

Identification of SNPs in ATP1A1 Gene and their Association with Heat Tolerance in Cattle



THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
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FOR THE AWARD OF THE DEGREE OF

MASTER OF VETERINARY SCIENCE

IN

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BY

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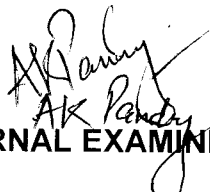
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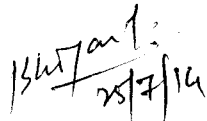
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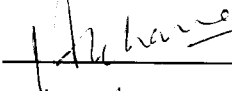
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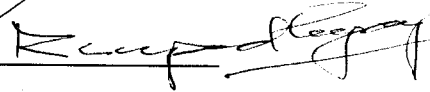

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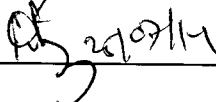
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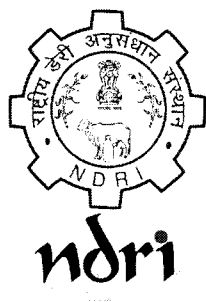
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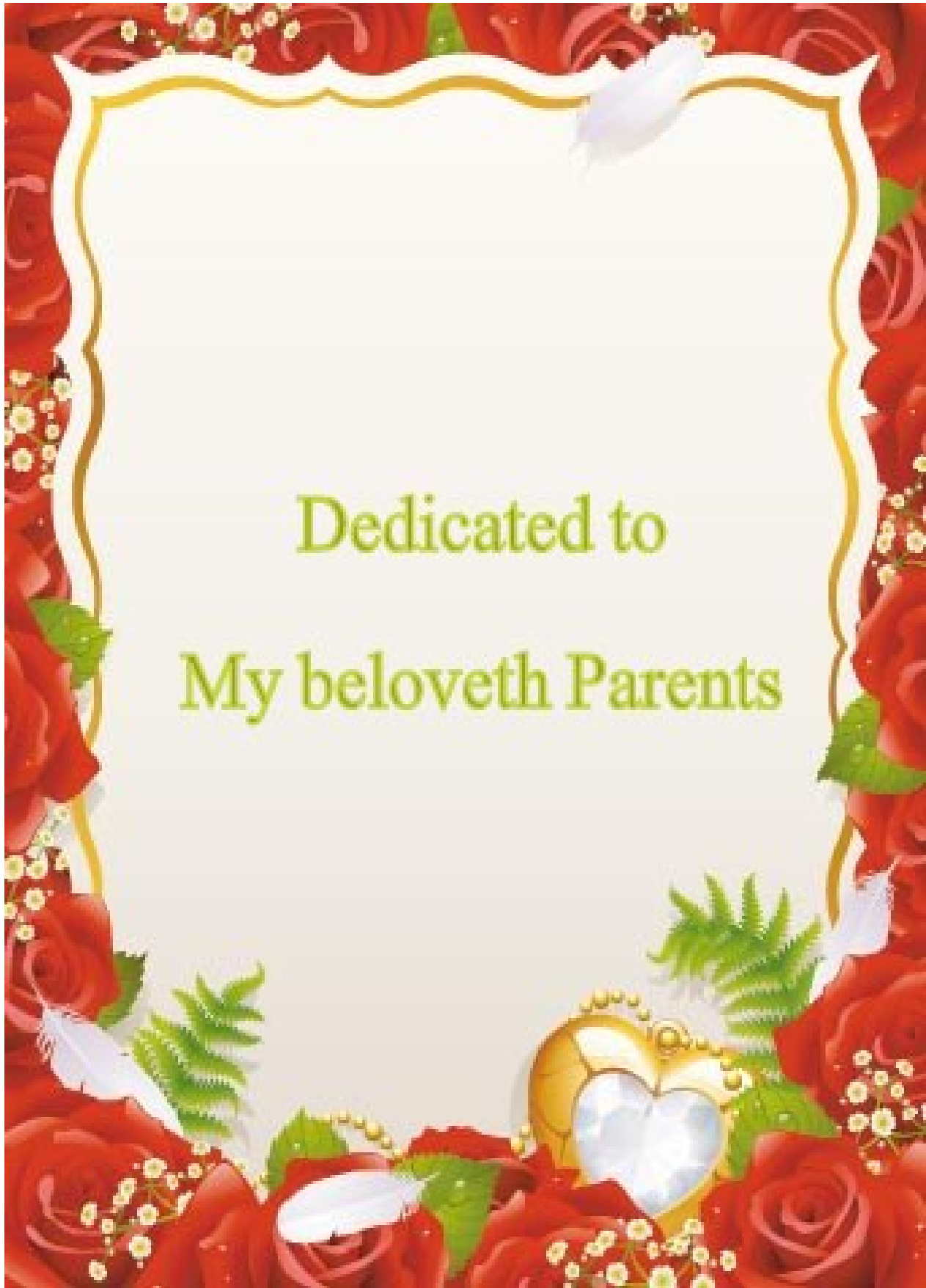
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This is to certify that the thesis entitled, **"Identification of SNPs in ATP1A1 gene and their association with heat tolerance in cattle"** submitted by **Mr. Ramendra Das** in partial fulfilment of the award of the degree of **MASTER OF VETERINARY SCIENCE in Animal Genetics and Breeding** of the National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

(I. D. Gupta)
Major Advisor & Chairman

Dated: 9th July, 2014



Dedicated to
My beloveth Parents

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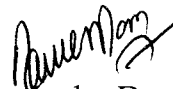
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ABBREVIATIONS

A	:	Adenine
A ₂₆₀	:	Absorption at 260 nano meters
A ₂₈₀	:	Absorption at 280 nano meters
ATP1A1	:	Sodium Potassium ATPase 1 alpha 1
bp	:	Base pair
C	:	Cytisine
EDTA	:	Ethylene Diamine Tetra acetic Acid
EtBr	:	Ethidium bromide
G	:	Guanine
GLM	:	General linear model
HSP	:	Heat shock protein
HTC	:	Heat tolerance coefficient
LSM	:	Least square mean
mg	:	Milligram
µg	:	Microgram
µl	:	Microlitre
MgCl ₂	:	Magnesium chloride
ng	:	Nano gram
OD	:	Optical Density
PCR	:	Polymerase Chain Reaction
pmol	:	PicoMole
rpm	:	Revolution Per Minute
RR	:	Respiration rate
RT	:	Rectal temperature
SE	:	Standard error
SNP	:	Single Nucleotide Polymorphism
TBE	:	Tris Borate EDTA
T	:	Thymine
T _a	:	Annealing temperature
TE	:	Tris EDTA
T _m	:	Melting temperature
UV	:	Ultra violet
V	:	Volt

ABSTRACT

Global warming and climate change have become the major threat to the sustainability of livestock production systems worldwide. In the plasma membrane the ion gradients formed by $\text{Na}^+ - \text{K}^+$ -ATPase enzyme is necessary for Na^+ - coupled transport of metabolites, nutrients and ions and represents a plausible candidate for heat tolerance traits. ATP1A1 gene encodes for α subunit of $\text{Na}^+ - \text{K}^+$ - ATPase enzyme, has been mapped on BTA 3 and spans about 22.77 kb and includes 23 exons and coding sequence of 3066 nucleotides. Present study was carried out in Sahiwal (51), Karan Fries (50) and Jersey crossbred (35) cows with the objectives of identification of SNPs in the targeted regions of ATP1A1 gene and to analyze their association with respiration rate and rectal temperature. Genomic DNA was isolated from blood samples of all cows. Two sets of primers were designed using DNASTAR software by referring bovine ATP1A1 gene sequence number ENSBTAG00000001246. Two amplicons of 491 and 465 bp were obtained successfully from all DNA samples using primer set 1 and 2, respectively. PCR products were subjected to custom sequencing and results for the respective region were visualized using Chromas Lite software. Each sequence was aligned with corresponding reference sequence using ClustalW multiple sequence alignment program to identify SNPs. A total of six SNPs (T27008243C, A27008223G, T27008097A, C27008016T, G27008015A and C27007790A) were found in all studied breeds. All six SNPs were observed in Sahiwal and Karan Fries cows whereas SNP T27008243C was not observed in Jersey crossbred cattle. Respiration rate and rectal temperature was recorded once during probable extreme hours in winter, spring and summer season. Association of each SNP genotypes was analyzed using GLM procedure in SAS. Sahiwal cows with CC genotype at T27008243C locus had highest RR (19.24 ± 1.52^a) compared to TT (15.91 ± 1.89^b) and TC (18.25 ± 1.77^{ab}) genotypes while heat tolerance coefficient (HTC) for TT genotype at T27008097A locus was highest (1.95 ± 0.05^a) compared to TA (1.79 ± 0.06^b) and AA (1.65 ± 0.11^c) genotypes. RR for GG genotype at A27008223G locus was highest (32.37 ± 2.51^a) compared to AA (28.85 ± 1.96^b) and rectal temperature (RT) for TT genotype at T27008097A locus was highest (38.32 ± 0.14^a) compared to AA (37.56 ± 0.32^b) in Karan Fries cows. In Jersey crossbred cows RT (38.01 ± 0.16^a) for AA and GG (37.86 ± 0.14^a) genotype at A27008223G locus did not differ significantly while RR for CA genotype at C27007790A locus was highest (21.76 ± 1.68^a) as compared to CC (19.25 ± 1.155^b) and AA (16.91 ± 1.57^{ab}) genotypes and HTC for CA genotype at C27007790A locus was highest (1.94 ± 0.07^a) as compared to CC (1.83 ± 0.05^b) and AA (1.72 ± 0.07^{ab}) genotypes. It is concluded that AA and TA genotype at 27008097 position in Sahiwal and Karan Fries cows were desirable for heat tolerance coefficient and rectal temperature respectively, whereas, AA and GG genotype at 27008223 position in Karan Fries and Jersey crossbred cows were desirable for respiration rate and rectal temperature respectively.

ATP1A1 जीन में SNPs की पहचान और उनके पशुओं में गर्मी सहने की क्षमता के साथ संबंध

सारंश

ग्लोबल वार्मिंग एवं जलवायु परिवर्तन दुनिया भर में पशुधन उत्पादन प्रणालियों की स्थिरता के लिये खतरा है। एवं ग्रीष्म सहनशीलता के लिये Na^+ आयन युग्मित मेटाबोलाईट, पोषक तत्वों और आयन परिवर्तन के लिए Na^+-K^+ ATPase एन्जाइम द्वारा प्लाज्मा झिल्ली में आयन ढलान आवश्यक होता है। ATP1A1, Na^+-K^+ ATPase एन्जाइम के एल्फा सबयूनिट को संकेतिक करता है। और ATP1A1 को भी मैच करता है। यह वंशाणु 22.77 केबी लम्बा है, जिसमें 3066 न्यूक्लियोटाइड अनुक्रम के कुल 23 एक्जोन शामिल हैं। वर्तमान अध्ययन साहीवाल (51), करनफीज (50) एवं जर्सी संकर (35) गायों का ATP1A1 जीन के लक्षित क्षेत्र में SNPs की पहचान एवं इसका स्वसन दर, गुदा तापमान के साथ संबंध के उद्देश्य के साथ किया। प्राइमर के दो सेट गो जातीय ATP1A1 जीन अनुक्रमों में संख्या ENSBTAG00000001246 से DNASTAR साफ्टवेयर का उपयोग करके बनाया गया है। प्राइमर सेट 1 एवं 2 का प्रयोग करके क्रमशः 491 और 465 bp एम्पलीकन सभी DNA नमूनों से सफलतापूर्वक प्राप्त किया गया। पी.सी. आर. उत्पादों के कस्टम अनुक्रमों को क्रोमास साफ्टवेयर के द्वारा चिह्नित किया गया। SNPs की पहचान के लिए Clustal W एकाधिक अनुक्रमों में संरेखण प्रोग्राम का उपयोग संदर्भित अनुक्रमों के प्रत्येक अनुक्रमों के साथ श्रेणीबद्ध किया गया है। कुल छह SNPs (T27008243C, A27008223G, 27008097A, C27008016T, G27008015A एवं C27007790A) सभी सुविचारित नस्लों में पाये गये। सभी छह SNPs साहीवाल, करन फीज गायों में पाये गये जबकि SNP T27008243C जर्सी संकर गायों में नहीं पाया गया। स्वसन दर एवं गुदा तापमान सर्दी, वसंत एवं गर्मी के संभावित चरण समय के दौरान एक बार दर्ज किये गये। प्रत्येक SNP जीनोटाइप का संबंध विश्लेषण SAS में GLM प्रक्रिया द्वारा किया गया। साहीवाल गायों में TT (15.91 ± 1.89^b) एवं TC (18.25 ± 1.77^{ab}) जीनोटाइप स्वसन दर की तुलना में CC जीनोटाइप के T27008243C लोकस में सर्वाधिक स्वसन दर (19.24 ± 1.52^a) पायी गयी, जबकि ग्रीष्म सहनशीलता गुणांक (HTC) TA (1.79 ± 0.06^b) एवं AA (1.65 ± 0.11^c) जीनोटाइप की तुलना में TT जीनोटाइप के T27008097A लोकस पर सर्वाधिक (1.95 ± 0.05^a) पाया गया। करन फीज गायों में AA (28.85 ± 1.96^b) स्वसनदर की तुलना में GG जीनोटाइप के A27008223G लोकस के लिए स्वसनदर सर्वाधिक (32.37 ± 2.51^a) पाया गया और गुदा तापमान के लिए AA (37.56 ± 0.32^b) की तुलना में TT जीनोटाइप T27008097A लोकस पर सर्वाधिक (38.32 ± 0.14^a) पाया गया। जर्सी संकर गायों में AA जीनोटाइप A27008223G पर (38.01 ± 0.16^a) एवं GG जीनोटाइप (37.66 ± 0.14^a) गुदा तापमान में सार्थक अन्तर नहीं मिला। जबकि स्वसन दर के लिए CC (19.2 ± 1.16^b) एवं AA (16.91 ± 1.57^{ab}) की तुलना में CA जीनोटाइप के C27007790A लोकस पर सर्वाधिक (21.76 ± 1.68^a) पाया गया। ग्रीष्म सहनशीलता गुणांक के लिये CC जीनोटाइप (1.83 ± 0.05^b) और AA (1.72 ± 0.07^{ab}) की तुलना में CA जीनोटाइप के C27007790A लोकस पर सर्वाधिक 1.94 ± 0.07^a पाया गया। इस अध्ययन से निष्कर्ष निकाला गया कि साहीवाल एवं करनफीज गायों में ग्रीष्म सहनशीलता गुणांक एवं गुदातापमान के लिए 27008097 स्थिति क्रमशः AA और TA जीनोटाइप वांछित है। जबकि करनफीज एवं जर्सी संकर गायों में स्वसन दर एवं गुदा तापमान के लिए 27008223 स्थिति में क्रमशः AA एवं GG जीनोटाइप वांछित थे।

CONTENTS

S. No.	Chapter	Page No.
1.0	Introduction	1-2
2.0	Review of Literature	3-22
2.1	Heat Stress	3
2.2	Temperature Humidity Index (THI)	3
2.3	Thermoneutral Zone	5
2.4	Thermoregulation	5
2.5	Acid Base Homeostasis	6
2.6	Effects of heat stress in dairy cattle	7
2.6.1	Effects on Production	7
2.6.2	Effects on Reproduction	9
2.7	Single-nucleotide polymorphisms (SNPs)	11
2.8	Heat shock protein (HSP)	11
2.9	Heat Shock Response (HSR)	12
2.10	Heat stress and physiological parameters	13
2.10.1	Rectal Temperature (RT)	13
2.10.2	Respiration Rate (RR)	14
2.11	ATP1A1 Gene	15
2.11.1	Biological function of ATP1A1 gene	15
2.11.2	Chromosomal localization, structure and molecular function of Bovine ATP1A1 Gene	16
2.11.3	Na ⁺ - K ⁺ - ATPase pump	19
2.12	Association studies of ATP1A1 Gene Polymorphism with Heat Tolerance Traits in Dairy Cattle	20
3.0	Material and Methods	23-34
3.1	Experimental Animals	23
3.2	Physiological Parameters Recorded	23
3.2.1	Respiration Rate	23
3.2.2	Rectal Temperature	23
3.3	Recording frequency of Physiological parameters	24
3.4	Physiological Parameters Estimated for Heat tolerance	24
3.4.1	Heat Tolerance Coefficient (HTC)	24
3.5	SNP Genotyping of Experimental Animals	24
3.5.1	Isolation of Genomic DNA	24

Contd...

S. No.	Chapter	Page No.
3.5.1.1	Blood Collection	24
3.5.1.2	DNA Extraction	25
3.5.2	Quality and Quantity Check of DNA	26
3.5.3	Preparation of Working Solution of DNA	27
3.5.4	Polymerase Chain Reaction (PCR) Amplification of Targeted Region of Bovine ATP1A1 gene	27
3.5.4.1	Primers	27
3.5.4.2	Reaction mixture	28
3.5.4.3	PCR programme	28
3.5.4.4	Agarose Gel Electrophoresis to Check PCR Amplification	28
3.5.5	DNA Sequencing	30
3.5.6	Sequence Data Analysis for SNP Detection and genotyping	30
3.6	Statistical analysis	30
3.6.1	Allele and genotype frequencies	30
3.6.2	Fixed effects considered in association analysis	30
3.6.2.1	Temperature Humidity Index (THI)	30
3.6.2.2	Parity	32
3.6.2.3	SNP locus	32
3.6.3	Association analysis of SNPs with rectal temperature, respiration rate and heat tolerance coefficient in Sahiwal, Karan fries and Jersey crossbred cows	32
4.0	Results and Discussion	35-48
4.1	Genomic DNA isolation	35
4.2	Polymerase Chain Reaction (PCR) Standardization	35
4.2.1	Template DNA Concentration	35
4.2.2	Primer Concentration	35
4.2.3	Annealing Temperature (Ta)	36
4.3	Polymerase Chain Reaction	36
4.4	Quality of PCR product	36
4.5	Analysis of DNA sequencing data	36
4.6	Allele and Genotype frequencies of ATP1A1 gene	37
4.7	Association analysis of SNPs with rectal temperature, respiration rate and heat tolerance coefficient	37
4.7.1	Association analysis of SNPs with rectal temperature, respiration rate and heat tolerance coefficient in Sahiwal cows	40

Contd...

S. No.	Chapter	Page No.
4.7.2	Association analysis of SNPs with rectal temperature, respiration rate and heat tolerance coefficient in KF cows	43
4.7.3	Association analysis of SNPs with rectal temperature, respiration rate and heat tolerance coefficient in Jersey crossbred cows	46
5	Summary and Conclusions	49-51
	Bibliography	i-xiv
	Appendix	i-iv

List of Tables

S. No.	Tables	Page No.
2.1	Temperature and Humidity combinations yielding a THI of 72	4
2.2	Effect of Environment on Nutrient Requirements of Dairy Animals	9
2.3	ATP1A1 Gene in different species	17
2.4	Nomenclature of ATP1A1 gene in cattle	18
2.5	Exon and Intron Length of ATP1A1 Gene	18
3.1	Experimental animals for the study	23
3.2	Frequency of recording the physiological parameters in Sahiwal, Karan Fries and Jersey cross cattle	24
3.3	Sequence of primers, their respective number of bases, target region and amplicon sizes of Bovine ATP1A1 gene	29
3.4	PCR programme for Primer set 1 and Primer set 2 for ATP1A gene	29
3.5	THI for all days in each season during which physiological parameters were recorded in Sahiwal and Karan Fries cows	31
3.6	Subclasses of fixed effects considered in association analysis	33
4.1	Sequence of primer fragment - 1 of ATP1A1 gene and SNPs screened in Sahiwal, Karan Fries and Jersey crossbred cattle	After 38 th page
4.2	SNPs and Genotypes for primer fragment – 1 in Sahiwal, Karan Fries and Jersey crossbred cattle	
4.3	Sequence of primer fragment - 2 of ATP1A1 gene and SNPs screened in Sahiwal, Karan Fries and Jersey crossbred cattle	
4.4	SNPs and Genotypes for primer fragment – 2 in Sahiwal, Karan Fries and Jersey crossbred cattle	
4.5	Allelic frequency at each SNP locus of ATP1A1 gene in Sahiwal, Karan Fries and Jersey crossbred cows	38
4.6	Genotypic frequency at each SNP locus of ATP1A1 gene in Sahiwal, Karan Fries and Jersey crossbred cows	39
4.7	ANOVA of linear model for dependent variable RR, RT and HTC in Sahiwal cows	41
4.8	ANOVA of fixed effects for dependent variable RR, RT and HTC in Sahiwal cows	41

Contd...

S. No.	Tables	Page No.
4.9	Least squares means of subclasses of different fixed effects for RR, RT and HTC in Sahiwal cows	42
4.10	ANOVA of linear model for dependent variable RR, RT and HTC in Karan Fries cows	44
4.11	ANOVA of fixed effects for dependent variable RR, RT and HTC in Karan Fries cows	44
4.12	Least squares means of subclasses of different fixed effects for RR, RT and HTC in Karan Fries cows	45
4.13	ANOVA of linear model for dependent variable RR, RT and HTC in Jersey crossbred cows	47
4.14	ANOVA of fixed effects for dependent variable RR, RT and HTC in Jersey crossbred cows	47
4.15	Least squares means of subclasses of different fixed effects for RR, RT and HTC in Jersey crossbred cows	48

LIST OF FIGURES

S. No.	Figure	After Page No.
2.1	Critical temperatures and thermo-neutral zone in dairy cattle	5
2.2	Effect of Heat Stress on Dietary Electrolyte Balance (DEB)	6
2.3	Physiological Response of cattle to Heat Stress	8
2.4	Mechanisms of effects of heat stress on reproduction in the lactating cow	9
2.5	Organization and Location of ATP1A1 Gene	18
2.6	Mechanism of action of Na ⁺ - K ⁺ ATPase Pump	20
4.1	Multiple Sequence Alignment of PCR product of ATP1A1 Gene obtained by Primer 1 in Sahiwal cows	36
4.2	Chromatograph showing T27008243C SNP in Sahiwal	36
4.3	Chromatograph showing A27008223G SNP in Sahiwal	36
4.4	Multiple Sequence Alignment of PCR product of ATP1A1 Gene obtained by Primer 2 in Sahiwal cows	36
4.5	Chromatograph showing T27008097A SNP in Sahiwal cows	36
4.6	Chromatograph showing C27008016T SNP in Sahiwal cows	36
4.7	Chromatograph showing G27008015A SNP in Sahiwal cows	36
4.8	Chromatograph showing C27007790A SNP in Sahiwal cows	36
4.9	Multiple Sequence Alignment of PCR product of ATP1A1 Gene obtained by Primer 1 in Karan Fries cows	36
4.10	Chromatograph showing T27008243C SNP in Karan Fries cows	36
4.11	Chromatograph showing A27008223G SNP in Karan Fries cows	36
4.12	Multiple Sequence Alignment of PCR product of ATP1A1 Gene obtained by Primer 2 in Karan Fries cows	36

Contd...

