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

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Abstract: *Corynebacterium pseudotuberculosis* is the etiological agent of caseous lymphadenitis (CLA), a chronic small ruminant's disease. *C. pseudotuberculosis* Hsp60 expressed in *E. coli* was purified and tested as a vaccine candidate against CLA. Immunization of BALB/c mice with recombinant Hsp60 (rHsp60) induced a significant anti-Hsp60 IgG response, with greater production of IgG1 than of IgG2a. Cell-mediated immune responses induced by immunization were characterized by elevated production of gamma interferon (IFN-γ) and interleukin (IL)-10, while IL-4 concentrations were not significantly increased. Otherwise, mice challenged with 10^6 c.f.u. of a virulent *C. pseudotuberculosis* strain developed abscesses and other signs of morbidity at the site of inoculation. The rate of survival of the animals immunized with rHsp60 was slightly higher than that of mice immunized with PBS; however, all the animals died within two weeks after challenge. We concluded that subcutaneous administration of rHsp60 does not induce effective protection against intraperitoneal infection with *C. pseudotuberculosis*.

INTRODUCTION

Corynebacterium pseudotuberculosis, a facultative intracellular bacterium, is the etiological agent of caseous lymphadenitis (CLA), a chronic and contagious disease that affects sheep and goats worldwide [1-3]. CLA is characterized by the formation of abscesses, typically located in superficial lymph nodes and the lungs, resulting in significant economic losses [4-6]. Transmission among animals occurs mainly through contamination of superficial wounds, which can appear during common procedures, such as shearing, castration and ear tagging, or through injuries of the animal's bodies generated by other traumatic events. Not infrequently, contaminated sheep cough bacteria onto skin cuts of other sheep, constituting another means of transmission [3].

Treatment and control of this disease is difficult because the bacteria are protected inside granulomes, safe from effective concentrations of antimicrobial agents [7]. Consequently other treatment options, such as immunoprophylaxis, are needed to control CLA. Various strategies have been tested to develop an efficient vaccine against *C. pseudotuberculosis,* including the use of attenuated and inactivated bacteria [8-12], and DNA-based vaccines [13]. Among the different formulations tested, the toxoid phospholipase D (PLD) is the most frequently used antigen; it induces partial protection in sheep and goats. However, many undesirable side effects are associated with the use of this exotoxin [1, 14, 15]; consequently, the search continues for identification of effective and safe *C. pseudotuberculosis* vaccine antigens.

Heat-shock proteins (HSPs), a highly conserved category of microbial and mammalian proteins, can elicit both humoral and cellular immune responses [16-18], which are crucial to initiate a specific response against pathogens. According van Eden [19], there are three factors to explain the immunogenicity of Hsps: (i) they are highly conserved molecules, consequently the immune response against conserved epitopes of non-related bacteria is more effective; (ii) they are highly expressed during stress, being easily recognized by the host's immune system; (iii) during infection, the chaperones can be associated to the released antigens during antimicrobial action, the complex antigen-Hsps is captured, processed and presented to immune system, generating an efficient response against the pathogen.

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Significant protection against bacterial infections has been achieved with vaccines made with HSPs [20-25], including Hsp65 from *Mycobacterium leprae*; this species is phylogenetically related to *C. pseudotuberculosis* [26].

All these statements encouraged us to test recombinant *C. pseudotuberculosis* Hsp60 (rHsp60) as an immunogen against CLA, in a murine model.

METHODS

Mice

BALB/c females, 6-8 weeks old, were obtained from the Federal University of Minas Gerais (UFMG) animal facility. This mouse strain was chosen due to its susceptibility to *C. pseudotuberculosis* infection [27-28].

Bacterial Strains, Growth Conditions and Plasmids

Escherichia coli Top10 was purchased from Invitrogen. *C. pseudotuberculosis biovar ovis* (T1 strain) was obtained from the Cellular and Molecular Genetics Laboratory's (UFMG) stock collection. *Escherichia coli* and *C. pseudotuberculosis* were aerobically grown in Luria Bertani (LB) and Heart and Brain Infusion (BHI) broths, respectively, at 37°C. The pTOPO plasmid (Invitrogen) was used for cloning and sequencing of the *C. pseudotuberculosis hsp60* gene. The pProEx-Hta plasmid (Gibco BRL) was used for production of the rHsp60 protein.

