Molecular characterization of the Corynebacterium pseudotuberculosis hsp60-hsp10 operon, and evaluation of the immune response and protective efficacy induced by hsp60 DNA vaccination in mice

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Abstract

Background: Heat shock proteins (HSPs) are important candidates for the development of vaccines because they are usually able to promote both humoral and cellular immune responses in mammals. We identified and characterized the hsp60-hsp10 bicistronic operon of the animal pathogen Corynebacterium pseudotuberculosis, a Gram-positive bacterium of the class Actinobacteria, which causes caseous lymphadenitis (CLA) in small ruminants.

Findings: To construct the DNA vaccine, the hsp60 gene of C. pseudotuberculosis was cloned in a mammalian expression vector. BALB/c mice were immunized by intramuscular injection with the recombinant plasmid (pVAX1/hsp60).

Conclusion: This vaccination induced significant anti-hsp60 IgG, IgG1 and IgG2a isotype production. However, immunization with this DNA vaccine did not confer protective immunity.

Findings

Corynebacterium pseudotuberculosis is a facultative, intracellular, Gram-positive bacterium of the class Actinobacteria, which also includes the genera Mycobacterium, Nocardia and Rhodococcus. The latter three genera, together with Corynebacterium, form a group of potentially pathogenic species termed the CMN group. Corynebacterium pseudotuberculosis is the etiological agent of caseous lymphadenitis (CLA), or cheesy gland, which affects small ruminants (sheep and goats) and occasionally other hosts. This chronic disease is pathognomically characterized by the formation of suppurative abscesses in superficial and internal lymph nodes. In severe cases, these abscesses are also found in internal organs, such as the lungs, kidneys, liver and spleen, characterizing visceral CLA [1]. The economic relevance of CLA, its widespread occurrence, and a lack of knowledge regarding its molecular mechanisms of virulence, have prompted the investigation of its pathogenesis with the aim to develop efficient treatment strategies against this disease [2].

Chemotherapeutic treatment of CLA is difficult because the bacteria are shielded within granulomas, where they are relatively protected from antibiotic drugs [3]. Therefore, attempts to control CLA usually entail immunoprophylaxis by vaccination. Various strategies have been used for developing vaccines against CLA, including the use of inactivated or attenuated C. pseudotuberculosis strains [4,5], fractions of bacterial cells containing bacterial antigens, antigens from culture supernatants, and DNA vaccines [6]. None of the
Currently commercially available vaccines for *C. pseudotuberculosis* provide effective protection against CLA [7].

Heat shock proteins (HSPs), or molecular chaperones, are traditionally classified according to their molecular weight; they are highly conserved proteins, abundantly expressed in eukaryotic and prokaryotic organisms [8]. These proteins are expressed in unstimulated cells at low levels, and play an important role in cell survival both under normal physiological conditions, during various phases of the cell cycle, cellular differentiation and growth, and under stress conditions, such as heat shock [9]. Heat shock proteins are considered immunologically important due to the fact that they are recognized by the host in bacterial, fungal, and parasitic infections and are therefore capable of inducing strong humoral and cellular immune responses in mammals [10].

Several studies have shown that these versatile proteins can be used as antigens for the development of vaccines against diseases. In the case of infectious diseases, HSPs could play a dual role in vaccine development. Pathogen-derived HSPs can be used as vaccine antigens, and host- and pathogen-derived HSPs can be used as adjuvants [11,12]. Strategies to more effectively induce immunity with HSPs include the use of DNA vaccines. HSP-based DNA vaccines have been effective in several immunization trials against diseases. In the case of infectious diseases, HSPs can be used as vaccine antigens, and host- and pathogen-derived HSPs can be used as adjuvants [11,12]. Strategies to more effectively induce immunity with HSPs include the use of DNA vaccines. HSP-based DNA vaccines have been effective in several immunization trials against *Mycobacterium spp.* infection [12].

The Hsp60 protein of *C. pseudotuberculosis*, using a protein subunit as immunogen against CLA, failed to confer protection against infection with *C. pseudotuberculosis* in mice [13]. Using an alternative strategy, we assessed the feasibility of using DNA encoding *hsp60* for protection against experimental challenge with *C. pseudotuberculosis*.

### Methods

#### Bacterial strains, growth conditions and plasmids

All bacterial strains, plasmids and PCR primers used in this study are listed in Table 1. *Escherichia coli* TOP10 was grown in Luria-Bertani broth (LB, Difco Laboratories, Detroit, USA) at 37°C with stirring for 18 h. Plasmid-containing transformants were selected by the addition of ampicillin (Invitrogen, San Diego, CA) and X-Gal (Invitrogen, San Diego, CA) to the media. The supplement concentrations were ampicillin (100 μg/mL) and X-Gal (40 μg/mL).

Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a final concentration of 1 mM in complementation experiments.

*Corynebacterium pseudotuberculosis* biovar *ovis* strain T1 was aerobically grown in brain heart infusion broth (BHI, Acumedia Manufacturers, Inc., Baltimore, MD, USA) and on 1.5% (w/v) BHI agar plates at 37°C for 48-72 h [14].

#### DNA isolation

All DNA templates were prepared with genomic DNA isolated by collecting a bacterial cell pellet from culture, as previously described [14]. Briefly, an aliquot of 20 mL from a 48 to 72 h culture was centrifuged at 4°C and 2000 × g for 20 min. Cell pellets were resuspended in 1 mL of Tris/EDTA/RNase [10 mM Tris/HCl (pH 7.0), 10 mM EDTA (pH 8.0), 300 mM NaCl, 50 mg RNaseA mL⁻¹] and centrifuged again under the same conditions. Supernatants were discarded and the pellets were resuspended in 1 mL of TE/lysozyme. Samples were then incubated at 37°C for 30 min; 30 μL of 30% (w/v) sodium N-lauroylsarcosine (sarcosyl) were added, and the mixture was incubated for 20 min at 65°C, followed by incubation for 5 min at 4°C. DNA was purified using phenol/chloroform/isoamyl alcohol and precipitated with ethanol.

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**Table 1 Strains and plasmids used in this study.**

| Description or sequence | Strain | Plasmid |
|-------------------------|--------|---------|
| *Corynebacterium pseudotuberculosis* biovar *ovis* | T1 (virulent strain isolates from goats; obtained from the Universidade Federal da Bahia, UFBA, Brazil). MIC-6 (virulent strain isolate from goats; obtained from the Laboratório de Genética Celular e Molecular -LGCM, UFMG, Brazil). | pTopo® Cloning vector |
| *E. coli* | TOP10 (F- merA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE16 galK16 rpsL(StrR) endA1 Δ3397) Invitrogen. 8178 groEL44 mutant (ΔgroEL44) characterized by Zeilstra-Ryalls et al [19]. | Cloning vector - CoE1/Ap⁺ - Invitrogen. |
| | | pVAX1® Vector |
| | | Eukaryotic expression vector - pUC/Km⁻ - Invitrogen. |
| | | pTopo/hsp60 |
| | | Cloning vector with the *C. pseudotuberculosis* hsp60 gene inserted in the BamHI and HindIII restriction sites of the vector. |
| | | pProEx-Hta/hsp60 |
| | | Prokaryotic expression vector containing the *C. pseudotuberculosis* hsp60 gene [13]. |
| | | pVAX1/hsp60 |
| | | Eukaryotic expression vector containing the *C. pseudotuberculosis* hsp60 gene. |
with ethanol. DNA concentrations were determined spectrophotometrically.

PCR amplification and cloning
PCR reagents, restriction endonucleases, and ligation reagents used in this study were all purchased from Invitrogen, San Diego, CA. Both genes were amplified by PCR using genomic DNA of *C. pseudotuberculosis* as a template, with primers that were designed based on the DNA sequence of paralogs of *C. diphtheriae* NCTC 13129 (NCBI Acc. GeneID: 2648771 and NCBI Acc. GeneID: 2648772). The *C. pseudotuberculosis* hsp60 gene was amplified by PCR using the following primers: 5'-GATGGCAAAGCTGATTGCA -3' (sense orientation) and 5'-TTAGTGGTGAGGTAGGTTG -3' (antisense orientation). The PCR assays were carried out in a final reaction volume of 50 μL, containing 20 ng genomic DNA, 2 μM of each of the primers, 1 × PCR Buffer II and 1 U AccuPrime taq DNA polymerase. Amplification was run in a thermal cycler (PTC-100, MJ Research, Inc.) as follows: one cycle of 95°C for 5 min; 29 cycles of 95°C for 1 min, 50°C for 40 s, and 68°C for 2 min 30 s; and a final extension step at 68°C for 7 min. The hsp60 fragment was purified from bands in 1.0% (w/v) agarose gels using the Consert TM Rapid Gel Extraction System kit (Gibco-BRL, Gaithersburg, MD, USA). The retrieved DNA fragment was then ligated into the PCR®2.1-TOPO® vector, as described in the manufacturer’s protocol. The recombinant plasmid (PCR®2.1-TOPO®/hsp60) was then introduced into competent *E. coli* TOP10 cells, and single recombinant colonies were selected. Plasmid DNA was isolated from cells by the alkaline lysis method [15]. The presence of the inserted DNA fragment was confirmed by sequencing, using the DYEnamic ET Dye Terminator kit (Amersham Biosciences, Piscataway, NJ, USA).

