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Molecular characterization of the *HSPA1A* gene by single-strand conformation polymorphism and sequence analysis in Holstein-Friesian crossbred and Deoni cattle raised in India

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Abstract: A group of proteins called heat shock proteins (HSPs) are synthesized during heat stress. They are expressed at high levels when exposed to stress. Polymorphism in HSPs has been reported to be associated with heat tolerance and reproductive performance in cattle. The present study aimed to investigate the polymorphisms in the coding region of the *HSPA1A* gene in Holstein-Friesian (HF) crossbreds and the Deoni breed of cattle. Genomic DNA was extracted from 94 animal blood samples and was subjected to polymerase chain reaction-single strand conformation polymorphism analysis, which revealed 14 band patterns in Deoni cattle and 8 band patterns in HF crossbreds. Sequence data were analyzed using BioEdit software for detecting single nucleotide polymorphisms. Sequence analysis showed 12 single nucleotide polymorphisms in the coding region of the *HSPA1A* gene, which included 5 transitions (G456A, A972G, A1098G, C1766T, and G1788A) and 2 transversions (C312G and G2033C) in Deoni cattle and 2 indels (C at positions 574–575 and 624–625), 2 transitions (A480G and A1098G), and 1 transversion (C312G) in HF crossbred cattle. The study indicated a high degree of genetic variability in the *HSPA1A* gene in the cattle populations under study.

Key words: *HSPA1A* gene, Deoni cattle, Holstein-Friesian crossbreds, genetic variability, single-strand conformation polymorphism analysis

1. Introduction

Heat shock proteins (HSPs) form a primary system for intracellular self-defense. Their function is necessary for the homeostasis of the living cell and becomes especially important in a stressful environment. They are highly conserved and apparently ubiquitous among eukaryotic organisms. Nonetheless, the genes encoding HSPA1A (HSP 70) are chromosomally dispersed, may or may not contain introns, and may be constitutively expressed or inducible by a variety of cellular stressors (1,2). HSPA1A is reported to protect cells, tissues, and organs from stress by promoting the folding of nascent polypeptides and by correcting the misfolding of denatured proteins (3). Heat shock-induced HSPA1A expression has a role in the antiapoptotic pathway (4). HSPs are grouped in families according to their molecular weight, and the HSPA1A family consists of proteins of around 70 kDa (5). *HSPA1A* is a single exon gene consisting of 1926 nucleotides located on chromosome 23 of bovine (BTA 23). *Bos indicus* cattle survive and perform better under heat stress compared to temperate breeds or their crossbreeds (6). Das et al. (7) reported that the Deoni breed is one of the important dual-purpose breeds of the southern region of India, having specific qualities like disease resistance, heat tolerance, the ability to survive and reproduce under stress, low feed input, and the potential for improvement in dairy traits. Holstein-Friesian (HF) crossbreds are reared for high milk production in India and they suffer from heat stress during the summer.

The genetic variation in HSP genes among breeds and the central role that HSPA1A has in coordinating thermal tolerance suggest that *HSPA1A* is a candidate gene for identification of genetic markers and that there is an opportunity to improve thermal tolerance through marker-assisted selection (8). We conducted a study to evaluate the genetic variability in the coding region and to identify single nucleotide polymorphisms (SNPs) in the *HSPA1A* gene in Deoni cattle and HF crossbred cattle.

2. Materials and methods

2.1. Experimental animals and DNA isolation

Deoni cattle are medium-sized dual-purpose *Bos indicus* cattle found in parts of the Maharashtra, Karnataka, and

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Andhra Pradesh states of India. Blood samples (8–10 mL) were collected aseptically by jugular vein puncture using vacutainers containing over 15% ethylene diamine tetraacetic acid from Deoni cows (n = 56) maintained at the Southern Regional Station (SRS) of the National Dairy Research Institute (NDRI), Bangalore, and from HF crossbreds (n = 38) maintained by progressive farmers in the Bangalore district.

