Advances
in
Bioresearch

ORIGINAL ARTICLE

Hyalomma dromedarii small Heat Shock Protein: Molecular Cloning and structural analysis

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ABSTRACT
Polyclonal antibodies were used to immunoscreen a λZAP ExpressII H. dromedarii cDNA library. Sequencing revealed the identification of clones carrying incomplete sequences from salivary glands, genes of nuclear proteins as well as replicates of the heat shock protein gene. Studying the genetic sequence of the heat shock protein gene showed that all clones are identical containing 877 nucleotides, of which 519 nucleotides encode for 172 amino acids with a molecular weight of 19.49 kD and IP of 7.3. Comparison of the deduced amino acids sequence of clones with protein data bank showed that the identified polypeptide belongs to the alpha crystalline small heat shock proteins superfamily and shows similarity of 91%, 70% and 59% with homologues from single-host ticks such as Rhipicephalus and two-host ticks such as Ixodesand Ornithodorus, respectively. Accordingly, the protein was identified as H. dromedarii small heat shock protein (Hd sHSP). The Hd sHSP was expressed in E. coli under the control of T7 promotor of the pET30b vector, then purified under native conditions and its immunogenicity was evaluated using immunoblotting. The protein was shown to be strong immunogen. To prove the stability of cellular proteins on thermal stress, the purified recombinant protein (rHd sHSP) showed protection to other bacterial proteins, in vitro, when exposed to temperatures up to 60°C. We have also been able to study how this protein interferes with metal ions like Zn and the acid radical ions as CO_3 and PO_4, using molecular docking tools. Zinc ions were found to bind amino acids in the hydrophobic region of the protein.
Keywords: Hyalomma dromedarii, Cloning, small heat shock proteins, Immunoscreening, Salivary glands.

INTRODUCTION

Ticks (phylum Arthropoda, order Acarina) are obligate blood-feeding ectoparasites that infest numerous vertebrate hosts including camel, cattle, humans and dogs. They are able to ingest very large blood volumes [1]. While sucking their hosts' blood, ticks act as vectors for many human and animal pathogens causing tick-borne diseases (TBDs). Hard ticks *Hyalomma dromedarii* (Ixodidae) are considered among the most important ectoparasites affecting camel (preferred host) but sheep, cattle, horses may be also infested. Tick feeding, on their hosts, varies from days to weeks based on several factors like host, tick species and the tick life stage [2].

Camel infestation by ticks affects milk production, causes weight loss, irritation and anemia in addition to the transmission of tick-borne pathogens resulting in several diseases. *Hyalomma dromedarii* transmits the blood parasite, *Theileria annulata* that cause the tropical theileriosis. More recently, the aerobic Gram-negative *Francisella spp* tick-borne bacteria was detected in *H. dromedarii*[3]. Since camel constitute one of the major livestock species in many countries including Saudi Arabia, TBDs causes devastating economical losses in these countries [4, 5]. Moreover, failure to control ticks and tick-borne disease is a major factor limiting livestock production world-wide.

Tick control represents the most important part of the current global strategy to control major TBDs [5]. The currently used methods to control tick infestation rely principally on a chemical approach [6] during which acaricides are administered to animals by dipping or spraying. However, this chemical approach is now strongly challenged due to the development of resistance to acaricides by ticks [7-11], the presence of their residues in meat and milk and the increase in legislation to combat the detrimental effect of chemicals on the environment [12]. The immunological control of ticks- through vaccination- is currently the most sustainable and practical alternative control method. The identification and cloning of potential tick vaccine antigens is the main route to develop a safe, effective and non-living vaccine [13].

Cloning and expression of the Bm86 gene, a gene coding for a glycoprotein on the surface of mid-gut cells of *Boophilus (Rhipicephalus) microplus*, resulted in the development of two commercial vaccines against this species known as TickGARD™ (Biotech Australia, together with the Commonwealth Scientific and Industrial Research Organization (CSIRO)) and GAVAC™ (developed in Cuba and commercialized by Heber Biotec S.A., Havana). The vaccine affects the female fertility causing a successive reduction in tick numbers [14]. Both vaccines are environmentally safe, leave no toxic residues in milk and meat and have low production cost. However, the effectiveness of both TickGARD ™ and GAVAC ™ vaccines against other tick species appears unsatisfactory to dates [15].

However, some strains of *B. microplus* had developed resistance to both TickGARD™ and GAVAC™ vaccines, as reviewed by [16, 17]. Search for novel tick antigens that may be used as vaccines outlined the importance of tick salivary gland and salivary secretions. The salivary glands are the largest glands in ticks and play a major role in feeding [6]. The salivary secretions, besides delivering pathogens to the host, contain numerous components affecting the vertebrate's host haemostatic, inflammatory and immune systems that help the tick to start and finish a blood meal [18] by suppressing the host immune response to the bite. The feeding process requires therefore a substantial shift in gene expression, including an increase in transcription of genes encoding attachment of cement proteins, proteins that counter the host immune responses and proteins that help to digest the blood meal [19].All these properties of tick salivary glands and salivary proteins expressed during feeding may be used in two different ways for vaccine development: either the identification and neutralization of salivary immunomodulators (secretory proteins) that affect the establishment of the parasite to its host preventing its rejection or searching for salivary proteins that may induce an immune response if administered to animals resulting in indirect death of the parasite or reduction of its female fecundity [18]. Till now, there is no clear candidate vaccine from tick saliva that can control pathogen transmission. However, recent studies identified, through cloning and sequencing, novel salivary gland proteins that are up regulated during feeding in *Ixodes scapularis* tick that may lead to vaccine development[6].Shahein [20] described a novel molecule that is expressed in the salivary glands of the Egyptian cattle tick *B. annulatus*.

This study aims to identify a new salivary protein in *H. dromedarii* tick in an attempt to control tick infestation of camels and to prevent transmission of TBDs. Development of vaccines from multiple antigens that could target a broad range of tick species will contribute to the control of tick and tick borne-diseases.

MATERIALS AND METHODS
Protein determination
To determine the protein concentration, a standard curve of protein concentration was established using Coomassie blue G-250 (CBB) and BSA, according to Bradford [21].
Preparation of rabbit anti-*H. dromedarii* salivary proteins in Rabbits
The salivary protein extract was used as immunogen to produce rabbit antibodies. A male rabbit (~3 Kg) was immunized intramuscularly with 20 µg of salivary protein extracts mixed with an equal volume of Freund's complete adjuvant. The rabbit was boosted with 15µg of salivary protein extracts mixed with Freund's incomplete adjuvant after 14 and 28 days from the first dose. Fourteen days after each dose, the rabbit was bled from the marginal ear vein. Immunoglobulin production was titered by direct ELISA according to Ricoux et al. [22]and confirmed by immunoblot according to Towbin et al. [23].
Immunoscreening of *H. dromedarii* cDNA expression library
A titer of $5×10^5$ plaque-forming units from *H. dromedarii* cDNA library was immunoscreened with 0.5 mg/ml chromatographically purified polyclonal rabbit anti-*H. dromedarii* salivary antigens in phosphate buffered saline (PBS) containing 5% dry milk following the protocol described by Sambrook and Russell[24].
Expression and purification of the cloned *H. dromedarii* gene:

The pET30b expression vector (Novagen Inc, Madison, USA) was used to express the *H. dromedarii* small heat shock protein gene. From the sequence of the identified *H. dromedarii* sHSP gene, two primers HSP *Bam*HI (5'-CGG**GGATCC**CGGGATGGCCTGCGCG-3') and HSP *Eco*RI (5'-CGG**GAATTC**GGTTTGTCCTTCGGG-3'), were designed for PCR amplification of the coding 492 bp of the identified gene. These two restriction sites are also present as unique sites in the cloning region of the pET 30b vector. Expression and purification in E. coli BL21(DE3) were carried out following the protocol described by Shahein *et al.*[25].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE)
The electrophoretic analysis was performed in the Mini-ProteanII Dual-Slab Cell, (BioRad). Preparation of gels, samples, and electrophoresis was carried out as described byLaemmli [26]**.**

Immunoblotting
The method of Towbin et al.[23], was performed using the polyclonal anti-rHd sHSP at a dilution of 1:1000. The reaction of antigen-antibody was detected by incubating the PVDF membranes with anti-rabbit IgG peroxidase conjugate at a dilution of 1:2000. The 4-chloro 1-naphthol was used as a substrate in the developing solution.

