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Molecular characterization of HSP70 Gene in local chicken of Poonch

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Abstract
Heat shock protein 70 (HSP70) perform many vital functions in the cells and is responsible for cytoprotection under stressfull conditions. In this study, molecular characterization of HSP70 gene in local chicken of Poonch was undertaken with the objectives of amplifying and sequencing of HSP70 gene and studying the genetic similarity and distance between different species with respect to local chicken of Poonch. Fresh blood was collected from chicken and used for RNA extraction. cDNA was synthesized by reverse transcription. HSP70 gene was amplified using specific primers designed by Primer 3 software. The amplified product (1986 bp) was purified and ligated in pGEM T Easy vector. The ligated product was cloned using DH5α competent cells and then sequenced. MEGA X and DNA STAR softwares were used for sequence analysis of the HSP70 gene. The comparison of HSP70 CDS sequence demonstrated 100%, 95.96%, 95.85%, 95.70%, 92.60%, 91.44% 91.39%, 91.39%, 81.47%, 81.41% and 81.36% similarity with chicken, quail, turkey, guinea fowl, pigeon, ostrich, emu, duck, goat, sheep and pig, respectively indicating sequence homology among different species was high. The percentage identity of translated protein sequence of 634 residues of HSP70 gene was 100%, 98.26%, 98.11%, 98.11%, 97.95%, 97.95%, 97.48%, 97.32%,92.36%, 91.82% and 91.51% similar to chicken, quail, turkey, guinea fowl, pigeon, duck, emu, ostrich, pig, sheep and goat, respectively. The phylogenetic tree drawn by MEGA X software showed that the gene is evolutionarily conserved. Z test was conducted which showed that the gene might have evolved by purifying selection (dS>dN).

Keywords: HSP70 gene, molecular characterization, sequencing, homology, purifying selection, Poonchi chicken

Introduction
Increased temperatures in the summer can cause high mortality, reduced intake of feed, rate of growth, consistency of egg shell, decreased weight gain and egg production in domestic birds (Cahaner *et al*., 2008; Melesse *et al*., 2011) [2, 10]. In order to combat stress, Heat shock proteins are formed. HSPs' main role is to protect cells from the adverse stressful conditions (Timperio *et al*., 2008; Kalmar and Greensmith, 2009) [18, 6]. Further, HSPs are also identified to be the confirmatory biomarker for assessing the thermo-tolerance of the livestock species (Sejian, 2013; Archana *et al*., 2017) [15, 1]. HSPs have the ability to be used as cellular damage markers, as well as for diagnostic and therapeutic purposes. HSPs are a form of molecular chaperone that were first discovered in Drosophila larvae when their expression was induced during heat stress (Ritossa, 1962) [13]. There are various families of HSPs like HSP27, HSP60, HSP70, HSP90, and HSP110/104. This classification is based on their molecular weight (Kregel, 2002) [7]. HSP70 proteins are the most abundant among all the HSPs. Acquired thermotolerance, was one of the first physiological functions connected to HSP70 (Landry *et al*., 1982) [8]. HSP70 can be present in the nucleus, cytosol, mitochondria, endoplasmic reticulum, and other cellular compartments (Flaherty *et al*., 1990) [3]. The HSP70 family contains several proteins, few of them are only present under stress (high inductive), while remaining others are present in cells under normal growth conditions and are not thermal inductive (constitutive) (Flaherty *et al*., 1990; Snutch *et al*., 1988) [3, 16]. HSP70 has a molecular weight of almost 70000 Daltons (69,913 Daltons). HSP70 gene is located on chromosome number 5 in chicken. The CDS region has a span of 1905 nucleotides. HSP70 protein consists of 634 amino acids. In all species, HSP70 is involved in three activities: preventing protein aggregation, promoting folding of misfolded proteins back to their original form, and solubilizing and refolding of aggregated proteins. HSPs bind to protein sequences that is water resisting (hydrophobic) that would otherwise interact with neighbouring proteins,

resulting in protein dysfunction. In J&K, indigenous chicken of Poonch region is locally called as Poonchi chicken. It has low initial investment and high economic returns. It is less explored population and no information regarding HSP70 gene in Poonchi chicken is available.