Genomic DNA was isolated by the high-salt method as described by Miller et al. (9), with minor modifications. The quality and quantity of DNA was checked by agarose gel electrophoresis and UV spectrophotometer. The samples showing an optical density (OD) ratio (260/280 nm) of between 1.6 to 1.8 were stored at –20 °C, used for further analysis, and diluted to 100 ng/µL for utilization as a DNA template in polymerase chain reaction (PCR).

2.2. Synthesis and confirmation of PCR amplification

Based on the bovine *HSPA1A* gene sequence (NCBI accession number U09861), 5 sets of overlapping primers were designed using Primer3 (V.0.4.0) online software (10) for amplifying the entire coding region of the *HSPA1A* gene and were procured from Eurofins MWG Operon, Bangalore, India. The details of the primers, location, annealing temperature, and expected product sizes are summarized in Table 1. PCR conditions were optimized for each primer set. PCR was carried out on about 100 ng of genomic DNA in 25 µL per reaction volume. The PCR reaction mixture consisted of 200 µM of each dNTP, 10X Taq polymerase assay buffer, 1 U of Taq polymerase enzyme, and 20 pM of each primer. The thermocycler conditions began with an initial denaturation at 94 °C for 2 min, followed by 35 cycles with denaturation at 94 °C for 30 s with varying annealing temperatures based on a specific pair of primers (Table 1) and an extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were electrophoresed at 100 V in 1.5% agarose gel in 1X TBE buffer containing 0.5 µg/mL ethidium bromide along with a DNA molecular size marker. The gels were visualized and documented using a gel documentation system (Gel Doc 1000, Bio-Rad, USA).

2.3. PCR-SSCP analysis

The genetic variants were determined by single strand conformation polymorphism (SSCP) analysis. Ten microliters of amplified PCR products were further diluted in denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue, and 20 mM EDTA) and denaturation was carried out at 94 °C for 8 min followed by rapid chilling on an ice block for 20 min and loaded on 10% acrylamide:bisacrylamide (29:1) in 1X TBE buffer for 6 h (200 V) at 4 °C. The gels were silver-stained as described by Sambrook and Russell (11). Band patterns were characterized by the number of bands and mobility shifts, and each pattern was scored manually. Each single strand conformation polymorphism pattern was carefully chosen and the 72 PCR products showing different band patterns were custom sequenced using an automated ABI DNA Sequencer (Amnion Biosciences Pvt. Ltd., Bangalore, India) to confirm the mobility shift in each pattern. Sequence data were analyzed using BioEdit software (12) for detecting SNPs.

3. Results

In the present investigation, PCR-SSCP analysis of 5 fragments that covered the entire coding region of the bovine *HSPA1A* gene was carried out to determine the genetic variability in the *HSPA1A* gene. After silver staining of gels, different SSCP band patterns in various fragments were scored manually. The analysis revealed 14

Table 1. Primer sequence, location, product size, and annealing temperature of the *HSPA1A* gene.

Gene fragments	Primers (5'→3')	Primer location	Product size (bp)	Ta (°C)
Fragment 1	F- TATCTCGGAGCCGAAAAGG R-TCATCTTGGTCAGCACCATC	125–538	413	62
Fragment 2	F-TGGTGCTGACCAAGATGAAG R-GTGCTGGACGACAAGGTTCT	520–989	469	60
Fragment 3	F-GCCAAGAGAACCTTGTCGTC R-CTCGTACACCTGGATCAGCA	963–1488	525	60
Fragment 4	F-TGCTGATCCAGGTGTACGAG R-TTCTTGGCAGACACCCTCTC	1468–1775	307	58
Fragment 5	F-GAGAGGGTGTCTGCCAAGAA R-ACATGAGCAATCCAGGGAAG	1755–2107	352	60

F = Forward, R = reverse, Ta = annealing temperature.

