Evaluation of Glyceraldehyde 3-Phosphate Dehydrogenase (GADPH) and Luteinizing Hormone Receptor (LHR) Gene Polymorphisms in Buffaloes and Cows

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Abstract: The present work evaluates whether buffalo and cattle have different sequences of luteinizing hormone receptor (LHr) and glyceraldehyde 3-phosphate dehydrogenase (GADPDH) genes. DNA was extracted from the peripheral blood of 38 animals (17 buffaloes and 21 cows) and the ovarian granulosa cells of 13 cows. Primers used for amplification were reported in the literature. The PCR products obtained were analyzed via electrophoresis on 1.5% agarose gels and sequenced via the Sanger technique. The electropherograms were analyzed via DNA Baser software, and the sequences were aligned via MEGA5 software. The quality of the electropherograms was evaluated via UGENE software.The edited contigs corresponding to the GAPDH gene were 100 nucleotides long, whereas those of the LHR gene reached 151 nucleotides. The most relevant changes were observed in the following positions: valine for isoleucine at position 65; asparagine for cysteine at position 67; alanine for glycine at position 70; threonine for proline at position 72; glycine for arginine at position 88; and alanine for aspartic acid at position 89. In the analyzed region, a variation was identified at position 446, where buffaloes preferentially present threonine, whereas in cows, alanine or valine.It is reported for the first time that there are differences in the LHr and GAPDH genes between buffaloes and cattle. The bioinformatic analysis of these sequences may explain whether the changes may affect the function of the genes and whether these may be responsible for the differences observed in the reproduction of the species analyzed.

Keywords: Buffaloes, cows, glyceraldehyde 3-phosphate dehydrogenase, luteinizing hormone receptor, polymorphisms.

INTRODUCTION

Buffaloes were domesticated around 3000–6000 years ago and have substantial economic significance as meat, dairy, and draught animals. However, they have remained underutilized in developing a well-annotated and assembled reference genome de novo [1].

There is great interest in improving milk and meat production through reproductive biotechnologies, such as artificial insemination and *in vitro* embryo production (IVP). The first involves the use of superior bulls. The second is the production of embryos *in vitro* (IVP) due to the existing problems and good results in obtaining the embryos *in vivo* [2] and the need to increase pregnancy rates in case of fixed-time artificial insemination [3], it is critical to obtain good results increase the number of oocytes and blastocysts [4].

The main limitations of the commercial application of IVP in buffaloes are the low quantity of oocytes obtained after ovarian aspiration, the low cleavage rate, and the poor resistance to cryopreservation, which is reflected in the observation of poor-quality embryos; it has been proposed that buffalo oocytes and embryos are sensitive to oxidative stress due to the high quantity of lipids they contain [5]. Later studies demonstrated that supplementing culture media with cysteamine increases the blastocysts obtained from this species [6].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme in glycolysis, and its role is associated with the catalysis of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. In the buffalo species *Bubalus bubalis*, it has also been used as a constitutive gene control for gene expression studies because of its stable expression in different tissues; it also has a role in reproductive physiology, the immune system, and disease resistance [7].

Enriching *in vitro* fertilization media with thiol-derived compounds such as cysteamine and cystine improved embryo production in buffalo by increasing glutathione synthesis [6].

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ISSN: 1927-5196 / E-ISSN: 1927-520X/25

The luteinizing hormone (LH) receptor (LHr) is a protein with seven transmembrane domains associated with G proteins. Its large ectodomain contains several leucine repeats, essential for binding human chorionic gonadotropin (HCG) or LH hormone. In humans, the ligand-receptor relationship is highly specific; it does not bind with LH from other primates. In rodents, it is not very specific [8]. Its characteristics in buffaloes are not known [9].

The LHR gene is highly conserved in vertebrates. It is located on chromosome 11 in cattle; it is expressed in the gonads together with the FSH receptor (FSHR), and it has an alternative splicing producing different variants of the gene [8, 9] lacking exon 10 or a partial deletion of exon 11and exon 3 [8] have been reported in cattle. These changes will produce changes in the function of the protein; variants with partial deletions of exon 11 can be translated into proteins, but these variants do not cross the cell membrane [8]. Therefore, it is reasonable to think that differences in response to LH are associated with the LHr gene expression [10] since, in humans, the function of the LHris lost when mutations in exons 8 and exon 10 appear or when exon 10 is deleted, there is no response to LH [8].

