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Identification of genetic variants in *HSF1* gene and their association with heat tolerance in Murrah buffaloes

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ABSTRACT

The study was carried out to identify Single Nucleotide Polymorphism (SNPs) and their association with thermos-tolerance traits in 200 murrah Buffalos. Genomic DNA was extracted from frozen/thawed blood samples collected in Beckton-Dickinson vacutainer containing 0.5% (10 µl/ml of blood) anticoagulant EDTA, using phenol-chloroform extraction method. Further the samples were processed for checked quality of DNA on 0.8% agarose gel electrophoresis while its quantification was done using Biospec-nano spectrophotometer method. Custom sequencing results revealed 7 SNPs at the position of A15732G located in intron 4, C17061T in intron 6, C17202T and A17226G in intron 7, G17454A in intron 8, C17605T in exon 9 and T18421C in intron 9 of *HSF1* gene sequence. Association analysis showed that the thermal tolerance trait in Murrah buffaloes was significantly affected by three SNP locus, viz. A15732G, C17061T and T18421C. The association among the different genotype of this SNP locus with thermo-tolerance was analyzed using Generalized Linear Model procedure in Statistical Analysis System. Animals of GG genotype at locus A15732 G, TT genotype at locus T18421C and C17061T locus showed lower respiration rate and least HTC was observed in animals belonging to GG genotype at locus A15732G. At linkage, disequilibrium and haplotype construction were analysed using SHEsis software. Haplotypes (49) were constructed, and out of these seven haplotypes (>3 sample size) were considered for association studies. The individuals with Hap6 (ACCAGCC) haplotype combination had lower respiration rate (RR) than other haplotype combinations and these individuals may have better thermal adaptability in comparison to others animals.

Key words: Haplotype, Heat shock protein, HSF1, SNP, Thermo-tolerance traits

Global warming has negative impact on productivity of livestock, especially on buffaloes due to their sensitivity to temperature variations. Buffaloes have poor heat tolerance capacity compared to other domestic ruminants which could be due to scarcely distributed sweat glands, dark body colour and sparse hair on body surface. However, minimization of effects of thermal stress in animals through modification in feeding and housing system is practiced with short period success (Armstrong 1994, West 1999 and Berman 2008). So, long term success could be possible through genetic evaluation of dairy animals for economic traits along with heat tolerance for sustainability in dairy farming system (Boonkum *et al*. 2011). The 'thermo-tolerance' trait is a quantitative trait as controlled by multiple genes (Gaughan *et al*.2010, Li *et al*. 2011 and Liu *et al*. 2011). However, only one report on *HSF1* gene polymorphism associations with thermo-tolerance traits is present for Chinese Holstein Cattle (Li *et al*. 2011). Selection of thermally adapted animals is very important in tropical and subtropical climates as global warming continuously causes negative impacts on animal productivity.

Heat shock factors (HSFs) are master transcriptional regulators activated by various proteotoxic stress stimuli and regulate stress-inducible synthesis of HSPs during development, growth and adaptation (Morimoto *et al*. 1994). *HSF1* gene has been mapped on *Bos Taurus* autosome no.14 (BTA14) and spans about 19.7 kb including 12 introns and 13 exons, coding sequence of 2021 bps nucleotide with 525 amino acids. *HSF1* is activated by external stimuli and combined with the promoter of HSPs, switch on gene transcription and express heat shock protein. Scanty information was available regarding association of *HSF1* with thermo-tolerance trait. So, the present study is carried out with the objectives to identify the SNPs and association study was done with physiological parameters (RR, RT and HTC) to select the best heat tolerance animal that can be used as marker for selection of better thermo-tolerance animals.

MATERIALS AND METHODS

The study was carried out in 200 randomly selected clinically healthy Murrah buffaloes maintained at the

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Livestock Research Centre, National Dairy Research Institute, Karnal, Haryana (29.68°N 76.98°E). The experiment was approved by institutional animal ethics committee.