S. No.	Figure	After Page No.
4.13	Chromatograph showing T27008097A SNP in Karan Fries cows	36
4.14	Chromatograph showing C27008016T SNP in Karan Fries cows	36
4.15	Chromatograph showing G27008015A SNP in Karan Fries cows	36
4.16	Chromatograph showing C27007790A SNP in Karan Fries cows	36
4.17	Multiple Sequence Alignment of PCR product of ATP1A1 Gene obtained by Primer 1 in Jersey crossbred cows	38
4.18	Chromatograph showing A27008223G SNP in Jersey crossbred cows	38
4.19	Multiple Sequence Alignment of PCR product of ATP1A1 Gene obtained by Primer 2 in Jersey crossbred cows	38
4.20	Chromatograph showing T27008097A SNP in Jersey crossbred cows	38
4.21	Chromatograph showing C27008016T SNP in Jersey crossbred cows	38
4.22	Chromatograph showing G27008015A SNP in Jersey crossbred cows	38
4.23	Chromatograph showing C27007790A SNP in Jersey crossbred cows	38

LIST OF PLATES

Plate No.	Title	After Page No.
4.1	Genomic DNA of Sahiwal, Karan Fries and Jersey crossbred cows	36
4.2		
4.3		
4.4	Sahiwal PCR Products: Primer- 1 (491 bp) & Primer- 2 (465 bp)	
4.5		
4.6	Karan Fries PCR Products: Primer- 1 (491 bp) & Primer- 2 (465 bp)	
4.7		
4.8	Jersey crossbred PCR Products: Primer- 1 (491 bp) & Primer- 2 (465bp)	
4.9		

CHAPTER - 1

Introduction

1. Introduction

The weather conditions prevailing in an area in general or over a long period time is termed as climate, which is a combination of elements that include temperature, humidity, rainfall, air movement, radiation, barometric pressure and ionization (Johnson, 1987). In 2007, the Intergovernmental Panel on Climate Change (IPCC) reported that the decadal earth's temperature increases by 0.2°C per decade and also predicted that the global average surface temperature would be increased to 1.4-5.8°C by 2100. Global warming and climate change have become the major threat to the sustainability of livestock production systems worldwide (Gaughan *et al.*, 2010). India is currently losing nearly 2% of total milk production, amounting to Rs 2661 crores due to heat stress among dairy cattle and buffalo. The negative impact of temperature rise due to global warming on total milk production for India was also estimated about 3.2 million tonnes in year 2020 and more than 15 million tonnes in year 2050. (Upadhyay *et al.*, 2009) which might be due to decreased availability of quality fodder as well as decrease production due to effect of heat stress on dairy animal physiological function lowering productivity. Environmental, psychological, nutritional, pathological and managerial stressors cause significant heat stress to livestock resulting in reduced feed consumption (Bernabucci *et al.*, 1999), decreased milk production (Sharma *et al.*, 1988) and a lower reproductive success rate (Cavestany *et al.*, 1985).

The strategies for reducing the impact of heat stress on cattle productivity include modification of the environment, nutritional management and genetic selection of animals with thermo tolerance (Beede and Collier, 1986; Hansen and Arechiga, 1999; Ravagnolo and Misztal, 2000). Genetic selection for core body temperature regulation is one potential strategy to mitigate effects of heat stress on dairy cows so that an animal with genetic adaptations that make for more effective regulation of core body temperature will experience a less severe reduction in production (Sharma *et al.*, 1988). 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, 2004; Lacetera *et al.*, 2006). The Indian cattle breeds, also known as 'zebu cattle' are broadly categorized into milch, draft and dual purpose breeds based upon their utility. The zebu cattle breeds have specific qualities like disease resistance, heat

tolerance, ability to survive and reproduce under low feed input (Muralidhar *et al.*, 2004). Indigenous cattle (*Bos indicus*) survive and perform better as compared to temperate breeds or their crossbreds under heat stress (Collier *et al.*, 2008).

Heat Shock protein (HSP) are the best-suited examples of genes whose expression is effected by heat stress, it has been apparent in recent years that thermal stress also leads to induction of a substantial number of genes not traditionally considered to be HSP genes. Approximately 50 genes not traditionally considered to be HSPs have been found to undergo changes in expression in response to heat stress (Sonna *et al.*, 2002). The Na⁺ - K⁺ ATPase enzyme accounts for at least 30% of the resting energy metabolism of the brain, utilizing energy derived from the hydrolysis of ATP to pump Na⁺ out of cells in exchange for K⁺ (Albers *et al.*, 1989). The ATP1A1 gene encodes the α 1 isoform, the major isoforms of α subunit of Na⁺ - K⁺- ATPase pump. ATP1A1 gene is responsible for establishing the electrochemical gradient of Na⁺ and K⁺ across the plasma membrane. These gradients are essential for osmoregulation, for sodium-coupled transport of a variety of organic and inorganic molecules and for electrical excitability of nerve and muscle. In the plasma membrane the ion gradients formed by Na⁺ - K⁺-ATPase enzyme is necessary for Na⁺- coupled transport of metabolites, nutrients and ions and represents a plausible candidate for heat tolerance traits (Geering *et al.*, 1987). ATP1A1 gene has been mapped on BTA 3 and spans about 22.77 kb and includes 23 exons and coding sequence of 3066 nucleotides (<http://www.ensembl.org>).

There are few reports in India regarding association of ATP1A1 gene polymorphism with heat tolerance in Tharparkar and Cross bred cattle (Kashyap, 2012), Deoni cattle (Barani, 2013) and Murrah buffaloes (Jayakumar, 2014) and abroad in Holstein cow (Liu *et al.*, 2010; Liu, *et al.*, 2011). These studies indicated importance of ATP1A1 gene polymorphism and its association with heat tolerance parameters. There is no report available about identification of ATP1A1 gene variants and their association with thermo-tolerance in Sahiwal, Karan Fries and Jersey crossbred cattle. Therefore the present study was planned with objectives:

1. Identification of SNPs in the targeted regions of ATP1A1 gene in Sahiwal and crossbred cattle
2. Analysis of association of genetic variants with respiration rate and rectal temperature

CHAPTER - 2

Review of Literature

2. Review of Literature

2.1 Heat Stress

Stress is reaction of body to stimuli that disturb homeostasis often with detrimental effects (David *et al.*, 1990). Livestock undergo various kinds of stress such as physical, nutritional, chemical, psychological and thermal stress. Among all heat stress is most concerning now a days in the ever changing climatic scenario, caused by a combination of environmental factors (temperature, relative humidity, solar radiation, air movement, and precipitation). In tropical and sub tropical regions high ambient temperature is the major constraint on animal production (Marai *et al.*, 2007; Nardone *et al.*, 2010). Much of studies on heat stress in livestock have focused mainly on temperature and relative humidity (Igono *et al.*, 1985; Igono and Johnson, 1990; Ravagnolo and Misztal, 2000; Bouraoui *et al.*, 2002; St-Pierre *et al.*, 2003; West, 2003; Correa-Calderon *et al.*, 2004) as these records can be usually obtained from meteorological stations.

Heat production study on adult cattle indicated that the heat produced by Zebu cattle was 62.0 Kcal/hr/100 Kg body weight during summer season against 80 Kcal/hr/100 Kg during hot-humid and winter season which clearly indicate the higher energy needs during winter and rainy season than summer due to extra energy expenditure during rainy season and winter season (Rao *et al.*, 1979).

2.2 Temperature-Humidity Index (THI)

The Temperature Humidity Index (THI) was developed by Thom (1959) and later adapted for dairy cows by Kibler (1964). Fuquay (1981) observed that the THI was commonly used method to know the degree of heat stress in animal. It is currently used to estimate cooling requirements of dairy cattle in order to improve the efficiency of management strategies to alleviate heat stress.

Temperature Humidity Index (THI) is a single value which represents the combined effects of air temperature and humidity, used to estimate the degree of thermal stress experienced by dairy cows. This index has been developed as a weather safety index to monitor and reduce heat stressed related losses. It is now widely used in livestock industry particularly in cattle (Mount, 1979). Different animal species have different sensitivities to ambient temperature and the amount of

moisture in the air depending on thermoregulatory mechanism (Yousef, 1985). The Livestock Conservation Institute evaluated the biological responses to varying THI values and categorized them into mild, moderate and severe stress levels for cattle (Armstrong, 1994). THI map of India based on more than 100 locations has been developed (Upadhyay *et al.*, 2008) indicated that on an average the THI values in coastal regions exceeds the comfortable conditions during most part of the year, which affects production, reproduction and health of the animals. It was recorded that milk yield of crossbred cows in India (e.g., Karan Fries, Karan Swiss and other Holstein and Jersey crosses) were negatively correlated with temperature-humidity index (Mandal *et al.*, 2002). Lactating dairy cows experienced heat stress when THI rises above 72, with severe heat stress occurring when THI exceeds 88 (Thatcher *et al.*, 2010). The table below (Table 2.1) illustrates various temperatures and humidity values that yield a THI of 72, the point at which signs of heat stress begin to develop in the dairy cow. For example, a temperature of 80° F, with a relative low humidity value of 35%, will yield a THI of 72 and create conditions that could impact the health and performance of a dairy cow (Zwald, 2007).

Table 2.1 Temperature and Humidity combinations yielding a THI of 72

Temperature (°F)	Relative Humidity (%)
72	100
74	80
76	60
80	35
84	15

The upper limit of THI at which cattle may maintain stable body temperature is ranges from 72 – 76 (Igono *et al.*, 1992; Ravagnolo and Misztal, 2000). Livestock are comfortable at THI between 65 and 72, under mild stress from 72 to 78 and above 80 under severe stress. High thermal stress (THI > 78) requires greater efforts to dissipate heat (Upadhyay *et al.*, 2008).

2.3 Thermoneutral Zone

The thermoneutral zone (TNZ) is defined as the range of ambient temperature within which thermoregulation is achieved only by control of sensible heat loss, i.e., without regulatory changes in metabolic heat production or evaporative heat loss (IUPS Thermal Commission, 2001). The Zone of Thermoneutrality can also be defined as the range of temperature within which no additional energy above maintenance is expended to heat or cool the body. For livestock this TNZ ranges between – 5 and 25°C, and were called as lower critical temperature (LCT) and upper critical temperature (UCT) (West, 2003). Lower critical temperature (LCT) is the ambient temperature below which the rate of metabolic heat production of a resting animal must be increased by shivering and or non shivering thermogenesis in order to maintain thermal balance (IUPS Thermal Commission, 2001). Upper critical temperature (UCT) is the ambient temperature above which the rate of evaporative heat loss of a resting thermo regulating animal must be increased (e.g. tachypnea or sweating) in order to maintain thermal balance. It has also been used to define the ambient temperature above which the mechanisms of heat transfer to the environment of a resting animal are exceeded and the core temperature is forced to rise and, as a consequence, metabolic energy transformation, i.e., the internal heat load, is further increased (IUPS Thermal Commission, 2001). Figure 2.1 shows LCT and UCT for dairy cattle (West, 2003). Thermoneutral zone of animals depends on the age, breed, feed intake, diet composition, previous state of temperature acclimatization, production, and housing and stall conditions, tissue (fat, skin) insulation and external (coat) insulation, and the behaviour of the animal.

2.4 Thermoregulation

Acclimation is a phenotypic response developed by the animal to an individual source of stress within the environment (Fregley, 1996). Heat is produced in the animal's body by metabolic activities and may also be gained from the environment. Heat is lost from the body by radiation, conduction, convection, evaporation of water from skin and respiratory passages and excretion of faeces and urine. The degree to which an animal resists change in body temperature varies with different species because of differences in their heat regulating mechanisms. A thermal steady state exists, when the heat gain and the heat loss are balanced. Body temperature in homeotherms is maintained by the thermoregulatory system within 1°C of its normal

temperature under ambient conditions that do not impose heat stress. In homeotherms, the various thermoregulatory mechanisms consist of a series of physiological adjustments that serve to establish a thermal steady state at the level of normal body temperature, which consequently struggle to maintain equality in heat gain and heat loss. A rise in the core body temperature increases heat loss by panting and sweating. These responses are physiological strategies to transfer heat from the cow's body to the environment. In order to maintain homeotherms, an animal must be in thermal equilibrium with its environment, which includes radiation, air temperature, air movement and humidity. Acclimation to high environmental temperatures involves responses that lead to reduce heat load. The immediate responses are the reduction of feed intake, increase in respiration rate and water intake and changes in hormonal signals that affect target tissue responsiveness to environmental stimuli (Collier and Zimbelman, 2007).

2.5 Acid-Base Homeostasis

Heat stress is cyclic in nature, with cows generally being at the peak of their stress by mid-afternoon and cooling in the evening and early morning hours (West, 1999). This cyclic nature induces a nycthermal pattern of acid-base homeostasis. It was first proposed by Dale and Brody in cattle (Dale and Brody, 1954). *Bos taurus* and *Bos indicus* cattle was found to have similar patterns of the nycthermal changes in acid-base and electrolyte balance, but in case of later the changes was being less pronounced (Beatty *et al.*, 2006). The acid-base balance of animals is dependent upon the balance between anions and cations in the blood affecting animal performance (Figure 2.2). High ambient temperature in addition with relative humidity elevates body temperature and increase respiration rate in cattle (Schneider *et al.*, 1984). Due to increased frequency of respiration there was increased loss of CO₂ through the lungs results into decreased blood carbonic acid content. As a consequence, animal suffered from respiratory alkalosis. However, to compensate such condition, animal excrete more bicarbonate in the urine to maintain the carbonic acid: bicarbonate ratio. Compensation results in urinary bicarbonate loss in excess (Benjamin, 1978). Thus, the acid-base chemistry of the heat stressed cow differs significantly from the normothermic cow. All the reactions evoked in response to climatic changes are directed towards maintaining or restoring thermal balance (Kibler and Brody, 1953). Heat stress can also results in chronic hyperthermia,

Figure 2.1 Critical temperatures and thermo-neutral zone in dairy cattle (West, 2003)

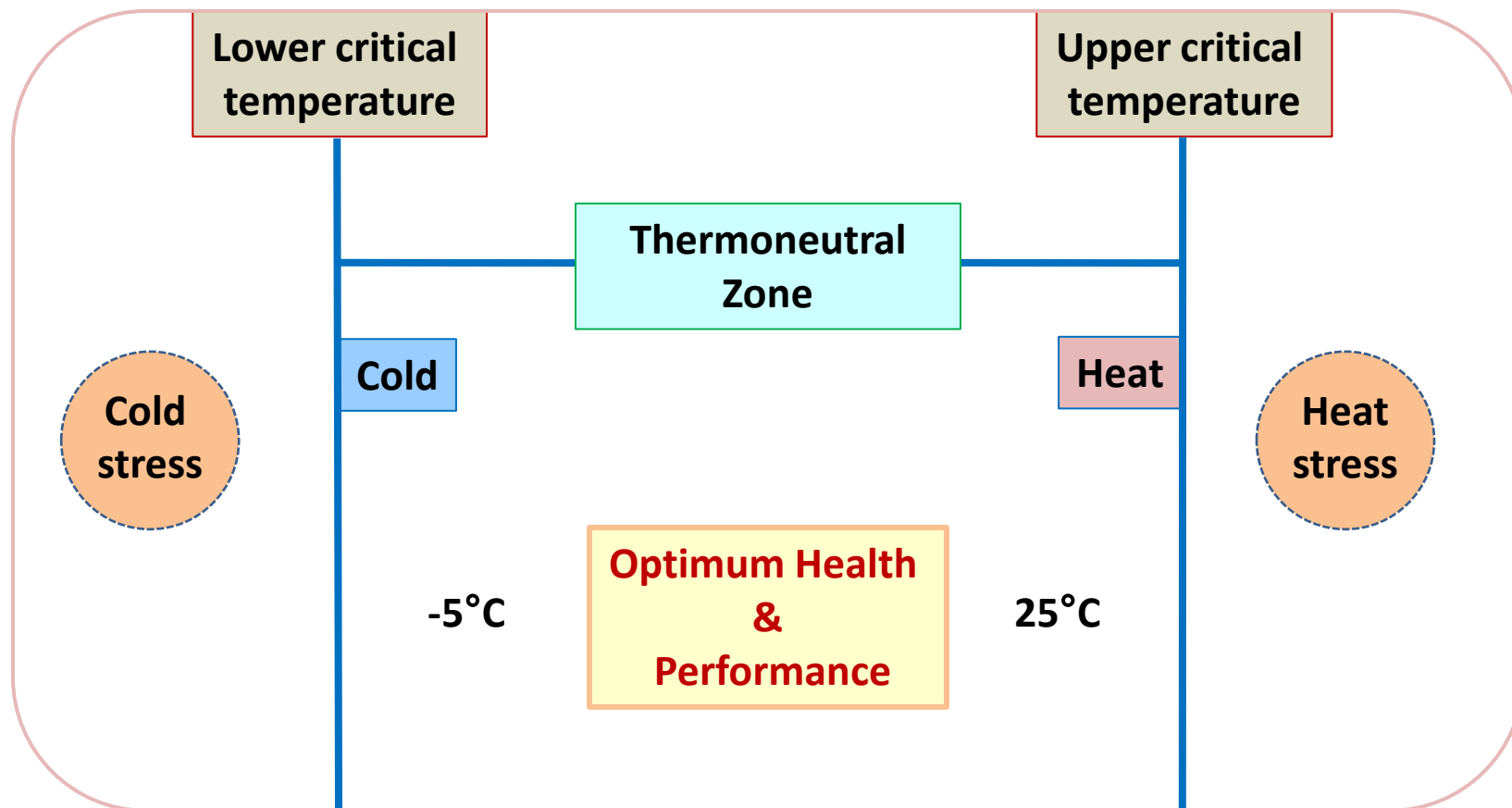
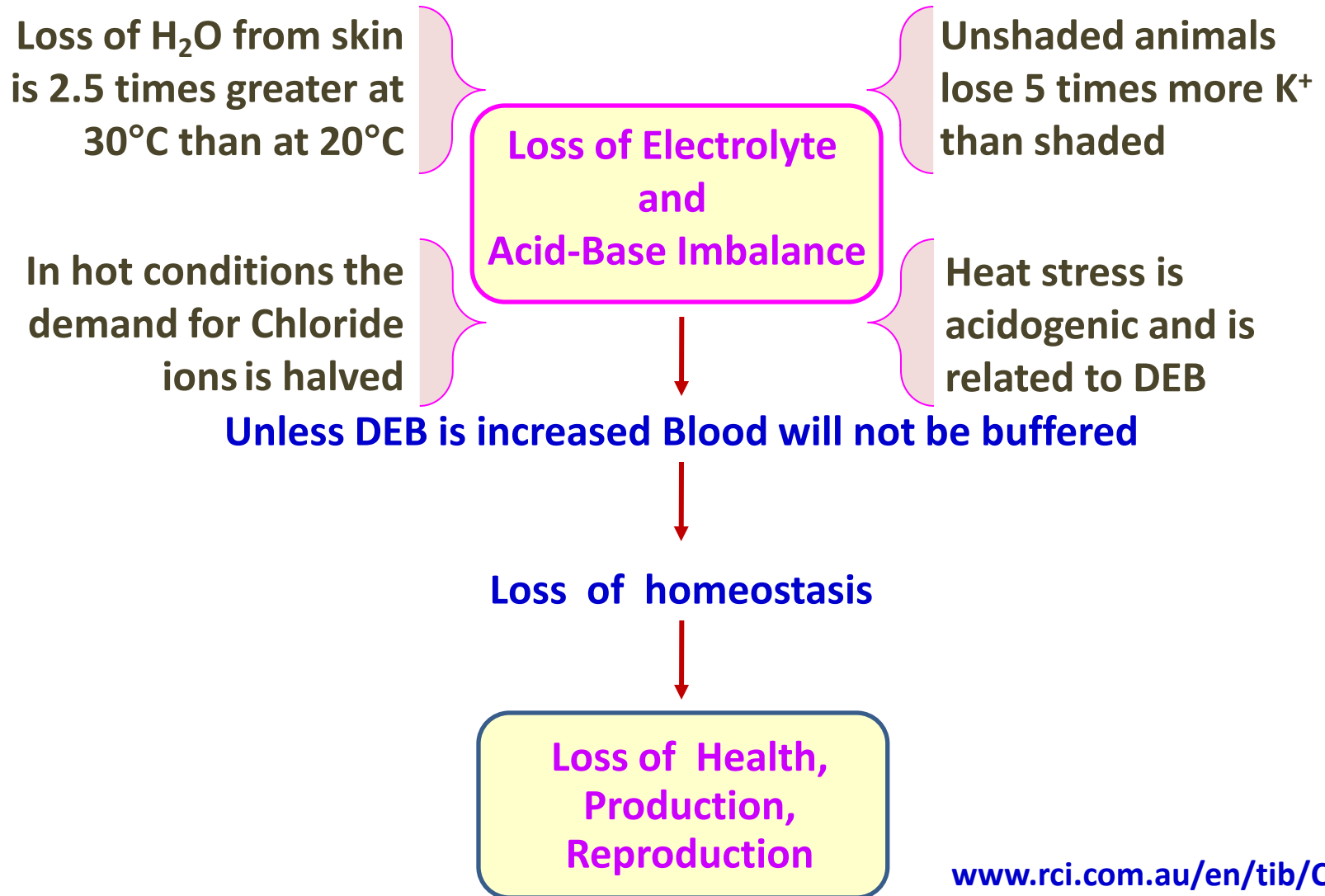


Figure 2.2 Effect of Heat Stress on Dietary Electrolyte Balance (DEB)



causing severe or prolonged inappetence (Bell *et al.*, 1989), which may further aggravate the increased supply of total acid in the rumen and decrease ruminal pH resulting into rumen acidosis.

2.6 Effects of heat stress in dairy cattle

Heat stress was a worldwide problem that causes heavy economic losses and affects about 60% of the world cattle population. Increase in environmental temperatures causes severe damage to the physiology, the metabolism and to the healthiness of animals. The physiological responses of animals in higher temperature were shown in Figure 2.3. Increased heat load decreases nutrient uptake in almost all species and in case of cattle, the nutrient uptake decreases upto 30% of dry matter intake (Rhoads *et al.*, 2013), which impairs the production (growth, milk quantity and quality), reproductive performance, metabolism, health status and immune response of farm animals.