Despite the tremendous phylogenetic and physiological closeness between buffaloes and cows, significant differences have been observed. They have fewer primordial follicles, 10,000-19,000 and ~150,000, and a higher proportion of atresia, ~92-95% vs. 70%, respectively. Consequently, this is part of the explanation for the observation that equal environmental and management conditions have different reproductive parameters. This opens the possibility that various sequences and forms of gene expression associated with follicular development are responsible for these differences [11].

Understanding the reasons for these gene differences and their functions will help facilitate the application of reproductive biotechnologies in buffalo species to develop the industry. The comparison-based research model is relatively new and is based on the premise of high phylogenetic closeness between the species and the reported high homology of their genes. The objective of the present work was to evaluate whether there are differences in the sequences of two genes (LHr and GADPDH) between buffalo and cattle to propose explanations for the differences observed in reproductive parameters and *in vitro* embryo behavior between the two species.

MATERIALS AND METHODS

For sequencing, DNA was extracted from the peripheral blood of 51 animals (17 buffaloes and 21 cows) and the ovarian granulosa cells of 13 cows via a commercial kit (Bionner, USA).The DNA was quantified via a spectrophotometer (Nandrop1000, Thermo Fisher Scientific, USA). DNA fragments were obtained under standardized optimal conditions for amplification of extracted DNA in a SimpliAmp™ thermal cycler (Thermo Fisher Scientific, USA.), with*Homo sapiens* DNA used as a positive control. The PCR products obtained were analyzed via electrophoresis on 1.5% agarose gels via an intercalating agent (GelRed Nucleic Acid Gel Stain) in TAE buffer at 80V for 30 minutes. The gels were visualized in a UV transilluminator at 365nm.

The obtained fragments were sequenced via the Sanger technique and sent for sequencing to Macrogen (Korea). The electropherograms were analyzed via DNA Baser software (www.dnabaser.com), and the sequences were aligned via MEGA5 software.

The Ethics Committee of the Amazonia Campus of Universidad Nacional de Colombia (A.3-05-123) endorsed the project.

Data Analysis

The quality of the electropherograms was evaluated via UGENE software [12]. Only those regions with good resolution were considered for assembly, from which contigs were constructed for each sequence. The contigs obtained, and the reference sequences available in GenBank were subsequently aligned via the ClustalW algorithm [13] via MEGA 11 software [14]. This same program was used to calculate genetic distances at the nucleotide and amino acid levels and to estimate nonsynonymous/synonymous substitution rates (Ka/Ks).

The reference sequences selected corresponded to the complete or longest possible CDS for each protein: AB098934 from *B. Taurus*and XM006065800 from *B. Bubalis* for GAPDH; NM174381 (*B. Taurus*) and DQ858168 (*B. Bubalis*) for LHR. To assess the possible structural implications of the observed amino acid changes, BLAST analysis of the sequences was performed, and the location of the substitutions in the predicted three-dimensional structure was determined via AlphaFold software [15], which is available on the BLAST platform.

RESULTS

The edited contigs corresponding to the GAPDH gene were 100 nucleotides long, whereas those of the LHR gene reached 151 nucleotides. These fragments span positions 58-90 amino acids (aa) for GAPDH and 433-481 aa for LHr relative to the *B. Taurus* open reading frames (ORFs), according to the reference sequences AB098934 (GAPDH) and NM174381 (LHr).

Fifteen high-quality sequences of GAPDH were obtained, of which five corresponded to *B. Bubalis* and 10 to *B. Taurus*. In addition, two reference sequences were included in the alignment: one from *B. Taurus* (AB098934) and one from *B. Bubalis* (XM00606565800) (Figure **1A**).

The most relevant changes were observed in the following positions: valine (V) for isoleucine (I) at position 65; asparagine (N) for cysteine (C) at position 67; alanine (A) for glycine (G) at position 70; threonine (T) for proline (P) at position 72; glycine (G) for arginine (R) at position 88; and alanine (A) for aspartic acid (D) at position 89. According to the prediction of the three-dimensional structure, these changes are found in regions of beta sheets (Figure **1B**).