band patterns in Deoni cattle while 8 band patterns were detected in HF crossbred cattle. Among the 14 observed band patterns, 6 bands were common in both Deoni and HF crossbred cattle, while 8 bands were unique to Deoni cattle and 2 bands were unique to HF crossbred cattle. The band patterns of the *HSPA1A* gene and their respective frequencies in Deoni and HF crossbred cattle are shown in Table 2. Fragment 1 showed 3 band patterns, named as A, B, and C (Figure 1a), in Deoni cows, while only 2 types of band patterns, named as E and F, were found in HF crossbred cattle (Figure 1b). Fragments 2, 3, and 5 showed 5, 3, and 3 SSCP band patterns, respectively, in Deoni cows, and 2 band patterns were observed in fragments 1, 2, 3, and 5 in HF crossbred cattle. Fragment 4 was monomorphic in both Deoni and HF crossbred cattle. In fragment 1, the genotypic frequency of the A band pattern

Table 2. Different single-strand conformation polymorphism band patterns and genotypic frequency of the *HSPA1A* gene in Deoni and HF crossbred cattle.

Fragment number	Type of band patterns observed, Deoni	Genotypic frequency	Type of band patterns observed, HF crossbreds	Genotypic frequency
1	A	0.79	E	0.80
	B	0.11	F	0.20
	C	0.10		
2	A	0.32	A	0.55
	B	0.04	B	0.45
	C	0.27		
	AC	0.24		
	D	0.13		
3	A	0.50	A	0.90
	B	0.37		
	AB	0.13	AB	0.10
4	Monomorphic	--	Monomorphic	--
5	A	0.51	A	0.40
	B	0.44		
	AB	0.05	AB	0.60

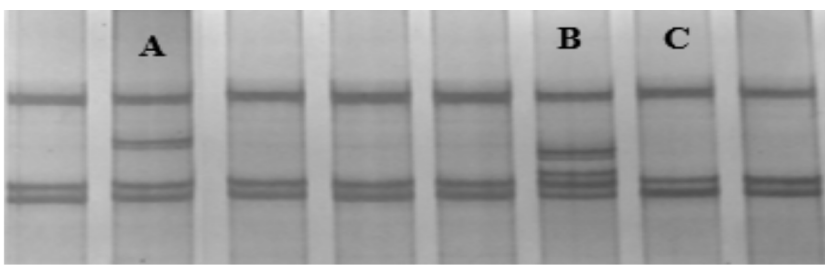

Figure 1a. Detection of 3 different SSCP band patterns in fragment 1 of the *HSPA1A* gene in Deoni cattle.

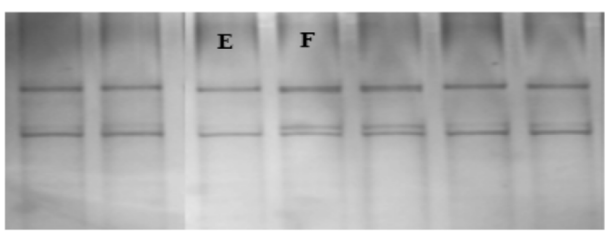

Figure 1b. Detection of 2 different SSCP band patterns in fragment 1 of the *HSPA1A* gene in HF crossbred cattle.

was 0.79 in Deoni cattle. In HF crossbred cows genotypic frequency of the E band pattern was 0.80. In fragment 2, the genotypic frequency of the A pattern was higher in HF crossbred cows as compared to Deoni cows. In fragment 3, the genotypic frequency of the A pattern was 0.90 in HF crossbred cattle while the frequency of the A pattern in Deoni cows was 0.50. In fragment 5, the genotypic frequency of pattern AB was 0.60 in HF crossbred cattle, whereas in Deoni cattle the pattern appeared with a low frequency of 0.05 (Table 2).

Comparison of the observed sequence with the bovine *HSPA1A* gene sequence (NCBI accession number U09861) revealed 7 SNPs in Deoni cattle and 5 in HF crossbred cattle. The base change, location, and amino acid change in the protein have been summarized in Table 3.

