



Genetic Polymorphism within Exon 3 of *HSP90AA1* Gene and its Association Studies in Sahiwal and Crossbred Cows

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ABSTRACT

Background: Heat shock proteins (*HSPs*) are molecular chaperones that play a critical role in recovering cells from stress and form a primary system for intra cellular self defense. They are highly conserved and play a crucial role in cellular thermo tolerance and heat stress response. Though there are many *HSP* genes, thermo tolerance is mainly correlated with *HSP70* and *HSP90* genes in Livestock species. Polymorphisms in these genes have shown an association with heat tolerance, milk production, fertility and disease susceptibility in livestock. They can be used as genetic markers for the selection of animals with better climate resilience, immune response and superior performance.

Methods: The present study was carried out in Sahiwal (n=50) and Crossbred cows (n=50) with the objective to identify polymorphisms in *HSP90AA1* gene. A 450 bp fragment of bovine *HSP90AA1* gene covering exon3 was subjected to Polymerase Chain Reaction-Single-Strand Conformation Polymorphism (PCR-SSCP) technique to identify the polymorphism. PCR-SSCP patterns were correlated with the physiological, productive and reproductive traits in Sahiwal and crossbred cows using the univariate GLM model of SPSS 25.

Result: The PCR-SSCP of exon 3 of *HSP90AA1* gene yielded two conformational patterns AA and AB corresponding to two allelic variants A and B in both Sahiwal and crossbred cows. The allele frequencies of A and B were 0.78 and 0.22 and 0.84 and 0.16 in Sahiwal and crossbred cows, respectively. The association analysis of SSCP patterns revealed that genotype AA had higher lactation length in Sahiwal cows and higher total lactation milk yield and peak yield in crossbred cows.

Key words: Crossbred cows, Heat stress, *HSP90AA1*, Polymorphisms, Sahiwal.

INTRODUCTION

Heat shock proteins (*HSPs*) are molecular chaperones that help cells recover from stress and provide cytoprotection, which protects them from further assaults. They defend stressed cells by recognizing nascent polypeptides, unstructured protein regions and exposed hydrophobic stretches of amino acids. *HSP* genes are those that code for heat shock proteins. Despite the fact that there are many *HSP* genes, thermo tolerance in livestock animals is mostly linked to the *HSP70* and *HSP90* genes. *HSP90* is the most prevalent and temperature-sensitive and it is thought to play a key role in environmental stress and thermal adaptation (Gade *et al.*, 2010). Higher levels of expression of proteins of the *HSP70* and *HSP90* families have been observed in various livestock species in summer season (Archana *et al.*, 2017). Heat tolerance, milk production, fertility and disease susceptibility in livestock have been linked to polymorphisms in the *HSP70* and *HSP90* genes (Shergojry *et al.*, 2014; Kumar *et al.*, 2015; Bhat *et al.*, 2016). They could be useful candidate gene markers for identifying animals with improved climatic resistance, immunological response and performance (Hassan *et al.*, 2019).

Although differences in thermo tolerance at the physiological and cellular level have been documented in both *Bos indicus* and *Bos taurus* cattle (Collier *et al.*, 2006; Chaiyabutr *et al.*, 2008; Wilson and Crandall, 2010 and Dalcin *et al.*, 2016), data on polymorphism of *HSP* genes in Sahiwal cattle and Holstein Friesian crossbreds is limited. There are only a few reports from India about the relationship

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of *HSP* gene polymorphism with heat tolerance in Tharparkar cattle (Bhat *et al.*, 2016), Deoni cattle (Kerekoppa *et al.*, 2015), Jersey crossbred cows (Sailo *et al.*, 2015) and from abroad in Holstein cow (Li *et al.*, 2011). Therefore polymorphisms of *HSP90AA1* gene and their association with various physiological, production and reproduction traits in Sahiwal and crossbred cows were investigated in the present study.