Thermal stability
Thermal stability was evaluated according to the method described by Kim *et al.*[27].

Metal Binding
The ion binding ligand prediction server (https://zhanglab.ccmb.med.umich.edu/IonCom/), was used to show the possible binding sites of 13 small ligand molecules including 9 metal ions (Zn^{+2}, Cu^{+2}, Fe^{+2}, Fe^{+3}, Ca^{+2}, Mg^{+2}, Mn^{+2}, Na^+, K^+) and 4 acid radical ions (CO_3^{-2}, NO_2^-, SO_4^{-2}, PO_4^{-3}) [28].

RESULTS
Immunogenecity, production of anti-salivary protein and antibody titration
Arabbit (3kg) was immunized with total salivary proteins (20 μg as an initial dose and 15 μg in two booster doses at two weeks intervals). Sera were collected before injection and two weeks after each injection. To determine the titration of anti- salivary proteins antibodies, wells of the ELISA plate were coated with 1.5 μg of total proteins of the salivary glands and the ELISA test was performed using serial dilutions from 3.7×10^{-4} to 0.2×10^{-4} of the collected sera. Diluted sera were incubated in the presence of the total salivary proteins or in its absence (as blank). Serum before immunization was used as negative control (normal rabbit serum). Anti- rabbit IgG conjugated to alkaline phosphatase was used as secondary antibody and the titration was constructed according to absorbance values at 405 nm after 30 minutes incubation with soluble ALP substrate p- NPP.

Antibodies titers of rabbit anti- salivary proteins were 1: 2810, 1: 3000 and 1: 3950 after the first, second and third immunization doses, respectively while the normal rabbit serum cross- reacted with salivary proteins giving a titer of 1: 810.

Immunoscreening of *H. dromedarii* cDNA expression library
Immunoscreening of a λZAP ExpressII cDNA library previously constructed from the cDNAs of adult *H. dromedarii* whole ticks, salivary glands, gut, ovaries and eggs with purified polyclonal rabbit anti- *H. dromedarii* salivary glands antigens revealed several positive clones from a total of 5×10^5 pfu screened. Several cDNA clones were isolated. These were subjected to dot blot hybridization using the longest cloned cDNAs as probes to put them in homogenous groups. The library was further screened by plaque hybridization technique to isolate full length cDNAs. Identified clones were isolated and sequenced in their entirety. Table 1 shows sample of the identified clones.

Table 1. Nucleotide and translated nucleotide blasts of some identified clones

Clone No.	Size (bp)	ORF (bp)	Nucleotide Hit	Protein Hit
1	936	423	No hits	Amblyomma hypothetical protein
2	943	321	No hits	Dermacentor G-3P dehydrogenase
3	968	NA	Rhipicephalus SINE	--
4	935	513	Rhipicephalus HQ05	*R. annulatus* HQ05/*I. scapularis* salivary protein
5	1004	369	No hits	Amblyomma salivary protein
6	888	561	--	Ixodes secreted salivary protein
7	906	294	No hits	No hits
8	991	543	Various hits	Ixodes fibronectin domain
9	988	Short	Ixodes Calmodulin	--
10	916	447	No hits	No hits

Molecular cloning and structural analysis of the isolated *H. dromedarii* clone

Among the sequenced clones, 7 clones contained 877 bp sequence each, without a start codon (ATG) and the stop codon (TGA) was found downstream of the 5' cDNA end at position 517thus forming an open reading frame (ORF) of 519 bp. The nucleotide sequence carries78 bp at the 3' untranslated region (3' UTR) showing one possible site of polyadenylation signal AATAAA at 571 bp. The polyA tail, however, was included within the cloned and sequenced fragment. The complete nucleotide sequence of the identified gene and its deduced amino acid sequence are shown in figure 1.

The identified ORF encodes a polypeptide of 172 amino acids, of calculated molecular mass of19.5 kiloDalton and of calculated isoelectric point of 7.3. The amino acids sequence was analyzed using the prediction server of the CBS. The deduced amino acid sequence of the sequenced ORF is predicted to belong to a non- secretory protein [29, 30]. The Scanprosite server of the "PROSITE database of proteins domains, families and functional sites"[31, 32]predicted that the identified protein belongs to the small Heat Shock protein 20 family and its conserved alpha crystallin domain ranges from the 59th to the 164th amino acid. The polypeptide showed no possible N-glycosylation sites while in the case of *Rhipicephalus annulatus* one potential glycosylation site at N121. However, The O-glycosylation was found in P22, N26 and E108 residues. The Scanprosite server predicted that, among a total of 29 serine, 18 threonine and 2 tyrosine residues within the amino acid sequence, only 1 serine (S73) and 5 threonine residues (T24, T46, T102, T125 and T134) present high probability of being phosphorylated.

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tttctggtaccggtccggattcccgggatggcctgcgcgcggcgaaacctctggaggcgc     60
 F   L   V   P   V   R   I   P   G   M   A   C   A   R   R   N   L   W   R   R
accccagccaccatcaacgacgtgttcggcgagatggaccggcagctgcggcacttcgag    120
 T   P   A   T   I   N   D   V   F   G   E   M   D   R   Q   L   R   H   F   E
cacgagatgtcgcgcaccttccgcgacctcgaccgatcgggcgcgttcggaccggcgttt    180
 H   E   M   S   R   T   F   R   D   L   D   R   S   G   A   F   G   P   A   F
cgctggctgcgctcgcgcgacgtgcccgtcgagtcgagctcgggcgacaagtttcaagtg    240
 R   W   L   R   S   R   D   V   P   V   E   S   S   S   G   D   K   F   Q   V
cagctggacgtgagcaagttcaggccggaggatgtgaaggtttcgctgtcgggcaaccag    300
 Q   L   D   V   S   K   F   R   P   E   D   V   K   V   S   L   S   G   N   Q
ctgaccgtgcgcgcccgctccgaagtcaaggagggcagctcgacgtacgtgcgcgagttt    360
 L   T   V   R   A   R   S   E   V   K   E   G   S   S   T   Y   V   R   E   F
tcgcacaccgtgacgctgcccgaagacgtggacccggacactgtgcgttccctgctgctc    420
 S   H   T   V   T   L   P   E   D   V   D   P   D   T   V   R   S   L   L   L
gctgacgggtcgctgtgcatcgaggcacccccgcgcgctgccggaacccaaggaagtgccc    480
 A   D   G   S   L   C   I   E   A   P   R   A   L   P   E   P   K   E   V   P
atcgagaaagcttccacggagccccccgaaggacaaa***tga***gactctcggtgctacggtgtg    540
 I   E   K   A   S   T   E   P   P   K   D   K   –
aatgtagtttttgtgtatgcttagtgtcta**<u>aataaa</u>**tacgcctatgtcttcacggtgaaa    600

