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Immunopathological evaluation of recombinant mycobacterial antigen Hsp65 expressed in *Lactococcus lactis* as a novel vaccine candidate

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Summary

Bovine tuberculosis (TBB) is a zoonotic disease distributed worldwide and is of great importance for public health and the livestock industry. Several experimental vaccines against this disease have been evaluated in recent years, yielding varying results. An example is the Bacillus Calmette-Guérin (BCG) vaccine, which has been used extensively in humans and tested in cattle showing mixed results related to protection (0-80%) against *Mycobacterium bovis*. In this study, we used the food-grade bacterium *Lactococcus lactis* as an expression system for production of mycobacterial protein Hsp65. For this purpose, the construction of a replicable plasmid in strain NZ9000 *L. lactis* (pVElepr) was conducted, which expressed the *Mycobacterium leprae* Hsp65 antigen, and was recognized by traded anti-Hsp65 antibodies. The strain NZ9000-pVElepr was applied to calves that were negative to tuberculin test and the immune response was monitored. The results showed that immune response was not significantly increased in calves with NZ9000-pVElepr with respect to control groups, and no injury was observed in any lung or lymph of the calves. Finally, this study suggest that the recombinant NZ9000 strain of *L. lactis* may protect against the development of *M. bovis* infection, although studies with longer exposure to this pathogen are necessary to conclude the matter.

Key words: Hsp65, *Lactococcus*, *Mycobacterium leprae*, Vaccination

Introduction

Bovine tuberculosis (TBB) is an infectious and contagious disease caused by *Mycobacterium bovis*, which is a member of the *Mycobacterium tuberculosis* complex and has an economic impact on dairy cattle in Mexico by reducing the production by 17%: in Mexico, only 28% of the produced milk is pasteurized (NOM-031-ZOO-1995). In dairy cattle herds, such as those located in Tijuana, Baja California, Mexico, TBB is a severe problem due to its high prevalence (16.5%) (according to SAGARPA in 2012), indicating that actions contemplated on Mexican Official Standard NOM-031-ZOO-1995 had been insufficient in reducing this disease in a significant way. Thus, it is necessary to implement several alternatives to achieve the reduction of TBB in a short time. Studies about vaccinations on bovines using a BCG vaccine show a reduction of severity and quantity of injuries (P>0.05) when used in a dose of 5×10^3 and 5×10^6 colony-forming units (CFU) for a later challenge using the *M. bovis* pathogen at a rate of (5×10^3 CFU) (Buddle *et al*., 1995; Buddle *et al*., 2003). Meanwhile, Lopez *et al*. (2009) observed that, when using a BCG vaccine in a dose of 1×10^6 CFU in field conditions, the frequency of cases with TBB on those vaccinated was 9.7% compared with 22.7% on those not vaccinated.

The immunological strategy, as an alternative against several pathogens, is the use of Gram-positive bacteria, food-grade or commensal, particularly those based on the model of lactic acid bacteria (LABs). Some studies showed the possibility to modulate both systemic and mucosal immune response (Bahey-El-Din *et al*., 2010; Bermúdez-Humarán *et al*., 2011). Among the group of LABs, *Lactococcus lactis* can be found; its genome has been completely sequenced by Bolontin *et al*. (2001). Additionally, several plasmids had been studied for the expression of heterologous proteins induced by nisin (a grade food natural preserver) and selected antibiotics. Profiling this kind of system provides excellent potential to obtain recombinant grade food live vaccines, with adequate efficiency and extensive use (Nouaille *et al*., 2003; Santos *et al*., 2011).

Recombinant expression of the highly preserved chaperonin Hsp65 of *Mycobacterium leprae*, has great potential for a variety of biotechnological, medical and

therapeutic applications (Azevedo *et al*., 2012). Studies have shown that using 100 µg of Hsp65-DNA intramuscularly in mice induced a statistically significant adaptive and innate response (P>0.01), as well as a reduction at the quantity of CFU in 100% of animals that received three immunizations (Lowrie *et al*., 1999; Lima *et al*., 2003; Lowrie, 2006). Actually, the vaccination system using *L. lactis* as a vector for protein and DNA expression has been successfully used for its prophylactic and therapeutic effects in several animal models. An example of this is a study to treat the 16 type human papillomavirus (HPV-16) in mice, which showed a reduction of 35% in the frequency of this cancer induced by the virus (Bermúdez-Humarán *et al*., 2011). Recombinant *L. lactis* used to immunize mice induced a statistically significant cellular immune response (P>0.05) on assays against avian influenza virus HA1 antigens compared with the effect of *L. lactis* without the recombinant fragment (Lei *et al*., 2011). Azevedo *et al*. (2012), using a model similar to the one proposed in this study, produced approximately 7 mg/L of recombinant Hsp65.

