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Association of GSTP1 gene polymorphisms with performance traits in Deoni cattle

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Heat stress directly and indirectly stimulate excessive intra/inter cellular toxicants such as malondialdehyde (MDA) and reactive oxygen species (ROS). Glutathione S-transferase Pi (GSTP1) plays the central role in the detoxification of ROS. In this investigation, we studied the genetic variation in GSTP1 gene using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) technique in 70 Deoni breed cows. All the seven exons of GSTP1 gene were amplified by PCR using a total of six sets of primers. The genetic variants were determined by PCR-SSCP technique. Two unique SSCP patterns were observed in fragment 1, 3, 5 and 6 of GSTP1 gene. Sequence analysis with reference to GenBank Acc. no AC_000186.1 revealed polymorphisms at position 210, 746, 2438, 2439, 2443, 2507, 2695 and insertions between positions 707 and 708, 2700-2701 and 2775-2776. All the observed variations in coding regions were silent mutations. The cows with SSCP pattern B of fragment-5 had higher age at first calving while the cows with pattern A had higher lactation length and lactation yield as compared to pattern B (P≤0.05). The animals with pattern A of fragment 6 in GSTP1 gene had higher lactation length and lactation yield as compared to animals with pattern B. There was no significant difference in enzyme activity and calving interval in cows with different patterns in different SSCP fragments.

Key words: Deoni, GSTP1, polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), single nucleotide polymorphism (SNP), heat stress.

INTRODUCTION

Heat stress has adverse effects on reproduction and milk production of dairy cattle. Milk production in cattle is very susceptible to high ambient temperature due to their weak heat tolerance ability which has strongly restricted the development of dairy industry in tropical regions and caused considerable economic loss (St-Pieree et al., 2003; Bernabucci et al., 2010). Heat stress has large effects on reproduction and milk production in dairy cattle (West, 2003; Ominski et al., 2002; Ahmad and Tariq 2010; Khodaei et al., 2011). It is reported that there was a 0.4 kg decrease in milk production among dairy cattle due to heat stress (Bouraoui et al., 2002). One potential mechanism for the destructive effect on organism is that the heat stress can directly and indirectly stimulates excessive intra/inter cellular toxicants such as malondialdehyde (MDA) and reactive oxygen species (ROS) (Zuo et al., 2000; Yang and Lv, 2006). MDA and ROS have long been considered to be toxic, harmful byproducts of living organisms in an aerobic environment. The heat tolerance ability in animals is partially genetically controlled (Ravagnolo and Misztal,

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Abbreviations: MDA, Malondialdehyde; **ROS,** reactive oxygen species; **HSPs,** heat shock proteins; **GSTs,** glutathione s-transferases; **SNPs,** single nucleotide polymorphisms; **AFC,** age at first calving; **CI,** calving interval; **LL,** lactation length; **LY,** lactation yield; **PAGE,** polyacrylamide gel electrophoresis; **EtBR,** ethidium bromide.

2000). However, along with heat shock proteins (HSPs), it is estimated that glutathione S -transferases also contribute to heat tolerance in animals.

The glutathione S-transferases (GSTs) are a multigene family of dimeric isoenzymes exhibiting a number of catalytic activities, including the conjugation of glutathione with hydrophobic electrophiles and the reduction of fatty acid and DNA hydroperoxides. Based on their amino acid sequence and substrate specificity, 5 classes of GSTs namely GST alpha, mu, pi, theta, zeta have been identified to be involved in conjugation of electrophilic compounds to reduced glutathione (Chronopoulou and Labrou, 2009). Single nucleotide polymorphisms (SNPs) of *GSTP1* gene have been widely reported in some human tumours such as prostate cancer (Millar et al., 1999) and endometrial cancer (Chan et al., 2005). A total of 31 variations were reported among the 15 sequenced Holstein Friesian cattle consisting of 3 deletions, 1 insert, 24 transitions and 3 transversions (Yao et al., 2011). Bovine GSTP1 gene shows > 85% homology of amino acid sequence with human (Hernando et al., 1992). GSTP1 plays positive role under heat stress in controlling cellular toxicants and to alleviate the destructive effect on cattle. Very few reports are available on the association of GSTP1 gene and heat tolerance in cattle. No reports are available on the association of GSTP1 gene variants with productive and reproductive performances of cattle and enzyme activity. Deoni is one of the important dual purpose cattle breed found in parts of Maharashtra, Karnataka and Andhra Pradesh states of India. Hence the present study was undertaken to study the association of GSTP1 gene variation with productive and reproductive performances and enzyme activity in Deoni cattle.

