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In silico characterization of heat shock protein HSP27 in Mammals

[a] Samar Bano, [a] Lubna Rasool *, [a] Abdur Rauf, [b] Shabbir Hussain

[a] Department of Chemistry, University of Sahiwal, Sahiwal, Pakistan,

[b] Khwaja Fareed University of Engineering and Information Technology, Rahim Yar Khan, Pakistan.

Authors' Contribution: **Bano, S.,** conducted the preliminary research work, swiss-modeling, WoLF-PROST and reference management was performed by **A. Rauf**. **S. Hussain** performed TB tool and MEGA 7 analysis. Physiochemical characterization of protein and Protein- Protein Interaction Study was done by **L. Rasool.**

*Corresponding Author's Email Address: lubnarasool@uosahiwal.edu.pk

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ABSTRACT

Dairy animals have a significant role in every country's agricultural economy. Livestock is the major sub-sector of the agricultural industry and the value of dairy products is increasing globally. Under pathological circumstances heat shock proteins (HSPs) promote protein repair, folding, refolding of misfolded peptides, and possible breakdown of irreparable proteins. The heat shock protein 27 (HSP27) is a member of the small heat shock protein family (sHSPs) also called HSPB1. The HSP27 is crucial in the regulation and progression of different types of tumors and also acts as an oncotherapeutic target in humans and other mammals especially sheep. By using computational tools, a detailed *in silico* investigation of HSP27 sequences from humans and sheep has been examined with respect to their structural, functional, expression and phylogenetic properties. An extensive study has been made to compare the human protein with that of other mammals using computational tools. However, the sequences of HSP27 in humans and sheep are closely linked with each other. Although the sequences of HSP27 vary, their structural characteristics and functional properties remain the same. In this study, hydrophilic proteins with an average molecular weight of 27kDa were discovered both in humans and sheep. This study helps to understand the economic worth of dairy animals worldwide. HSP27 phosphorylation has been predicted as an anticancer agent. Recent research shows that new strategies have been developed for cancer treatment based on therapy of HSP27. This research will highlight the characterization and significant role of HSP27 in sheep.

Keywords: Physiochemical properties, motif analysis, phylogenetic analysis, SWISS modeling, protein structure analysis.