Based on the above-described results, the present study was aimed to build a *L. lactis* strain that expressed a mycobacterial Hsp65 protein for further characterization. Additionally, a brief preliminary experimental assay was conducted in order to evaluate its capability of induction of the innate and adaptive immune response in the experimentally challenged calves.

Materials and Methods

Bacterial strains and growing conditions

The *L. lactis* strain NZ9000 was grown in a Difco M17 broth medium, containing 0.5% glucose (GM17), at 30°C overnight without agitation. If required, 10 µg/ml of chloramphenicol was added to the broth medium. *Escherichia coli* DH5α strain was aerobically cultured at 37°C in a LB broth medium supplemented with 100 µg/ml ampicillin or 10 µg/ml chloramphenicol, when needed.

Plasmid construction

An *EcoR1* fragment DNA from the *M. leprae hsp65* gene (provided by Laboratorio de Inmunología Molecular y Biotoxinas, CICESE, Ensenada, BC, Mexico) was cloned into pCR-Blunt II-TOPO® (GenBank: M14341.1) and then transformed in *E. coli* DH5α. Selection was done on LB agar containing 50 µg/ml of Kanamycin.

The sub cloning in the pVE5547 vector (Dieye *et al*., 2001; Cortes-Perez *et al*., 2003) was done by using a high fidelity enzyme (iProofTM. high fidelity DNA polymerase, BioRad), amplifying the *hsp65* gene by PCR with SalI and NheI enzyme sites (primer pveleprcF: 5´ TGGGA GGAAA AATTA CAAA GAACA-3´ and pveleprcR: 5´-CTTCT AAAGC TAGCA AGTCC ATAC-3´) and inserting the amplified fragment (Hsp65) in the pVE5547 plasmid, which was previously partially digested with the same restriction enzymes and purified with the S.N.A.P. Miniprep™ Kit (Invitrogen Inc.) method, obtaining a fragment of 4791 pb.

Sub cloning in the pSEC/E7 vector is replicable on *L. lactis* (Bermúdez-Humarán *et al*., 2002). Once the pVE5547 vector contained the Hsp65 sequence, it was inserted inpSEC/E7 vector by means of digestion with BglII-NheI restriction enzymes, selecting a fragment of 3403 pb for its final sub cloning. The result of this was the construction of the pVElepr plasmid replicable on *L. lactis* as shown in Fig. 1.

