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ISOLATION, PCR AMPLIFICATION AND CLONING OF HEAT SHOCK PROTEIN GENE FROM SALIVARY GLANDS OF *Hyalomma dromedarii* TICKS FROM *Camelus dromedarius*

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ABSTRACT

A molecular study was carried out to isolate the heat shock protein gene of *Hyalomma dromedarii* ticks of camels, which play important role in their survival under harsh environmental conditions. Engorged adult *H. dromedarii* ticks were collected from camel herds in the Bikaner district of Rajasthan. Total genomic DNA and RNA was isolated from the salivary glands of ticks. Primers were designed for amplification of heat shock protein gene from *H. dromedarii* by using base sequence of *Haemaphysalis longicornis*. The heat shock protein gene of *H. dromedarii* was successfully amplified from genomic DNA and cDNA and was identified on the basis of its size in agarose gel electrophoresis as 560 bp. The amplicon of expected size was purified from the 1% low melting agarose gel. DNA fragment of interest was then ligated to the pGEM- T Easy vector and ligated mixture was transformed into *Escherichia coli* JM109 strains for cloning. Screening of recombinants was done by restriction enzyme digestion of plasmid DNA and by colony PCR for quick screening of plasmid insert directly from *E. coli* colonies in the presence of insert specific primers.

Key words: Amplification, *Camelus dromedarius*, cloning, heat shock protein gene, *Hyalomma dromedarii*, salivary gland

Tick infestation of camels is a universal problem and all age groups are prone to it. The ixodid tick *Hyalomma dromedarii* is a three host tick and found in India and other tropical and sub-tropical countries. *H. dromedarii* acts as vector for transmission of a wide range of pathogens such as protozoa like *Theileria annulata* (De Kok *et al,* 1993), *Theileria equi, T. annulata* and *Babesia caballi* (El- Kammah *et al,* 2001), bacteria and tick borne infections such as animal dermatophilosis and human cases of haemorrhagic fever in Saudi Arabia due to a flavivirus, alkhurma haemorrhagic fever virus (Charrel *et al,* 1971). The *Hyalomma* sp. ticks are ectoparasites with irritating bites causing 'tick paralysis' in man and animals, probably due to the secretion of toxic substances in their saliva. Tick saliva contains a wide array of bioactive proteins and lipid molecules exhibiting a range of pharmacological properties to thwart the defence mechanisms elicited by the host in response to the tick bite (Bowman *et al,* 1997).

Despite the importance of ticks as vectors of diseases very little is known about their ability to survive in harsh conditions. In terms of evolution, the tick heat shock proteins play important role in their adaptation to harsh climatic conditions. Heat shock protein genes are a subset of a larger group of genes coding for proteins that are involved in 'house-keeping' functions in the cell (Sorensen *et al,* 2003). The level of expression of HSPs is different on exposure of different stresses such as radiation exposure, chemical exposure, infections etc. (Lakhotia and Prasanth, 2002; Mahroof *et al,* 2005). Ticks in response to stresses like heat, cold, hunger etc. undergo dispause i.e. physiological state of dormancy (Rinehart *et al,* 2007). Heat shock proteins belong to a multigene family and range in molecular size from 8 to 150 kd. They typically are named according to their molecular size. The 70-kd protein is referred to as Hsp70, and the gene coding for that protein would be hsp70. HSPs have emerged as attractive immune-stimulating components for the development of subunit vaccines against many infectious pathogens. They possess antigenic peptides that mediate the maturation and stimulation of dendritic cells necessary for secretion of inflammatory cytokine (Suto and Srivastava, 1995). Control of ticks is very

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important all over the world in order to limit the economic losses and health hazards to both livestock and human beings. Control of ticks by conventional methods using acaricides has become increasingly difficult owing to emergence of acaricide resistant strains of ticks (Nolan, 1990). This has stimulated research on alternative method for control of ticks.

The salivary gland proteins from the ticks are the important molecules as vaccine target for the ticks control programme from the various reports. This study was therefore, undertaken to provide information about isolation of the heat shock protein gene from camel tick, *Hyalomma dromedarii* so as to find out the suitable strategy for its control.

Materials and Methods

Engorged adult male and female ticks were collected from the camels in and around Bikaner city at environmental temperature around 40°C and 75% relative humidity. Morphological features of *H. dromedarii* were examined under stereo microscope and identified using the guide to identification of species (Estrada-Pena *et al,* 2004, Apanaskevich *et al,* 2008). Dissection of ticks for collection of salivary glands was done under a dissecting microscope (10x-15x) following the method of Purnell and Joyner (1968). These salivary glands were transferred to separate labelled micro centrifuge tubes, washed 3 times with (PBS) and stored at -80°C with 100 µl of 70% ethyl alcohol until used for extraction of DNA. Then total genomic DNA was isolated from the fine suspension of ground salivary glands of adult *H. dromedarii* by the phenol-chloroform extraction followed by ethanol precipitation method as suggested by Sambrook and Russel (2001). Then the concentration and purity of DNA sample was determined by Spectrophotometric method and Agarose Gel Electrophoresis. RNA isolation was done by using Promega SV Total RNA isolation system and cDNA synthesis was done using Clontech RT-PCR Kit. This genomic DNA and cDNA both were used for amplification by Polymerase Chain Reaction using gene specific forward primer 5′ GGTGTGGGTTTCTACTCTGCC 3′ and reverse primer 5′ TCTTCCCAGTCGTTCGTGAG 3′ designed from published sequence of *Haemaphysalis longicornis* (Accession No. JQ346697.1) using the primer designing tool at NCBI. PCR amplification was performed by cycling conditions as initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 45 sec, extension at 72°C for 1 min. and 30 sec, and final extension for 10 min at 72°C. The PCR amplified products were checked with 1 kb DNA molecular weight marker in 1.2% agorose gel.

