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RESEARCH ARTICLE

# R159Q Polymorphism in Leptin Gene and Its Correlation with Semen Quality Parameters in Nili-Ravi Buffalo Bulls

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**ABSTRACT**

Genome analysis in animals has introduced new criteria for selection and development of strategies for increasing herd productive and reproductive potential through breeding. Leptin gene has been established as a potential candidate for marker assisted selection of animals. This study was aimed to find polymorphism in leptin gene and its relationship with male reproductive traits and semen quality parameters in 99 Nili-Ravi buffalo breeding bulls. DNA was extracted from whole blood for genome analysis of each experimental bull. PCR product was sequenced and data was analyzed for polymorphism. Results revealed the presence of R159Q polymorphism at position c.523. This polymorphism was heterozygous and the change of guanine to adenine (G>A) nucleotide was noted in experimental bulls. Sequence comparison of leptin gene revealed two genotypes, GG and GA, with frequencies of 68.69 and 31.31%, respectively. The allelic frequencies for A and G allele were 15.66 and 84.34%, respectively. Codon CGG was changed into CAG, which resulted in glutamine production instead of arginine (R>Q). Further analysis of data showed that the R159Q polymorphism was significantly negatively correlated with sperm motility after freezing. However, it had non-significant correlation with ejaculatory volume, mass activity, sperm concentration, individual sperm motility, sperm motility after dilution, live and dead sperm percentage. Findings of present study suggest that R159Q polymorphism of leptin gene might be considered as genetic marker for selection of buffalo bulls. Further study is needed to explore the mutations in other regions of this gene which may affect semen quality parameters in males of this specie.

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## INTRODUCTION

Leptin (LEP) gene is one of the potential genes that play an important role as markers for the identification of elite animals, leading to better adaptability and improved production (Moravcikova *et al.,* 2012). This gene is positioned on chromosome 4q32 in bovine. More than 15000 base pairs (bp) have been found in DNA sequence of this gene, with 3 exons and 2 introns (Qureshi *et al.,* 2015). However, only two exons are translated into protein (Javanmard *et al.,* 2008).

The leptin gene regulates secretion of leptin hormone (Denver *et al.,* 2011), which plays an important role in feed intake (Trujillo *et al.,* 2013), productive (Silva *et al.,* 2014) and reproductive performance (Singh *et al.,* 2013) in different animals. Genetic differentiation of leptin gene was first reported in mice (Halaas *et al.,* 1995), followed by humans (Ohshiro *et al.,* 2000). Later, possible associations between the polymorphism of this gene and productive traits were investigated in different farm animals.

Leptin gene polymorphism and its effects on reproductive traits were evaluated in Sahiwal and Frieswal cattle, with polymorphism C/NruI/T was found to have significant relationship with the age at first service and calving (Singh *et al.,* 2013). In another study, LEP/Sau3AI polymorphism and its effects on age at first calving, calving interval, days open, and insemination interval in Pinzgau and Slovak Spotted cows were

monitored. It was noted that *LEP/Sau3AI* polymorphism significantly (P<0.01) affected the age at first calving in these cows (Trakovická *et al.*, 2013).

Investigation regarding the effects of LEP gene polymorphism on reproductive traits using *Dra*I restriction enzyme in Polish Holstein-Friesian bulls revealed presence of four polymorphisms, including LEP-C(-963)T, LEP-R25C, LEP-Y7F and LEP-A80V. Three polymorphisms LEP-C(-963)T, LEP-R25C and LEP-A80V influenced the estimated breeding values in daughters of bulls and were linked with the non-return rate, with the effect of LEP-A80V was highly significant (Komisarek, 2010).

According to Giblin *et al.* (2010), association of single nucleotide polymorphisms (SNPs) in leptin gene with reproductive parameters was investigated in Holstein-Friesian sires. Results showed that all SNPs were segregating in that sample population. The LEP-963, LEP-1238, R25C and Y7F SNPs were significantly linked with gestation length, while LEP-963 and R25C SNPs were significantly linked with difficult calving in daughters of bulls. However, G allele of LEP-1238 tended to be associated (P<0.1) with difficulty in calving. Similarly, the allele T (LEP-2470) and G allele of LEP-1238 were significantly linked with calf mortality.

