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RESEARCH ARTICLE

In silico analysis of Stress Inducible 70 kDa Heat Shock Protein of Bubalus bubalis

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Abstract

Heat shock protein 70 (HSP70) is a molecular chaperone that is expressed in response to stress. HSP70 binds to its protein substrates and stabilize them against denaturation or aggregation until the conditions improve. In addition to its functions during a stress response, HSP70 has multiple responsibilities during normal growth; it assists in the folding of newly synthesized proteins, the subcellular transport of proteins and vesicles, the formation and dissociation of complexes 5 and the degradation of unwanted proteins. The present study describes the cloning, sequence analysis and 3D structure prediction of Bubalus bubalis HSP70. The cloned sequence consists of an open reading frame of 641 amino acid residues. Sequence alignment and predicted 3Dstructure revealed that the HSP70 consists of the signature sequences of the HSP super family. The present study reports the molecular modeling / 3D-structure prediction of HSP70.

INTRODUCTION

The ability to control the site-specific delivery of drugs on demand may circumvent many of the pharmacological dilemmas in achieving desired cytotoxicity to malignant tumors or sites of diseased tissue versus reducing systemic toxicity. Given the relatively nonselective nature of most anticancer agents, the therapeutic index is often narrow for otherwise highly potent drugs. We have approached this problem by postulating that drug delivery may be better controlled if specific targets could be reliably altered by external means [1]. The class of well-characterized heat shock proteins (HSP) may be used as key targets to enhance intracellular drug delivery. The high affinity ligand for such inducible proteins as a targeting moiety conjugated to another compound to form a suitable HSP-targeted drug complex [2,3].

Heat shock proteins are phylogenetically conserved gene products that catalyze the proper refolding of client proteins presumably altered during heat denaturation or other physicochemical events, collectively described as “stress” [4-8]. These proteins require the proper conformation and the action of HSPs are essential for the survival during environmental changes as well as in maintaining the normal biochemical activities of the cell. However, “these pro-survival functions of HSPs appear to have been usurped by malignant cells which render these gene products as potentially attractive pharmacologic targets” [9-12]. HSPs have been implicated in tumor cell proliferation, differentiation, invasion, and metastasis with important clinical consequences for cancer therapy [10,12-14]. Among the well-characterized HSPs, the 70 kDa group collectively termed “HSP70,” has been shown to exert strong cytoprotective effects by limiting aggregation of intracellular proteins and directly antagonizing apoptosis pathways [13,15-17].

Concomitant with increased cellular proliferation rate, high baseline levels of HSP70 expression have been observed in variety of human tumors [19-23]. Interestingly, lower levels of HSP70 expression correlates with poor clinical prognosis in breast, endometrial, cervical, and bladder carcinomas and is implicated in the development of drug resistance [10]. Structural analysis of HSP70 has revealed that the N-terminus ATPase domain is involved in substrate engagement and protein refolding [24]. Using this domain as a potential pharmacologic target, Williamson et al. [4] synthesized several adenosine analogs and found a few derivatives to bind HSP70 in vitro in the low micromolar range. Using these compounds as a starting point for producing HSP70- selective ligands, it can be used for enhancing the intracellular targeting of an inducible protein on demand. This study describes conserved regions

of HSP70, phylogenetic analysis, structure prediction along with the biochemical and biophysical properties, homology modeling and its validation.

MATERIALS AND METHODS

Bacterial strains and plasmids:

Escherchia coli strain DH5α was used as host for cloning experiment, TA cloning vector was used.

Genomic DNA isolation and PCR reaction:

DNA was extracted from the buffy coat of centrifuged blood sample containing leukocytes by standard phenol/chloroform method [25]. Concentration and purity of DNA was assessed by UV spectro-photometry using Nanodrop system. PCR was performed on the genomic DNA as a template using forward (5' CGC CCATGG **CC** ATGGCGAAAAACATGGCTATCGGC 3' and reverse (5' CGC CTCGAG CTAATCCACCTCCTCAATGGTGGGGC 3') primers containing NcoI and XhoI restriction endonuclease sites, respectively.

Cloning and sequencing:

PCR amplified product was purified and ends were ligated with TA cloning vector with T4 DNA ligase and transformed into Competent E. coli DH5α cells. The transformants were analyzed by colony PCR and were further confirmed by restriction enzyme digestion for the release of the insert. The positive recombinant clones were sequenced by using Automated Fluorescent DNA Sequencing method at "DNA sequencing facility" at Department of Biochemistry, Delhi University, New Delhi (India).

Sequence Analysis

Bubalus bubalis HSP70 sequence was deduced and deposited to GenBank and the accession number was obtained (JF502845). Sequence analysis tools of the ExPASy Molecular Biology Server of Swiss Institute of Bioinformatics were used to process nucleic acid sequence for the deduced amino acid sequence. The multiple sequence alignment at amino acid level for HSP70 across the species was performed using ClustalW (Version 1.83) [26]. The phylogenetic tree was inferred using MEGA 4.0 package. The antigenicity prediction of HSP70 protein sequence was achieved using the method of Jameson and Wolf [27] (DNASTAR, Madison, Wisconsin, USA)

Structure Modeling and Visualization of Model:

The most appropriate template for Homology modeling of B.bubalis HSP70 (Accession No. JF502845) was identified using BlastP analysis. Bos taurus HSP70 in the Protein Database (PDB) (PDB entry 2qw9, resolution =1.85, R value =0.204) was used as the reference organism [28]. The target and the template sequences were aligned using ClustalW. Homology modeling program Swiss-Model was employed to generate a comparative 3D- structure model of B.bubalis HSP70 [29]. No other refinements were applied. Swiss PDB viewer software [31] was employed as a tool to envisage the generated structural model.