To verify operon structure, the hsp10 gene and intergenic regions of the operon were amplified using the following primer pair: 5'-GTGGCTAACGTCAAATATCAAGCC -3' (sense orientation) (designed based on the DNA sequence of paralogs of *C. diphtheriae* NCTC 13129) and 5'-TTTAGTGGTGAGGTAGGTTG -3' (antisense orientation) (designed based on the DNA sequence of the *C. pseudotuberculosis* hsp60 gene). The PCR assays were carried out in a final reaction volume of 50 μL containing 20 ng genomic DNA, 2 μM of each of the primers, 1 × PCR Buffer II and 1 U AccuPrime taq DNA polymerase. Amplification was performed with a thermal cycler (PTC-100, MJ Research, Inc.) as follows: one cycle of 95°C for 3 min; 29 cycles of 95°C for 1 min, 50°C for 40 s, and 72°C for 1 min 30 s; and a final extension step at 72°C for 5 min. The amplicon containing hsp10 was also purified, cloned, transformed, and sequenced as described above.

**Bioinformatics and comparative genomics**
Sequence homology analysis was carried out against nucleotide and protein databases available in GenBank http://www.ncbi.nlm.nih.gov/, using the Blast tool http://www.ncbi.nlm.nih.gov/BLAST/ [16]. Multiple sequence alignments and analysis were performed using the ClustalW algorithm http://www.ebi.ac.uk/clustalw/. The predicted amino acid sequences were analyzed to identify conserved motifs, using the Conserved Domains program http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi and the ExPaSy ScanProsite program http://www.expasy.org/tools/scanprosite/. For three-dimensional modeling, we employed MODELLER http://salilab.org/modeller/modeller.html [17]. The isoelectric point (Ip) and molecular weight (Mw) were predicted with software found on the ExPaSy website http://web.expasy.org/compute_pi/ [18].

**Functional characterization of the *C. pseudotuberculosis* hsp60 gene**
To examine the functional activity of the *C. pseudotuberculosis* hsp60 gene, *E. coli* B178ΔgroEL44 mutant (ΔgroEL44) was transformed with a pProEx-Hta/hsp60 plasmid constructed by Pinho et al [13]. The *E. coli* ΔgroEL44 mutant is a strain that bears a temperature-sensitive allele of groEL, namely groEL44. This mutant strain is unable to propagate bacteriophages γ, T4 or T5 [19]. Two phenotypes were analyzed in this complementation assay: heat stress resistance and inability of γlβ2 phage to grow without the protein coded by the hsp60 (groEL) gene, as described by Kumar et al [20], with adaptations for use in our study. First, the empty vector (pProEx-Hta) and the recombinant vector (pProEx-Hta/hsp60) were transformed into the *E. coli* wild-type B178 and ΔgroEL44 strains. To investigate the effect of temperature on protein expression, the bacteria were grown in LB broth containing ampicillin and IPTG. The protein was visualized in an SDS-polyacrylamide gel [21]. Aliquots of 3 μL of the induced cultures were plated on LB, with and without IPTG, in dilutions from 10^-1 to 10^-6, and the plates were incubated at 30 and 42°C. Finally, to assess the growth of the γlβ2 phage, *E. coli* ΔgroEL44 was transformed with the empty and recombinant vectors (groEL44[pProEx-Hta] and groEL44 [pProEx-Hta/hsp60]), while the wild-type *E. coli* was transformed with the empty vector (B178[pProEx-Hta]). Dilutions of the phage (10^-1, 10^-2, 10^-3 and 10^-4) were made. Aliquots of the dilutions were plated on LB containing these transformants, with and without IPTG, and the plates were incubated at 30°C for 18 h.

**Plasmid construction for the DNA vaccine**
The hsp60 gene was amplified using primers containing restriction sites and the Kozak consensus sequence...
within the primer: 5’- AAGCTTACCATGGCAAA-
GATTGCAATT - 3’ (sense orientation) and 5’-
GGATCCTTAGTGGTGATGGTG - 3’ (antisense
orientation), which include the HindIII and BamHI sites
(underlined), respectively. The hsp60 gene was ampli-
cified, cloned and transformed as described above. The
recombinant plasmid (pCR®2.1-TOPO®/hsp60) and the
eukaryotic expression vector (pVAX1®) were submitted
to HindIII/BamHI double digestion. The digested sam-
ples were analyzed by 1% agarose gel electrophoresis.
DNA fragments were purified as described above. The
purified DNA fragments were then subcloned into the
pVAX1 vector. The components and conditions of the
ligation reaction were according to the manufacturer’s
instructions. The presence and the correct size of the
insert were confirmed by restriction enzyme digestion
(EcoRI/HindIII). Both the empty vector (control) and
the recombinant vector (pVAX1/hsp60) were introduced
into competent E. coli TOP10, and single recombinants
were selected. Control and pVAX1/hsp60 vectors were
isolated and purified using the EndoFree Plasmid Giga
kit (Qiagen, Valencia, CA, USA). DNA concentration and purity were determined by absorbance at 260 and
280 nm.