MATERIALS AND METHODS

Animals

The study was conducted on Deoni cattle (N=70) maintained at Cattle yard, NDRI Southern Campus, Bangalore, India. The reproductive performance data viz., age at first calving (AFC) and calving interval (CI) and the productive performance data viz., lactation length (LL) and lactation yield (LY) was collected from history-cum-pedigree sheets.

Isolation of DNA

Genomic DNA was extracted from whole blood by adopting high salt method as described by using modified high salt method (Miller et al., 1988) with minor modifications. After checking the quality and quantity of DNA, it was diluted to a final concentration of 50 ng/µl and stored at -20°C.

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis

A total of six sets of primers were designed based on the 2868 bp sequence for bovine GSTP1 gene (Gen Bank Acc.noAC_000186.1) using Primer 3 software and were procured from Chromous Biotech Bangalore, India to amplify the GSTP1 gene fragments (Table 1). The PCR-SSCP analysis involved PCR amplification of the gene fragments, resolution in non-denaturing polyacrylamide gel electrophoresis (PAGE) and visualization using ethidium bromide (EtBR) staining. The details of the primers, location, annealing temperature and the expected product sizes are summarized in Table1. PCR conditions were optimized for each primer by testing a number of variables such as master mix concentration, thermal cycling conditions among others. The combinations giving the best amplification were used for further studies. PCR amplifications were performed in a 25 µl reaction mixture containing 100 ng of DNA, 3.0 µl 1X PCR buffer, 1.2 µl dNTPs, 10 pM of each primer and 1.0 unit of Taq DNA polymerase. The thermal cycling conditions involved an initial denaturation at 94°C for 5 min, followed by 35 cycles with initial denaturation at 94°C for 1 min, with required annealing temperature (Table 1) for 40 s and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1.5% agarose gel at 100 V for 45 min along with 100 bp DNA ladder and visualized under Gel Doc System (Bio Rad, USA). The amplified GSTP1 PCR products were resolved by SSCP analysis (Figure 1) (Markoff et al., 1997) with minor modifications using vertical slab gel unit (Biometra, USA). Various factors such as the amount of PCR product, denaturing solution, time, acrylamide concentration, glycerol, voltage, running time and temperature were optimized for SSCP analysis. Each PCR product was diluted in denaturing solution (95% formamide, 10 mM NAOH, 0.05% bromophenol blue, 20 mM EDTA), denatured at 95°C for 8 min, chilled on ice and resolved on optimized concentration of non-denaturing polyacrylamide gels. The electrophoresis was carried out in a vertical electrophoresis chamber (SCIE-PLAS, U.K) in 1X TBE buffer. The gels were EtBr stained for 15 min and destained with distilled water for 30 min. EtBr stained SSCP gels were dried and documented for detecting mobility shifts in different fragments of GTP1 gene in Deoni breed of cattle.

Sequencing

The PCR products giving unique SSCP patterns were further analyzed by direct sequencing (Xcelris Pvt. Ltd. Ahmedabad, India). Sequence data was analyzed using DNA Baser and Bio edit software (Hall 1999) Clustal W multiple alignments for detecting SNP's. Our sequences were compared to reference sequence of GSTP1 in NCBI Gene Bank with accession number AC_ 000186.1.

Enzyme estimation

Sigma GST enzyme assay kit was used for the estimation of glutathione S-transferase enzyme activity in the erythrocyte lysate of Deoni cattle. The enzyme activity was estimated as per the manufacturers' guidelines using absorption spectrophotometer by linear kinetic method. Erythrocyte lysate was extracted within 24 h after collection of blood samples in a vacutainer tube contatining 0.5% EDTA. Spectrophotometer was used to read absorbance at 340 nm, using a temperature- regulated cuvette holder set at 25°C. The increase in absorbance is directly proportional to the GST activity. The linearity of the reaction was determined by plotting the absorbance values against time. Change in absorbance (∆A340)/ min was calculated, in the linear range of the plot for the sample and for the blank using the equation:

$$(\Delta A_{340})/\text{min} = \frac{A_{340}\ (\text{final read}) - A_{340}\ (\text{initial read})}{\text{Time (Final time)} - \text{Time (Initial time)}}$$

Table 1. Description of primers used for PCR amplifications of GSTP1 gene.