Validation of the generated model:

The generated 3D-model was assessed with various structure verification servers viz. PROCHECK [32] that relies on Ramachandran plot [33].

RESULTS AND DISCUSSION

Sequence Analysis and evolutionary status of HSP70

PCR amplification was carried out using extracted DNA. Agarose gel electrophoresis revealed 1926-bp PCR product of HSP70 gene on 1.5% agarose gel (Figure 1).

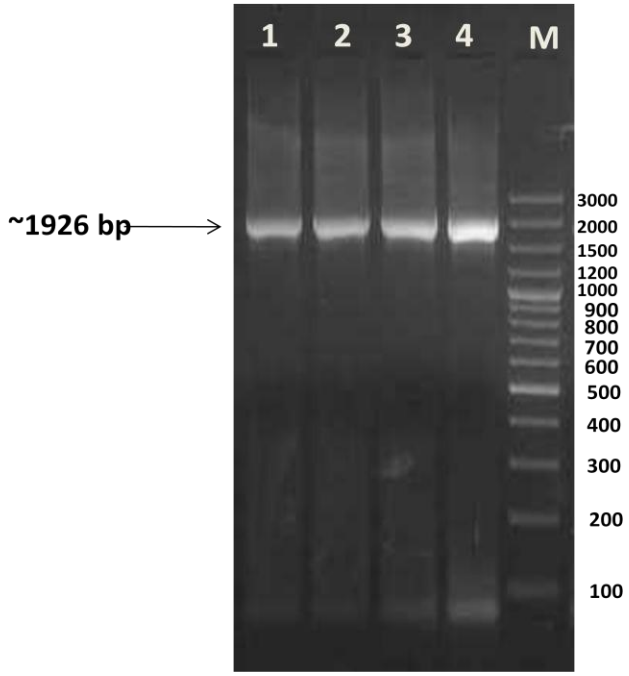


Figure 1 *PCR amplification of complete HSP70 gene from buffalo*
Lane 1,2,3,4: PCR amplicon of HSP70 gene from different buffalo breeds
M: GeneRuler™ 100 bp plus DNA ladder (Fermentas, USA)

The sequence analysis revealed 1926bp of B. bubalis HSP70, mRNA (GenBank Id: JF502845). The homology status of HSP70 at the nucleotide and amino acid levels was assessed across the species using ClustalW (Version 1.83). The entire nucleotide sequence of buffalo HSP70 gene shows 98% homology with cattle, goat and sheep, 96% with camel, 95 % with pig, 94% with dog, 93% with human and 84% with chicken, quail and fish sequences indicating close evolutionary relationship (Figure 2).