aaaaaaaaaaaaaaaaaaaaaaaaaaa    628
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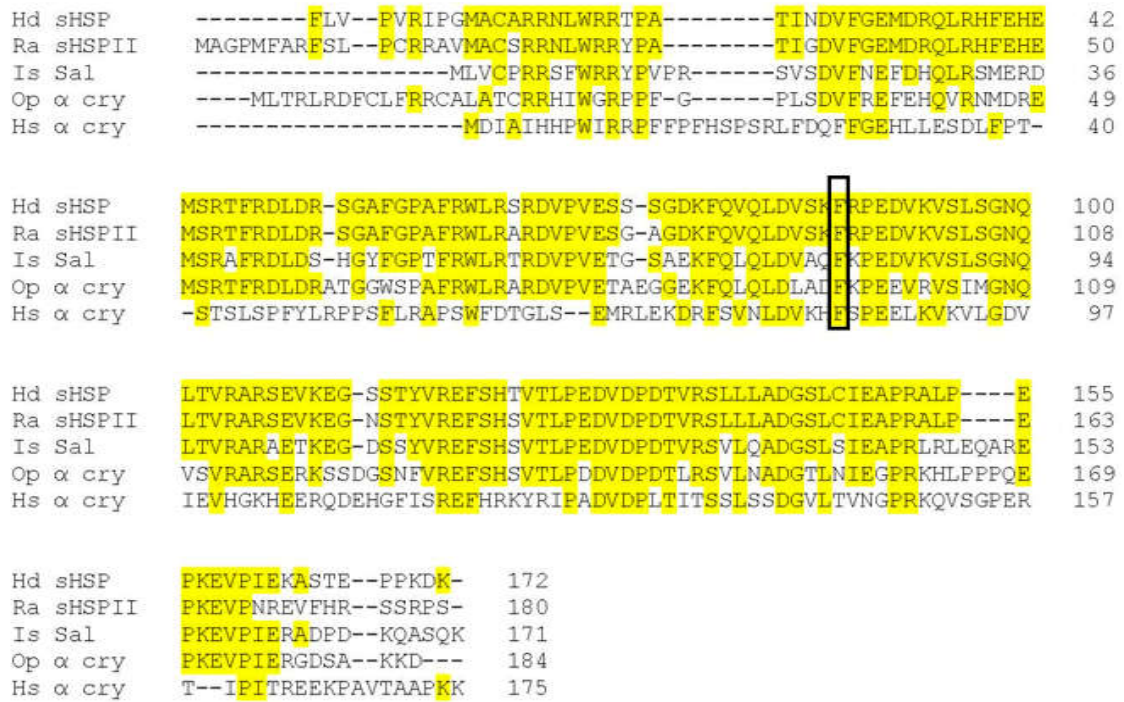
Fig. 1. cDNA sequence and deduced amino acid sequence of the tick *H. dromedarii* small heat shock protein gene (Hd sHSP). The first base in the sequence is part of the coding region and the stop codon TGA is in bold italic. Underlined bold characters denote the possible site of polyadenylation signal. This sequence has been submitted to GenBank and assigned the accession number MH229947.

Blast algorithm of the GenBank database was used to identify homologues of the deduced amino acid sequence. The amino acid sequence showed high degree of similarity to previously identified alpha crystallin heat shock proteins of some tick species such as *Ixodes scapularis* and *Ornithodorosparkeri*, and Homo sapiens' B chain of α crystallin. Consequently, multiple alignment of the deduced amino acid sequence with these peptides using the Dialign software of the Expasy web site strongly reinforced the suggestion that this sequence codes for an alpha crystallin member of the heat shock protein family. **Figure 2** shows multiple sequence alignment of the amino acid sequence of *Hyalomma dromedarii* small heat shock protein (HdsHSP) with other heat shock proteins and **table (2)**and **figure 3**show the degree of similarity of the HdsHSP to other similar proteins and the phylogenetic tree, respectively. The protein was

thus named Hd sHSPand its cDNA sequence was submitted to GenBank and assigned the accession number MH229947.

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Hd sHSP      --------FLV--PVRIPGMACARRNLWRRTPA--------TINDVFGEMDRQLRHFEHE    42
Ra sHSPII    MAGPMFARFSL--PCRRAVMACSRRNLWRRYPA--------TIGDVFGEMDRQLRHFEHE    50
Is Sal       ------------------MLVCPRRSFWRRYPVPR------SVSDVFNEFDHQLRSMERD    36
Op α cry     ----MLTRLRDFCLFRRCALATCRRHIWGRPPF-G------PLSDVFREFEHQVRNMDRE    49
Hs α cry     ------------------MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFPT-    40

Hd sHSP      MSRTFRDLDR-SGAFGPAFRWLRSRDVPVESS-SGDKFQVQLDVSKFRPEDVKVSLSGNQ   100
Ra sHSPII    MSRTFRDLDR-SGAFGPAFRWLRARDVPVESG-AGDKFQVQLDVSKFRPEDVKVSLSGNQ   108
Is Sal       MSRAFRDLDS-HGYFGPTFRWLRTRDVPVETG-SAEKFQLQLDVAQFKPEDVKVSLSGNQ    94
Op α cry     MSRTFRDLDRATGGWSPAFRWLRARDVPVETAEGGEKFQLQLDLADFKPEEVRVSIMGNQ   109
Hs α cry     -STSLSPFYLRPPSFLRAPSWFDTGLS--EMRLEKDRFSVNLDVKHFSPEELKVKVLGDV    97

Hd sHSP      LTVRARSEVKEG-SSTYVREFSHTVTLPEDVDPDTVRSLLLADGSLCIEAPRALP----E   155
Ra sHSPII    LTVRARSEVKEG-NSTYVREFSHSVTLPEDVDPDTVRSLLLADGSLCIEAPRALP----E   163
Is Sal       LTVRARAETKEG-DSSYVREFSHSVTLPEDVDPDTVRSVLQADGSLSIEAPRLRLEQARE   153
Op α cry     VSVRARSERKSSDGSNFVREFSHSVTLPDDVDPDTLRSVLNADGTLNIEGPRKHLPPPQE   169
Hs α cry     IEVHGKHEERQDEHGFISREFHRKYRIPADVDPLTITSSLSSDGVLTVNGPRKQVSGPER   157

Hd sHSP      PKEVPIEKASTE--PPKDK-   172
Ra sHSPII    PKEVPNREVFHR--SSRPS-   180
Is Sal       PKEVPIERADPD--KQASQK   171
Op α cry     PKEVPIERGDSA--KKD---   184
Hs α cry     T--IPITREEKPAVTAAPKK   175
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Fig.2. Alignment of the deduced amino acid sequence of *H. dromedarii* sHSP with *Rhipicephalus annulatus* sHSPII (HM149782), *Ixodesscapularis* fed tick salivary protein 5 (Is sal, accession number (AY788855)), *Ornithodorosparkeri* alpha crystallin (Op α cry, accession number (EF633915)), and *Homo sapiens* α crystallin of the basic type (Hs α cry, accession number (NM001885)). Amino acids similarities between species are highlighted while the conserved phenylalanine residue in the metazoan alpha crystallin domain but not in the non-metazoan is squared.