Subcloning of the *C. pseudotuberculosis* hsp60 Gene

Genomic DNA from C. *pseudotuberculosis* was isolated, as described by Pacheco *et al.* [29]. The *C. pseudotuberculosis hsp60* gene was amplified by PCR from genomic DNA with the following primers: 5'- GGG<u>GGATCC</u>GATGGCAAAGCTGATTGCA-3' (sense orientation) and 5'- GGG<u>AAGCTT</u>TTAGTGGTGGTGATGGTG-3' (antisense orientation), which include, respectively, *Bam*HI and *Hind*III sites (underlined). The parameters for the PCR reaction were as follows: 1 cycle: 95°C, 5 min; 29 cycles: 95°C, 1 min, 58°C, 40 s, 68°C, 2 min; 1 cycle: 68°C, 7 min. The PCR fragment (1,626 pb), corresponding to the amplified *hsp60* gene, was isolated from agarose gels using the Concert™ Rapid Gel Extraction System (Gibco BRL) and digested with *Bam*HI and *Hind*III (according to manufacturers recommendations). After DNA digestion with *Bam*HI and *Hind*III, the digested DNA was inserted into a pTOPO plasmid that had been previously digested with these same enzymes, generating a pTOPOhsp60 construction. Subsequently, the *hsp60* gene was sequenced using MegaSequencing Systems (GE Healthcare) and subcloned at the *Bam*HI/*Hind*III sites, into the pProEx-HTa expression vector, generating the pPROEx-HTahsp60 construction. Constructs were transformed into *E. coli* electrocompetent cells, according to standard procedures [30], using the gene Pulser System™ (Bio-Rad). *Escherichia coli* transformants harboring the constructed plasmids were screened on LB agar plates containing ampicillin (100 µg ml^{-1}) and X-Gal (40 µg ml^{-1}). The full-length sequence of *C. pseudotuberculosis hsp60* DNA was deposited in GenBank, accession number AY_781285.

Expression and Purification of Recombinant Hsp60

The recombinant Hsp60 was expressed in a prokaryotic system using the pProEX-Hta expression vector. Proteins are expressed with an in-frame six-histidine N-terminal tag in this expression vector. An *E. coli* Top 10 culture (500 ml) containing the recombinant plasmid was grown at 37°C to an optical density at 600 nm of 0.5, and expression of rHsp60 was induced by 1 mM IPTG. After 4 h of induction, the bacterial cells were harvested by centrifugation at 4,000 *g* for 20 min. The pellet was resuspended in 35 ml of 10 mM Na$_2$HPO$_4$, 10 mM NaH$_2$PO$_4$, 0.5 M NaCl and 10 mM imidazole. Subsequently, the cells were submitted to three cycles of sonication lasting 30 s each and centrifuged at 5400 *g* for 20 min. The rHsp60 was recovered as inclusion bodies and solubilized in 50 ml of 8 M urea, 10 mM Na$_2$HPO$_4$, 10 mM NaH$_2$PO$_4$, 0.5 M NaCl, and 10 mM imidazole. The protein was purified by affinity chromatography on a Ni-Sepharose column using an AKTAexplorer chromatograph (GE Healthcare) under denaturing conditions, according to the manufacturer's protocol. Fractions containing rHsp60 were pooled and dialyzed against decreasing concentrations of urea (6M, 4M, 2M and 1M) in PBS buffer, followed by dialysis against PBS buffer. The dialyses were carried out at 4°C using a Spectra/Por2 membrane (6 to 8 kDa; Spectrum Medical). The purity of the rHsp60 was checked on 12% SDS-PAGE, followed by Coomassie blue staining [30], and protein concentration was quantified using the Bradford method [31]. This recombinant protein was used as an antigen for experimental immunization of the mice.

Immunoblotting

The polyacrylamide gel of purified rHsp60 was electroblotted onto a nitrocellulose membrane (Amersham Biosciences), based on Towbin *et al.* [32]. The membrane was blocked with TBS-T (0.5 M NaCl, 0.02 M Tris [pH 7.5], 0.05 % (w/v) Tween 20) containing 10% (w/v) skimmed milk powder for 16 h at room temperature. Subsequently, the membrane was incubated in a 1:2000 dilution of mouse alkaline phosphatase (AP) conjugated anti 6xHis antibody (Invitrogen) in TBS-T plus 10% (w/v) skimmed milk powder for 1 h at room temperature. After three washes with TBS-T, the blot was developed using NBT (nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl-1-phosphate) detection reagents (Gibco BRL), according to the manufacturer's protocol (Gibco BRL). After the alkaline phosphatase reaction had developed, the membrane was washed with distilled water and dried on filter paper.

