA Ab. However, the attenuated live *B. melitenses* Rev1 increased the production of IFN- γ more than DNA, whereas *B. melitenses* Rev1 DNA increased the production of anti- ds DNA antibody more than attenuated live vaccine.

References

1. Godfroid, J., Cloeckaert A., Liautard J. P., Kohler S., Fretin D., Walravens K., Garin-Bastuji B., and Letesson J. **2005**. From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. *Vet. Res*., 36:313–326.
2. ReCorbel MJ and Morgan WJB. **1984**. Genus *Brucella* Meyer and Shaw. In: Holt JG, Ed. Bergey's Manual of Systematic Bacteriology, Springer, New York.
3. Franco, M. P., Mulder M., Gilman R. H. and Smits H. L. **2007**. Human brucellosis. *Lancet Infect. Dis*., 7:775–786.
4. Memish, Z. A., and Balkhy H. H. **2004**. Brucellosis and international travel. *J. Travel Med.*, 11:49–55.
5. United States Agency for International Development. **2004**. Iraq reconstruction and humanitarian relief weekly update number 34.
6. Bechtol D, Carpenter LR, Mosites E, Smalley D and Dunn JR. **2011**. *Brucella melitensis* infection following military duty in Iraq. *Zoonoses Public Health*, 58(7):489-492.
7. Smither S, Perkins S, Davies C, Stagg A, Nelson M, and Atkins H. **2009**. Development and Characterization of Mouse Models of Infection with Aerosolized *Brucella melitensis* and *Brucella suis*. *Clin Vaccine Immunol.*, 16(5): 779–783.
8. González D, Grilló M-J, De Miguel M-J, Ali T, Arce-Gorvel V, Delrue R-M, Conde-Álvarez R, Muñoz P, López-Goñi I, Iriarte M, Marín C-M, Weintraub A, Widmalm G, Zygmunt M, Letesson J-J, Gorvel J-P, Blasco J-M and Moriyón I. **2008**. Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. *PLoS ONE*, 3(7): e2760.
9. Sambrook, J. Russell, D.W. **2001**. *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Press.
10. Tavakoli Z, Ardestani S, Lashkarbolouki T , Kariminia A and Tavassol N. **2009**. DNAs from Brucella strains activate efficiently murine immune system with production of cytokines, reactive oxygen and nitrogen species. *Iran J Allergy Asthma Immunol*., 8(3): 127- 134.
11. Yamamoto, S., Yamamoto, T., Shimada, S., Kuramoto, E., Yano, O., Kataoka, T. and Tokunaga, T. **1992**. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. *Microbiol. Immunol*., 36:983-997.
12. Al Mathkhury H, Utba N and A-Alusi N. **2012**. Low GC and high GC bacterial DNA impact on anti-DNA antibodies, IL-6 and IL-12 levels in rat. *Int. J. Sci. Technol*., 7(2): 90-94.
13. Deng, G. and Tarkowski, A. **2000**. The features of arthritis induced by CpG motifes in bacterial DNA. *Arthritis Rheum*., 43(2): 356–364.
14. Elson G, Dunn-Siegrist I, Daubeuf B and Pugin J. **2007**. Contribution of Toll-like receptors to the innate immune response to Gram-negative and Gram-positive bacteria. *Blood*, 109: 1574-1583.
15. Oliveira SC, Harms JS, Rech EL, Rodarte RS, Bocca AL, Goes AM and Splitter GA. **1998**. The role of T cell subsets and cytokines in the regulation of intracellular bacterial infection. *Braz J Med Biol Res.*, 31: 77-84.

16. Müller T, Hamm S and Bauer S. **2008**. TLR9-mediated recognition of DNA. *Handb Exp Pharmacol*., (183):51-70.
17. Klinman DM, Yi AK, Beaucage SL, Conover J and Krieg AM. **1996.** CpG motifs expressed by bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and IFN-γ. *Proc Natl Acad Sci USA*, 93(7):2879-83.
18. Munoz L, Gaipl U and Herrmann M. **2008**. Predictive value of anti-dsDNA autoantibodies: Importance of the assay. *Autoimmunity Reviews*, 7(8), 594–597.