Table 2. Similarity of heat shock proteins from various species to Hd sHSP.

| Species	Protein	Accession number	*H. dromedarii* small Heat Shock Protein	
			Nucleotide Sequence	Protein Sequence
Rhipicephalus annulatus	Heat Shock Protein	HM149782	88%	91%
Ixodes scapularis	Fed tick salivary protein 5	AY788855	76%	70%
Ornithodoros parkeri	αcrystallin	EF633915	65%	59%
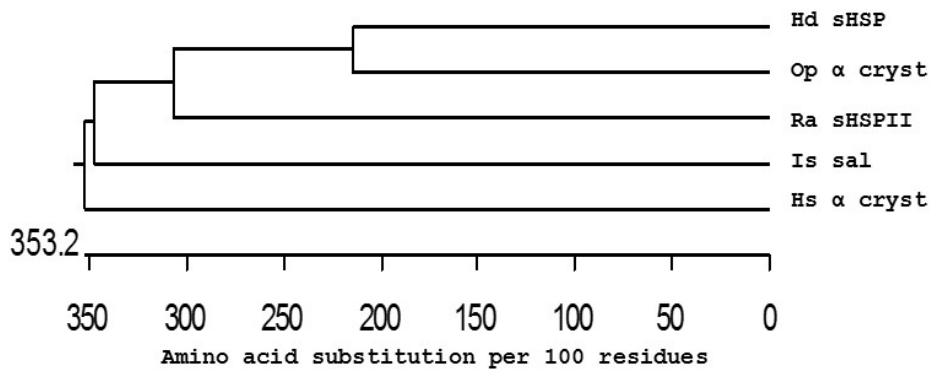
Fig.3.Phylogenetic tree between species.

To investigate the presence of transmembrane hydrophobic regions and/ or surface hydrophilic regions that may have antigenic properties within the Hd sHSP, a Kyte-Doolittlehydropathy plot was generated using the Lasergene-DNAstar **(Fig. 4)**. Most domains within the protein possess strong negative scores and thus possess strong hydrophilic characters. This, in turn, indicates the absence of hydrophobic trans-membrane regions. The Hd sHSP protein may, therefore, be located predominantly within the cytosol.
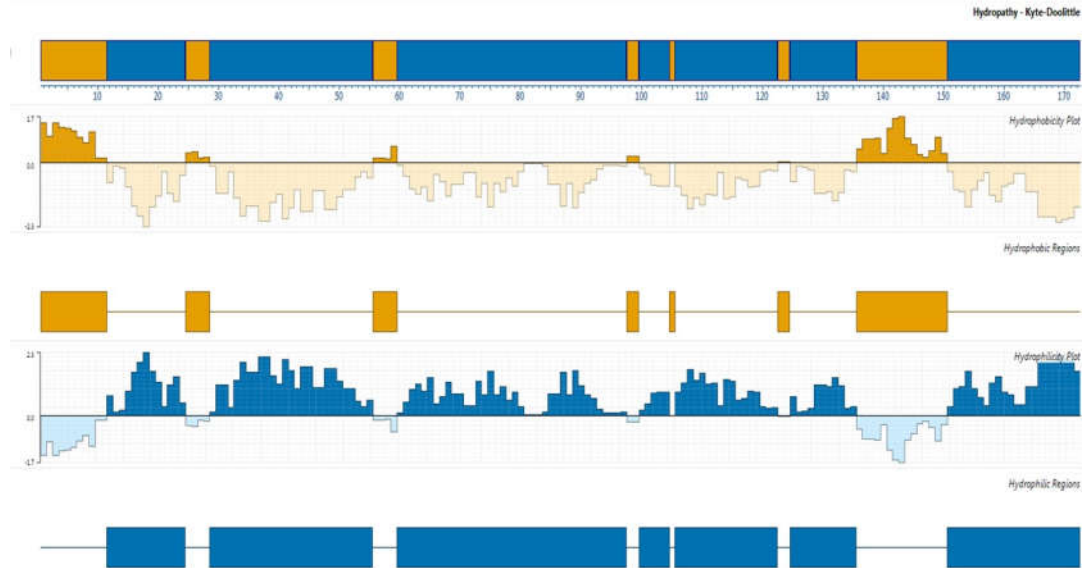
Fig. 4. Kyte-Doolittle hydropathy of the deduced amino acids of the *H. dromedarii* small heat shock protein.

Despite the absence of amino acids around eight, according to similarity index with *R. annulatus*, in the N-terminal signal peptide sequence, and strong hydrophobic regions within the Hd sHSP protein, the protein was found to have a NN- score (Neural Network score) more than 0.5 (thus exceeding the threshold NN- score by 0.2) according to the Secretome P-2 server of the CBS (predicting non- classically secreted proteins)[33]. This result indicates that the protein may represent a "leaderless" secretory protein, i.e. the Hd sHSP may follow an unusual pathway for secretion.