Materials and methods

Collection of blood and Isolation of DNA

Collection of blood (2ml) was done from the wing vein of Poonchi Chicken under aseptic conditions in sterile K3 EDTA coated vacutainer (Vacutech, Labtech disposables, India). RNA was isolated from the blood samples using All Blood RNA Purification Kit (HiPura). Isolated RNA was stored at -80°C. The quality of RNA was checked through 1% horizontal submarine agarose electrophoresis. Electrophoresis was carried out at 75 volts for 40 min and then UV trans-illuminator was used to visualize gel. Three intact bands of 28s, 18s and 5s with smearing indicated RNA was intact and of good quality. The purity of genomic RNA stock samples were quantified by using Nano-drop spectrophotometer (ND-1000) at 260 nm and 280 nm. The ultraviolet (UV) absorbance was checked at 260 nm and 280 nm for checking concentration and purity of RNA. Samples having OD ratio (260/280 nm) of approximately 2 were used for cDNA synthesis.

First strand cDNA synthesis

cDNA was synthesized from RNA template using RevertAid first strand cDNA synthesis kit. 20µl mixture was prepared by mixing 8µl nuclease free water, 3µl template RNA, 1µl random hexamer, 4µl reaction buffer (5X), 1µl RiboLock RNase inhibitor (20U/µl), 1µl RevertAid RT(200U/µl), 2µl dNTP (10mM). The mixture was incubated in PCR machine first at 25°C for 5 minutes, followed by 42 °C for 60 minutes. The reaction was terminated by heating at 70 °C for 5 minutes. The cDNA was chilled on ice and stored at -20 °C.

Designing of primers and PCR Amplification

Specific primers for Poonchi chicken HSP70 were designed based on available sequence of chicken HSP70 gene (Accession number: NM 001006685.1) using Primer3 software available at NCBI. The amplicon size was of 1986 bp. The sequence of the primer pair used were HSP70 Forward: 5'GGCTGACTGACCAAGAGGAA3' and HSP70 Reverse: 5'AGCAAAGAATGGAGTGACTGT 3'. 50µl reaction mixture was prepared. The standardised reaction mixture concentrations of the different components which gave optimum result for HSP70 gene consisted of 25µl PCR master mix, 5µl forward Primer, 5µl reverse primer, 4µl template cDNA, 11µl distilled water. PCR amplification included one cycle of initial denaturation at 95 °C for 3 min followed by 34 cycles each of denaturation (95 °C for 30 sec), annealing (54 °C for 1 min) and extension (72 °C for 2 min) followed by final extension at 72°C for 5 min. 1% agarose gel in 1X TAE buffer was used for analysis of PCR product. Ethidium bromide was added in the agarose (1%solution@ 5µl/100ml). The run was performed at constant voltage at 75V for 40 minutes. Along with the test samples Gene DireX Kplus DNA Ladder RTU (Ready-to-use) was also run in one lane. UV transilluminator was used to visualize the amplified product.

Gel purification and ligation, cloning and transformation of PCR product

The PCR product was purified using GenElute Gel extraction kit. The product after purifying was ligated into pGEM-T Easy vector by preparing a ligation mixture consisting of 5µl 2X Rapid Ligation Buffer, 1µl pGEM-T Easy vector, 3µl PCR product and 1µl T4 DNA. The components in the tube were mixed by pippeting and left for overnight incubation at 4 °C. Ligated product was transformed and cloned using DH5α competent cells. Recombinant clones produced white colonies whereas non recombinant clones produced blue colonies. Randomly white colonies were picked and inoculated to 10 ml LB broth containing ampicillin and incubated at 37 °C overnight in a shaking incubator. The colony was used as template and to make up volume upto 50 µl remaining PCR reagents were added to tube. The colony PCR was carried out under same conditions that were used in amplification of the HSP70 gene. The PCR product from clones was run in 1% agarose gel with DNA marker was used.

