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GENETIC CHARACTERIZATION OF CHOLISTANI BREED OF CATTLE FOR ATP1A1 GENE AND ITS ASSOCIATION TO HEAT TOLERANCE TRAITS

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Climate change and effects of heat stress on animals are evident on different biological systems in form of oxidative stress and at cellular membrane level as Na^+-K^+-ATPase gradient. The present study was planned for genetic characterization of Cholistani cattle (N=25) using three primers for exon14, intron 14, exon 16 and exon and intron 17 regions of ATP1A1 gene. Rectal temperature (RT), respiration rate (RR), vaginal temperature (VT) and ear skin temperature (outer surface) (EST) was recorded in the morning and afternoon of the same day while heat tolerance coefficient (HTC) was calculated for each animal using recorded physiological parameters and adaptability of animals was observed in heat stress. The genetic characterization was done using molecular technique polymerase chain reaction (PCR). The means and standard error values of response variable traits RR, RT and VT were 23.34 ± 4.41 and 25.14 ± 4.41 bpm, 38.01 ± 0.43 and 38.81 ± 0.35 °C, 38.11 ± 0.37 and 38.86 ± 0.34 °C and corresponding HTC values were 2.01 ± 0.09 and 2.11 ± 0.13 in the morning and afternoon, respectively. The average values of RR using primer[1], in genotype AA (23.51 ± 0.53), AB (24.97 ± 0.84) and BB (24.48 ± 0.86 bpm) were not statistically different from one another. The RT and VT for the genotype AA (38.41 ± 0.04 and 38.44 ± 0.05 °C), AB (38.37 ± 0.04 and 38.47 ± 0.05 °C) and BB (38.49 ± 0.03 and 38.61 ± 0.03 °C) were not statistically different from one another. Significant ($p < 0.05$) effect of genetic variant (BB) of ATP1A1 gene (primer[1]) on VT (higher) of Cholistani cattle was found. Diurnal variations of temperature humidity index (THI) (higher in afternoon) showed significant increase ($p < 0.05$) in rectal temperature and respiration rate. These genotypes and relative traits can be used as biological indicators for assessment of heat stress tolerance in Cholistani cattle.

Keywords: ATP1A1 gene, thermotolerance, Cholistani, Na^+-K^+-ATPase

INTRODUCTION

Livestock production systems are facing many threats (infectious diseases, nutritional stress, heat stress, climate change stress) which create hindrance in getting maximum output (Batool *et al*., 2019; Butt *et al*., 2019; Firyal *et al*., 2019; Marwan *et al*., 2019; Saber *et al*., 2020; Siregar *et al*., 2019; Zafar *et al*., 2019). Climate change is a big challenge for existing agricultural and livestock production systems because climatic variables like solar radiations, temperature and humidity have been recognized as hazards after threshold level for health, growth, production and reproduction of animals (Bernabucci *et al*., 2010; Yatoo *et al*., 2012; Ganaie *et al*., 2013). Threshold for heat stress in cattle is THI equals 72 (Ravagnolo *et al*., 2000) which in summer usually surpasses and cause the heat stress in animals. Large animals particularly cattle being homeotherms can regulate their body temperature using heat dissipation phenomenon to match environmental conditions (Zumbach *et al*., 2008). Capacity of thermal tolerance in animals considerably varies at specie and breed levels as presented by Hall (2004) and McManus *et al*. (2008). Cellular and physiological variations in animals do exist regarding thermo tolerance (Lacetera *et al*., 2006). Considerable genetic differences have been observed in cattle at higher temperature humidity indices (Ravagnolo and Misztal, 2002). Most of the previous studies have been conducted for thermo-tolerance focusing mainly on temperature, relative humidity or the index of both called temperature humidity index (West, 2003). In dairy cows, higher levels of ambient temperature can cause the heat stress of moderate level, oxidative stress and reduced milk production (Bernabucci *et al*., 2002; Kaldur *et al*., 2014). Sodium and potassium level were influenced by the heat stress (El-Nouty *et al*., 1980). Plasma markers of oxidative stress presented weak negative effects of thermal stress (Calamari *et al*., 1999). The Na^+-K^+-ATPase gene may be used as candidate for thermo tolerance traits. Only one SNP was reported in ATP1A1 gene (Hawken *et al*., 2004). Significant association of genetic polymorphism in ATP1A1 gene with HTC, RT, daily milk yield and 305 day milk yield was revealed; level of Na^+ and K^+ in plasma is affected by the heat stress (Srikandakumar and Johnson, 2004). The α1