**Immunization and challenge**

BALB/c mice (6-8 weeks old) were divided into two
groups of five mice each. Mice were pretreated with 10
μM cardiotxin five days before the first DNA immuni-
zation, as previously described [22]. Each animal was
immunized by injecting the quadriceps muscle with four
doses of 100 μg at 15-day intervals, with an empty vec-
tor (pVAX1; control) or a recombinant vector pVAX1/
hsp60. Blood samples from the mice were collected by
retro-orbital plexus puncture 15, 30, 45 and 60 days
after the first immunization. All mice were challenged
intraperitoneally 21 days after the fourth vaccination
with an infectious dose of 1 ± 10^6 CFU of the MIC-6
strain of *C. pseudotuberculosis*. After challenge, the mice
were monitored daily and the protective effect of the
DNA vaccine was assessed by evaluation of the survival
rate of the immunized animals. This experiment was
performed twice to confirm the results.

**Determination of antibody levels**

To examine the humoral responses induced by pVAX1/
hsp60, the levels of specific anti-hsp60 IgG, IgG1 and
IgG2a isotypes in individual mouse sera were deter-
dined by ELISA. Flat-bottomed 96-well plates were
coated with the *C. pseudotuberculosis* Hsp60 protein in
0.05 M carbonate/bicarbonate buffer, pH 9.6, (5 μg/mL
per well) at 4°C for 18 h. The plates were washed three
times with PBS-0.05% T20 (1X PBS, pH 7.4, 0.05%
Tween 20) and then blocked with PBS-0.05% T20
containing 10% bovine serum albumin - BSA (250 μL
per well) at room temperature for 2 h. Plates were then
washed three times with PBS-0.05% T20 before addition
of a 1 in 100 serum dilution (100 μL per well) from
immunized mice. The plates were then incubated at
room temperature for 1 h, and subsequently washed
three times with PBS-0.05% T20. Afterwards, 100 μL of
peroxidase-conjugated anti-mouse IgG (1 in 5000), IgG1
(1 in 5000) and IgG2a (1 in 2000) antibodies were
added per well (Southern Biotechnology, Birmingham,
AL), and the plates were incubated for 1 h. The reaction
was developed by adding 200 pmol orthophenyldiamine
(Sigma-Aldrich, Bornem, Belgium) for 15 min and
stopped by the addition of 50 μL of 6% H2SO4 to each
well. The plates were read at 492 nm with an automatic
microplate reader (Bio-Rad, Hercules, CA).

**Statistical analysis**

All data were expressed as means ± standard deviation
(S.D.) and analyzed using GraphPad Prism, version 4.03,
for Windows (GraphPad Software, San Diego, CA). Sta-
tistical differences between groups were identified using
one-way ANOVA. A one-tailed Student’s t-test was
used to determine if there were significant differences
between the experimental and control groups. A P value
of 0.05 or less was considered significant.

**Results**

**Isolation and characterization of the *C. pseudotuberculosis* hsp60 and hsp10 genes**

The *C. pseudotuberculosis* hsp60 gene was amplified by
PCR using primers that were designed based on the
genome of *C. diphtheriae*, because of the phylogenetic
proximity between these two species. The full lengths of
the DNA sequences that were amplified were 1,626
(hsp60) and 297 (hsp10) nucleotides. The nucleotide
sequences of the *C. pseudotuberculosis* strain T1 60 kDa
chaperonin and 10 kDa chaperonin GroES coding
sequences were deposited in GenBank under accession
numbers AY781285 and DQ869271, respectively. These
two genes are separated by a small sequence of 11 bp
(Figure 1). In order to obtain information on similarity
with genes of other species, the corresponding
sequences were subjected to BlastN. Similarity searches
of nucleotide sequences of both genes revealed signifi-
cant identity with other *Corynebacterium* species. The
hsp60 gene was predicted to encode a putative protein
consisting of 541 amino acid residues, with a predicted
molecular weight (MW) of 57.4 kDa and a theoretical
isoelectric point (Ip) of 4.91. The hsp10 gene was pre-
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MW of 10.6 kDa and an Ip of 4.49. In order to search
for the putative identities and functions of these genes,
the sequences were subjected to BlastX searches against
the protein database of GenBank. The putative Hsp60 protein displayed greater similarity to the Hsp60 protein of *C. diphtheriae* (89%) and *C. glutamicum* (82%), when compared to those of *Nocardia* (66.9%) and *Mycobacterium* (61%). The comparative alignment of primary amino acid sequences of Hsp60 homologues can be observed in Figure 2. The Hsp60 putative protein shows a motif characterized by a histidine-rich C-terminal. In order to compare the structure of Hsp60 protein of *C. pseudotuberculosis* with that of other chaperonins, a three-dimensional (3D) model was predicted based on PDB-related structures (Figure 3). The protein was found to have high sequence similarity (54% identity) with chaperonins of other bacteria.