Sequence analysis

After the confirmation, recombinant colony was selected and was sent for Sanger sequencing with "Primer Walking" at Agri Genome Labs Pvt. Ltd. Kochi India. The obtained sequence of Poonchi chicken HSP70 was subjected to BLAST analysis in order to confirm that the obtained sequence is of HSP70 or not. The obtained sequence of HSP70 gene of Poonchi chicken was analysed by MEGA X software and comparison was made with other CDS (Coding sequences) of different poultry species present at the the NCBI (National Center for Biotechnology Information) site. ClustalW method using MEGA X software was used for multiple sequence alignment. Based on the aligned sequences, phylogenetic tree was constructed using Neighbor-Joining method (Saitou and Nei, 1987) [14]. The evolutionary distances between each pair of sequences were estimated by calculating the proportion of nucleotide differences between the sequences. The evolutionary distances can also be estimated on basis of proportion of amino acid differences. Maximum Composite Likelihood model (Tamura *et al*., 2004) [17] was used for analyses. The frequencies of the four nucleotides (nucleotide composition) and that of the twenty amino acid residues (amino acid composition) were computed for all the sequences used. Z test was conducted in order to test whether positive selection is operating on a gene or not. Nei-Gojobori method (Nei and Gojobori, 1986) [12] was used for analysing and comparing the relative abundance of synonymous (dS) and non-synonymous substitutions (dN) t in the gene sequences. Null hypothesis is that H0: $dN = dS$ Alternate hypothesis are HA: $dN \neq dS$ (test of neutrality, $dN > dS$ (positive selection), $dN < dS$ (purifying selection).

Results and discussion

HSP70 gene characterization

The obtained sequence of Poonchi chicken HSP70 was subjected to BLAST analysis in order to confirm that the obtained sequence is of HSP70 or not. Following BLAST, different sequences of HSP70 gene of different species were retrieved. This confirms that the sequence obtained is of HSP70. Further BLAST analysis was done for comparing the percentage identity of Poonchi chicken HSP70 CDS sequence with CDS of other species and the percentage identity of translated protein sequence of Poonchi chicken HSP70 with other species. The comparison of HSP70 CDS sequence demonstrated 100%, 95.96%, 95.85%, 95.70%, 92.60%, 91.44% 91.39%, 91.39%, 81.47%, 81.41% and 81.36% similarity with chicken, quail, turkey, guinea fowl, pigeon,

ostrich, emu, duck, goat, sheep and pig, respectively indicating a high homology of sequence that exsisted between different species. The percentage identity of translated protein sequence of 634 residues of HSP70 gene was 100%, 98.26%, 98.11%, 98.11%, 97.95%, 97.95%, 97.48%, 97.32%,92.36%, 91.82% and 91.51% similar to chicken, quail, turkey, guinea fowl, pigeon, duck, emu, ostrich, pig, sheep and goat, respectively

Similar results were shown by Morimoto *et al*., 1986. The chicken HSP70 gene had only one exon with a 1,905-bp coding region. They found that chicken HSP70 cDNA sequence is 80% similar to human HSP70 cDNA sequence and 73% similar to Drosophila HSP70 sequence of cDNA. However, chicken HSP70 amino acid sequence is 80% similar to human HSP70 but only 71% similar to amino acid sequence of Drosophila HSP70.

Gaviol *et al*., 2008 [5] investigated the HSP70 gene in *Japanese quail* and found that the quail HSP70 sequence had a 98 percent similarity with chicken HSP70 and a 99 percent homology with the helmeted Guinea fowl (*Numida meleagris*) sequence of HSP70. The amino acid sequence of chicken (*Gallus gallus*) was 98 percent identical to quail. The quail HSP70 sequence of amino acid is 99 percent similarl to the HSP70 sequence of the guinea fowl.

According to Mathew *et al*., 2013 [9], the sequence of chicken HSP70 gene shared 96.4 percent similarity with *C. coturnix*, 96.2% with *N. meleagris*, 96.4% with *C. japonica*, 91.8% with *A. platyrhynchos*, 81.1% with *C. hircus*, 81.6% with *C. l. familiaris*, 76.7% with *E. caballus*, 81.7% with *M. musculus*, 75.5% with *O. aries*, 75.2% *with B. taurus*, 75.4% with *R. norvegicus* and 81.7% *O. cuniculus* in a nucleotide sequence BLAST quest.

