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Research Article

Correlation of Vaginal Cytology with Estrous Behavior and Genetic Markers (GDF9 and BMP15) in indigenous Goat Breeds

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ABSTRACT

This study aimed to investigate the cytological, behavioral, and molecular aspects of the estrous cycle in three indigenous goat breeds; Gulabi, Pateri, and Tapri of Sindh, Pakistan. A total of 45 healthy does were monitored under uniform semi-intensive management and grouped to ensure diversity in reproductive history. The estrous cycle was tracked over 21 days using behavioral observations and vaginal cytology, identifying distinct cellular patterns between proestrus and estrus phases. Proestrus was characterized by a mixed population of epithelial cells and moderate leukocytes, while estrus showed over 85% superficial cornified cells, minimal leukocytes, and clinical signs of receptivity. Concurrently, genomic DNA was extracted from blood, and exon–intron regions of two key fecundity genes, GDF9(799 bp) and BMP15 (503 bp), were amplified using PCR. Gel electrophoresis confirmed successful amplification with high primer specificity, yielding clean, expected-size bands without non-specific products. These genes were then sequenced for further analysis. The combined cytological, behavioral, and genetic data provided a robust framework for reproductive monitoring in goats. This study supports the utility of vaginal cytology as a non-invasive, reliable method to detect estrous phases and highlights the molecular profiling of GDF9 and BMP15 as potential tools for improving reproductive efficiency in indigenous goat breeds.

Keywords: Estrus Cycle, Goat Breeds, Genes, Molecular Markers, Proestrus.

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INTRODUCTION

The productivity (milk production and the number of offspring) of the certain mixed-breeds is quite low. The low productivity may in relation to reproductive disorders, undirected selection, low quality seeds and inbreeding. The reproductive efficiencies including proestus, corpus luteum and pregnancy are linked to estrus cycle in goat species. Estrus detection is helpful in offspring production and milk yield. Estrus identification can be performed using vaginal smears. In general, estrus detection in goats can be detected by changes in the behavior of farm animals. The reproductive phase of female goats can be identified using vaginal smears (Jha et al., 2020; Sumaryanti, 2024). Estrus behavioral changes can be detected using extrinsic and/or exfoliative of vaginal cells of goat species. Exfoliative vaginal cells have been screened through the vaginal smear method in ovine and caprine species (Setiawan et al., 2017; Sitaresmi et al.,2019; Sumaryanti, 2024). In fact, the estrus cycle stage, estrogen hormonal and physiological variations may affect pH dynamics in goats (Antonov, 2014). Bone Morphogenetic Protein Receptor Type 1B (BMPR1B), and growth

differentiation factor 9 (GDF9) have been identified for their genetic potential and involved in fecundity of goat breeds (Mishra et al. 2017; Wang et al. 2019; Faris et al., 2025). GDF9 and BMP15, genes are associated to prolificacy and growth of cells, transforming growth factor beta (TGF-β) and ovarian folliculogenesis (Galloway et al., 2000; Hanrahan et al.,2004; Sanfins et al., 2018). Overall, oocyte driven factor produces profound effect on ovarian follicles the development and physiology (Sanfins et al., 2018; Nesbit et al., 2020). Considering physiological, behaverial and vaginal cellular changes during proestrus and estrus cycles in goat breeds, the present study is designed to evaluate differences using genetic markers for the fertility regulation and reproductive traits in Tapri, Pateri, and Gulabi indigenous goat breeds.