**Functional characterization of hsp60**

The GroEL mechanism is universally conserved in prokaryotic species; consequently, paralogous copies of GroEL may result in redundancy of chaperonin function in these organisms. In *E. coli*, the development of phage lambda (γ) requires a functional GroEL/S system. GroEL was originally identified as the host factor responsible for phage γ capsid protein assembly and was subsequently shown to be essential for cell viability [20]. We used a complementation test to functionally characterize the hsp60 gene (groEL gene) of *C. pseudotuberculosis* (Figure 4). Two *E. coli* strains were used for this purpose: the wild-type B178 strain and a ΔgroEL44 mutant. We observed that the mutant transformed with the pProEx-Hta/hsp60 vector only grew at the 10⁻¹ dilution, when incubated at 30°C. When incubation was at 42°C, bacterial growth was not observed (Figure 4b). The ΔgroEL44 mutant, incubated at 30°C, was also not phenotypically restored in a phage experiment. The expression of *C. pseudotuberculosis* hsp60 (groEL) did not promote phage development (Figure 4c).

**Anti-Hsp60 antibody response and protective efficacy of the DNA vaccine in a murine model**

Vaccination with plasmid DNA encoding hsp60 induced significant levels of anti-Hsp60 IgG, IgG1 and IgG2a antibodies in BALB/c mice, after the second immunization, compared to levels in mice vaccinated with the empty vector (Figure 5). Increased antibody responses following boosting were observed for all the isotypes. The levels of IgG1 and IgG2a at 15, 30, 45 and 60 days after the first immunization and the IgG1/IgG2a ratio were recorded (Table 2). The results suggest a Th2-type immune response, induced by pVAX1/hsp60 vaccination, at 15 days after the first immunization, with a decrease of the IgG1/IgG2a ratio at 30, 45 and 60 days after the first immunization.

**Protection studies**

DNA vaccine protection studies were carried out in mice to test the potential of hsp60 for this purpose. All the animals died 12 days after challenge with the wild-type *C. pseudotuberculosis* MIC-6 strain. The mice began to display clinical signs of morbidity three days after infection; during the first two weeks the animals...
showed cachexia, piloerection, cyanosis, hypothermia and ascitis, all characteristic signs and symptoms of a C. pseudotuberculosis infection.

**Discussion**

This study was the first to characterize the immunogenic potential of the hsp60 DNA vaccine for protection against the veterinary pathogen C. pseudotuberculosis. The hsp60 DNA vaccine induced a cellular immune response but failed to confer protective immunity to the host, which corroborates a previous study that found that an Hsp60 protein subunit vaccine also did not confer protection [13].

**Characterization of the hsp60-hsp10 bicistronic operon**

We found that in C. pseudotuberculosis these genes are arranged in a bicistronic hsp60-hsp10 operon, separated by a small sequence of 11 bp. The size and organization of the hsp60 gene in C. pseudotuberculosis (1,626 bp) was similar to those described for other bacterial species, such as C. glutamicum (1,617 bp) [23] and R. equi (1,623 bp) [24]. The hsp10 gene (297 bp) has a start codon (GTG), and the predicted molecular weight is 10.6 kDa. These characteristics are conserved in the hsp10 genes of other species [25]. Comparative DNA sequence analysis of the hsp10 and hsp60 genes showed significant similarity with genes of microorganisms with
phylogenetic proximity, especially between two hsp60 paralogs (groEL1 and groEL2) within the Corynebacterium genus [26]. Alignment of amino acid sequences coded by hsp60 revealed a higher identity at the N- and C-terminal regions. According to Barreiro et al [26] some microorganisms have different functional motifs at the C-terminal ends of the proteins coded by hsp60; there is a string of histidines in groEL1, while groEL2 has a glycine-glycine-methionine (GGM) motif. This putative protein showed a motif containing eight...
histidine residues at the C terminus, which is characteristic of hsp60 paralog groEL1 protein in actinomycetes [27]. The predicted tertiary structure of the Hsp60 protein showed three functionally distinct domains that are very characteristic of chaperonins: an α-helical equatorial, a small intermediate, and a highly flexible apical domain [28].