MATERIALS AND METHODS

Experimental animals

The present study included a total of 50 Sahiwal cows maintained at the Livestock Farm Complex, College of Veterinary Science Rajendranagar and 50 crossbred cows (Holstein Friesian × Sahiwal) crosses maintained at the Military Dairy Farm.

Weather conditions

The capital city of Telangana State, Hyderabad, is located at 17.366°N Latitude and 78.476°E Longitude. It is situated at a height of 536 metres (1607 feet) above mean sea level. During the experimental period, data on weather conditions, such as maximum and minimum temperatures (°C), dry and wet bulb readings (°C) and relative humidity (%), were collected from the Agriculture Climate Research Center, ARI, Hyderabad. During the 2018-19 study year, average environmental temperatures ranged from 27.92°C in December, the coldest month, to 41.24°C in May, the hottest month. In the months of July, August and September, Hyderabad received moderate rainfall. The average annual rainfall was 766 mm and the average relative humidity ranged from 45% in the summer to 78% during the monsoon. The winters were mild, with temperatures ranging from 15°C to 31°C.

Genomic DNA isolation

10 ml of blood was collected aseptically from the external jugular vein of each cow into a sterile vacutainer tube containing 0.5 per cent EDTA. The tubes were transported on ice to the laboratory, stored at 4°C and processed within 24 hours. Each animal's genomic DNA was extracted from blood samples using the standard phenol-chloroform extraction method described by Green and Sambrook (2012), with minor modifications. The purity of the genomic DNA samples was determined by measuring the optical densities (OD) at 260 nm and 280 nm against a blank using Nanodrop (Thermo Fisher Scientific) and storing them at -20°C until further use.

Physiological parameters

The physiological parameters, respiration rate (RR) and rectal temperature (RT) of each animal in the current study were recorded twice daily for 30 days in each of the three seasons, i.e. during May (2018) for summer, August (2018) for rainy and from mid-December (2018) to mid-January (2019) for winter and the average was taken as the final reading for each cow in association analysis. The physiological parameters were recorded at 8 AM. and 2 PM. The heat tolerance coefficient (HTC) was calculated for each animal based on respiration rate and rectal temperature using Benezra's formula (1954).

Production and reproduction traits

Data on each animal about various aspects such as Animal no., Sire no., Dam no., Date of birth, Date of calving, Lactation length and Lactation milk yield, etc., were collected

from the history sheets/daily farm registers. From the available data, the various production and reproduction traits such as total lactation milk yield (TLMY), peak yield (PY), lactation length (LL), service period (SP), dry period (DP) and calving interval (CI) were calculated in both Sahiwal and crossbred cows.

PCR primers and amplifications

To amplify the targeted region, primers (purchased from BioServe Biotechnologies Pvt Ltd, Hyderabad) specific for the desired region (450 bp) of the *HSP90AA1* gene covering exon 3 were used. The primer sequence, length of the primer (bp) and melting temperature (T_m) are listed below.

Gene fragment	Sequence (5'-3')	Length of the primer (bp)	Melting temperature (T_m , °C)
Exon3 450bp	F GCGTCATCACGTGTCATCTT	20	52
	R CCT CCTTTGGGGTTCCAGT	19	53

F = Forward; R = Reverse.

The PCR reactions were carried out on a total of 12.5 µl volume containing 1 µl of template DNA of (50-100 ng/µl), 1.0 µl each of forward and reverse primers (10pM), 2.5 µl of 10X Taq buffer, 0.8 µl of dNTPs (10 mM), 0.125 µl of Taq Polymerase (5 units/µl) and 6.075 µl of Nuclease free water. Amplification was done in a pre-programmed thermo cycler (Prima-Duo, Himedia labs). The PCR cycling conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of initial denaturation at 95°C for 30 seconds, annealing at 51°C for 45 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 10 minutes. PCR products were detected by electrophoresis on 2% agarose gel stained with ethidium bromide.