MATERIALS AND METHODS

This study was performed on goats of three common indigenous breeds; Gulabi, Pateri and Tapri in province Sindh of Pakistan. The research experiments were performed at Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tandojam. A total of 45 apparently healthy goats divided in three experimental sub-groups; i.e. A, B, and C. Each subgroup was consisted of similar age five physically and clinically healthy goats in order to decrease the age related influence particularly physiological variation. Animals were tagged by Radio Frequency Identification (RFID) method for the identification and accurate data collection. During the experiment diverse representation of reproductive traits of three common indigenous breeds; Gulabi, Pateri and Tapri were kept in consideration for further analyses under similar experimental conditions. The reproductive history of the experimental animals related to births of quadruplets, triplets, twins and single was included. The selected goats were of approximately similar age to minimize the influence of age-related physiological variability on the study results. Special consideration was given to the reproductive history of the animals, and individuals with a record of single, twin, triplet, and quadruplet births were purposefully included. This ensured a diverse representation of litter sizes for a more comprehensive assessment of reproductive traits across the three breeds. Experimental animals were maintained and housed under similar management and environmental conditions. The animals were provided balanced diet and water according to standards institutional husbandry practices and guidelines. Strict preventive measures including disease monitoring, de-worming and vaccinations were adopted during the entire experimental period.

Management of experimental animals

The experimental animals were kept and reared under semi-intensive conditions. The animals were fed open field graze natural herbs consisted of small trees, shrubs and herbs; local roughages palai, wanda, Berseem (Trifolium alexandrinum) and Jantar (Sesbania bispinosa). In addition, concentrated ration was given to the all experimental animals to maintain and meet protein and energy requirements during entire period. Routinely, the estrous cycle of the experimental animals was monitored for 21 days. Initial estrus cycle was noted using external physical signs including tail wagging, frequent urination, restlessness, mucus discharge and swollen vulva. In addition, estrous diagnosis was performed using viginal cytology for accurate detection and conformation. For vaginal cytology, the sears were obtained from vagina to identify estrus cycle phase in respect of predominant cell types for confirmation of reproductive status of the animal.

Confirmation of estrus cycle status through exfoliated vaginal cytology

Adult female goats exhibiting normal reproductive behavior and no signs of vaginal infection or systemic illness were selected. The animals were restrained gently in a standing position by an assistant to reduce stress and ensure safety during sampling. The external genital area was cleaned with sterile gauze soaked in physiological saline (0.9% NaCl) to remove dirt and contaminants. Care was taken to prevent contamination of samples with feces or urine. A sterile cotton swab was gently inserted approximately 5–7 cm into the vaginal canal at a slight angle to avoid trauma. The swab was rotated gently against the vaginal mucosa for 10–15 seconds to collect exfoliated epithelial cells. The swab was immediately rolled onto a clean, labeled glass slide to transfer the cells. For fluid samples, drops were placed on slides and evenly spread using another slide.The slides were air-dried at room temperature for 5–10 minutes. Fixation was performed by immersing the slides in absolute methanol for 2–3 minutes. Staining was done using the Giemsa stain, prepared fresh by diluting Giemsa stock solution 1:10 in phosphate buffer saline (pH 7.2). Slides were immersed in the stain for 20–30 minutes. After staining, slides were rinsed gently with distilled water and air-dried. Stained slides were examined under a light microscope at 100× and 400× magnifications. For each slide, at least 10 fields were observed to identify and count the different cell types.Parabasal cells: Small, round with large nuclei; predominant in diestrus. Intermediate cells: Larger than parabasal with smaller nuclei. Superficial cells: Large,

polygonal, often anucleated or with pyknotic nuclei; predominant in estrus. Leukocytes: Present mainly during diestrus and metestrus. The relative proportions of the cell types were used to determine the stage of the estrous cycle: Proestrus: Increased intermediate cells and some superficial cells. Estrus: Predominantly superficial cells, often anucleated. Metestrus: Mix of parabasal, intermediate cells, and leukocytes. Diestrus: Mainly parabasal cells and leukocytes. The percentage of each cell type was recorded for every sample.Vaginal cytology results were correlated with behavioral observations and, where applicable, ultrasonographic findings to confirm estrous phases.In addition to cytological examination, close behavioral observation of animals was conducted to identify clinical signs of estrus, such as restlessness, tail raising, vulvar swelling, and increased vocalization. The combined analysis of vaginal cytology and behavioral cues allowed accurate prediction and confirmation of the animals' estrus cycle phases.