About 10 ml of blood was collected from the animal in a sterile Beckton-Dickinson vacutainer containing 0.5% (10 µl/ml of blood) anticoagulant Ethylene Diamine Tetra Acetic Acid (EDTA) and stored at –20°C. Genomic DNA was extracted from frozen/thawed blood samples using phenol–chloroform extraction method described by Sambrook and Russell (2001) with minor modifications. Quality of DNA was checked on 0.8% agarose gel electrophoresis while its quantification was done using Biospec-nano spectrophotometer method. The DNA sample with OD_{260} and OD_{280} ratio of 1.8 was considered good and utilized for further study. The DNA was diluted to a final concentration of 50 ng/µl and stored at –20°C.

Physiological data recording: Respiration rate (RR) and rectal temperature (RT) of animals were recorded in winter (January, 6–8 AM), spring (March, 12–2 PM) and summer (June, 12–2 PM) three times consequently and average was taken as final reading for association analysis. Heat tolerance coefficient (HTC) which is measure of heat load intensity was also calculated with the following equation developed by Benezra (1954).

HTC (Benezra Coefficient of Heat Adaptability) =
RR/23 + RT\38.33

In the equation, the denominator 23 and 38.33 are normal RR and RT under ideal conditions. According to Benezra (1954) lower the value determined by the equation, higher the degree of adaptability.

Temperature-humidity index (THI) is a combined effect of environmental temperature and relative humidity which were calculated for all days in three seasons, viz. winter (69.92), spring (71.15) and summer (69.80) during which physiological parameters were recorded and the value was determined based on temperature of wet bulb (Wb) and dry bulb (Db). The following formula was used for calculation of THI value developed by National Research Council, 1971.

$$THI = 0.72\ (Wb + Db) + 40.6$$

where Wb, wet bulb; Db, dry bulb.

Primer designing and PCR amplification: Primers (6 sets) were designed by primer 3.0 Software and synthesized by Eurofins Genomics Pvt. Ltd. Company (India) which is illustrated in Table 1 along with the different annealing temperature of *HSF1* gene (Ensembl gene ID: ENSBTATOOOOO034656.2). Each PCR tube containing 25 µl of reaction volume consisted of template DNA of 3 µl (50 ng/µl), 0.5 µl of forward (F) and reverse (R) primer, PCR Master Mix (2×) (Fermentas) of 12.5 µl and 8.5 µl of water. Amplification of DNA was performed in a Thermal cycler (Bio-Rad T100). The PCR condition consisted of an initial denaturation step at 95°C for 3 min, followed by 34 cycles of denaturation at 94°C for 30 Second, annealing temperature standardized for different primers (Table 1) and extension at 72°C for 25 sec for all the targeted region of the *HSF1* gene. The reaction was terminated by a final extension at 72°C for 8 min. The PCR products were run in 2% agarose gel electrophoresis stained with ethidium bromide for confirmation of amplification. Further, amplified PCR products were directly sequenced by outsourcing (Merck Specialties Pvt. Ltd. Bengaluru) for detection of identification of SNPs in the population. The raw sequence was analyzed and also ClustalW multiple sequence alignment programme was used to align respective sequence with reported *Bos taurus* sequence (ENSBTAT00000027654.4) to detect any nucleotides changes.

Table 1. Primer Sequences, targeted region and amplicon sizes of *HSF1* gene

Set	Primer Sequence, 5'to 3'	Product length (bp)	Melting Temperature (T_m)
1	F GACCCATCATCTCCGACATC R CCTGGAAGGCACTCACTTGT	443	64
2	F CGGGCCCTTTCTGCTATATCC R CGAAGTTCTTTCTGGAACCCT	337	56.1
3	F GGGCAGGCCTAATCTAT R ATGGCTTGTCAGCATGGTC	453	58.5
4	F TCTTCCCTGCAGCTGTTC R CTGAGTCTGGGCTGCTTT	238	56.9
5	F GCTCATCCAGTTCCTCATCTC R CTCCTCCTTTACGCCGAACC	583	58.8
6	F TCGCTTGTCAAGAAGACA RGTTGACTTTCTGTTGCTG	422	56.9