isoform of α-subunit of Na^+-K^+-protein complex were reported to present in RBCs were encoded by ATP1A1 gene in bovines. This bovine ATP1A1 gene is of 3065 nucleotides having 23 exons located on autosome 3 in *Bos taurus* spanning a total length of 22768 base pairs and mRNA length of 3746 bases. Heat shock effects have been reported to affect the synthesis of DNA and gene expression (Lacetera *et al*., 2009). ATP1A1 gene may possibly affect the heat tolerance in cattle, novel SNP can potentially be used for marker assisted genetic selection in dairy cattle (Liu *et al*., 2011). Exposure of animals to higher temperature causes changes in the activities of Na^+-K^+-ATPase in tissues (Levy *et al*., 2005). The genetic variation of ATP1A1 gene has been found to affect the feed intake in cattle (Barendse *et al*., 2007). Environmental temperature and humidity affect the electrolyte balance of plasma (Banerjee and Ashutosh, 2011) particularly significant change in sodium-potassium enzymes (ATPase) activity (Kashyap *et al*., 2014). This enzyme regulates the electrochemical gradient across plasma membrane, representing a plausible candidate for heat tolerance traits (Geering *et al*., 1987). The Cholistani breed of cattle is mainly found in the warm areas of Punjab, Pakistan which can be used as model to study response to thermal stress (Farooq *et al*., 2010). Cholistani cattle is relatively new breed emerged from crosses of Sahiwal and local non-descript cattle, having ability to withstand severe heat stress (Khan *et al*., 2005) and Cholistani cattle breed was registered in Pakistan livestock census in 2006 as a new breed (Khan *et al*., 2008). Yet, no study has been reported on Cholistani cattle at physiological response as well as for genetic basis of thermotolerance. Keeping in view the importance of Cholistani breed in harsh climate of Cholistan and role of ATP1A1 gene in regulatory mechanism during heat stress, the present study was carried out on Cholistani cattle to identify genetic polymorphism in ATP1A1 gene and its association with tolerance traits.

MATERIALS AND METHODS

The study was conducted during months of March to September on Cholistani cattle (*Bos indicus*) (N=25) having at least one complete lactation record, with good body conditions at Government Livestock Farm (GLF) Jugait Pir, District Bahawalpur. The blood was collected from jugular vein in EDTA coated vacutainers under hygienic conditions. Blood samples at immediate were shifted to icebox and transported to lab for storage at -20 °C till DNA extraction.

The Cholistani cattle were evaluated for rectal temperature (RT) and vaginal temperature (VT) (Suthar *et al*., 2013), using mercury glass thermometer inserted in rectum and vagina for 60 seconds, ear outer skin surface temperature (EST) using infrared digital thermometer (AR-320, Intell instruments) and respiration rate (RR) from the chest movement of animals twice for a period of 30 seconds to estimate RR per minute for 5 days in the morning and afternoon. The calculation of heat tolerance coefficient (HTC) based on respiration rate and rectal temperature using index by Benezra (1954) as presented in equation (1)

$$\text{HTC} = \frac{\text{RR}}{23} + \frac{\text{RT}}{38.3} \qquad (1)$$

Where, RT is average rectal temperature of each animal and 38.33 ºC is normal rectal temperature of cattle. RR is average respiration rate per minute while 23 are taken as normal respiration rate in cattle. The HTC index was calculated to assess the heat stress adaptability for individual cow, animal with lower HTC value have better adaptability traits as compared to animals with high values of HTC.

Data on ambient temperature (°C) and relative humidity (%) was recorded on experimental site as well as from the meteorological data center facility in the morning and afternoon. Temperature humidity index (THI) was calculated using equation (2) (Garcia-Ispierto *et al*., 2007).

$$\text{THI} = \left[(0.8 \times \text{Tdb}) + \left\{\left(\frac{\text{RH}}{100}\right) \times (\text{Tdb} - 14.4)\right\} + 46.4\right] \qquad (2)$$

Genomic DNA was extracted from whole blood using method (Sambrook *et al*., 1989) in Molecular Genetics Lab, Institute of Animal and Dairy Sciences, Faculty of Animal Husbandry, University of Agriculture, Faisalabad, Pakistan. Primers used for this study were based on bovine ATP1A1 gene sequence available at GenBank database (GenBank accession No. NC_007301.3) as reported by (Liu *et al*., 2010; Liu *et al.*, 2011). The primers used to amplify different sized fragments are presented in the Table 1.

