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Research Article

The *Leishmania* HSP20 Is Antigenic during Natural Infections, but, as DNA Vaccine, It does not Protect BALB/c Mice against Experimental *L. amazonensis* Infection

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Protozoa of the genus *Leishmania* are causative agents of leishmaniasis, an important health problem in both human and veterinary medicine. Here, we describe a new heat shock protein (HSP) in *Leishmania*, belonging to the small HSP (sHSP) family in kinetoplastids. The protein is highly conserved in different *Leishmania* species, showing instead significant divergence with sHSP's from other organisms. The humoral response elicited against this protein during *Leishmania* infection has been investigated in natural infected humans and dogs, and in experimentally infected hamsters. *Leishmania* HSP20 is a prominent antigen for canine hosts; on the contrary, the protein seems to be a poor antigen for human immune system. Time-course analysis of appearance of anti-HSP20 antibodies in golden hamsters indicated that these antibodies are produced at late stages of the infection, when clinical symptoms of disease are patent. Finally, the protective efficacy of HSP20 was assessed in mice using a DNA vaccine approach prior to challenge with *Leishmania amazonensis*.

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1. INTRODUCTION

Leishmania spp., kinetoplastid protozoan parasites, cause a diverse collection of human diseases (known as leishmaniasis) ranging in severity from a spontaneously healing skin ulcer to overwhelming visceral disease. Leishmaniasis currently affects an estimated 12 million people in 88 countries, with approximately 500 000 new cases of visceral leishmaniasis (VL) per year and 1.5 million for cutaneous leishmaniasis [1]. The clinical manifestations of leishmaniasis depend on complex interactions between the virulence characteristics of the infecting *Leishmania* species and the host immune response [2, 3]. Thus, CL is caused mainly by *Leishmania major* and *Leishmania mexicana*, whereas VL is essentially caused by *Leishmania donovani* and *Leishmania infantum* (also called *Leishmania chagasi* in Latin America). However, a single *Leishmania* species can produce more than one clinical outcome; for example, *Leishmania amazonensis* is known to be associated with cutaneous, diffuse cutaneous, and visceral leishmaniasis in South and Central America. There are no vaccines available at present to control any form of leishmaniasis, despite considerable laboratory efforts [4]. Current chemotherapy is far from satisfactory [5] and new reagents to improve diagnosis would be desirable.

Leishmania is transmitted by the bite of infected sandflies, where the parasite lives as extracellular promastigote in the insect gut, to mammalian hosts. The promastigotes invade macrophages, where they differentiate and replicate as obligatory intracellular amastigotes. During transmission, the parasite is subjected to a drastic change of environmental temperature from the ambient temperature in the insect vector to higher temperatures in the mammalian host. The heat shock response, mediated by the induction of the heat shock proteins (HSPs), is a homeostatic mechanism that

protects cells from the deleterious effects of thermal and other environmental stresses [6]. Given these circumstances, HSPs, in *Leishmania*, are believed to play essential roles in the host-pathogen interaction. Remarkably, many immunogenic *Leishmania* antigens are members of different HSP families [7]. Thus, HSP60 [8], HSP70 [9–13], Grp78 [14], HSP83/90 [11, 12, 15], and Grp94 [16] have been described as prominent antigens recognized by sera from leishmaniasis patients. The preponderance of HSPs as molecules recognized by human immune system was illustrated in a recent study [17]: from 242 protein-producing clones, which were identified by immunoscreening with sera from VL patients, 118 (49%) contained sequences coding for members of the HSP70 and HSP83/90 families. However, among the main groups of HSPs, there exists a notable absence, that is there is not any report describing members of the small heat shock protein (sHSP) family as antigens during *Leishmania* infections. Indeed, no descriptions of such genes have been published so far.

The sHSP family comprises the most widespread but also the most poorly conserved group of HSPs. sHSPs have been found in bacteria, archaea and eukaryotes [18]. Although proteins belonging to the sHSP family are diverse in sequence and size, they share characteristic features: (i) a conserved α-crystallin domain of approximately 90 residues; (ii) a small molecular mass of 12–43 kDa; (iii) tendency to form large oligomers; (iv) increased synthesis by stress conditions; (v) chaperone activity in suppressing protein aggregation [19].

The main aim of this work was to characterize possible sHSPs existing in *Leishmania*. For this purpose, we took advantage of the recently completed sequence of the *L. major* genome [20]. In this database, we found sequence information that helped us to identify the *L. amazonensis* HSP20, which represents in turn the first description of a member of the sHSP family in kinetoplastids. Furthermore, the *HSP20* gene was expressed in bacteria and the purified recombinant protein was found to be antigenic during *Leishmania* infection. Finally, the immunoprotective properties of this protein, administered as a DNA vaccine, were analyzed in the murine model of *L. amazonensis* infection.

2. MATERIALS AND METHODS

2.1. Parasites

Two *L. amazonensis* strains were used in this work: strain IFLA/BR/67/pH-8 for gene cloning and strain MHOM/77/LTB0016 for infection of mice. The virulence of the parasite was maintained by regular passage through BALB/c mice. Parasites were cultured at 25°C in Schneider's medium supplemented with 10% fetal bovine serum (FBS). *L. infantum* promastigotes (M/CAN/ES/96/BCN150) were cultured at 26°C in RPMI 1640 medium supplemented with 10% FBS.

