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Identification of Polymorphism within Exon 8 of Bovine *HSP90AA1* Gene using PCR-SSCP Technique

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Abstract

Heat shock proteins (*HSP*s) are a type of molecular chaperones that aid in the recovery of stressed cells and serve as a major system for intracellular self-defense. A study was conducted during the year 2018–19 at College of Veterinary Science Rajendranagar, Hyderabad, Telengana State, India to find polymorphisms in exon 8 of the bovine *HSP90AA1* gene in Sahiwal (n=50) and crossbred (n=50) cows. Blood samples were collected from the experimental animals and genomic DNA was isolated. Physiological parameters like body temperature and respiration rate for each animal were taken during the experimental period and the heat tolerance coefficient was calculated. The data on production and reproduction traits were obtained from the history sheets of the animals. To detect the polymorphism, a 539 bp fragment of the *HSP90AA1* gene covering exon 8 was subjected to the Polymerase Chain Reaction-Single-Strand Conformation Polymorphism (PCR-SSCP) technique.The PCR-SSCP of exon 8 of *HSP90AA1* gene yielded two genotypic patterns AA and AB corresponding to two allelic variants with frequencies of 0.85, 0.15, 0.81 and 0.19 in Sahiwal and crossbred cows, respectively. The PCR-SSCP patterns obtained were correlated with the physiological, productive, and reproductive traits in both Sahiwal and crossbred cows. The association analysis of SSCP patterns of the exon 8 of *HSP90AA1* gene revealed non-significant effect in Sahiwal cows, although the AB genotype had a significantly longer service period in crossbred cows.

Keywords: Crossbred cows, Exon 8, Heat stress, *HSP90AA1*, Polymorphisms, Sahiwal

1. Introduction

Heat shock proteins (*HSP*s) are molecular chaperones that assist cells in recovering from stress and providing cytoprotection, which protects them from further attack. They protect stressed cells by identifying nascent polypeptides, unstructured protein regions, and exposed hydrophobic amino acid stretches. Heat shock proteins are encoded by *HSP* genes. Though there are many *HSP* genes, thermo tolerance in livestock animals is primarily associated with the *HSP70* and *HSP90* genes. The most common and temperature-sensitive is *HSP90*, which is thought to play an important role in environmental stress and thermal adaptation (Gade et al., 2010; Aritonang et al., 2017; Puteri et al., 2018). In summer, higher levels of expression of proteins of the *HSP70* and *HSP90* families have been observed in a variety of livestock species (Archana et al., 2017). Polymorphisms in the *HSP90* genes have been associated to heat

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tolerance, milk production, fertility, and disease susceptibility in livestock (Shergojry et al., 2014a; Kumar et al., 2015; Bhat et al., 2016). They may be useful as candidate gene markers for identifying animals with enhanced climatic resistance, immunological response, and performance (Hassan et al., 2019).

Holstein Friesian is extensively used for crossbreeding in India (Wakchaure et al., 2015). Several crossbred populations with Friesian inheritance ranging from 35.93% to 93.75% were investigated and performance evaluated. It was observed that crossbreds with 62.5% of Friesian inheritance were found to be superior to all other crosses (Bhaduria and Katpatal, 2003, Lakshmi et al., 2010).

Sahiwal is among the best milch breeds of the Indian sub-continent (Nivsarkar et al., 2013), with an average lactation milk yield of 1880 kg (Verma et al., 2016). They are highly tolerant of tropical diseases and are known for their adaptability to heat stress. However, their number is fast dwindling due to indiscriminate crossbreeding and is resulting in the loss of unique heat stress genes due to genetic recombination (Singh, 2016). Therefore, there is an urgent need to characterize these unique genes in Sahiwal and other indigenous cattle breeds.

Although differences in thermo tolerance have been documented at the physiological and cellular level in both *Bos indicus* and *Bos taurus* cattle (Collier et al., 2006; Chaiyabutr et al., 2008; Behl et al., 2010; Wilson and Crandall, 2010, and Dalcin et al., 2016), data on polymorphism of *HSP* genes in Sahiwal cattle and Holstein Friesian crossbreds is limited. Only a few reports on the relationship 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., 2015a), and Holstein cows (Sailo et al., 2015b; Li et al., 2011) have been published.