Immunization Protocol

Two groups of 5 mice each were immunized subcutaneously in the nape of the neck on days 0, 15 and 30. Each animal of the experimental group was injected with 200 µl of a formulation containing 25 µg of rHsp60 in complete Freund's adjuvant during the first immunization and 25 µg of rHsp60 in incomplete Freund's adjuvant during the second and third immunizations. The control animals underwent the same vaccination protocol, but with PBS and without rHsp60. Blood samples were collected from the retro-orbital sinus of each mouse 15 days after each dose of the vaccine. Sera samples were stored at 20°C until use.

Challenge of Mice with Virulent *C. pseudotuberculosis*

Three weeks after the last immunization, mice were challenged *via* intraperitoneal inoculation with 1x10^6 c.f.u. of virulent *C. pseudotuberculosis*. The protection level was

evaluated by the survival rate of the rHsp60 vaccinated group, compared to the control group. Mice were killed by cervical dislocation when moribund.

Analysis of Humoral Responses to rHsp60

Sera obtained from five mice of each group (rHsp60 vaccinated and PBS control groups) were collected two weeks after each immunization. Detection of specific anti-Hsp60 antibodies was performed by ELISA. Briefly, Max-isorp 96-well microtiter plates (Nunc) were coated with 5 µg rHsp60 ml^{-1} in carbonate–bicarbonate buffer, pH 9.6, (100 µl/well) for 16 h at 4°C. The plates were then washed with PBS-T (PBS plus 0.05 % (v/v) Tween 20) and blocked for 2 h at room temperature with 200 µl per well of PBS-T plus 10% (v/v) fetal bovine serum. Sera from immunized mice were diluted 1:200 in PBS-T, added to plates (100 µl/well), and incubated for 1 h at room temperature. Plate-bound anti-body was detected by peroxidase-conjugated anti-mouse IgG (Promega), IgG1 (Sigma) and IgG2a (Sigma) diluted 1:5000, 1:5000 and 1:2000, respectively, in PBS-T. The colorimetric reaction was developed by addition of 100 µl per well of 200 pmol o-phenylenediamine (OPD, Sigma) in citrate–phosphate buffer, pH 5.0, plus 0.04% (v/v) H_2O_2, for 10 min and stopped with 50 µl of 5% (v/v) sulfuric acid per well. The absorbance was read in a spectrophotometer (Bio-rad) at 492 nm.

Cytokine Analysis

One week after the last immunization, five mice of the rHsp60 immunized group, non-challenged, were sacrificed. Splenocytes isolated from the macerated spleens were washed twice in sterile PBS and then adjusted to $1X10^6$ cells mL^{-1} in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum, 100 U penicillin G sodium ml,$^{-1}$ 100 µg streptomycin sulfate ml,$^{-1}$ 250 ng amphotericin B ml^{-1} and 10 µg polymyxin B ml^{-1}. Splenocytes were main-tained in RPMI medium alone (negative control) or stimu-lated with rHsp60 (50 µg ml^{-1}), concanavalin A (ConA) (5 µg ml^{-1}) or purified *E. coli* LPS (Sigma) at the same concen-tration of endotoxin measured in rHSP60 (2.5 µg ml^{-1}). The 96-well plates (Nunc) were maintained in an incubator at 37°C with 5% CO_2. Culture supernatants were collected after 24 h of ConA stimulation, and 48 h (IL-4) or 72 h (IFN-γ and IL-10) after rHsp60 treatment. Assays for measuring IL-4, IFN-γ and IL-10 were performed using Duoset ELISA kits (R&D Diagnostic), according to the manufacturer's protocol.

Statistical Analysis

Statistical analysis was performed with the Student's t-test or ANOVA using the computer software package GraphPad Prism 2.0 (GraphPad Software).