For the LHr gene, 28 high-quality sequences were obtained, of which nine corresponded to *B. Bubalis* and 19 to *B. Taurus*. The reference sequence (NM_174381) from *B. Taurus* contains the complete ORF, and a partial sequence (DQ858168) from *B. Bubalis* was also included in the alignment. In the analyzed region, a variation was identified at position 446, where buffaloes preferentially present threonine (T), whereas in cows, alanine (A) or valine (V) was observed (Figure **2A**). According to structural prediction, this change is located within an alpha helix (Figure **2B**).

DISCUSSION

Bubaline and cattle are two species of the Bovinae subfamily tribe Bovini with remarkable similarities in their reproductive physiology and differences in their reproductive parameters when placed under the same management and environmental conditions [16]. An approach to explain this situation and learn more about the reproductive biology of the buffalo species is by contrasting the species. This paper compares two genes, GADPH associated with glucose metabolism and LHr with ovulation. The coding regions of the reference sequences and those previously reported in

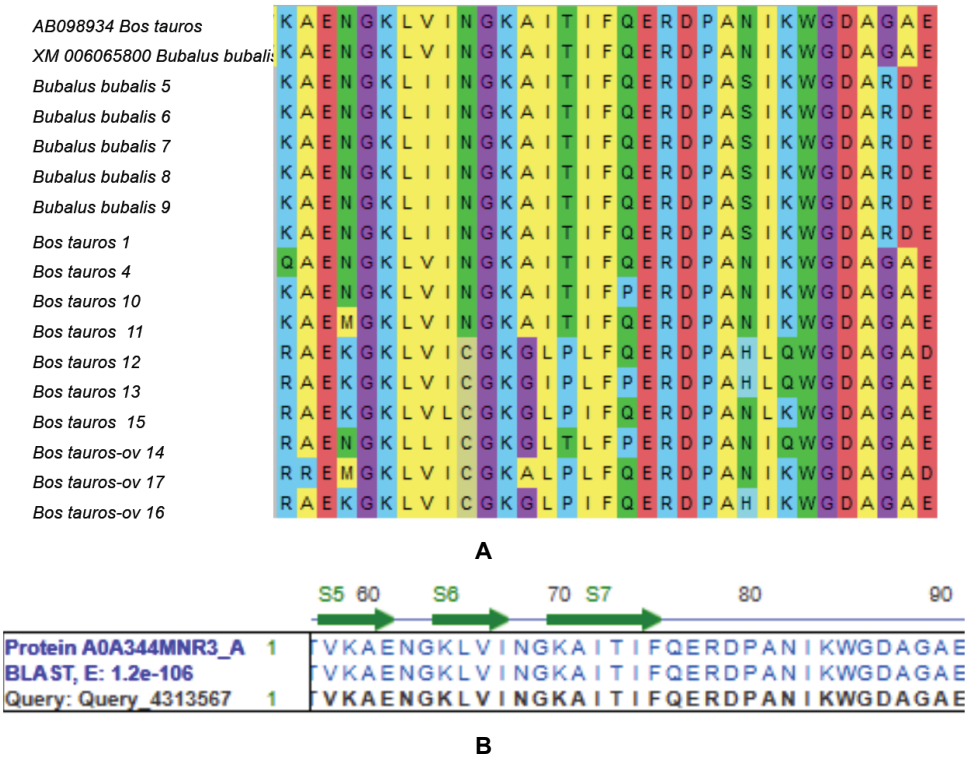

Figure 1: Amino acid alignment of GAPDH: This alignment includes 17 sequences between positions 58 - 90 aa, 15 of which correspond to the contigs identified by the project, and two reference sequences, AB098934 and XM006065800, for *B. Taurus* and *B. Bubalis*, respectively. **A**: Primary sequence of the protein. **B**: Predicted sequence structure by Blastp with AlphaFold structure AB098934 *B. Taurus* again *B. Bubalis*.