In order to generate a model of the secondary structure of the Hd sHSP protein, its sequence was submitted to the Phyre2 web portal for protein modeling [34].The model of three dimensional structure of the Hd sHSP was generated using 152 residues (88% of the sequence) with 99.9% confidence by the single highest scoring template of the tsp36 model (*Taenia saginata* protein 36) of the metazoan small heat shock protein **(Fig.5 A and B)**.
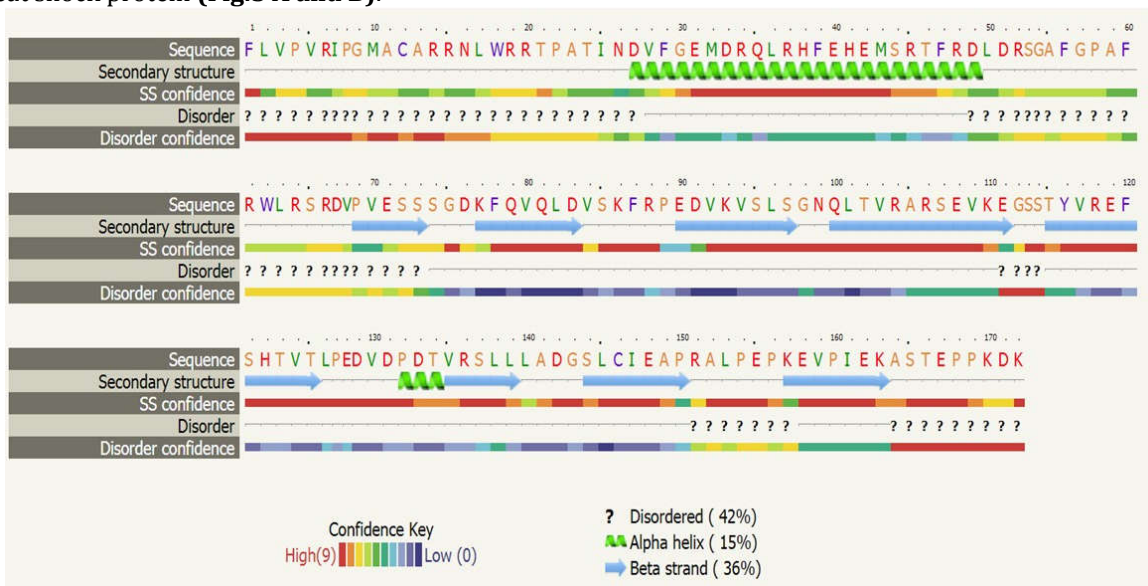
A:
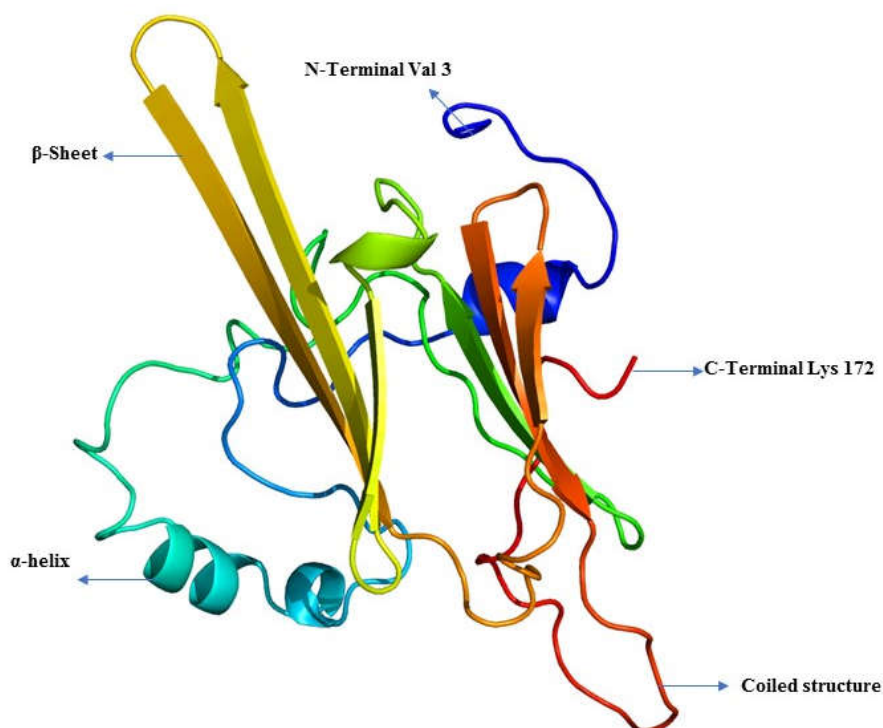
B:
Fig.5. A. Secondary structure and domain percentages. B. Predicted model of the three dimensional structure of the Hd sHSP protein showing two α- helices;7 antiparallel ß-sheets andirregular coiled structures. Model dimensions (A): X: 38.814, Y: 58.175 and Z: 55.305.

Expression and purification of the cloned *H. dromedarii* gene
RNA extraction from *H. dromedarii* ticks and reverse transcription:
The procedure was carried out using the Tri RNA isolation reagent in order to obtain good RNA preparation from salivary glands of adult *H. dromedarii* ticks suitable for RT-PCR. The quality of the RNA was confirmed by 1% agarose gel electrophoresis stained by ethidium bromide and ratio of A260/280 and reverse transcription was carried out.
Hd sHSP gene amplification, double digestion and purification:
In order to construct a prokaryotic expression system for the Hd sHSP gene, part of the gene was amplified by RT- PCR (as described under the "Materials and Methods" section) using the prepared cDNA as template and the specific primers "HSP *Bam*HI" as forward primer and "HSP *Eco*RI" as reverse primer, carrying the *Bam*HI and *Eco*RI restriction sites, respectively. Since these primers flanked a region of 516 bp from the whole ORF, the size of the amplified gene fragment was expected to be 537 bp. Indeed, agarose gel electrophoresis of the RT-PCR revealed a specific band around 537 bp from *H. dromedarii* cDNA clone **(Fig.6A).**Subsequently, the band was excised and purified as described in the materials and methods section.
After purification of the amplified gene fragment, double digestion using the *Bam*HI and *Eco*RI restriction enzymes and the subsequent purification of the digested product, the digested purified gene fragment was successfully recovered.
Construction of the prokaryotic expression system:
Ligation of the double digested and purified Ha sHSP gene recovered from the previous step to the IPTG inducible pET30b vector previously digested with the same restriction endonucleases and the subsequent transformation into the BL21 (DE3) strain of *E.coli*, resulted in growth of several colonies on LB agar-Kanamycin plates. This result was confirmed by *Bam*HI and *Eco*RI double digestion of the miniprep **(Fig.6B)** that revealed two fragments of 5.4 kb and 513 bp corresponding to the linear double digested pET30b plasmid and the sHSP gene fragment, respectively.
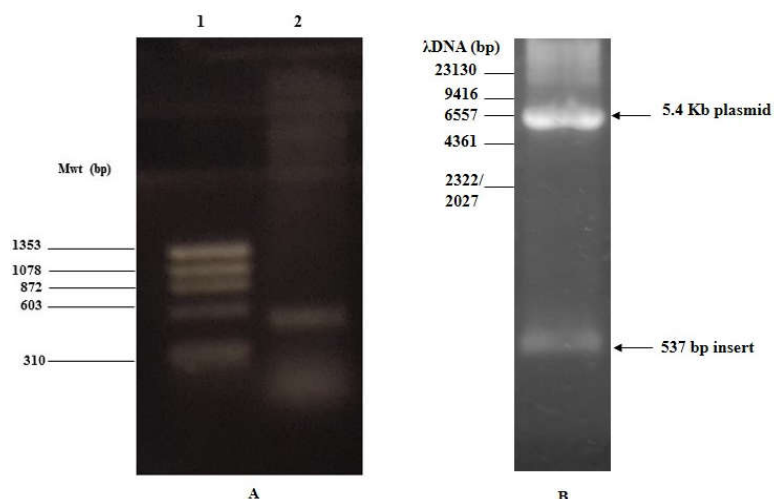
Fig.6. A. 1% agarose gel electrophoresis of RT-PCR of 513 bp fragment of the *H. dromedarii* sHSP gene. Φ_{X174} molecular weight marker, lane (1); gene amplification of 537 bp of the ORF of *H.dromedarii*, lane (2). B. Double digestion of plasmid miniprep from a positive clone transformed with pET 30b-Hd sHSP construct.

Expression of the Hd sHSP gene:
Expression of Hd sHSP gene was induced by IPTG at a final concentration of 1mM for five hours at 37ºC in *E. coli* BL21(DE3**)**. The recombinant (rHd sHSP) was predicted to have a theoretical molecular weight of 27.2 kDa and to include 242 aminoacids. Bacterial cells lysate was analyzed by 12% SDS- PAGE, before and after addition of IPTG, where an overexpressed protein having a molecular weightaround the expected molecular mass was detected **(Fig.7, lane 3)**.

