Volume Number 10 || Issue Number 3 || Year 2025 || Page 21 || * Corresponding Author: shaistaghumro007@gmail.com ||

ISSN (Online) = 2522-6754 ISSN (Print) = 2522-6746

WORLD JOURNAL OF BIOLOGY AND BIOTECHNOLOGY

Research Manuscript www.sciplatform.com Peer review

Genetic diversity of the BMP-4 gene and associated amino acid variations in indigenous Bari goats of Khairpur Mir's, Sindh, Pakistan

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Contribution | **Shaista Ghumro, S., and S. A. Jakhrani** contributed equally

Bone morphogenetic protein 4 found in goats, cattle, and humans and is encoded by the BMP4 gene. BMP4 is found on chromosome 10q in goats. BMP4 gene is the subfamily of the superfamily transforming growth factor beta. It is an evolutionary conserved member of the BMPs family. BMP-4 is a protein coding gene that concerns with connective and soft tissues of the body structure. It plays main role in goats, cattle, human and other animal's bodies for maintaining the body activities such as cell proliferation, differentiation, apoptosis, and migrations. The aim of this study was to investigate the novelty of single nucleotide polymorphisms (SNPs) in the BMP-4 gene and their potential impact on body growth and development. Blood samples were collected from the Animal Hospital in District Khairpur using sterile techniques under hygienic conditions. The samples were then transported on dry ice in a cooling box to the Molecular Genetics Laboratory, Department of Zoology for further analysis. Using a commercial DNA extraction kit, DNA was extracted from whole blood samples in the first step, and the results were displayed on gel electrophoresis. The PCR results were then sent for DNA sequencing in order to identify SNPs. According to the analysis, the Bari goat breed has roughly 19 mutations. The different base pair (bp) was where these mutations were found. While genetic codon changes result in the conversion of non-essential amino acids into essential amino acids, which improves the quality and quantity of milk and meat, this is a very positive sign. The milk of Bari goats was used for analysis and the results revealed the highest percentage of essential amino acids was Leucine 3.15% while the lowest Methiodine 2.40%. While in non-essential amino acids the highest percentage was Glutamic acid 2.98% and lowest was Glycine 1.01%. According to the results the Bari goat breed can be inferred from breed admixture and genetic markers can help with breeding selection.

Keywords: DNA Extraction, PCR, DNA sequencing, Point Mutations, Amino acids

INTRODUCTION: Goat (*Capra hircus*) is a domesticate animals and also found in wild areas. The goats are the members of the Bovidae family, tribe *Caprini*, it is closely associated with the sheep species. More than 300 distinctive breeds of goats have been named (Hirst, 2008). Bari goat breed is also called Bar-bari goat breed. It is a small goat breed found in the Pakistan province of Sindh such as Hyderabad, Dadu, Larkana, Khairpur, Nawabshah and Jacobabad. It is a very beautiful breed that looks like a deer by its face and has attractive eyes. They are medium sized animals with compact body form. They are pure white colored bodies with small brown, red spots. They have short ears, small face, and small hair on the body. Their reproduction rate is slow and their gestation period is also very low because they give very few progenies. Their average weight ranges from 24 to 29kg. They are fitted to the domesticated household, dual for meat and milk purpose, but they are also used for their amusement and fancy. The milk yield is approximately 107 liters in a lactation of about 150 days. It is a seasonal breeder and is used for intensive farming (Barbari, 2017). BMP-4 means Bone Morphogenetic Protein. It is an evolutionary conserved associates of the BMP's family, it belong to superfamily of transforming growth factor beta (Mangino *et al.,* 1999). BMP-4 gene is considered a master gene that controls many aspects of development in all species, including palate production, sight development, and various others. Many regulatory genes, such as BMP-4 and hox genes are conserved in this way. Orofacial cleft and Microphthalmia diseases have occurred due to the two mutations appeared that are concerning with this gene protein in humans and animals. The encoded protein also performed a main part in pathophysiology with a number of cardiovascular illnesses and malignancies in humans (Oida, 1995). Like other bone morphogenetic proteins it is involved in the formation of connective tissue development, particularly osteo-inductive expansion, as well as the repairing of bones. They also serve a crucial function in human endochondral bone development. Muscles growth, bone mineralization and ureteric buds formation (Oida, 1995). Amino acids were made up of multiple different kinds of elements. Protein aids in the formation of bodily tissues, growth, and the formation of many bioactive substances. Analysis of amino acid can be found in many areas of research and most important purpose is to assess their nutritional values of food and other organic products (Cao, *et al.,* 2021). Developed countries including Norway peoples are willing to pay more than 50 % for best quality of meat with essential amino acids and 25% for a better percentage of essential amino acids because it will be considered as the second best and healthy food. Recently, the profiles of amino acids are used for the assessment of certain diseases including lung cancer by the differentiation between cancerous and adjacent normal tissues (Liu, *et al.,* 2020).