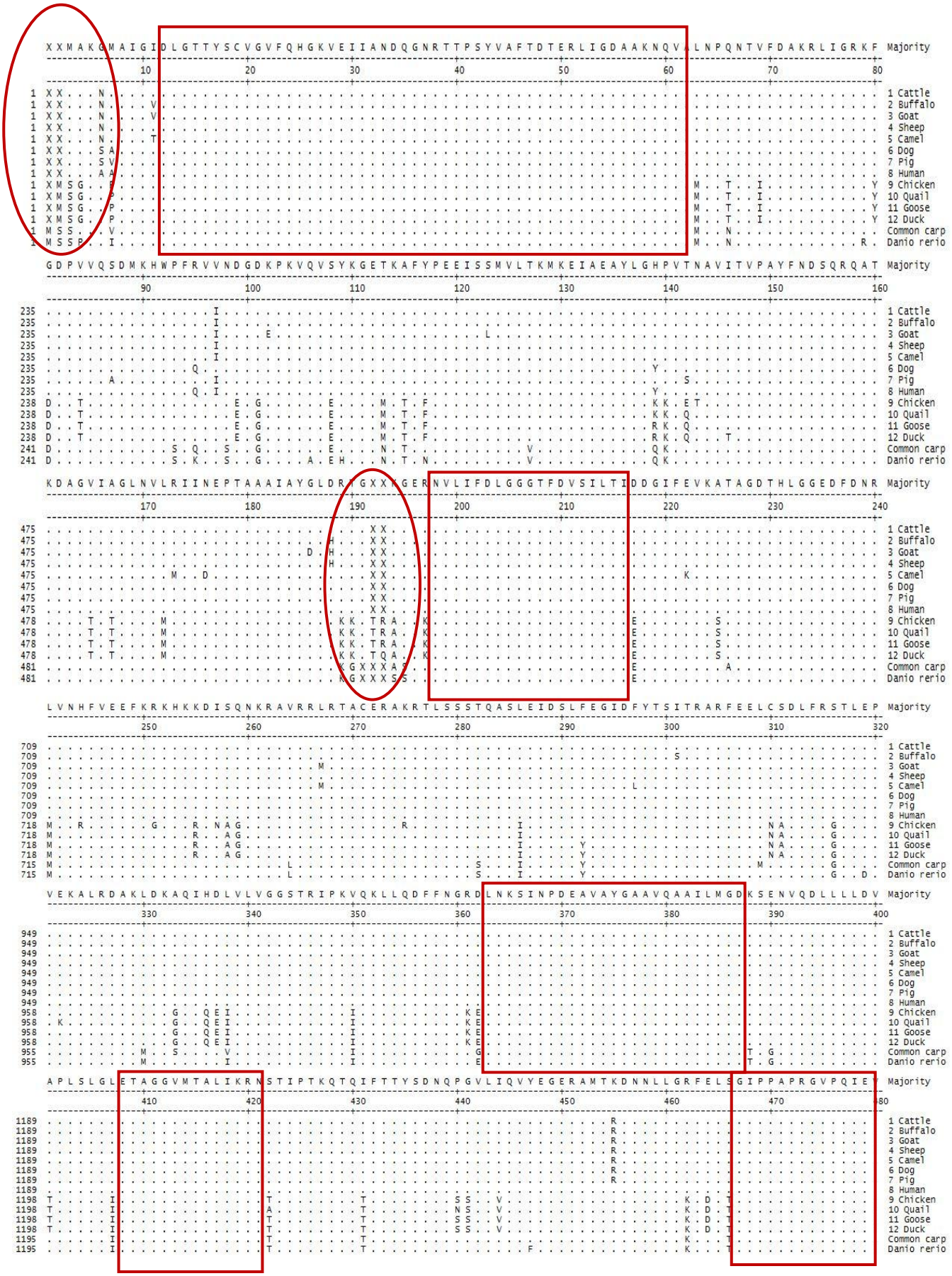
Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		99.5	98.9	99.8	98.9	98.8	99.1	98.8	83.6	84.1	84.1	84.0	85.8	85.3	1	1 Cattle
2	0.5		99.1	99.7	98.6	98.3	98.6	98.3	83.2	83.6	83.6	83.5	85.3	84.9	2	2 Buffalo
3	1.1	0.9		99.1	98.6	97.7	98.0	97.7	82.6	83.0	83.0	82.9	84.7	84.4	3	3 Goat
4	0.2	0.3	0.9		98.8	98.6	98.9	98.6	83.5	84.0	84.0	83.8	85.6	85.2	4	4 Sheep
5	1.1	1.4	1.4	1.3		97.7	98.0	97.7	82.6	83.0	83.0	82.9	84.7	84.4	5	5 Camel
6	1.3	1.7	2.4	1.4	2.4		98.6	98.9	84.0	84.4	84.4	84.3	86.1	85.2	6	6 Dog
7	0.9	1.4	2.1	1.1	2.1	1.4		98.5	83.5	84.0	84.0	83.8	85.6	85.0	7	7 Pig
8	1.3	1.7	2.4	1.4	2.4	1.1	1.6		83.6	84.1	84.1	84.0	86.0	85.3	8	8 Human
9	16.3	16.9	17.7	16.5	17.7	15.9	16.5	16.3		98.1	98.3	98.0	84.1	83.2	9	9 Chicken
10	15.7	16.3	17.1	15.9	17.1	15.3	15.9	15.7	1.9		99.2	98.9	84.6	84.0	10	10 Quail
11	15.7	16.3	17.1	15.9	17.1	15.3	15.9	15.7	1.8	0.8		99.7	85.0	84.4	11	11 Goose
12	15.9	16.5	17.3	16.1	17.3	15.5	16.1	15.9	2.1	1.1	0.3		84.9	84.3	12	12 Duck
13	14.0	14.6	15.4	14.2	15.4	13.7	14.2	13.9	15.9	15.3	14.7	14.9		95.8	13	Common carp
14	14.6	15.2	15.8	14.8	15.8	14.8	15.0	14.6	17.1	16.1	15.5	15.7	4.4		14	Danio rerio
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		

Figure 2 *Percent identity of protein sequences between buffalo HSP70 with other species*

Inferred amino acid sequence of 641 residues of buffalo HSP70 gene was 99.1% similar to cattle, 98.9% to goat, 98.9% to sheep, 98% to camel, 95.9% to pig, 95.1% to dog, 94.7% to human 75% to chicken, 76.3% to quail, 76.6% with common carp and 78.8% to Zebra fish sequence (Figure 3 and 4).


Majority
1 Cattle
2 Buffalo
3 Goat
4 Sheep
5 Camel
6 Dog
7 Pig
8 Human
9 Chicken
10 Quail
11 Goose
12 Duck
Common carp
Danio rerio