Association of LEP gene (HinfI) markers with litter size (LS), mummified piglets (MM) and weaning to estrus interval (WEI) was evaluated in Landrace and Yorkshire sows. Genotypes of the LEP gene affected LS, MM and WEI significantly. Statistically significant additive and dominance effects were seen only in LEPI gene. Addition of allele T decreased MM, but increased WEI. However, the heterozygous genotype CT was associated with increased LS (p=0.009). These results indicated that LEP gene markers could be used in selection programs in swine (Suwanasopee and Koonawootrittriron, 2011).

Kmieć *et al.* (2003) monitored the effects of HinfI polymorphism of LEP gene on some semen quality traits in AI boars and reported a significant (P≤0.05) predominance of LEP CT and TT genotypic boars with respect to ejaculatory volume, live sperm percentage and sperm concentration. Kanchan *et al.* (2014) examined i*n-vitro* capacitated and fresh ejaculated spermatozoa of buffalo bulls for the existence of leptin gene transcript. On the basis of band intensity, it was noted that gene expression was slightly higher in *in-vitro* capacitated than fresh ejaculated spermatozoa, which suggested the role of LEP gene in the regulation of sperm capacitation.

However, there is relatively little information in the existing literature on leptin gene polymorphism and its relation with reproductive potential of Nili-Ravi buffalo bulls. Therefore, the present study was planned to find out any correlation of single nucleotide polymorphisms of leptin gene with some reproductive traits and semen quality parameters of Nili-Ravi bulls in order to provide the data which could be of use in MAS of buffalo bulls for breeding purposes.

## MATERIALS AND METHODS

**Experimental animals:** This study was conducted at two Semen Production Units (SPUs) managed by the provincial government of Punjab, Pakistan i.e. Qadirabad, District Sahiwal and Kraniwala, District Bahawalpur. Breeding bulls of high genetic potential are kept at these SPUs. Semen collected and processed at these SPUs is supplied to different artificial insemination (AI) centers/ sub centers throughout the Punjab province. A total of 100 adult Nili-Ravi breeding buffalo bulls were selected. However, sequencing results of one bull could not be obtained and the data of 99 bulls was used in the final analysis.

**Scrotal circumference and testicular length measurements:** Measurements were taken with the help of tape measure (Younis *et al.*, 2003).

**Assessment of libido and number of mounts per ejaculate:** Mating behavior of each bull was videotaped and by watching videotapes libido was assessed in terms of reaction time (Dilbar *et al.*, 2014). Reaction time was taken by the bull up to giving the first ejaculation after its entry into the semen collection panel. Similarly, total no of attempts taken by the bull to ejaculate semen were also recorded to calculate the number of mounts per ejaculate.

**Semen collection and evaluation:** Semen samples were collected from each bull twice a week for 10 weeks, by using artificial vagina. After collecting semen, samples were immediately shifted to a water bath at 37°C and evaluated for ejaculatory volume, mass activity, sperm motility percentage, sperm concentration, live and dead sperm percentage. Ejaculatory volume was noted from graduated semen collection tube. For estimation of mass activity, a drop of fresh semen was placed on the warmed glass slide and examined under bright field microscope (Salisbury *et al.*, 1985). By seeing the wave pattern, score of mass activity ranging from 0 to 5 was assigned to every sample (Nazir, 1988). The procedure described by Hulet and Ercanbrack (1962) was used to determine individual sperm motility percentage of semen samples. For this purpose, a semen drop diluted with sodium citrate (2.9%) was placed on the pre warmed slide, covered with a cover slip and examined under the microscope (40X). Spermatozoa which showed linear and forward movement were taken as motile, and those spermatozoa which showed circular movement or were oscillating at one place were taken as non-motile. Sperm concentration in each sample was directly measured by using Accucell Bovine Photometer (Model: 014475, IMV- technologies, France). The eosin-nigrosin staining protocol was adopted for the estimation of live and dead spermatozoa percentage (Swanson and Bearden, 1951).