Statistical analysis: Genotypic frequencies, allelic frequencies, Hardy-Weinberg equilibrium χ^2 test, heterozygosity (He), polymorphism information contents (PIC), effective numbers of alleles (Ne) and Shannon's Information index (I) were calculated using POPGENE version 1.31. The association of the identified genetic variations with RR, RT, and HTC in Murrah buffaloes was analysed using Generalized Linear Model (GLM) procedure in Statistical Analysis System (SAS) according to the following linear model:

$$Y_{ijkl} = \mu + P_i + T_j + G_k + e_{ijk}$$

where, Y_{ijkl}, the observed value of thermo-tolerance traits; µ, overall mean, P_i fixed effect of parity; T_j, fixed effect of THI; G_k, fixed effect of genotypes and e_{ijkl} random residual error associated with Y_{ijkl} observation and assumed to be NID (0, $\sigma^2 e$).

Haplotype analysis: Haplotypes were constructed using DNASP5 software. Association analysis of haplotype was done (>3sample size) by using Generalized Linear Model (GLM) procedure in Statistical Analysis System (SAS) according to the following linear model:

$$Y_{jkl} = \mu + T_j + H_k + e_{ik}$$

where, Y_{ijkl}, observed value of thermotolerance; µ, overall

mean; T_j, fixed effect of THI; H_k, fixed effect of haplotypes; e_{jkl}, random residual error associated with Y_{jkl} observation and assumed to be NID (0, $\sigma^2 e$). Linkage disequilibrium analysis was performed using SHEsis Software (Shi and He 2005).

RESULTS AND DISCUSSION

Screening of sequence data and SNP Detection: In this study, the targeted regions of *HSF1* gene was amplified by 6 pairs of primer. Amplified products were sent for sequencing by outsourcing. Sequencing results were compared and aligned with the edited sequences of other Murrah buffaloes by ClustalW software for detection of SNPs. ClustalW multiple alignments revealed 7 SNPs at the position of A15732G located in intron 4, C17061T in intron 6, C17202T and A17226G in intron 7, G17454A in intron 8, C17605T in exon 9 and T18421C in intron 9 of *HSF1* gene sequence (Fig. 1).

Genetic parameter analysis: Distribution of genotypes and alleles of the nucleotide sequence polymorphisms of *HSF1* gene is illustrated in Table 3. After chromatogram analysis all possible genotypes were confirmed in most of the SNP locus except in C17061T and A17226G, as CC and GG were not observed respectively, in the screened population. Molecular Genetic Data as effective number alleles (Ne), Shannon's information index (I) Polymorphism Information Content (PIC) were given in Table 2. Pair-wise linkage disequilibrium analysis showed that out of seven SNPs only two SNPs (Fig. 2) were in strong linkage disequilibrium (D>0.75).

Haplotype construction and pair-wise linkage disequilibrium analysis: Total 49 haplotypes combinations were found in the present study. However, only seven haplotypes (>3 sample size), Hap1 (ATTAACT), Hap2 (ATTGATT), Hap3 (ACTAACT), Hap4 (ACTAGCC), Hap5 (ACCAACT), Hap6 (ACCAGCC) and Hap7 (ACCAACT) were considered for association studies. The frequencies of haplotypes were 16.5, 9.5, 14.5, 4.5, 6, 6 and 4.75% respectively that shows highest frequency of animals belonging to Hap1 (ATTAACT) group in studied population.

Table 2. Data on polymorphism information contents (PIC), heterozygosities (He) and effective number of alleles (Ne) of SNP locus of *HSF1* gene in Murrah buffalo

Locus	Ne	I	He	PIC	P
G15732A	1.2985	0.3911	0.2299	0.4982	P<0.01
C17202T	1.7592	0.6230	0.4315	0.2262	
C17061T	1.2375	0.3413	0.1919	0.4352	
A17226G	1.7649	0.6250	0.4334	0.3318	
G17454A	1.9560	0.6819	0.4888	0.42	
C17605T	1.3676	0.4397	0.2688	0.4872	
T18421C	1.9950	0.6919	0.4988	0.2688	

Association between SNP, haplotype combination of HSF1 *gene and heat tolerant performance:* The association between SNPs, haplotypes and heat tolerant traits,viz. Respiration rate (RR), Rectal temperature (RT) and Heat tolerance coefficient (HTC) was analysed for Murrah buffaloes. However, no association among parity of the cows with these traits was observed. The association studies performed for the identified genotypes with physiological parameters is depicted in Table 3. The increase in RR is a very important thermo-regulatory response to heat stress