Blood for DNA isolation and hormonal detection

DNA Extraction:

Genomic DNA was extracted from whole blood samples using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, 200 µL of blood was lysed using the supplied lysis buffer and Proteinase K. The lysate was then mixed with ethanol and applied to a silica spin column to bind DNA. After washing to remove contaminants, DNA was eluted in 50 µL of elution buffer. The concentration of the extracted DNA was quantitatively measured using the Qubit 4 Fluorometer (Thermo Fisher Scientific). Extracted DNA samples were stored at –20°C until further analysis.

Primers

Primers specific to the GDF9 and BMP15 genes were designed based on the nucleotide sequences available in the NCBI GenBank database. Reference sequences for each gene were carefully selected to cover regions of interest. Primer design was conducted using Oligo Explorer and Oligo Analyzer software tools, which helped optimize primer length, melting temperature (Tm), GC content, and minimize secondary structures such as hairpins and dimers to ensure high specificity and amplification efficiency. The designed primers (listed in Table-1) were commercially synthesized. Primer sequences were validated in silico for specificity using BLAST against the Capra hircus genome to avoid non-specific amplification. The primers were then used in PCR assays to amplify the target gene regions for downstream applications.

Amplification of GDF9 and BMP15 Genes

The amplification of GDF9 and BMP15 genes were performed. The PCR reaction was conducted in a total of 20 µL in micro centrifuge tube. The mixture comprised of 2.0 µl of 10 X buffer) 2.0 µl of dNTP, 1.20 µl $MgCl_2$, 1.5 U Taq DNA polymerase, template, forward and reverse primers (each 10 pM) and DEPC water was adjusted to total volume of 20 µl product. The thermal cycle reaction was set up; initial denaturation 95 °C for 5 min; then 95 °C for denaturation for 30 sec; annealing 63-65 °C for 45 sec., extension at 72 °C for 30 sec and Final extension at 72 °C for 5 min. The amplified DNA product was subjected to electrophoresis and run on 2% agarose gel containing ethidium bromide. 1kb marker was used for the detection of band size. The DNA bands were observed using UV trans-illuminator (Cleaver Scientific, United Kingdom).

Table 1. Primer sequences (5′–3′), amplicon length (bp), primer melting temperature (Tm) and corresponding GenBank accession numbers of GDF9 and BMP15 genes.

Gene	Sequence	Amplicon Length (bp)	Annealing Temperature (TA)	Accession Number
BMP15	for: 5´-ACTCCGCTTCGTATGTCAG-3´ rev: 3´-ACTGTATGCTTCGCCTCA-5´	503	58^{o}C	JQ350890.1
GDF9	for: 5´- GATTGATGTGACGGCTCCT-3´ rev: 3´- CTCCCAAAGGCATAGACAGG-5´	799	56°C	KY780296.1

dominance of superficial cornified epithelial cells, constituting approximately 85% of the smear. These cells often exhibited pyknotic or absent nuclei, indicative of complete cornification driven by peak estrogen concentrations. Parabasal and intermediate cells were rare or absent, and leukocytes are minimal or nonexistent, creating an optimal environment for mating. The vaginal mucus during estrus was clear, watery, and abundant. Clinically, does demonstrated typical signs of standing heat, including vulvar swelling, redness, tail flagging, and receptivity to mating.

The clear cytological and behavioral differences between proestrus and estrus highlighted the usefulness of vaginal

cytology as a reliable, non-invasive tool for monitoring reproductive status in goats.

Sequencing of GDF9 and BMP15 Genes

The PCR products were purified using kit and sent to Liaqat Medical University and Health sciences for sequences. The sequences were subjected to Basic Local Alignment Search Tool (BLAST) using services available on https://sky-blast.com/blast/n.

RESULTS

During proestrus, vaginal smears displayed a mixed population of cells, including parabasal, intermediate, and superficial types. Superficial cells generally account for about 35% of the sample, while parabasal and intermediate cells comprise roughly 65%. A moderate presence of leukocytes suggests inflammatory processes associated with epithelial turnover. The vaginal mucus appeared slightly turbid and sticky. Behaviorally, does showed increased restlessness, vocalization, and frequent tail movements, reflecting rising estrogen levels, although full sexual receptivity is not yet evident.