Then, each sample was diluted with egg yolk diluent and deep frozen, as described previously (Andrabi *et al.*, 2008). Sperm motility percentage after dilution and freezing was also assessed by the similar technique as described for the fresh undiluted semen.

**Collection and processing of blood samples:** About 5 ml blood was taken from every bull in sterilized vials containing EDTA as anticoagulant. These samples were stored at -20°C till further processing for DNA extraction. Standard organic protocol (Sambrook and Russell, 2001) was used for DNA extraction. Amplification was done using simple PCR protocol. The PCR program was set at

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95°C for 5 minutes, followed by 35 cycles at 95°C for 30 seconds, at 54°C for 30 seconds and at 72°C for 60 seconds. Final extension was completed at 72°C for 10 minutes. Once the PCR amplification was completed, Sanger chain termination sequencing method (Sanger *et al.,* 1977) was used for the sequencing of amplified fragment of leptin gene of buffalo bulls.

**Data analysis:** Verification of single nucleotide polymorphism (SNP) was done with the help of Bio-Edit (V7.2.6.1) and genotype frequencies of leptin gene were counted directly. The difference in expected and observed genotypic frequencies was tested by Chi square ($\chi 2$) test to confirm whether the population followed Hardy-Weinberg equilibrium or not. Pearson's correlation coefficients were calculated to describe the association of polymorphism with semen quality parameters.

## RESULTS

Mean values (±SE) for reproductive traits and semen quality parameters of 99 Nili-Ravi buffalo bulls were recorded. Mean values of scrotal circumference and testicular length were 32.07±0.34 and 15.29±0.16 cm, respectively. Mean reaction time was 7.28±0.14 minutes, while overall mean of number of mounts per ejaculate was 2.8±0.02.

Mean ejaculatory volume was 3.09±0.12 ml, while mean mass activity was 1.87±0.09. Similarly, mean values of individual sperm motility percentage and sperm concentration were 62.08±1.68% and 1249.87±29. $31\times10^6$/ml, respectively. Mean values of motility after dilution and freezing semen samples were 67.67±1.19 and 51.93±0.41%, respectively. Mean values of live and dead sperm were 76.38±1.43 and 23.62±1.43%, respectively.

**Sequenced results of leptin gene:** Single nucleotide change of guanine to adenine (G>A) was noted in R159Q polymorphism at positions c.523. As this polymorphism was exonic, the change of codon CGG into CAG was also observed. R159Q polymorphism was non-synonymous. As this change was polymorphic, so all the samples were analyzed and aligned for variation analysis. HWE value and genotype frequencies were calculated using Chi-square test for allele distribution. The observed p-value was non-significant and genotypes distribution was not deviating from Hardy-Weinberg Principle, as shown in Table 1.

Data analysis was done by using Pearson's correlation to find out the associations of R159Q polymorphism of leptin gene with different male reproductive traits and semen quality parameters included in the study. It was noticed that correlation among male reproductive traits and R159Q polymorphism was non-significant (Table 2). Similarly, analysis of the data on semen quality parameters indicated non-significant correlation with R159Q polymorphism, except sperm motility after freezing. Polymorphism R159Q was negatively correlated with sperm motility after freezing (r=-0.22; P<0.05), as shown in Table 3.

## DISCUSSION

Marker assisted selection (MAS) is comparatively modern technique which is used as an alternate to traditional selection of animals for breed improvement through genetic gains. In MAS, DNA based biomarkers are used for early identification of elite animals for production and breeding purposes. Availability of information on genetic makeup and functions of various genes in the data bank is necessary for the modern gene based breeding programs for livestock improvement (Nassiry *et al.*, 2008). Leptin gene (LEP) is one of the potential genes used for the identification of elite animals, leading to improved production (Agarwal *et al.,* 2009).