2.2. Cloning and purification of the recombinant HSP20

The full-length open reading frame of *L. amazonensis HSP20* gene was PCR amplified from genomic DNA (strain IFLA/BR/67/pH-8) with specific primers: HSP20d, 5′-CCAAGCTTATGTGGAGCCCGAGCAACAA-3′; HSP20r, 5′-CGGGATCCTTAGTCGATGGTGACTGAGT-3′ (underlined are restriction sites included in the primers for cloning purposes). The PCR product was cloned into pCR2.1 vector (Invitrogen Corp., San Diego, Calif., USA) to generate pCRHSP20La clone. The *HSP20* gene in pCRHSP20La was removed by *Hin*dIII plus *Not*I double digestion and cloned in the corresponding restriction sites of the pET-28b prokaryotic expression plasmid (Novagen, Madison, Wis, USA) to generate pETHSP20La clone. This clone was used to express the *L. amazonensis* HSP20 in *Escherichia coli* (BL21 strain). The recombinant protein (rHSP20), which contains an N-terminal His-tag, was purified on a Ni-NTA agarose column, following the methodology provided by the supplier (Qiagen, Hilden, Germany). The tag was not removed after purification.

The *L. amazonensis* HSP20 coding sequence contained in the pCRHSP20La clone (see above) was obtained by *Hin*dIII-*Bam*HI double digestion and subcloned in the corresponding sites of the eukaryotic expression plasmid pcDNA3 (Invitrogen) to produce clone pcDNA3-LaHSP20. The authenticity of the different clones and the fidelity of the PCR-amplified fragments were verified by nucleotide sequencing (Parque Científico de Madrid, UAM, Madrid). The nucleotide sequence data for *L. amazonensis* HSP20 coding sequence have been deposited at the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession no. **AM712297**.

2.3. Phylogenetic analysis

The genome databases for different *Leishmania* species (http://www.genedb.org) were used to determine sequence homologues to members of the sHSP family. Protein sequences were scanned for the occurrence of patterns stored in PROSITE-SWISS-PROT database (http://www.expasy.org). For phylogenetic analysis, 39 sHSP sequences were used, choosing representative members for the main groups of organisms: archaea, bacteria, fungi, plant, animal, and kinetoplastids. According to previous studies [18], bacterial sHSPs can be grouped within class A or class B, and representative sequences for both groups have been included in our analysis. The alpha-crystallin domain from the sHSP sequences were aligned using the ClustalW algorithm and the data set was used to build a phylogenetic tree with the MEGA3 software [21]. The trees were made using the neighbor-joining and the minimum evolution algorithms, with Poisson-corrected amino acid distances. The reliability of clustering patterns in the tree was tested by bootstrapping (1000 replicates).

2.4. Sera and enzyme-linked immunosorbent assays (ELISA)

Sera of golden hamsters (*Mesocricetus auratus*) were obtained from animals experimentally infected with *L. infantum* (strain M/CAN/ES/96/BCN150). Blood samples were collected monthly. Further details of these sera have been

published elsewhere [22]. Human sera were obtained from patients with VL living in Spain. All patients had clinical signs of leishmaniasis and the sera were positive for *Leishmania* by indirect immunofluorescent tests. The presence of parasites was further demonstrated in smears of bone marrow aspirates. Also, canine sera from naturally *Leishmania*-infected dogs from the Comunidad de Madrid (Spain) were assayed. For all animals, *Leishmania* infection was determined by serological and parasitological methods.

Total *Leishmania* antigen was prepared from *L. infantum* promastigotes by incubation of parasites in lysis buffer (1% Triton X-100; 150 mM NaCl; 10 mM Tris-HCl, pH 8.0; 1 mM PMSF) for 15 minutes. Afterwards, the suspension, kept on ice, was sonicated until a decrease in viscosity was observed. The insoluble material was pelleted at 10 000 g for 5 minutes and the supernatant stored at -70°C until use. ELISA assays were carried out using standard conditions. Microplate wells (Nunc A/S, Roskilde, Denmark) were coated with either 0.5 μg/ml of purified rHSP20 (see above) or 2 μg/ml of total *Leishmania* antigen. Primary antibodies were assayed at 1 : 200 dilution. Secondary antibodies (horseradish peroxidase immunoconjugates; Nordic Immunological Laboratories, Tilburg, The Netherlands) against human, hamster, or dog total IgGs (H + L chains) were used at 1 : 1500 dilution. Finally, ortho-phenylenediamine (Dako, Glostrup, Denmark) was used in the developing solution and the reaction product was read at 450 nm.

For each particular assay, cut-off values (Cov) were established from the reactivity values of control-negative sera, calculating the 99% confidence interval for the mean of a normal population. Thus, the Cov values were calculated with the following formula: $\mathrm{Cov} = \mu + 2.576\,\sigma\,(N)^{-1/2}$, where μ is the mean value, σ is the standard deviation, and N is the number of control sera. At least, ten control sera were used for each assay.