In contrast, estrus cytology was marked by a dominance of superficial cornified epithelial cells, constituting approximately 85% of the smear. These cells often exhibited pyknotic or absent nuclei, indicative of complete cornification driven by peak estrogen concentrations. Parabasal and intermediate cells were rare or absent, and leukocytes are minimal or nonexistent, creating an optimal environment for mating.

The vaginal mucus during estrus was clear, watery, and abundant. Clinically, it demonstrates typical signs of standing heat, including vulvar swelling, redness, tail flagging, and receptivity to mating. The clear cytological and behavioral differences between proestrus and estrus highlighted the usefulness of vaginal cytology as a reliable, non-invasive tool for monitoring reproductive status in goats.

PCR Amplification of GDF9 Gene

The specific exon and intron regions of GDF9 gene were targeted for amplification of the fragment. The targeted fragment region comprised of approximately 799 bp (base pairs). Electrophoresis analyses revealed presence of expected size band on agarose gel. There were no non-specific bands were observed on the agrose gel confirmed the primer specificity and successful amplification of GDF9 gene fragment consisted of exon and intron regions.

PCR Amplification of BMP15 Gene

The specific exon and intron regions of BMP15 gene were targeted for amplification of the fragment. The targeted fragment region comprised of approximately 503 bp. Electrophoresis analyses revealed presence of expected size band on agarose gel. There were no non-specific bands were detected on the agrose gel confirmed the primer specificity and successful amplification of BMP15 gene fragment consisted of exon and intron regions.

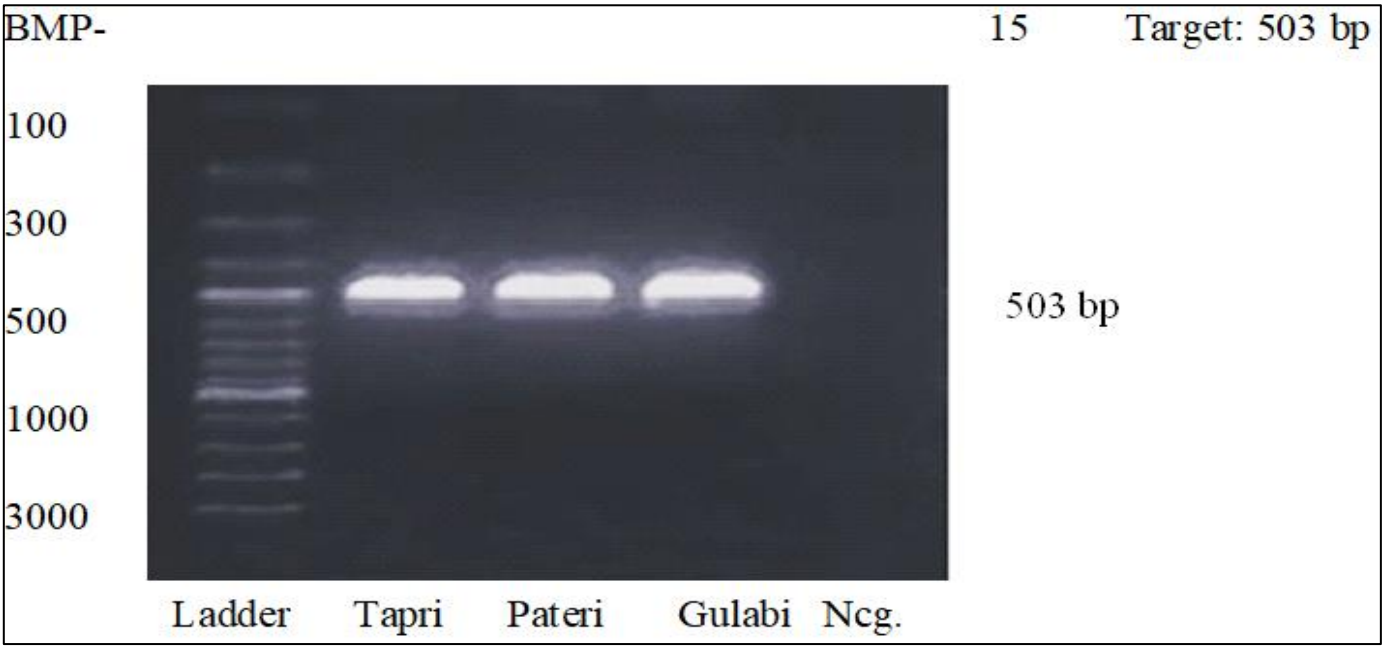

Figure 1. The expression of BMP15 gene in goats is categorized by litter size groups.