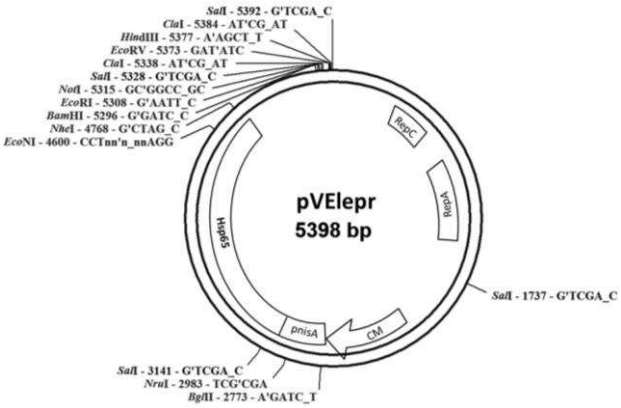

Fig. 1: Final plasmid constructed, replicable in *L. lactis*

SDS-PAGE and Western blot

Lactococcus lactis NZ9000 with pVElepr or pSEC/E7 was cultured in 5 ml of M17 broth with or without 10 ng/ml of nisin (Sigma, USA) expression inducer for 1 h to reach a $O.D._{600}$= 0.6. Cellular lysis was performed according previous reference (Sambrook and Russell, 2001). Forty µg of NZ9000/pVElepr or pSEC/E7 protein samples, 5 µg of total Hsp65 purified protein (Stressgen, USA) and 100 µg of BSA were running at 100 volts in a 10% SDS-polyacrilamide gel and stained with 0.2% coomassie brilliant blue (Laemmli, 1970). The proteins were then transferred to a nitrocellulose membrane for 1 h at 25 V in a Trans-Blot SD semi Dry Transfer Cell (BioRad, USA) with transference buffer (25 mM Tris base, 192 mM glycine and 20% methanol, pH = 8.3) at room temperature. The blocking of reactive free sites was done with 5% skim milk on PBS for 1.5 h at 37°C and the membranes was washed five times with 0.01% PBS Tween 20 (PBS-T) for 5 min. IgG anti-Hsp65 was utilized as a primary antibody (Biothechologies, Stressgen) in a 1:1000 dilution for 1 h at room temperature. The secondary antibody was a rabbit anti-mouse IgG Horseradish peroxidase conjugate (SIGMA, USA) in a 1:4000 dilution, which was incubated for 1 h with the membrane at room temperature. Finally, membranes were developed with 3,3´,5,5´ Tetramethylbenzidine (TMB) liquid substrate system for membranes (Sigma, USA) for 15 min.

Experimental units

Twelve calves (three months of age, of the Holstein

Friesian breed, males, castrated), derived from a local herd that has been free of TBB for 30 years, were tested with intradermic tuberculin test, and were found to be negative. Three calves were vaccinated with *L. lactis* pSEC/E7 (control group) and three calves were vaccinated with *L. lactis* pVElepr (vaccinated group "V"). Both were vaccinated with a dose of 2×10^6 CFU (one dose intranasal and one dose via the intramuscular pathway on the neck) followed by two boosters at intervals of two weeks using the same dose and route. Two new groups were formed, each having the same conditions as the first two. However, the difference with the previous groups lies in a challenge conducted at month two by the intratracheal pathway with *M. bovis* strain 138 (Martínez-Vidal *et al*., 2011) in a 1×10^4 CFU dose; thus, forming the challenged control group, "Control + R", and challenged vaccinated group, "V + R", respectively.

Testing

Previously, all animals were tested with double comparative intradermal tuberculin tests conducted at the start of the study (the results of which were negative) under the established rules of the Mexican Official Standard (NOM-031-ZOO-1995) and established protocols of the Bioethics Committee from the IICV/ UABC (Asociación Médica Mundial, 2005). These standards guided everything related to experimentation proceedings on live animals, while considering facilities and appropriate equipment to maintain biosafety standards required by the experiment.

Sample collection

Heparinized and non-coagulant tubes were used for blood sample collection starting at month three, collecting at intervals of 28 days for six months from the coccygeal vein for a total of eight samplings for each animal.

Antibody evaluation

Plates were coated with 10 ng per well with a commercial *M. bovis* recombinant Hsp65 protein (Stressgen, USA) on a carbonate-bicarbonate buffer solution pH = 9.6 (1.53 g/L $NaCO_3$, 2.93 g/L $NaHCO_3$, adjusted with 37% hydrochloric acid) at 4°C for 24 h. *Mycobacterium bovis* Hsp65 was used because it shows a 99.81% identity with *M. tuberculosis* Hsp65, and *M. tuberculosis* Hsp65 shows high cross-reactivity with *M. leprae* Hsp65 (Mustafa *et al*., 1999). The plates were blocked with 200 µL per well of 3% non-fat milk on sterile PBS-T at room temperature. Each serum of the study was added at a rate of 1:100, using 50 µL per well for 2 h at 37°C. For detection of specific antibodies, IgG anti-bovine in mice (SIGMA) was used at 1:20,000 rates for 2 h at 37°C. Fifty µL of a TMB solution (20 ml of pH = 4.0 citrate solution, 100 µL TMB stock solution (0.1 g TMB, 10 ml DMSO)), 10 µL H_2O_2 (30%) was added to each well. The reaction was stopped after 15 min with an acid solution using 50 µL per well of 0.5 M H_2SO_4. Optical density (OD) was read at 450 nm on a spectrophotometer. Antibodies levels were recorded in duplicate and were determined by subtracting the absorbance at 450 nm of blank values to the values obtained in the samples.

Interferon gamma assay

To determinate the interferon gamma assay (γ-IFN), 750 µL of heparinized whole blood was cultured in borosilicate tubes, adding 50 µL of antigen (CSL, Australia) per sample before being incubated at 37°C in high ambient relative humidity for 24 h. The supernatant was collected and the γ-IFN level was measured using the immunoenzymatic assay package BOVIGAM (CSL, Melbourne, Australia).