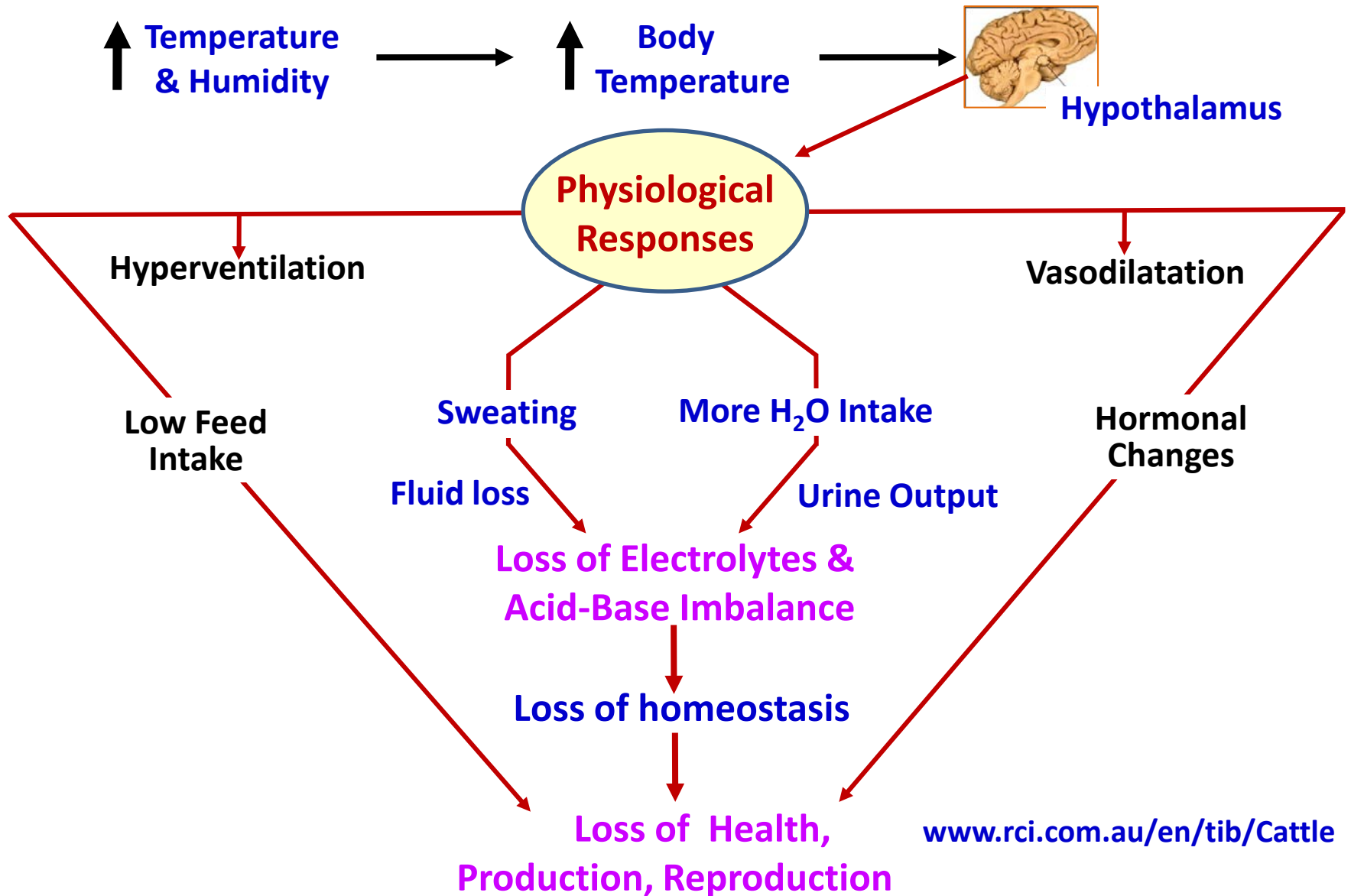
2.6.1 Effects on Production

A thermal environment was a major factor that negatively affects milk production in dairy cows, especially in animals of high genetic merit has been documented globally by several researchers (Hahn, 1995, 1999; Nienaber *et al.*, 1999; Sprott *et al.*, 2001; St-Pierre *et al.*, 2003; Hahn *et al.*, 2003; Upadhyay *et al.*, 2008). Umphrey *et al.* (2001) reported that the partial correlation between milk yield and rectal temperature for cows was 0.135. The sensitivity of dairy cattle to heat stress increases with increase in milk production thus reducing the “threshold temperature” when milk loss begins to occur (Berman, 2005). This might be due to increased metabolic heat output as production levels increases. When milk production was increased from 35 to 45 kg/d the threshold temperature for heat stress was reduced by 5°C (Berman, 2005). Effect of a given temperature on milk production was maximal between 24 and 48 hours following heat stress (Collier *et al.*, 1981; Spiers *et al.*, 2004). Dairy cows automatically reduce their feed intake during heat stress and this pattern follows as weather becomes hotter with further reduction in feed intake. This reduction in feed intake was the major cause of decrease in milk production because it directly associated with negative energy balance, regardless of the stage of lactation (Wheelock *et al.*, 2010). The stage of lactation was an important factor affecting dairy cows responses to high temperature.

Johnson *et al.* (1988) observed that the mid-lactating dairy cows were the most sensitive compared to their early and late lactating counterparts, mid-lactating dairy cows showed a higher decline in milk production (-38 %) when the animals were exposed to heat. West *et al.* (2003) reported that ambient weather conditions two days prior to milk yield measurement had the greatest correlation to decreases in milk production and dry matter intake. The effects of the ambient environment on cow performance have been measured by establishing critical ambient temperatures for the cow (Berman *et al.*, 1985; Johnson, 1987; Igono *et al.*, 1992), an equivalent temperature index incorporating temperature, humidity, and air velocity (Baeta *et al.*, 1987), and temperature humidity index (THI), which incorporates the combined effects of temperature and relative humidity (NOAA, 1976). Many studies have shown the significant negative correlation between THI and DMI for cows in the south-eastern U.S (Holter *et al.*, 1996; Holter *et al.*, 1997), and the effect of THI was probably mediated through the effects of increasing body temperature on cow performance. Johnson *et al.* (1962) showed a linear reduction of dry matter intake (DMI) and milk yield when THI exceeded 70 and they reported a reduction of -0.23 and -0.26 kg/day per unit of THI for DMI and milk yield, respectively. Studies, carried out in climatic chambers under heat stress conditions revealed a decrease in milk yield of 14% in early lactating dairy cows and of 35% in mid-lactating dairy cows (Lacetera *et al.*, 1996). Research has shown that the total number of hours when THI is greater than 72 or 80 over a 4-day interval had the highest correlation with milk yield (Linville and Pardue, 1985). Different ambient temperature has different Temperature Humidity Index (THI) and changes in THI causes decreases in dry matter intake (DMI) but increases in water intake. Relative changes expected in dry matter intake (DMI) and milk yield and water intake with increasing environmental temperature published by National Research Council, 1981 is listed in the Table 2.2.

The milk production in Holstein Friesian cows was very susceptible in high ambient temperature due to their weak thermotolerance ability which has strongly restricted the development of dairy industry in tropical region and caused considerable economic loss, estimated total economic loss was found between 1.69 and 2.36 billion US dollars was spend to control the impact of heat stress out of which 58% of loss occurred in dairy industry, 20% in the beef industry, 15% in pigs and the remaining 7% was in the poultry industry (St-Pieree *et al.*, 2003). The

Figure 2.3 Physiological Response of cattle to Heat Stress



reduction in dry matter intake could account for about 40 to 50% of the reduction in milk yield when cows were under heat stress conditions and that the remaining 50 to 60% could be explained by other changes induced by heat stress (Rhoads *et al.*, 2009). In India decreases in milk production in cattle was ranges from 10-25% (Upadhyay *et al.*, 2009). India is currently losing nearly 2% of total milk production, amounting to Rs 2661 crores due to rise in heat stress among dairy cattle and buffalo because of the global warming (Upadhyay *et al.*, 2009). Hot environment was also found to have negative effects not only on milk yield but also on milk quality (Bernabucci and Calamari, 1998; Calamari and Mariani, 1998). Above 72 THI value milk protein content declines, whereas the response of fat yield seems delayed.

Table 2.2 Effect of Environment on Nutrient Requirements of Dairy Animals

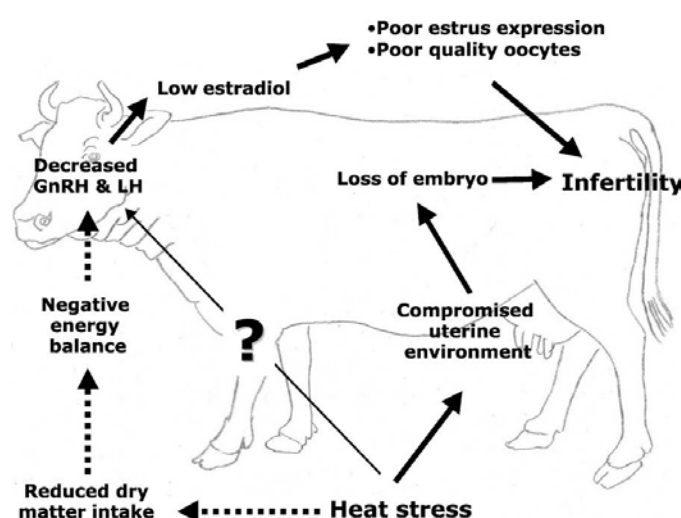
Temperature (°F)	Expected intakes and milk yields		
	Dry matter intake (lbs)	Water Intake(Gal)	Milk yield (lbs)
68	40.1	18.0	59.5
77	39.0	19.5	55.1
86	37.3	20.9	50.7
95	36.8	31.7	39.7
104	22.5	28.0	26.5

2.6.2 Effects on Reproduction

High environment temperatures compromise reproductive efficiency of farm animals in both the sexes. Hyperthermia directly alters and impairs the cellular functions of various parts or tissues of the reproductive system include: the oocyte, granulosa cells, particularly theca cells within the preovulatory follicle, the developing embryo during early stages of development, the corpus luteum, the uterine endometrium etc. A schematic description of the possible mechanisms for the effect of heat stress on reproduction is given in Figure 2.4 (Rensis and Scaramuzzi, 2003).

Heat stress compromises oocyte growth in cows by altering progesterone, the secretion of luteinizing hormone and follicle-stimulating hormone and dynamics during the oestrus cycle (Ronchi *et al.*, 2001). A lower plasma progesterone and higher prolactin concentration during oestrus cycle was reported in Murrah buffalo heifers and concluded that prolactin and progesterone profiles during the summer and winter months are directly correlated with the reproductive performance of buffaloes (Roy and Prakash, 2007). Heat stress was also found to be associated with reduced fertility of dairy cows in summer by poor expression of oestrus due to a reduced estradiol secretion from the dominant follicle developed in a low luteinizing hormone environment (De Rensis and Scaramuzzi, 2003). Conception rates was found to dropped from about 40–60% in cooler months to 10–20% or lower in summer, depending on the severity of the thermal stress (Cavestany *et al.*, 1985). Conception rates was drop by about a 20–27% in summer (Chebel *et al.*, 2004; Lucy, 2002) and/or a decrease in 90-day non-return rate to the first service in lactating dairy cows (Al-Katanani *et al.*, 1999). High ambient temperature was also been associated with impairment of embryo development and increased embryo mortality in cattle (Benyei *et al.*, 2001; Wolfenson *et al.*, 2000; Hansen, 2007). Semen concentration, number of spermatozoa and motile cells per ejaculate of bulls are lower in summer than in winter and spring (Mathevon *et al.*, 1998). Nichi *et al.* (2006) reported higher percentage of major sperm defects during summer than the winter in Simmental and Nellore bulls.

Figure 2.4 Mechanisms of effects of heat stress on reproduction in the lactating cow (Rensis and Scaramuzzi, 2003)



2.7 Single-nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs) are stably inherited, highly abundant, and distributed throughout the genome. These variations are associated not only with diversity within and among populations, but also with individual responses to medication and susceptibility to diseases (Strachan and Read, 2004). SNPs are useful as markers in population genetics and evolutionary studies and to identify genes implicated in complex multigenic traits by using linkage disequilibrium (Vignal *et al.*, 2002). SNPs might rapidly become the marker of choice for many applications in population ecology, evolution and conservation genetics, because of the potential for higher genotyping efficiency, data quality, genome-wide coverage and analytical simplicity (e.g. in modeling mutational dynamics).

2.8 Heat shock protein (HSP)

At the cellular level, stress results in the synthesis of a family of proteins termed heat shock proteins (Lindquist and Craig, 1988). Heat shock proteins (HSPs) are highly conserved molecular chaperones response to environmental and/or metabolic stressors belonging to distinct multigenic families. The ubiquitous nature of the heat shock response as well as its phylogenetic conservation suggests that the HSPs are essential for survival during or after stress (Morimoto *et al.*, 1990). According to their molecular weight, they can be classified into several major categories, e.g., HSP100, HSP90, HSP70, HSP60 etc. Many HSPs are also expressed constitutively in the absence of stress and thus appear to play a vital role in normal cellular processes. All organisms share a common molecular response that includes a dramatic change in the pattern of gene expression and the elevated synthesis of a family of heat shock or stress-induced proteins (Lindquist and Craig, 1988; Morimoto *et al.*, 1990). Their synthesis is triggered by stressful cellular conditions such as high temperature, anoxia, tissue trauma, ischemia, heavy metals, radiation, calcium increase, glucose deprivation, cancer, and bacterial infection (Iwama *et al.*, 1998; Feder and Hofmann, 1999; Robert, 2003). Heat shock proteins ensure survival under stressful conditions that, if left unchecked, would lead to irreversible cell damage and ultimately to cell death. As molecular chaperones, HSPs play a key role in cell protection by preventing protein aggregation, facilitating folding of newly synthesized proteins, stabilizing and refolding damaged proteins, and targeting misfolded proteins to specific degradative pathways.

2.9 Heat Shock Response (HSR)

The Heat Shock Response (HSR) is compatible with the development of rapid transient thermotolerance subsequent to acute Heat stress. When a cell is challenged by temperatures above the physiological level, it launches a heat shock response to adapt to these stressful conditions. A rise in body temperature increases the transcription of the heat shock genes ultimately leading to augmentation of their level in the cell (Moseley *et al.*, 1993; Palmon *et al.*, 1994; Flanagan *et al.*, 1995). It has been observed that cells subjected to mild heat stress conditions acquire thermotolerance to subsequent severe heat stress that would otherwise be lethal (Parsell and Lindquist, 1993; Kaufman, 1999). The predominant features of heat shocked cells were disorganization of the nucleolus, accumulation of electron-dense granules in the cytoplasm as well as alterations in organization and distribution of organelles, including mitochondria on animal, plant and yeast cells (Neumann *et al.*, 1984; Welch and Suhan, 1985; Webster and Watson, 1993). The protective HSR cascade started within minutes (Laszlo, 1992), although it was temporal value in the whole, intact body, as well as in cells in culture. In cells, thermotolerance was best observed 12–24 hr after heat stress (HS), whereas in intact animals, it was fully expressed 24 hr after the given stress. It was then manifested by a significant increase in the ability to withstand HS (Weshler *et al.*, 1984). Most changes in the cellular ultrastructure were reversible after shift-down to normal temperature conditions (Neumann *et al.*, 1984; Webster and Watson, 1993; Collier *et al.*, 1993). The heat shock response induced gene expression, in eukaryotic cells this response involves transcriptional activation mediated by a transcription factor known as heat shock factor (HSF). In unstressed cells, HSF was present in both the cytoplasm and nucleus in a monomeric form that has no DNA binding activity. In response to heat shock and other physiological stresses, HSF get activated and bind to the heat shock element (HSE) and assembled into a trimer and accumulates within the nucleus. A specific DNA recognition sequence located in the 5'-flanking sequences of heat shock-responsive genes was detected within minutes of temperature elevation (Westwood *et al.*, 1991). HSF exhibits a stress-dependent phosphorylation which modulated its transcriptional activity (Larson *et al.*, 1988). The heat shock transcriptional response attenuates upon prolonged exposure of cells at intermediate heat shock temperature (42°C) or upon return to physiological temperature (37°C)

this attenuation is accompanied by conversion of the active trimeric form of HSF to the non DNA binding monomer and returns to the normal sub cellular distribution. Heat Shock protein (HSP) are the best-suited examples of genes whose expression is effected by heat shock, it has been evident in recent years that thermal stress also leads to stimulation of a considerable number of genes not traditionally considered to be HSPs. Approximately 50 genes not traditionally considered to be HSPs have been found to undergo changes in expression in response to heat stress (Sonna *et al.*, 2002) among those ATP1A1 is one of the most important gene controlling thermal stress of animals.

2.10 Heat stress and physiological parameters

Rectal temperature (RT) and respiration rate (RR) are the most sensitive indices of heat tolerance among the physiological reactions studied (Verma *et al.*, 2000) which usually increase at high ambient temperature and humidity and reflect the degree of stress imposed on animals by climatic parameters. These responses have been used as a measure of dairy cow comfort and adaptability to an adverse environment or as a sensitive physiological measure of environmental modification (Roman-Ponce *et al.*, 1977).

2.10.1 Rectal Temperature (RT)

Core body temperature is commonly ascertained by measuring rectal temperature of animals. Rectal temperature of animals was recognized as an important measure of physiological status as well as ideal indicator for assessment of stress in animals (Lefcourt *et al.*, 1986) even though there is considerable variation among different parts of the deep body core at different parts of the day. Even a rise of less than 1°C in rectal temperature was enough to reduce performance in most livestock species (McDowell *et al.*, 1976). A relationship between ambient temperature and rectal temperature has indicated that a rise in ambient temperature beyond 19-20°C, increases RT at a faster rate than at low temperature. *Bos indicus* cattle were better able to maintain body temperature in response to heat stress than *Bos taurus* cattle (Hammond *et al.*, 1996; Hansen, 2004).

At an ambient temperature of 11.77°C, the rectal temperature of Karan Fries was 37.80 ± 0.3°C and 38.3 ± 0.2°C in Sahiwal cow which increased to 38.9 ± 0.4°C

and $38.6 \pm 0.2^{\circ}\text{C}$ at an ambient temperature of 22.6°C in KF and Sahiwal cows respectively (Singh and Upadhyay, 2009). The difference in two breeds may be attributed to better adaptability of Sahiwal cows than KF cows at higher ambient temperature. The magnitude of increase ($p < 0.01$) in rectal temperature during heat stress was lowest in Australian milking zebu (AMZ) cows (0.38°C) followed by Holstein cows (0.47°C) and Jersey cows (0.70°C), which indicates that jersey cows are least capable in maintaining their normal body temperature compare to other two breeds (Srikandakumar and Johnson, 2004).

Heritability of RT in dairy cattle was reported to range from 0.15 to 0.31 (Seath, 1947) which can be used to improve thermotolerance of dairy cattle by selection for this trait. The increase in milk yield of dairy cows and changes in housing conditions does not appear to have altered the heritability of RT substantially (Dikmen *et al.*, 2012).

2.10.2 Respiration Rate (RR)

Respiratory rhythm proved to be a more sensitive index under tropical conditions for assessing the animal response to environmental changes and evaluating heat tolerance (Williams *et al.*, 1960). Increase respiration rate was the first reaction when animals were exposed to environmental temperature above the thermo neutral zone (Gaalas, 1945; Seath and Miller, 1946). The combination of relative humidity and air temperature caused variation in body temperature and respiration rate of animals (Bhatnagar and Choudhary, 1960). Respiration rate under heat stress significantly increase enabled the animal to dissipate the excess body heat by vaporizing more moisture in the expired air and accounts for about 30% of the total heat dissipation (McLean, 1963). The respiration frequency of Lactating Karan Fries and Sahiwal cows was 25 ± 2 and 21 ± 1 breaths/ min, respectively at the lower ambient temperature (range $10\text{-}20^{\circ}\text{C}$, average 11.76°C). The RR in KF cows doubled (51 breaths/ min) whereas, in Sahiwal animals the increased was around one and half times at an average ambient temperature of 23.3°C . RF further increased to 59 and 56 breaths/min in KF and Sahiwal cattle at ambient temperature conditions (31°C and above). Initially, the RF increased linearly with the increase of ambient temperature but with the further increased in ambient temperature 31°C and above causes a rise in RF comparatively at a slower rate (Singh and Upadhyay,

2009). A linear rise in RF of cattle to a certain limit of ambient temperature was also reported by Hahn (1997).

Salem (1980) reported an increase in respiration rate of buffaloes and crossbreds cattle during summer compared to other seasons. A highly significant correlation between RR and seasonal air temperature in swamp buffalo and Holstein cows was reported when the relative humidity was kept constant, whereas no such significant correlation was observed between RR and relative humidity when the air temperature was kept constant (Chikamune and Shimizu, 1983). Exposure of bull calves to severe heat (40°C) resulted in increase in ventilation by means of rapid and shallow breathing upto 79.0/minute (Regan and Richardson 1938). Respiration rate increased from 29 to 59/minute when male buffalo calves exposed to 40.5°C (Joshi and Tripathy, 1991). Lemerle and Goddard (1986), reported that although rectal temperature only increased when THI was greater than 80, the respiration rate would begin to increase above a THI value of about 73 and would probably increase steeply at THI values of 80, which suggests that homeostatic mechanisms including increased respiration can prevent a rise in rectal temperature until the THI reaches 80. Jersey cows had the highest increase ($p < 0.01$) in their respiration rates (25 units) compare to Holstein and AMZ cows (only 20 units) which reveals that Jersey cows are less capable in maintaining their normal body temperature compare to these two breeds (Srikandakumar and Johnson, 2004).