Single strand conformation polymorphism (SSCP)

Polymorphism in exon 3 of *HSP90AA1* gene was screened using the single-strand conformation polymorphism (SSCP) technique using the amplified PCR products. About 5 µl of PCR products were taken in sterile PCR tubes and 15 µl of formamide denaturing dye was added and mixed properly to each sample. The tubes were sealed by parafilm and placed in a water bath heated to 95°C for 5 minutes. Immediately they were snap cooled on ice for 15-20 minutes. The denatured products were then loaded into the gel and electrophoresis was carried out at 4°C at a constant 15-20 mA current and 110V for 10-12 hrs. The gel mix was prepared by adding the required components (acrylamide: bisacrylamide (49:1) solution, ammonium persulfate (APS) 10%, Glycerol, 1X TBE and TEMED in the required proportions and was prepared freshly.

The variants were identified basing on the band pattern observed in the SSCP gels after silver staining (Bassam *et al.*, 2007). The most common band pattern identified was named as A. If there are more bands, in addition to the

common bands, they were marked as B, C, etc., depending on the band pattern.

Genotype and allele frequencies

Genotype frequencies for variant genotypes were calculated using the formula:

$$\text{Genotype frequencies} = \frac{\text{No. of animals with specific genotypes (AA, AB or BB)}}{\text{Total no. of animals}}$$

Allele frequencies were calculated as follows:

$$\text{Allele frequencies of A} = \text{AA} + \frac{1}{2} \text{AB}$$

$$\text{Allele frequencies of B} = \text{BB} + \frac{1}{2} \text{AB}$$

Where,

AA and BB = Genotype frequencies of homozygotes.

AB = Genotype frequency of heterozygote.

A and B = Allele frequencies.

Association analysis

The association of each SSCP genotype on physiological, production and reproduction traits in Sahiwal and crossbred cows was studied statistically. The data on physiological traits was corrected for the season effect and then used for association analysis. The univariate GLM model of SPSS 25 was used to perform the analysis according to the following statistical model:

$$Y_{ijk} = \mu + G_i + P_j + e_{ijk}$$

Where,

Y_{ijk} = Dependent variable (respiration rate, rectal temperature, heat tolerance coefficient, total lactation milk yield, peak yield, lactation length, gestation period, service period, dry period and calving interval).

μ = Overall mean,

G_i = Effect of i^{th} SSCP genotype ($i = 1 \dots n$)

P_j = Effect of j^{th} parity of the animal at the time of blood collection ($j = 1 \dots n$)

e_{ijk} = Random error assumed to be distributed normally and independently with mean zero and variance σ_e^2 .

Significant differences between the means of different genotypes and parities were tested by Duncan's Multiple Range Test (DMRT). Values were considered significant at $P \leq 0.05$ and presented as means \pm standard errors.

RESULTS AND DISCUSSION

The PCR reactions were set for all the animals (50 each of Sahiwal and crossbreds) with species-specific primers available in the literature for the amplification of exon 3 of *HSP90AA1* gene. Amplification of desired size was noticed in all the tested samples. The representative figure showing the PCR amplified products of exon 3 of *HSP90AA1* gene, showing the size of 450 bp are presented in Fig 1, while the PCR-SSCP polyacrylamide gel image is presented in Fig 2.

The 450bp fragment covering exon 3 of the *HSP90AA1* gene was found to be polymorphic in both Sahiwal and crossbred cows. Two SSCP genotypes namely AA and AB were documented and consequently at this locus two alleles A and B were present in both genetic groups with allelic frequency of 0.78 and 0.22 in Sahiwal and 0.84 and 0.16 in crossbred cows. The frequencies of AA and BB genotypes were estimated to be 0.56 and 0.44 in Sahiwal and 0.68 and 0.32 in crossbred cows, respectively. Kumar *et al.* (2015) also observed a higher amount of polymorphism in the same 450 bp fragment and detected three genotypes AA, AG and GG with respective frequencies of 0.23, 0.50 and 0.27 within the exon 3 of *HSP90AA1* gene in Sahiwal cows. Badri *et al.* (2018) identified five single nucleotide polymorphisms in Chinese Holstein lactating cows: one in the promoter, three in the coding region and one in 3' - UTR region of *HSP90AA1* gene.

