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Single nucleotide polymorphisms in Heat Shock Protein (HSP) 90AA1 gene and their association with heat tolerance traits in Sahiwal cows

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ABSTRACT

Heat Shock Proteins (HSPs) are group of proteins which are synthesized during heat stress. HSP genes have been reported to be associated with heat tolerance and production performance in cattle. HSP90AA1 gene has been mapped on *Bos taurus* autosome 21 (BTA 21) and spans nearly 5368 bp comprising 11 exons out of which first exon does not translate. The present study was carried out in Sahiwal cows (*n=100*) with the objectives to identify SNPs in targeted regions (exon 3, 7, 8 & 11) of HSP90AA1 gene and to analyze their association with heat tolerance traits in Sahiwal cows. Respiration rate (RR) and rectal temperature (RT) was recorded once during probable extreme hours in winter, spring and summer season. Further, heat tolerance coefficient (HTC) also calculated to see the adaptability of the animals during the period of heat stress. On the basis of comparative sequence analysis, total five SNPs were revealed at position of A1209G, A3292C, T4935C, T5218C and A5224C in the targeted region of HSP90AA1 gene. Out of these, only two SNPs at A1209G and A3292C loci were found significantly associated with heat tolerance traits in Sahiwal cows. Whereas, THI has a highly significant associated with RR, RT and HTC in all the seasons. At A1209G locus in Sahiwal cows for traits RR, genotype AA (18.40±0.46^{a}), AG (19.60±0.85^{b}) and GG (21.18± 0.64^{ab}) and trait HTC AA (1.78±0.04^{a}), AG (1.85±0.03^{b}) and GG (1.91± 0.02^{c}) differ significantly ($p<0.01$) while trait RT, AG (38.32±0.10^{b}) and GG (38.27±0.08^{ab}) didn't differ significantly. For a locus A3292C for traits RR, genotype AA (21.55±1.01^{a}), AC (19.66±1.59^{b}) and CC (18.40±1.03^{c}) differ significantly ($p<0.05$), while traits RT, genotype AA (38.41±0.12), AC (38.28±0.20) and CC (37.96±0.15) didn't differ significantly. For trait HTC, AA (1.93±0.04^{a}), AC (1.85±0.06^{b}) and CC (1.79±0.05^{c}) found significantly ($p<0.05$) differ with each other. Our study indicated that Sahiwal cows of AA and AC genotype had better thermo-tolerance capacity, which had been useful for genetic improvement of Sahiwal cattle for heat tolerance traits.

Key words: Heat stress, HTC, HSP90AA1, Polymorphism, Sahiwal.

INTRODUCTION

Global warming and climate change have become the major threats to the sustainability of livestock production systems (Gaughan *et al*., 2010). In tropical and sub tropical regions high ambient temperature is the major constraint on animal production (Marai *et al.*, 2007; Nardone *et al*., 2010). The negative impact on total milk production was estimated and predicted to be about 3.2 million tonnes in year 2020 and more than 15 million tonnes in year 2050 (Upadhyay *et al*., 2009). The loss of milk production due to heat stress in monetary terms amounts to a whopping Rs 2661.62 crore per year (Upadhyay, 2010). Indigenous (*Bos indicus*) cattle survive and perform better under heat stress as compared to temperate breeds or their crossbreds due to high prevalence of heat shock protein gene (Collier *et al*., 2008; McManus *et al*., 2013). Genetic differences in thermo-tolerance at the physiological and cellular levels are documented by number of studies on *Bos indicus* and *Bos taurus* cattle breeds (Paula-Lopes *et al.*, 2003; Hansen *et al*., 2004; Lacetera *et al.*, 2006). Suitable breeding programs can help to achieve animal population that could cope with effects of heat stress (Hoffmann, 2010).

At the cellular level, mammals respond to heat stress by transcriptional activation of a set of proteins known as heat shock proteins (HSPS) (Kregel, 2002). Among members of the HSP family, HSP70 and HSP90 are the most abundant proteins in eukaryotic cells. HSP90 act as important molecular chaperones that are constitutively expressed as a consequence of heat or stress induction (Chen *et al*., 2006). The protective function of HSPs depends on their chaperone activity. The chaperone known as 90-kDa heat shock protein, is one of the most abundant proteins in eukaryotic cells, comprising 1–2% of cellular proteins under non-stress conditions. There are two major isoforms of HSP90 which have arisen by gene duplication, HSP90α or HSP90AA1 (inducible form) and HSP90β or HSP90AB1 (constitutive form). HSP90AA1 gene has been mapped on *Bos taurus* autosome 21 (BTA 21) and spans nearly 5368 bp comprising 11 exons out of which first exon does not translate. The 'trait' heat tolerance is a quantitative trait (Gaughan *et al*., 2010;