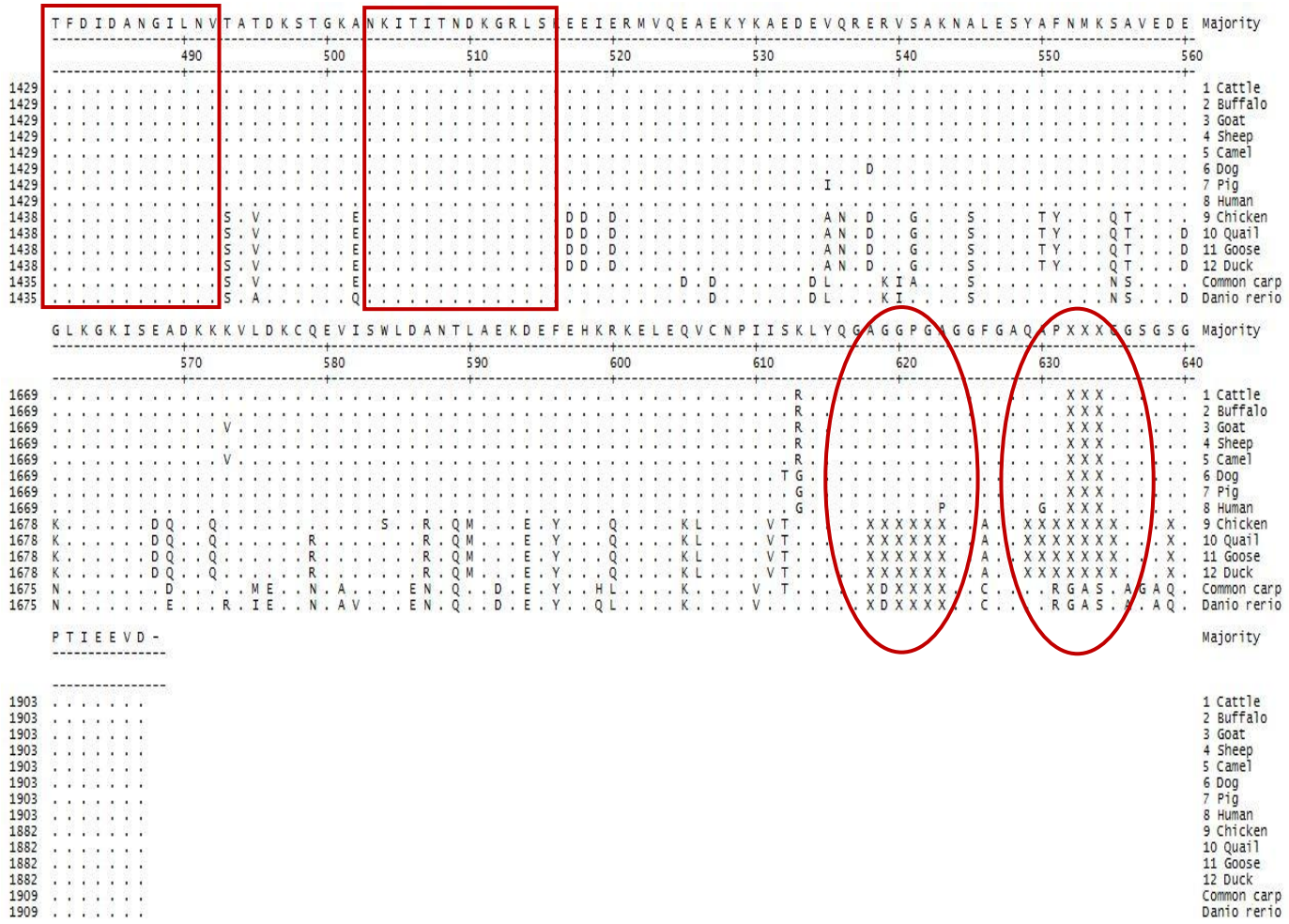


Figure 3 *Amino acid sequence alignment of buffalo HSP70 with other species. Boxes in alignment show the conserved regions and circles shows the gaps between the sequences.*