The reaction mixture for PCR was of 25 µL, prepared using reaction ingredients as genomic DNA (50 ng/µL), forward and reverse primers both (10 pM), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM), *Taq* polymerase (5 U/µL), 17 µL of double distilled deionized water (d_3H_2O) and PCR Buffer (10X) with $MgCl_2$. The thermo cycler PCR profile in step one was denaturation

Table 1. Primers used for amplification and the fragment sizes

Primers	Primer sequence	F.L*	Targeted site	Reference
ATP1A1 gene Primer[1]	F:5'-TGAGCAACCAACGCAACACT-3' R:5'-TGGAACTGCAATCACTGAGGTC-3'	330	exon 14 and parts of intron 14	Liu *et al*., 2010
ATP1A1 gene Primer[2]	F:5'-AATGACTCCCCGGCTTTGAAGA-3' R:5'-GGCCACGGGGACCCAGAGAAC-3'	491	Partial exon 16 -Intron 17	Das *et al*., 2015
ATP1A1 gene Primer[3]	F:5'-ACAAACAAAAGGGTCACAACAT-3' R:5'-CTTACCCTAGATCCTGGCTCAT-3'	301	Exon 17	Liu *et al*., 2011

*F.L. = fragment length

at 95 °C for 5 minutes, then in step two cycles (n=30) of 94 °C (30 s), 58 °C (30 s) and 72 °C (60 s) and in step three final extension temperature was 72 °C for 10 minutes and programed for hold on at 4 °C till to use for gel electrophoresis. The PCR amplified products were observed using gel electrophoresis (1.0% agarose added with ethidium bromide) (1.0 µL) and finally documentation using UV-trans-illuminator gel doc system (Uvipro, Gas 7300NP, Uvitec, UK).

***Statistical analysis*:** For the genotypic and allelic frequency calculations POPGENE software was used (Yeh *et al*., 1999). The associations of genetic variants of ATP1A1 with the traits (RT, VT, EST, RR and HTC) were calculated using general linear model (GLM) procedure of SAS, 2008. The following model was used (3),

$$Y_{ijk} = \mu + G_i + T_j + e_{ijk} \quad (3)$$

Where, Y_{ijk} is phenotypic value for traits, µ is overall mean, G_i is the effect of $_i$th genotype, T_j is effect of time of the day and e_{ijk} is random error associated with individual observation. The parity and age of cows did not have significant effect on response variables so were not included in model. The respiration rate, rectal temperature, vaginal temperature, ear skin surface temperature and heat tolerance coefficient were taken as response variables while THI and genetic variant of ATP1A1 gene were independent variables.

RESULTS AND DISCUSSION

The maximum temperature humidity index (THI) calculated was 83.78 and 88.72 for morning and afternoon, respectively while corresponding minimum THI was 47.08 and 62.31. Overall, the results revealed an increasing trend in respiration rate (RR), ear skin surface temperature (EST) and rectal temperature (RT) with the increase in THI. Effect of THI on RT and vaginal temperature (VT) in Cholistani cattle was significant ($p < 0.05$). Effect of time of the day (morning and afternoon) was significant ($p < 0.05$) on the RT, VT, and RR in Cholistani cattle. Non-significant ($p > 0.05$) effect of THI was recorded for respiration rate in Cholistani cattle.

The PCR products of primers of ATP1A1 gene were successfully amplified in Cholistani cattle. Allelic and genotypic frequencies are presented in Table 2. Primer^1 was used to amplify a 330 bp fragment from exon14 and part of intron14 ATP1A1 gene. Primer^2 was to target 491 bp in partial exon and 16-17 intron of ATP1A1 gene. The primer^3 was for targeted amplification of 301 bp fragment of exon 17 of ATP1A1 gene. The PCR products of all three primers showed genetic polymorphism (AA, AB and BB) in Cholistani cattle.

The genetics variants of ATP1A1 gene did not show significant effects on RT and RR. Significant ($p < 0.05$) effect of genetic variant (BB) of ATP1A1 gene (primer^1) on the VT of Cholistani cattle was found. Correlation of the RT and VT was significant ($p < 0.05$) while correlation of RR and parity with RT and VT was non-significant ($p > 0.05$).

The mean values with standard error of RR using primer^1 genotype AA (23.51 ± 0.53), AB (24.97 ± 0.84) and BB (24.48 ± 0.86 bpm) were not statistically different from one another. The RT and VT for the genotype AA (38.41 ± 0.04 and 38.44 ± 0.05 °C), AB (38.37 ± 0.04 and 38.47 ± 0.05 °C) and BB (38.49 ± 0.03 and 38.61 ± 0.03 °C) were not statistically different from one another. Significant ($p < 0.05$) differences were observed for the ear skin surface temperature in Cholistani cattle for the genetic variants based on primer^3. Animals with genetic variant BB showed the least HTC values although differences among genotypes were non-significant. The minimum values of HTC index among all the animals was 1.87 while maximum HTC value was 2.23. The parity and age have non-significant effects on VT, RT and HTC and non-significant correlations with these parameters. The identification of genotypes associated with the physiological parameter variation as a response to environmental variables stress will be helpful to identify animals with desirable alleles for that stress factor (Collier *et al*., 1993). In domestic animals among all physiological variables, respiration rate and rectal temperature has been reported as the most sensitive indicators of thermal stress tolerance (Verma *et al*., 2000).