2.11 ATP1A1 Gene

2.11.1 Biological function of ATP1A1 gene

Apart from maintenance of electrochemical gradient of Na^+ and K^+ across the plasma membrane, the ATP1A1 gene also facilitates the following functions:

- Mineral absorption
- Cardiac muscle contraction
- Adrenergic signalling in cardiomyocytes
- Insulin secretion
- Thyroid hormone synthesis
- Aldosterone-regulated sodium reabsorption
- Endocrine and other factor-regulated calcium reabsorption
- Proximal tubule bicarbonate reclamation
- Salivary secretion

- Pancreatic secretion
- Carbohydrate digestion and absorption
- Protein digestion and absorption
- Bile secretion
- Gastric acid secretion

2.11.2 Chromosomal localization, structure and molecular function of Bovine ATP1A1 Gene

ATP1A1 gene is responsible for establishing the electrochemical gradient of Na^+ and K^+ across the plasma membrane. These gradients are essential for osmoregulation, sodium-coupled transport of a variety of organic and inorganic molecules and for electrical excitability of nerve and muscle. The ATP1A1 gene is also named as sodium/potassium-ATPase subunit alpha-1, sodiumpump-1, Na^+ - K^+ ATPase 1, Na, K-ATPase alpha-1 subunit, sodium pump subunit alpha-1, Na^+ , K^+ -ATPase alpha subunit, Na, K-ATPase, alpha-A catalytic polypeptide, sodium-potassium-ATPase, alpha 1 polypeptide and Na, K-ATPase catalytic subunit alpha-A protein. The genomic organization and evolutionary history of the Na^+ , K^+ -ATPase are particularly intriguing given the highly conserved sequences of ATP1A1 gene and their ubiquity in biological systems (Liu *et al.*, 2010). The ATP1A1 gene encodes α_1 isoform, the major isoform of α subunit of Na^+ - K^+ ATPase pump. In the plasma membrane the ion gradients formed by Na^+ , K^+ -ATPase enzyme is necessary for Na^+ coupled transport of metabolites, nutrients and ions and represents a plausible candidate for heat tolerance traits (Geering *et al.*, 1987). ATP1A1, highly expressed Na^+ - K^+ ATPase α subunit isoforms in cerebrum and cerebellum, is a major mediator of cellular transmembrane sodium and potassium ionic gradients and plays an important role in central nervous system nervous signal transduction (Horisberger and Geering, 2009). The reduction of ATP1A1 gene expression level may cause the amount and functional deficiencies of its coded Na^+ - K^+ ATPase α_1 subunit isoforms on cell surface, and may impact the transmembrane ionic gradients of the cell, and then reduce the capability of neural signal transduction. The decreased expression of ATP1A1 cause a reduction in the amount of functional deficiency of the α_1 isoforms on the cell membrane which leads to imbalance of ionic osmolarity across the cell membrane that further causes cell damage and trigger apoptosis. Heat stress produces oxidative stress and affects K^+ and Na^+ level across the plasma

membrane. Since Na⁺ - K⁺ ATPase is sensitive to oxidative stress and critical for maintaining the homeostasis of these two ions.

ATP1A1 gene has been mapped on *Bos taurus* chromosome no. 3 (BTA 3) and spans about 22.77 kb and includes 22 introns, 23 Exons and coding sequence of 3066 nucleotides with 87 base pair size of 3 prime UTR (Figure 2.5).

There are reports from China (Liu *et al.*, 2010; Liu *et al.*, 2011) and India (Kashyap, 2012, Barani, 2013 and Jayakumar, 2014) indicating importance of ATP1A1 gene polymorphism and its association with heat tolerance parameters. ATP1A1 Gene has been found to be conserved among different species (Table-2.3). The nomenclature and sizes of exon and introns are given in Table 2.4.and 2.5 (<http://www.ensembl.org>).

ATP1A1 is one of the house keeping genes involved in homeostasis of Na⁺ and K⁺ in all the animal cells. Suzuki-Yagawa *et al.* (1992) identified and characterized the cis- acting elements that regulate the expression of ATP1A1 gene. The identified cis element was named the ATP1A1 regulatory element. The core sequence of the element was found in several other genes involved in cellular energy metabolism, suggesting that the sequence was a common regulatory element responsible for the state of energy metabolism.

Table 2.3 ATP1A1 Gene in different species

Species	Accession no.	Chromosome	No. of base pairs	
			Transcript	CDS
<i>Homo sapiens</i>	NM_000701.7	1p	3754	3072
<i>Mus musculus</i>	NM_144900	3	3720	3072
<i>Rattus norvegicus</i>	NM_012504	2q	3636	3072
<i>Gallus gallus</i>	NM_205521	1	3535	3066
<i>Equus caballus</i>	NM_001114532	5	3232	3066
<i>Sus scrofa</i>	NM_214249	4	3429	3066
<i>Bos taurus</i>	NM_001076798	3	3152	3066

(<http://www.ncbi.nlm.nih.gov>; <http://www.ensembl.org>)

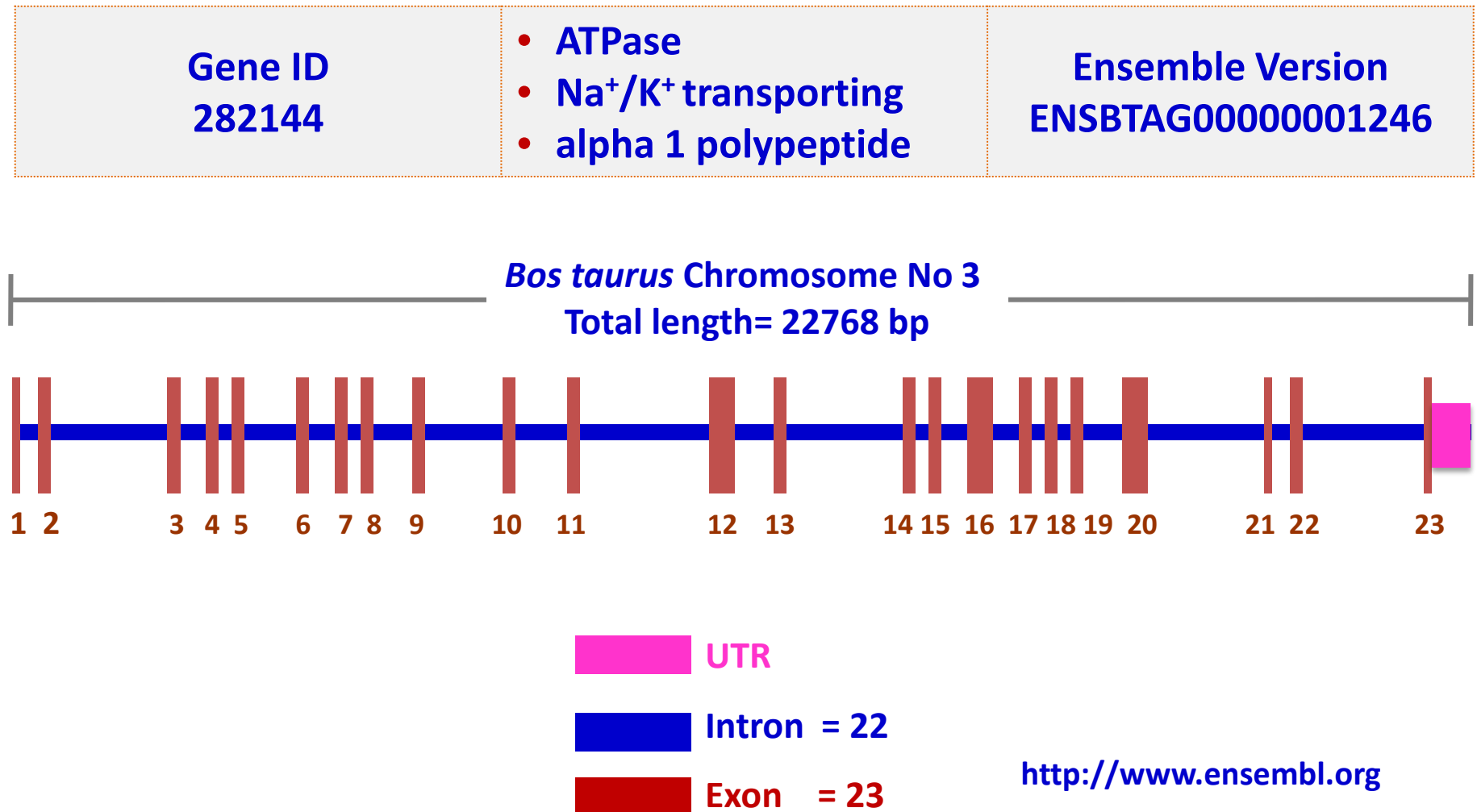
Table 2.4 Nomenclature of ATP1A1 gene in cattle

Accession no	NM_001076798
Gene ID	282144
Synonyms	ATPase, Na ⁺ /K ⁺ transporting, alpha 1 polypeptide
Chromosome	3
Ensemble Version	ENSBTAG000000001246
Total length	22768 bp
Exons	23
Introns	22
mRNA length	3152 bases
CDS length	3066 bases

Table 2.5 Exon and Intron Length of ATP1A1 Gene

Exon	Size (bp)	Intron	Size (bp)
1	12	1	2102
2	105	2	620
3	60	3	2078
4	204	4	672
5	114	5	380
6	135	6	109
7	118	7	451
8	269	8	531
9	199	9	251
10	110	10	1927
11	135	11	661
12	193	12	2212
13	176	13	985
14	137	14	1248
15	151	15	596
16	169	16	114
17	155	17	371
18	124	18	1146
19	146	19	125
20	131	20	489
21	102	21	2063
22	92	22	486
23	115		

Figure 2.5 Organization and Location of ATP1A1 Gene



2.11.3 Na^+ - K^+ ATPase pump

The first P-type ATPase was discovered by Jens Skou in 1957, (Skou, 1957). The Na^+ - K^+ ATPase pump belongs to a widely distributed class of P-type ATPase found in both prokaryotic and eukaryotic cells, are responsible for transporting a variety of cations like H^+ , Na^+ , Mg^{2+} , K^+ , Ca^{2+} , Cu^{2+} , Cl^- , PO_4^{3-} and SO_4^{2-}), substrates (glucose and amino acids), and neurotransmitters across cell membranes by active transport (Jorgensen, 1990; Skou, 1990; Sachs and Munson, 1991; Lutsenko and Kaplan, 1995; Glynn, 1993). The Na^+ - K^+ ATPase is a heterodimer characterized by a complex molecular heterogeneity that results from the expression and differential association of multiple isoforms of an α and β -subunit (Lingrel and Kuntzweiler, 1994). The α subunit is a multi-spanning membrane protein with a molecular mass of 112,000 Da contains the binding sites for the cations, ATP and the inhibitor ouabain, and is responsible for the catalytic and transport properties of the enzyme (Figure 2.6). The β -subunit is a polypeptide of molecular weight of 40,000 to 60,000 Da and is essential for the normal activity of the enzyme. In addition, in vertebrate cells, the β -subunit may act as a chaperone, stabilizing the correct folding of α -polypeptide to facilitate its delivery to the plasma membrane (Shull *et al.*, 1986; Herrera *et al.*, 1987; Urayama *et al.*, 1989; McDonough *et al.*, 1990; Geering, 1991; Jaisser *et al.*, 1992; Lutsenko and Kaplan, 1993; Eakle *et al.*, 1994; Lingrel and Kuntzweiler, 1994; Chow and Forte, 1995; Pressley, 1996; Therien *et al.*, 1997; Arystarkhova *et al.*, 1999). A third protein termed as γ -subunit has also been identified which is a small, hydrophobic polypeptide of 8–14 kDa (Mercer *et al.*, 1993; Minor *et al.*, 1998). The α -subunit has four isoforms namely α_1 , α_2 , α_3 and α_4 that are each encoded by the different genes namely ATP1A1, ATP1A2, and ATP1A3 and ATP1A4 respectively. Likewise β -subunit is also having three isoforms namely β_1 , β_2 and β_3 each encoded by the different genes like ATP1B1, ATP1B2 & ATP1B3 respectively (Sweadner, 1989; Mercer, 1993; Lingrel and Kuntzweiler, 1994; Mobasher *et al.*, 2000).

Na^+ - K^+ ATPase is the key component of cell membrane to regulate cellular homeostasis (Richards *et al.*, 2007). The main function of the α subunit of Na^+ - K^+ ATPase is to pump 3 Na^+ out of cells in exchange for 2 K^+ to maintains the high internal K^+ and low internal Na^+ concentrations (Shamraj and Lingrel, 1994) which accounts for at least 30% of the resting energy metabolism of the brain, utilizing energy derived from the hydrolysis of ATP (Albers *et al.*, 1989).

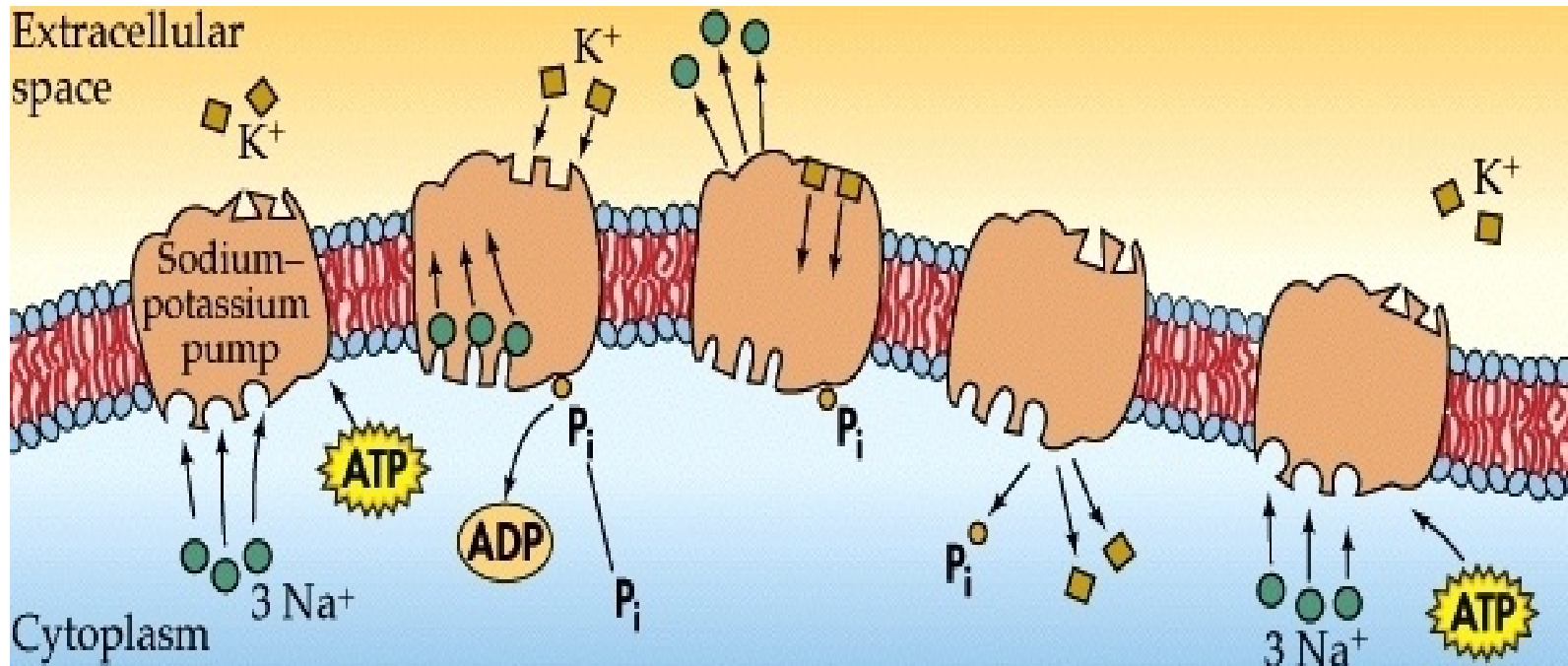
The electrochemical gradient generated by $\text{Na}^+ - \text{K}^+$ ATPase is critical for maintaining the osmotic balance of the cell, the resting membrane potential of most tissues, and the excitable properties of muscle and nerve cells. In addition, the Na^+ gradient provides the energy that fuels the Na^+ -coupled transporters. The reduction of $\text{Na}^+ - \text{K}^+$ ATPase activity affects directly neurotransmitter signalling, neural activity and whole body behaviour through reduction in nor-epinephrine, dopamine and serotonin uptake and increasing acetylcholine release (Blasi *et al.*, 1988; Vatta *et al.*, 2004; Steffens and Feuerstein., 2004). In the kidney the $\text{Na}^+ - \text{K}^+$ -ATPase plays a primary role in driving the reabsorption of Na^+ and water. Thus, the enzyme is essential in the maintenance of body fluid and electrolyte homeostasis (Jorgensen, 1990).

2.12 Association studies of ATP1A1 Gene Polymorphism with Heat Tolerance Traits in Dairy Cattle

Liu *et al.* (2010) studies polymorphism of the ATP1A1 gene in 160 Holstein cows using polymerase chain reaction low ionic strength single-strand conformation polymorphism and DNA sequencing methods. G to A at position 14103 in exon 14 and C to T at position 14242 in intron 14 of the bovine ATP1A1 gene were identified, but the former single nucleotide polymorphism was silent with respect to the amino acid sequence of the protein. However, they found significant correlations between ATP1A1 gene polymorphism and the coefficient of heat tolerance ($P < 0.01$) and with respiratory rate ($P < 0.01$). Genotype AC was the most favourable genotype for heat tolerance and concluded that polymorphism site has potential as a genetic marker for heat tolerance traits in dairy cattle breeding.

Liu *et al.* (2011) studied ATP1A1 Gene by PCR low ionic strength single-strand conformation polymorphism (PCR-LIS-SSCP) and DNA sequencing methods to analyze the polymorphisms within the coding region of bovine ATP1A1 gene. One novel C to A mutation was identified at the nucleotide position 2789 of the ATP1A1 but it was silent with respect to the amino acid sequence of the protein. The LIS-SSCP banding pattern was divided into three kinds of genotypes named CC, CA, and AA. and the association of the novel SNP of ATP1A1 gene with heat tolerance traits was studied and found that the individuals with genotype CC showed significantly ($P < 0.05$) higher heat resistance than those of genotype CA. The mRNA levels of ATP1A1 gene in lymphocytes of peripheral blood in dairy cows among

Figure 2.6: Mechanism of action of Na^+ - K^+ ATPase Pump



various temperature groups and genotypes were also analyzed which showed that the expression of ATP1A1 mRNA was highest in heat-stressed cows with CC genotype among the three genotypes ($P<0.01$), and the ATP1A1 mRNA level at temperature 32.5°C was higher than that at optimal temperature 12.5°C in dairy cows ($P<0.01$). They have concluded that the identified SNP could be a potential genetic marker for anti-heat stress trait in dairy cow breeding.

Kashyap (2012) identified ATP1A1 gene polymorphism within two regions i.e. 272 bp (exon 14) and 301 bp (exon 17) by PCR-SSCP analysis in 64 Tharparkar and 100 crossbred adult cattle. Seven genotypes namely AA, AB, AD, BC, CD, BD and AC were documented for the 272 bp amplified fragment (fragment I). The BD (0.52) genotype was most frequent in Tharparkar breed whereas, BD (0.24) and AD (0.23) were frequent in crossbred cattle. Genotype BC was absent in Tharparkar. Three genotypes namely CC, CA and AA were documented in both in Tharparkar and crossbred cattle at the amplified region of 301 bp fragment (fragment II). At this locus, two alleles namely A and C were present. The frequencies of CC, CA and AA genotypes and C and A alleles were estimated. The CA genotype was most frequent in Tharparkar (0.49) and crossbred cattle (0.47) whereas, CC and AA genotypes were equally present in both populations. The C allele in Tharparkar (0.51) and crossbred cattle (0.52) were present nearly equal to A allele (0.49 and 0.48, respectively) in these populations. Various SSCP genotypes observed were sequenced by the Sanger's dideoxy chain termination sequencing method. One single nucleotide change was observed on sequence analysis of 301 bp fragment which showed highly significant association with various heat tolerance parameters. Genotype CC showed a significantly ($p<0.01$) higher HTC in both Tharparkar (97.82 ± 0.33) and crossbred cattle (98.23 ± 0.19). The overall breed analysis showed higher heat tolerability in Tharparkar (94.19 ± 0.31) than in crossbred cattle (93.74 ± 0.18) in summer season. It was concluded that animals with CC genotypes are more heat tolerant in both the breeds.

Barani (2013) studied ATP1A1 Gene in Deoni breed of cattle using a total of nineteen sets of primers by PCR-SSCP analysis. The association of identified polymorphisms with thermotolerance, productive and reproductive performances was investigated and analysed using GLM procedure of SAS software. A total of 16 SNPs was identified out of which 11 were in introns (2, 3, 4, 5, 6, 10, 12 and 20) and

5 were in exons (5, 10, 11, 17 and 20). All the observed SNPs were silent mutation without any amino acid change in the translated products. Out of all those identified SNPs, C to A transversion at position 15739 in exon 17, positively affected thermo-tolerance in Deoni cattle which was also previously reported by Liu *et al.* (2011) and Kashyap (2012) and concluded that ATP1A1 gene could be used as a candidate gene for developing marker for heat tolerance and performance traits in cattle.

Jayakumar (2014) studied ATP1A1 gene in 160 Murrah Buffaloes. The exon 16, 17, 18 and 19 of ATP1A1 gene were amplified using 4 sets of reported primers (Liu *et al.*, 2011). He found that SNP locus G19333T were significantly associated with physiological parameters viz. respiration rate and pulse rate.

CHAPTER -3

Materials and methods

3. Materials and Methods

3.1 Experimental Animals

The present study was conducted on Sahiwal and Karan Fries cows maintained at Cattle Yard of National Dairy Research Institute (Karnal) and Jersey crossbred cows maintained at College of Veterinary Science (Assam Agriculture University, Khanapara, Guwahati) and its adjoining hilly area in Meghalaya.

Table 3.1: Experimental animals for the study

Type of cattle	Breed	Number
Indigenous	Sahiwal	51
Crossbred	Karan Fries	50
Crossbred	Jersey cross	35
Total		136

3.2 Physiological Parameters Recorded

3.2.1 Respiration Rate

Respiration rate (RR) of each animal was recorded by visual observation of inward and outward flank movement for one minute without disturbing the animal. One inward and outward movement was counted as one respiration and respiration rate was expressed in number per minute. The respiration per minute was recorded three times consequently and average was taken as final reading for further analysis.

3.2.2 Rectal Temperature

Rectal temperature (RT) was recorded with a digital thermometer by keeping the thermometer in contact with rectal mucosa for about 2 minutes. The same procedure to calculate rectal temperature was performed three times consequently and average were taken for association studies. The observations were recorded in degree Celsius (°C).

3.3 Recording frequency of Physiological parameters

Respiration rate and rectal temperature were recorded once in each of the three seasons at the probable extreme hours of day. Frequency of recording the physiological parameters in Sahiwal, Karan Fries and Jersey cross cattle is shown in Table 3.2.