OBJECTIVES: To analysis the amino acid profile from the milk sample of the Bari goat breed of Khairpur District Sindh Pakistan.

MATERIALS AND METHODS: **Ethics committee approval:** Ethics approval for this study was obtained from Animal Hospital of District Khairpur as well as Departmental permission from Shah Abdul Latif University Khairpur. Consent for sample collection was also secured from the relevant veterinary authorities and animal owners where applicable. The study complies with the ARRIVE (Animal Research: Reporting of in Vivo Experiments) guidelines to ensure transparency and reproducibility in animal research (Percie *et al.,* 2020).

Animals and sample collections: Approximately, thirty blood samples were collected from indigenous Bari goat breed from Animal's Hospital of district Khairpur Mir's by applying careful sample techniques. The age of breed was identified by using dentition method and most of goats were about 1 to 2 years old. About (10mL) of sterilized disposable syringe had been used for taking the blood from goat's jugular vein of each animals. Subsequently, the blood from the syringe was transferred into 250uL (0.5M) EDTA (ethylenediaminetetraacetic acid) tubes that protect the blood samples from anticoagulation. After that EDTA tubes were transported into a cool box which contains dry ice, then preserved the blood samples into the fridge at -20°C for further process of DNA extraction at the Molecular Genetic laboratory Department of Zoology, Shah Abdul Latif University Khairpur Mir's Sindh, Pakistan.

Extraction of deoxyribonucleic acid: Extraction of Deoxyribonucleic acid (DNA) from collected blood samples by using of MQ Blood Genomic DNA Extraction Kit (MOLEQULE-ON® (MQ-Kit), followed the manufacturer's standard protocol. The procedure involved cell lysis, removal of proteins and other contaminants and subsequent binding of DNA to the silica membrane column. After a series of wash steps, high purity DNA was eluted using the provided elution buffer.

Quantification of DNA: Nanodrop™ 1000 Spectrophotometer (Thermo Fisher Scientific) was used to quantify extracted DNA at the "Jamil-Ur-Rahman" Center for Genome Research at Karachi University, Sindh Pakistan. Quantification of DNA had been done to ensure the presence of DNA is extracted samples from blood, which could be used for further processing of amplification. Furthermore, purity of DNA was measured by using the ratio of 260/280nm absorbance (absorbance calculated by taking 260nm divided by absorbance taken at 280nm). DNA is regarded as pure if it had reading between 1.7-1.8 (MacNeil and Newman, 1994; Sadaf *et al.,* 2019; Majida *et al.,* 2021).

Primer design and synthesizing: Initially, BMP-4 gene sequences have been used to retrieve for the formation of primers designing on the website of National Center for Biotechnology Information (NCBI). Primers Forward (5'ACCACGAAGGTCAGTCCCTA 3') and reverse primer (5' TCCCCAGCGATCTTGGAAAC 3') obtained from particular website and blast was again through NCBI website for

checkered specificity. Primers which were handpicked for amplification of PCR and their application were commercially synthesized from Bionics Company Islamabad, Pakistan.

The PCR amplification and confirmation of BMP4 gene: The reagents included into mixture were genomic DNA template (5uL), absolute red PCR Master Mix (7uL) from MOLEQULE-ON® Company, forward primer (2uL), reverse primer (2uL), PCR grade water or molecular DDH2O (4uL). Consequently, specific tubes of PCR were retained in Thermal Cycler Machine (Bio Rad T-100) for Amplification of PCR. PCR reactions were accomplished through already issued procedure defined by (Zhang *et al.,* 2007). The PCR protocol began with an initial denaturation at 95°C for 5 min., followed by 35 amplification cycles consisting of denaturation at 94°C for 30 sec., annealing at 61-65°C for 30 sec., and extension at 72°C for 30 sec. A final extension step was carried out at 72°C for 9 min. PCR amplified products were visualized on 1.5% of agarose gel run for 60 min. at 70 volts. A 1000 kb DNA ladder was loaded into the first well as a molecular size marker, while the remaining wells contained the DNA samples mixed with loading dye. The gel was documentation system using a Bio-Rad gel documentation system equipped with ultraviolet illumination.