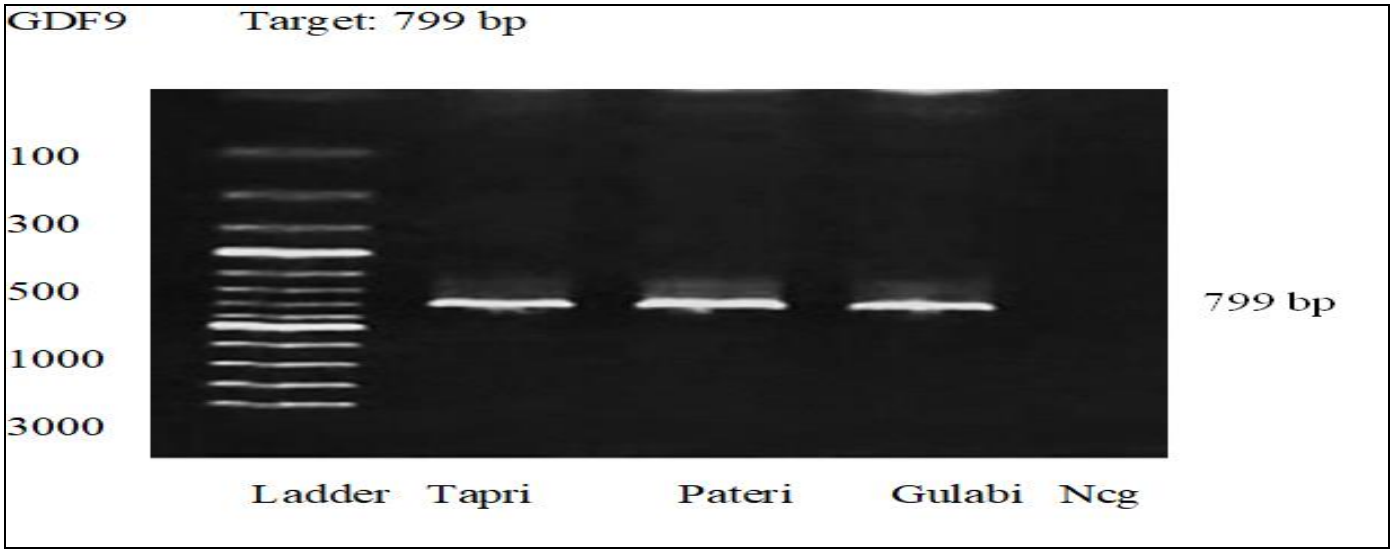

Figure 2. The expression of GDF9 gene in goats categorized by litter size groups.