The high level of similarity between hsp60 of C. pseudotuberculosis and those of other important pathogens, such as M. leprae and M. tuberculosis, is important
because other studies have indicated that mycobacterial Hsp60 is a potential immunodominant target of the humoral and T-cell response in mice and humans [29]. Additionally, high sequence homology of HSPs between different species results in HSPs that have cross-reactive epitopes [30].

Despite high homology between C. pseudotuberculosis hsp60 and E. coli GroEL, our complementation assay failed to complement the GroEL defect in E. coli. There are several explanations for these results. First, the hsp60 expressed in E. coli may have improperly folded and thus lacked activity to complement GroEL. Second, since HSPs have been reported to adversely affect protein homeostasis and vital intracellular functions [11], overexpression of hsp60 may have reduced cell viability at all concentrations except at the 10⁻¹ dilution at 30°C. Third, protein-protein interaction between E. coli GroEL and GroES (the corresponding hsp60 and hsp10 paralogs) is also necessary for cell viability [31]. The GroEL mutant E. coli complemented with the C. pseudotuberculosis hsp60 gene did not grow, indicating that there may be little protein-protein interaction between the Hsp60 and E. coli GroES proteins. Finally, lack of growth may indicate that the Hsp60 protein cannot function inside E. coli despite protein homology because species-specificity of chaperone proteins is complex and is not explained simply by protein homology [32], because the regulatory mechanism of the heat shock response differs among species [33].

E. coli GroEL is also required for the proper growth of bacteriophages, including capsid formation [27,34]. The inability of the C. pseudotuberculosis hsp60 gene to support the growth of ? phage in the E. coli GroEL mutant suggests that the Hsp60 protein does not perform the specific function of capsid formation inside E. coli, again possibly due to incorrect folding.

### Immune response to hsp60 DNA vaccine in mice

Due to the success of DNA-based vaccines using genes encoding HSPs to induce immunity against a variety of pathogens [24], we chose the C. pseudotuberculosis hsp60 gene for the development of a DNA vaccine. Vaccination with the recombinant C. pseudotuberculosis hsp60 antigen induced significant production of specific anti-Hsp60 antibodies. These data indicate that the DNA vaccine (pVAX1/hsp60) generated both IgG1 and IgG2a responses, when administered to BALB/c mice, however, with a tendency towards a Th1-type immune response after 30 days of the first immunization based on reduced IgG1/IgG2a ratio. Nevertheless, DNA immunization with pVAX1/hsp60 conferred no protection against challenge with the pathogen; it did not prevent infection.

The protective efficacy of DNA vaccines has been studied extensively for Mycobacterium tuberculosis. For example, a DNA vaccine encoding the M. leprae hsp65 induced protective immunity against tuberculosis challenge in a mouse model [35], and a DNA vaccine with the M. avium hsp65 plasmid elicited a strong protective immune response in lambs and protected against M. avium subspecies paratuberculosis infection [36]. However, other studies have shown that a high antibody response induced by DNA vaccines does not always result in protective immunity [24], as we also observed in our study.

In summary, intramuscular administration of hsp60 DNA vaccine in mice induced an immune response but failed to confer protection against infection with C. pseudotuberculosis.

### Table 2 IgG1 and IgG2a immune profile induced by vaccination with pVAX1/hsp60 or pVAX1 vectors.

| Days | Groups          | IgG1     | IgG2a     | IgG1/IgG2a ratio |
|------|-----------------|----------|-----------|-----------------|
|      | pVAX1/hsp60     | pVAX1    | pVAX1/hsp60| pVAX1           |
| 15   | 0.06 ± 0.039b   | 0.002 ± 0.001 | 0.03 ± 0.011b | 0.003 ± 0.002 | 2.00 |
| 30   | 0.23 ± 0.094b   | 0.003 ± 0.001 | 0.27 ± 0.149b | 0.002 ± 0.001 | 0.85 |
| 45   | 0.24 ± 0.133b   | 0.004 ± 0.002 | 0.43 ± 0.114b | 0.004 ± 0.001 | 0.56 |
| 60   | 0.32 ± 0.165b   | 0.003 ± 0.002 | 0.49 ± 0.160b | 0.004 ± 0.003 | 0.65 |