Table 3.2: Frequency of recording the physiological parameters in Sahiwal, Karan Fries and Jersey cross cattle

Season	Time
Winter (9-12, Jan)	6-8 am
Spring (25-28, Mar)	12-2 pm
Summer* (27-29, May)	12-2 pm

* RR and RT of Jersey cross cattle were recorded in the month of June

3.4 Physiological Parameters Estimated for Heat tolerance

3.4.1 Heat Tolerance Coefficient (HTC): HTC was calculated based on heat tolerance index developed by Benezra (1954). The formula is based on both respiration rate and rectal temperature. In the equation, the denominator 38.33 is defined as the normal body temperature of cattle and the denominator 23 is considered to be the normal RR of the cattle under ideal conditions. The lower the value determined by the equation higher the degree of adaptability.

$$\text{HTC: } \text{RR}/23 + \text{RT}/38.33$$

3.5 SNP Genotyping of Experimental Animals

3.5.1 Isolation of Genomic DNA

3.5.1.1 Blood Collection

Blood samples were collected randomly from Sahiwal, Karan Fries and Jersey-cross cattle. 10 ml blood was collected aseptically from jugular vein puncture in a sterile Beckton-Dickinson vacutainer containing 0.5 per cent (10 µl/ml of blood) anticoagulant Ethylene Diamine Tetra Acid (EDTA). After proper labeling, the samples were transported to the laboratory in an icebox and stored at - 20°C till further processing for DNA isolation.

3.5.1.2 DNA Extraction

Genomic DNA was extracted from blood of Sahiwal, Karan Fries and Jersey-cross cattle using phenol- chloroform extraction method described by Sambrook and Russell (1989) with minor modifications. The steps involved in this method of DNA isolation were as follows:

Cell Lysis

1. 10 ml Blood was taken in an Oakridge tube with two volumes of ice-cold RBC lysis buffer, mixed well by vortexing and the tubes were kept on ice for 10 minutes.
2. Centrifuged at 8500 rpm for 10 minutes at 4°C and the supernatant were decanted.
3. WBC pellet was re-suspended in one volume of RBC lysis buffer and kept on ice for 10 minutes and centrifuged as given in above step.
4. 2nd and 3rd steps were repeated for complete lysis red blood cells until a clear pellet of white blood cells was obtained.
5. WBC pellet was re-suspended in DNA extraction buffer (equal to the volume of blood sample collected) by vortexing.
6. SDS (20%) was added @ 200µl per 10ml of whole blood. The mixture was gently mixed, and Proteinase-K was added @ 40 µl (20 mg/ml stock solution) per 10 ml of blood and incubated at 56°C overnight.

Phenol Extraction

1. Equal volume of Tris (pH 8.0) saturated phenol was added to the above mixture and mixed gently by inversion to form a uniform suspension (5-10 minutes).
2. The mixture was centrifuged at 10,000 rpm for 15 minutes at 20°C.
3. Upper aqueous phase was gently aspirated using a wide bore sterile Pasteur pipette, without disturbing the interphase of protein, and transferred to a fresh Oakridge tube.

4. A mixture of saturated phenol, chloroform and isoamyl alcohol in a ratio of 25:24:1 was added in equal volume and mixed gently by inverting the tube until a uniform suspension was formed.
5. The mixture was centrifuged at 10,000 rpm for 15 minutes at 20°C and the upper aqueous phase was gently aspirated using a wide bore sterile Pasteur pipette without disturbing the interphase of protein and transferred into a fresh Oakridge tube.
6. A mixture of chloroform and isoamyl alcohol at a ratio of 24:1 was added and mixed properly by inverting the tube. The mixture was centrifuged again at 10,000 rpm for 15 minutes and the aqueous phase was aspirated into a fresh sterile glass tube.

DNA Precipitation

1. Sodium acetate (3 M, pH 5.2) was added to the aqueous layer to a final concentration of 0.33 M, mixed gently, and two volumes of ice-cold ethanol was added and tilted gently for DNA precipitation.
2. The precipitated DNA was spooled out with the help of bent Pasteur pipette and transferred to eppendorf tube with 1.00 ml of 70% ethanol, mixed well and centrifuged at 10,000 rpm for 5 minutes at 4°C. This step was repeated three times.
3. The DNA was dried by keeping the eppendorf tubes open in a sterile incubator at 37°C. The DNA was dissolved in 500 µl TE buffer (pH 8.0), covered with parafilm, kept in a water bath maintained at 65°C for 30 minutes, after DNase treatment, the tubes were kept at 4°C for 4-5 days and finally stored at - 20°C.

3.5.2 Quality and Quantity Check of DNA

Agarose gel electrophoresis was carried out to check the quality of DNA. The gel plates and combs were cleaned with distilled water and 70% ethanol and air-dried. The edges of the plate were sealed with cellophane tape to avoid leakage of the gel. The combs were fixed and the entire assembly was kept on a level stage. The gel and combs were handled with gloved hands to avoid any fingerprints. The agarose gel was prepared using 0.60 gm of agarose powder dissolved in 100 ml 1x TBE solution. This mixture was then heated in microwave for about 2 minutes till it

became colorless and allowed to cool to 50°C and finally 1% ethidium bromide was added @ 2 µl/100 ml of gel. This mixture was then poured in gel plate for solidifying and two combs were placed for making valves. Care was taken to avoid bubble formation while pouring the gel. The gel was allowed to solidify for about half an hour. After solidification the combs are removed gently without damaging the wells. The solidified gel was placed in the electrophoresis chamber containing 650 ml of 1X TBE buffer. 2 µl DNA mixed with 2 µl of 6X gel-loading dye were loaded slowly in separate wells. The chamber was closed with the lid and power supply turned on to 100 volts for 45 minutes. At the end of run, power supply was turned off and the gel was carefully removed from the chamber and placed on trans-illuminator, viewed under UV light and photographs were taken with the help of gel documentation system (BioRad). Sharp bands were considered as correct concentration of the DNA. Quality and quantity of DNA was also estimated by Biospec-nano spectrophotometer. The ratio between OD₂₆₀ and OD₂₈₀ was observed for each sample. DNA sample with ratio of 1.8 was considered good and considered for further analysis. The sample possessing a ratio of less than 1.7 and more than 1.9 was subjected to Proteinase- K digestion and DNA extracted with phenol chloroform isoamyl alcohol as described previously.

3.5.3 Preparation of Working Solution of DNA

Working solution was prepared by diluting the stock genomic DNA with autoclaved double distilled water to arrive at a final concentration of 50-100 ng/ul.

3.5.4 Polymerase Chain Reaction (PCR) Amplification of Targeted Region of Bovine ATP1A1 gene

3.5.4.1 Primers

In silico primer designing for target region of Bovine ATP1A1 gene was carried out using DNASTAR software and gene specific sequence (ENSBTAG00000001246) available at ensemble genome browser (<https://www.ensembl.org>). Two sets of forward and reverse gene-specific oligonucleotide primers were designed. While designing the primers, oligonucleotide melting temperature, oligonucleotide length, GC content, primer-dimer possibilities, PCR product size, positional constraints within the source sequence and miscellaneous constraints were considered. Primers were procured from Sigma

Aldrich Chemicals Pvt. Ltd (Bangalore). The sequence of primers, their respective nucleotide numbers, target region and amplicon sizes are given in Table 3.3.

3.5.4.2 Reaction mixture

- The working solutions of both forward and reverse primers were prepared from stock solutions by adding primer and autoclaved double distilled water to obtain final concentration of 10 pmol for each primer.
- Template DNA stock solution was diluted using autoclaved double distilled water to working concentration of 50 to 100 ng/ μ l.
- Each PCR reaction cocktail was prepared using PCR Master Mix (2X) (Fermentas) purchased from M/s Genetix Biotech Asia Pvt. Ltd. (New Delhi).
- Final reaction cocktail (25 μ l)

Forward primer	:	0.5 μ l
Reverse primer	:	0.5 μ l
PCR Master Mix (2X)	:	12.5 μ l
Water	:	8.5 μ l
Template DNA	:	3.0 μ l

The above contents of PCR reaction cocktail were mixed thoroughly by vortexing followed by brief spin.

- All the tubes were marked with respective DNA sample number.

3.5.4.3 PCR programme

The PCR amplification was performed using Thermal cycler (MJ research and BioRad T100). Each 0.2 ml tube containing PCR reaction cocktail was kept in Thermal cycler for amplification of target region of Bovine ATP1A1 gene. Detailed PCR programme and annealing temperatures are given below in Table 3.4.

3.5.4.4 Agarose Gel Electrophoresis to Check PCR Amplification

After PCR amplification, the PCR product was checked on 2% agarose gel to verify the amplification of target region. The gel plates and combs were cleaned with distilled water and 70% ethanol and air-dried. The edges of the plate were sealed with cellophane tape to avoid leakage of the gel. The combs were fixed and the

entire assembly was kept on a level stage. The gel and combs were handled with gloved hands to avoid any fingerprints. The agarose gel was prepared using

Table 3.3 Sequence of primers, their respective number of bases, target region and amplicon sizes of Bovine ATP1A1 gene

Primer set	Sequence (5'-3')		No of base	Targeted Region (Nucleotide position on gene)	Amplicon size (bp)
1	F	AATGACTCCCCGGCTTTGAAGA	22	Partial exon 16 - 17 Intron (27008667 - 27008177)	491
	R	GGCCACGGGGACCCAGAGAAC	21		
2	F	GGTCCCCGTGGCCAGCATTTG	21	Partial Intron 17- Intron 18 (27008189 - 7007725)	465
	R	GGCAGACAGCACCCCTACAGATACTT	26		

Table 3.4 PCR programme for Primer set 1 and Primer set 2 for ATP1A gene

S. No	Steps	Temperature	Time	No of Cycles
I	Initial Denaturation	95°C	3.0 minutes	One
II	Denaturation	94°C	30 seconds	34
	Annealing	*	30 seconds	
	Extension	72°C	25 seconds	
	Final extension	72°C	8.0 minutes	
IV	Hold	4°C	Forever	

*Annealing temperature for both primer sets were 56.5°C

2 g of agarose powder dissolved in 100 ml 1 x TBE solution .This mixture was then heated in microwave for about 2 minutes till it became colorless and allowed to cool to 50°C and finally 1% ethidium bromide was added @ 2 µl/100 ml of gel. This mixture was then poured in gel plate for solidifying and two combs were placed for

making wells. Care was taken to avoid bubble formation while pouring the gel. The gel was allowed to solidify for about half an hour. After solidification the combs are removed gently without damaging the wells. The solidified gel was placed in the electrophoresis chamber containing 650 ml of 1X TBE buffer. 5 µl of PCR product freshly mixed with 2 µl of 6X gel-loading dye were loaded slowly in separate wells and the 100bp marker was loaded in the middle well of the gel. The chamber was closed with the lid and power supply turned on to 100 volts for 45 minutes.

At the end of run, power supply was turned off and the gel was carefully removed from the chamber and placed on trans-illuminator, viewed under UV light and photographs were taken with the help of gel documentation system (BioRad). A single sharp band indicated proper amplification of the target DNA.

3.5.5 DNA Sequencing

The Amplified PCR products from 2 sets of primers were sent to 1st base sequencing INT (Singapore) and Merck Specialties Pvt. Ltd. (Bangalore) for purification and custom sequencing from both ends (5' and 3' ends).

3.5.6 Sequence Data Analysis for SNP Detection and genotyping

DNA sequencing results for the respective region of Bovine ATP1A1 gene were visualized and edited using BioEdit software. Each edited sequence was aligned with corresponding reference sequence using ClustalW multiple sequence alignment program for DNA (www.ebi.ac.uk/tools/msa/clustalw2) to identify SNPs.

3.6 Statistical analysis

3.6.1 Allele and genotype frequencies

Allele and genotype frequencies were calculated using POPGENE software package (Yeh *et al.*, 1999).

3.6.2 Fixed effects considered in association analysis

3.6.2.1 Temperature Humidity Index (THI)

THI measures the combined effects of ambient temperature and relative humidity (RH) to ascertain heat load intensity (Berry *et al.*, 1964). THI can be used as an indicator of thermal climatic conditions as suggested by McDowell *et al.* (1976). The THI is based on dry and wet bulb temperatures and it is calculated according to formula developed by National Research Council (NRC), 1971:

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

Where, Wb is wet bulb and Db is dry bulb temperature in °C

THI values for all days in each season during which physiological parameters were recorded in Sahiwal and Karan Fries cows are shown in table 3.5. THI values for Jersey cross cows were obtained from College of Veterinary Science (AAU, Khanapara, Guwahati). The three subclasses of THI 69.92, 71.15 and 69.80 of Jersey cross cows were considered in association analysis (Table 3.6).

Table 3.5 THI for all days in each season during which physiological parameters were recorded in Sahiwal and Karan Fries cows

		Dry Bulb	Wet Bulb	THI	Relative Humidity	T °C Max	T °C Min
Winter (Jan.9-12)	Day 1	08.4	08.4	52.70	100	18.5	07.3
	Day 2	04.4	04.4	46.94	100	17.0	03.4
	Day 3	06.0	06.0	49.24	100	16.2	04.7
	Day 4	06.6	06.2	49.82	94	17.5	05.0
*Avg. = 49.70							
Spring (Mar.25-28)	Day 1	19.0	15.4	65.37	66	23.0	13.0
	Day 2	17.4	16.0	64.65	86	30.0	17.0
	Day 3	17.4	16.6	65.08	92	27.7	15.5
	Day 4	16.2	15.6	63.5	94	25.0	13.8
*Avg. = 64.65							
Summer (May 27-29)	Day 1	36.6	24.0	84.23	32	37.0	22.0
	Day 2	38.4	25.0	86.25	31	38.8	23.0
	Day 3	40.6	26.4	88.84	30	40.2	23.5
*Avg.= 86.44							

3.6.2.2 Parity

Records upto 9 parities were considered for Sahiwal and upto 7 for Karan Fries cows while records of parity were not considered in analysis of Jersey cross cows (Table 3.6).

3.6.2.3 SNP locus

SNP genotypes of polymorphic loci were considered in all three cattle breeds. SNP loci and their respective genotypes are shown in Table 3.6

3.6.3 Association analysis of SNPs with rectal temperature, respiration rate and heat tolerance coefficient in Sahiwal, Karan fries and Jersey crossbred cows

Association of each SNP genotype with rectal temperature, respiration rate and heat tolerance coefficient in Sahiwal, Karan fries and Jersey cross Cows was analyzed using GLM procedure of Statistical Analysis System (SAS) software.

The significant effect of SNP variants on physiological parameters were analyzed using the following model in Sahiwal cows:

$$Y_{ijklmnopq} = \mu + P_i + S_j + SNP1_k + SNP2_l + SNP3_m + SNP4_n + SNP5_o + SNP6_p + e_{ijklmnopq}$$

Where,

$Y_{ijklmnopq}$ = q^{th} observation on RR/RT/HTC of Sahiwal cows in i^{th} parity, j^{th} seasons (THI), k^{th} SNP1, l^{th} SNP2, m^{th} SNP3, n^{th} SNP4, o^{th} SNP5, p^{th} SNP6

μ = Overall mean

P_i = Effect of i^{th} parity ($i = 1$ to 9)

S_j = Effect of j^{th} seasons ($j = 1$ to 3)

$SNP1_k$ = Effect of k^{th} genotype of SNP T27008243C ($k = 1$ to 3)

$SNP2_l$ = Effect of l^{th} genotype of SNP A27008223G ($l = 1$ to 3)

$SNP3_m$ = Effect of m^{th} genotype of SNP T27008097A ($m = 1$ to 3)

$SNP4_n$ = Effect of n^{th} genotype of SNP C27008016T ($n = 1$ to 3)

$SNP5_o$ = Effect of o^{th} genotype of SNP G27008015A ($o = 1$ to 3)

$SNP6_p$ = Effect of p^{th} genotype of SNP C27007790A ($p = 1$ to 3)

$e_{ijklmnopq}$ = Random error associated with $Y_{ijklmnop}$ observation and assumed to be NID ($0, \sigma^2_e$)

Table 3.6 Subclasses of fixed effects considered in association analysis

	Subclass			Remark
Effect	1	2	3	
Seasons (THI)	49.7	64.65	86.44	Sahiwal and KF
	69.92	71.15	69.80	Jersey crossbred
T27008243C	TT	TC	CC	All three breeds
A27008223G	AA	GG	AG	
T27008097A	TT	TA	AA	
C27008016T	CC	CT	TT	
G27008015A	GG	GA	AA	
C27007790A	CC	CA	AA	
Parity	1 to 9			Sahiwal and KF

The significant effect of SNP variants on physiological parameters were analyzed using the following model in Karan fries cows:

$$Y_{ijklmnopq} = \mu + P_i + S_j + SNP1_k + SNP2_l + SNP3_m + SNP4_n + SNP5_o + SNP6_p + e_{ijklmnopq}$$

Where,

$Y_{ijklmnopq}$ = q^{th} observation on RR/RT/HTC of KF cows in i^{th} parity, j^{th} seasons (THI), k^{th} SNP1, l^{th} SNP2, m^{th} SNP3, n^{th} SNP4, o^{th} SNP5, p^{th} SNP6

μ = Overall mean

P_i = Effect of i^{th} parity ($i = 2$ to 7)

S_j = Effect of j^{th} seasons ($j = 1$ to 3)

$SNP1_k$ = Effect of k^{th} genotype of SNP T27008243C ($k = 1$ to 2)

$SNP2_l$ = Effect of l^{th} genotype of SNP A27008223G ($l = 1$ to 2)

$SNP3_m$ = Effect of m^{th} genotype of SNP T27008097A ($m = 1$ to 2)

$SNP4_n$ = Effect of n^{th} genotype of SNP C27008016T ($n = 1$ to 3)

$SNP5_o$ = Effect of o^{th} genotype of SNP G27008015A ($o = 1$ to 3)

$SNP6_p$ = Effect of p^{th} genotype of SNP C27007790A ($p = 1$ to 3)

$e_{ijklmnopq}$ = Random error associated with $Y_{ijklmnop}$ observation and assumed to be NID ($0, \sigma^2_e$)

The significant effect of SNP variants on physiological parameters were analyzed using the following model in Jersey crossbred cows:

$$Y_{ijklmno} = \mu + S_i + \text{SNP2}_j + \text{SNP3}_k + \text{SNP4}_l + \text{SNP5}_m + \text{SNP6}_n + e_{ijklmno}$$

where,

$Y_{ijklmno}$ = o^{th} observation on RR/RT/HTC of Jersey crossbred cows in i^{th} seasons (THI), j^{th} SNP2, k^{th} SNP3, l^{th} SNP4, m^{th} SNP5, n^{th} SNP6 on RR/RT/HTC

μ = Overall mean

S_i = Effect of i^{th} seasons ($i = 1$ to 3)

SNP2_j = Effect of j^{th} genotype of SNP A27008223G ($j = 1$ to 2)

SNP3_k = Effect of k^{th} genotype of SNP T27008097A ($k = 1$ to 2)

SNP4_l = Effect of l^{th} genotype of SNP C27008016T ($l = 1$ to 3)

SNP5_m = Effect of m^{th} genotype of SNP G27008015A ($m = 1$ to 2)

SNP6_n = Effect of n^{th} genotype of SNP C27007790A ($n = 1$ to 3)

$e_{ijklmno}$ = Random error associated with Y_{ijklmn} observation and assumed to be NID (0, σ^2_e)

CHAPTER- 4

Results and discussion

4. Results and Discussion

The present investigation was carried out to find SNPs in targeted region of ATP1A1 gene and to study their relationship with heat tolerance in Sahiwal, Karan Fries and Jersey crossbred cows. The results obtained from the experiment are presented along with the discussion under the following sections.

4.1 Genomic DNA isolation

Blood samples of Sahiwal (51) and Karan Fries cows (50) were collected from Livestock Research Centre, National Dairy Research Institute, Karnal, Haryana and that of Jersey crossbred cows (35) from College of Veterinary Science, Khanapara, Guwahati and its adjoining hilly area in Meghalaya. Genomic DNA was extracted from blood samples by phenol-chloroform extraction method reported by Sambrook and Russell (1989) with minor modifications. Quality of DNA was confirmed with 0.7% agarose electrophoresis. Plate 4.1, Plate 4.2 and Plate 4.3 shows the representative genomic DNA of Sahiwal, Karan Fries and Jersey crossbred cows, respectively. The quality of DNA obtained from his method was found to be good.

4.2 Polymerase Chain Reaction (PCR) Standardization

4.2.1 Template DNA Concentration

DNA template concentrations varied between 150 to 300 ng per 25 µl reaction volume with increments of 25 ng. There was no apparent difference in the yield and specificity of the DNA samples observable by visual appraisal in agarose gel in case of different samples. Hence, a concentration of 150 to 300 ng per 25 µl reaction volume was used.

4.2.2 Primer Concentration

The concentration of forward and reverse primer was optimized by setting up PCR trials. The optimum level of primer used was 10pmol. When primers were used at less than optimum level, lower yield of the desired product was obtained. This might be due to limited amount of the primers (Sambrook and Russel, 1989).

4.2.3 Annealing Temperature (Ta)

One of the most critical factors is the annealing temperature (Ta) which affects both specificity and yield of the PCR product. Both primer sets were optimized at 56.5°C. At lower annealing temperatures, non-specific products were formed while at higher than optimal temperature yield of the products were reduced.

4.3 Polymerase Chain Reaction

Two sets of primers were used to amplify target regions of ATP1A1 gene in Sahiwal, Karan Fries and Jersey crossbred cattle. For the first primer set the target region on ATP1A1 gene was partial exon 16 - 17 Intron (nucleotide position 27008667..27008177) while that for second primer set it was partial Intron 17- Intron 18 (nucleotide position 27008189..27007725). PCR reactions were set up for a total volume of 25 µl for each sample. PCR was done for all 136 DNA samples of all three cattle breeds.

