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Lactococcus-based vaccine against brucellosis: IgG immune response in mice with rOmp16-IL2 fusion protein

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Abstract
This study was designed to introduce the recombinant *Lactococcus lactis* MG1363 as a cell factory candidate for production of recombinant *Brucella melitensis* Omp16-Human IL2 (r-Omp16-IL2) and to suggest it as a promising safe, non-pathogenic mucosal live vaccine against brucellosis. Three groups of BALB/c mice (10 mice per group) were intragastrically administrated with phosphate-buffered saline (PBS), *L. lactis* harboring the empty pAMJ2008 plasmid and with *L. lactis* expressing rOmp-IL2. The first two groups were classified as control groups and the third one is indicated as treatment group. Another group was injected by the intraperitoneal (i.p.) route with purified rOmp16-IL2 protein. The total serum IgG of each group was assessed with indirect ELISAs at two days before immunization and also two weeks after the last immunization. Results showed that BALB/c mice intragastrically administrated with *L. lactis* expressing rOmp-IL2 had dominant IgG response compared to the control (PBS administrated) group ($P < 0.05$). The level of IgG was significantly increased by intraperitoneally injection of recombinant Omp-IL2 in adjuvant compared to the intragastrically administration of PBS and *L. lactis*/pAMJ2008 as control groups, and also compared to *L. lactis*/pAMJ2008-rOmp-IL2 ($P < 0.05$). Our findings provide the use of *L. lactis* rOmp16-IL2 as a new promising alternative safe strategy than presently live attenuated vaccines toward developing an oral vaccine or subunit-based vaccine against brucellosis.

Keywords *Brucella* · *Lactococcus lactis* · Omp16-IL2 protein · Expression pAMJ2008 vector

Abbreviations
GIT   Gastro intestinal tract
OMP   Outer membrane protein
IFA   Incomplete Freund's adjuvant
Ig   Immunoglobulin
IL   Interleukin
LAB   Lactic acid bacteria

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Introduction

The species of *Brucella* genus are small facultative intracellular Gram-negative pathogens that causes the brucellosis infection (Liautard et al. 1996). Brucellosis infection is one of the major zoonotic endemic diseases in many of the countries (Colmenero et al. 2007) and is transmitted commonly through contact with contaminated animal tissues and secretion (He 2012). It is mainly acquired in humans by consumption of raw milk and cheese made from unpasteurized milk and is considered an economically important infection of animals (He 2012) Due to the high incidence of brucellosis in the Middle East, vaccination of domestic animal is the best way to prevent this disease and to diminish the incidence of brucellosis infection in human (Gonzalez

et al. 2008). All current live attenuated vaccines are not suitable for human use (Izadjoo et al. 2004) due to some disadvantages such as diagnosis interference (Alton 1987), abortions in administered pregnant animals (Minas et al. 2004). So, a new generation of safer, easier to administer and more cost-effective mucosal administered vaccines as an alternative strategy than the currently brucellosis vaccines may be required to induce the immune response at the site of primary infection (Pontes et al. 2003). Nowadays, due to the high potential of *Lactococcus lactis* expressing the viral and bacterial antigens as a live vector, the researchers were encouraged to use the mucosal delivery and probiotic-based system (Wyszyńska et al. 2015). The Omp16 could be considered as a suitable surface-exposed candidate (Tibor et al. 1999) due to good expression and simple subsequent purification because of its suitable molecular mass (approximately 32 kDa) for fusing with Human IL2.

The *B. Melitensis* Omp16 protein as one of the low-molecular weight (Tibor et al. 1999) outer membrane proteins (omps) has been considered as an immunodominant antigen (Rezaei et al. 2019). The main reason for selection of the Omp16 selected in our study was due to have more advantages e.g. more antigenicity and more accessibility than other cytoplasmic and periplasmic antigens of *Brucella* previously investigated (e.g. L7/L12 (Ribeiro et al. 2002), GroEL heat-shock protein (Miyoshi et al. 2006), Cu, Zn superoxide dismutase (Sáez et al. 2012)). Additionally, it has the suitable molecular weight (approximately 32 kDa) when fusing with Human IL-2 with a potential to be used as an ideal choice for good expression and simple subsequent purification process. The immunogenicity of the Omp16 as a suitable candidate for the development of the subunit-based vaccine predicted by comprehensive bioinformatics analysis (Rezaei et al. 2019) Therefore; the advantages described above encouraged us to evaluate the *L. lactis* MG1363 as an appropriate host cell for producing the rOmp16-IL2 fusion protein instead of *Escherichia coli*.