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|      | pVAX1/hsp60     | pVAX1    | pVAX1/hsp60| pVAX1           |
| 15   | 0.06 ± 0.039b   | 0.002 ± 0.001 | 0.03 ± 0.011b | 0.003 ± 0.002 | 2.00 |
| 30   | 0.23 ± 0.094b   | 0.003 ± 0.001 | 0.27 ± 0.149b | 0.002 ± 0.001 | 0.85 |
| 45   | 0.24 ± 0.133b   | 0.004 ± 0.002 | 0.43 ± 0.114b | 0.004 ± 0.001 | 0.56 |
| 60   | 0.32 ± 0.165b   | 0.003 ± 0.002 | 0.49 ± 0.160b | 0.004 ± 0.003 | 0.65 |

* Days after the first immunization.

b Significantly different compared to mice immunized with the pVAX1 vector.
References
1. Dorella FA, Pacheco LGC, Oliveira SC, Miyoshi A, Azevedo V: Corynebacterium pseudotuberculosis: microbiology, biochemical properties, pathogenesis and molecular studies of virulence. Vet Rev 2006, 37:1-18.
2. Baird GJ, Fontaine MC: Corynebacterium pseudotuberculosis and its role in ovine caseous lymphadenitis. J Comp Path 2007, 137:179-210.
3. Moura-Costa LD, Bahia RC, Carminati R, Vale VLC, Paule BJA, Portela RW, Freire S M, Nascimento J, Schaer R, Barreto LMS, Meyer R: Evaluation of the humoral and cellular immune response to different antigens of Corynebacterium pseudotuberculosis in Caninde goats and their potential protection against caseous lymphadenitis. Vet immunol immunopathol 2008, 126:131-141.
4. Eggleton DG, Middleton HD, Doidge CV: Immunization against ovine caseous lymphadenitis: comparison of Corynebacterium pseudotuberculosis vaccines with and without bacterial cells. Aust Vet J 1991, 68:317-319.
5. Ellis JA, Hawk DA, Mills KW: Antigen specificity and activity of ovine antibodies induced by immunization with Corynebacterium pseudotuberculosis culture filtrate. Vet Immunol Immunopathol 1991, 28:303-316.
6. Chaplin PJ, De Rose R, Boyle JS, McWaters P, Kelly J, Tennent JM, Lew AM, Scheelnicl JPY: Targeting improves the efficacy of a DNA vaccine against Corynebacterium pseudotuberculosis in sheep. Infect Immun 1999, 67:6434-6438.
7. Williamson LH: Caseous lymphadenitis in small ruminants. Vet Clin North Am Food Anim Pract 2001, 17:359-371.
8. Hauet-Brière F, Wieten L, Guichelsa T, Berlo S, van der Zee R, van Eden W: Heat shock proteins induce T cell regulation of chronic inflammation. Ann Rheum Dis 2005, 65:665-668.
9. Prohászka Z, Fust G: Immunological aspects of heat-shock proteins—the optimum stress of life. Mol Immunol 2004, 41:29-44.
10. Udvarmoly K, Czervenak L, Uray K, Hudecz F, Georgievski I, Schiebelin JPY: Antibodies against C-reactive protein cross-react with 60-kilodalton heat shock proteins. Clin Vac Immunol 2007, 14:335-341.
11. Pockley AG: Heat shock proteins in health and disease: therapeutic targets or therapeutic agents? Exp Rev Mol Med 2001, 2:31-21.
12. Segal BH, Wang XY, Dennis CG, Youn R, Repaiaky EA, Manijii MH, Subjeck JR: Heat shock proteins as vaccine adjuvants in infections and cancer. Drug Discovery Today 2006, 11:534-540.
13. Pinho JMR, Dorella FA, Coelho KS, Fonseca CT, Cardoso MC, Meyer R, Portela RWD, Oliveira SC, Miyoshi A, Azevedo V: Immunization with recombinant Corynebacterium pseudotuberculosis heat-shock protein (Hsp)-60 is able to induce an immune response in mice, but fails to confer protection against infection. Open Vet J Sci 2009, 3:22-27.
14. Pacheco LGC, Pena RR, Castro TLP, Dorella FA, Bahia RC, Carminati R, Freita MNL, Oliveira SC, Meyer R, Alves FS, Miyoshi A, Azevedo V: Multiplex PCR assay for identification of Corynebacterium pseudotuberculosis from public cultures and for rapid detection of this pathogen in clinical samples. J Med Microbiol 2007, 56:480-486.
15. Sambrook J, Russell DW: In Molecular Cloning: a Laboratory Manual. 2nd edition. Edited by: Cold Spring Harbor. New York: Cold Spring Harbor Laboratory; 2001.
16. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 1997, 25:3389-3402.