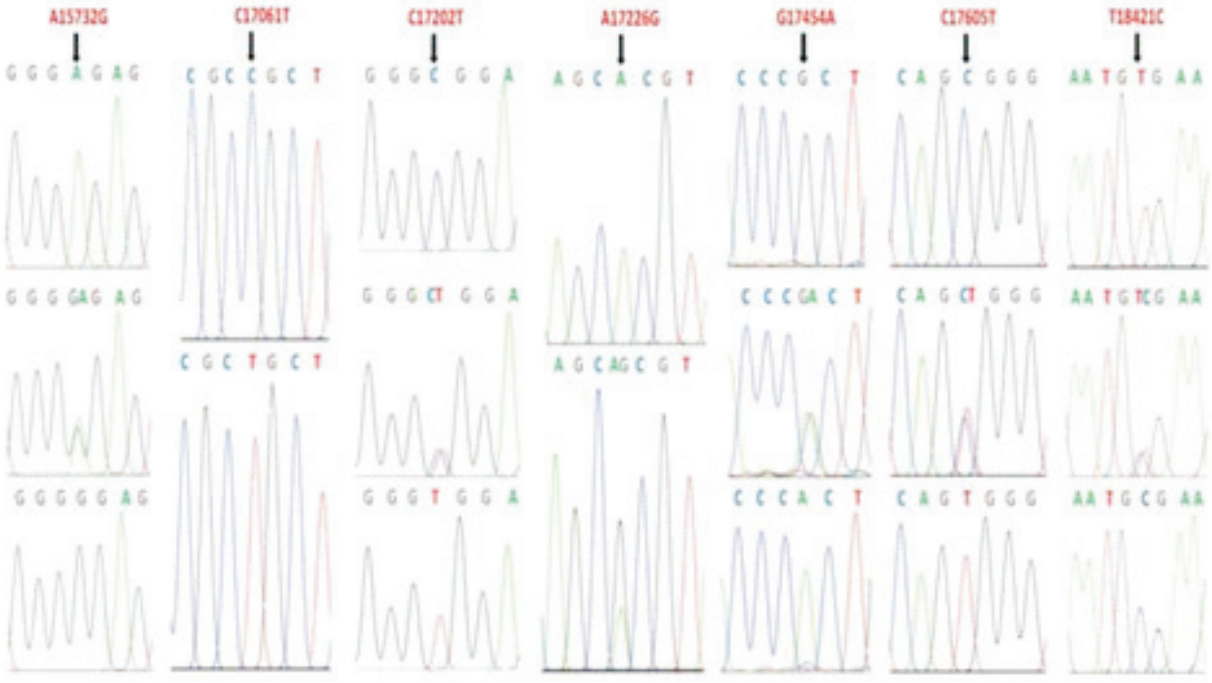

Fig.1. Sequence analysis of different genotypes of *HSF1* gene in Murrah buffalo

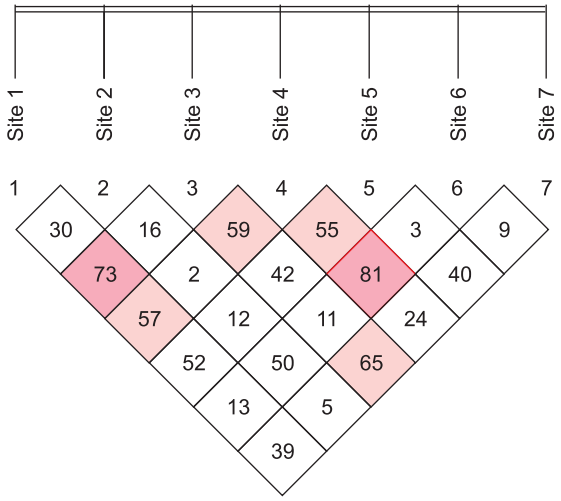

Fig. 2. D^2 value of pairwise linkage disequilibrium analysis in *HSF1* gene

Table 3. Least squares mean (LSM) and standard errors (SE) for heat tolerantperformanceindex of *HSF1* gene in Murrah buffalo