INTRODUCTION: Dairy animals play a significant role in subcontinent's agricultural economy due to their capacity to adapt to challenges climatic circumstances, their resistance to tropical diseases, and their ability to endure inadequate feeding and management techniques. The demand for dairy products is increasing globally. According to Precedence Research, in 2021 the global dairy product market size was valued at 481.7 billion US dollars. Pakistan is also an agricultural country and livestock is major sub-sector of agriculture with a 61.9% contribution to the growth agriculture sector and 14.04% to the national GDP during 2021-22 (Tariq, 2021). The two main items in the livestock sector are milk and meet, and demand for livestock products is increasing quickly (Rehman *et al.*, 2017). In Pakistan, milk is the most significant agricultural product and is referred to as the "white gold"(Shahzad, 2022). Pakistan is the eleventh largest producer of sheep in the world, with 28 well known sheep breeds (Saleem *et al.*, 2018). Small ruminants are required to produce meat and dairy products in dry locations. One of the most significant domestic animals in terms of economy, culture and society is the sheep *(Ovis arise)* (Abousoliman *et al.*, 2020). Sheep play a significant role in the agricultural economy, particularly in these hilly regions, because to their many uses for wool, meat, milk, skins, and dung. The agricultural industry is seriously threatened by the rising demands of dairy products and frequently hot weather. The most pressing issue at the moment, given the constantly shifting weather conditions is heat stress. Heat stress causes a reduction in natural immunity, development, reproduction, production, milk quality and quantity and growth, leading animals more susceptible to illness and even death (Jaiswal *et al.*, 2019). Heat stress in cells gives rise to the synthesis of specific proteins called heat shock proteins (HSPs) (De Miguel *et al.*, 2009). These HSPs work to prevent or counteract denaturation or unfolding of cellular proteins in response to stress or temperature (Zhang *et al.*, 2021). Cells produce heat shock proteins (HSPs)in response to stressor such as high or low temperature, anoxia, UV light, hypoxia, toxins exposure, chemical agents, and other hemostatic disturbances that result in protein denaturation (Chatterjee and Burns, 2017). This response mechanism is the most highly conserved genetic system known, appearing in all known creatures from archaebacteria to eubacteria, from mammals to plants. Ritossa initially identified heat shock proteins (HSPs) in the salivary glands of Drosophila chromosomal larvae in 1962 (Afzal *et al.*, 2022). Since then, have been widely identified in prokaryotic and eukaryotic cells (Jagla *et al.*, 2018). According to the molecular weight, HSPs are divided into following families: small HSPs (sHSPs), HSP40, HSP60, HSP70, HSP90 and massive HSPs. HSPs can range in size from 10 to 110KDa. When small HSPs like HSP27 are being discussed, another tiny molecular chaperone with significant heat-shock-like characteristics is clusterin, which is frequently mentioned (Ischia and So, 2013). Proteins play important role in homeostasis and thermos tolerance and they also play significant role in cell proliferation, drug resistance and tumorigenesis also act as chemotherapeutic agent to diagnose cancer. A tiny specific heat shock protein is called HSP27 (sHSP). There are 10 members of mammalian sHSP family, also called the HSPB family. HSP27 is a member of the family of ATP-independent chaperon and is encoded on the HSPB1 gene and is present in both the nucleus and cytoplasm (Fuller, 1994) metastasis of cancer and cell apoptosis and its overexpression is associated with disease prognosis (Shiota *et al.*, 2013). High expression levels of HSP27 result in many cancers including breast cancer, lung cancer and skin cancer in human and according to a recent report HSP27 causes skin cancer in sheep and acts as potential biomarker of mammary neoplastic in sheep. It was demonstrated that HSP27 overexpression facilitated recovery from heat- induced nuclear protein aggregation, indicating that HSP27 was partially in charge of subsequent cell survival (Kokolakis *et al.*, 2008). HSP27 has been recognized as a key oncotherapeutic target, due to its anti-apoptotic and cytoprotective functions against chemotherapeutic medicines (Heinrich *et al.*, 2016).

OBJECTIVES: Initially biophysical and biochemical properties, expression of HSP27 in different cell organelles was carried out. Furthermore, Swiss modeling, protein proteins interaction studies and phylogenetic evolution were studied.

MATERIALS AND METHODS: **Physicochemical properties analysis:** The DNA, CDS and Heat Shock protein HSP27 sequences for sheep and humans were obtained from National Center for Biotechnology Information (NCBI) protein database. The corresponding sequences of all the selected organisms such as Humans (Homo Sapiens), Domestic Sheep *(Ovis Aries)*, Arabian Camel *(Camelus Dromedarius)*, Cattle *(Bos Taurus)*, Goat *(Capra Hircus)*, Buffalo *(Bubalus Bubalis)* were retrieved using Basic Alignment Search Tool (BLASTp). Accession numbers and no of amino acids of the following proteins are displayed in table 1.

Organisms name	GenBank accession number	No of amino acids
Homo Sapiens (Human)	AAA62175.1	199
Ovis Aries (sheep)	XP_027817273.1	201
Camelus Dromedarius (Camel)	XP_031288550.1	201
Bos Taurus (Cattle)	NP_001020740.1	204
Capra Hircus (Goat)	AFK93550.1	183
Bubalus Bubalis (Buffalo)	XP_006057191.1	201

Table 1: No of amino acids and accession numbers of HSP27 protein.