RESULTS AND DISCUSSION

Production of Recombinant Hsp60

To confirm that *C. pseudotuberculosis* rHsp60 was ex-pressed in *E. coli* as a 6xHIS-tag fusion, SDS-PAGE (Fig. **1a**) and Western blot (Fig. **1c**) analyses were performed, revealing a band of approximately 60 kDa, corresponding to the estimated molecular mass for rHsp60 (58.2 kDa). This recombinant protein was recovered as inclusion bodies and purified under denaturing conditions in 8 M urea using a nickel affinity column (Fig. **1b**). Refolding in renaturing

PBS buffer resulted in loss of protein in the precipitated form; however, it yielded enough soluble recombinant pro-tein (1.6 µg µl^{-1}) for the immunization experiments.
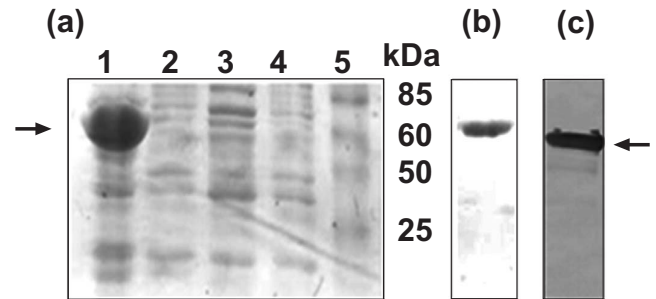

Fig. (1). SDS-PAGE and Western blot analysis of the recombinant HIS-Hsp60 fusion protein (rHsp60). (**a**) Coomassie Blue stained SDS-12% PAGE profile of *E. coli* lysate expressing the pProEx-Hta-hsp60 construct. Pellet and supernatant of the culture induced by IPTG (lanes 1 and 2, respectively). Pellet and supernatant of the uninduced culture (lanes 3 and 4, respectively). Molecular weight marker (lane 5). (**b**) Coomassie Blue stained SDS-12% PAGE profile of the purified rHsp60. (**c**) Western blot of the purified rHsp60 using anti-6xHIS antibody. Arrow indicates the rHsp60.

Humoral Responses to rHsp60

In order to assay for specific anti-rHsp60 IgG antibodies, sera from the vaccinated mice were tested by ELISA. Re-combinant Hsp60 vaccinated mice developed high and sig-nificant titers of IgG anti-rHsp60 compared to the PBS con-trol group, mainly after the second immunization (Fig. **2**). The same high titers were maintained after the third immuni-zation, demonstrating that the immunization with rHsp60 stimulated the production of specific antibodies.
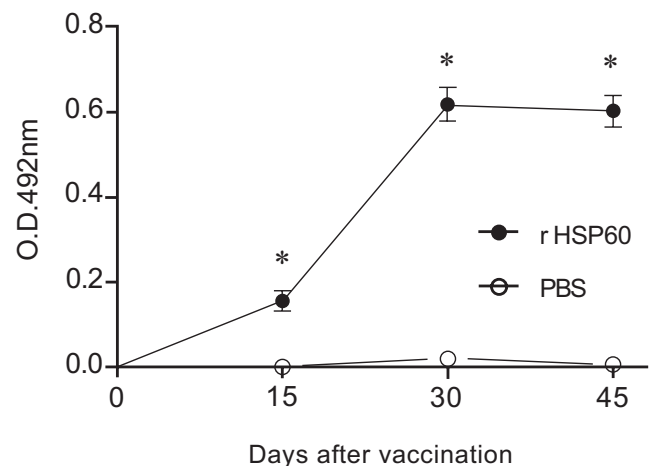

Fig. (2). Kinetics of anti-rHsp60 specific IgG responses in rHsp60-immunized mice. Sera of five immunized mice were collected at days 15, 30 and 45 after the first immunization (day 0) and assayed by ELISA for anti-Hsp60 antibodies. Results are presented as means of each immunization group. Error bars indicate standard deviations of the means. *Significantly different from the PBS control group (p<0.05).