A high copy number expression vector containing the P170 promoter and the SP310mut2 signal sequence (Glenting et al. 2007; Madsen et al. 1999) was used in this study. The protein can be expressed in *L. lactis* MG1363 under control of the acid-inducible promoter P170. The expression is induced by low pH during transition from post exponential to stationary phases of glucose grown cultures (Madsen et al. 1999). We used recombinant *L. lactis* harboring pH inducible expression pAMJ2008 vector to express an Omp16 antigen of *Brucella* fused with Human-IL2 (pAMJ2008-rOmp16-IL2) and analyzed the IgG immune response in mice after oral administration of *L. lactis*/pAMJ2008 and recombinant *L. lactis*/pAMJ2008-rOmp-IL2. The anti-rOmp16-IL2 IgG response in the separate group of mice injected with intraperitoneal purified rOmp16-IL2 protein was also assessed.

Materials and methods

Construction of *L. lactis*/pAMJ2008-rOmp16-IL2

The recombinant plasmid pAMJ-rOmp16-IL2 was correctly transformed into *L. lactis* MG1363 strain (from Mashhad University of Medical Science) by electroporation method, as described previously (Rezaei et al. 2019). The positive transformed *L. lactis* MG1363/pAMJ-rOmp16-IL2 were selected via using the erythromycin-resistant gene in pAMJ2008 and identified by colony PCR using the designed primers:

Forward primer: 5′TTAGATCTATGAAGAACCTTC CGAATAATGCCG3′

Reverse primer: 5′TTTGTCGACTCAATGATGATGATG ATGATGAGTCAGTGTTGA3′.

PCR was performed at the optimized conditions (preheating at 94 ºC for 5 min; followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 1 min, and extension at 72 ºC for 1 min, the final extension was performed at 72 ºC for 10 min).

The Lactococcus expression vector, pAMJ2008 was provided by Bioneer A/S (Horsholm, Denmark). To select recombinant *L. lactis* harboring the recombinant pAMJ2008, erythromycin (Sigma-Aldrich), 5 µg/ml was added for *L. lactis*. Following the expression of fusion protein under pH inducible promotor p170 of pAMJ2008 in stationary phase, the His6-labeled protein was purified with Ni-NTA and was identified for further specificity. The purified fusion protein (Omp16-IL2) from the *L. lactis*/pAMJ2008-Omp-IL2 was detected on 12% SDS-PAGE and was verified by Western blot analysis. The purified protein aliquots were stored at − 20 ºC until use for enzyme-linked immunosorbent assay (ELISA).

Preparation of *Lactococcus lactis* cells for immunization

The *L. lactis*/pAMJ2008 (Harboring the empty vector) as a naive control and *L. lactis*/pAMJ2008-Omp-IL2 (expressing recombinant omp-IL2) strains are grown in M17 medium (Biolife, Italy) supplemented with 1% glucose and 5 µg erythromycin (Sigma-Aldrich) per ml at 30 ºC without shaking to an optical density at 600 nm of 1.0. Then the harvested cells are washed twice by centrifugation (4000*g* at 4 ºC) in sterile, ice-cold PBS before being suspended in vaccine buffer (0.2 M sodium bicarbonate, 5% casein hydrolysate, and 0.5% wt/vol glucose) at 5×10^{10} cfu/ml dose described by Steidler (1998).

Animals and feeding procedure

The three experimental groups ($n = 10$) of 6–8 weeks old BALB/c mice weighing 25 to 30 g were classified as following: *L. lactis/*pAMJ2008-Omp16-IL2 strain as treatment group and *L. lactis*/pAMJ2008 strain (harboring the empty vector) were intragastrically administered with (5×10^{10} CFU/mouse in 200 µl of vaccine buffer) by stainless feeding tube (Steidler et al. 1998). A control group of non-treatment mice was also included in the experiment and were intragastrically administered with PBS. All mice groups are administrated on days 0, 7, 14, 28 (4 doses of the experimental vaccine). Serum IgG response was detected by indirect ELISA as described below. Another experimental group ($n = 10$) of 6–8-weeks-old BALB/c mice weighing 25 to 30 g, were immunized by the intraperitoneal (i.p.) route with 100 µg of rOmp16-IL2 protein in Incomplete Freund's adjuvant (IFA) (Sigma, St. Louis, MO). Mice were injected on days 0 and 15. Serum samples of all groups were taken at 2 days before of each immunization and 15 days after the last immunization.