To *author's* knowledge, leptin gene polymorphism in buffalo bulls and its association with semen quality parameters has not been reported so far. In this study, R159Q polymorphism at position c.523 was noted and it was a heterozygous change (G>A). Codon CGG changed into CAG, which resulted in glutamine production instead of arginine. Frequencies of genotypes were assessed after

**Table 1:** Chi-square test for significance of relation of R159Q polymorphism in Nili-Ravi buffalo bulls

| Leptin gene change | Genotype and Allele | Observed changes | | Expected changes | | Chi-square value | P- value |
|---|---|---|---|---|---|---|---|
| | | n=99 | % | n=99 | % | | |
| R159Q polymorphism | GG | 68 | 68.69 | 70.4 | 71.11 | 3.41 | 0.07 |
| | AG | 31 | 31.31 | 26.1 | 26.36 | | NS |
| | AA | 0 | 0 | 2.4 | 2.42 | | |
| | G | 167 | 84.34 | | | | |
| | A | 31 | 15.66 | | | | |

NS: Non-significant.

**Table 2:** Pearson's correlation coefficients among R159Q polymorphism and various male reproductive traits

| Change | Male reproductive traits | | | |
|---|---|---|---|---|
| R159Q polymorphism | Scrotal circumference | Testicular length | Reaction time | Number of mounts per ejaculate |
| Correlation coefficient | -0.152 | -0.155 | -0.058 | 0.040 |
| Level of sig… | 0.134 | 0.124 | 0.566 | 0.693 |

* = Significant (P<0.05).

**Table 3:** Pearson's correlation coefficients among R159Q polymorphism and various semen quality parameters

| Change | Semen quality parameters | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| R159Q polymorphism | Ejaculatory volume | Mass activity | Individual sperm motility | Sperm conc. | Sperm motility after dilution | Sperm motility after freezing | Dead sperm | Live sperm |
| Correlation coefficient | -0.033 | -0.052 | -0.099 | 0.000 | -0.033 | -0.220* | 0.113 | -0.113 |
| Level of sig… | 0.747 | 0.609 | 0.329 | 0.997 | 0.745 | 0.029 | 0.266 | 0.266 |

* = Significant (P<0.05).

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sequencing the PCR product. When Chi-square test was applied on R159Q SNP, the difference between observed and expected changes was non-significant, indicating that population was in Hardy Weinberg equilibrium (HWE). Orru *et al.* (2007) also reported SNP R159Q as G3434A in Italian and Egyptian River buffaloes. Frequencies of G and A alleles were 0.984 and 0.015%, respectively, while in the present study frequency of G allele was 84.34% and that of A allele was 15.66% in Nili-Ravi buffalo bulls. However, Orru *et al.* (2007) did not find any association of this SNP with productive or reproductive parameters.

In the past, associations of age of the bull and season with reproductive performance parameters such as scrotal circumference and libido of Nili-Ravi buffalo bulls have been checked (Younis *et al.*, 2003). In Current study, analysis of the data on scrotal circumference, testicular length, reaction time and number of mounts per ejaculate of Nili-Ravi buffalo bulls indicated non-significant correlation with R159Q polymorphism. Sadeghi *et al.*, (2014) also noticed a non-significant relation between the detected haplotypes of leptin gene and scrotal circumference in Makooei sheep.

Previously, studies have been carried out to monitor the effect of gene polymorphism on semen quality parameters. In this study, results of the semen quality parameters in buffalo bulls revealed non-significant ($P>0.05$) correlation between leptin gene polymorphism and ejaculatory volume. Kmieć *et al*. (2003) investigated the effect of HinfI polymorphism of leptin gene and reported a significant ($P<0.05$) predominance of LEP CT and TT genotypic boars in respect to ejaculatory volume. However, association of FSH beta gene and prolactin gene polymorphism with semen volume was non-significant (Ghasemi and Ghorbani, 2014; Hasanain *et al.*, 2017).