Purification of the recombinant Hd sHSP protein (rHd sHSP):
The rHd sHSP protein was purified using the IMAC-Qiagen Ni-NTA agarose matrix. The Hd sHSP protein was successfully eluted under native conditions **(Fig. 7, lanes 4-9)**.
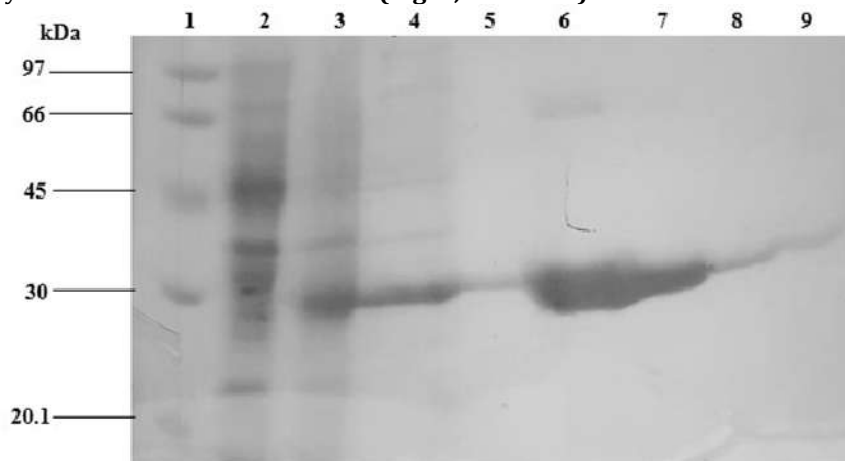
Fig.7. Induction of Expression and Purification of the recombinant Hd sHSP protein under native conditions using Ni-NTA column. 12% SDS- PAGE of: Low molecular weight marker, lane (1); cell lysate of IPTG induced bacteria containing the empty plasmid, lane (2); cell lysate of IPTG induced bacteria containing the recombinant plasmid, lane (3); column flow through, lane (4); and the purified sHSPelution fractions, lanes (5) to (9). Elution was carried out using 100 mM HEPES and 500 mM imidazole.

Immunogenicity of the recombinant Hd sHSP
To confirm the immunogenicity of the rHd sHSP protein, rabbit anti-rHd sHSP antisera were raised in a rabbit as previously mentioned in Materials and methods. The antibodies (1:1000) were reacted with the purified rHd sHSP protein in immunobloting. The rabbit anti- rHd sHSP anti-sera reacted with the purified rHd sHSP **(Fig. 8)**.

Fig. 8. Immunoblotting of 12% SDS- PAGE of purified rHd sHSP using rabbit anti- rHd sHSP antisera.

Thermal Stability

The rHd HSP and rRa GST proteins (as a control) were overexpressed in BL21(DE3) cells. Following the expression, bacterial cell lysates were subjected to heat treatment. Bacterial cell extracts expressing the rHd HSP showed thermal tolerance than extracts from cells expressing the rRa GST. At temperatures 50°C and 60°C, several proteins were degraded or precipitated in the insoluble fractions. At temperatures 80°C and 100°C, no significant differences were observed (Fig. 9).
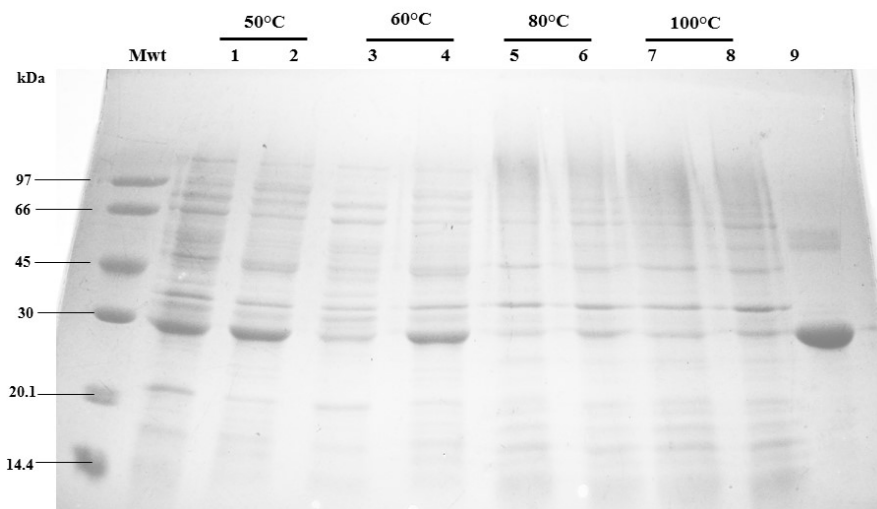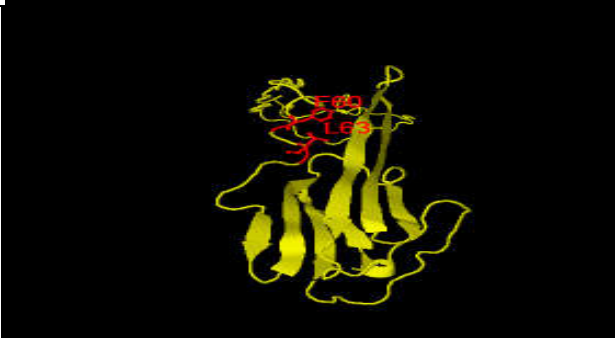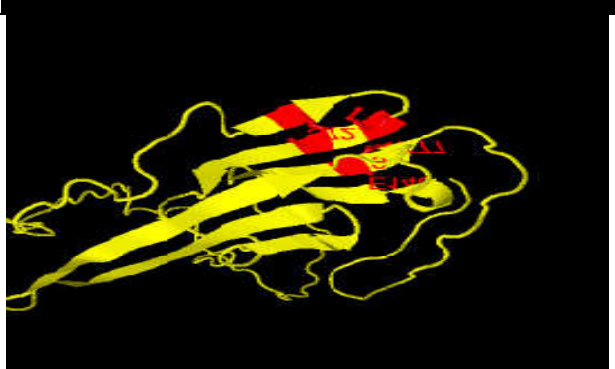

Fig. 9. Thermal stability of crude extracts of BL21(DE3) expressing Hd sHSP. Lane 1 is low molecular weight marker; lanes 1, 3, 5, and 7, BL21(DE3)/recombinant *Rhipicephalus annulatus*-GST cell extracts heated at 50, 60, 80, and 100°C, respectively; lanes 2, 4, and8, BL21(DE3)/rHd sHSP cell extracts heated at 50, 60, 80, and 100°C, respectively. Lane 9 is the purified rHd sHSP as a reference.

Metal Binding

Hyalomma dromedarii small heat shock protein sequence was submitted to the ion binding ligand prediction server (https://zhanglab.ccmb.med.umich.edu/IonCom/). The algorithm shows the possible binding sites of 13 small ligand molecules including 9 metal ions (Zn^{+2}, Cu^{+2}, Fe^{+2}, Fe^{+3}, Ca^{+2}, Mg^{+2}, Mn^{+2}, Na^+, K^+) and 4 acid radical ions (CO_3^{-2}, NO_2^-, SO_4^{-2}, PO_4^{-3}) [28]. Table 3 shows the potential ion ligand binding sites of *H. dromedarii* sHSP. No binding sites were detected for ions:Cu, Fe^2, Fe^3, Ca, Mg, Mn, K, NO_2, and SO_4.

Table 3. Potential binding sites of small ligand molecules on Hd sHSP.

Ion	Amino acid Number	3D Model
Zinc	R6 C12 D91 S107 E108 V109 D142 E148	
CO$_3$	F60 L63	
PO$_4$	S72 K77 Q79 E148	

DISCUSSION

For several decades, the efforts dedicated to the study of animals and arthropods molecular biosciences were very limited if compared to those devoted to similar research areas in humans and plants [35]. This situation is changing progressively due to the raised awareness of the scientific community on the importance of blood feeding arthropods and the way they affect both animal and human health.