Fragment	Primer sequence 5'- 3'	Location	Annealing temperature (T_a) (°C)
Fragment-1 (Exon 1 to 2)	F 5'-CACCTTTACCGACTTCCCCGACT-3' R 5'-TTGAACCGGGAAGTAGACGATGGTGTA-3'	45-307	66.0
Fragment-2 (Exon 2 to 3)	F 5'-ACTGGCTTCTTTGTGCATCC-3' R 5'-ATGGCTACGACCTCCTCCTT-3'	248-651	54.0
Fragment-3 (Exon3 to 4)	F 5'-AGAGCTGGAAGGAGGAGGTCGTA-3' R 5'-CGCAGGATGGCATTGGACTGGTACA-3'	624-879	66.0
Fragment-4 (Exon 5)	F 5'-AATGGAGGCGTGTGGAGGTT-3' R 5'-CCACCCAGAACCAGAAGCAGC-3'	1017-1667	56.3
Fragment-5 (Exon 6 to 7)	F 5'-GCCAGGAGGATGATACCCAG-3' R 5'-TGTTTCCCATTGCCGTTGAT-3'	2290-2784	55.3
Fragment-6 (Exon 7)	F 5'-CTCCTTTGCGGACTACAACC-3' R 5'-GTCCTGGGGAAAGGAAGAAC-3'	2601-2846	56.0

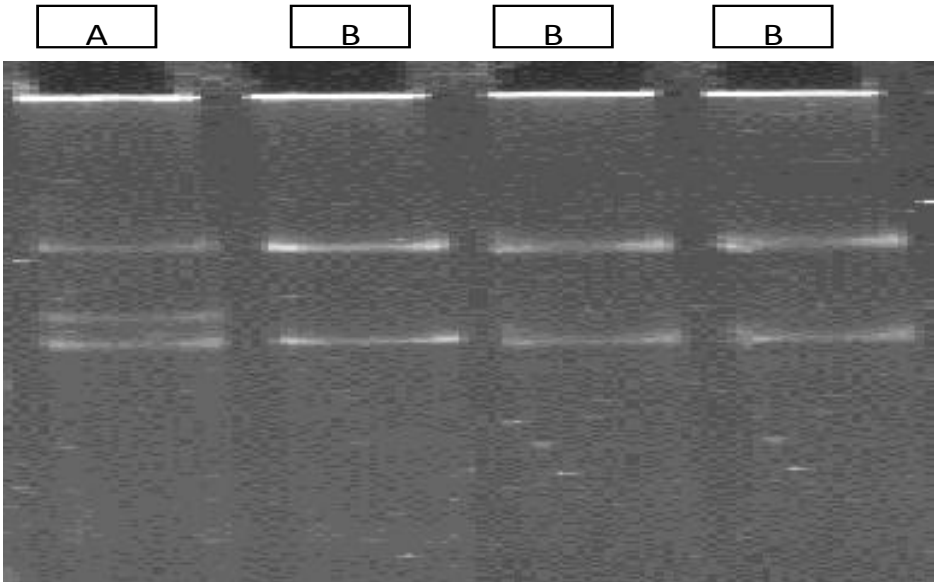

Figure 1. EtBr stained PAGE for PCR-SSCP band patterns (A, B) in fragment-3.

GST specific activity was calculated using the equation:

$$\mu mol\ /ml/min = \frac{(\Delta A_{340})/min \times v\ (ml) \times dil}{\varepsilon mM \times V_{enz}\ (ml)}$$

Where, *dil* is the dilution factor of the original sample; εmM (mM^{-1} cm^{-1}) is the extinction coefficient for CDNB conjugate at 340 nm. Test in 1 ml cuvette = 9.6 mM^{-1} (path length - 1 cm).

Statistical analysis

Statistical procedures were carried out as described by Snedecor and Cochran (1994) and tests were performed using statistical analysis system (SAS) Version 9.2 to find out any significant difference at 5% level ($P \leq 0.05$) (SAS Inc. 2003). The association between GSTP1 gene genetic variants and reproductive performance, productive performance and enzyme activity were analyzed with the general linear model (GLM) procedure of SAS Version 9.2 by using the model:

$$Y_{ij} = \mu + P_i + e_{ij}$$

Where, Y_{ij} is the reproductive/productive/enzyme activity trait of j^{th} animal belonging to i^{th} pattern; μ, is the overall population mean; P_i is the effect of i^{th} pattern; e_{ij} is the random error associated with Y_{ij} observations.

RESULTS AND DISCUSSION

SNPs identification

All the seven exons of GSTP1 gene were amplified by PCR using a total of six sets of primers. The genetic variants were determined by SSCP analysis of amplified

Table 2. Genotypic frequencies and genetic diversity of SSCP fragments of GSTP1.