Postmortem examination

At the end of the study (six months post inoculation), animals were sent to the Federal Inspection slaughterhouse (TIF) for sacrifice and inspection. After the federal inspection, lungs and lymph nodes (retropharyngeal, bronchial and mediastinal) were collected for a second examination in the laboratory. They were stored in biological bags at 4°C and identified with the animal number for transportation. Tissues were sectioned at 0.5-1 cm thickness by means of standard procedures and cut at 4 µm and stained with hematoxilin and Ziehl-Neelsen stains.

Lungs

Pulmonary lobes were examined individually (apical, cardiac and diaphragmatic left lobes and apical, cardiac, diaphragmatic and accessory right lobes). For each lobe, the following scoring system was applied: 0, no visible lesion; 1, no macroscopic lesion but lesion at cutting; 2, <5 macroscopic lesions with <10 mm of diameter; 3, <6 lesions with diameters < than 10 mm; 4, <1 distinctive macroscopic lesion with < than 10 mm of diameter; 5, coalescence macroscopic lesions. Individual lobe scores were added to calculate a pulmonary score (Lyashchenko *et al*., 2004).

Lymph nodes

The macroscopic severity of the disease observed in lymph nodes was evaluated under the following score system: 0, no necrosis or visible lesion; 1, small focal lesion (1-2 mm in diameter); 2, several small focal lesion or necrotic areas, at least 5 × 5 mm in diameter; 3, multiple necrotic areas at least 5 × 5 mm in diameter distributed along the node, or necrotic areas affecting 5% of the node. The individual score of lymph nodes was added to calculate a lymphatic nodes score. Lymphatic node score and pulmonary score were considered to determine a complete pathological score per animal. The score was established by the same personnel in the slaughterhouse for all animals in the study to insure consistency (Lyashchenko *et al*., 2004).

Statistical analysis

The statistical difference between humoral and cellular immune responses was estimated with

MINITAB software, where statistical significance was determined at P<0.05. Correlations between immune response and lesions scale grade were estimated by non-parametric analysis (Spearman rank test).

Results

Lactococcus lactis NZ9000 was used to construct "*L. lactis*/pVElepr" (Fig. 1), and was analyzed to determinate Hsp65 expression by a Western blot (Fig. 2), showing Hsp65 expression as a band of 70 kDa, not present on the *L. lactis*/pSEC/E7 strain. Also, the expression capacity and antigenic character of the Hsp65 protein expressed on "*L. lactis*/pVElepr" strain was probed.

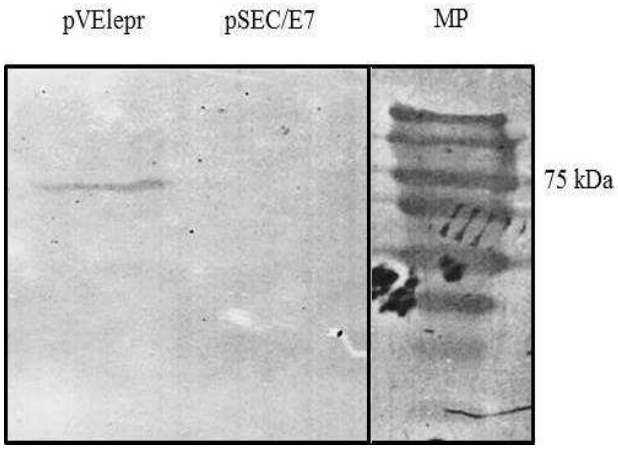

Fig. 2: Antigenic recognition of Hsp65 protein of *Mycobacterium leprae* expressed on NZ9000 strain-pVElepr. *Lactococcus lactis* pSEC/E7 strain (primary plasmid) was used as a negative control. Hsp65 protein was expressed and recognized by anti-Hsp65 antibodies after 1 h post nisin induction. Protein shows approximately 70 kDa of weight

An enzyme-linked immunosorbent assay (ELISA) to measure specific antibodies (anti-Hsp65) by spectro-photometry at 450 nm was made (Fig. 3), which showed no significant statistical difference between the control group and vaccinated group without challenge (P>0.05), with standard deviations of 0.045 and 0.079, respectively. The results for the control group "*L. lactis*pSEC/E7", and the vaccinated group "*L. lactis*/pVElepr" were both challenged by *M. bovis* strain 138 and showed no significant statistical difference (P>0.05) with standard deviations of 0.101 and 0.093, respectively, although a small activation of the humoral immune response against Hsp65 was observed at the end of study (seven and eight samples). The gamma interferon assay, used to measure cellular immune response during the study, showed no statistical difference between vaccinated and control groups (P>0.05), with a standard deviation of 0.159. The same tendency was observed in Control + R and V + R groups, although comparing those vaccinated with those challenged showed a tendency to maintain a cellular immune response superior to those vaccinated non-challenged (sampling 6, 7 and 8).