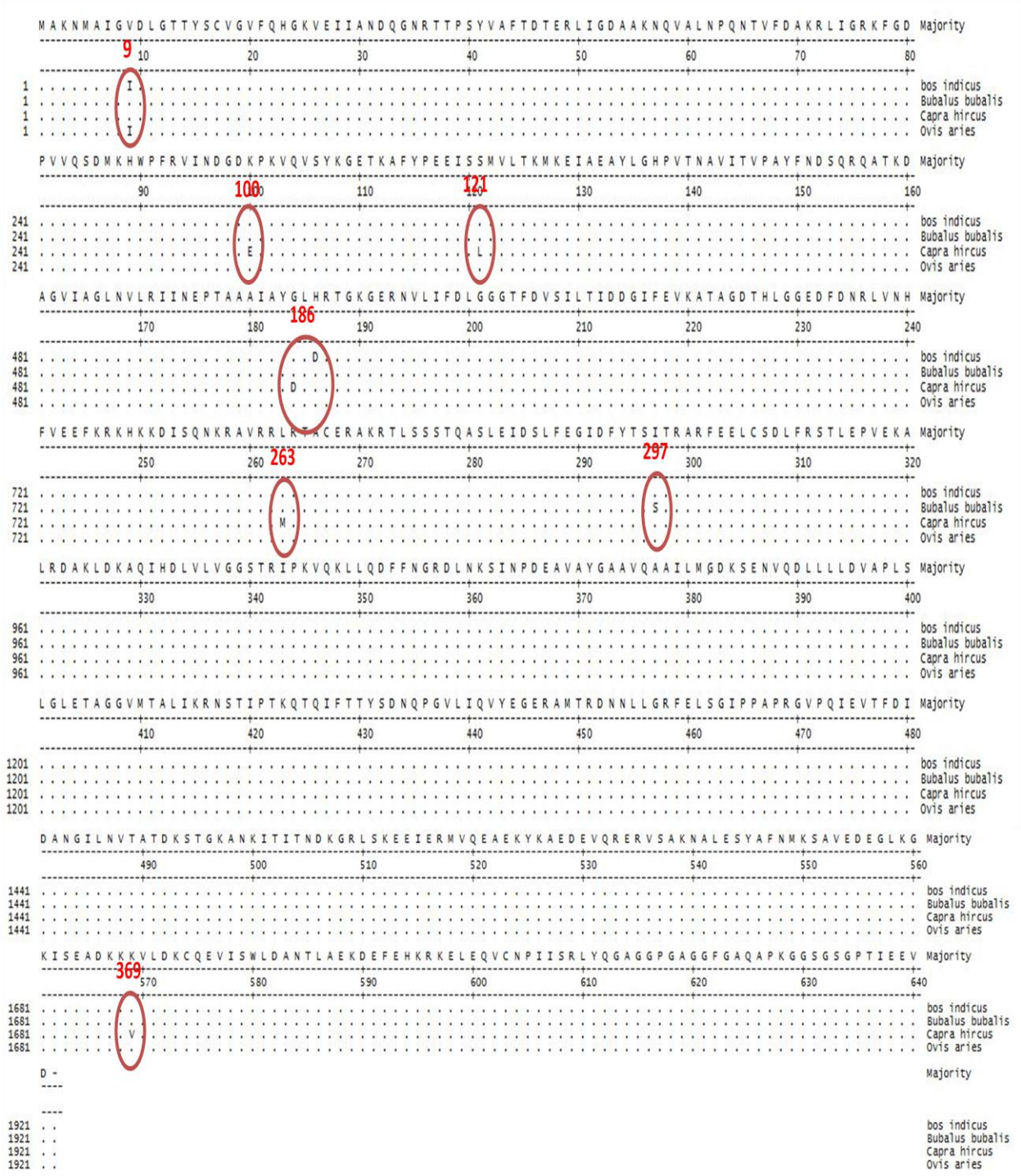


Figure 4 *Amino acid alignments of buffalo HSP70 with the only ruminant species*

The results indicate that buffalo HSP70 nucleotide and deduced amino acid sequence is highly conserved across the species. Pelham [34] reported that HSP proteins are highly conserved both in protein coding sequence and in regulatory sequence. Morimoto et al [35] characterized chicken HSP70 gene and found that chicken HSP70 cDNA sequence and deduced amino acid sequence is 80% identical to human and 73% identical to Drosophila. Our

results correlated with studies showing 90%–95% sequence similarity between HSP70 in canine and bovine, human, and mouse proteins [36]. For the alignment of the HSP70 amino acid sequence different species sequences were used including ruminants, monogastric and avian species.

Phylogenetic analysis:

Based on the nucleic acid sequences of HSP70 full length orf, phylogenetic tree was drawn by Mega 4.0 [37]. For phylogenetic analysis, Neighbor-Joining, Maximum parsimony and UPGMA (unpaired group mean average) methods were used as described. Both the neighbor joining and the maximum parsimony trees were consistent in generating trees with similar topologies. The neighbor joining tree with relatively higher bootstrap values provided clear resolution of all the nodes (Figure 5).

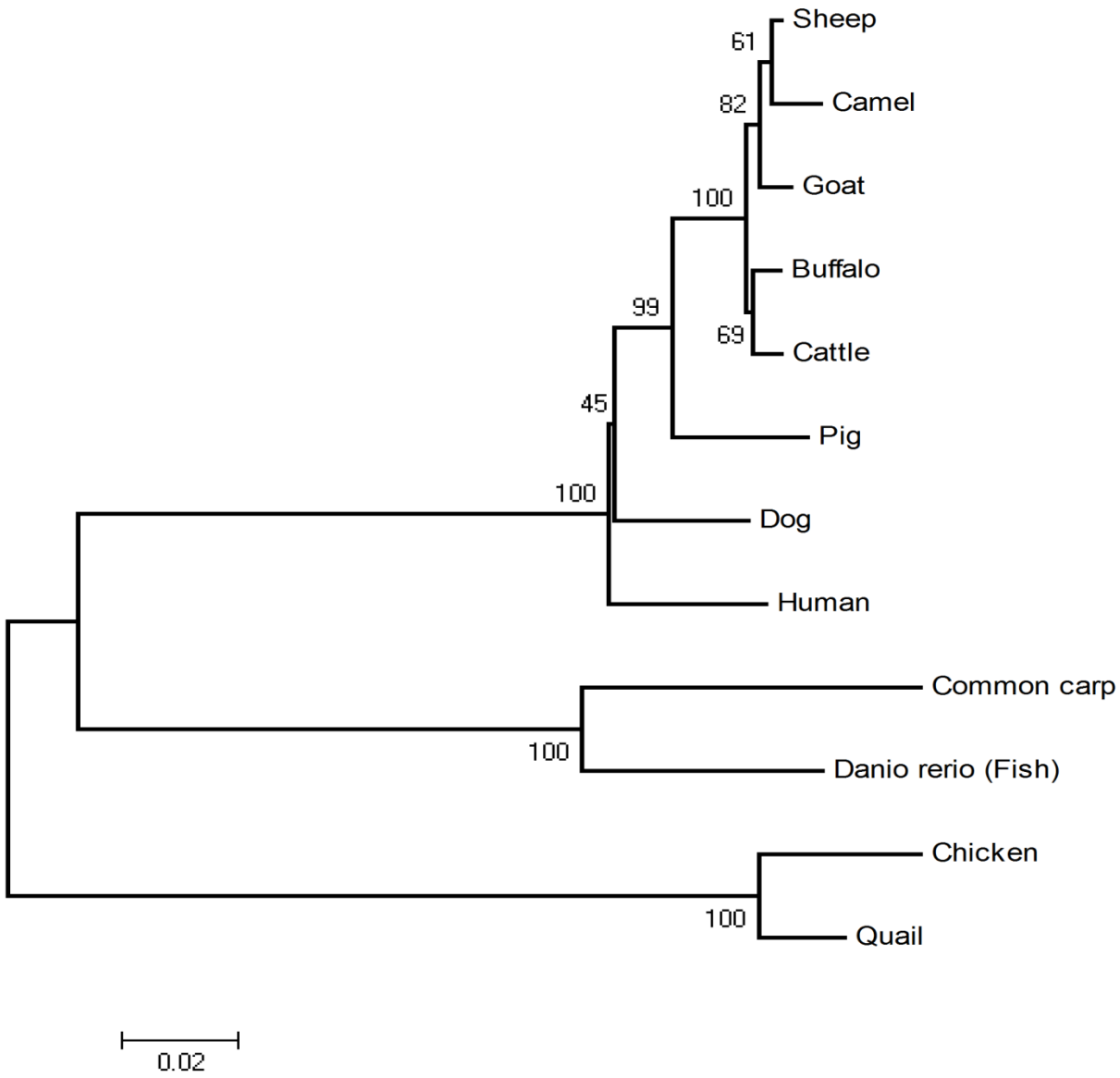


Figure 5 *Phylogenetic tree showing relationship between buffalo and other species of HSP70*

