The Haryana Veterinarian (June, 2025) **64(1),** 26-29 ISSN 0033-4359 (P) **Research Article**

MOLECULAR CHARACTERIZATION OF GLUTATHIONE PEROXIDASE 5 EXON-2 IN SAHIWAL BULL (*BOS INDICUS*)

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Received: 15.06.24; Accepted: 18.11.24

ABSTRACT

Lipid peroxidation generates highly reactive malonaldehyde (MDA), which is responsible for sperm DNA damage in the absence of GPX5 protein, preventing the spermatozoa from ROS harmful effect. The study was aimed at the identification of polymorphism in GPX5 gene exon-2. The ~171 bp fragment of exon-2 of GPX5 gene was successfully amplified in *Bos indicus* (Sahiwal) bull. PCR-RFLP of the amplicon was performed by MseI and exon-2 exhibited a uniform restriction pattern, resulting in two fragments of ~114 and ~57 bp. All the Sahiwal bulls showed a similar pattern of allelic profile exhibiting the absence of polymorphism at the Mse I exon-2 locus of the GPX5 gene with monomorphism genotyped as TT with the fixation of the T allele. Results of the study indicated that the "T" allele is a dominant allele in GPX5 exon-2 of Sahiwal bull.

Keywords: GPX5, Polymorphism, ROS, Sahiwal bull

How to cite: Alone, C.D., Shende, A.M., Kannake, A.S., Bonde, S.W., Borkar, S.D., Tembhurne, P.A., Kale, D.S. and Ramteke, S.S. (2025). Molecular characterization of glutathione peroxidase 5 exon-2 in Sahiwal bull (*Bos indicus*). *The Haryana Veterinarian* **64(1)**: 26-29.

Half of the human population pregnancy failures are attributed by male infertility or decreased male fertility (Lee and Foo, 2014), the story of animal breeding is nothing but same (Kwon *et al.*, 2015). Nearly 50 percent of infertility cases are due to genetic defects. Whereas only 50 percent of artificial inseminations result in pregnancies (Park *et al.*, 2012; Park *et al.*, 2013).

Frozen semen straws are used in Artificial Insemination (AI) programs. Freezing imparts physiological stress, which affects the quality of semen (Mohammadzadeh *et al.*, 2020). During the cryopreservation process, semen is subjected to peroxidation damage and oxidative stress due to rapid cooling, leading to the dysfunction of mitochondria, a decline in semen quality, and a decreased conception rate (Wu *et al.*, 2021). Oxidative stress caused by Reactive Oxygen Species (ROS) is balanced by an antioxidants enzyme system consisting of Glutathione peroxidase (GPX), Superoxide dismutase (SOD) and catalase (Kowalczyk, 2022). The porcine GPX5 gene may be used as a potential candidate gene for the genetic improvement of litter size traits in the pig breeding industry (Kumar *et al.*, 2021). Kmiec *et al.* (2007) observed GPX5 polymorphism, pigs with the 2B2B genotype are associated with better semen quality, higher ejaculate volume, live sperm percentage, and live sperm content concerning the 1B1B genotype. Michos *et al.* (2021) reported a significant and positively correlated relationship between GPX5 and farrowing rates in pigs. Noblanc *et al.* (2012) reported that the mice spermatozoa lacking nucleus glutathione peroxidase 4 (snGPX4) and GPX5, showed sperm abnormalities like delayed and defective nuclear compaction, nuclear instability and DNA damage. Hence, GPX5 mainly maintains sperm quality in swines (Polasik *et al.*, 2017, Huang *et al.*, 2024).

The Sahiwal cattle, one of the important milch breed with average lactation milk yield between 1500-2500 kg (Joshi *et al.*, 2001), good/heat tolerance (Madalena *et al.*, 2022) and also resistance to tick parasites (Narwaria *et al.*, 2015) is most suitable in Indian sub-continent and is widely usedby Governmentaswellasfarmersfora breeding program. Therefore, the selection of breeding males is extremely important for the successful fertilization and genetic benefits of progeny in upcoming generations. The polymorphism in the GPX5 gene and its association with gene expression and enzyme stability might be one of the landmark tools for the selection of Elite Sahiwal bull in the artificial insemination program worldwide.

MATERIALS AND METHODS

A total of 50 Sahiwal bulls were randomly selected from the dairy farms around the Nagpur region of Maharashtra, India. About 3 mL of blood was collected aseptically from each animal's jugular vein in EDTA vacutainers and immediately transported to the laboratory in an ice box at 4º C for further processing.