The IgG1 and IgG2a subclasses were also analyzed in order to determine the pattern of immune response. Immuni-zation with rHsp60 induced production of both IgG1 (Fig. **3a**) and IgG2a (Fig. **3b**) anti-Hsp60 antibodies. However, higher levels of specific IgG1anti-Hsp60 were produced than

of IgG2a. Similar results were obtained after BALB/c immunization with *Rhodococcus equi* rHsp60, a *C. pseudotuberculosis* philogenetically related microorganism [33].
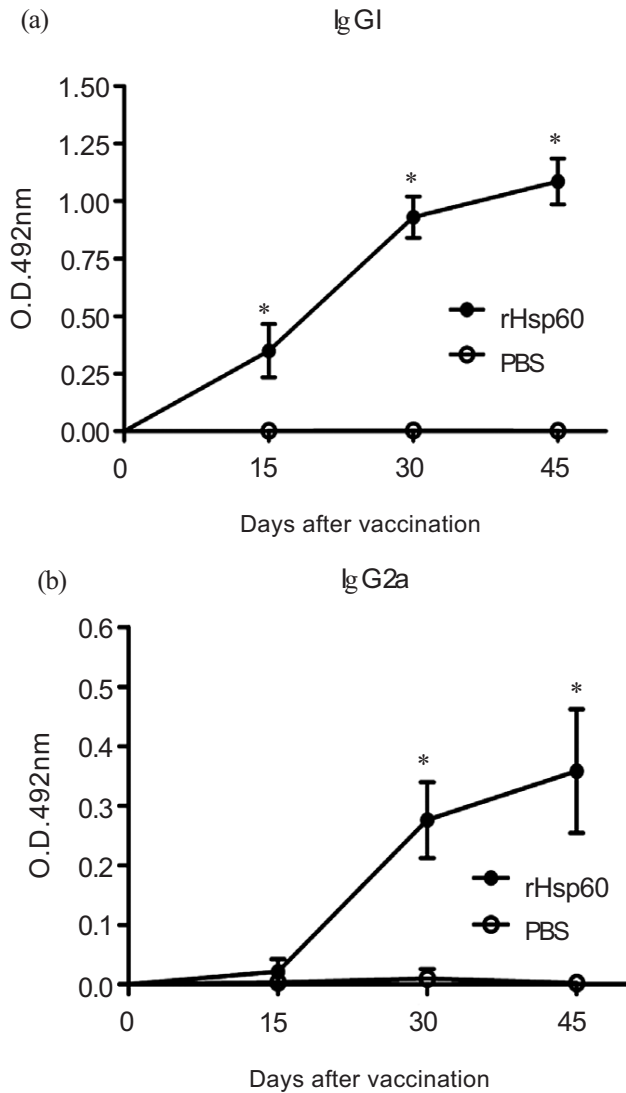

Fig. (3). Levels of IgG1 and IgG2a isotypes following rHsp60 immunization. Kinetics of anti-rHsp60 specific IgG1 (**a**) and IgG2a (**b**). Results are presented as means of each immunized group (n = 5). Error bars indicate standard deviations of the means. *Significantly different from the PBS control group (p<0.05).

Cytokine Profile Induced Following rHsp60 Vaccination

Production of IFN-γ, IL-4 and IL-10 in splenocyte culture supernatants from immunized mice was measured by ELISA (Fig. **4**). We observed high levels of IFN-γ production in cells stimulated with rHsp60 and these levels were significantly greater than in cells cultured with medium and LPS alone. The IL-10 production was significantly elevated too, while IL-4 production levels were almost undetectable. This cytokine profile, with the production of antagonistic cytokines IFN-γ and IL-10, was not expected because the high production of IL-10 inhibits the production of IFN-γ. We suggest that the cellular response was immunomodulated by rHsp60. The epitope recognition produced an inflamma-

tory response, characterized by IFN-γ production. Otherwise, the immune system could recognize conserved sequences between prokaryotes and eukaryotes Hsps, giving rise to an immune suppressor response, with IL-10 production [34-35].
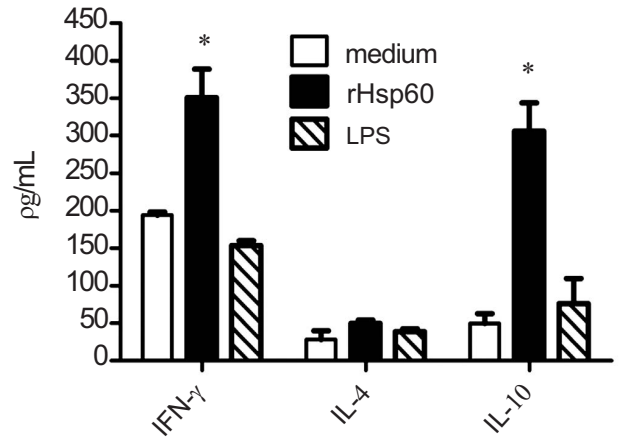

Fig. (4). Cytokine profile of mice immunized with rHsp60. One week after the third immunization (day 45) splenocytes were isolated and assayed for production of IFN-γ, IL-4 and IL-10 in response to rHsp60 (50 μg ml^{-1}), and LPS (2.5 μg ml^{-1}) or RPMI medium alone (controls). The results are presented as means of each group (n= 5). Error bars indicate standard deviations of the means. * Significantly different from the medium and LPS control groups (p<0.05).