Number of fragment	Pattern name	I*	Genotypic Frequency
Fragment 1	A (26)	0.6597	0.3714
	B (44)		0.6286
Fragment 3	A (15)	0.5196	0.2143
	B (55)		0.7857
Fragment 5	A (19)	0.5847	0.2714
	B (51)		0.7286
Fragment 6	A (22)	0.6225	0.3143
	B (48)		0.6857

N, Number of animals; I*, Shannon's information index. Figures in parenthesis indicates the number of animals.

PCR products. The PCR-SSCP analysis of GSTP1 gene amplification products revealed varying degree of genetic polymorphism with respect to each of the GSTP1 gene fragments analyzed. PCR-SSCP analysis of fragments 2 and 4 showed monomorphism in Deoni cattle. Thus the GSTP1 gene fragment 2, and fragment 4 showed absence of polymorphisms indicating the lack of mutations and suggesting high degree conservation in the exon 2, 3 and 5 region of GSTP1 gene in Deoni breed of cattle. Analysis of fragments 1, 3, 5 and 6 of GSTP1 gene revealed two unique SSCP patterns in each fragment with different mobility shifts viz. pattern A and pattern B, respectively. Out of the total 70 Deoni animals genotyped, the genotypic frequency of pattern A and pattern B for all the fragments are shown in Table 2 along with calculated Shannon's information index for the each fragment. The genotypic frequencies observed in the present investigation suggests that the Deoni breed of cattle have a diverse type of SSCP patterns for fragments 1, 3, 5 and 6 of GSTP1 gene comprising of exon 1 and 2, exon 3 and 4, exon 6 and 7 and exon 7, respectively in the sampled population indicating the existence of high degree of variability.

Based on the differences in the SSCP patterns, allelic variants were selected and corresponding PCR products were custom sequenced to confirm polymorphisms. Our sequences were compared to sequence of GSTP1 gene in Gen Bank accession number AC_000186.1 for *Bos taurus* cattle by DNA Baser sequence alignment tool. The analysis of fragment 5 comprising of exon 6 and 7 revealed three variations in intron 6 of GSTP1 were G → T transversion at position 2438, C → T transition at 2439 position and C → G transversion at 2443 position, one variation in exon 7 consisting of G → A transition at 2507 position. All observed variations in intron 6 and exon 7 of fragment 5 were silent mutations. The analysis of fragment 6 comprising exon 7 of GSTP1 gene revealed G → A transition at 2695 position which was a silent mutation. Insertions were observed in exon 7 at 2775-2776 position of fragment 5 and 2700 to 2701 position of fragment 6 (Table 3).

Association of SSCP patterns with GST enzyme activity

Association of SSCP patterns with GST enzyme activity was analyzed by using a general linear model (GLM) procedure of SAS system 9.2 version (SAS Inc. 2003). The enzyme activity in erythrocyte lysate of Deoni cattle ranged from 17.8 to 69.59 nmol / ml / min with an overall least squares mean of 46.51 ± 2.20 nmol / ml / min. Analysis revealed lack of association between SSCP patterns in fragments 1, 3, 5 and 6 of GSTP1 gene and enzyme activity.

Association of SSCP patterns with reproductive performance

The reproductive traits viz., AFC and CI were analyzed for association of different PCR-SSCP patterns of GSTP1 gene. The AFC in Deoni cattle ranged from 26 to 69 months with an overall least squares mean of 43.76 ± 1.08 months which is higher than the earlier reports of 38 months by Das et al. (2011) and 40 months by Shergojry et al. (2011) in the same breed and the calving interval in Deoni cattle ranged from 330 to 690 days with an overall least squares mean of 463.27 ± 9.02 days which is higher than earlier reports of 447 days (Singh et al., 2002; Das et al., 2011) and 432 days (Shergojry et al., 2011). Studies on the association of observed SSCP patterns of GSTP1 gene with reproductive traits viz., age at first calving and calving interval revealed no significant difference in age at first calving and calving interval for the fragment 1, 3 and 6 of GSTP1 gene. The animals with pattern B of fragment-5 in GSTP1 gene had higher AFC (45.43 ± 1.20 months) as compared to animals with pattern A (38.31 ± 2.00 months ($P \leq 0.05$) (Table 4). The results indicate that pattern A had positive influence on AFC. Further, the number of animals with A pattern was very few. Thus there is scope for decreasing AFC using animals possessing A pattern in fragment 5. The calving interval was similar in cows with different patterns (A, B) in fragment 5 of GSTP1 gene. No earlier reports are available to compare or contrast the present findings.