Studies aimed at SNP discovery in the coding region of the *HSPA1A* gene showed 7 SNPs including 5 transitions (G456A, A972G, A1098G, C1766T, and G1788A) and 2 transversions (C312G and G2033C) in Deoni cattle, and 5 SNPS including 2 indels (C at positions 574–575 and 624–625), 2 transitions (A480G and A1098G), and 1 transversion (C312G) in HF crossbred cattle, indicating a high degree of genetic variability in the *HSPA1A* gene in the population under study. Sequence data obtained in the study of *HSPA1A* have been submitted to the European Molecular Biology Laboratory Nucleotide Sequence Database (Accession No. HF559382 for *Bos indicus* partial *HSPA1A* gene and Accession No. HF559383 for *Bos taurus* partial *HSPA1A* gene).

The observed SNPs in fragment 1 at position 312 (C→G) in Deoni cattle and HF crossbreds, 456 (G→A) in Deoni cattle, and 480 (A→G) in HF crossbreds were silent mutations. In the coding region of fragment 2 at base position 574–575 an insertion of C was detected in HF crossbred cattle, which resulted in no amino acid change in the translated product, whereas at base position 624–625, an insertion of C was detected in HF crossbred cattle, which exhibited altered amino acid from alanine to arginine in the peptide chain (Table 3). In fragment 3, the observed base changes at positions 972 (A→G) in Deoni cattle and 1098(A→G) in Deoni and HF crossbred cattle were detected, which resulted in silent mutations. In fragment 5, detected base changes (Figure 2) at positions 1766 (C→T) and 2033(G→C) in Deoni cattle resulted in an altered amino acid change from serine to phenyl alanine and glycine to alanine, respectively (Table 3), and 1788 (G→A) conversion in Deoni cattle exhibited silent mutation.

4. Discussion

In the present study, 7 SNPs, including 5 transitions and 2 transversions, were detected in the coding region of the *HSPA1A* gene in the Deoni breed of cattle, while 5 SNPs, including 2 indels, 2 transitions, and 1 transversion, were observed in HF crossbred cattle, indicating a high degree of genetic variability in the *HSPA1A* gene in the breeds under study. Rosenkrans et al. (13) reported that SNPs in the promoter region of the bovine *HSPA1A* gene resulted in 11 SNPs, which included 1 deletion at position 895, 7 transitions (G1013A, G1045A, C1069T, A1096G, G1117A, T1134C, and T1204C,) and 3 transversions (A1125C, G1128T, and C1154G), in crossbred Brahman

Table 3. SNPs in the *HSPA1A* gene of Deoni and HF crossbred cows.

Fragment	Breed	Polymorphism[A]	Amino acid change[B]
1	Deoni and HF crossbred	C312G	Gly (no change)
1	Deoni	G456A	Lys (no change)
1	HF crossbred	A480G	Lys (no change)
2	HF crossbred	574–575IC	Thr (no change)
2	HF crossbred	624–625IC	Ala to Arg
3	Deoni	A972G	Arg (no change)
3	Deoni and HF crossbred	A1098G	Leu (no change)
5	Deoni	C1766T	Ser to Phe
5	Deoni	G1788A	Ser (no change)
5	Deoni	G2033C	Gly to Ala

[A]G- Guanine; A- adenine; C- cytosine; T- thymine; I- insertion.
[B]Based on the transeq predict database of EMBL-EBI, Thr- threonine; Ala- alanine; Arg- arginine; Leu- leucine; Gly- glycine; Ser- serine, Phe- phenylalanine; Lys- lysine.