The results obtained from the association studies of SSCP genotypes on physiological, production and reproduction traits are presented in Tables 1, 2 and 3 respectively.

Association analysis of *HSP70* gene polymorphism with physiological traits

The SSCP genotypes of exon 3 of the *HSP90AA1* gene had no significant effect on the physiological parameters studied in both Sahiwal and Crossbred cows. However, Kumar *et al.* (2015) found that AA genotype of exon 3 of *HSPAA1* gene had lower heat tolerance coefficient as compared to AG and GG genotypes in Sahiwal cows and GG genotype had lower mean respiration rate, rectal temperature and HTC in Karan Fries cows (Kumar *et al.*, 2016).

Association with production traits

The SSCP genotypes of exon 3 of *HSP90AA1* gene obtained in the present study had a significant effect on lactation length with AA genotype having higher lactation length in Sahiwal cows and higher total lactation milk yield and peak yield in crossbred cows. The genetic polymorphisms in exon 3 of *HSP90AA1* gene and its association with milk production

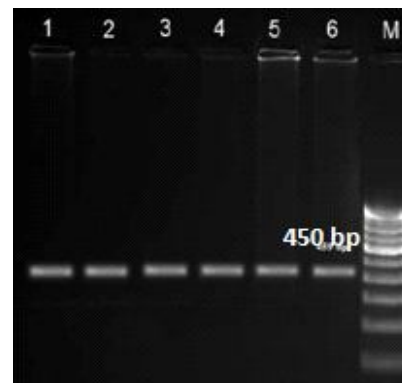


Fig 1: Agarose gel electrophoresis image showing PCR amplified product (450bp) of *HSP90AA1* gene.

traits reveal their importance as a potential genetic marker for milk production traits in Sahiwal and Crossbred cows.

Association analysis with reproduction traits

The differences obtained in reproductive traits due to different genotypes were not statistically significant in both

Sahiwal and crossbred cows. The results could not be compared as there were no studies pertaining to the association of *HSP90* gene polymorphisms in indigenous or crossbred cattle breeds with respect to the reproduction traits.



Fig 2: Polyacrylamide gel electrophoresis showing PCR-SSCP patterns for exon 3 of *HSP90AA1* gene. Lane 1-8 Sahiwal; Lane 9-16 Crossbred cows.

Table 1: Means of *HSP90AA1* exon 3 genotypes and parity effects for physiological traits in Sahiwal and Crossbred cows.

Effect	Sahiwal							Crossbreds						
	n	RR		RT (°C)		HTC		n	RR		RT (°C)		HTC	
		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE
Genotype	50	24.15	0.21	38.29	0.02	2.05	0.01	50	30.92	0.38	38.57	0.04	2.35	0.02
AA	28	24.27	0.28	38.27	0.03	2.05	0.01	34	31.31	0.44	38.58	0.04	2.37	0.02
AB	22	24.03	0.31	38.31	0.03	2.04	0.01	16	30.53	0.60	38.57	0.06	2.33	0.03
Parity														
1	13	24.63	0.39	38.35	0.04	2.07	0.02	5	31.57	1.25	38.67	0.12	2.38	0.05
2	11	24.31	0.43	38.30	0.04	2.05	0.02	7	30.87	0.97	38.50	0.09	2.35	0.04
3	18	23.83	0.34	38.25	0.04	2.03	0.01	12	30.79	0.71	38.53	0.07	2.35	0.03
4	8	23.84	0.51	38.27	0.05	2.03	0.02	9	31.11	0.83	38.65	0.08	2.36	0.04
5	-	-	-	-	-	-	-	9	30.28	0.81	38.64	0.08	2.33	0.04
6	-	-	-	-	-	-	-	8	29.27	0.82	38.60	0.08	2.28	0.04

Table 2: Means of *HSP90AA1* exon 3 genotypes and parity effects for production traits in Sahiwal and Crossbred cows.