The genomic DNAwas isolated by the Phenol: Chloroform-Isoamylalcohol method (P:C:I in the ratio of 25:24:1) (Sambrook and Russell, 2006). 13 ml chilled RBC lysis buffer added in 2 ml blood sample was mixed and

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centrifuged at 2000 rpm for 15 min at 4º C. The supernatant was discarded again, and 10 ml chilled RBC lysis buffer was added and centrifuged at 2000 rpm for 10 min at 4º C.

The supernatant was discarded, and then the remaining WBC pellet was washed with PBS buffer 3 times and centrifuged at 2000 rpm for 10 min at 4º C. The clear WBC pellet was transferred to a 2 ml microcentrifuge tube. 30 µl of proteinase K (10 mg/ml), 40 µl 20% of SDS and 500 µl TNE buffer were added and the solution was then mixed thoroughly and incubated in a dry water bath for 3 Hr at 55º C. 250 µl phenol and 250 µl chloroform: isoamyl alcohol were added to the above solution and incubated at room temperature for 30 min. followed by centrifuged at 14000 rpm for 25 min at 4º C. The upper aqueous layer was transferred to another microcentrifuge tube, 900 µl of 95% chilled ethanol, 0.3 M sodium acetate pH 5.2 at 1/100 volume were added, mixed and centrifuged at 14000 rpm for 15 min at 4º C. The supernatant layer was discarded and 900 µl 70% ethanol was added, mixed, and centrifuged at 14000 rpm for 10 min at 4º C. The pellet was allowed to air dry for 10-15 min at room temperature, followed by reconstituted in 250 µl nuclease-free water and stored at -20º C till further use. The DNA data bank of Japan (DDBJ) accession number GJ060503 revealed that GPX5 gene is located on chromosome 1 from (1295037. 1303993). Amplification of the GPX5 gene was done using *Bos taurus* (NC_037328) as a reference sequence obtained from NCBI comprising 5 exons with an exon-2 size of 153 bp. The pair of primers (F: GATGGATTGCTACAAAG ATG, R: GACTATGAACTCTTACCAGGA) was designed using online primer-3 (https://bioinfo.ut.ee/primer3-0.4.0/) for a size of 171 bp including partial intron 2 and 3 for getting the maximum length of exon-2 in the study. Amplification of GPX5 exon-2 was done as follows initial denaturation at 95º C for 5 min, denaturation at 95º C for 1min, Annealing at 62º C for 1 min., Extension at 72º C for 45 sec. and Final extension at 72º C for 10 min in a Thermal cycler (Himedia, Prima-TRIO™, India). PCR amplification was analyzed by 2% agarose gel, using 50 bp DNA ladder (Himedia, India). The bands were captured under UV light and the gel appearance was documented by a gel documentation system (SYNGENE G:BOX, USA). The nucleotide sequence of 171 bp was retrieved from genebank by primer used in the study. The sequence was analyzed in-silico for restriction pattern using NEB cutter V2.0 (https://www.labtools.us/nebcutter-v2-0/). The amplicon was further subjected to the restriction fragment length polymorphism (RFLP) analysis by MseI restriction enzyme as follows: PCR amplicon 15 µl, Mse I (10 IU/µL 1 µL, 10X buffer 5 µL and distilled water 29 µL and incubated in the water bath at 37º C for 3 Hrs. Agarose gel electrophoresis of the digested product was done on 2% agarose gel, and 50 bp DNA Ladder (Himedia, India). The bands were captured under UV light, and the gel appearance was documented by the gel documentation system (SYNGENE G: BOX, USA). Further, The PCR products were sequenced at Eurofins Genomics India Pvt. Ltd. (India).

RESULTS AND DISCUSSION

The research studies have reported that the freeze-thaw process may decrease sperm motility by 25-75% after mitochondrial damage in the mid-piece of spermatozoa. The ROS have been responsible for sperm DNA damage during the freeze-thaw process (Najafi *et al.*, 2019). The GPX5 gene is one of the antioxidant genes/enzymes present in sperm, which prevents or protects the spermatozoa from reactive oxygen species (Chabory *et al.*, 2009). GPX5 gene is expressed in the epididymis and spermatozoa (Drevet, 2006), which connects to the sperm surface during epididymal pass-through and protects the spermatozoa from peroxide-mediated attack during their maturation (Aitken, 2009). The above reports suggest that the expression and stability of GPX5 are important to maintain plasma membrane integrity and prevent oxidative damage (Aitken *et al.*, 1998; Chatterjee and Gagnon, 2001). In keeping with the importance of the antioxidant gene GPX5, this study has been carried out to characterize the GPX5 gene using PCR-RFLP in Sahiwal bull.