Table 3. Summary of Single nucleotide polymorphisms observed in GSTP1 gene in Deoni cattle.

Gene	Segment	Position[a]	Variation[b]
GSTP1	Intron 1 of fragment 1	210	GGGCC G/AGTGTC
	Intron 3 of fragment 3	707-708	CCCGTCCCCGG
		746	TGGGAG/AGCGCA
	Intron 6 of fragment 5	2438 and 2439	GGTGAG/TC/TGGCCC
	Intron 6 of fragment 5	2443	GCTGGC/GCCCAC
	Intron 6 of fragment 5	2507	CACGGG/AAGTCA
	Intron 6 of fragment 5	2695	CCTAC G/A TGGCC
	Exon 7 of fragment 5	2775-2776	GCAATGGGGGGA
			GCAATGGGGGA
	Exon 7 of fragment 6	2700-2701	TGGCCCTCGTCT

[a]Based on the sequence from the NCBI GenBank accession number AC_000186.1; [b], polymorphic residues underlined (common nucleotide followed by the variant).

Table 4. Effect of SSCP patterns in fragment- 5 of GSTP1 gene on reproductive performance in Deoni cattle.

Dependent variable	Pattern	LSM ± S.E
Age at fight calving (months)	A (19)	38.31 ± 2.00[a]
	B (51)	45.43 ± 1.20[b]
Calving Interval (days)	A (19)	456.05 ± 14.80[a]
	B (51)	467.82 ± 10.95[a]

Superscripts with different alphabets (a,b) differ significantly (P ≤ 0.05). Figures in parenthesis indicates the number of animals.

Table 5. Effect of SSCP patterns in fragment 1, 5 and 6 of GSTP1 gene on productive performance in Deoni cattle.

Number of fragment	Dependent variable	Pattern	LSM ± S.E
Fragment 1	Lactation Length (Days)	A (26)	263.19 ± 214.48[a]
		B (44)	225.68 ± 14.60[a]
	Lactation Yield (Kg)	A (26)	1244.96 ± 102.83[a]
		B (44)	959.48 ± 92.6[b]
Fragment 5	Lactation Length (Days)	A (19)	282.58 ± 16.59[a]
		B (51)	223.61 ± 12.84[b]
	Lactation Yield (Kg)	A (19)	1390.45 ± 117.85[a]
		B (51)	944.27 ± 81.46[b]
Fragment 6	Lactation Length (Days)	A (22)	271.77 ± 13.89[a]
		B (48)	224.87 ± 13.89[a]
	Lactation Yield (Kg)	A (22)	1299.82 ± 110.70[a]
		B (48)	957.93 ± 86.81[a]

Superscripts with different alphabets (a,b) differ significantly (P ≤ 0.05). Figures in parenthesis indicates the number of animals.

Association of SSCP patterns with productive performance

The productive parameters viz., lactation length (LL) and lactation yield (LY) were analyzed for association of different PCR-SSCP patterns of GSTP1 gene (P ≤ 0.05). The lactation length in Deoni cattle ranged from 75 to 386 days with an overall least squares mean of 239.61 ± 10.79 days and the LY ranged from 235 to 2549 kg with an overall least squares mean of 1065.38 ± 71.11 kg. The observed SSCP patterns in fragments 1, 3, 5 and 6 of GSTP1 gene were associated with productive traits viz., lactation length and lactation yield and analysis shown that the animals with pattern A of fragment 1 in GSTP1 gene had higher Lactation yield (1244.96 ± 102.83 kg) as compared to animals with pattern B (Table 5). There

was no significant difference in lactation length and lactation yield between the two patterns in fragment 3. The animals with pattern A of fragment 5 in GSTP1 gene had higher lactation length (282.58 ± 16.59 days) as well as higher lactation yield (1390.45 ± 117.85 kg) as compared to animals with pattern B (Table 5). The animals with pattern A of fragment 6 in GSTP1 gene had higher LL (271.77 ± 13.89 days) and LY (1299.82± 110.70 kg) as compared to animals with pattern B (Table 5). As the insertion mutations can change the amino acid sequence, the insertions observed in fragments 5 and 6 are majorly responsible for the significant variations in productive performance of the animals.

In conclusion, the present study indicate the usefulness of genetic characterization of GSTP1 gene in association studies between productive, reproductive and enzyme activity traits and SSCP patterns. The results indicate that the GSTP1 gene in Deoni breed of cattle is highly conserved. Further studies on SNPs in particularly in fragment 5 covering exon 6 and exon 7 of GSTP1 gene using large number of animals is required to develop prognostic markers for heat stress in cattle.

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