2.5. Production of mouse antisera against rHSP20

Female 6-8-week-old BALB/c mice (Harlan Interfauna Ibérica, Barcelona, Spain) were intradermically injected with a mixture of 20 μg of rHSP20 and 50 μg of oligonucleotide ODN-1826 (5′-TCCATGACGTTCCTGACGTT-3′), emulsified 1 : 1 with incomplete Freund's adjuvant (Gibco-BRL, Grand Island, NY, USA). Oligonucleotides containing CpG sequences, such as that ODN-1826, have immunostimulating properties for the mouse immune system, and they are being used in vaccination assays for inducing stronger humoral and cellular responses against coadministered antigens [23]. Injections were repeated (using only rHSP20 emulsified 1 : 1 with IFA) at 2-week intervals for a total of three immunizations. Antibody IgG-titres, evaluated by ELISA against rHSP20, were higher than 200 000.

2.6. Expression of Leishmania HSP20 in pcDNA3-LaHSP20-transfected cells

COS7 cells were harvested from cultures in the late-logarithmic phase of growth (85% confluence); cells were separated from plates by a short treatment (1-2 minutes at 37°C) with 0.002% EDTA and 0.25% trypsin. Afterwards, cells were washed with DMEM (supplemented with 10% FBS) and resuspended in the same medium containing 10 mM HEPES. Two hundred μl of the cell suspension (3×10^6 cells) were transferred into a 0.4-cm cuvette. After adding 5 μg of plasmid DNA, 100 μg of salmon sperm DNA and 5 μl of 1.5 mM NaCl, cells were electroporated using a capacitance of 960 μF and a voltage of 200 V. Immediately, cells were transferred to a 100-mm culture dish containing 10 ml of DMEM (with 10% FBS) and incubated for 72 hours at 37°C with atmosphere of 5% CO_2. Afterwards, cells were harvested, washed two times with ice-cold PBS and immediately lysed by addition of Laemmli's load buffer. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham, Little Chalfont, United Kingdom). The blots were probed with the mouse anti-HSP20 antibody diluted at 1 : 500 (see above). As secondary antibody, an antimouse IgGs (horseradish peroxidase immunoconjugate) was used at 1 : 2000 dilution. The blot was developed using the ECL system (Amersham, Little Chalfont, United Kingdom).

2.7. Vaccination and infection of mice

Endotoxin-free plasmid DNA, prepared using the EndoFree Plasmid Giga Kit (Qiagen, Hilden, Germany), was used for immunizations. BALB/c mice were obtained from the Centro Nacional para la Producción de Animales de Laboratorio (CENPALAB, La Habana, Cuba). Mice were 6–8 weeks of age when DNA immunizations were initiated. Groups of 6 mice were injected twice at 2-week interval intramuscularly in the left quadriceps with 100 μg of pcDNA3-LaHSP20 DNA plasmid. As negative controls, groups of mice were injected with either PBS or empty vector (pcDNA3, Invitrogen). Two weeks after the last injection, the mice were challenged with 10^5 stationary-phase *L. amazonensis* promastigotes that were suspended in 50 μl of PBS and injected into the left hind footpad. The development of lesions was monitored weekly using a digital calliper. The contralateral footpad of each animal represented the control value, and the swelling was calculated as follows: thickness of the left footpad—thickness of the right footpad.

3. RESULTS

3.1. Identification of Leishmania sHSPs and sequence analysis

We began this study by performing a BLASTP search of the *L. major*, *L. infantum*, and *L. braziliensis* databases (http://www.genedb.org) using as query sequence the *Saccharomyces cerevisiae* HSP26 sequence (UniProtKB/Swiss-Prot entry P15992). Interestingly, for each *Leishmania* species a single entry was retrieved: LmjF29.2450(*L. major*), LinJ29_V3.2560 (*L. infantum*), and LbrM29_V2.2420(*L. braziliensis*). The BLAST scores were significant (114 or higher), suggesting that they may represent sHSP members in these *Leishmania* species. In fact, these entries in the different *Leishmania* databases are annotated as putative HSP20 proteins. Based on these sequences, we designed PCR primers to amplify the

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LaHSP20    --MWSPS-NKRDSLINSDDALFPFLLH--FPGLHPRQMFSSFFAS    40
LiHSP20    --MWSPS-NKRDSLINSDDALFPFLLH--FPGLHPRQMFSSFFSS    40
LmjHSP20   --MWSPS-NKRDSLINSDDTFFPFLFH--FPGSHPRQMLSSFFAS    40
LbHSP20    --MWSPN-NKGDSLSNSDDGLFPFLFP--FPDLRPLQMFNTFFSS    40
ScHSP260   MSFNSPFFDFFDNINNEVDAFNRLLGEGGLRGYAPRRQLANTPAK    45

LaHSP20    RSRG-----------------------------SWIP--------    48
LiHSP20    RSRG-----------------------------SWIP--------    48
LmjHSP20   RSRG-----------------------------SWIP--------    48
LbHSP20    RSRG-----------------------------SWIP--------    48
ScHSP26    DSTGKEVARPNNYAGALYDPRDETLDDWFDNDLSLFPSGFGFPRS    90

LaHSP20    ---AVDILEQDDGYTLVADLPEVK-KEDLRV-YTESSSIICISGN    88
LiHSP20    ---AVDISEQDDGYTLVADLPEVR-KEDLRV-YTESSSIICISGN    88
LmjHSP20   ---AVDISEQDDGYILVADLPEVK-KEDLRV-YTESSSIICISGN    88
LbHSP20    ---AVDLSQQDDGYTLVADLPEVK-KEDLRV-YTESASIICISGN    88
ScHSP26    VAVPVDILDHDNNYELKVVVPGVKSKKDIDIEYHQNKNQILVSGE    135