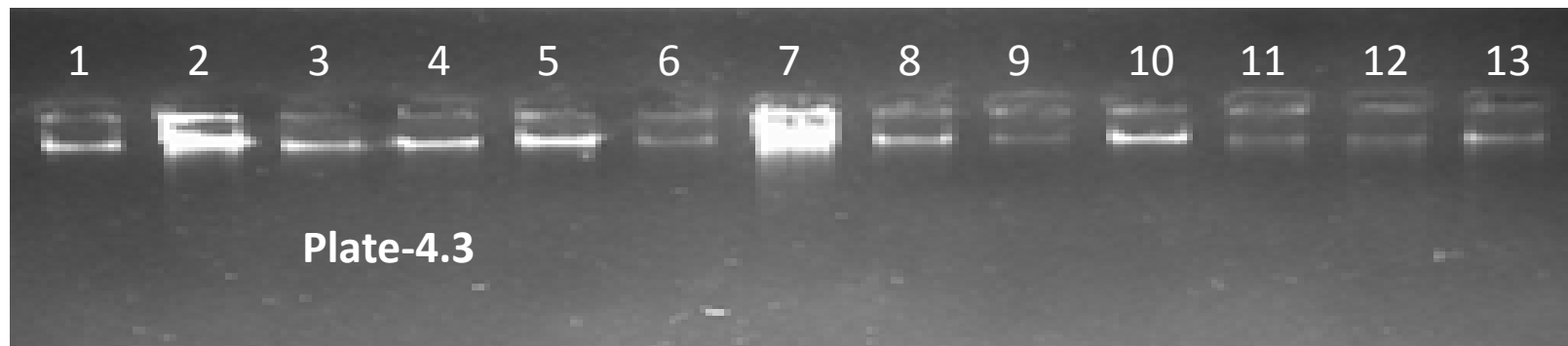
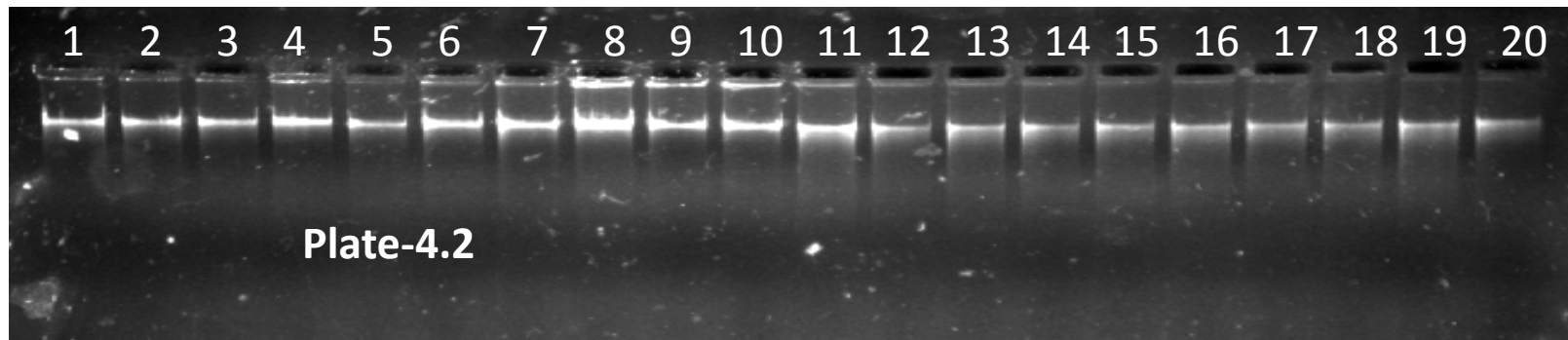
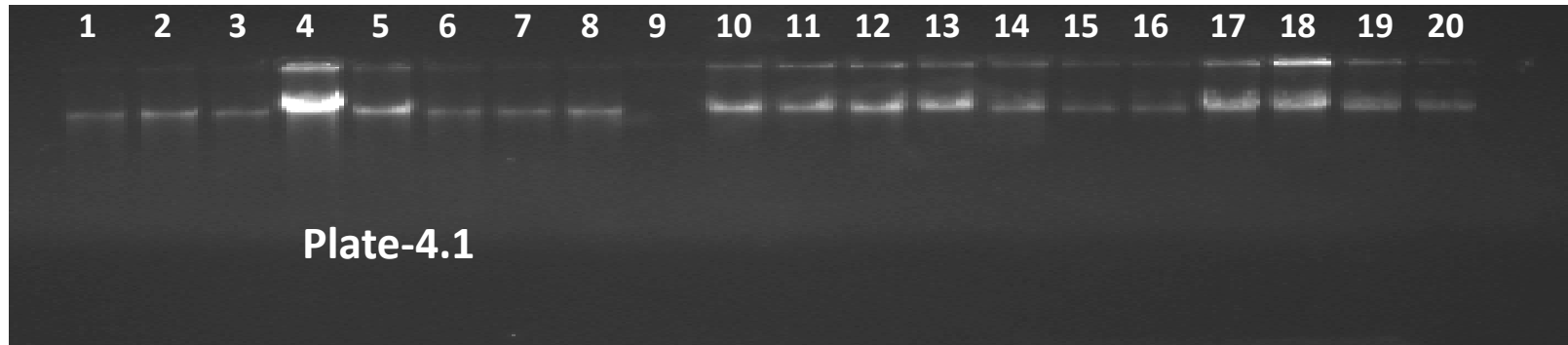
4.4 Quality of PCR product

Targeted genomic regions of both primer sets were successfully amplified in all the animals considered in the present study. Quality and sizes of PCR products of both target regions were checked on 2% agarose gel electrophoresis. The PCR products of primer set 1 and 2 of Sahiwal, Karan Fries and Jersey crossbred cows are shown in Plate 4.4 - 4.9.

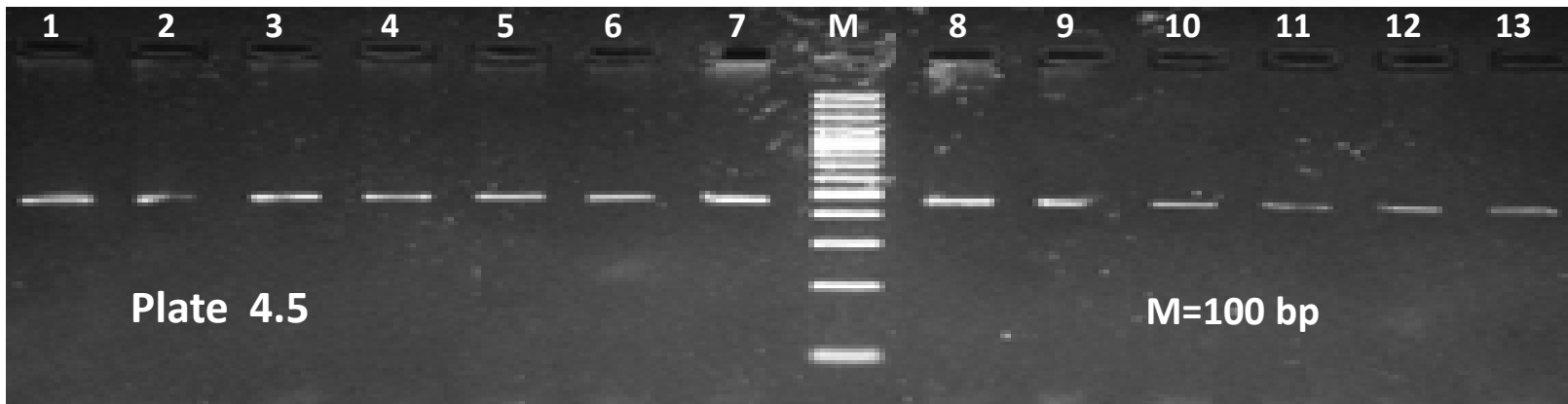
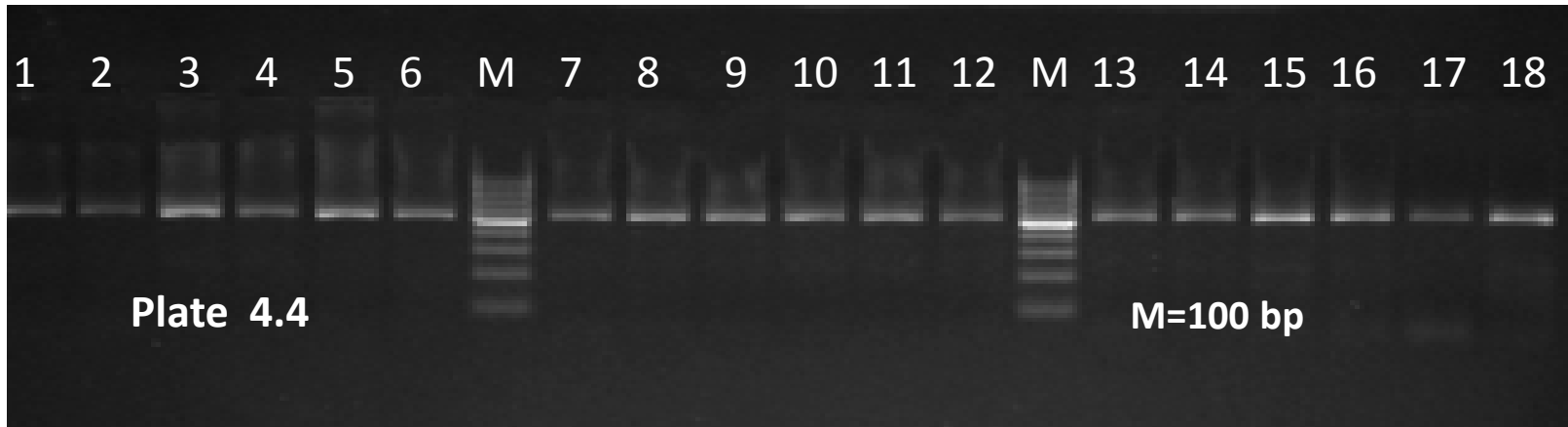
4.5 Analysis of DNA sequencing data

PCR products of both primer sets of all 136 DNA samples of all three cattle breeds were sent for purification and custom sequencing (both forward and reverse) to 1st base sequencing INT (Singapore) and Merck Specialties Pvt. Ltd. (Bangalore). Sequencing data was analyzed using BioEdit software. For determining SNPs, reference sequence ENSBTAG00000001246 of *Bos taurus* available at ensemble genome browser (www.ensembl.org) was aligned with sequencing results of each animal for each target region using ClustalW multiple alignment programme. Total six SNPs (T27008243C, A27008223G, T27008097A, C27008016T, G27008015A and C27007790A) were found in studied breeds. Multiple sequence alignment was performed for both targeted regions of ATP1A1 gene between reference *Bos taurus* sequence (ENSBTAG00000001246) and observed sequences of Sahiwal (Figure 4.1 and 4.4), Karan Fries (Figure 4.9 and 4.12) and Jersey crossbred cattle (Figure

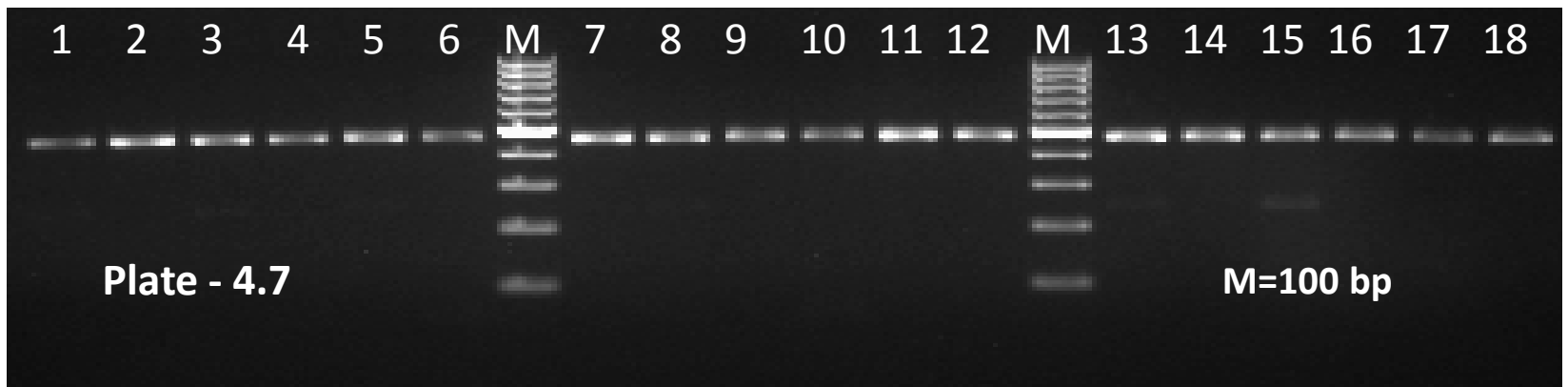
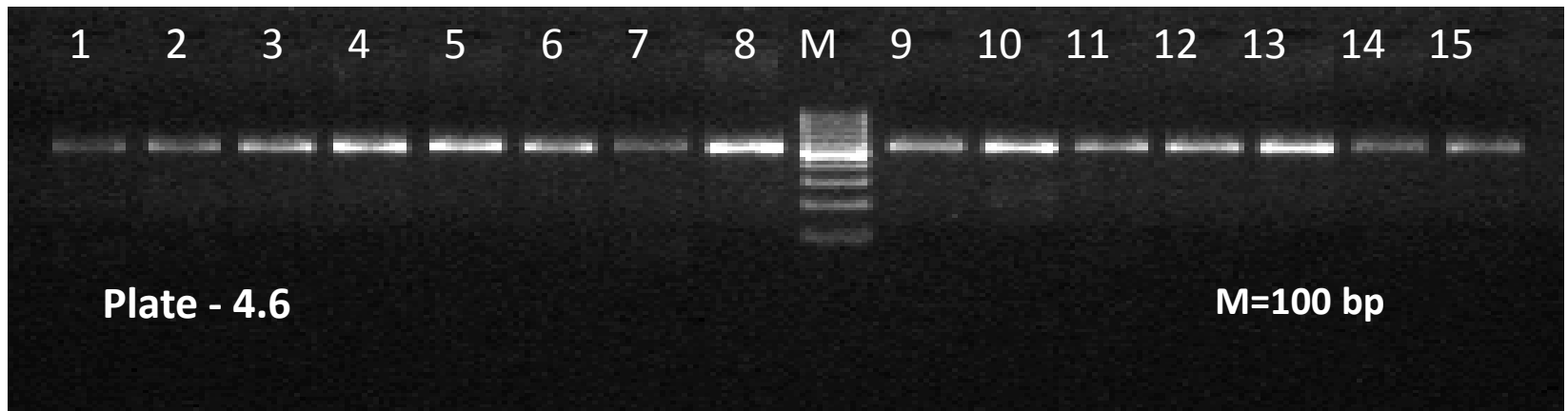
Genomic DNA of Sahiwal, Karan Fries and Jersey crossbred cows



Sahiwal PCR Products: Primer- 1 (491 bp) & Primer- 2 (465 bp)



Karan Fries PCR Products: Primer- 1 (491 bp) & Primer- 2 (465 bp)



Jersey crossbred PCR Products: Primer- 1 (491 bp) & Primer- 2 (465 bp)

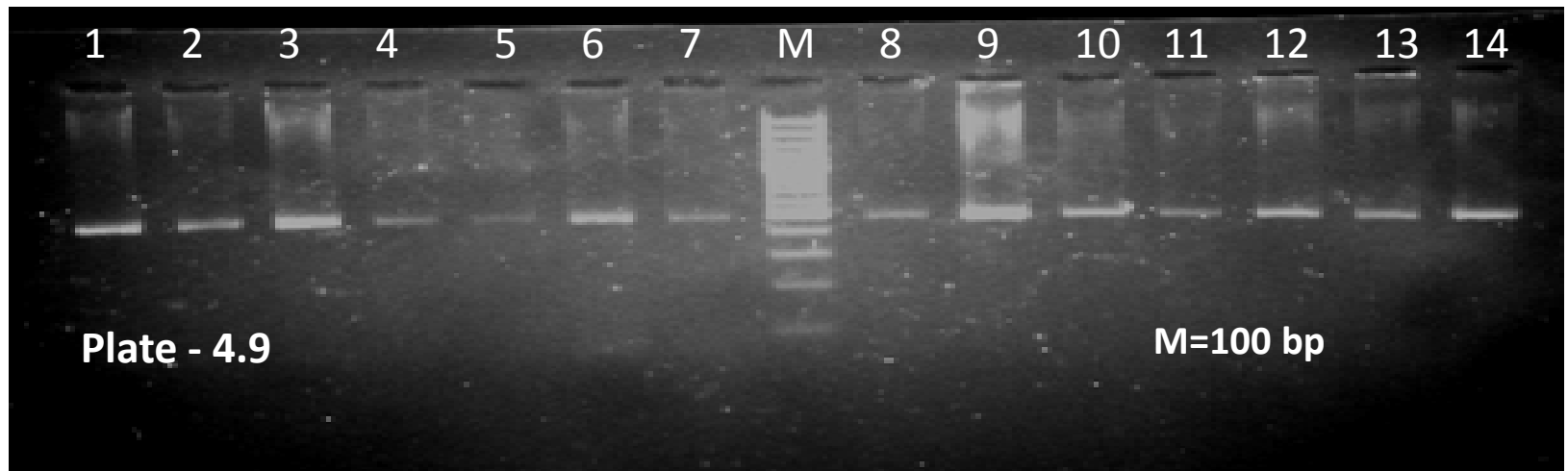
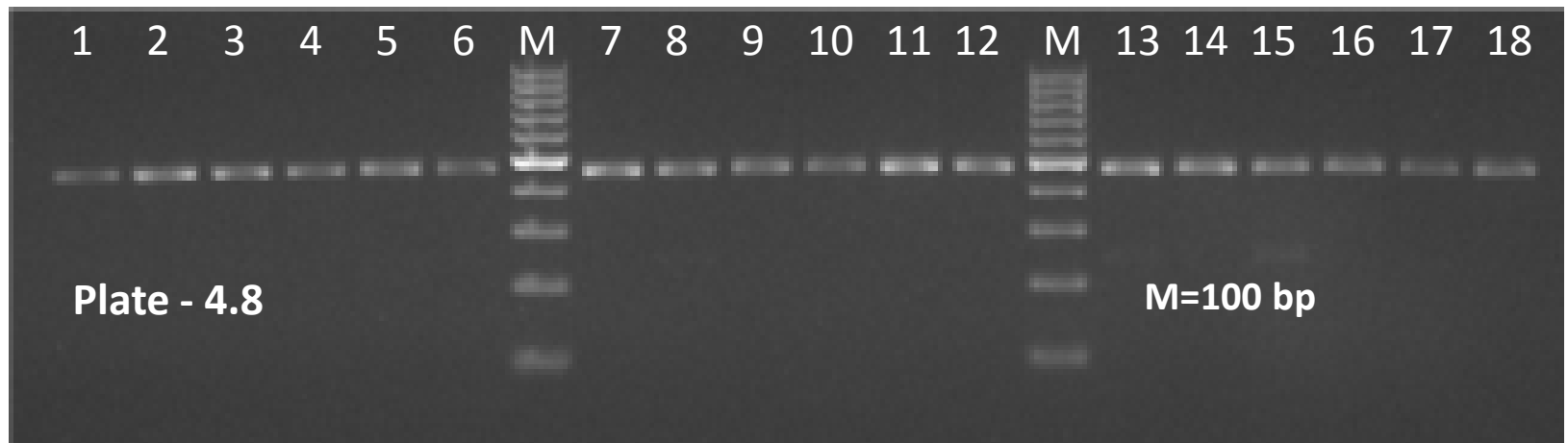


Figure 4.1 Multiple Sequence Alignment of PCR product of ATP1A1 Gene obtained by Primer 1 in Sahiwal cows

```

BTA      --AATGACTCCCCGGCTTTGAAGAAGGCAGACATTGGGGTTGCCATGGGGATTGCAGGCT 58
S_R1F    -----TTGG-GGTGC-ATGGGGAT-GCAG-CT 25
S_R1Rc   TAATGACTNCCCCGGCTTTGAAGAAGGCAGACATTGGGGTTGCCATGGGGATTGCAGGCT 60
          **** * *** ***** **

BTA      CAGACGTGTCTAAGCAAGCTGCTGACATGATTCTCCTAGATGACAACCTTTGCCTCAATTG 118
S_R1F    CAGACGTGTCTAAGCAAGCTGCTGACATGATTCTCCTAGATGACAACCTTTGCCTCAATTG 85
S_R1Rc   CAGACGTGTCTAAGCAAGCTGCTGACATGATTCTCCTAGATGACAACCTTTGCCTCAATTG 120
          *****

BTA      TGA CTGGAGTAGAGGAAGGTGAGAGTGCTGCGTGAAACCTGGTCCAGATGCTGCTGAGCA 178
S_R1F    TGA CTGGAGTAGAGGAAGGTGAGAGTGCTGCGTGAAACCTGGTCCAGATGCTGCTGAGCA 145
S_R1Rc   TGA CTGGAGTAGAGGAAGGTGAGAGTGCTGCGTGAAACCTGGTCCAGATGCTGCTGAGCA 180
          *****

BTA      GTATCCATTTCTGCACGTCATTTCCGGTCCTTTTCATTAAGCTCTACATTA AAAACAATC 238
S_R1F    GTATCCATTTCTGCACGTCATTTCCGGTCCTTTTCATTAAGCTCTACATTA AAAACAATC 205
S_R1Rc   GTATCCATTTCTGCACGTCATTTCCGGTCCTTTTCATTAAGCTCTACATTA AAAACAATC 240
          *****

BTA      TTGCTTTCCAGGTCGTCTGATCTTTGATAACTTGAAGAAATCCATTGCTTATACCTCA 298
S_R1F    TTGCTTTCCAGGTCGTCTGATCTTTGATAACTTGAAGAAATCCATTGCTTATACCTCA 265
S_R1Rc   TTGCTTTCCAGGTCGTCTGATCTTTGATAACTTGAAGAAATCCATTGCTTATACCTCA 300
          *****

BTA      CCAGTAACATTCTGAGATTACCCCTTCCTGATATTTATTATTGCAAACATTCCACTCC 358
S_R1F    CCAGTAACATTCTGAGATTACCCCTTCCTGATATTTATTATTGCAAACATTCCACTCC 325
S_R1Rc   CCAGTAACATTCTGAGATTACCCCTTCCTGATATTTATTATTGCAAACATTCCACTCC 360
          *****

BTA      CTCTGGGGACCGTCACCATCCTCTGCATCGACTTGGGAACGGACATGGTAAGTGACTGGC 418
S_R1F    CTCTGGGGACCGTCACCATCCTCTGCATCGACTTGGGAACGGACATGGTAAGTGACTGGC 385
S_R1Rc   CTCTGGGGACCGTCACCATCCTCTGCATCGACTTGGGAACGGACATGGTAAGTGACTGGC 420
          *****

BTA      ACACATTCACACATCACAGTGCTAAAGTG TG GGCATTAGCACTCACTACTGTGTTCTCTG 478
S_R1F    ACACATTCACACATCACAGTGCTAAAGTG TG GGCATTAGCACTCACTACTGTGTTCTCTG 445
S_R1Rc   ACACATTCACACATCACAGTGCTAAAGTG NG - CATTGANN CNNTNNNN----- 468
          *****

BTA      GGTCCCCGTGGCC---- 491
S_R1F    GGTCCCCGTGGCAAAA 462
S_R1Rc   -----