Primary structure prediction: Expert Protein Analysis System (EXPASY) is a proteomic server of Swiss Institute of Bioinformatics (SIB) was applied by Protparam to identify the primary structural

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analysis of HSP27 in humans and sheep (Gasteiger *et al.*, 2005). The physicochemical properties include molecular weight (Mw) composition and number of amino acids, theoretical isoelectric point (PI), negative and positive charged residues, absorbance, extension coefficient (EC-quantitative study of protein- protein interactions) (Gill and Von Hippel, 1989), instability index (Guruprasad *et al.*, 1990), aliphatic index (AI-how much volume of protein covered by aliphatic side chains) (Ikai, 1980), grand average hydrophobicity (GRAVY-hydrophobic and hydrophilic properties of each amino acids of all the protein sequences) (Kyte and Doolittle, 1982), half-life and expression of HSP27 in different organelles.

Secondary and tertiary structure analysis: The PSIPRED 4 server was employed to estimate the secondary structures of protein sequences of humans and sheep HSP27 (Jones, 1999). Secondary structure parameters such as alpha helix, beta bridge, beta turn, extended strand and random coil are thoroughly studied by a software called SOPMA which also gives percentage composition of secondary structure parameters. Swiss Model software was utilized to predict tertiary structure of HSP27 protein and the validation of this model was checked via Ramachandra's plot (Waterhouse *et al.*, 2018).

Motifs and domain analysis: To identify motifs in the protein sequence of all the organisms, a tool called MEME suite was used (Bailey *et al.*, 2006). Inter-ProScan tool (Jones *et al.*, 2014) was used to identify family membership in protein sequences, presence of functional sites and domains within all the organisms.

Phylogenetic analysis: Phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis (MEGA) version 7. This analysis helps to recognize the evolutionary relationship among organisms. Phylogenetic tree was constructed by applying maximum likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 6 amino acid sequences. All positions contain gaps and missing data were eliminated.

Protein-protein interaction study: The STRING database server was utilized to forecast the interaction of human and sheep HSP27 proteins with other proteins closely related proteins respectively. Based on the direct and indirect linkages for both the proteins, a critique of the protein-protein interaction was created (Szklarczyk *et al.*, 2015).

RESULTS: Percentage genetic homology: The HSP27 protein of humans and sheep has similarities with other species which was determined by the Basic Local Alignment Search Tool (BLASTp) online server. The HSP27 of humans was revealed to be 89%, 89%, 88%, 88%, and 88% identical to goats, sheep, camels, cattle, and buffalo, respectively. In the same way, HSP27 of sheep also has % similarities such as 89%, 99%, 95%, 99%, and 98% to humans, goats, camels, cattle and buffalo. The highest percent similarity of human protein was with sheep and goat and sheep protein resembled with goat and cattle.

Analysis of primary structure: Human HSP27 has 199 amino acids, sheep HSP27 has 201 amino acids, goat HSP27 has 183 amino acids, camel HSP27 has 201 amino acids, cattle HSP27 has 204 amino acids, and buffalo HSP27 has 201 amino acids (table 1). The amino acids composition of the HSP27 protein of humans, sheep and other species which was analyzed by the Protparam online server is shown in table 1. Heat shock protein 27 (HSP27) of human and sheep with distinct physicochemical characteristic are shown in table 1. Theoretical pi (isoelectric point) of a molecule is a pH having net charge zero and is electrically neutral. A buffer for purification and crystallization of proteins can be chosen with the help of PI. Those proteins whose pH value is higher than PI are negatively charged while positively charged proteins show their pH lower than PI. The theoretical pI of the human HSP27 protein was 7.83 which indicates that it is basic in nature while pI for the sheep HSP27 protein was 6.22 which shows that it is below 7 and mild acidic in nature. Instability index of proteins higher than 40 indicates that these proteins may be unstable while instability index lower than 40 showed that these protein mat be stable in nature. The values of instability index for both the proteins. For both the HSP27 proteins, the value of instability index was higher than 40 which reveal its unstable nature. The relative volume of proteins covered by aliphatic side chains is known as aliphatic index. Structural and thermal stability of proteins depends on the aliphatic index values and aliphatic amino acids are hydrophobic and non-polar in nature. Aliphatic index was higher than 50% which reveals that 50% volume of the selected protein was occupied by aliphatic amino acids. The grand average of hydropathy (GRAVY) value represents solubility of proteins and thoroughly described the hydrophilic and hydrophobic properties of each amino acid chain. Proteins that are hydrophobic are indicated by a positive value while hydrophilic proteins are indicated by a negative value. The GRAVY score for all the HSP27 proteins is negative which indicates that both proteins are hydrophilic in nature and the estimated half-life of each protein is around 30 hours. HSP27 is also expressed in different organelles as in mitochondrial matrix or mitochondrial inner membrane, cytoplasm, nucleus, peroxisome, cytoplasm-nucleus and also secreted in extracellular matrix as in figure 1.