The integrity of isolated genomic DNA was analyzed by 0.8% agarose gel electrophoresis (Fig. 1), whereas purity and quantification were by Bio Spectrometer (Eppendorf, USA). The sharp high molecular weight bands of DNA indicates that DNA was of good quality and the ratio of the optical density at 260 nm and 280 nm ranged from 1.7 to 1.9, indicating good quality of DNA without contamination (Patel *et al.*, 2007, Suguna *et al.*, 2014, Al-Shuhaib, 2017). The consistent and good quality PCR amplification results were obtained at 62º C annealing temperature having product size ~171 bp for GPX5 exon-2. The nucleotide sequence of 171 bp was analyzed in-silico for restriction pattern using NEB cutter V2.0 revealed, MseI restriction enzyme recognized the sequence (Fig. 2). It cut the DNA sequence at ~114 and ~57 positions. The MseI PCR-RFLP of ~171 bp amplicon revealed only one restriction pattern, resulting in two fragments of ~114 and ~57 bp in all the DNA samples investigated during the study (Fig. 4). Allelic profile showed the absence of polymorphism at MseI exon-2 locus of GPX5 gene in Sahiwal bulls genotyped as 'TT' with only the 'T' allele with a frequency of 1.0.

The obtained sequence data was edited manually using Bio Edit Sequence Alignment Editor and submitted to NCBI (OQ656378) compared with the sequences from the GenBank assembly using the BLAST program

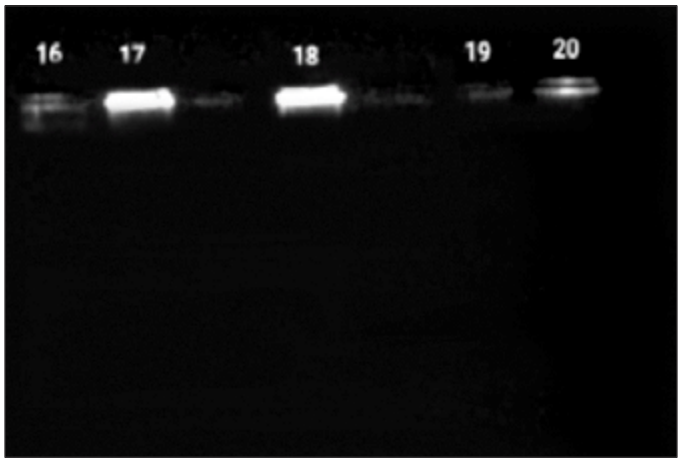

Fig. 1. Analysis of genomic DNA

Fig. 2. Restriction site recognized and digested by MseI enzyme

(www.ncbi.nlm.nih.gov/blast/). It showed 100 per cent identity with Bos taurus (OY997251.1), Bos mutus (CP027091.1), Bos javanicus (XM_061398689.1), Bison bisonbison (XM_010857311.1): whereas 98.71, 98.06 percent identity with *Bubalus kerabau*, *Bubalus bubalis*, respectively and 96.77 per cent identity with *Ovis aries*, *Capra hircus* GPX5 exon-2. The substitution mutations between Bos indicus and Bubalus bubalis need to be characterized for their possible effect on gene stability and protein function. Mackowski *et al.* (2004) found that the 1B2B genotype was related to higher sperm concentration, also suggest that the GPX-5 gene is linked to an unknown QTL controlling sperm production. The above finding suggests that GPX5 exon-2 is well conserved in the Bos genus and similarly affects protein function. Therefore, characterization of the rest of the 4 GPX5 exons is needed to identify polymorphism and their effects on Glutathione peroxidase enzyme functionality and its relation with sperm quality in Sahiwal bulls.

CONCLUSION

The PCR-RFLP of GPX5 gene exon-2 with MseI revealed the site is well conserved and the frequency is one. DNA sequencing of exon-2 in Sahiwal bulls (*Bos indicus*) showed 100 percent identity with *Bos taurus* indicating exon-2 is having a similar effect on protein function in both.

REFERENCES

Aitken, R.J., De Iuliis,G.N. and McLachlan, R.I. (2009). Biological and clinical significance of DNA damage in the male germ line. *Int. J. Androl.* **32(1)**: 46-56.