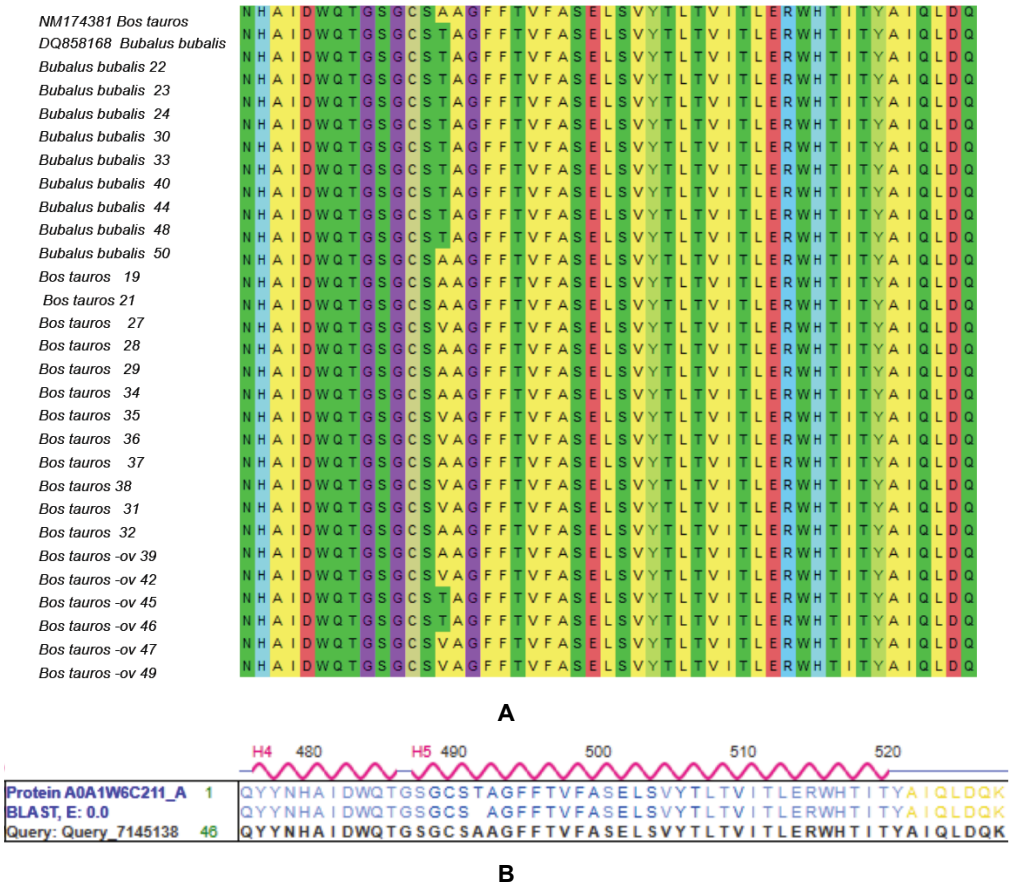

Figure 2: Amino acid alignment of LHr: This alignment included 30 sequences between positions 58 - 90 aa and 28 corresponding to the sequences identified by the project and two reference sequences, NM174381 and DQ858168, for *B. Taurus* and *B. Bubalis*, respectively. **A**: Primary sequence of the protein. **B**: Secondary structure predictionby Blastp with AlphaFold structure NM174381*B. Taurus* again *B. Bubalis*.

the literature were compared in terms of nucleotide and amino acid similarity.

GADPH is highly conserved but has a mutation that differs between buffaloes and cows. In this case, it would be talking about glycolysis; however, the region in which the contigs for GAPDH are aligned is conserved at the amino acid level in the reference sequences. The sequences obtained in this study show variations between buffaloes and cows [17]. These differences need to be evaluated in depth to explain the high susceptibility to oxidation of buffalo embryos beyond the cytoplasmic lipid content.GAPDH indicates that the sequence changes present in *B. Bubalis* may not confer adaptive advantages [18].

From the GAPDH gene, the amino-terminal ends of the *B. Taurus* and *B. Bubalis* proteins are highly conserved. Moreover, most of the differences are concentrated at the carboxy-terminal end. The *B. Bubalis* protein appears longer than that of *B. Taurus*; however, this sequence comes from a computational prediction based on whole-genome sequencing data (XP_00606065862), so its actual length is unknown. In the ORF common segment, which corresponds to 489 nucleotides and 162 amino acids, 47 nucleotide differences resulting in 16 amino acid substitutions were identified. A Ztest was applied to evaluate the neutral selection hypothesis via the dN/dS ratio, obtaining a statistical value of -1.283, suggesting a possible purifying selection action. However, the value of p=0.202 did not reach statistical significance, so it cannot be concluded with certainty that this region of the gene is under purifying selection pressure (Figure **3A**).