The analysis of macro and microscopic lesions compatible with TBB during slaughter inspection TIF did not show visible lesions. Of the animals in the study, 100% were classified as both pulmonary and lymphatic nodes with a score of 0. Similarly, a microscopic evaluation of tissues did not show any suggestion of an inflammatory process.

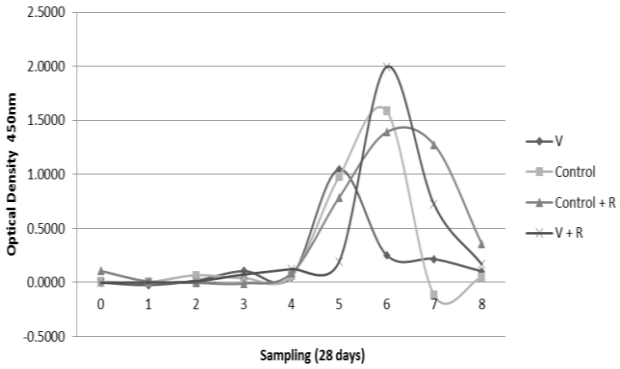

Fig. 3: Measurement of cellular immune response γ-IFN

Discussion

The results obtained in the present study are not as strong as those in which Hsp65-DNA was used as an inductor of innate and acquired immune response on immunized mice and challenged later using 10^5 CFU of *M. tuberculosis* H37Rv intramuscularly. In that study, a statistically significant stimulation of both immune responses (P<0.01) was obtained after i.m. injection (three doses of 100 µg) and a reduction of CFU quantity (P<0.001) was determined comparing vaccinated group versus non-immunized or challenged control groups (Lowrie *et al*., 1999; Lima *et al*., 2003).

This finding may be due to an uninterrupted expression on several organs of the host when the DNA vaccine was applied, which can be a determinant to activate a strong immune response. Similarly, the study differs from the results obtained by Bermúdez-Humaran *et al*. (2011), because they reported that a *L. lactis* strain expressing an antigen against HPV-16 on a mouse model showed an activation of the humoral immune response, and concluded that vaccine result to be prophylactic and therapeutically acceptable. This may be due to specific IgA production induced by the exposition of the mucosae to antigens produced by *L. lactis* resulting in protection against HPV-16 infection, in contrast to the cellular immune response needed to protect against infections such as TBB. In this study, expression of the antigenic Hsp65 protein using "*L. lactis*/pVElepr" as the vector proved to be antigenic by the recognition of specific antibodies anti-Hsp65 (Stressgen, USA) (Fig. 2). Nevertheless, activation of immune response after nasal and intramuscular administration of "*L. lactis*/pVElepr" was not sufficient to observe a significant statistical difference between "*L. lactis*/pVElepr" and "*L. lactis*/pSEC/E7" groups. It was also shown that

expression or exposition of immune system cells to Hsp65 was also insufficient. An unexpected finding was that *L. lactis* NZ9000 strain administration induces a humoral and cellular immune response, and, due to this, it wasnot possible to establish an immunogenic differentiation between study groups. This may be due to the presence of similar chaperonin to Hsp65 on *L. lactis*, as *groEL* of 60 kDa that is expressed on its surface (Bolontin *et al*., 2001).

An important aspect in relation to infective dose is that it could appreciate the establishment of infection after performing the tuberculin double comparative test at the end of the study, where all groups were challenged with the field strain *M. bovis* 138. Groups had a positive result after infection and were kept in this immunologic status until the end of the study, as compared with non-challenged animals that had a negative result to the same test (data not shown). This suggests that the infective dose used was not sufficient to induce macroscopic or microscopic lesions on analyzed organs, or that the NZ9000 strain can (by itself) protect against the development of lesions at least in the infective doses used and at the time of post infection in which the experiment was conducted. Finally, we observe the need for a new experiment using the constructed strain in this study, a large number of experimental units, a control group without *L. lactis* NZ900 strain, a superior infective dose and a long-term experimentation interval to evaluate (with higher precision) the protective effectiveness of *L. lactis* NZ9000/pVElepr.

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Conflict of interest

The authors declare that they have no competing interests. We gave assurance that neither the submitted materials nor portions of the article have been published previously or are under consideration for publication elsewhere.

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