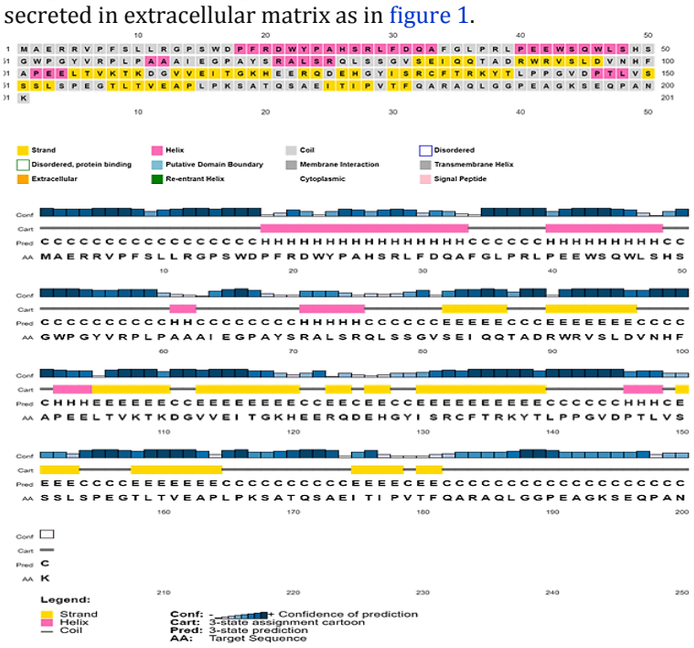

Figure 1: Secondary structure prediction of sheep Hsp27 protein using PSIPRED.

Secondary and tertiary structure prediction: Using the PSIPRED and SOPMA online servers, a thorough prediction of the secondary structure of the amino acid sequence of humans and sheep HSP27 and the percentage composition of these proteins were determined. The examination of the comparative structures of all the organisms' HSP27 proteins revealed that random coil content predominated, followed by strand, and then helix. Secondary structure of humans and sheep HSP27 protein is shown in figures 2 and figure 1 respectively.

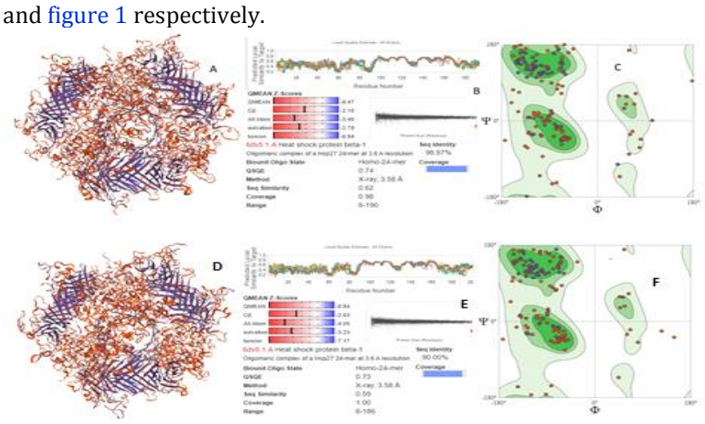

Figure 2: The 3D Structure of human HSP27 protein (A), Swiss modeling of human HSP27 (B), structure validation by Ramachandran Plot (C), 3D Structure of sheep HSP27 protein (D), Swiss modeling of sheep HSP27 (E) and its structure validation by Ramachandran Plot (F)

