Molecular Characterization and Polymorphism of Inhibin (INHβA) Gene in Water Buffalo (Bubalus bubalis) Bulls

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The study characterized inhibin-βA (INHβA) gene of 12 water buffalo (Bubalus bubalis) bulls [two Philippine Carabao (PC), five Bulgarian Murrah (BM), and five Italian Murrah (IM)] using DNA extracted from semen. Using MEGA 7.0 software and Signal P® version 4, the sequences were assembled and aligned and amino acid sequences were determined, respectively. BM bull sequence showed 100% similarity with B. bubalis from the National Center for Biotechnology Information (NCBI) database. However, a lower similarity was seen on both PC and IM at 98%. The translated amino acid sequence of PC, BM, and IM had 95, 97, and 93% similarity with B. bubalis sequence from NCBI, respectively. The phylogeny tree revealed that both PC and IM were closely similar in their gene sequence, while BM was more similar with B. bubalis from NCBI. Their similarities in nucleotide sequence suggest that INHβA gene was conserved in bulls. The signal peptide was observed in BM and B. bubalis from NCBI. BM has an amino acid exchanged from threonine to alanine, while in B. bubalis has an amino acid exchanged from histidine to aspartic acid. This polymorphism from their cleaving site could cause an interaction in the reproduction, growth, and maintenance of the bull.

Keywords: Bubalus bubalis, Bulgarian Murrah, INH gene, Italian Murrah, Philippine Carabao, water buffalo

Inhibin (INH) was first described by McGullagh (1932) and considered as the main regulator in the secretion of follicle-stimulating hormone (FSH) that is involved in follicular development and steroidogenesis in females (Tabor et al. 2001). Many experiments were performed in female cattle and buffalo to improve its reproductive performance using the INH gene that was correlated to some phenotypic characteristics in reproduction but poorly studied in males. Moreover, INH is recognized as a testicular regulator and has a promising marker to male and female fertility (Bhardwaj et al. 2012) and influences the hypothalamic-pituitary function (Parraguez et al. 2012). INH has two structures [INH A (α-βA) and INH
B (α-βB)] that are coded by three genes [INHa, INHβA, and INHβB] (Bernard et al. 2001).

Inherent to water buffalo reproductive problems like delayed maturity, older age at first calving, long post-partum, anestrus period, long inter-calving period, silent heat coupled with the poor expression of estrus, seasonality in breeding, and low conception rate (Terzano et al. 2012) may be further elevated by the impact of environmental challenges today. The objective of this study is to contribute baseline information on the genetic characteristics of the INHβA gene in water buffalo bulls in the Philippines. Specifically, characterization of the INHβA gene in water buffalo bulls and determine polymorphisms in different water buffalo bull breeds in the Philippines. Future identification of polymorphism of this gene can be used to select breeder male water buffaloes with superior sperm quality.

Frozen semen samples from 12 water buffalo bulls from Nueva Ecija were selected from 45 bulls for the conduct of the study. This is composed of two PC, five BM, and five IM bulls. Semen samples were placed in 1.5-mL microtubes and labeled. Briefly, DNA was extracted following the protocol of the Biosafety and Environment Section Laboratory of the Philippine Carabao Center (PCC). Polymerase chain reaction (PCR) was performed in a total reaction volume of 10 µL containing 0.2 µL of each set of primers (Table 1), 0.8 µL dNTP, 2 µL buffer. 0.8 µL MgCl₂, 0.1 µL Taq-DNA polymerase (Promega, USA), and 1 µL of genomic DNA as the template. PCR condition used was at 95 °C for 1 min initial denaturation, 35 amplification cycles were performed comprising denaturation at 95 °C at 30 s, annealing temperature of 55 °C for 1 min, extension at 72 °C for 30 s, and final extension temperature of 72 °C for 7 min and 4 °C at holding. After amplification, 3 µL of PCR product were electrophoresed in 2% agarose gel and visualized under an ultraviolet transilluminator. Sequences were aligned using MEGA® ver. 7.0 software and were compared to the stored INH sequences in NCBI BLAST. A phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replications using MEGA® ver. 7.0 software.