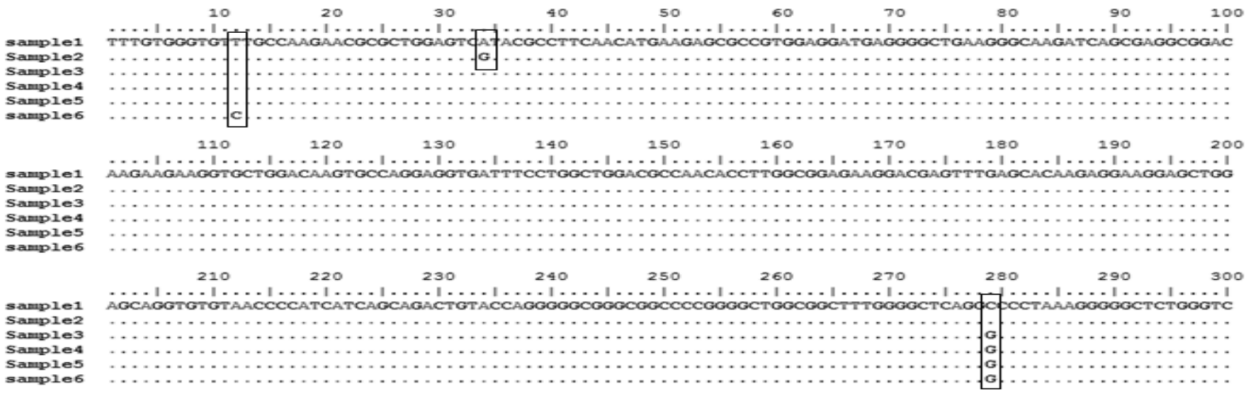

Figure 2. Multiple sequence alignment of fragment 5 (*HSPA1A* gene) showing 3 SNPs, i.e. C1766T transition, G1788A transition, and G2033C transversion, respectively. Reference sequence (NCBI accession number U09861).

cows. Among them, the base positions 895, 1125, and 1128 were related to calving percentages. The deletion of cytosine at base position 895 had the greatest effect on the average Julian calving date. Only 8% of cows homozygous with the cytosine deletion calved, and those cows that calved had an average calving date of 109 days, which was approximately 35 days longer than cows without the deletion. An earlier report suggested that HSP gene expression was related to embryonic survival and overall pregnancy success (14). SNPs of the *HSP70* gene, such as the base change at G2033C of the *HSPA1A* gene that results in an amino acid change from glycine to alanine in the translated products, had an effect on milk yield and milk content (15). Li et al. (16) identified 5 novel SNPs in the CDS and 3′-UTR region and 11 different genotypes in the *HSP70.1* gene of Chinese Holstein cattle. Sodhi et al. (17) reported a total of 54 SNPs among 3 species in the *HSP70.1* gene. Hansen et al. (18) reported that high thermal temperatures can negatively impact fertility in cattle, and there is evidence that the oocyte and embryo are targets of heat stress. Paula-Lopes et al. (19) reported that breed differences between *Bos indicus* and *Bos taurus* cattle contribute to heat tolerance and improved fertility under heat stress conditions. Muralidhar et al. (20) reported that Indian breeds have specific qualities like disease resistance, heat tolerance, ability to survive and reproduce under stress, and low feed input. Blackshaw et al. (6) also reported that the indigenous (*Bos indicus*) cattle survive and perform better under heat stress as compared to temperate breeds or their crossbreeds. Earlier studies showed association between observed SNPs in the *HSPA1A* gene and economically important traits like heat tolerance and reproductive performance in cattle (13,15). Our findings in this study indicate that there is high variability in the *HSPA1A* gene in Deoni and HF crossbred cattle, which suggests that *HSPA1A* could be used as a candidate gene for identifying markers for heat tolerance. Further studies on the association between SNPs in the *HSPA1A* gene with heat tolerance and economically important traits could result in identification of markers for thermotolerance.

Screening of Deoni and HF crossbred cattle for SNPs in the coding region of the *HSPA1A* gene revealed 7 SNPs in Deoni and 5 SNPs in HF crossbred cattle. Polymorphisms identified in the *HSPA1A* gene could be a step towards identification of genetic markers for selecting cattle for heat tolerance.

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