LHr has more variations and differs between buffaloes and cows. In this case, regarding function in the ovulatory process, LHr indicates that the observed changes could confer an adaptive advantage, which may mean that buffaloes naturally have a better chance of ovulating than cattle.In both cases, we have described the difference between genes, but to move forward, we would have to see if these differences

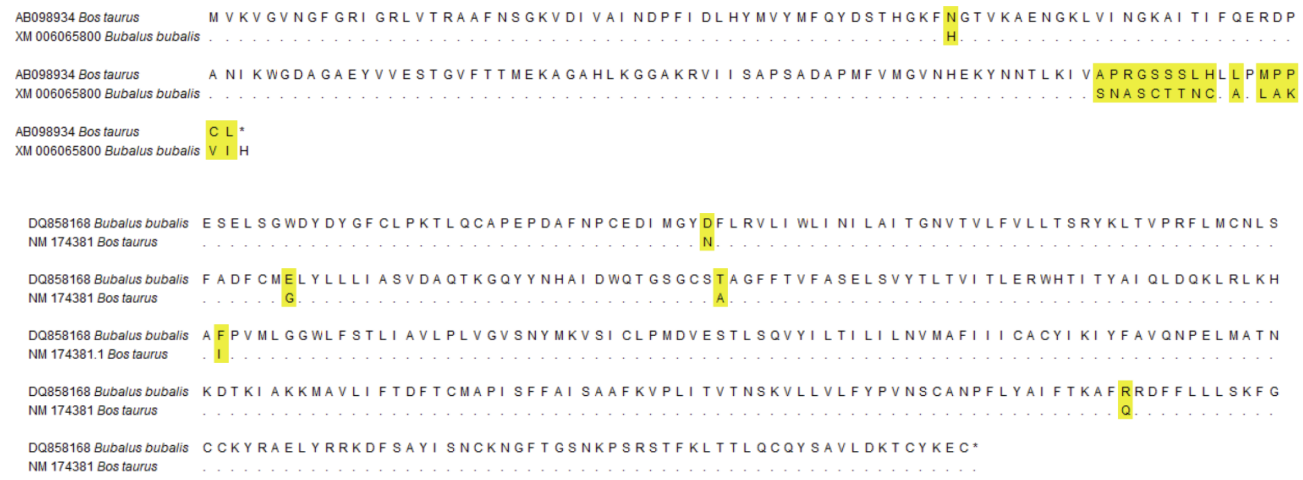

Figure 3: Variable regions of the GAPDH and LHr proteins in the reference sequences. The dots indicate conserved amino acids, and the variable regions between the reference ORFs are highlighted in yellow. AB098934 from *B. Taurus* and XM006065800 from *B. Bubalis* for GAPDH; NM174381 (*B. Taurus*) and DQ858168 (*B. Bubalis*) for LHr.

affect the sequence of the proteins if they are positive, and if they have any effect on the function of the protein.

Comparisons between the reference sequences for the ORF of the LHr gene revealed that both proteins are the same length, with 377 amino acids and 1134 nucleotides. In the complete alignment of these ORFs, five differences were identified at both the nucleotide and amino acid levels. A Ztest was applied to detect selection (dN/dS ratio), obtaining a statistical value of 2.1671, suggesting the existence of positive selection. Furthermore, the value of p=0.0322 was statistically significant, supporting the hypothesis that this gene region is under positive selection pressure (Figure **3B**).

Indeed, this is a pioneering study, but its realization has significant limitations, one of them being the scarce information on the genomics of the buffalo; to date, the entire genome of the buffalo has not been sequenced. However, it is true that, as a species, it is essential, but the technification of the production system does not yet allow us to have much interest in the species. Within the same study, the GADPH gene is used more as a housekeeping gene than for its role in the embryo's metabolism.

CONCLUSION

Differences in the LHr and GAPDH genes between buffaloes and cattle are reported for the first time.The bioinformatics analysis of these sequences may explain whether the changes may affect the function of the genes and whether these changes may be responsible for the differences observed in the reproduction of the species analyzed.

CONFLICT OF INTEREST

We hereby declare that there is no conflict of interest concerning the publication of this manuscript.

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Received on 10-04-2025 Accepted on 21-06-2025 Published on 07-07-2025

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