From the 12 samples, three contiguous sequences were completed – one representative per buffalo breed. Sequences aligned consist of 685 bp from PC, 613 bp from BM, and 717 bp from IM. The nucleotide BLAST of INHβA coding nucleotide sequence of BM bull showed 100% similarity with B. bubalis from NCBI. However, low similarity (98%) of nucleotide sequence was seen on both PC and IM.

Neighbor-joining algorithm with 1000 bootstrap replications showed clustering of buffalo’s INHβA and other ruminant farm animals (Figure 1). PC and IM were clustered together 100% bootstrap. While BM clustered with B. bubalis from the NCBI database and cattle with 67%. O. aries and C. hircus were clustered together with 99%. This clustering revealed the evolutionary origin of INHβA gene in bulls, PC and IM had more nucleotide base differences than BM. Moreover, PC is more related to IM because based on their nucleotide sequence they have more similarities than BM. On the other hand, BM and B. bubalis have more similarities in their nucleotide sequence.

Single nucleotide polymorphism was predicted at the cleaving site and was found in exon 1 of the gene between positions 18 and 19 of the amino acid sequence of BM. PC and IM INHβA genes had no predicted signal peptide in their sequence; however, it was observed that bulls have a similar nucleotide sequence in the predicted polymorphic site. Nucleotide site numbers 139–141 and 142–144 (Figure 2A) were the codons identified corresponding to positions 18 and 19 (Figure 3A). There was an observed change of nucleotide from A to G and T to C at sites 139 and 144, respectively, which may indicate polymorphic sites in the coding sequence of the gene.

On the other hand, the cleaving site of B. bubalis from NCBI was located between positions 20 and 21 of the amino acid sequence. Positions 20 and 21 (Figure 2B) were identified at sites 145–147 and sites 148–150 of the nucleotide sequence, respectively (Figure 3B). Furthermore, nucleotide change of G to C and T to C at sites 139 and 144 of the B. bubalis sequences in NCBI from the sequences derived from this study.

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**Table 1. Primers for INHβA gene used in the study.**

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
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| INHβA-1 | Forward: 5ˈ - GGA TGC CCT TGC TCT GG -3ˈ  
Reverse: 3ˈ - GAT GTG CCA GGT GCT CTT -5ˈ | 520 |
| INHβA-2 | Forward: 5ˈ - AGG AGG CTG AGG AAG TGG -3ˈ  
Reverse: 3ˈ - GGC TGG GCG CTG TAT GA -5ˈ | 721 |
Figure 1. Phylogenetic tree of INHβA gene in ruminant farm animals.

Figure 2. [A] INHβA nucleotide sequence of Exon 1 of BMB. Nucleotide site numbers 139, 140, and 141 plus 142, 143, and 144 code the positions 18 and 19 of the amino acid sequence respectively; [B] INHβA nucleotide sequence of Exon 1 of B. bubalis from NCBI. Nucleotide site numbers 145, 146, and 147 plus 148, 149, and 150 code the positions 20 and 21 of the amino acid sequence, respectively.
These differences in nucleotide frequencies command varying effects of the INHβA gene and in other biochemical processes – which may interact in male reproduction, growth, and maintenance of the bubaline bull. Jaeger and Hiendleder (1994) reported that in 1000 lambing records, INHβA had influenced the litter size in sheep. Furthermore, Hiendleder et al. (1996) reported that TaqI RFLP in sheep was associated with litter size. Sang et al. (2011) further reported the significant association of polymorphism of INHβA C7639T (rs 43408735) with semen volume and sperm concentration and its effects on motility in bovine bulls. However, in pigs, INHβA was found to adversely affect sperm quality with a significant observation of plasma droplets and abnormal sperm rate (Lin et al. 2006).