The chaperone *HSP90* is one of the most abundant proteins in eukaryotic cells, comprising 1–2% of cellular proteins under non-stress conditions (Young et al., 2001). There are two major cytoplasmic *HSP90* isoforms, the inducible form (*HSP90AA1*/*HSP90*α) and the constitutive form (*HSP90*AB1/*HSP90*β), which have arisen by gene duplication (Chen et al., 2005).

HSP90AA1 gene is located on *Bos taurus* autosome 21 (BTA 21) and spans nearly 5368 bp comprising of 11 exons out of which the first exon does not translate. In the current study, polymorphisms in the exon 8 of the *HSP90AA1* gene and their associations with various physiological, production, and reproduction traits in Sahiwal and crossbred cows were investigated.

2. Materials and Methods

2.1. Experimental animals

A total of 50 Sahiwal cows were used in this study.

2.2. Genomic DNA isolation

Genomic DNA was extracted from blood samples of the experimental animals (50 Sahiwal cows maintained at the Livestock Farm Complex, College of Veterinary Science Rajendranagar, and 50 crossbred cows (Holstein Friesian X Sahiwal) maintained at the Military Dairy Farm, Secunderabad) using the standard phenol-chloroform extraction method as 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.

2.3. 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 season, respectively, and the average was taken as the final reading for association analysis in the study. 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 the formula given by Benezra (1954).

2.4. Production and reproduction traits

Data on each animal about different aspects like Animal no., Sire no., Dam no., Date of birth, Date of calving, Lactation length and Lactation milk yield, etc., were collected from the history sheet/daily farm registers maintained at the concerned farms. The various production and reproduction traits like Total Lactation Milk Yield (TLMY), Peak Yield (PY), Lactation length (LL), Service Period (SP), Dry Period (DP) and Calving Interval (CI) were calculated from the available data in both Sahiwal and crossbred cows.

2.5. PCR primers and amplifications

Primers (procured from BioServe Biotechnologies Pvt Ltd, Hyderabad) specific for the desired fragment (539bp) of the *HSP90AA1* gene covering exon 8 region were used to amplify the targeted region. The details of primer sequences, length of the primer (bp), melting temperature (Tm) are presented in Table 1.

The PCR reaction mixture was prepared for a 12.5 µl reactionusing the components as given in Table 2 and the PCR cycling conditions used are given in Table 3.

The PCR products were detected by electrophoresis on 2%

Table 1: Sequence of primers, their respective number of bases and melting temperature of bovine HSP gene fragment

Gene fragment		Sequence (5'-3')	Length of the primer (bp)	Melting Temperature (°C)
Exon 8 539bp	F	CCCATGGGAACAGTTGAGTG	20	54
	R	GCTTTAAGCTCCTTTTAAGTTCG	23	52

F: Forward; R: Reverse

Table 2: Components of PCR reaction mixture

Components	Volume (µl)
Taq polymerase (5 units μl^{-1})	0.125
dNTPs (10 mM)	0.8
Primer-forward (10 pM)	1.0
Primer-reverse (10 pM)	1.0
10× Taq buffer	2.5
Nuclease-free water (NFW)	6.075
Template DNA	1.0
Total	12.5

Table 3: Conditions of PCR reaction

Steps	Temperature (°C)	Time
Initial denaturation	95	5 min
Cyclic denaturation	95	30 sec
Primer annealing	51	45 sec
Cyclic extension	72	30 sec
Steps 2 to 4 were repeated for 35 cycles		
Final extension	72	10 min
Hold	4	Forever

agarose gel stained with ethidium bromide.

2.6. Single strand conformation polymorphism (SSCP)

Polymorphism in exon 8 of *HSP90AA1* gene was screened using the single-strand conformation polymorphism (SSCP) technique using the amplified PCR products. The variants were identified basing on the band pattern observed in the SSCP gels after silver staining (Bassam and Gresshoff, 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.

2.8. Genotype and allele frequencies

Genotype frequencies for variant genotypes were calculated using the formula:
Genotypefrequency=(No.ofanimals with specific genotype (AA, AB or BB))/Total No.ofanimals

Allele frequencies were calculated as follows:

Where,
AA and BB=Genotype frequencies of homozygotes
AB=Genotype frequency of heterozygote
A and B=Allele frequencies

2.9. Association analysis

Statistical analysis was done to study the association of each SSCP genotype on physiological, production, and reproduction traits in Sahiwal and crossbred cows. Data on physiological traits was corrected for season effect and 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…n)
Pj=Effect of j^{th} parity of the animal at the time of blood collection (j= 1…n)
e_{ijk}=Random error assumed to be distributed normally and independently with mean zero and variance σ^2_e.