Table 2. Allelic and genotypic frequency for various locus of ATP1A1 gene in Cholistani cattle

Primers	Genotype	Frequency	Allele	Frequency
Primer^1	AA	0.369	A	0.61
	AB	0.477		
	BB	0.154	B	0.39
Primer^2	AA	0.108	A	0.33
	AB	0.441		
	BB	0.451	B	0.67
Primer^3	AA	0.270	A	0.52
	AB	0.499		
	BB	0.231	B	0.48

Overall least square means of rectal temperature (°C), vaginal temperature (°C), ear skin surface temperature (°C), respiration rate (bpm) and heat tolerance coefficient were 38.33 ± 0.56, 38.41 ± 0.51, 33.23 ± 2.09 °C, 23.96 ± 4.48 bpm and 2.057 ± 0.09. The response variable traits RR, RT, VT were 23.34 ± 4.41 and 25.14 ± 4.41 bpm, 38.01 ± 0.43 and 38.81 ± 0.35 °C, 38.11 ± 0.37 and 38.86 ± 0.34 °C and corresponding HTC values were 2.01 ± 0.09 and 2.11 ± 0.13 in the morning and afternoon (Table 3).

Various gene has been presented as candidate gene for heat tolerance traits (Olson *et al*., 2003) among them ATP1A1 gene has its importance due to involvement in physiological roles. The polymorphism in ATP1A1 gene has been reported to be associated with feed intake reduction in cattle (Barendse *et al*., 2007). Na^+-K^+-ATPase activity was associated with

Table 3. Least square means for RT, VT, EST, RR and HTC for different genotypes and overall

Effects	Subclass	RT (°C)	VT (°C)	EST (°C)	RR (bpm)	HTC
Overall mean		38.33±0.56	38.41±0.51	33.23±2.09	23.96±4.48	2.06±0.09
THI (AM)	83.78	38.01±0.43	38.11±0.37	31.48±1.09	23.34±4.41	2.00±0.09
THI (PM)	88.72	38.81±0.35	38.86±0.34	34.98±1.19	25.14±4.41	2.11±0.13
Primer[1]	AA	38.41±0.04	38.44±0.05	32.92±0.17	23.51±0.53	2.02±0.02
	AB	38.37±0.04	38.47±0.05	33.46±0.34	24.97±0.84	2.09±0.04
	BB	38.49±0.03	38.61±0.03	33.38±0.40	24.48±0.86	2.07±0.03
Primer[2]	AA	38.42±0.04	38.50±0.04	32.86±0.21	23.96±0.68	2.04±0.03
	AB	38.41±0.03	38.46±0.05[a]	33.50±0.25	25.05±0.58	2.09±0.03
	BB	38.42±0.08	38.50±0.09[b]	34.04±0.48	23.22±0.76	2.01±0.04
Primer[3]	AA	38.43±0.03	38.48±0.03	33.13±0.19[a]	24.15±0.59	2.05±0.02
	AB	38.37±0.02	38.45±0.07	33.39±0.21[b]	24.04±0.63	2.04±0.03
	BB	38.45±0.07	38.53±0.06	33.32±0.66[c]	24.86±1.23	2.08±0.06

(Different asterisk presents significant differences in column, LS Means were compared using Tukey test)

heat resistance (Yang, 2007) in cattle. The HTC and RT has been used as a factor for assessment of thermo tolerance (McManus *et al*., 2005; Verma *et al*., 2000). In Chinese Holstein cows with CC genotypes showed a higher ability to adapt heat stressed environment, and ATP1A1 gene can play important role in heat shock response (Liu *et al*., 2011). Higher level of the mRNA was observed in cows at 32.5 as compared to 12.5 °C temperature (Liu *et al*., 2011). Different levels of ATP1A1 gene expression revealed its association with animal's ability to respond to environmental variables (Kashyap *et al*., 2014). The threshold THI for cattle was 72 (Ravagnolo *et al*., 2000) while the level of the present study THI was above the comfort zone of cattle. The ability of the cows to regulate its physiological activities and body temperature under heat stress response is in part under the genetic control in relevance to ATP1A1 gene.

***Conclusion*:** In this study genetic polymorphism of ATP1A1 gene in Cholistani cows was observed. The variation in vaginal temperature and heat tolerance coefficient among different genotypes indicate that ATP1A1 gene contributes to the heat tolerance traits. The genetic variants of the gene and response variables RT, RR and HTC can be used as biological marker for assessment of thermotolerance in Cholistani cattle.

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