Indirect ELISA for detection of anti rOmp16-IL2 IgG responses

The serum IgG of each group was assessed with indirect ELISAs at 2 days before of each immunization and 15 days after the last immunization. The purified rOmp16-IL2 was diluted to 5 µg/ml in carbonate buffer (pH 9.6) and used to coat the wells of a polystyrene plate (100 µl/well). After overnight incubation at 4 ℃, the plates were washed with PBS containing 0.05% (w/v) Tween 20 and were blocked with 5% skim milk in PBS containing 0.5% Tween 20, for overnight at 4 ℃ to prevent nonspecific binding. The blocking buffer was removed, after that, plates were incubated with 100 µl of serum samples diluted 1:100 in blocking buffer 2 h at room temperature with rocking. After three more washes performed with PBS-Tween between incubations the goat Anti-mouse IgG (Bio-Rad Cat no: 170-6516) conjugated with HRP was added to the wells at a dilution 1:2000 in PBS-Tween and incubated at room temperature for 1 h. After a final washing step, specific reactivity was determined by the addition of 50 µl/well of the enzyme substrate TMB for 30 min at 37 ℃. The reaction was stopped by adding 2 M H_2SO_4 to each well. The optimal density at 492 nm (OD 492) was measured after 10 min. All assays were performed in triplicate.

Statistical analysis

The statistical difference between two groups was analyzed by the *t* test and the data among several groups were analyzed by one factor analysis of variance (ANOVA) and Turkey's post hoc test in SPSS. *P* values < 0.05 were considered significantly different.

Results

Confirmation of transformed pAMJ-rOmp16-IL2 in *L. lactis* MG1363

The recombinant plasmid pAMJ-rOmp16-IL2 was confirmed by direct colony PCR and double digestion analysis by two restriction enzymes. Then, the recombinant plasmid was subjected to DNA sequencing and was assigned the GenBank nucleotide sequence Accession number MH734194.1. The sequence was confirmed by comparison (Blastn) to GenBank Accession numbers CP001488.1 and NG_016779.1.

Purification of the fusion protein

The production of the fusion protein in *L. lactis* MG1363/pAMJ-rOmp16-IL2 and *L. lactis* MG1363/pAMJ2008 as control was analyzed on 12% SDS-PAGE using the method described by Laemmli (1970). The purification of the fusion protein was confirmed by 12% SDS-PAGE and Western blot analysis, as previously reported (Rezaei et al. 2019).

The analysis of the IgG titer

Sera were collected from the 3 groups of immunized BALB/c mice with intragastrically *L. lactis*/pAMJ2008, *L. lactis*/pAMJ2008-Omp16-IL2 and PBS administrated and also from forth group of mice which immunized with intraperitoneal injection of the purified rOmp16-IL2 protein.

The changes in IgG antibody response in group immunized with intragastrically *L. lactis*/pAMJ2008-Omp16-IL2 are reported as significantly different between before (week 0) and after last immunization (Week 5) ($P = 0.004$) and also the analysis of the IgG response showed a significant increase in compared to the control (PBS) administrated group ($P = 0.002$) (Fig. 1).

The group administrated with PBS did not produce any significant antibodies after the fifth week ($P = 0.168$). The serum IgG antibody levels in the group administrated with *L. lactis*/pAMJ2008 were slightly higher than that in PBS administrated although the increases were not significant ($P = 0.3$) (Fig. 1) and were lower than the group administrated with *L. lactis*/pAMJ2008-Omp16-IL2 but without significant differences ($P = 0.12$) (Fig. 1).

A significant increase in the antibody response between before (week 0) and after last immunization (week 5) was observed ($P < 0.05$) when mice were intraperitoneally (i.p.) immunized with rOmp16-IL2 antigen in Freund's
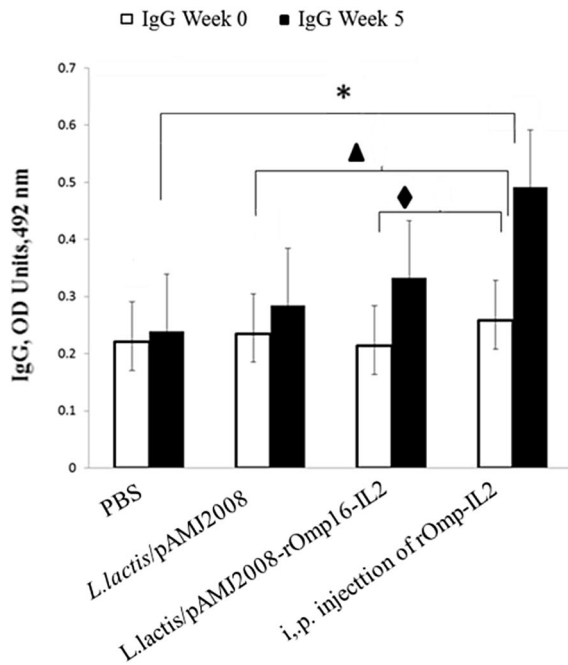
Fig. 1 Comparison of Serum IgG response between four groups. Data represent the mean A492 ± SD from each group of mice. The level of IgG was significantly increased by i.p injection of rOmp-IL2 in compared to the other groups (PBS, *L. lactis*/pAMJ2008 and *L. lactis /* pAMJ2008-Omp16-IL2). The statistical significance is represented by: *$P < 0.05$, compared with the control PBS group ▲$P < 0.05$, compared with *L. lactis*/pAMJ2008 group ♦$P < 0.05$, compared with *L.lactis /*pAMJ2008-Omp16-IL2 group