According to our results on the semen quality parameters, a non-significant correlation was seen between leptin gene polymorphism and mass activity. The same was true for individual sperm motility. Hasanain *et al*. (2017) evaluated the effect of prolactin (PRL) gene polymorphism on semen characteristics of buffalo bulls and reported non-significant association between the genotypes of prolactin gene and the semen quality parameters. Dalvi *et al.* (2018) reported non-significant effect of FSH beta gene polymorphism on seminal attributes in cattle bulls. However, Gafer *et al.* (2015) worked on association of heat shock protein70 (HSP70) promoter gene with semen quality traits in buffalo bulls and reported that bulls with TT genotype of SNP 812 had significantly higher ($P<0.05$) individual sperm motility than bulls with TC genotype. In another study Ghasemi and Ghorbani (2014) reported that AA and AB genotypes of FSH beta gene were significantly associated with fresh sperm motility and total sperm in semen. Our results on semen quality parameters in buffalo bulls indicated non-significant ($P>0.05$) correlation between R159Q polymorphism and sperm concentration. Similarly, Hasanain *et al.* (2017) and Dalvi *et al.* (2018) reported non-significant ($P<0.05$) association of sperm concentration with prolactin and FSH beta gene, respectively. However, Kmieć *et al*. (2003) reported a significant ($P<0.05$) predominance of LEP CT and TT genotypic boars in respect to sperm concentration in their ejaculates.

The present findings presented a non-significant correlation of leptin gene polymorphism with live and dead sperm percentage. However, Gafer *et al.* (2015) reported significant ($P<0.05$) association of TT genotype of heat shock protein70 (HSP70) promoter gene with sperm membrane integrity and viability index in buffalo bulls. In another study, Kianpoor *et al.* (2018) analyzed the association of melatonin receptor 1A (MTNR1A) gene with reproductive parameters in Sanjabi rams. A significant association was observed between CA genotype of MTNR1A and normal sperm morphology ($P<0.05$). However, MTNR1A polymorphism was not associated with most traits of sperm quality. Similarly, live and dead sperm percentage was not associated with aromatase cytochrome p450 gene (Kianpoor *et al.*, 2018), prolactin gene (Hasanain *et al.*, 2017) and FSH beta gene polymorphism (Dalvi *et al.*, 2018).

Correlation between R159Q polymorphism and sperm motility after dilution was also non-significant in our finding. Meanwhile, value of sperm motility after dilution was greater than the mean value of individual sperm motility percentage. This could have been due to the fact that only samples with lower individual sperm motility percentage values were included for the analysis of that trait. However, such samples were excluded for further analysis.

Kanchan *et al.* (2014) examined i*n-vitro* capacitated and fresh ejaculated spermatozoa of buffalo bulls for the presence of leptin gene transcript. On the basis of band intensity, it was noted that gene expression was slightly higher in *in-vitro* capacitated spermatozoa compared to fresh ejaculated spermatozoa, which suggests the role of leptin gene in sperm capacitation. However, in this study, sperm motility after freezing was negatively correlated ($P<0.05$) with G>A change of R159Q polymorphism of leptin gene in Nili-Ravi buffalo bulls. Lower value of sperm motility in the bulls of GA genotype as compared to those of GG genotype indicates that allele 'A' is linked with lower sperm motility after freezing in Nili-Ravi buffalo bulls. In another study, Ghasemi and Ghorbani (2014) determined non-significant association of FSH beta gene with post thaw sperm motility. Similarly, sperm motility after freezing showed non-significant association with prolactin gene (Hasanain *et al.*, 2017) and FSH beta gene (Dalvi *et al.*, 2018).

**Conclusions:** Based on results of the present study, it is concluded that leptin gene polymorphism (R159Q) at position c.523 in Nili-Ravi buffalo bulls is negatively associated ($P<0.05$) with sperm motility after freezing. Further studies are suggested to find the polymorphisms in other regions of leptin gene and their effect on feed intake, energy production, energy consumption and stress tolerance in order to establish the genotypes as a reference for screening and selection of breeding bulls.

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**Authors contribution:** GHD worked as main researcher in this study and performed all experiments. ZIQ and HJ provided the guidance in semen collection and helped in evaluation of reproductive parameters of bulls. TJ guided in collection and transportation of blood samples and helped in analysis of reproductive data. MEB guided and helped in DNA extraction, PCR and analysis of sequencing results. All authors critically reviewed the manuscript.

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