Data analysis, purification and sequencing: The amplified product of PCR samples were send to Macrogen, company, Korea for purification, sequencing and data analysis. DNA sequence's data were examined by using of available genome browser Ensemble.org and blast the interrogated alignment of BMP-4 gene of nominated goat varieties by using of alignment sequence tools (Smigielski *et al.,* 2000; Sadaf *et al.,* 2019; Majida *et al.,* 2021).

Amino acid profiling: Amino acid profile had been done on Bari Goats breed in which 200 mL of milk sample was taken and transferred to the commercial company named as Gene Janch Biotechnology, Karachi Sindh Pakistan. Amino acid analyzer is procedure which based on ions exchange liquid chromatography used in a wide range of areas and provide several of application in the form of qualitative and quantitative compositional analysis of protein. Amino acid analysis was carried out to analysis of essential, semi essential and non-essential amino acids.

RESULT AND DISCUSSION: DNA quantification was carried out by using a Nano Drop spectrophotometer, which measured the concentration of DNA samples, ranging from 9.080 to 9.011ng/uL. The obtained quantities were sufficient for successful PCR amplification. The purity of the isolated DNA was also confirmedwith A260/A280 ratios ranging from between 1.70 and 1.81nm indicating acceptable levels of purity (table 1).

Sample ID	Breed Name	DNA Concentration (ng/uL)	DNA Purity (A260/A280)
S4	Bari goat	9.080ug/ml	1.81
S5	Bari goat	20.878ug/ml	1.78
S6	Bari goat	12.309ug/ml	1.70
S7	Bari goat	9.011ug/ml	1.70

Table 1: DNA quantification and purity analysis.

Gel electrophoresis and PCR amplification: Gel electrophoresis was used to visualize the amplified of PCR products of the BMP-4 gene from the selected goat breeds, confirming the expected product size of approximately 1000bp. The DNA ladder bands were loaded into first well of the gel, while the amplified samples were loaded from the second well onward (figure 1A).

Identification and sequencing of SNPs: The target gene's nucleotide sequences from each sample of goat breed have been analyzed by using mutation detector online software (ensemble.org). This procedure was used alignment of sequence tool or blast tool (figure 1B).

About 19 mutations were recognized in the Exon 1 region of BMP-4 gene in Bari goat breed. While the mutations based on the variant into genetic codon. In Bari goat breed (16) Missense Mutation, (2) Nonsense Mutation, (1) Deletion Mutation were found (table 2).

Amino acid profile: Analysis of amino acid was carried out by using of an automatic amino acid analyser for essential and non-essential amino acids. Experimental data were processed by using of software of SPSS.10 statistical program package. Statistical analysis was done by LSD-test and results were shown in mean ± standard deviation.

High milk production is one of a dairy farmer's top concerns. In addition to this milk composition features a new breeding goal has been established to meet the demands of a better diet (Krovvidi *et al.,* 2013). As a result, choosing farm animals with better dairy implementation is crucial for dual breeders and consumers. DNA markers are important in animal breeding programs. Genetic mapping and gene of all animal and plant generally has been transformed by DNA markers. SNPs also called "snips" are the extremely predominant variety of genetic variations found in mammals.