LaHSP20    RKHVLKQDEH-QLLVAERGTGRFERCFDLP--TPVDSSKIKATFN    130
LiHSP20    RKHILKQDEH-QLLVAERGAGRFERCFDLP--TSVDSSKIKASFN    130
LmjHSP20   RKHILKQDEH-QLLVAERGAGRFERCFDLP--TSVDSSKIKASFN    130
LbHSP20    RKSVLKQDER-QLLVAERGFGRFERCFELP--TSVDNSKIKATFN    130
ScHSP26    IPSTLNEESKDKVKVKESSSGKFKRVITLPDYPGVDADNIKADYA    180

LaHSP20    DQQLNVSIPKLRNSRSGTSNSVTID                        155
LiHSP20    DQQLNVSIPKLRNSRSGTSNTVTID                        155
LmjHSP20   DQQLNLSIPKLRNSKSGASNSVTID                        155
LbHSP20    DHQLSVSIPKMRNTKSGASNSVTID                        155
ScHSP26    NGVLTLTVPKLKPQKDGKNHVKKIEVSSQESWGN               214
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Figure 1: Amino acid sequence alignment of HSP20 from *Leishmania* spp. and *S. cerevisiae* HSP26. Protein sequences of *L. amazonensis* (LaHSP20, this work; EMBL accession number **AM712297**), *L. infantum* (LiHSP20, GeneDB identifier **LinJ29_v3.2560**), *L. major* (LmjHSP20, GeneDB identifier **LmjF29.2450**), *L. braziliensis* (LbHSP20, GeneDB identifier **LbrM29_V2.2420**) sHSPs, and *S. cerevisiae* HSP26 (UniProtKB/Swiss-Prot entry **P15992**) were aligned using the default settings of ClustalW (DNAstar program). Amino acid residues conserved in all sequences are shaded; those conserved in more than 50% of the sequences are boxed. The position of the α-crystallin domain is indicated by a horizontal line. The domains were defined using the Prosite utility at the ExPASy Proteomics Server (http://www.expasy.org); high scores with the HSP20/α-crystallin family profile (accession number **PS01031**) were obtained.

HSP20 gene homologue for *L. amazonensis*. Figure 1 shows the alignment of the various *Leishmania* HSP20s and the *S. cerevisiae* HSP26. *Leishmania* HSP20s share a high sequence identity (higher than 80%); instead, *Leishmania* HSP20s and yeast HSP26 show ≈30% sequence identity. However, all proteins present the signature motif of sHSPs, that is the HSP20/α-crystallin domain (Figure 1). In addition, we performed a phylogenetic analysis using representative sHSP sequences from the different groups of organisms (Figure 2). This analysis showed a sHSP grouping consistent with previous reports [18]. However, it should be kept in mind that phylogenetic analyses based on sHSP sequences are not adequate to establish evolutionary relationships between distant groups of organisms. Thus, the valid conclusion of our analysis was that *Leishmania* HSP20s are close to each other and distant from all other sequences. Although with a low bootstrap value, our data showed that *Leishmania* HSP20s, together *Trypanosoma brucei* HSP20, form a monophyletic group, separated from the other groups of organisms. This agrees with the belief that kinetoplastids evolved early in evolution from the rest of eukaryotes [24]. Furthermore, even though the bootstrap values are very low, our analyses showed that kinetoplastid sHSPs are closer to homologues from plants than to the corresponding proteins from animals. Remarkably, some plant-like traits in kinetoplastids have been reported in previous studies [25].

3.2. *Recognition of Leishmania HSP20 by sera from infected animals and leishmaniasis patients*

As shown in Figure 1, the HSP20 sequence is well conserved among the different *Leishmania* species. For instance, *L. amazonensis* and *L. infantum* HSP20s share 147 out of the 155 amino acids (95% of identity), and 4 out of the 8 amino acid changes are conservative (97.4% of similarity). With this remarkable degree of sequence conservation, it is expected that both proteins are essentially cross-reactive. Indeed, as shown below, the *L. amazonensis* HSP20 was recognized by all sera from *L. infantum* infected dogs. Thus, the *L. amazonensis* HSP20, expressed in *E. coli* as a His-tagged recombinant protein (rHSP20), was used in ELISA assays to determine whether antibodies against HSP20 are elicited in