The PCR products from Low melting point agarose slices were purified by illustra GFX PCR DNA and Gel Band Purification Kit (GE healthcare, USA) using the manufacturer's protocol. The DNA fragment of heat shock protein gene and the pGEM- T Easy vector (Promega, USA) in which it is to be cloned were digested with T4 DNA ligase enzyme to generate compatible ends for ligation. The ligation was done (as per the Promega protocol with slight modification) in the reaction volume of 20 µl containing 10µl of 2X Rapid ligation T4 DNA Ligase buffer [400mM Tris-HCl, 100mM $MgCl_2$, 100mM DTT, 5mM ATP (pH 7.8 at 25°C)], 6 µl PCR product, 2 µl pGEM- T Easy vector and 2 µl of T4 DNA ligase. The contents were vortexed, spun down in a micro centrifuge for 3-5 seconds and incubated for overnight at 4°C . The ligation mix was then used directly for transformation in JM109 competent cells (Promega, USA). After incubation of transformation culture 100 µl of transformation culture was plated onto antibiotic agar plates and incubated at 37°C for overnight (18 hr). Colonies harbouring the recombinant plasmid were inoculated into LB broth and incubated at 37°C overnight with horizontal shaking. The plasmids DNA were extracted from culture using illustra plasmid prep mini spin kit (GE healthcare, USA) according to the manufacturer's instructions. The positive clones were identified by Restriction Enzyme digestion of plasmid DNAs with EcoR1 and Colony PCR of plasmid colonies.

Results

The genomic DNA and RNA was analysed in 0.8% analytical agarose gel and was found to be intact without much shearing. Gene specific forward and reverse primers were used for amplification and the amplicons were analysed by agarose gel electrophoresis (Fig 1). An intensely amplified DNA was seen in lane 2, 3 and 5, 6 which was absent in the controls (Lane 4 and7) using genomic DNA and cDNA, respectively. The intense band did correspond to the heat shock protein gene of *H. dromedarii*. The size of the intense band was deduced from the standard curve drawn between the log molecular sizes of the marker bands against their respective mobility and found to be 560 bp. There were several white colonies along with a few blue colonies were found after cloning of amplified PCR product. The blue colonies represent the presence of vector alone but few blue colonies may contain vector with insert. The white colonies may represent recombinant clones

carrying insert in the plasmid. Two well separated DNA bands were seen in case of plasmid isolated from positive colonies upon digestion with EcoRI, the less intense lower band may correspond to the insert (Fig 2). Release of DNA fragments of around 560 bp for heat shock protein gene was found after restriction enzyme digestion. Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies and amplification was found in wells of white colonies and also in blue colony (Fig 3).

Discussion

Heat shock proteins originally were discovered when it was observed that heat shock produced chromosomal puffs in the salivary glands of *Drosophila* sp. (Whitley *et al*, 1999). In the past years heat shock proteins (HSPs) have received much attention in model organisms undergoing experimental stresses in the laboratory. The expression of HSPs in organisms is essential to normal growth and development (Bond and Schlesinger, 1987). The molecular characterisation of heat shock protein gene from *H. dromedarii* tick of camel is one of its kinds of work and has never been undertaken elsewhere in the world. In the present study, newly designed primers for amplification of *H. dromedarii* heat shock protein gene was confirmed as specific amplicon and the two important variables such as primer annealing temperature and $MgCl_2$ concentration were considered for the optimisation of the PCR conditions. Gradient PCR was used for the optimisation of the PCR conditions. Different concentrations of $MgCl_2$ ranging from 1.0mM to 4.0mM were tried for the PCR trouble shooting. It was found that primer annealing temperature and $MgCl_2$ concentration for the PCR condition were found to be 52°C and 3.0mM, respectively. The amplicon obtained in the PCR reaction would be the specific target region as there was no amplification in the negative control included in the reaction (negative control included all the components of the PCR mix without template). In the agrose gel DNA profile, the expected size of the amplicon (560 bp) was obtained. Gene encoding the members of this protein family has already been found in several species of hard ticks. Hussein *et al* (2014) reported the identification of a small HSP gene from the salivary glands of *Rhipicephalus annulatus* ticks. The identified cDNA contained a 742 bp sequence with 543 bp open reading frame (GenBank accession number is JX912231). Similarly, Shahein *et al* (2010) did the immunoscreening of a cDNA expression