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Li *et al.,* 2011). HSP90AA1 gene in Deoni cattle (*Bos indicus*) has been found to be polymorphic and showed significant association with productive and reproductive parameters (Shergojry *et al.,* 2011). In sheep, polymorphisms within HSP90AA1 gene were also investigated. A single nucleotide polymorphism (SNP) located at position 660 in the 5' flanking region was associated with different thermal conditions (Marcos-Carcavilla *et al*., 2010).

However, no report is available on HSP90AA1 gene variants and their association with thermo-tolerance in Indian dairy cattle breeds. Keeping in view the importance of HSP90AA1 gene the present study has been undertaken to determine the genetic polymorphism in targeted regions (exon 3, 7, 8 &11) of HSP90AA1 gene in Sahiwal breed of cattle and to associate the observed genetic polymorphisms with heat tolerance traits.

MATERIALS AND METHODS

Geographical and climatic description: The animals for conducting research were taken from Livestock Research Complex, ICAR-National Dairy Research Institute, Karnal, located at 29.68°N latitude and 76.98°E longitude with altitude ranging from 235 to 252 meters above mean sea level. The climate is sub-tropical with temperature in summer months i.e. April to June, ranging between 24°C to 44°C and experiences moderate rainfall from July to September with annual precipitation of 744 mm. Whereas, winter months i.e. October to January, are extremely cold with temperature ranging from 4°C to 32°C.

Ethical approval: The experimental and plan of study was duly approved by Institution Animal Ethics Committee of National Dairy Research Institute, Karnal, Haryana, India.

Experimental animals and DNA extraction: About 10 ml of blood from each Sahiwal cow (*n=100*) was collected in EDTA coated vacutainer tube and stored at -20°C until DNA isolation. Genomic DNA of each animal was extracted from 5 ml of blood sample using phenol- chloroform extraction method described by Sambrook and Russell, (1989). The quality of DNA was checked by 1.5 % agarose gel electrophoresis. The ratio of OD_{260} and OD_{280} for different DNA samples were observed between 1.7 to 1.9 by Biospec-nano spectrophotometer. DNA samples with ratio of 1.8 were considered good and utilized for further exploration. The genomic DNA was diluted to a final concentration of 30 ng /µl and stored at - 20°C.

Physiological parameters recorded: Respiration rate (RR) and rectal temperature (RT) were recorded once daily for three days consecutively during probable extreme hours in winter, spring and summer season and average was taken as final reading for association analysis. Heat tolerance coefficient (HTC) was calculated based on heat tolerance index developed by Benezra, (1954). The formula is based on both respiration rate and rectal temperature.

HTC: RR/23 + RT/38.33

THI measures the combined effects of ambient temperature and relative humidity (RH) to ascertain heat load intensity (Berry *et al*., 1964). Temperature humidity index (THI) were calculated for all days in three seasons *viz*. winter (48.77), spring (64.86) and summer (90.96) during which physiological parameters were recorded and used in the association analysis as fixed variables. THI calculation using dry bulb temperature (Db) and wet bulb temperature (Wb) to estimate the magnitude of heat stress (Thom, 1959), were the most common compare to other methods.

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

Where, Wb and Db are wet and dry bulb temperatures in °C, respectively.

PCR primers and amplifications: Polymerase Chain Reaction (PCR) amplification of targeted region (exon 3, 7, 8 & 11) of bovine HSP90AA1 gene was carried out using four sets of forward and reverse gene-specific oligonucleotide primers. The Primers were designed for target region of bovine HSP90AA1 gene (NCBI GenBank AC_000178.1) using Primer3 software. The sequence of primers, their respective nucleotide numbers, targeted region and amplicon sizes are given in (Table 1). The PCR reactions were carried out in a total of 25 µl volume of containing template DNA of 3 µl (30 ng/ µl), 10 pmole of each primer, PCR Master Mix (2X) (Fermentas) of 12.5 µl and 8.5 µl of water. The thermal cycling conditions of all primers having initial denaturation at 95°C for 3 min, followed by 35 cycles

Table 1: Primer Sequences, targeted region and amplicon sizes of Bovine HSP90AA1 gene in Sahiwal cows