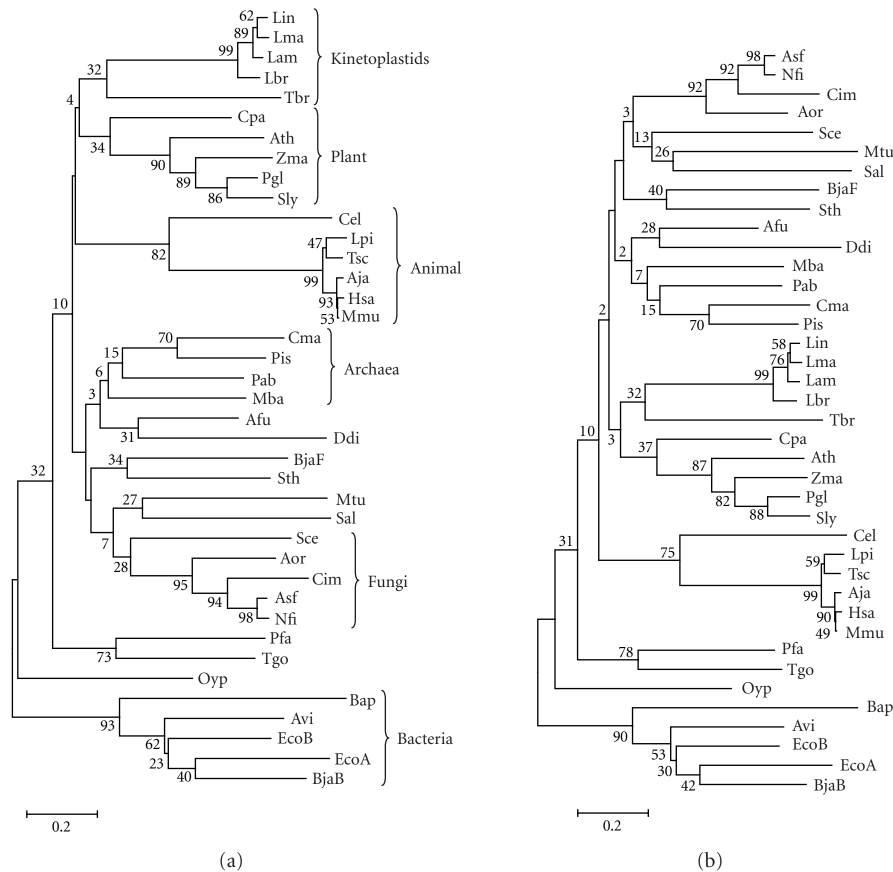

Figure 2: Phylogenetic analysis of sHSPs. Phylogenetic trees were constructed on the basis of α-crystallin/HSP20 domains by Neighbor Joining (a) and Minimum Evolution (b) using the MEGA 3 program as described in the Material and Methods section. Sequences (UniProtKB/TrEMBL entry): Afu, *Archaeoglobus fulgidus* (**O28308**); Aja, *Artibeus jamaicensis* (**P02482**); Aor, *Aspergillus oryzae* (**Q2TXY8**); Asf, *Aspergillus fumigatus* (**Q4WV00**); Ath, *Arabidopsis thaliana* (**O81822**); Avi, *Azotobacter vinelandii* (**P96193**); Bap, *Buchnera aphidicola* (**P57640**); BjaB, *Bradyrhizobium japonicum* (HspB; **P70918**); BjaF, *Bradyrhizobium japonicum* (HspF; **O69243**); Cel, *Caenorhabditis elegans* (**Q7JP52**); Cim, *Coccidioides immitis* (**Q1E6R4**); Cma, *Caldivirga maquilingensis* (**A8MB44**); Cpa, *Carica papaya* (**Q69BI7**); Ddi, *Dictyostelium discoideum* (**Q54I91**); EcoA, *Escherichia coli* (ibpA; **P0C054**); EcoB, *Escherichia coli* (ibpB; **P0C058**); Hsa, *Homo sapiens* (**P02489**); Lam, *L. amazonensis*; Lbr, *L. braziliensis*; Lin, *L. infantum*; Lma, *L. major*; Lpi, *Lygodactylus picturatus* (**Q6EWI0**); Mba, *Methanosarcina barkeri* (**Q46E59**); Mmu, *Macaca mulatta* (**P02488**); Mtu, *Mycobacterium tuberculosis* (**P0A5B7**); Nfi, *Neosartorya fischeri* (**A1DEG0**); Oyp, *Onion yellows phytoplasma* (**P81958**); Pab, *Pyrococcus abyssi* (**Q9V1L0**); Pfa, *Plasmodium falciparum* (**Q8IB02**); Pis, *Pyrobaculum islandicum* (**A1RRY3**); Pgl, *Picea glauca* (**Q40852**); Sal, *Streptomyces albus* (**Q53595**); Sce, *S. cerevisiae* (**P15992**); Sly, *Solanum lycopersicum* (**O82545**); Sth, *Streptococcus thermophilus* (**P80485**); Tbr, *Trypanosoma brucei* (**Q57V53**); Tgo, *Toxoplasma gondii* (**Q6DUA8**); Tsc, *Trachemys scripta* (**Q91517**); Zma, *Zea mays* (**P24632**). The scale represents mutational changes per residue.

experimentally infected hamsters. Remarkably, 62% of the *Leishmania* infected animals developed significant humoral responses against the HSP20 (Figure 3). No correlation was observed between total antigen and HSP20 reactivity values for individual sera. Furthermore, we analyzed the time course of appearance of anti-HSP20 antibodies along the infection process. As illustrated in Figures 3(b) and 3(c), the anti-HSP20 reactivity was detected long time later than the antibodies against total *Leishmania* proteins.

In order to know whether the *Leishmania* HSP20 is also immunogenic during natural *Leishmania* infections, the reactivity against rHSP20 of a collection of sera from canids with VL and leishmaniasis patients was determined by ELISA (Figure 4). Interestingly, all sera from dogs with VL reacted with rHSP20, showing reactivity values similar to those observed using total *Leishmania* proteins (Figure 4(a)). In contrast, few sera from leishmaniasis patients showed a positive reactivity against rHSP20, suggesting that this protein is poorly antigenic for human immune system (Figure 4(b)).