```

Figure 4.2 Chromatograph showing T27008243C SNP in Sahiwal cows

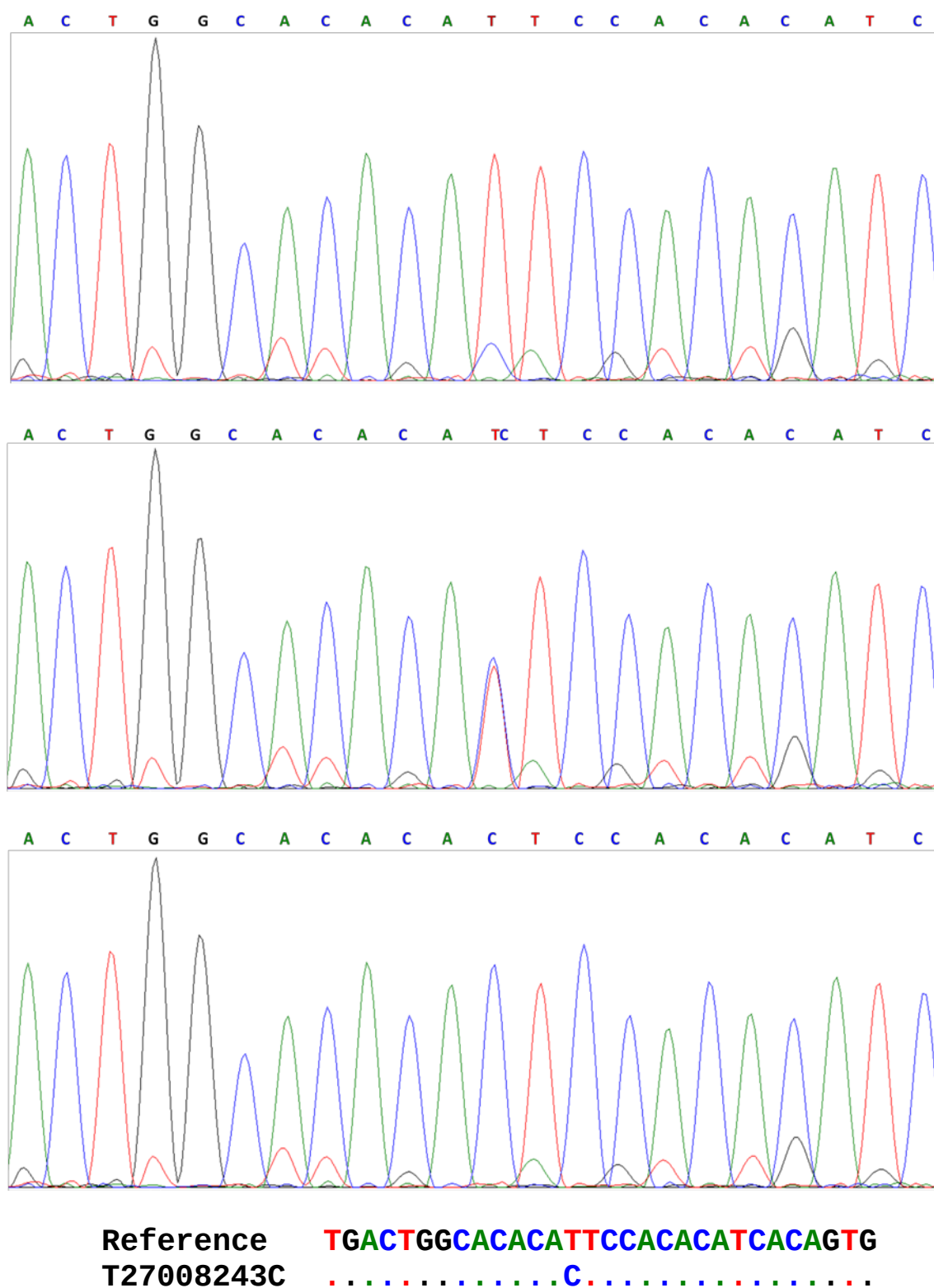


Figure 4.3 Chromatograph showing A27008223G SNP in Sahiwal cows

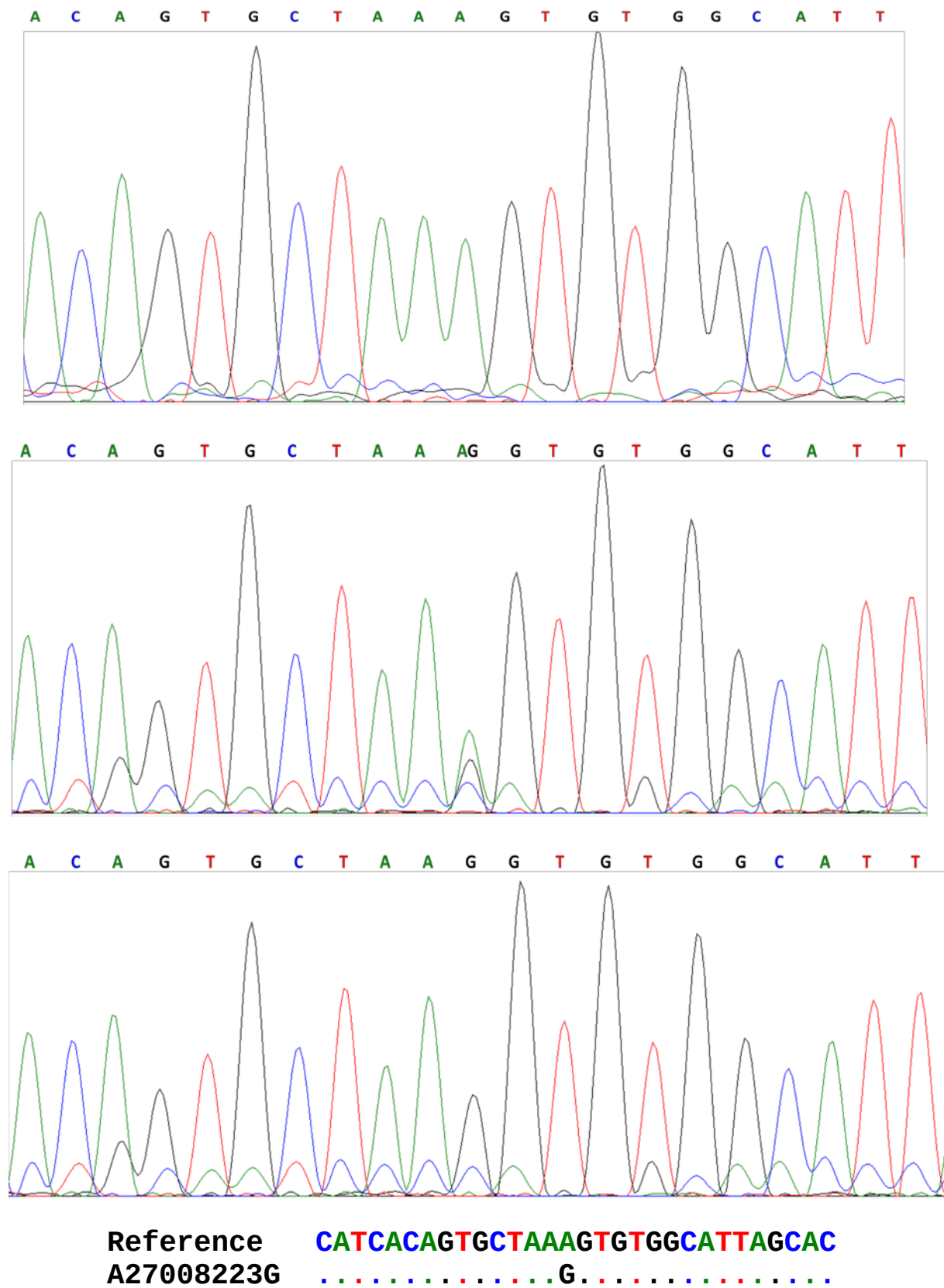


Figure 4.4 Multiple Sequence Alignment of PCR product of ATP1A1 Gene obtained by Primer 2 in Sahiwal cows

```

BTA      - - - - -GGTCCCGTGGCCAGCATTTGGAAATATCACTACCGAGAATTTCTTGCCCTGTGT 55
S_R2F    - - - - -GCCTAATTTTTCTATA - - -CACTGTCGAGA - -TTCTTGCCCTGTGT 41
S_R2Rc   TTTGGGTCCCACATGGCCAGCATTTGGAAATATCACTACCGAGAATTTCTTGCCCTGTGT 60
          * * *      * *      * * *      * * *      * * *      * * *

BTA      GTGGCAGCCTCAGCATCAAAGATTCAGTGTTAATCATTTCTTAAGACAGGTGTCAATACC 115
S_R2F    GTGGCAGCCTCAGCATCAAAGATTCAGTGTTAATCATTTCTTAAGACAGGTGTCAATACC 101
S_R2Rc   GTGGCAGCCTCAGCATCAAAGATTCAGTGTTAATCATTTCTTAAGACAGGTGTCAATACC 120
          *****

BTA      TATTTATCCGATAGAAATCTTCCCTTTTGTATCATTTTGCCCTTTTTTTAAAAAAGACG 175
S_R2F    TATTTATCCGATAGAAATCTTCCCTTTTGTATCATTTTGCCCTTTTTTTAAAAAAGATA 161
S_R2Rc   TATTTATCCGATAGAAATCTTCCCTTTTGTATCATTTTGCCCTTTTTTTAAAAAAGATA 180
          *****

BTA      GGGGACTTGTAATATCTCATTATGGAAAACAGTTTTTTTTAAGGGAGAGCAAGAATTTGAG 235
S_R2F    GGGGACTTGTAATATCTCATTATGGAAAACAGTTTTTTTTAAGGGAGAGCAAGAATTTGAG 221
S_R2Rc   GGGGACTTGTAATATCTCATTATGGAAAACAGTTTTTTTTAAGGGAGAGCAAGAATTTGAG 240
          *****

BTA      AACAAACAAAAGGGTCACAACATGAGCTTGCCAGTTTCTGATGACTTCTCGCCCTCCTT 295
S_R2F    AACAAACAAAAGGGTCACAACATGAGCTTGCCAGTTTCTGATGACTTCTCGCCCTCCTT 281
S_R2Rc   AACAAACAAAAGGGTCACAACATGAGCTTGCCAGTTTCTGATGACTTCTCGCCCTCCTT 300
          *****

BTA      CAGGTCCCTGCTATCTCCTTGGCTTATGAGCAGGCTGAGAGCGACATCATGAAGAGGCAG 355
S_R2F    CAGGTCCCTGCTATCTCCTTGGCTTATGAGCAGGCTGAGAGCGACATCATGAAGAGGCAG 341
S_R2Rc   CAGGTCCCTGCTATCTCCTTGGCTTATGAGCAGGCTGAGAGCGACATCATGAAGAGGCAG 360
          *****

BTA      CCCAGAAACCCCCAGACGGACAAACTCGTGAACGAGCGGCTGATCAGCATGGCCTATGGA 415
S_R2F    CCCAGAAACCCCCAGACGGACAAACTCGTGAACGAGCGGCTGATAAGCATGGCCTATGGA 401
S_R2Rc   CCCAGAAACCCCCAGACGGACAAACTCGTGAACGAGCGGCTGATAAGCATGGCCTATGGA 419
          *****

BTA      CAGATTGGTGAGCCAGCAGATTTCAAGTATCTGTAGGGGTGCTGT-CTGCC- - - - 465
S_R2F    CAGATTGGTGAGCCAGCAGATTTCAAGTATCTGTAGGGGTGCTGTTCTGCAAAAAA 457
S_R2Rc   CAGATTG- -GATCCCGCATACCCAAATTTTTTTT- - - -TACCGG- - -GCG- - - - 460
          ***** **

```

Figure 4.5 Chromatograph showing T27008097A SNP in Sahiwal cows

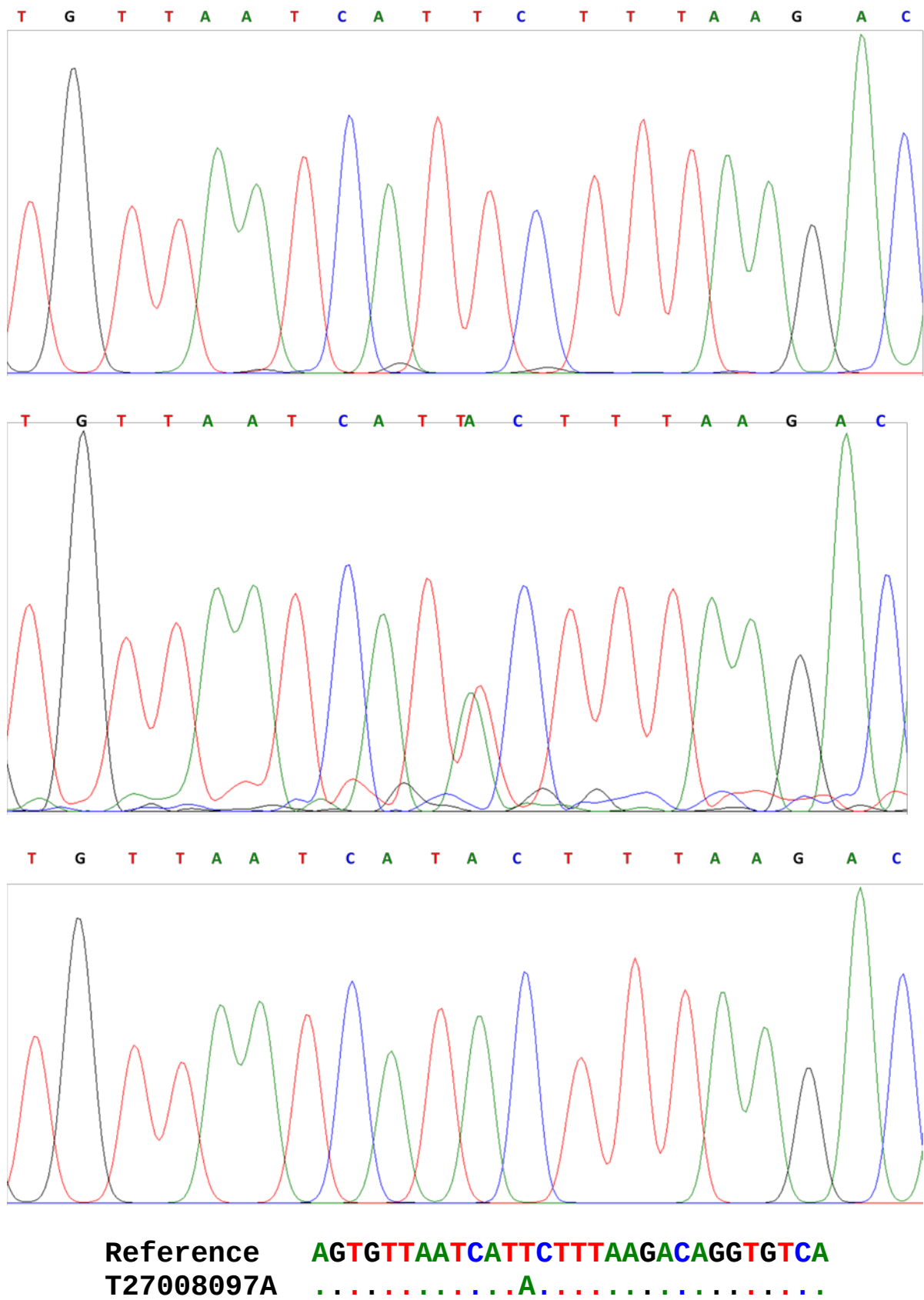
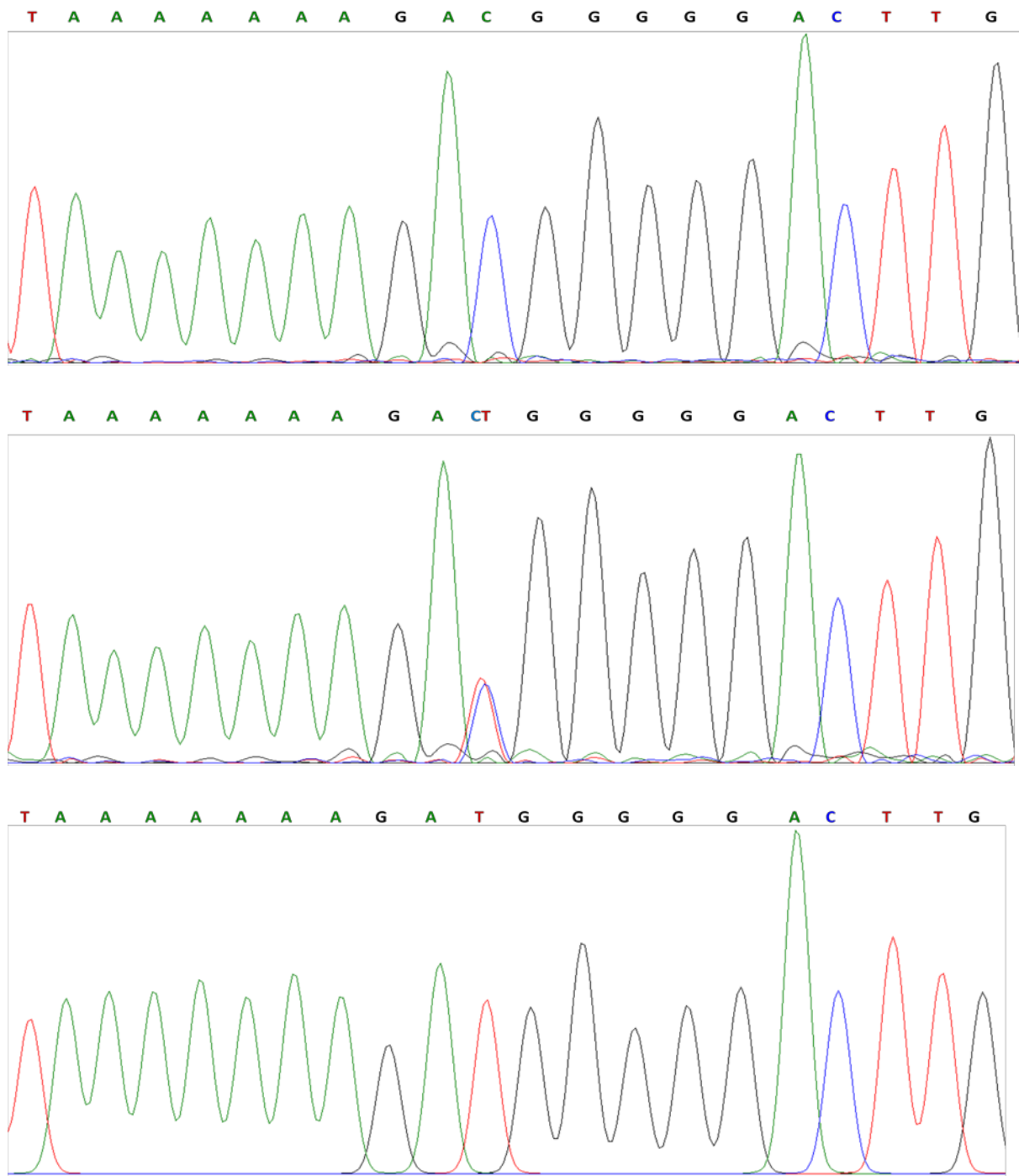


Figure 4.6 Chromatograph showing C27008016T SNP in Sahiwal cows



Reference TTTTAAAAAAGACGGGGGACTTGTAAATAT
C27008016T T.....