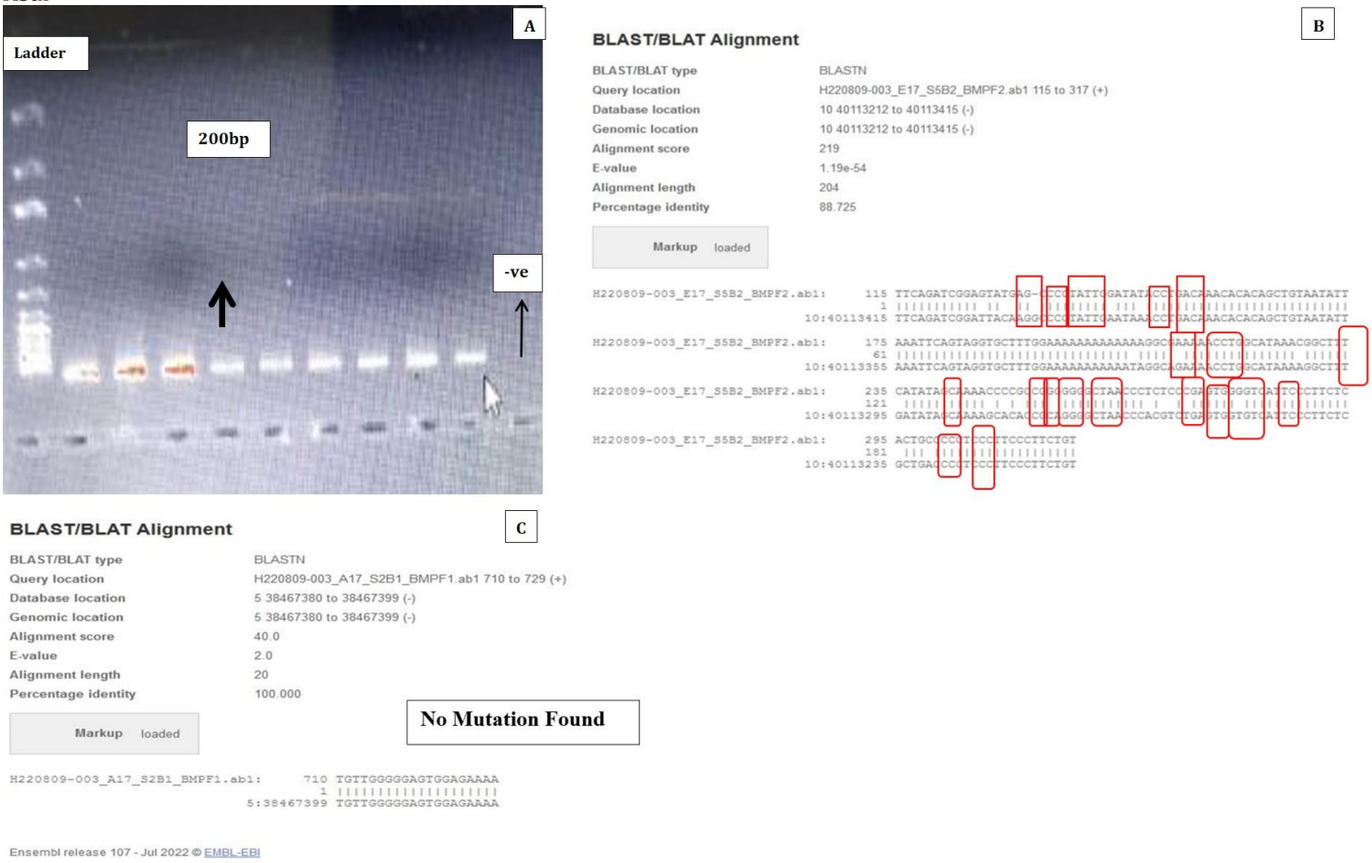

Figure 1: Amplified product of PCR (A), the Blast query of Primary data (B) and the BlAST query of primary data (C) of BMP-4 gene.

SNPs stand for single nucleotide polymorphism. The majority of the time, these modifications is found between genes. SNPs are recognized as biological markers that help researchers find genes associated with disease or genes involved in the expression of

characteristics. SNPs that are located inside or close to a gene's regulatory domain may affect how genes function more directly. These mutations (SNPs) may cause sickness or occasionally have good effects such as increasing the output of milk or beef (Dodgson *et al.,* 1997).

Sample ID	Nucleotide Position	Original codon	Mutant codon	Original amino acid	Mutant amino acid	Type of point mutation
	127	GTA	TTA	Valine	Leucine	Missense
	130	TGA	CCA	Stop Codon	Glutamine	Nonsense
S5B5	134	-CC	GCC	Deletion	Alanine	Missense
	144	GAT	AAT	Aspartic Acid	Asparagine	Missense
	148	TAC	AAC	Tyrosine	Asparagine	Missense

Table 2: Missense and nonsense mutations identified in Bari goat breed.

Sample ID	Nucleotide Position	Original codon	Mutant codon	Original Amino acid	Mutant Amino acid	Type of Point Mutation
	207	AAG	ATG	Lysine	Methiodine	Missense
	212	GAA	AGA	Glutamic Acid	Arginine	Missense
	229	ACG	AAG	Threonine	Lysine	Missense
S5B5	236	CAT	GAT	Histidine	Aspartic acid	Missense
	248	CCC	GCA	Proline	Alanine	Missense
	250	CCC	CAC	Proline	Histidine	Missense

Table 3: Missense mutations identified in Bari goat breed.

Sample ID	Nucleotide Position	Original Codon	Mutant Codon	Original Amino acid	Mutant Amino acid	Type of Point Mutation
	252	CGC	CAC	Arginine	Histidine	Missense
	256	GGG	CAG	Glycine	Glutamine	Missense
	269	CTC	CAC	Leucine	Histidine	Missense
	271	TCC	GTC	Serine	Valine	Missense
	274	CGA	TGA	Arginine	Stop codon	Nonsense
	281	GGT	TGT	Glycine	Cysteine	Missense
S5B5	296	ACT	GCT	Threonine	Alanine	Missense
	300	CCC	ACC	Proline	Threonine	Missense

Table 4: Missense and nonsense mutation identified in Bari goat breed.