3.3. Immunoprotective potential of *Leishmania* HSP20 as a DNA vaccine

The potential of parasite HSP20 to induce an immunoprotective response was assessed in DNA vaccination experiments using the infection model of *L. amazonensis* in mice. For that purpose, the *L. amazonensis HSP20* gene was cloned into the eukaryotic expression vector pcDNA3 and its appropriate expression was assessed in COS7 cells transfected with

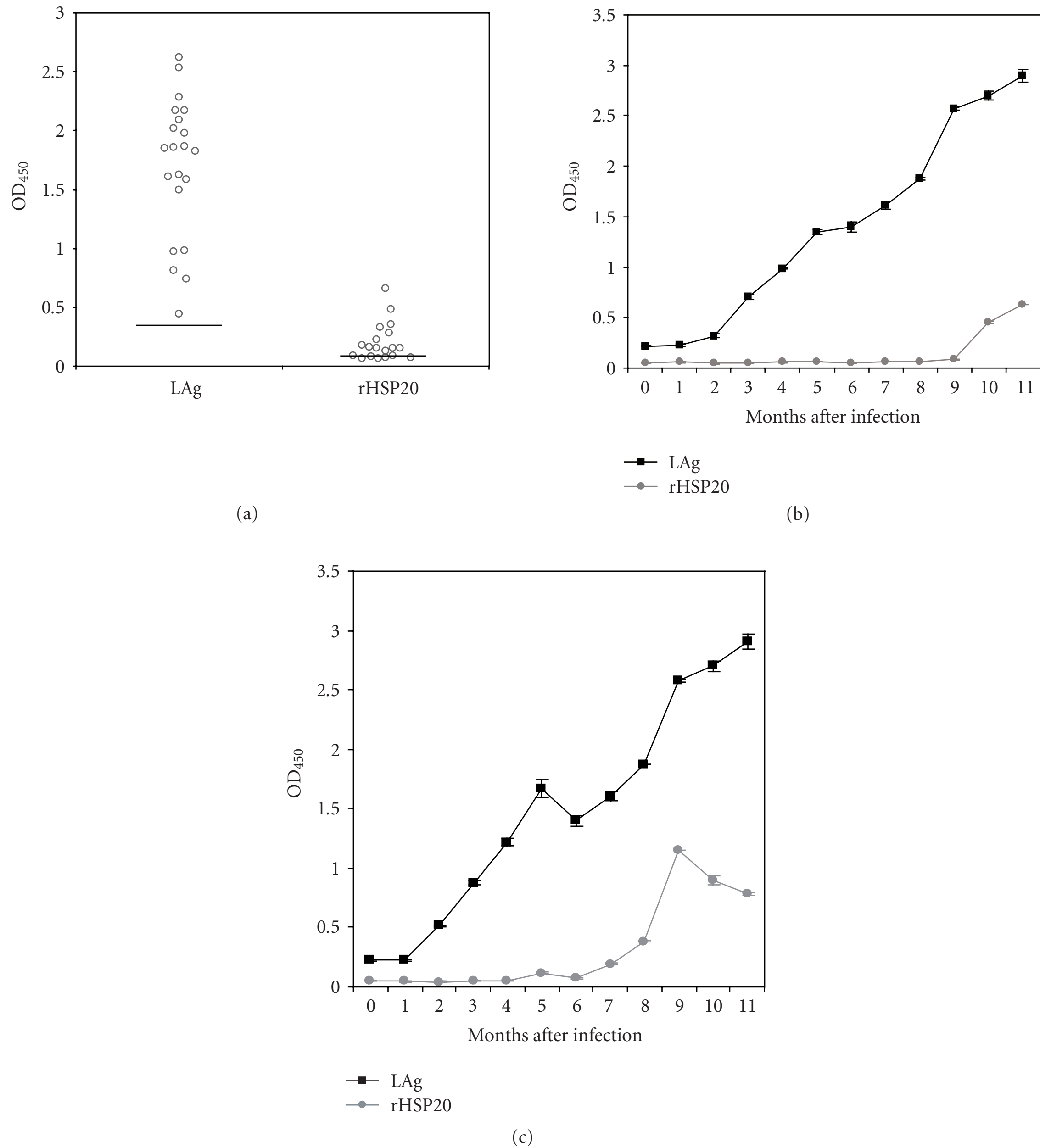

FIGURE 3: Antigenicity of *Leishmania* HSP20 in experimentally infected hamsters. (a) Reactivity against total *L. infantum* proteins (LAg) or *L. amazonensis* recombinant HSP20 (rHSP20) of sera from *L. infantum* infected hamsters is indicated as the mean optical density read at 450 nm (OD_{450}). Horizontal lines represent the cut-off values for the assay, which were 0.286 for LAg and 0.067 for rHSP20. (b) and (c) Time course of the humoral response against total antigens (LAg) or rHSP20 elicited in two experimentally infected hamsters.

the construct (Figure 5(a)). Groups of BALB/c mice were immunized with either pcDNA3-LaHSP20 DNA, empty plasmid DNA or PBS (see materials and methods for further details). A very low, but detectable, reactivity against rHSP20 protein was observed in the sera of LaHSP20-DNA vaccinated mice (data not shown). After challenge, *L. amazonensis* infection was evaluated by measuring lesion development in the infected footpads (Figure 5). Lesions progressed similarly in the three groups with no statistical differences. Thus, it was concluded that no significant protection or reduction in lesion development was induced in the animals immunized with the *HSP20* DNA vaccine in the experimental conditions used in this assay.