Gade *et al*., 2010 [4], conducted the research work on the entire sequence of nucleotide of goat HSP70-1 gene which shows 99.4% homology with sheep (partial), 96.3% homology with buffalo, 97.5% with yak, 97.8% with cattle, 94.4% with horse, 95.3% with pig and 94.1% with human which indicates there is close evolutionary relationship. The sequence of amino acid of HSP70 gene of goat was 100% similar to sheep (partial), 95.9% similar to buffalo, 98.6% similar to cattle, 98% to pig, 98.4% to yak, 98.1% to horse, and 97.7% similar to human sequence.

Multiple Alignment of Poonchi chicken HSP70 CDS with coding sequence of different species by ClustalW method

All CDS sequences of HSP70 in different species were aligned using ClustalW method which reveals the nucleotide substitutions. There were 77 changes in sequence of Poonchi chicken HSP70 gene when compared with *Coturnix japonica*, 164 changes with *Anas platyrhynchos* and 367 changes when compared with *Sus scrofa*. The HSP70 gene showed 76, 151 and 321 changes in the amino acid sequence when compared with *Coturnix japonica, Anas platyrhynchos and Sus scrofa*.

Nucleotide and Amino acid compositions

The composition of nucleotide and amino acid was calculated using MEGA X. GC content of the Poonchi chicken HSP70 CDS sequence was 52.13%. By using DNA STAR software it was found that the translated protein of Poonchi chicken HSP70 sequence (1905 bp) has molecular weight of 69749.85 Daltons with 634 amino acids. Out of which 95 are strongly acidic (-) amino acids (D, E) and 86 strongly basic (+) amino acids (K,R), 200 hydrophobic amino acids (A, I, L, F, W, V) and 159 polar amino acids (N,C,Q,S,T,Y).

Phylogenetic tree analysis

MEGA X software at was used to construct phylogenetic tree at nucleotide level. Neighbor-Joining method (Saitou and Nei, 1987) [14] was used to deduce evolutionary history. Chicken and turkey were in the same clade. Guinea fowl, quail along with chicken and turkey might have evolved from same common ancestor. Goat, emu and ostrich were in the same clade. Pig shows dissimilarities suggesting different ancestry.

Mathew *et al*., 2013 [9] showed a high homology of sequence among different species showing that the HSP70 gene is evolutionarily conserved. In the HSP70 gene was non synonymous substitution (dN) were less than synonymous substitution (dS), indicating that positive selection is not operating on gene.

In a study by Gade *et al*., 2010 [4], it was found that (dN>dS) at 5% level of significance might be responsible for evolution of HSP70-*1* gene as dN is greater than dS.

Estimating evolutionary distances using pairwise distance

The pair wise distance between sequences aligned with ClustalW method was estimated by MEGA X software by calculating the differences in proportion of nucleotide between each pair of sequences. The pairwise distance estimates how divergent evolutionary sequences are. Maximum Composite Likelihood model (Tamura *et al*., 2004) [17] was used for conducting analyses. Maximum divergence from Poonchi chicken HSP70 CDS was observed with *Sus scrofa* with value of 0.2349431948 and minimum divergence was observed with *Meleagris gallopavo* with value of 0.0412556317.

Z test for Test of neutrality

The above test was conducted on MEGA X software and the alternate hypotheses (a) dN ≠ dS (test of neutrality) and (c) dN<dS (purifying selection) are accepted and so the selection is of purifying type. There is no positive selection. It means that the number of non-synonymous nucleotide changes are low as compared to synonymous changes. The selection against non-synonymous substitutions at the DNA level is called as purifying selection. In this case, the evolutionary distance based on synonymous substitutions is expected to be greater than the distance based on non-synonymous substitutions.

Conclusions

It was concluded that CDS of HSP70 gene of Poonchi chicken was of 1905 bp having molecular weight of 69749.85 Daltons with 634 amino acids, the HSP70 gene might have evolved by purifying selection (dS>dN). On the basis of genetic similarity of Poonchi chicken HSP70 gene, it was found to be highly conserved among different species. Phylogenetic analysis revealed that HSP70 CDS sequence is most close to turkey and divergent to pig.

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