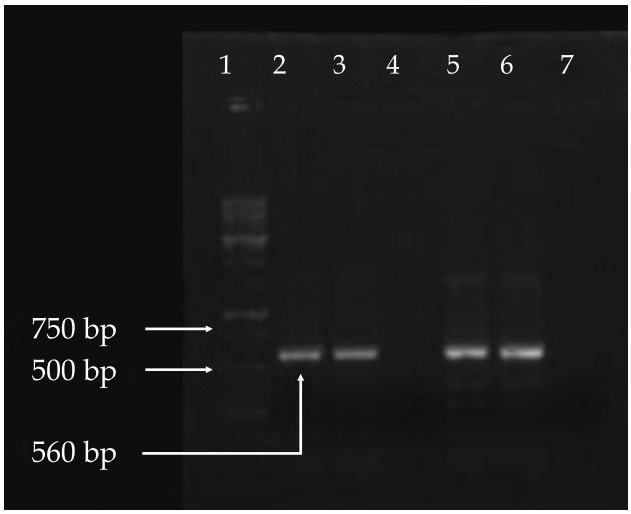

Fig 1. Amplified heat shock protein gene of *H. dromedarii*. Lane 1 : 1kb DNA ladder, Lane 2 and 3 : amplicons using genomic DNA, Lane 5 and 6: amplicons using cDNA, Lane 4 and 7 : Negative controls.

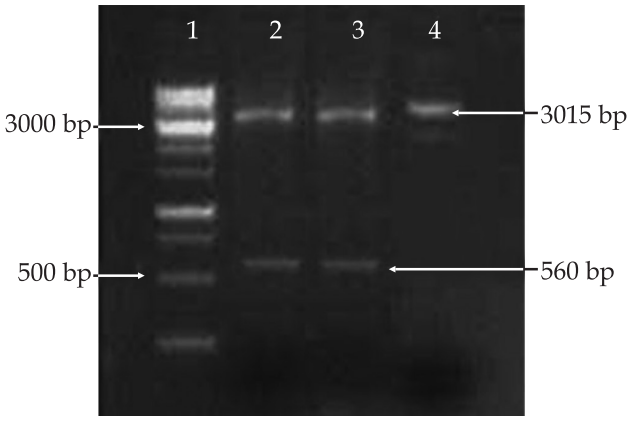

Fig 2. Heat shock protein gene fragments after restriction enzyme digestion. Lane 1 : 1kb DNA ladder, Lane 2 and 3 : released clones of heat shock protein gene, Lane 4 : undigested plasmid.

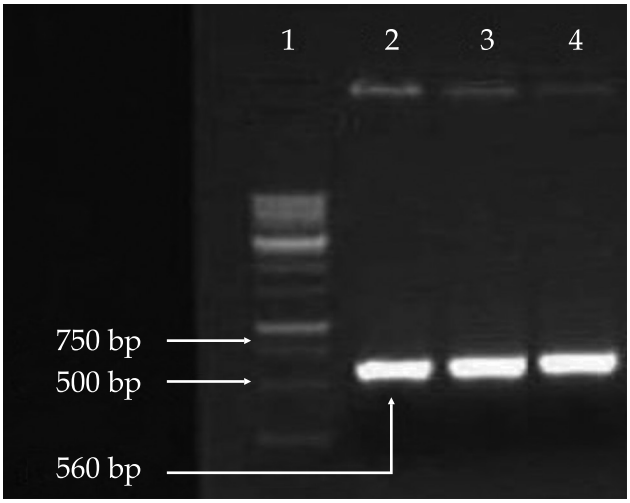

Fig 3. Amplification of heat shock protein gene by colony PCR. Lane 1 : 1kb DNA ladder, Lane 2 and 3 : amplicons from white colonies, Lane 4 : amplicon from blue colony.

library of the Rhipicephalus (*Boophilus annulatus*) tick which led to the identification of a 661 bp sequence (GenBank accession number is HM149782). Tiana *et al* (2011) identified a *Haemaphysalis longicornis* heat shock protein 70 (HLHsp70) from a cDNA library synthesized from tick eggs. The HLHsp70 cDNA is 2311 bp in length and encodes 661 amino acid residues with the predicted molecular weight of 72.5 kDa (GenBank accession number is EU289295). Shim *et al* (2006) isolated a heat shock cognate 70 (hsc70) gene from the two-spotted spider mite, *Tetranychus urticae*, a serious agricultural pest. The hsc70 cDNA is 2275 bp and contains a 1962 bp open reading frame. However, in this study, the transcripts of the gene isoforms were not categorically identified. It was proving that the DNA fragment amplified in the PCR reaction was of expected size (560bp) and highly target specific region of heat shock protein gene of *H. dromedarii*. With the cloning and sequencing of heat shock protein gene of *Hyalomma dromedarii* and expression of this protein it can make a great impact on the discovery of new protective antigen. It would be an ideal vaccine target or drug target in its own right for the control of *Hyalomma* genus or some different tick species of India.

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