Effect	Subclass	RR	RT	HTC
	Overall Mean	20.40±0.42	101.53±1.50	3.53±0.04
Season (THI)	Winter (THI=49.70)	13.48±0.23	98.85±0.11	1.51±0.01
	Spring (THI=64.65)	17.98±0.32	100.75±4.51	1.78±0.06
	Summer (THI=86.44)	29.20±0.91	101.58±0.05	2.22±0.04
Parity	1(68)	21.08±0.71	101.99±0.42	1.96±0.03
	2(75)	21.90±0.81	100.46±0.10	1.92±0.03
	3(29)	17.44±0.70	100.46±0.16	1.78±0.03
	4(09)	19.07±2.00	99.41±0.20	1.81±0.08
	5(10)	17.86±1.37	102.21±0.34	1.85±0.06
	6(06)	16.33±1.74	101.61±0.44	1.78±0.07
	7(03)	16.33±2.66	102.94±1.00	1.77±0.12
A15732G	AA(67)	23.89±1.02[a]	101.13±5.3	2.01±0.08[a]
	AG(77)	19.64±0.61[b]	100.06±0.10	1.77±0.02[b]
	GG(56)	18.43±0.59[c]	99.42±0.12	1.73±0.02[c]
C17061T	CC(136)	22.91±0.52[a]	101.16±2.21	1.79±0.03
	TT (64)	17.53±0.73[b]	102.29±0.12	1.80±0.03
C17202T	CC(154)	19.23±0.47	100.66±0.07	1.80±0.03
	CT(40	19.84±1.03	101.64±7.51	1.80±0.03
	TT(06)	21.59±2.69	97.88±0.49	1.77±0.05
A17226G	AA(159)	20.39±0.48	101.72±1.89	1.79±0.03
	AG(41)	20.05±0.88	101.74±0.15	1.80±0.03
G17454A	GG(113)	21.81±0.57	101.92±2.66	1.77±0.05
	AG(56)	21.05±0.81	101.40±0.12	1.80±0.03
	AA(31)	17.79±1.00	101.86±0.18	1.80±0.03
C17605T	CC(66)	19.51±0.69	99.16±0.10	1.73±0.03
	CT(100)	20.71±0.62	99.18±0.10	1.80±0.02
	TT(34)	20.45±1.05	101.84±8.83	1.90±0.13
C18421T	TT(15)	16.99±0.45[c]	102.16±1.99	1.80±0.03
	TC(34)	22.71±1.26[a]	100.58±0.16	1.79±0.04
	CC(151)	20.96±1.91[b]	102.44±0.29	1.78±0.04

[#]Data in parenthesis with different superscript differ significantly.

which helps in heat dissipation via evaporative cooling (Beatty *et al*. 2006). Therefore, a low RR may signify better thermo-tolerance in animals. It was found that average RR value of A15732G of GG genotype are significantly (P<0.01) lower than AA and AG in Murrah buffaloes. At another SNP point in C17061T, RR for genotype TT was significantly (P<0.05) different from CC genotypes (Table 3). Moreover SNP locus T18421C also shows significantly (P<0.05) least RR for animals belonging to genotype TT compared to CC and TC genotypes (Table 3). Furthermore, the GG genotype animals had significantly lower HTC (P<0.01) compared to the AA and AG genotypes (Table 3). Dikmen *et al.* (2012) estimated that 13 to 17% of the variation in rectal temperature in cows during heat stress is due to genetic differences. However, association of RT with different SNPs position were found non-significant (P>0.05) in the present study. Haplotypes combinations were also used for association analysis and showed that RR and HTC were significantly different in different haplotype combinations (P<0.05). For RT, no significant difference was observed in different SNPs position and haplotype combinations (Table 4). The haplotype combination Hap6 is significantly (P<0.05) associated with RR. The individuals with Hap6 (ACCAGCC) combination had lower RR than those other haplotype combinations and these individuals may have better heat resistance.