Primer set	Sequence (5'-3')	No.of base	Targeted region On gene (HSP90AA1)	Amplicon size (bp)
1.	F- GCGTCATCACGTGTCATCTT	20	Exon 3	450 bp
	R- CCTCCTTTGGGGTTCCAGT	19	(827..1276)	
2.	F- AAGGCGTTCATCTTTGGATTTT	22	Exon 7 & 8	632 bp
	R- ACTAAACTACGTGTACCACCAC	22	(2700..3331)	
3.	F- GCCTTGAGAGTGGGTATGATG	23	Exon 11	501 bp
	R- TGATAGCTAAACACTTCAGACCA	21	(4477..4977)	
4.	F- TGGTCTGAAGTGTTTAGCTATCA	23	Part of Exon 11	440 bp
	R- CCCCACAAATAAGACATCACACA	23	(4955..5394)	

with denaturation at 95°C for 30 sec, annealing temperature of primers set of 1, 2, 3 & 4 are 55.7°C, 61.7°C, 62.6°C and 61.8°C respectively for 45 sec, extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. PCR programme for both the primers was found to be same. PCR products were detected by electrophoresis on 1.5 % agarose gel stained with ethidium bromide.

Statistical Analysis: Allelic and genotypic frequencies were calculated using POPGENE software package (Yeh *et al*., 1999). The significant effect of SNP variants on physiological parameters were analyzed using the General Linear model (GLM) procedure of SAS Version 9.2, in Sahiwal cows:

$Y_{ijklmno} = \mu + T_i + SNP1_j + SNP2_k + SNP3_l + SNP4_m + SNP5_n + e_{ijklmno}$

$Y_{ijklmno}$ = O^{th} observation on RR/RT/HTC of cows in i^{th} THI, j^{th} SNP1, k^{th} SNP2, l^{th} SNP3, m^{th} SNP4 and n^{th} SNP5

μ = overall mean

T_i = Effect of i^{th} THI ($i_=$ 48.77, 64.86 and 90.96)

$SNP1_j$= Effect of j^{th} genotype of SNP A1209G (j= AA, AG and GG)

$SNP2_k$= Effect of k^{th} genotype of SNP A3292C (k= AA, AC and CC)

$SNP3_l$ = effect of l^{th} genotype of SNP T4935C (l=TT and CC)

$SNP4_m$= Effect of m^{th} genotype of SNP T5218C (m=TT, TC and CC)

$SNP5_n$= Effect of n^{th} genotype of SNP A5224C (n=AA, AC and CC)

eijklmno= Random error associated with Yijklmno observation and assumed to be NID ($0,\sigma^2e$)

RESULTS AND DISCUSSION

Detection of SNPs: Comparative sequence analysis revealed five SNPs *viz*., A1209G, A3292C, T4935C, T5218C and A5224C in Sahiwal cattle, three of which located in exon 11, and one in each exon 3 and exon 7 &8.

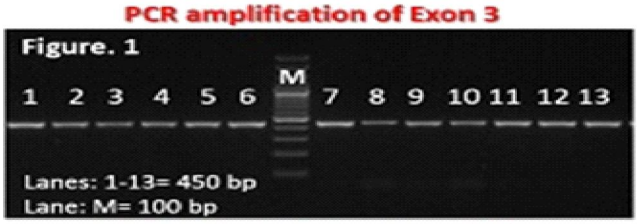
Association of heat tolerance traits with HSP90AA1 SNPs: The four PCR products with the length of 450 bp, 632 bp, 501 bp and 440 bp were successfully amplified in Sahiwal cows (Figure 1, 2, 3 and 4). The allelic and genotypic frequencies of HSP90AA1 gene are given in (Table 2). For association study RR, RT and HTC were taken as dependent variables. Relationship of each dependent variable with THI and each polymorphic variants of HSP90AA1 gene was analyzed separately.

Overall least square means of respiration rate per minute, rectal temperature (°C) and HTC were found to be 19.75±0.42, 38.22±0.05 and 1.85±0.02 respectively (Table 3). At A1209G locus in Sahiwal cows, Respiration

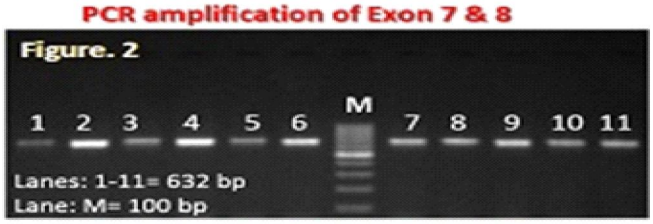
Table 2: Genotypic and allelic frequency at each SNP locus of HSP90AA1 gene