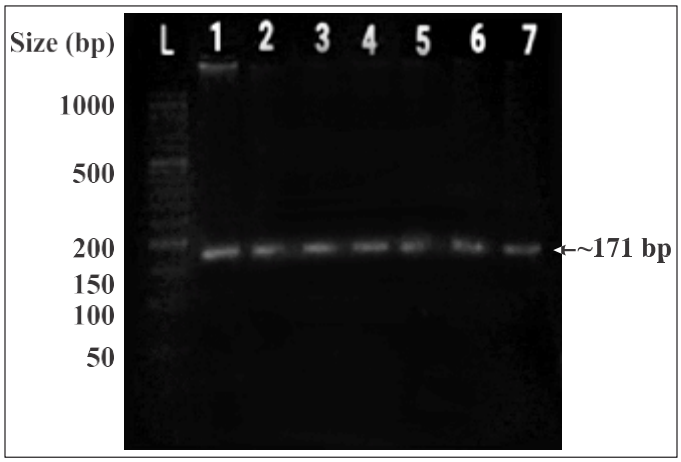

Fig. 3. PCR amplification of GPX5 gene exon-2 (~171bp)

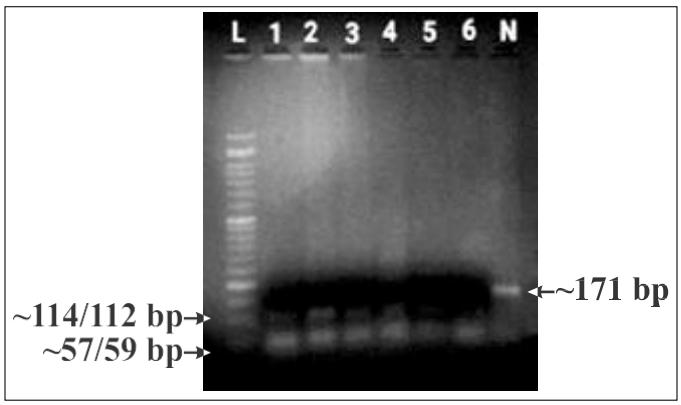

Fig. 4. PCR-RFLP of 171bp GPX5 gene exon-2 with MseI restriction enzyme

Aitken, R.J., Gordon, E., Harkiss, D., Twigg, J.P., Milne, P., Jennings, Z. and Irvine, D.S. (1998). Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol. Reprod.* **59(5)**: 1037-1046.

Al-Shuhaib, M.B.S.A. (2017). A universal, rapid and inexpensive method for genomic DNA isolation from the whole blood of mammals and birds. *J. Genet.* **96(1)**: 171-176.

Chabory, E., Damon, C., Lenoir, A., Kauselmann, G., Kern, H., Zevnik, B., Garrel, C., Saez, F., Cadet, R., Henry-Berger, J., Schoor, M., Gottwald, U., Habenicht, U., Drevet, J.R. and Vernet, P. (2009). Epididymis seleno-independent glutathione peroxidase 5 maintains sperm DNA integrity in mice. *J. Clin. Invest.* **119(7)**: 2074-85.

Chatterjee, S. and Gagnon, C. (2001). Evidence for the production of oxygen free radicals during freezing/thawing of bull spermatozoa. *Mol. Reprod. Dev.* **59(4)**: 451-458.

Drevet, J.R. (2006). The antioxidant glutathione peroxidase family and spermatozoa: a complex story. *Mol. Cell. Endocrinol.* **250(1-2)**: 70-79.

Huang, J., Li S., Yang, Y., Li, C., Zuo, Z., Zheng, R., Chai, J. and Jiang, S. (2024). GPX5-Enriched Exosomes Improve Sperm Quality and Fertilization Ability. *Int. J. Mol. Sci.* **25(19)**: 10569-10586.

Joshi, B.K., Singh, A. and Gandhi, R.S. (2001). Performance evaluation, conservation and improvement of Sahiwal cattle in India. *Ani. Gen. Reso.* **31**: 43-54.

Kmiec, M., Terman, A., Napierała, F. and Polasik, D. (2007). RFLP/ HinfI polymorphism within intron 1 of glutathione peroxidase-5 (GPX5) gene in AI boars. *Arch. Tierzucht.* **50**: 94-199.