The mammalian sequences (cattle, sheep, goat, camel, dog, pig and human) clustered into a monophyletic group. It was found that ruminants and monogastrics are derived from different clusters according to their closer

evolutionary relationship. Within this group the sequence in this study was found closest to the cattle sequence. The fish group (common carp and Zebra fish) was found to be closer to the mammalian group than the avian group (chicken and quail) which clearly clustered away from the mammalian group. This shows that cattle, goat, sheep, camel, pig and human are having similarity with buffalo having different lineage. Chicken, quail and fish sequences show dissimilarities suggesting varying ancestry.

Similar results were reported by Gade et al [38] in their findings on goat HSP70 molecular characterization that ruminants and monogastrics are derived from different clusters according to their closer evolutionary relationship.

Biochemical properties of predicted HSP70 protein

Predicted HSP70 protein of buffalo possesses molecular weight of 70353.67 Da with 641 amino acids of which 81 are Strongly Basic (+) Amino Acids (K,R) and 93 Strongly Acidic (-) Amino Acids (D,E) (Figure 6).

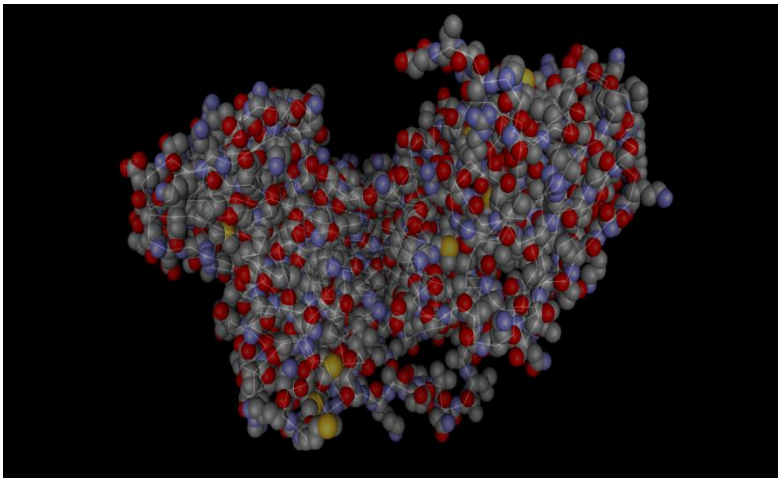


Figure 6 *Biochemical structure of Buffalo HSP70 protein*

Positively (blue) and negatively (red) charged atoms of standard residues of protein, carbon atoms (grey), sulphur (yellow) defined by PyMOL software

Further, 222 amino acids are hydrophobic (A,I,L,F,W,V) and 150 (N,C,Q,S,T,Y) are polar in nature. Isoelectric point of B. bubalis HSP70 is 5.924 and with a charge of -6.662 at pH 7.0. Molecular structure of HSP70 contains 44 kDa fragment (amino acid residues 1–386) at N-terminus contains 4 domains forming 2 lobes with a deep cleft between. 18-kDa fragment (amino acid residues 384–543) contains two 4-stranded antiparallel β-sheets and single α-helix [39] 10-kDa fragment (amino acid residues 542–640 for HSP-70) at C-terminus conserves EEVD terminal sequence. The N-terminal 44 kDa domain is ATPase domain; 18 kDa domain is peptide-binding domain; C-terminal 10 kDa fragment carries highly conserved EEVD terminal sequence, which is present in all eukaryotic HSP70 and HSP90 [40].

As there are only 3 amino acid difference (position 9, 186 and 297) as compared buffalo to cattle HSP 70 protein sequences predicted protein structure appears similar. Glut-204, Asp-10, Asp-199, Asp-206 and Glu-175 are catalytically essential acidic residues in the active site region of the ATPase fragment of HSP 70 which were found conserved in buffalo and cattle HSP 70 protein as well as comparison of archaeal, eubacterial, eukaryotic, and organellar HSP 70 sequences identifies C-terminal half of the linker (bHSP 70 388-396, VQDLLLLDV) as a well-conserved element in the HSP 70s.

Biophysical properties

The consensus predictions for some biophysical properties; primary and secondary structures identified HSP70 protein obtained by DNASTAR (Madison, Wisconsin, USA) program (Figure 7).