SNP	Genotypes	Genotypic frequency	Allele	Allelic frequency
	AA	0.23	A	0.48
A1209G	AG	0.50		
	GG	0.27	G	0.52
	AA	0.24	A	0.48
A3292C	AC	0.47		
	CC	0.29	C	0.52
	TT	0.72	T	0.72
T4935C	CC	0.28	C	0.28
	TT	0.49	T	0.62
T5218C	TC	0.26		
	CC	0.25	C	0.38
	AA	0.30	A	0.51
A5224C	AC	0.43		
	CC	0.27	C	0.49

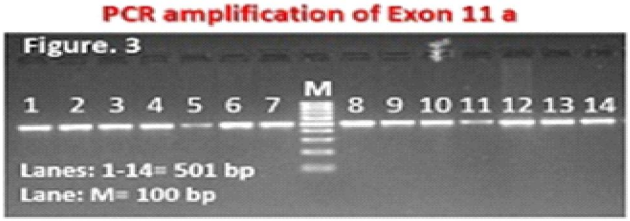
PCR amplification of Exon 3



PCR amplification of Exon 7 & 8



PCR amplification of Exon 11 a



PCR amplification of Exon 11 b

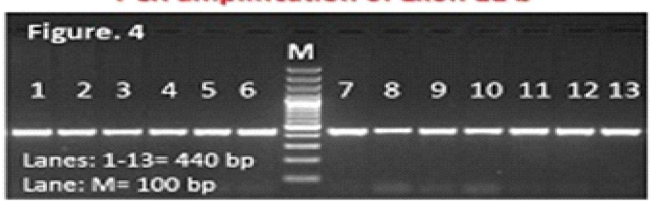


Table 3: Least squares means of subclasses of different fixed effects for RR, RT and HTC in Sahiwal cows

Effect	subclass	RR (times/min.) ± SE	RT(ºC)± SE	HTC± SE
Overall Mean		19.75±0.42	38.22±0.05	1.85±0.02
THI**	winter (48.77)	14.07±0.43^{a}	37.97±0.05^{a}	1.60±0.01^{a}
	spring (64.86)	18.56±0.43^{b}	38.23±0.06^{b}	1.80±0.01^{b}
	summer (90.96)	26.61±0.41^{c}	38.45±0.06^{c}	2.16±0.05^{c}
A1209G**	AA (23)	18.40±0.46^{a}	37.91±0.12^{a}	1.78±0.04^{a}
	AG(50)	19.60±0.85^{b}	38.32±0.10^{b}	1.85±0.03^{b}
	GG (27)	21.18±0.64^{c}	38.27±0.08^{b}	1.91±0.02^{c}
A3292C*	AA (24)	21.55±1.01^{a}	38.41±0.12^{a}	1.93±0.04^{a}
	AC (47)	19.66±1.59^{b}	38.28±0.10^{a}	1.85±0.06^{b}
	CC (29)	18.40±1.03^{c}	37.96±0.20^{a}	1.79±0.06^{c}
T4935C	TT (72)	20.18±0.73	38.32±0.04	1.87±0.04
	CC (28)	18.61±0.60	37.96±0.09	1.79±0.03
	TT (49)	19.81±O.42	38.20±0.05	1.85±0.01
T5218C	TC (26)	20.03±0.40	38.24±0.04	1.86±0.06
	CC (25)	19.33±0.48	38.22±0.06	1.83±0.02
A5224C	AA (30)	20.98±0.93	38.38±0.11	1.91±0.04
	AC (43)	19.74±0.92	38.27±0.12	1.85±0.05
	CC (27)	18.39±1.60	37.94±0.20	1.78±0.07

(Figures in parenthesis are number of animals; Figures with dissimilar superscript differ significantly, **P<0.01, *P<0.05; SE, standard error)

rates per minute of each genotype, AA (18.40±0.46^{a}), AG (19.60±0.85^{b}) and GG (21.18±0.64^{c}) and rectal temperatures (°C) of respectively genotype, AA (37.91±0.12^{a}), AG (38.32±0.10^{b}) and GG (38.27±0.08^{b}) were observed during winter, spring and summer seasons. The corresponding values of HTC of each genotype, AA (1.78±0.04^{a}), AG (1.85±0.03^{b}) and GG (1.91±0.02^{c}) were observed during probable extreme season. Similarly A3292C locus for traits RR, genotypic value of AA (21.55±1.01^{a}), CC (18.40±1.03^{c}) and AC (19.66±1.59^{b}) genotypes were observed. For traits RT, genotypic value of AA (38.41±0.12^{a}), AC (38.28±0.20^{a}) and CC (37.96±0.15^{a}) and trait HTC, AA (1.93±0.04^{a}), AC (1.85±0.06^{b}) and CC (1.79±0.05^{c}) were observed in respective season.