Essential Amino acid (grams)	Semi-essential Amino acids (grams)	Non-essential Amino acids (grams)
Threonine 3.13 ± 0.08g	Cysteine 2.28 ± 0.04g	Aspartic acid 1.41 ±0.04g
Valine 2.95 ± 0.04g	Tyrosine 2.61 ± 0.03g	Serine 1.22 ±0.02g
Methionine 2.40 ± 0.03g		Glutamic acid 2.98 ± 0.01g
Isoleucine 3.10 ± 0.03g		Proline 2.42 ± 0.02g
Leucine 3.15 ± 0.03g		Glycine 1.01 ± 0.03g
Phenylalanine 3.09 ± 0.05g		Alanine 1.44 ± 0.03g
Lysine 2.80 ± 0.02g		Arginine 2.42 ± 0.02g
Histidine 2.43 ± 0.02g		
Total 23.12 ± 0.3g		**Total 12.9 ±0.17g**

Table 5: The comparison between essential, semi essential, and nonessential amino acids in milk samples of Bari goat breed.

Researchers have previously looked into the relationship between SNPs in the BMP4 gene and the quantity and quality of milk globally (Singh *et al.,* 2015; Karuthadurai *et al.,* 2019; Bangar *et al.,* 2021). PCR-Gel electrophoresis followed by DNA sequence techniques revealed that about (19) point mutations were identified in Bari goat breed based on genetic codon maximum number of mutations were noted. This suggests that SNPs could affect positive or negative depend upon production of milk and quality in Bari goat breed. In future time period it may validated phenotypically. Maximum quantity of missense mutations have been found a very satisfactory symbol and could have a substantial impact on meat quantities and yielding of milk of that goat breed, meanwhile changed genomic codon produced alteration of non-essential amino acid into essential amino acid at numerous locations (Karuthadurai *et al.,* 2019). This result agreed with the research of (Bukhari *et al.,* 2013) and disagrees with study conducted by (Singh *et al.,* 2015). In this study there were not any kind of silent mutations were found because it would not be effective on the qualities and quantities of milk, subsequently, the phenotypically gene expression would not be affected because of the amino acid code by that gene endure similar. Non-sense mutations were found only 2 times then it remains good mark, while it's negatively consequence is the termination codon (stop codon) partly produced protein. Deletion mutation was found in only Bari goat breed. The type of change would have harmful effect on milk attribute then the mutated codons would not be code for any amino acid, shortening the structure of protein and reduction of milk characteristics and quantities. If any kind of mutation occurred in DNA sequences are less than 1% considered mutation and if it is greater than 1% than called SNPs. Result of that research specified that one sample of Bari goat. While none of them showed just type of mutations. From the result of Bari goat breed performed to be healthier breed for variety of admixture to progress milk and meat qualities and quantities. Therefore, this characteristic could also be very beneficial for breeding admixture to face changeable environmental conditions.

CONCLUSION: Present study revealed that about 19 mutations were found in the Bari goat breed, according to the current study. PCR sequencing methods based on genetic codons were used to identify the mutations. About (16) Missense Mutation, (2) Nonsense Mutation, (1) Deletion Mutation were found. Finding missense mutations were related to the different amino acids. Missense mutations have been found to be beneficial for improving an animal's morphogenetic expression, traits and body characteristics. Non sense mutations can lead to shortened, non-functional protein due to introduction of pre-mature stop codon, while deletion mutation can also influence the reproductive traits. The breed's meat and milk yields are improved as a result of these modifications. Different percentages of essential, non-essential and semi-essential amino acids were found in the milk of the Bari goat breed after an amino acid analysis was conducted.

ACKNOWLEDGEMENT: The authors would like to greatly appreciate the Department of Zoology, Shah Abdul Latif University Khairpur Mir's Sindh, Pakistan for providing the resources and assistance required to carry out this research work.

CONFLICT OF INTEREST: All the authors declared no conflict of interest.

LIFE SCIENCE REPORTING: In current research article no life science threat was reported

ETHICAL RESPONSIBILITY: This is original research, and it is not submitted in whole or in parts to another journal for publication purpose.

INFORMED CONSENT: The author(s) have reviewed the entire manuscript and approved the final version before submission.

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