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±standard errors.

3. Results and Discussion

3.1. PCR-SSCP of exon 8 of HSP90AA1 gene

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 8 of *HSP90AA1* gene. Amplification of desired size was noticed in all the tested samples. The representative figures showing the PCR amplified products of exon 8 of *HSP90AA1* gene, showing the size of 539 bp are presented in Figure 1, while the PCR-SSCP polyacrylamide gel imageis presented in Figure 2.

PCR-SSCP of exon 8 (539 bp)of the *HSP90AA1* gene revealed two different SSCP patterns AA and AB in both Sahiwal and crossbred cows. Consequently, at this locus two alleles namely A and B with allelic frequency of 0.85 and 0.15, respectively in Sahiwal and 0.81 and 0.19, respectively in crossbred cowswere present. The frequencies of AA and BB genotypes were estimated to be 0.70 and 0.30 in Sahiwal and 0.62 and

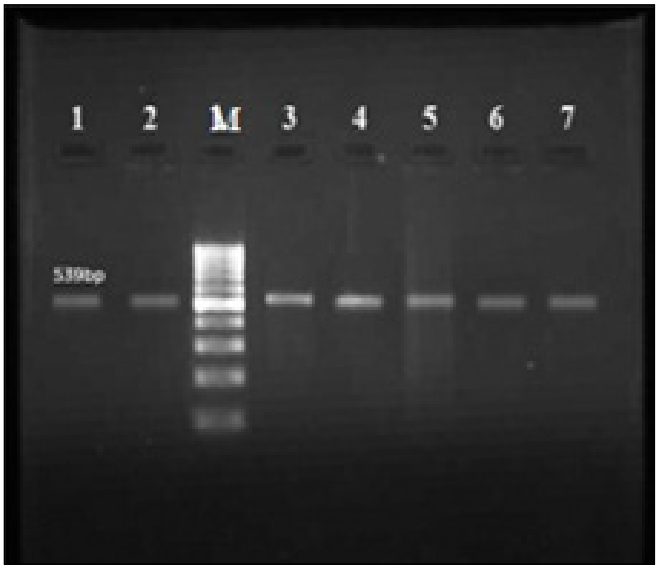

Figure 1: Agarose gel electrophoresis image showing PCR amplified product (539bp) of *HSP90AA1* gene

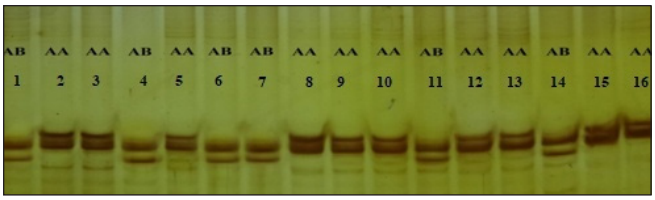

Figure 2: Polyacrylamide gel electrophoresis showing PCR-SSCP patterns for 539bp fragment of HSP90AA1 gene. Lane 1-8 Sahiwal; Lane 9-16 Crossbred cows

0.38 in crossbred cows, respectively. However, Shergojry et al. (2014a) analyzed the polymorphism in the same region (exon 8) of *HSP90AA1* gene using the PCR-SSCP technique in Deoni cattle and revealed three unique SSCP genotypes with frequencies of 0.250, 0.639 and 0.111 respectively. Shergojry et al. (2014b) also found two SSCP patterns in exon 9 region of*HSP*AA1 gene with frequencies of 0.153 and 0.847 and two PCR-SSCP patterns in exon 10 with frequencies 0.236 and 0.764 respectively in Deoni cows. Also 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.

3.2. Association analysis of HSP90AA1 gene polymorphism

The means obtained from the analysis of variance for the effect of exon 8 of *HSP90AA1* genotypes on the physiological, production and reproduction traits in Sahiwal and crossbred cows are presented in Tables 4, 5 and 6 respectively.