In the present study, 7 SNPs were identified distributed in different position in *HSF1* gene in Murrah buffaloes. Similar type of study was reported in Chinese Holstein Cattle by Li *et al*. (2011) that found two novel SNPs at T909C and G4693T locus and Liu *et al.* (2011) found one novel SNP in the *ATP1A1* gene that was associated with heat tolerance traits in dairy cows. Out of seven SNPs detected in present study, only one SNP locus C17061T was screened in the coding region and rest were observed in intronic region. This intronic region could be associated with eukaryotic gene expression; transcription, processing of primary transcripts, transport of mRNAs from nucleus to cytoplasm, translational efficiency, and detection and elimination of mRNAs with nonsense-coding errors (Zhao and Hamilton, 2007). Frequency of all the wild type of allele in different SNP locus was found to be predominant over mutant alleles. The value of PIC, He and Ne were calculated that are the index of evaluating the genetic variation in population, as higher values of PIC and He determine

Table 4. Least squares means for Respiration rate (RR), Rectal temperature (RT), Heat tolerance coefficient (HTC) of different HSF1 Haplotypes

Haplotype/sample	RR	RT	HTC
Hap1/64	18.30±0.50	100.19±0.11	1.40±0.02
Hap2/32	20.71±1.03	100.52±0.14	1.52±0.04
Hap3/55	20.11±0.77	100.19±0.12	1.48±0.03
Hap4/18	17.14±0.92	99.59±0.21	1.34±0.04
Hap5/24	20.23±1.2	101.38±0.15	1.83±0.32
Hap6/24	18.23±1.11	99.32±0.17	1.38±0.05
Hap7/18	21.59±1.68	100.50±0.21	1.56±0.07

greater levels of the genetic variation. In this study, it was moderate polymorphism (0.25<PIC<0.5) at all locus in Murrah buffaloes. This revealed that the genetic polymorphism was present in the studied population. If the value of Ne is close to the detected alleles of the absolute number, alleles in the population show more uniform distribution. So, SNP locus C17202T, A17226G, G17454A, T18421C was more uniform in comparisons to SNP locus G15732A, C17061T and C17605T. The χ^2 test results showed that all loci deviated from the Hardy-Weinberg disequilibrium, which implies significant (P<0.01) differences in genotypic and allelic distributions within the locus in the population. Thus, polymorphisms could be possibly affected by artificial selection, because selection may significantly change the genotypic and allelic distribution of the *HSF1* gene.

The identification of SNPs is associated with variation in sensitivity to thermal stress and therefore permits the screening of animals for presence or absence of desirable or undesirable alleles (Verma *et al.* 2015). Association analysis showed that the thermal tolerance trait was significantly affected by three SNP locus namely A15732G, C17061T and T18421C locus. Animals belong to GG genotype at locus A15732G has lower RR and higher HTC which suggest that animals belonging to this genotype have better thermal adaptability in comparison to others animals. Charoensook *et a*l. (2012) reported a low RR may indicate an improved thermo-tolerance adopted by animals. According to Benezra (1954), the lower the value determined by the equation higher the degree of adaptability. Genotype GG at locus A15732G showed less RR and HTC values, which determined that animals belonging to particular genotype, could be more adapted under thermal environment. Li *et a*l. (2011) found Chinese Holstein cows with Genotypes CC, CT at 909, and TT at the 4693 locus was advantageous genotypes for thermal adaptation. Our study also revealed that TT genotype at locus T18421C and CT genotype at C17061T has least respiration rate. The individuals with Hap7 (GCCAACC) haplotype combination also showed significantly (P<0.05) lower RR than those other haplotype combinations. Li *et a*l. (2011) reported that cows with H2H4 (TCTT) haplotype combination of T909C and G4693T SNPs had lower RT (P<0.05) and higher HTC (P<0.05) than those with H1H3 haplotype combination. Although, RT is significant physiological measure of heat stressed animals, despite, no association was found among this parameters and genetic variations in our present study. By applying genetic markers through marker-assisted selection; it can be improve the accuracy and efficiency of traditional selection methods. Finally, it may be concluded that the *HSF1* gene be considered as a DNA marker for thermo-tolerance traits of buffaloes in marker-assisted selection.

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