Animal infestation by ticks, one of the most dangerous blood sucking ectoparasites, representing a major veterinary problem not only in tropical and subtropical areas, but also in many temperate areas of the world [36]. In areas where animals are infested, animals are affected in their growth, develop anemia, reduced production of milk, and damage of leather. Moreover, a wide variety of pathogenic organisms are transmitted and seriously affects livestock industry causing impairment of an important source of animal protein and subsequently huge economic losses.

Several methods have been implemented for tick infestations control. The most successful and safe approach was the immunological vaccination approach applied against cattle ticks. Reports and observations had shown that repeated infestation by ticks results in tick resistance to the use of crude tick protein extract for immunization [12, 37-39]. Moreover, molecular cloning techniques are used for the production of recombinant protective antigens such as the rBm86 protein produced in *E.coli* [40]and *Pichia pastori* yeast [41] which was the first effective vaccine against cattle ticks.

It may be true that "vaccines often do not prevent infection but rather enhance immune response and thereby prevent clinical disease" [42]. However, vaccines against ticks affected directly the parasite by decrease of the total number of eggs, reduction of engorgement weight, and/ or the number of viable eggs, blocked molting and death of engorged ticks [43]. These effects result in a progressive reduction of tick population rather than a direct killing of ticks [14] in addition to impairment of the ability of ticks to

transmit pathogens [44]. In point of fact, all drawbacks of using acaricides, which is the currently principal method used for tick control, the anti- tick vaccines have overcome it nearly [6].

Although vaccination with rBm86 was shown to be effective under field conditions, shortcomings of the currently available vaccines have encouraged scientists to examine new protective molecules to develop a universal effective polyvalent vaccine against several tick species [15-17, 45]. The salivary glands of ticks offer a challenging and promising source of protective antigens due to their important role in tick physiology and pathogen transmission. Impairment of salivary gland function is expected to disrupt the feeding process and consequently to affect tick fertility and pathogen transmission [35, 46-48].

In the present study, in order to identify a salivary gland antigen that may present a candidate vaccine, a specific cDNA library of the camel ticks *H. dromedarii* was immunoscreened using purified polyclonal rabbit anti- *H.dromedarii* salivary glands antigens. Among the identified clones, seven clones contained a 877 bp sequence that forms a single open reading frame (ORF) of 519 bp.The identified ORF encodes a polypeptide of 172 amino acids, of calculated molecular weight 19.5 kDa. The Blast analysis in the GenBank database showed that this polypeptide belongs to the alpha crystallin heat shock proteins superfamily as confirmed by the Scanprosite server of the "PROSITE database of proteins domains, families and functional sites" [31, 32].Many sHSP conserved regions were contained in the sequence specifically the alpha- crystallin domain extending from the Pro69 to Lys163. The predicted protein sequence showed 91%, 70% and 59% similarity to *Rhipicephalus annulatus*, *Ixodes scapularis* fed tick salivary gland protein and *Ornithodoros parkeri* alpha crystallin protein, respectively. The high bit score in all three cases (299, 226 and and 184 respectively) in contrast to the low E- value (8e^{-102}, 3e^{-73} and 3e^{-56}, respectively) give indication of a good and statistically significant alignment. The protein was thus called Hd sHSP.

Small heat shock proteins (sHSP) are small molecular mass heat shock proteins (monomer ranging from 12 to 43 kDa), that function, in their oligomeric state, as molecular chaperones [49]. Their sequence shows considerable similarity to the alpha-crystallin vertebrate eye lens [50-53].They are usually characterized by the presence of an evolutionary conserved alpha-crystallin domain (formed of about 100 amino acids)[51,54] in the middle of an N- terminal arm (of variable length) and a poorly conserved C-terminal tail/ extension [49, 55]. To our knowledge, sHSPs in arthropods, including ticks, are not frequently investigated. However, these proteins are intensively studied and characterized in plants and bacteria.

Kyte-Doolittle hydropathy plot predicted the absence of strong hydrophobic domains within the Hd sHSP, indicating, in turn, the absence of transmembrane regions within the protein. According to the protein sorting pathways in eukaryotic cells, polypeptides carrying a hydrophobic N- terminal signal motif follow the classical secretory pathway; they are directed to the rough endoplasmic reticulum and then move via transport vesicles to the Golgi complex where they are either directed to the plasma membrane or to the lysosomes [56]**.** Other polypeptides may carry organelle- specific sequences. Polypeptides carrying neither N- terminal signal peptide nor organelle- specific sequences remain in the cytosol and are called non- secretory proteins [56]. Thus, it can be inferred that Hd sHSP may be a cytosolic non-secretory protein. However, the presence of the Hd sHSP in other cellular compartments or even as a transmembrane protein cannot be ruled out. This contradictory fact is reported by several authors for other sHSP. SHSP are known to be "ubiquitous in term of cellular localization as well as in the biological world" [49]. Some sHSP appears to be membrane- associated such as the HSP29, one of the 5 sHSPs, was found to be membrane-associated on immunostaining in *Toxoplasma gondii*[57]. Accordingly, the analysis of the Hd sHSP by the Secretome P-2 server [33] revealed thatthe Hd sHSP has a score above the threshold indicating that it may represent a "leaderless" secretory protein following non-classical secretory pathways. Interestingly, some HSPs may escape from cell interior and are called exogenous HSPs. These exogenous HSPs are important mediators of intercellular signaling[58]. Those authors have reviewed several HSPs in many cells and tissues lacking the signal sequences and are found associated with plasma- or organellar membranes or cross the plasma membrane. Numerous non- classically secreted proteins have been identified and are known to escape the network complex of ER-Golgi [58]. Possible interpretations to the unexpected cellular distribution of the sHSP include the suggestion that association to the membrane may reflect different membrane composition in specified locations to which the sHSP become associated or are able to "slip" [59]. Another possible explanation is that sHSP may be able to move from one cellular compartment to another. For example, the stress activation of specific pathways alters the nuclear accumulation and transcriptional activity of HSF1 in mammalian cells and ultimately modulates the actual level of HSPs, like HSP70 to translocate to either cell membrane, or secreted outside the cell, or moves to lysosome [58].

The predicted O-glycosylation and phosphorylation patterns of the Hd sHSP protein highlight a common and dynamic posttranslational modification of cytoplasmic and nuclear proteins [60]. Some authors suggest a regulatory role of glycosylation of some alpha B-Crystallin HSP as predicted by the high turnover rate of the carbohydrate moiety, which exceeds the turnover rate of the protein backbone by many fold [60]. However, if the Hd sHSP is a leaderless secreted protein, it would never be glycated despite the presence of putative N-glycosylation site (*in vivo*), as it would not enter the endoplasmic reticulum [33]. On the other hand, phosphorylation is thought to occur in a protein kinace- C independant manner in other HSPs (for example HSP 20) in cardiac myocytes as a mean to regulate the activity of sHSP following exposure to some immunological stimuli (such as Tumor Necrosis Factor (TNF) or Interleukin- 1 (IL- 1)) [59]. Phosphorylation of N- terminal serine residue in mammalian sHSPs through stress- sensitive signalling pathways is believed to modulate their chaperone function [61].