4. DISCUSSION

HSPs represent dominant antigens in many infections and autoimmune diseases, inducing strong humoral and cellular immune responses. Among the main HSP families, HSP60, HSP70, and HSP90 have been described as major antigens in a large number of infectious diseases caused by nematodes, protozoa, fungi, or bacteria. Furthermore, in various

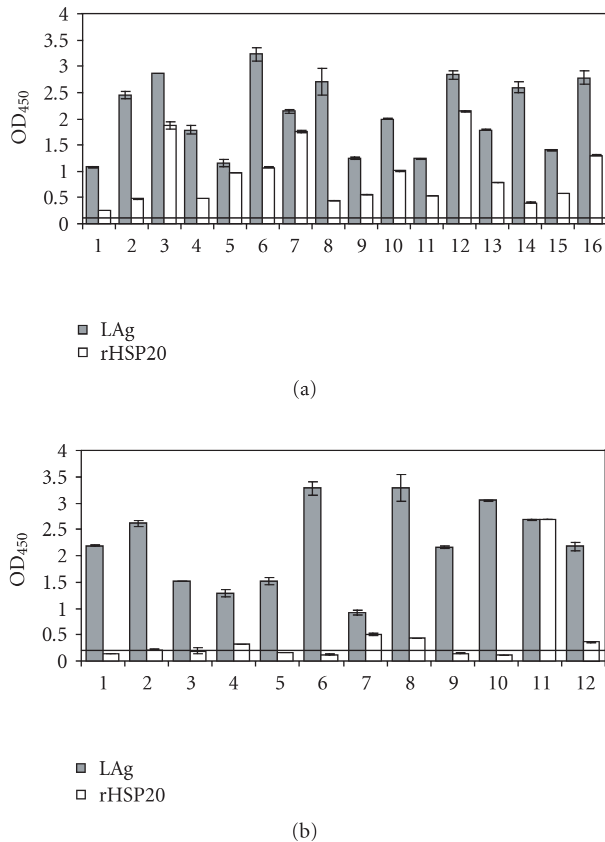

Figure 4: Antigenicity of *Leishmania* HSP20 in VL human patients and dogs. (a) Reactivity of 16 serum samples from VL dogs against total *L. infantum* proteins (LAg) or *L. amazonensis* recombinant HSP20 (rHSP20). (b) Reactivity of 12 sera from patients with VL against LAg or rHSP20. Horizontal lines represents the cut-off value of negative sera for the LAg antigen (0.122 for canine sera and 0.232 for human sera). All sera were tested in duplicate at a 1 : 200 dilution.

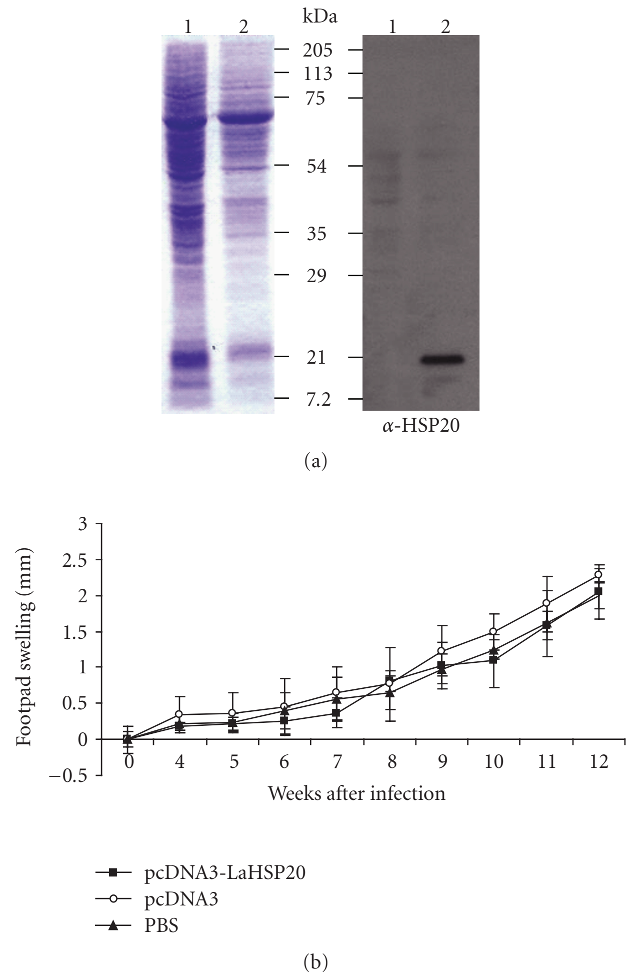

Figure 5: Analysis of protective effect of DNA vaccination with *L. amazonensis HSP20* gene. (a) Expression of *Leishmania* HSP20 in pcDNA3-LaHSP20 transfected COS7 cells. COS7 cells were transiently transfected by electroporation with either pcDNA3 DNA (lane 1) or pcDNA3-LaHSP20 DNA (lane 2). Protein extracts were analyzed by 12% SDS-PAGE and stained with Coomassie blue (left panel). The expression of *Leishmania* HSP20 was assayed by Western blotting using a mouse anti-HSP20 polyclonal antibody (right panel). (b) BALB/c mice (six per group) were immunized twice at 2-week interval with PBS, empty vector or pcDNA3-LaHSP20. After challenge with *L. amazonensis*, lesion development (footpad swelling) was monitored weekly. Each point represents the average and standard deviations for the group.

infectious disease models, vaccination strategies using HSPs have induced significant protection [26]. In addition, the unique and potent immunostimulatory properties of some HSPs have been applied to the development of new vaccines in which HSPs act as immunomodulatory/carrier agents [27]. However, there are few reports describing members of the sHSP family as antigens during infectious diseases [28, 29]. Unlike large HSPs, the sHSPs are highly divergent in both size and primary sequence; this feature has impaired the characterization of the coding genes and, in consequence, the analysis of the possible antigenicity of this class of proteins.