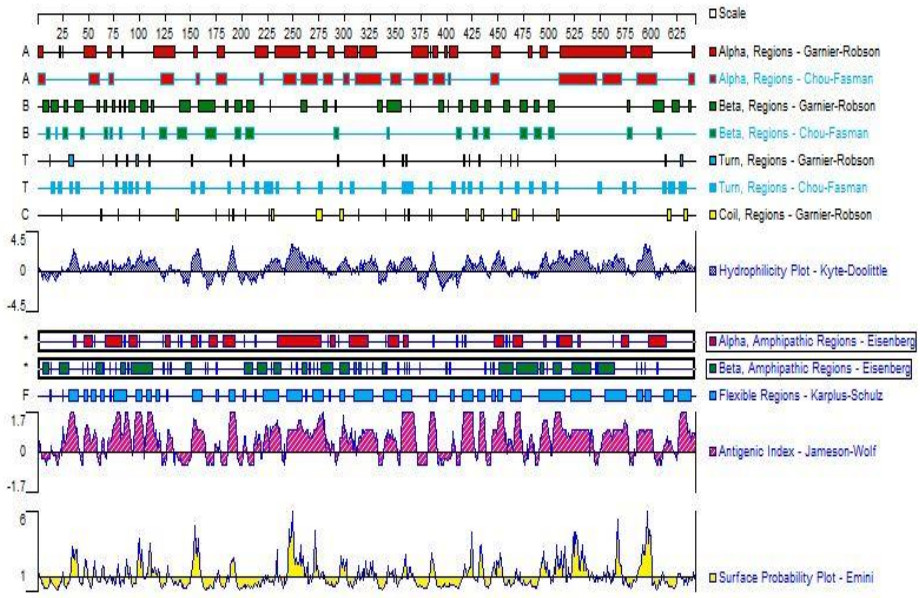


Figure 7 *The diagrams show the regions in the primary sequence of HSP70 protein of Bubalus bubalis containing predictions for primary and secondary structures (α-helix, β-sheet, turn or coil), hydrophilicity, flexibility, antigenic index, and surface probability.*

For amino acid sequence, the programs predicted tendencies for α-helix (40%) and random coil (60%) in its structure. This amphipathic α-helix is a feature of many peptide antibiotics from animals and plants [41]. The region is formed by cluster of negative/hydrophobic and positive/hydrophilic amino acids that displays a perfect helical wheel. The Jameson–Wolf method is well used in protein antigenic prediction [42-46]. The output of the former algorithm, the antigenic index, is used to create a linear surface contour profile of the protein, and it offers a reliable means of predicting potential antigenic determinants [27]. On the other hand, application of the latter to a large number of proteins has shown that the method can predict antigenic determinants with about 75% accuracy which is better than most of the other known methods [47].

Structural model and Overall Architecture of HSP70

X-ray resolved crystal structure of HSP70 from Bos taurus (PDB entry 2qw9) was available from Protein Data Bank (PDB). Based on the sequence alignment, Bos taurus HSP70 was used as the most appropriate template structure for homology modeling of the target sequence. The comparative 3D-structure model of B. bubalis HSP70 was generated by homology-modeling program Swiss-Model (Figure 8).

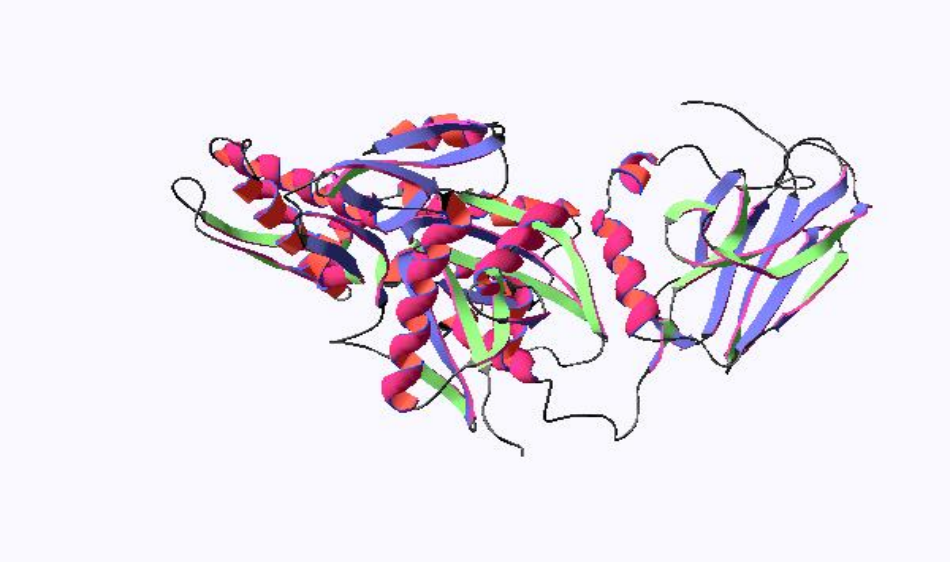


Figure 8 *Predicted protein structure of Buffalo HSP70*

The predicted model of buffalo HSP70 depicted in the form of ribbons is composed of fourteen α-helices and thirteen β-strands. The Ramachandran plot indicates the different conformations of the possible angles of the predicted B. bubalis HPS70, indicting its good quality (figure 9).