The SWISS-MODEL was used to estimate the tertiary structures of the HSP27 proteins. To predict the three dimensional structure of proteins, a template sequence that shares a lot of similarities with the query sequence is needed. A crystal structure of heat shock protein beta 1, 6dv5.1, was the template sequence chosen for this study. For the human model, the sequence identity between the query sequence and the template sequence was 98.97% while for the sheep model it was 90%. The oligomeric complex of both the human HSP27 protein and sheep HSP27 protein model was homo-mer-24 at 3.6 A resolution. A high quality model for humans *(Homo Sapiens)* and sheep *(Ovis Aries)* was chosen using QMEAN and Z scores in figure 2. Ramachandran plot analysis showed that 75.40% of the amino acids were in favor for the human HSP27

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model while for the sheep HSP27 model 74.49% of the amino acids were in favor as shown in figure 2 (C).

Domain analysis: Inter-ProScan is the basic online tool used to identify protein families, functional sites and domains of HSP27 in all the dairy animals. Alpha crystallin and small heat shock protein 20 were predicted by Inter-ProScan which belongs to the same family of proteins. The protein of selected organisms belongs to the same family with accession ID IPR001236 and the same homologous superfamily containing accession ID IPR008978. There were two DNA building domains in all the organisms with accession numbers IPR037876 and IPR002068.

Motif study: A region or sequence of protein or DNA that has a specific structure is known as motif. Motifs are also called as transcriptional building sites of a protein sequence which help for studying regular structure of gene. The presence of motifs is the indication of their potential roles as some of them have been recommended to work as locating nuclear signals that aid in phosphorylation of proteins. By using P- and E- values, the statistical significance of motif locating is determined. The statistical implication and the corresponding occurrence of the motifs are shown by E-value and p-value provides an estimation of close resemblance of their occurrence. By meme suit tool, it is observed that Motif 1, 2 and 3 are present in all the selected organisms. Motif 4 is found in *Ovis Aries*, *Bos Taurus, Bubalus Bubalis* and *Camelus Dromedarius* while Motif 5 is present in only two organisms like *Homo Sapiens*, and *Bos Taurus*. Motif 6 is found in only 2 organisms as *Homo Sapiens* and *Bubalus Bubalis*. Motif 1,2 and 3 are common in HSP27 of humans and sheep. Motif 4 is found in sheep only while motif 5 and 6 are present in humans only. P values of each protein sequence with their start and end point are shown in table 2 and E values are shown in figure 3.

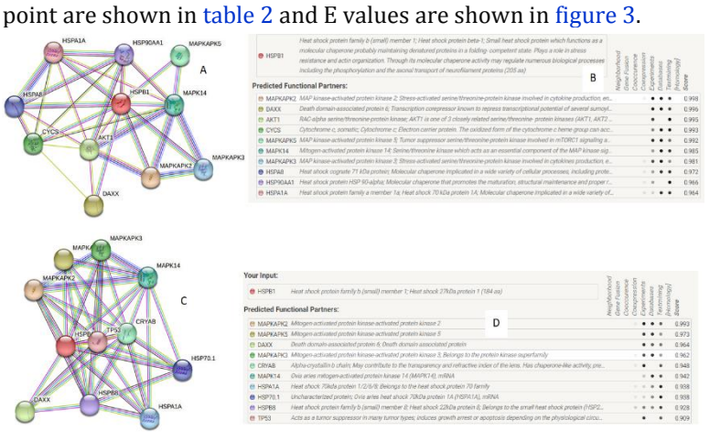

Figure 3: Tertiary structure of humans and sheep HSP27 protein (A) & (C) with Screenshot of interacting proteins with the query sequence from STRING database (B) & (D) respectively.