Effect	Sahiwal							Crossbreds						
	n	TLMY		PY		LL		n	TLMY		PY		LL	
		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE
Genotype	50	1759.15	104.68	10.05	0.39	268.24	12.55	50	2808.13	94.21	13.46	0.48	327.89	2.69
AA	28	1879.78	137.36	10.60	0.51	295.62 ^a	16.46	34	3025.11 ^a	108.84	14.46 ^a	0.56	332.65	3.10
AB	22	1638.52	153.53	9.49	0.57	240.85 ^b	18.40	16	2591.15 ^b	146.20	12.45 ^b	0.75	323.14	4.17
Parity														
1	13	1956.74	195.70	10.52	0.72	313.82	23.45	5	2597.33 ^c	305.81	13.34	1.57	336.25 ^a	8.72
2	11	1615.74	212.78	9.60	0.79	245.22	25.50	7	3408.80 ^a	236.44	16.40	1.21	337.95 ^a	6.74
3	18	1941.69	167.70	11.24	0.62	279.64	20.10	12	3210.51 ^a	174.88	14.00	0.90	321.25 ^c	4.99
4	8	1522.44	250.56	8.82	0.93	234.28	30.03	9	3382.87 ^a	202.81	16.43	1.04	320.11 ^c	5.78
5	-	-	-	-	-	-	-	9	3270.39 ^a	197.73	14.37	1.01	312.68 ^d	5.64
6	-	-	-	-	-	-	-	8	2995.00 ^b	199.67	14.00	1.02	329.61 ^b	5.70

Means with similar superscripts in a column do not differ significantly ($P \leq 0.05$).

Table 3: Means of *HSP90AA1* exon 3 genotypes and parity effects for reproduction traits in Sahiwal and Crossbred cows.

Overall	Sahiwal							Crossbreds						
	n	SP (days)		DP (days)		CI (days)		n	SP (days)		DP (days)		CI (days)	
		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE
	44	150.56	8.18	140.66	7.08	436.24	6.79	46	203.18	9.45	158.24	7.47	418.42	8.04
Genotype														
AA	24	160.68	10.65	138.34	9.22	437.16	8.66	32	184.97	10.89	150.03	8.60	407.07	9.27
AB	20	140.45	11.90	142.97	10.30	435.32	10.09	14	221.39	15.22	166.45	12.03	429.76	12.96
Parity														
1	13	191.30 ^a	14.16	144.41	12.26	464.01	11.70	5	185.26	30.00	157.26	23.71	443.55	25.54
2	11	147.10 ^b	15.40	168.34	13.33	420.08	12.72	7	247.96	23.19	135.96	18.33	428.73	19.74
3	14	138.55 ^{bc}	13.68	127.12	11.84	444.51	11.42	11	212.16	18.68	176.94	14.76	415.71	15.90
4	6	125.29 ^c	20.99	122.77	18.17	416.36	17.34	7	191.40	21.33	165.57	16.85	424.95	18.16
5	-	-	-	-	-	-	11.70	8	191.87	20.63	172.44	16.30	419.68	17.56
6	-	-	-	-	-	-	12.72	8	192.90	21.33	153.57	16.85	420.28	18.16

Means with similar superscripts in a column do not differ significantly ($P \leq 0.05$).

CONCLUSION

The present study revealed an association between the different SSCP genotypes of exon 3 of *HSP90AA1* gene and the production traits in Sahiwal and crossbred cows. These genetic polymorphisms reveal their importance as genetic markers in selecting cows for better production and reproductive performance. Further research is needed to establish the physiological mechanisms by which these polymorphisms determine the association between genotypes and performance of cows.

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