Proteins sharing sufficient sequence homology within one family may have conserved three dimensional structure [62]. Structure elucidation of one member of the protein family helps to generate a three dimensional model for other homologous proteins and this comparative modelling has been shown to be the most accurate method for three dimensional structure prediction [63]. The Phyre2 server employs the comparative modelling strategy to predict tertiary structure of target proteins [34] by identification of suitable template structure and its alignment with the target sequence prior to generation of the model and evaluation of its quality. The template used by the Phyre2 server was a crystallized sHSP of *Taenia saginata* called Tsp36 [64]. Tsp36 is a 314 amino acids sHSP; it possesses two alpha- crystallin domains, each having a local short segment adjacent to it considered to be its own N-terminal, in addition to a 80 residue global N-terminal of the whole molecule. The two alpha- crystallin domains form ß- sandwiches while the global 80 residues N-terminal form a series of α-helices [64]. Despite of the presence of only one alpha crystallin domain in Hd sHSP, the three dimensional model of the region extending from amino acid 69 to 163 showing a tertiary structure comparable to the Tsp36 template with 99.93% confidence **(Fig. 6)**; the model includes seven antiparallelß-sheets forming a ß- sandwich and two α-helices; one of them at its N- terminal followed by irregular coiled structures that are also seen at its C- terminal.

sHSPs perform many cellular functions and help the cell to withstand many types of stresses such as high temperature and oxidative stress [49]. Nevertheless, the most studied role of sHSP is their role as molecular chaperone that interact and stabilize proteins that are partially or totally unfolded [55]. They perform this role constitutively [55, 59] or under stress conditions (for example under high temperatures where different proteins become susceptible to misfolding and aggregation) [55]. sHSPs are thought to hold unfolded proteins in a soluble, folding- competent state until the intervention of high molecular weight HSPs for subsequent refolding in an ATP- dependant manner [49].

The quaternary structure of sHSP affect their *in vivo* mode of function; it has been shown that the mode of assembly of sHSP monomers in metazoa differ from that seen in non- metazoa [64]. This difference is largely based on the conservation of some amino acid residues in metazoa that are substituted by other amino acids of different spatial configuration in non- metazoa including plants. One of those residues is a conserved phenyl alanine, in the alpha crystallin domain [64]. Conservation of particular amino acids residues at defined locations suggests that the mode of dimerization unveiled for one metazoan sHSP may be applied to other metazoan sHSP [64]. Indeed, this phenyl alanine residue is also conserved in the Hd sHSP (squared residue **Fig. 3**). Accordingly, Hd sHSP monomers may interact the same way as Tsp36 and other metazoan sHSP and function in a similar oligomeric state. The proposed method of oligomerization outlines a flexible mode of assembly to an oligomeric state, the breakdown to smaller subunits upon substrate binding followed by reassembly to the large oligomeric sHSP- substrate complex [64].

Although the major sHSP function is as molecular chaperones, they contribute in other important cellular processes, particularly, in shaping and activating the mammalian immune response [65]. Stewart and Young claim that "both host and pathogen heat- shock proteins contribute to immunity by receptor- mediated activation of the innate immune response and by participation in the presentation of antigens for the adaptive immune response" [65]. Thus, ticks salivary sHSP may contribute to its immunomodulatory effect that facilitates completion of the blood meal. Immunoglobulins directed against one of those sHSP may significantly alter the tick feeding capacity and may represent an attractive option for vaccine development. These immunological roles of HSPs stand behind the suggestion of considering them as candidate anti- tick vaccines [6]and behind our trial to clone and express the Hd sHSP. Such vaccine is expected to affect tick feeding and to elicit a suitable immune response in camels.

In order to produce the candidate vaccine based on the Hd sHSP protein, the fragment of the Hd sHSP gene of 513 bp was cloned into the IPTG- inducible pET- 30b vector followed by transformation into BL21 (DE3) *E.coli* cells. The pET vectors are high copy number plasmid; more than 100 copy of the plasmid are

expected to occur in one bacterial cell [66] which increases the yield of the recombinant DNA. Subsequently, the purification of the bacterial lysate to obtain the rHd sHSP was carried out and the protein was successfully eluted under native conditions.

Purified rHd sHSP protein has an expected molecular mass of 30 kDa approximately due to the presence of the histidine tag at the 5' end of the inserted fragment, in addition to several amino acids belonging to the ORF of the vector until the stop codon (TGA) at the 3' end accounts for the difference in molecular weight between the cloned fragment of the Hd sHSP gene and the recombinant protein.

The rHd sHSP protein includes 172 amino acids from the first base of the ORF of the N-terminal. This means that, the rHd sHSP protein carries the N- terminal of the native non-recombinant protein in addition to the conserved alpha-crystallin domain. Both the N-terminal and the alpha-crystallin domains are immunologically important. Leroux *et al*.[55] assumed that the N-terminal domain plays vital role in substrate binding and stabilization whereas, Linder *et al*. [67] proposed that the C- terminal domain acts as solubilizer of the complex formed between the sHSP and its substrate inhibiting aggregation of sHSP-substrate complex. Therefore, the presence of the C-terminal domain in the Hd sHSP during expression facilitated its solubility. This character of Hd sHSP was in contrast with the findings of Shahein *et al.* [25]. They found that the Ra sHSPII of the cattle ticks was found as inclusion bodies and they were not able to elute the expressed protein under native conditions.

Polyclonal anti-rHd sHSP protein antibodies were raised in rabbit to assess the immunogenicity of the protein. Sera were collected and anti- rHd sHSP titers were assessed and we obtained satisfactory titer demonstrating an immunogenic character the rHd sHSP. On using the immunoblot technique, polyclonal rHd sHSP antibodies strongly reacted with the purified rHd sHSP that was used for immunization confirming its immunogenicity. The immunogenic character of this protein may be attributed to the presence of the N terminal domain; a fact supported by the study of Norimine *et al* [68] who claimed that the N-terminal domain of the HSP20 of Babesia is a vaccine.

Binding of heat shock proteins to other proteins inside the cells as molecular chaperones forming large multimeric structures is called a holdase action. The holdase action of the Hd sHSP was assessed in vitro. Following thermal stress at 50 and 60ºC the cellular proteins conferred thermal stability in those cells expressing the recombinant heat shock protein and cannot be attributed to other bacterial proteins since, the rRa GST protein with similar molecular mass failed to confer thermal stability to bacterial cell extracts at the same temperatures.

Several scattered works showed the importance of the bivalent metal ions in both structural and functional characters of alpha crystalline proteins [69, 70]. Metal ions were reported to have inhibitory effect on heat shock proteins promoting aggregation: like Ca^{+2}[71], Cu^{+2} was reported to be a structuring center, and Zn^{+2} may play a structural role [72]. The metal docking analysis using the prediction server, showed binding of the Hd sHSP to Zn^{+2} ions and did not show any binding potentials to Cu^{+2} or Ca^{+2} ions. Binding of heat shock proteins to zinc ions was demonstrated to have significant effect in exposing the hydrophobic groups. This exposure plays important role in enhancement of the chaperone structural integrity and hence its function. In table 3 most of predicted amino acids (R6, C12, S107, E108, D142 and E148) to bind zinc ions were found in the hydrophobic region of the molecule as shown in figure 5. This binding of zinc ions will modulate the hydrophobic interactions of the alpha-crystallin and may promote conformational changes, which affect the overall stability of the molecule.

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