Phylogenetic analysis: The evolutionary history was deduced by using maximum likelihood method based on the JTT matrix-based model. The tree is drawn to scale with branch lengths measurement. The analysis involved 6 amino acid sequences. All positions contain gaps and missing data were eliminated. There were a total of 182 positions in the final dataset. By using MEGA 7 software, a phylogenetic tree was created having two clades. According to phylogeny, human HSP27 showed more resemblance with sheep and camel sequences while sheep HSP27 was more similar to goat, cattle and buffalo sequences as in figure 3. No doubt sheep HSP27 sequence is forming independent clade with goat, cattle and buffalo but it also shows similarity with human as its clade is originating from human's clade.

Protein–Protein interaction study: In general, protein works by interacting to form protein complexes. Various parameters such as gene fusion, gene co-expression, co-occurrence, neighborhood, text mining, protein homology and database were used to create protein interaction with 10 possible interacting protein companions for HSP27 of human and sheep. The nearest interacting protein for human HSP27 was MAP kinase-activated protein kinase 2 (MAPKAPK2), death domain-associated protein 6 (DAXX) and MAP kinase- activated protein kinase 5 (MAPKAPK5) and MAP kinase-activated protein kinase 3 (MAPKAPK3) and estrogen receptor was the indirect associated proteins. For sheep HSP27, the nearest interacting protein was MAP kinase-activated protein kinase 2 (MAPKAPK2), death domain-associated protein 6 (DAXX) and MAP kinase- activated protein kinase 5 (MAPKAPK5) while MAP kinase-activated protein kinase 3 (MAPKAPK3), MAP kinase 14 (MAPK14) and HSP70.1 are the distinct interacting protein.