In *Leishmania*, several families of HSPs have been described as prominent antigens [7, 17], but no description of sHSPs as antigens exists. Thus, as first step, we analyzed whether sHSPs are encoded in the genome of *Leishmania*. For this purpose, we took advantage of the recent completion of the genome sequences for *L. major* [20], *L. infantum*, and *L. braziliensis* [30]. A bioinformatics analysis of the *L. major* database allowed us to identify a protein entry (LmjF29.2450) containing the α-crystallin motif, which characterizes sHSPs. The predicted protein (named HSP20) has 155 amino acids, accounting for a molecular mass of 17.5 kDa. This seems to be the sole sHSP family member in *Leishmania*, since no new protein entries were recovered from the *L. major* database when the LmjF29.2450 sequence was used as query in further bioinformatics studies. Homologous sequences were identified in the sequence databases for *L. infantum* (LinJ29_V3.2560) and *L. braziliensis* (LbrM29_V2.2420). Based on these sequences, we designed oligonucleotides that allowed us to PCR amplify the corresponding gene in *L. amazonensis*. Sequence alignments (Figure 1) and phylogenetic studies (Figure 2) revealed that HSP20 is well conserved among *Leishmania* species but it

is highly divergent when compared with sHSPs from other groups of organisms.

In order to analyze the antigenic properties of *Leishmania* HSP20, the *L. amazonensis* protein was expressed in *E. coli* and used to determine the presence of specific IgG in the sera of *Leishmania*-infected animals and human patients with VL. Remarkably, the protein was recognized by 100% of the sera from dogs with VL, suggesting that this protein would be useful for serodiagnosis of canine leishmaniasis. However, the protein has a limited antigenicity for the human immune system, since only about 30% of the assayed sera showed a positive reactivity. Also, we assayed the recognition of the *Leishmania* HSP20 by sera from experimentally infected hamsters. In this infection model, the protein was recognized by sera from about 62% of infected animals. In experimentally infected hamsters, analysis of the time-course appearance of anti-*Leishmania* humoral response indicated that anti-HSP20 antibodies seem to be produced late during infection (Figure 3), coinciding with the onset of disease symptoms. These findings would indicate that HSP20 behaves as a pathoantigen [31], and consequently, anti-HSP20 antibodies could be involved in the pathological processes leading to disease progression. In addition, these findings would point to a usefulness of this protein for serodiagnosis of active leishmaniasis disease.

Finally, we examined whether *Leishmania* HSP20 could elicit protective immunity. Recently, it has been reported that vaccination with *Toxoplasma gondii HSP30* gene, a member of the sHSP family, induced protection in mice against a challenge with the parasite [32]. We chose to use a DNA-based vaccination because this approach has been demonstrated to be adequate for inducing protection against diseases that require cell-mediated immunity such as intracellular protozoan parasites [33]. DNA vaccination has proven to be a successful approach for development immunoprotective responses in different *Leishmania*-infection models, particularly for protection against *L. major* [4]. However, our results indicate that immunization of susceptible BALB/c mice with the DNA encoding *Leishmania* HSP20 provided negligible protection against *L. amazonensis* infection. Thus, it appears that HSP20 does not represent a feasible vaccine candidate against *L. amazonensis* infection. Nevertheless, this finding does not exclude that HSP20 would be able to elicit an immunoprotective response against infection with other *Leishmania* species as occurs for other antigens [34]. In particular, given the strong humoral response elicited by HSP20 in naturally infected dogs, it would be of high-value testing in dogs the immunoprotective potential of this antigen against development of visceral leishmaniasis. On the other hand, the results showed here do not discard a possible use of the *Leishmania* HSP20 within a multivalent vaccine. For example, a vaccine combination, including DNA encoding P4 and HSP70, induced a significant protection in mice against *L. amazonensis*, but no protection was observed after administration of these genes separately [35]. Recently, it has been published that the *L. major HSP70* is not protective in murine models of cutaneous leishmaniasis using a prime-boost strategy [36]. Overall, these findings could be indicating that *Leishmania* HSPs alone, using conventional vaccination strategies, are unsuitable for inducing immunoprotection against *Leishmania* infections; however, these studies do not preclude their use as either immunomodulators or, alternatively, vaccine candidates if different vaccination approaches are followed.

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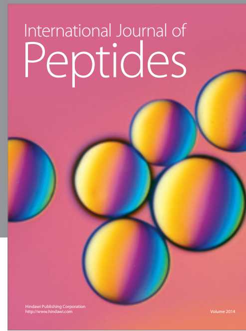

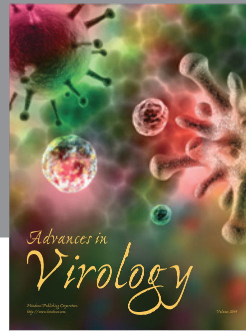

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