Protective Efficacy of rHsp60 Against Challenge

Three weeks after the last immunization, BALB/c mice were challenged with virulent *C. pseudotuberculosis*. Both vaccinated and control groups presented signs of morbidity and abscesses. All of the mice immunized with the PBS control were killed by cervical dislocation, due these morbidity signs, by day 3. The animals immunized with rHsp60 lived slightly longer, with 100% mortality occurring by day 11 (Fig. **5**). Although animals immunized with recombinant protein had presented a survival rate higher than control group, the time of survival was short to consider rHSp60 as a protective vaccine. These experiments were carried out twice, giving similar results.
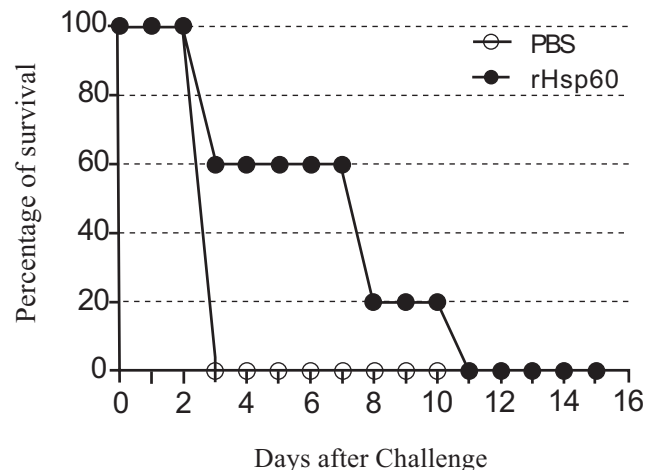

Fig. (5). Survival of BALB/c mice infected intraperitoneally with 1x10^6 c.f.u. of virulent *Corynebacterium pseudotuberculosis*. The data show a representative experiment, using five mice for each group, which were monitored daily for survival.

CONCLUSION

We concluded that subcutaneous administration of rHsp60 does not induce effective protection against intraperitoneal infection with *C. pseudotuberculosis*. Turner *et al.* [36] found similar results with vaccines directed against the Hsp60 molecule of *M. tuberculosis*. Immunity to CLA has been attributed to both a strong humoral component and to cell-mediated immunity mechanisms. Induction of a Th1-type T-cell response, characterized by IFN-γ production [37-39], has been found to be involved in protection against infection. In our study, vaccination with rHsp60 induced high levels of IL-10 and IFN-γ, suggesting an immunomodulatory response. This could be the reason why our immunization procedure was unable to confer protection, since IL-10 down-regulates the production of other cytokines, such as IFN-γ [40-43].

Despite the successful utilization of rHsp60 in immunization against different microorganisms [20, 44], there are some reports where the protection did not occur [45, 46]. There is not an explication why proteins highly conserved, like Hsp60, acts differently when used as vaccinal antigens. Moreover, is difficult to establish a comparison among Hsp60s from different microorganisms used as antigens since the protocols of immunization, doses and inoculation route were diverse.

Other strategies could be used in order to improve the protective response provided by vaccination with *C. pseudotuberculosis* Hsp60. Co-administration of immunostimulatory molecules, such as IL-12, improves the immune response and directs it towards a Th1 phenotype that is able to elicit protection against challenge by *Yersinia enterocolitica* [20]. Other alternatives consist include a DNA based-vaccine and immunization with an attenuated *C. pseudotuberculosis* strain overexpressing Hsp60. Besides, we can suggest the utilization of rHsp60 as an adjuvant in immunization with attenuated or inactivated *C. pseudotuberculosis* or even combined with other proteins of this bacterium.

ACKNOWLEDGEMENTS

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