DISCUSSION: The Heat shock protein 27 (HSP27) is a member of small heat shock proteins family (sHsps) also known as heat shock protein beta 1 (HSPB1). It maintains stable condition during stress conditions like hypoxia, high temperature, toxin exposure and oxidative injuries. It plays a vital role in tumor progression and also acts therapeutic agent for cancer treatment. This is a computational based study of HSP27 in humans and sheep. Dairy industry is under threat due to diseases and hot weather. Demand of dairy products is also increasing day by day (Abousoliman *et al.*, 2020). Different tasks were performed to explore role of HSP27 protein, discover physiochemical properties, pick out motifs and domains, generate phylogenetic tree, identify secondary structure parameters and percentage similarity among organisms, discover protein interaction with other proteins and also build 3D models of human and sheep along with their Ramachandran plots. The physicochemical properties include molecular weight, composition and number of amino acids, theoretical pi, absorbance, extension coefficient (Gill and Von Hippel, 1989), instability index, aliphatic index, GRAVY, half-life and expression of HSP27 in different organelles. The isoelectric point (pI) described the acidic and basic nature of the HSP. Theoretical PI value of *Homo sapiens* was above 7 which indicate basicity in this protein while pi for sheep was below 7 and mild acidic in nature. Both have prolein and leucine rich amino acid and sulphur containing amino acid methionine and cysteine were recorded in least quantity. Aliphatic index of both proteins were greater than 50% which indicates hydrophilic protein. Similar results were seen in literature with HSP27 (Kyte and Doolittle, 1982). Solubility of protein depends upon the GRAVY value. Similar results have been reported for solubility of protein and localization (Tripathy *et al.*, 2021; Amare and Kebede, 2023). Molecular chaperone have recognized as poly peptide protein stabilizer during their transport into subcellular organelle mitochondria to cytoplasm of the cell. Therefore, the subcellular localization of various forms of sHSPs was showed the major role in housekeeping of protein functions (Young *et al.*, 2004; Craig, 2018). Computational tool was used to find the localization of HSPs in cell organelles due to insufficient experimental data. Cytosolic, mitochondria and chloroplast HSPs each have distinct functions (Waters and Rioflorido, 2007). The current studies showed that the localization of the HSP27 more in mitochondria. It has been reported that the HSPs show different gene expression in different tissues (Tripathy *et al.*, 2021). Alpha crystalline (IPR037876) and ACD_HspB1(IPR002068) are two domains found in protein sequences of both humans and sheep. Homologous superfamily HSP20 (IPR008978) and family Alpha crystallin/ sHSP animal (IPR001436) are also common. Motif analysis predicted that all organisms' protein sequences have 3 common motifs. Motif 4 is found in all organisms except *Homo sapiens* and *capra hircus*. Percentage similarity among selected organisms' protein sequences was analyzed by BLASTp while in other article, it was visualized by jalview. Human's proteins showed greater percentage similarity with goat and sheep showed. Greater percentage similarity among sheep and humans indicates more similarity in their structure and functions. The evolutionary history was deduced by using maximum likelihood method based on the JTT matrix-based model (Kumar *et al.*, 2016). Hsp27 sequence is forming out group with others goat, cattle and buffalo. The examination of the comparative structures of all the organisms' Hsp27 proteins revealed that random coil content predominated, followed by strand, and then helix. Tertiary structures of human and sheep, HSP27 proteins are developed by Swiss model. For the selection of final model several parameters are considered like GMQE, QSQE, sequence identity, coverage, range, sequence similarity, Qmean value and Ramachandran's plot (Garnier *et al.*, 1996). QMEAN value of both models of sheep and humans has thumbs up sigh revealing that these are good models. According to string database, Hsp27 of sheep and humans form complexes through protein interactions. Various parameters such as gene fusion, gene co-expression, co-occurrence, neighborhood, text mining, protein homology and database were studied to explain interaction between Hsp27 proteins and other interacting protein. In another study HSP27 protein for humans and canines (Afzal *et al.*, 2022) has been reported in which different tasks were performed like physiochemical analysis, secondary structures prediction, 3D structure. Current study has additional analysis such as expression of Hsp27 in different organelles, motif analysis with

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P and E values; find homologous superfamily and domains, check hydrophilic and hydrophobic characters by hydrophobicity plot.

CONCLUSION: Heat shock proteins are the most evolutionary conserved class of proteins whose primary functions are cell protection from damage due to various factors like heat, cold, hypoxia and UV light exposure. These proteins also maintain cellular functions by assisting in the folding and remodeling of various nascent and non-native proteins. HSPs play an important role in tumorigenesis, cardiovascular and infectious diseases. They may be used as clinical biomarkers for cancer diagnosis and prognosis and as therapeutic targets. However, the most abundant and dispersed group of proteins among them are the small heat shock proteins that have protective role in many stresses. Heat Shock Protein 27 (HSPB1) plays an important role in tumor invasion and cell apoptosis and has been implicated as therapeutic targets in human and other dairy mammals like sheep and cattle. For better understanding the structural and functional properties of the proteins, several computational tools were utilized to discover physiochemical properties, pick out motifs and domains, generate phylogenetic tree, identify secondary structure parameters and percentage similarity among organisms, discover protein interaction with other proteins and also build 3D models of human sheep along with their Ramachandran plots. Through above analysis, it is predicted that human and sheep have maximum sequence similarity with each other as they have many common features like same motifs and domains. A computational comparative analysis of human and sheep HSP27 protein revealed that the protein is more conserved, flexible, unstable and hydrophilic having molecular weight of 27KDa and homology in their functional and structural properties reveals that they have common features. Future research on HSP27 explored that it can be helpful in therapeutic and diagnostic purposes assisting both medical and animal experts.

CONFLICT OF INTEREST: Authors have no conflict of interest.

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