incomplete adjuvant at 42 days post first immunization. It also showed that there are significant differences between intraperitoneally (i.p.) immunized mice with control groups (the intragastrically administration of PBS and *L. lactis*/pAMJ2008) and also compared to *L. lactis*/pAMJ2008-rOmp16-IL2 ($P < 0.05$) (Fig. 1).

Discussion

Due to the wide incidence of brucellosis in the Middle East and economic costs in livestock industry, vaccination is strictly recommended to prevent this disease (Dorneles et al. 2015). New vaccination strategies with the aims of good protective immunity, minimization of side effects, safe handling, and simple administration and, a low cost of production and delivery could hinder safety concerns and overcome the disadvantages of the currently used live attenuated strains of *Brucella* to control brucellosis (Saez et al. 2012). The attractive vaccination approaches rely on identifying and using new immunogenic antigens of *Brucella* for development of new recombinant vaccines (Vishnu et al. 2015).

Previous studies in the development of the preventive recombinant vaccine against brucellosis are restricted with

Escherichia coli (Contreras-Rodriguez et al. 2006; Guptaa et al. 2012). The present study purpose to introduce the *Lactococcus lactis,* as one of the safe, non-pathogenic mucosal live vaccines and to produce the immunogenic fusion rOmp-IL2 protein as a replacement machinery system for *E. coli* due to its advantages (Le Loir et al. 2005). We investigated the ability of *L. lactis* MG1363 harboring empty vector pAMJ2008 and rOmp16-IL2 to deliver the fusion protein antigen to gastrointestinal and subsequently immunization in the mice. However, for investigating the efficacy of oral vaccine, the challenge experiment in mice against virulent *Brucella* strain is required to address the hypothesis. The result indicated that fusion rOmp16-IL2 antigen is an effective immunogen to elicit specific antibodies and it was also confirmed the previous reports associated with high levels of IgG responses (Yang et al. 2011; Ghasemi et al. 2015).

Selection of the preferred epitopes of Omp16 due to containing only preferred immunogenic epitopes in future subunit-based vaccines instead of the entire pathogen could be ideal choices toward developing new vaccination strategies in comparison with live attenuated strains of *Brucella* with the risk of reversion to original virulent form (Delany et al. 2013). In the hypothesis that the Human IL2 can increase the efficiency of our designed vaccine due to its multiple roles in immune functions by contributing to the generation of antigen-specific immune responses and its adjuvant effect on de novo IgG and IgM antibody production (Capobianco et al. 2016), We postulated that the fusing the Omp16 as an immunogenic surface-exposed antigen (Rezaei et al. 2019) with the Human IL-2 as an adjuvant could be a reasonable choice toward developing vaccination studies based on protein subunit vaccine against Human brucellosis in future. Previously, Steindler had reported that the efficacy of a vaccine increased due to the administration of the immunoregulatory cytokines with the antigens (Steidler et al. 1998).

Conclusion

In conclusion, although the route is shown to different IgG response for immunized mice, both i.p. and oral administration of routes are acceptable. Nevertheless, a mucosal-administered vaccine based on *L. lactis* which do not colonize and can survive in the gastrointestinal tract (GIT) and possess the ability of resistance to gastric acid and adherence to the mucosal surface might be a rational choice for developing a controlling vaccine against brucellosis (steidler et al. 1998). In summary, the orally live Lactococcus-based vaccine can be considered as an alternative potential vaccine delivery vector for the currently used virulent live *Brucella* vaccines toward developing a safe, effective vaccine against human brucellosis in the future.

Declarations

Conflict of interest The authors declare no conflict of interest for this study.

Ethical approval The animal experiments were approved by the Ethical Research Committee in Arak university of Medical Science (Approval number for immunization of mice: 1396.99).

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