DISCUSSION

This research highlights behavioral and cytological distinctions between the proestrus and estrus phases of the estrous cycle of goat breeds of Sindh, and emphases the detection utility of vaginal cytology as a non-invasive method for reproductive monitoring. During proestrus; the appearance of parabasal, intermediate, and superficial epithelial cells (mixed cell population) may in relation to transitional phase characterized by early estrogenic influence and active epithelial proliferation. The findings of this study are supported by previous research demonstrated that superficial epithelial cells become more common during the proestrus and estrus phase than luteal phase, in West African Dwarf goats. Whereas, intermediate cells and parabasal and predominate in non-fertile or non-cycling phase (Ola et al., 2006). Similarly, superficial (cornified) cells enhance during the late estrus related to high level of desquamation of vaginal epithelium and mucus becomes appears, fern-like, clear, thin and variation in leukocytes may occur during the estrus cycle in Angora breed doe (Pretorius, 1977). It was observed proestrus, a mixed smear with aproximately35% superficial cells and moderate leukocytes, sticky mucus, and early behavioral restlessness, during the estrus, approximately 85% cornified superficial cells, minimal leukocytes, clear abundant mucus, and overt receptivity. In contrast to our findings in current research, previous study based on vaginal cytology using ultrasound and mucous examination predicted approximately 88% accuracy of estrus at the time of ovulation in small ruminants reported that is useful method but not perfect (Souza-Fabjan et al., 2021). Moreover, in an experiment during synchronization high level presence of superficial cells during estrus cycle can associate to higher estrogen level and shifts in intermediate/parabasal cells in Surti goats (Patel et al., 2023). It has been observed that during induced estrus in Beetal breed does, showed standing heat behaviors;, tail wagging, bleating and clear mucous and copious with high stretchability (spinnbarkeit) (Dogra et al., 2016).

In this study, amplification of regions comprising of exon and intron regions (799bp) of the GDF9 gene was successful, as evidenced by a single, accurate size band on agarose gel. The findings demonstrated efficient primer design and specificity and concurrent to GDF9 in chicken or reproductively gene amplification (Liu et al 2018). Similarly, amplification of in the exon 1 region (approximately 710 bp) of GDF9 gene related to fecundity in two Egyptian sheep breeds exhibited band pattern confirming the validation of amplification specificity (El Fiky et al., 2017). The amplification and sequencing of GDF9 gene and its association with litter size and inLuzhong mutton sheep (*Ovis aries*), have been confirmed (Wang et al., 2021). Overall, eamplificationxon–intron regions GDF9 gene and its relation with proestrus and estrus cycle of goat breeds Tapri, Pateri, and Gulabi of Sindh, and sequencing confirms the encompassing and internal sequence, in line with the methodological observed in prior GDF9 gene polymorphism research.Incurrent research, amplification of regions comprising of exon and intron regions (503bp) of the BMP15 gene was successful, as yielded a single, accurate size band on agarose gel, and suggested high specificity and accuracy of molecular method. Previous research studies amplification of exonic regions of BMP15 gene of Jining Grey goat breed detected discrete, expected size bands (Chu et al., 2007). DNA sequencing of the PCR amplicons were verified and confirmed exon–intron boundaries and detect SNPs in BMP15 gene and its association to litter size in Xinjiang Cele Black Sheep (Niu et al., 2021). Amplification of exon–intron regions BMP15

gene and its relation with proestrus and estrus cycle of goat breeds Tapri, Pateri, and Gulabi of Sindh, and sequencing confirms the encompassing and internal sequence, in line with the methodological observed in prior GDF9 gene amplification and sequencing studies. This study highlights both behavioral and cytological markers of the proestrus and estrous cycle with molecular validation using amplification of the *GDF9* and *BMP15* gene regions, and their involvement in reproductive regulation. These results not only support the use of vaginal cytology as a non-invasive method for estrus detection but also align with genetic studies linking *GDF9* and *BMP15* to fecundity traits in small ruminants

CONCLUSION

The study highlighted that integrating behavioral monitoring, vaginal cytology, and molecular techniques offered a thorough understanding of the estrous cycle in Sindh's indigenous goat breeds. Vaginal cytology was found to be a dependable and non-invasive method for differentiating proestrus and estrus phases, aligning closely with observed behavioral indicators of estrus. Analysis of the key fecundity genes, GDF9 and BMP15, showed precise amplification and specificity, providing a basis for future genetic research to improve reproductive performance. Overall, the results emphasized the importance of combining cytological, behavioral, and genetic data to assess and enhance reproductive efficiency in Gulabi, Pateri, and Tapri goats, supporting effective breeding programs and the preservation of local genetic resources.

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AUTHOR CONTRIBUTIONS

Research Ideas: Faiza Rubab; Methodology: Pershotam Khatri; Data Analysis & Writing: Shahid Hussain Abro; Preparation of Paper Draft: Hira Sajjad Talpur

COMPETING OF INTEREST

The authors declare no competing interests.

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