BM, IM, and B. bubalis from NCBI have the common bloodline of Murrah water buffalo from India (Alexiev 1998), wherein B. bubalis INHβA gene from NCBI was from a female Mediterranean buffalo; however, despite their origin of bloodline, individual reproductive performance of the bulls varies – which indicates that the action of the gene also varies overtime thus fluctuation of INH concentration occurs that may affect semen quality. Sanford et al. (1993) observed that INH regulation of FSH is more prominent in the breeding season vs. non-breeding season due to redevelopment of testis and a greater number of FSH receptors. Furthermore, positive correlation in seasonal variations in blood INH and testosterone concentration in rams. Razie et al. (2011) reported that INH B level affected spermatogenesis and other functions of the Sertoli cells after testicular trauma in rats. Moreover, PC was more similar to IM. This similarity may indicate that IM can comparably adapt to the local environment. Single nucleotide polymorphisms can alter functions of DNA, RNA, and proteins, and are generally classed by genomic locations. Non-synonymous SNPs alter the amino acid sequence of protein products through either amino acid substitution or introduction of nonsense/termination mutation. A variant may also affect the expression or translation of gene products, either by interrupting a regulatory region or by interfering with normal splicing and mRNA function. This variant can include SNPs in regulatory regions, synonymous SNPs, and intronic SNPs (Mooney 2004).

A predicted polymorphic site of the sequence was observed at a close distance of the sequence. Site numbers 139–150 of the nucleotide sequence could influence gene expression of INHβA that affects the performance of the bull. This effect may reduce the fertilizing ability of sperm cells and the volume of semen – which include levels of proteins like citric acid, ergothionine, fructose, glycerylphosphorylcholine, sorbitol, and other numerous enzymes that enhance the survival of sperm cells in vitro (Hafez and Hafez 2000).

Alberts et al. (2010) suggested that hormone serves as a receptor and regulatory gene that signals and transmits it to cells response machinery and binds to switch genes on or off. These activities could be illustrated by alternate actions of INH and activin in spermatogenesis, wherein INH regulates the production of FSH. When altered, effects on acceptable sperm concentration of the bull at approximately 0.8–1.2 billion sperm cells/mL may decrease (Hafez and Hafez 2000) from the water buffaloes fitness for semen donors. However, despite the possible effects of this nucleotide change, in vitro manipulation of
sperm cells was done to improve sperm quality.

Aside from the internal factor, INHβA, gene expression can probably depend on the age and breed of bulls. The arising environmental challenge may also elevate changes in the performance of bulls due to their natural pigmentation of the skin. The darker skin color of buffalo absorbs more heat than lighter skin. In addition, the influence of external factors aside from age and breed, the season was also considered in the evaluation of reproductive performance (Colenbrander et al. 1993).

INH protein can have the regulatory action on the gonocytes (spermatogonia to round spermatids) of immature and adult’s testis, and also influences Sertoli cell population (Barakat et al. 2008), which corroborates on the observation that individual bull’s reproductive performance in terms of semen quality of different buffalo breeds and age at the National Bull Farm varies. It has been further observed that in this study, predicted polymorphic sites were similar among bulls except the sequence of B. bubalis from NCBI that were from female Mediterranean buffalo. The predicted polymorphic site could affect, in male or female, the reproductive trait of buffalo. The nucleotide sequence of the INHβA gene from buffalo bulls was highly similar to that in the NCBI database. Nucleotide and amino acid composition vary between breeds. The polymorphisms determined on their signal peptide has changed the code for a specific protein in the sequence. The alteration occurs on the coding region that is vital in gene expression and this could be a link to influence bulls’ semen quality and sperm fertility. Therefore, this polymorphism of INHβA determined in the coding region could be a candidate marker for sperm quality and offer a tool in the selection of breeder bulls in the future.

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STATEMENT ON CONFLICT OF INTEREST

All authors declare not to have a conflict of interest.

REFERENCES