Table 4: Means of *HSP90AA1* exon 8 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
	50	23.97	0.24	38.28	0.03	2.04	0.01	50	30.95	0.38	38.57	0.04	2.35	0.02
Genotype														
AA	35	24.38	0.25	38.30	0.03	2.06	0.01	31	31.27	0.45	38.57	0.04	2.37	0.02
AB	15	23.56	0.45	38.25	0.05	2.02	0.02	19	30.63	0.58	38.57	0.06	2.34	0.03
Parity														
1	13	24.80	0.40	38.36	0.04	2.08	0.02	5	31.33	1.26	38.66	0.12	2.37	0.06
2	11	23.89	0.50	38.27	0.05	2.03	0.02	7	30.86	0.97	38.50	0.09	2.35	0.04
3	18	23.72	0.34	38.24	0.04	2.03	0.01	12	30.90	0.70	38.53	0.07	2.35	0.03
4	8	23.47	0.56	38.24	0.06	2.01	0.02	9	31.23	0.82	38.65	0.08	2.37	0.04
5	-	-	-	-	-	-	-	9	30.42	0.79	38.64	0.07	2.33	0.03
6	-	-	-	-	-	-	-	8	29.37	0.82	38.60	0.08	2.29	0.04

Table 5: Means of *HSP90AA1* exon 8 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
	50	1707.48	123.80	10.03	0.47	268.94	15.56	50	2837.11	97.55	13.64	0.50	328.48	2.73
Genotype														
AA	35	1841.91	125.71	10.19	0.47	273.94	15.80	31	2974.37	114.64	14.09	0.59	331.67	3.20
AB	15	1573.05	227.87	9.86	0.86	263.94	28.65	19	2699.86	148.53	13.20	0.76	325.28	4.15
Parity														
1	13	2017.72	203.34	10.63	0.77	317.84	25.56	5	2479.25c	319.67	12.85	1.65	333.60^{ab}	8.94
2	11	1470.35	253.30	9.38	0.95	237.73	31.85	7	3392.85a	247.18	16.29	1.27	337.64^{a}	6.91
3	18	1923.69	173.04	11.31	0.65	284.06	21.75	12	3285.80a	177.99	14.42	0.92	322.82^{c}	4.98
4	8	1418.17	285.08	8.80	1.07	236.13	35.84	9	3456.25a	208.75	16.79	1.07	321.69^{c}	5.83
5	-	-	-	-	-	-	-	9	3364.50a	200.01	14.90	1.03	314.65^{d}	5.59
6	-	-	-	-	-	-	-	8	3045.61b	208.75	14.21	1.07	330.74^{b}	5.83

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

Table 6: Means of *HSP90AA1* exon 8 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	150.73	10.41	135.46	8.74	432.90	8.76	46	203.74	9.23	158.00	7.44	417.267	8.119
Genotype														
AA	32	152.56	9.80	144.48	8.23	439.20	8.25	30	183.21b	10.68	150.29	8.61	409.163	9.393
AB	12	148.91	19.63	126.45	16.49	426.60	16.52	16	224.27a	14.70	165.71	11.85	425.372	12.932
Parity														
1	13	192.78	15.11	147.70	12.69	466.50	12.71	5	198.18	29.45	162.57	23.74	450.04	25.90
2	11	144.36	19.47	159.53	16.35	413.70	16.38	7	247.49	22.77	136.06	18.35	429.38	20.02
3	14	138.96	15.40	121.63	12.93	441.04	12.96	11	204.84	17.18	173.13	13.85	409.59	15.18
4	6	126.84	24.18	112.98	20.30	410.36	20.35	7	192.18	20.93	165.40	16.87	423.87	18.41
5	-	-	-	-	-	-	-	8	193.52	20.22	172.08	16.30	417.36	17.78
6	-	-	-	-	-	-	-	8	186.83	20.72	150.83	16.70	416.50	18.22

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

3.2.1. Associationwith physiological traits

The SSCP genotypes of exon 8 of the *HSP90AA1* gene obtained in the present study had no significant effect on the physiological parameters studied in both Sahiwal and crossbred cows. However, Kumar et al. (2015) found that other regions of *HSPAA1* gene were polymorphic and that AA genotype 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)

3.3. Association with production traits

The effect of genotype was found to be non significant in both genetic groups, while parity significantly influenced the total lactation milk yield and lactation length in crossbred cows. No studies were available to compare the results, with respect to the PCR- SSCP genotypes.

3.4. Association analysis with reproduction traits

The differences obtained in various traits due to different genotypes of exon 8 of the *HSP90AA1* gene were not statistically significant in Sahiwal cows, whereas in crossbred cows the effect of genotype was significant only on service period with AB genotype having longer service period. 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.

4. Conclusion

The SSCP patterns obtained in the current study for various fragments of *HSP* genes and their association analysis with physiological and production parameters revealed no significant differences between genotypes, implying that a larger sample size with a broad genetic base may be required to elucidate the polymorphism association.

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