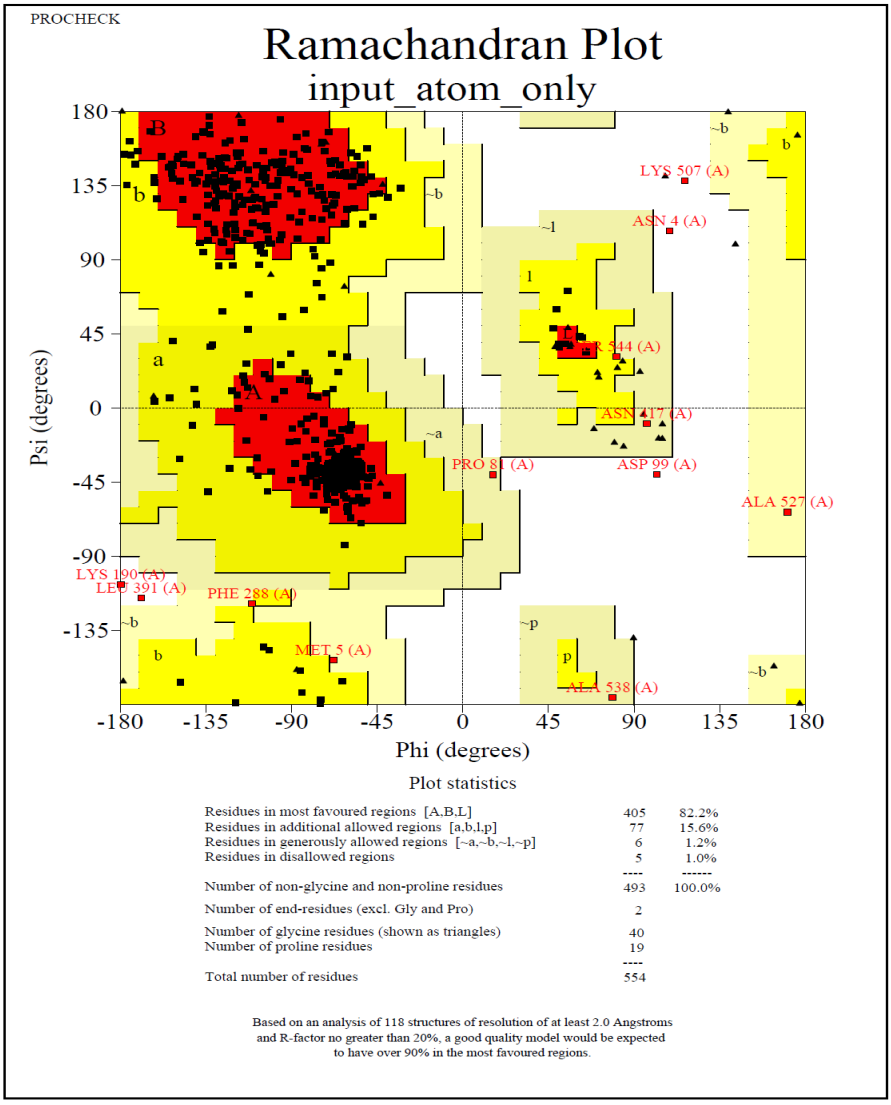


Figure 9 *Ramachandran plot of the predicted model of Buffalo HSP70 protein*

The main chain and side chain parameters for all of them were found to be concentrated/ convoluted in the 'better' region.

To define a model reliable, the score for G-factor (a log odds score based on the observed distribution of stereochemical parameters such as main chain bond angles, bond length and phi-psi torsion angles) should be above -0.50. The observed G-factor score for the present model was -0.06 for dihedrals bonds, 0.31 for covalent bonds and 0.09 overall. The distribution of the main chain bond lengths and bond angles were 99.6% and 98.5% within the limits, respectively. The modeled structure of Bubalus bubalis is comparable to the structurally resolved Bos taurus, wherein structural motifs have been identified to remain conserved. A superimposition of the Bubalus bubalis HSP70 onto the Bos taurus monomer shown in Figure 10.

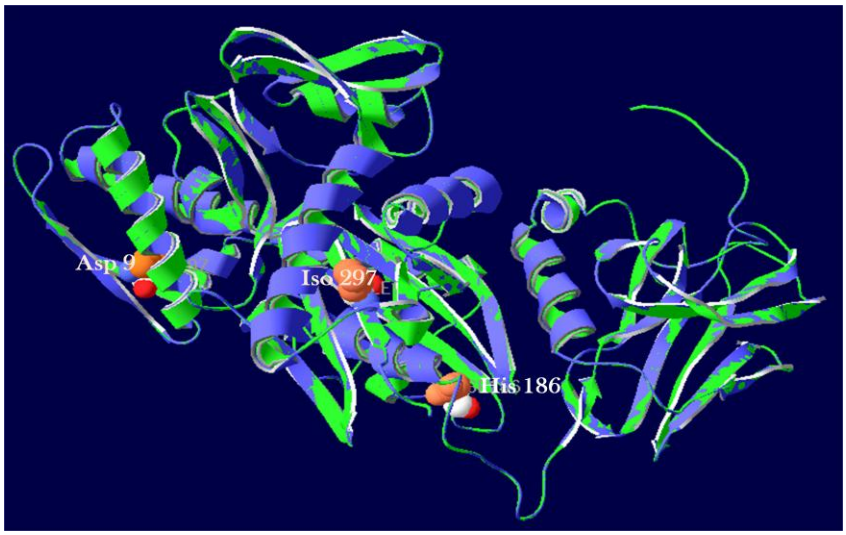


Figure 10 *Superimposition of homology modeled structure of Buffalo HSP70 onto Cattle HSP70 monomer (Blue coloured protein of Buffalo superimposed on green coloured cattle protein and orange coloured balls shows dissimilarity of amino acids at 9, 186 and 297 position)*

CONCLUSION

Bubalus bubalis heat shock protein 70 (HSP70) gene encoding 641 amino acid residues was found highly conserved among the domestic animals. The study of HSP70 gene described here may open the way to disclosure of functional characteristics and assessment of possible role in physiology.

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Conflict of interest statement:

The authors declare that they have no conflict of interest.

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