17. Marti-Renom MA, Stuart A, Fiser A, Sánchez R, Melo F, Sali A: Comparative protein structure modeling of genes and genomes. Annu Rev Biophys Biomol Struct 2000, 29:291-325.
18. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A: Protein identification and analysis tools on the ExPaSy Server. In: The Proteomics Protocols Handbook. 2nd edition. Edited by: John M Walker. NJ: Humana Press; 2005:571-601.
19. Zelisitsa-Ryllis J, Fayer O, Baird L, Georgopoulos C: Sequence analysis and phenotypic characterization of groEL mutations that block I and T4 bacteriophage growth. J Bacteriol 1993, 175:1139-1143.
20. Kumar CMS, Khare G, Srikanth CV, Syagi AK, Sardesai AA, Mande SC: Facilitated oligomerization of mycobacterial GroEL: evidence for phosphorylation-mediated oligomerization. J Bacteriol 2009, 121:6525-6538.
21. Sambrook J, Fritsch EF, Maniatis T: In Molecular Cloning: a Laboratory Manual. 2nd edition. Edited by: Cold Spring Harbor. New York: Cold Spring Harbor Laboratory; 1989.
22. Héchard C, Gépinede O, Rodolakis A: Molecular cloning of the Chlamydophila abortus groEL gene and evaluation of its protective efficacy in a murine model by genetic vaccination. J Med Microbiol 2004, 53:861-868.
23. Banerjo C, González-Lavado E, Pátek M, Martin JF: Transcriptional analysis of the groE5-groEL, groEL2 and dnaK genes in Corynebacterium glutamicum: characterization of heat shock-induced promoters. J Bacteriol 2004, 186:4813-4817.
24. Van damaged T, Barton MD, Heussenbrown MW: The immunogenicity of Rhodococcus equi GroEL2 based vaccines in a murine model. Vet immunol immunopathol 2004, 98:91-100.
25. Kim SN, Kim SW, Pyo SN, Rhee DK: Molecular cloning and characterization of groEL operon in Streptococcus pneumoniae. Mol Cells 2001, 11:360-368.
26. Barrejo C, González-Lavado E, Brand S, Tauch A, Martin JM: Heat shock proteome analysis of wild-type Corynebacterium glutamicum ATCC 13032 and a spontaneous mutant lacking GroEL1, a dispensable chaperone. J Bacteriol 2005, 187:864-869.
27. Ojha A, Anand M, Bhatt L, Jacobs WR, Hatfull G: GroEL: a dedicated chaperone involved in mycobacterial biosynthesis during biofilm formation in Mycobacteria. Cell 2005, 123:861-873.
28. Braig K: Chaperonins. Curr Opin Struct Biol 1998, 8:159-165.
29. Young RA: Stress proteins and immunobiology. Annu Rev Immunol 1990, 8:401-20.
30. Qamar R, Shekhar C, Mande SC, Coates AR, Henderson B: The unusual chaperonins of Mycobacterium tuberculosis. Tuberculosis 2005, 85:385-394.
31. Radulovic S, Rahman MR, Beier MS, Azad AF: Molecular and functional analysis of the Rickettsia typhi groEL operon. Gene 2002, 298:41-48.
32. Kuchnany-Argido D, Lipinska B: Cloning and characterization of the GroE heat-shock operon of the murine bacterium Vibrio Harveyi. Microbiology 2003, 149:1483-1492.
33. Eom CV, Kim E, Ro YT, Kim SW, Kim YM: Cloning and molecular characterization of groEL heat-shock operon in Myelotropic bacterium Myclophysoplosp. Strains SS1 DSM 11726. J Bacterim Mol Biol 2005, 38:695-702.
34. Lee WT, Tretelsky KC, Tabata FR: Cloning and characterization of two groEL operons of Rhodobacter sphaeroides: transcriptional regulation of the heat-induced groEL operon. Amer Soc Microb 1997, 170:487-495.
35. Silva CL, Bonato VL, Coelho-Castelo AA, Souza AO, Santos SA, Lima KM, Faccioli LH, Rodrigues JM: Immunotherapy with plasmid DNA encoding mycobacterial Hsp65 in association with chemotherapy is a more rapid and efficient form of treatment for tuberculosis in mice. Gene Ther 2005, 12:281-287.
36. Sechi LA, Mara L, Cappai P, Frothingam R, Ortu S, Leonia A, Ahmed N, Zanetti S: Immunization with DNA vaccines encoding different mycobacterial antigens elicits a Th1 type immune response in lambs and protects against Mycobacterium avium subspecies paratuberculosis infection. Vaccine 2006, 24:229-235.