The average RR value for a A1209G locus genotype, AA (18.40±0.46^{a}), AG (19.60±0.85^{b}) and GG (21.18± 0.64^{c}) and for trait HTC with genotype, AA (1.78±0.04^{a}), AG (1.85±0.03^{b}) and GG (1.91± 0.02^{c}) were highly significantly (P<0.01) associated with each other. Whereas, effect of RT didn't differ significantly with genotype, AG (38.32±0.10^{b}) and GG (38.27±0.08^{b}) in different season. Similarly, effect of SNP at A3292C locus traits RR, genotype AA (21.55±1.01^{a}), AC (19.66±1.59^{b}) and CC (18.40±1.03^{c}) and for trait HTC, AA (1.93±0.04^{a}), AC (1.85±0.06^{b}) and CC (1.79±0.05^{c}) were found significantly (p<0.05) associated with each other. But, the corresponding values of RT of each genotype AA (38.41±0.12), AC (38.28±0.20) and CC (37.96±0.15) were not found significantly associated in respective season.

Our results indicated that for a locus A1209G, the homozygotic animals with AA genotype had significantly (P<0.01) lower heat tolerance coefficient (1.78±0.04^{a}), as compared to both genotype AG and GG (1.85±0.03^{b} and 1.91±0.02^{c}) respectively. On the other hand, at the locus A3292C, the homozygotic animals with CC genotype had significantly (P<0.05) lower heat tolerance coefficient (1.79±0.06^{b}), as compared to both genotypes AA and AC (1.93±0.04^{a} and 1.85±0.05^{c}) respectively. So, our present study indicated that Sahiwal cows of AA and CC genotype at the locus (A1209G & A3292C) respectively found lower HTC which aids in intemperance of excess body moisture in the expired air (Beatty *et al*., 2006). Hence, lower HTC may indicate an improved thermo tolerance which had been useful for genetic improvement of Sahiwal cattle for heat tolerance traits.

Further, Efforts were done to link between the thermal-stress related phenotypes with genotypes. For example, polymorphisms detected in ATP1A gene at position "2789 and HSP70.1 at Position "895 were associated with the heat tolerance trait in dairy cattle (Liu *et al*., 2011; Deb *et al*., 2013). Effects of the SNP g.1524G>A, g.3494T>C and g.6601G>A within HSP70A1A affect thermo-tolerance in Chinese Holstein cattle (Li *et al*., 2011). In contrast allele T at SNP g.4338>C of HSP90AB1 gene was found to be associated with heat tolerance coefficient in Thai native cattle (Charoensook *et al*., 2013), Sahiwal and Frieswal cattle in India (Deb *et al*., 2013; Sajjanar *et al*., 2015). Recently in Jersey crossbred cows found that of CC genotype had better thermo-tolerance capacity at (T17872112C) locus of HSP90AB1 gene (Sailo *et al*., 2015). Similarly, AA genotype at (C27007790A) locus of ATP1A1 gene in Jersey crossbred cows were desirable for respiration rate and heat tolerance coefficient (Das *et al*., 2015). The results of the present study were first time reported, so no earlier reports are available to compare the present findings.

CONCLUSIONS

The study was carried out in Sahiwal cows (n=100) with the objectives to identify SNPs in the targeted regions (exon 3, 7, 8 & 11) of HSP90AA1 gene and to analyze their association with heat tolerance trait. On the basis of comparative sequence analysis total five SNPs revealed at position of A1209G, A3292C, T4935C, T5218C and A5224C in the targeted region (exon 3, 7, 8 & 11) of HSP90AA1 gene. Out of these, only two SNPs at A1209G and A3292C loci were found significantly associated with heat tolerance traits (RR, RT & HTC) in Sahiwal cows. Whereas, THI has a highly significant association with RR, RT and HTC in all the seasons. So, our present study indicated that Sahiwal cows of AA and CC genotype had better thermo-tolerance capacity, which had been useful for genetic improvement of Sahiwal cattle for heat tolerance traits, the same can also be utilized as a genetic marker to select appropriate animals for hot climatic conditions.

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