Kowalczyk, A. (2022). The role of the natural antioxidant mechanism in sperm cells. *Reprod. Sci.* **29(5)**: 1387-1394.

Kumar, R., Kadirvel, G., Khargharia, G., Deori, S., Doley, S. Das, M., Singh, N.U., Singh, M., Baruah, K.K. and Mishra, V.K. (2021). Polymorphisms in GPX5 gene and its association with production and reproduction traits in niangmegha pigs of eastern Himalaya. *Explor. Anim. Med. Res.* **11(2)**: 145-151.

Kwon, W.S., Rahman, M.S., Ryu, D.Y., Park, Y.J. and Pang, M.G. (2015). Increased male fertility using fertility-related biomarkers. *Sci. Rep.* **5**: 15654.

Lee, L.K. and Foo, K.Y. (2014). Recent insights on the significance of transcriptomic and metabolomic analysis of male factor infertility. *Clin. Biochem.* **47(10-11)**: 973-982.

Mackowski, M., Switonski, M., Mackowska, J. and Perz, W. (2004). Polymorphism of the GPX-5 gene and characteristics of boar semen. *Arch. Anim. Breed.* **47(2)**: 165-171.

Madalena, F.E., Toledo-Alvarado, H. and Cala-Moreno, N. (2022). *Bos indicus* Breeds and *Bos indicus*×*Bos taurus* Crosses. Encyclopedia of Dairy Sciences, (3rd Edn.). **1**: 30-47.

Michos, I., Maria, T., Boscos, C.M., Tsousis, G. and Basioura, A. (2021). Effect of boar sperm proteins and quality changes on field fertility. *Animals.* **11(6)**: 1813-1827.

Mohammadzadeh, M., Khalili, M.A., Ramezani, V., Hamishehkar, H. and Marvast, L.D. (2020). Does resveratrol affect prepared sperm parameters and chromatin quality in normozoospermic and asthenozoospermic patients before and after freezing. A lab trial study. *Int. J. Reprod. Biomed.* **18(9)**: 755-764.

Najafi, L., Halvaei, I. and Movahedin, M. (2019). Canthaxanthin protects human sperm parameters during cryopreservation. *Andrologia.* **51(10)**: e13389.

Narwaria, U.S., Mehla, R.K., Verma, K.K., Lathwal, S.S. and Yadav, R. (2015). Study of short lactation in Sahiwal cattle at organized farm. *Vet. World.* **8(5)**: 690-694.

Noblanc, A., Peltier, M., Damon-Soubeyrand, C., Kerckkove, N. and Chabory, E. (2012). Epididymis response partly compensates for spermatozoa oxidative defects in snGPX4 and GPX5 double mutant mice. *PLoS One.* **7(6)**: e38565.

Park, Y.J., Kim, J., You, Y.A. and Pang, M.G. (2013). Proteomic revolution to improve tools for tools for evaluating male fertility in animals. *J. Proteome. Res.* **12(11)**: 4738-4747.

Park, Y.J., Kwon, W.S., Oh, S.A. and Pang, M.G. (2012). Fertility-related proteomic profiling bull spermatozoa separated by percoll. *J. Proteome. Res.* **11(8)**: 4162-4168.

Patel, G., Patel, R.K., Soni, K.J., Singh, K.M. and Chauhan, J.B. (2007). Detection of ê-casein and â-lactoglobulin variants in holstein Friesian cattle by PCR-RFLP. *The Haryana Veterinarian* **46**: 48-51.

Polasik, D., Terman, A., Kumalska, M., Chomczyńska, O. and Urbanski, P. (2017). Effect of the polymorphism in GPX5 gene on reproductive performance traits in Large White × Landrace crossbreed sows. *Ani. Sci. Papers and Repo.* **35(1)**: 97-102.

Sambrook, J. and Russell, D.W. (2006). Molecular Cloning: ALaboratory Manual. (3rd Edn.), Vol. 1., Cold Spring Harbor Laboratory Press, New York, USA.

Suguna, A., Nandal, D.H., Kamble, S., Bharatha, A. and Kunkulol, R. (2014). Genomic DNA isolation from human whole blood samples by non-enzymatic salting out method. *Int. J. Pharm. Pharma. Sci.* **6(6)**: 198-199.

Wu, C., Dai, J., Zhang, S., Sun, L. and Liu, Y. (2021). Effect of Thawing Rates and Antioxidants on Semen Cryopreservation in Hu Sheep. *Biopreserv. Biobank.* **19(3)**: 204-209.

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