Figure 4.7 Chromatograph showing G27008015A SNP in Sahiwal cows

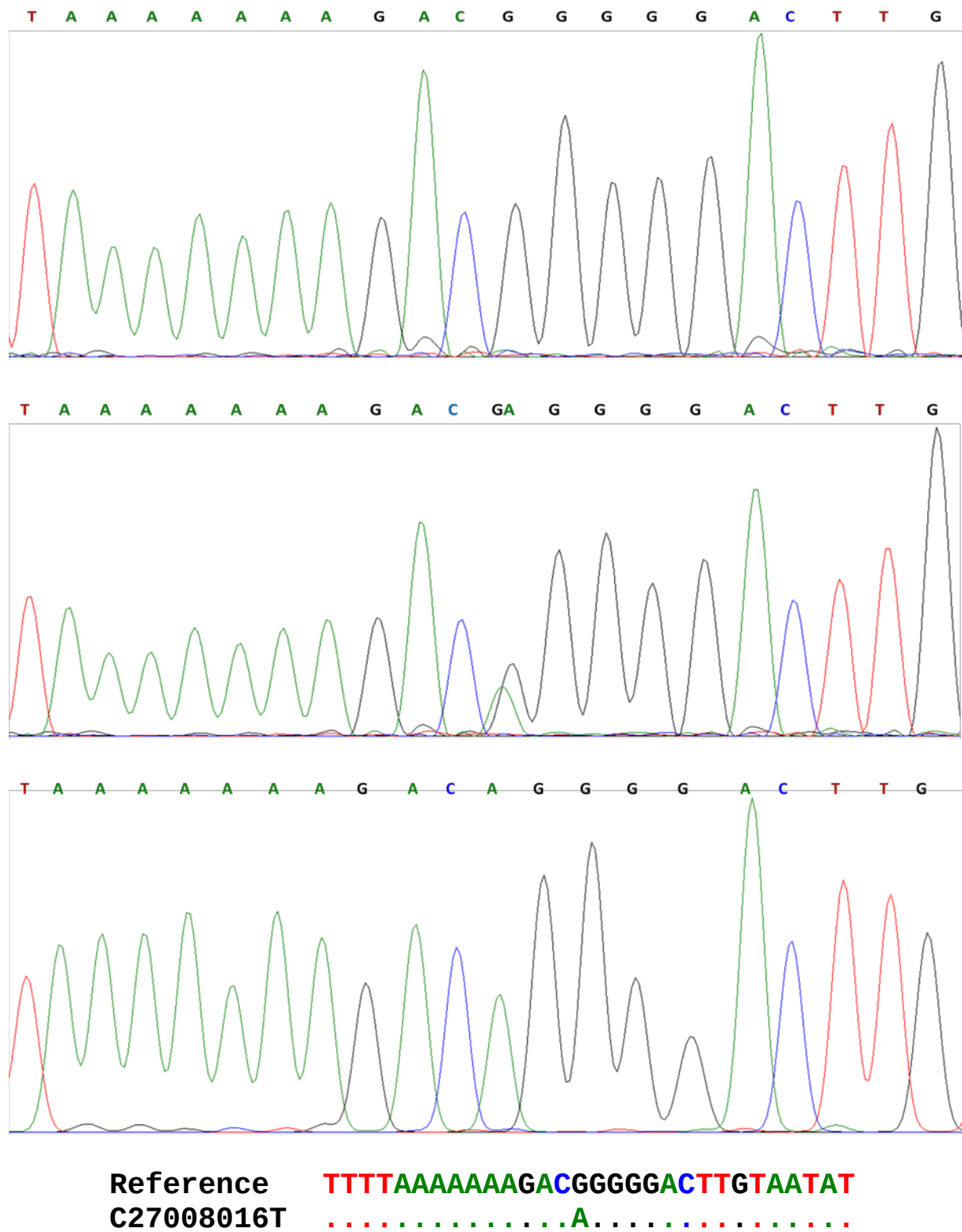
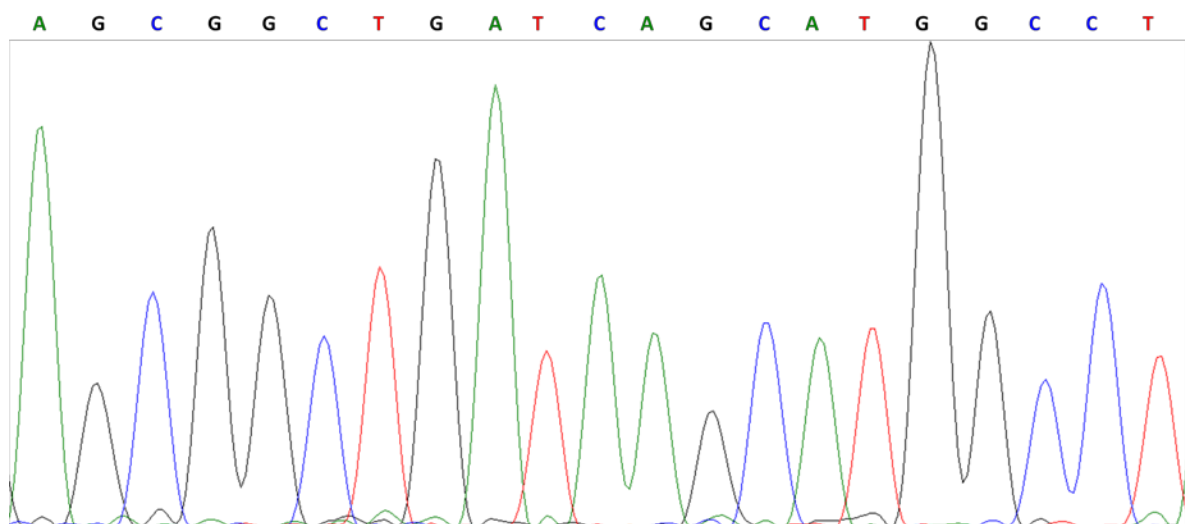
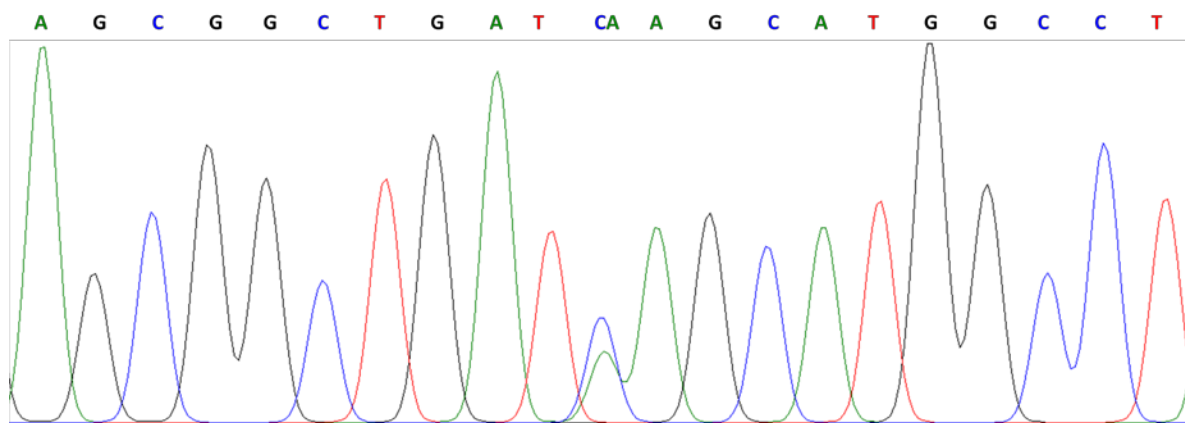
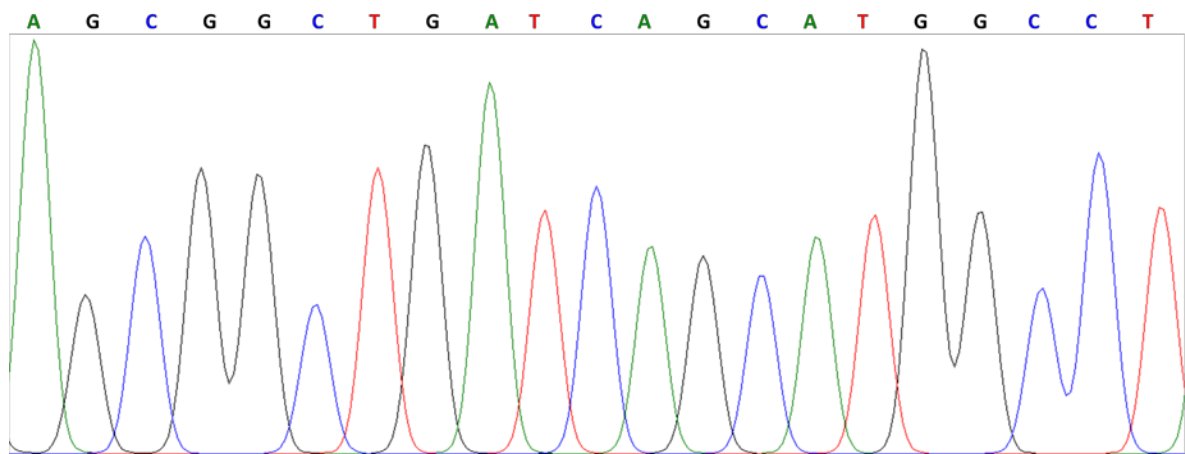


Figure 4.8 Chromatograph showing C27007790A SNP in Sahiwal cows



Reference **TCGTGAACGAGCGGCTGATCAGCATGGCCT**
 C27007790A A.....

Figure 4.9 Multiple Sequence Alignment of PCR product of ATP1A1 Gene obtained by Primer 1 in Karan Fries cows

BTA	--AATGACTCCCCGGCTTTGAAGAAGGCAGACATTGGGGTTGCCATGGGGATTGCAGGCT	58
KF_R1F	-----TTGGGGGTGC-ATGGGGAT-GCAGGCT	25
KF_R1Rc	TAATGACTNCCCCGGCTTTGAAGAAGGCAGACATTGGGGTTGCCATGGGGATTGCAGGCT	60
	***** ** *	
BTA	CAGACGTGTCTAAGCAAGCTGCTGACATGATTCTCCTAGATGACAACCTTTGCCTCAATTG	118
KF_R1F	CAGACGTGTCTAAGCAAGCTGCTGACATGATTCTCCTAGATGACAACCTTTGCCTCAATTG	85
KF_R1Rc	CAGACGTGTCTAAGCAAGCTGCTGACATGATTCTCCTAGATGACAACCTTTGCCTCAATTG	120

BTA	TGACTGGAGTAGAGGAAGGTGAGAGTGCTGCGTGAAACCTGGTCCAGATGCTGCTGAGCA	178
KF_R1F	TGACTGGAGTAGAGGAAGGTGAGAGTGCTGCGTGAAACCTGGTCCAGATGCTGCTGAGCA	145
KF_R1Rc	TGACTGGAGTAGAGGAAGGTGAGAGTGCTGCGTGAAACCTGGTCCAGATGCTGCTGAGCA	180

BTA	GTATCCATTTCTGCACGTCAATTCGGTCCTTTTCATTAAGCTCTACATTAACCAATC	238
KF_R1F	GTATCCATTTCTGCACGTCAATTCGGTCCTTTTCATTAAGCTCTACATTAACCAATC	205
KF_R1Rc	GTATCCATTTCTGCACGTCAATTCGGTCCTTTTCATTAAGCTCTACATTAACCAATC	240

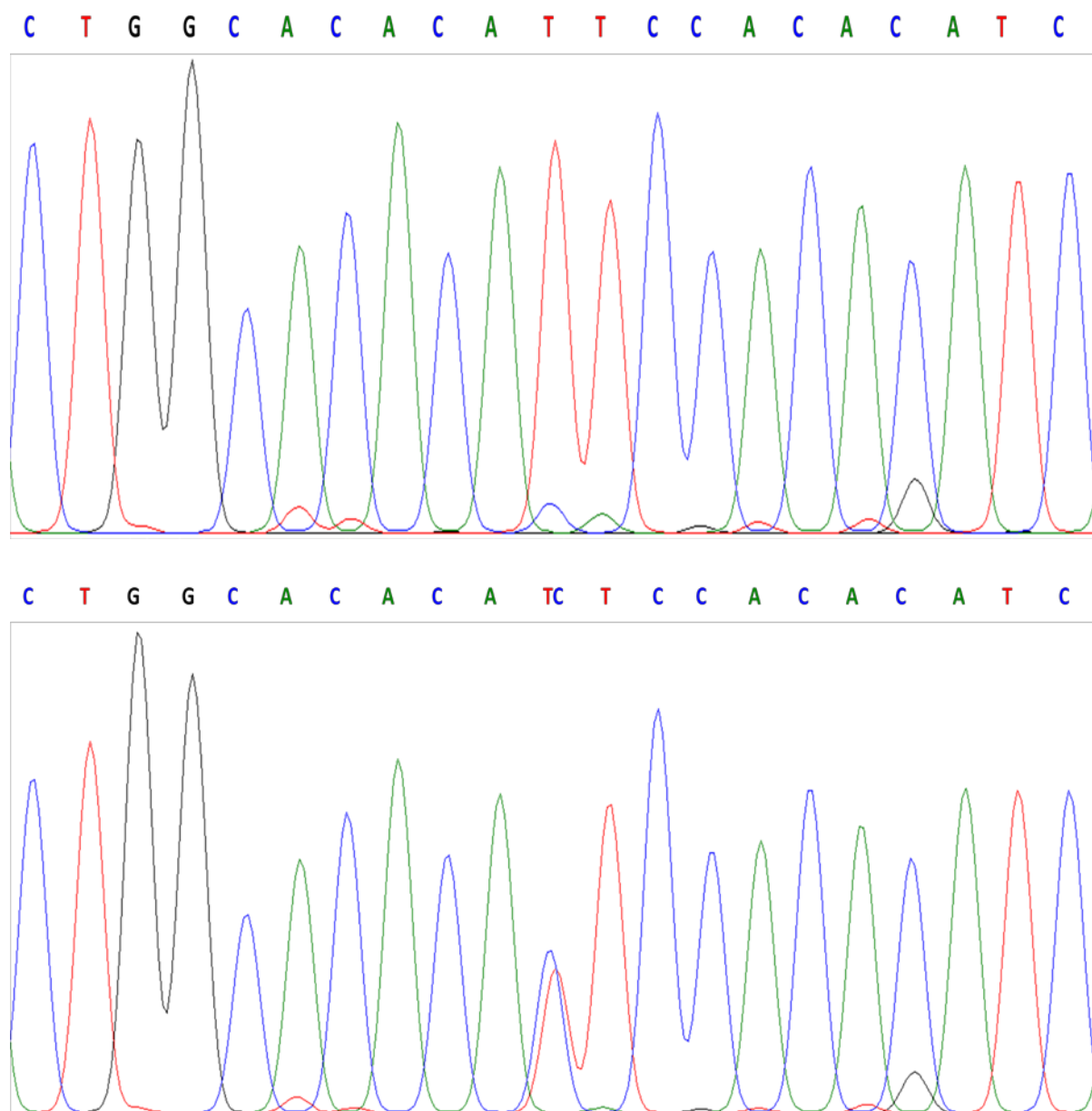
BTA	TTGCTTTCCAGGTCGTCTGATCTTTGATAACTTGAAGAAATCCATTGCTTATACCCTCA	298
KF_R1F	TTGCTTTCCAGGTCGTCTGATCTTTGATAACTTGAAGAAATCCATTGCTTATACCCTCA	265
KF_R1Rc	TTGCTTTCCAGGTCGTCTGATCTTTGATAACTTGAAGAAATCCATTGCTTATACCCTCA	300

BTA	CCAGTAACATTCTGAGATTACCCCTTCCTGATATTTATTATTGCAAACATTCCACTCC	358
KF_R1F	CCAGTAACATTCTGAGATTACCCCTTCCTGATATTTATTATTGCAAACATTCCACTCC	325
KF_R1Rc	CCAGTAACATTCTGAGATTACCCCTTCCTGATATTTATTATTGCAAACATTCCACTCC	360

BTA	CTCTGGGGACCGTCACCATCCTCTGCATCGACTTGGGAACGGACATGGTAAGTGACTGGC	418
KF_R1F	CTCTGGGGACCGTCACCATCCTCTGCATCGACTTGGGAACGGACATGGTAAGTGACTGGC	385
KF_R1Rc	CTCTGGGGACCGTCACCATCCTCTGCATCGACTTGGGAACGGACATGGTAAGTGACTGGC	420

BTA	ACACATTCCACACATCACAGTGCTAAAGTGTGGCATTAGCACTCACTACTGTGTTCTCTG	478
KF_R1F	ACACATTCCACACATCACAGTGCTAAAGTGTGGCATTAGCACTCACTACTGTGTTCTCTG	445
KF_R1Rc	ACACATTCCACACATCACAGTGCTAAAGTGTGGCATTAGCACTCACTACTGTGTTCTCTG	468
	***** ** *	
BTA	GGTCCCCGTGGCC----	491
KF_R1F	GGTCCCCGTGGCCAAAA	462
KF_R1Rc	-----	

Table 4.10 Chromatograph showing T27008243C SNP in Karan Fries cows



Reference **TGACTGGCACACATTCCACACATCACAGTG**
T27008243C **.....C.....**

Table 4.11 Chromatograph showing A27008223G SNP in Karan Fries cows

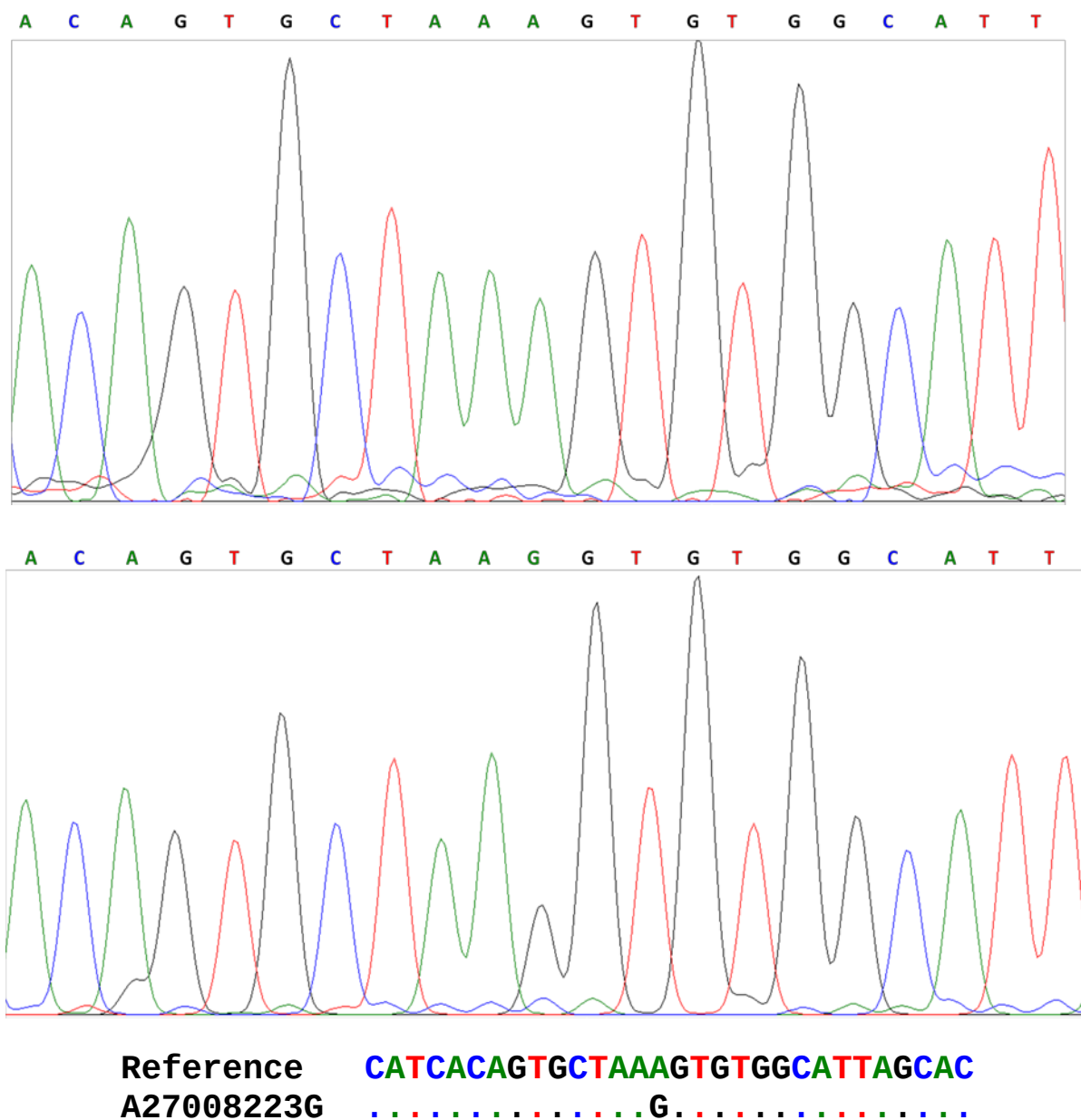


Figure 4.12 Multiple Sequence Alignment of PCR product of ATP1A1 Gene obtained by Primer 2 in Karan Fries cows

```

BTA      - - - - -GGTCCCCGTGGCCAGCATTGGAATATCACTACCGAGAATTTCTTGGCCTGTGT 55
KF_R2F   - - - - -CACC G - - GAAGATTTCTTGGCCTGTGT 25
KF_R2Rc  TTCTGGGTCCCCGTGGCCAGCATTGGAATATCACTACCGAGAATTTCTTGGCCTGTGT 60
          ***      **      *****

BTA      GTGGCAGCCTCAGCATCAAAGATTCAGTGTTAATCATTCTTTAAGACAGGTGTCAATACC 115
KF_R2F   GTGGCAGCCTCAGCATCAAAGATTCAGTGTTAATCATACTTTAAGACAGGTGTCAATACC 85
KF_R2Rc  GTGGCAGCCTCAGCATCAAAGATTCAGTGTTAATCATACTTTAAGACAGGTGTCAATACC 120
          *****

BTA      TATTTATCCGATAGAAATCTTCCCTTTTGTATCATTTTGCCTTTTTTTAAAAAAGACG 175
KF_R2F   TATTTATCCGATAGAAATCTTCCCTTTTGTATCATTTTGCCTTTTTTTAAAAAAGATA 145
KF_R2Rc  TATTTATCCGATAGAAATCTTCCCTTTTGTATCATTTTGCCTTTTTTTAAAAAAGATA 180
          *****

BTA      GGGGACTTGTAATATCTCATTATGAAAAACAGTTTTTTTAAGGGAGAGCAAGAATTTGAG 235
KF_R2F   GGGGACTTGTAATATCTCATTATGAAAAACAGTTTTTTTAAGGGAGAGCAAGAATTTGAG 205
KF_R2Rc  GGGGACTTGTAATATCTCATTATGAAAAACAGTTTTTTTAAGGGAGAGCAAGAATTTGAG 240
          *****

BTA      AACAAACAAAAGGGTCACAACATGAGCTTGCCAGTTTCTGATGACTTCTCGCCCCTCCTT 295
KF_R2F   AACAAACAAAAGGGTCACAACATGAGCTTGCCAGTTTCTGATGACTTCTCGCCCCTCCTT 265
KF_R2Rc  AACAAACAAAAGGGTCACAACATGAGCTTGCCAGTTTCTGATGACTTCTCGCCCCTCCTT 300
          *****

BTA      CAGGTCCTGCTATCTCCTTGGCTTATGAGCAGGCTGAGAGCGACATCATGAAGAGGCAG 355
KF_R2F   CAGGTCCTGCTATCTCCTTGGCTTATGAGCAGGCTGAGAGCGACATCATGAAGAGGCAG 325
KF_R2Rc  CAGGTCCTGCTATCTCCTTGGCTTATGAGCAGGCTGAGAGCGACATCATGAAGAGGCAG 360
          *****

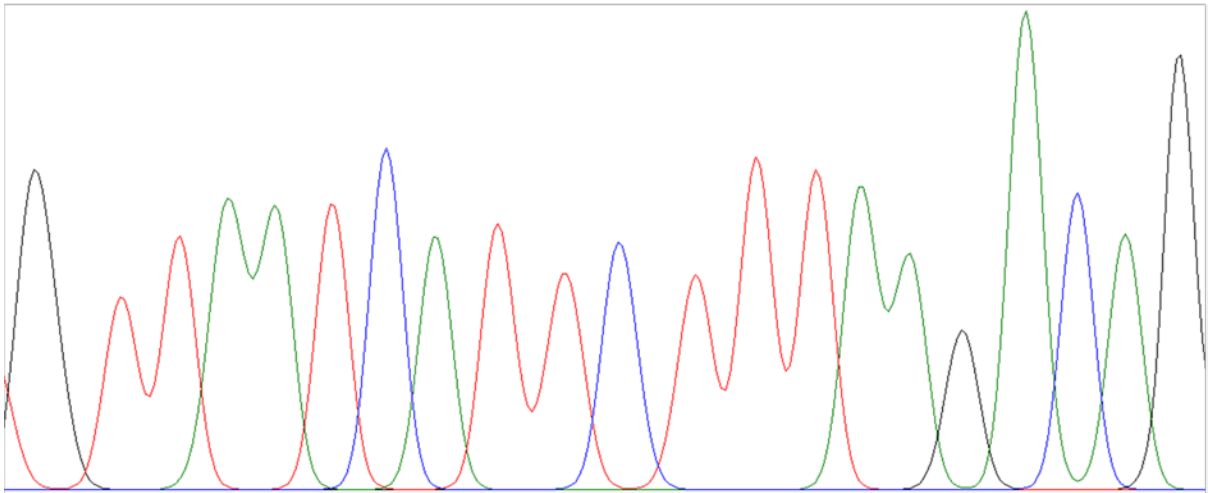
BTA      CCCAGAAACCCCGACGGACAAACTCGTGAACGAGCGGCTGATCAGCATGGCCTATGGA 415
KF_R2F   CCCAGAAACCCCGACGGACAAACTCGTGAACGAGCGGCTGATAAGCATGGCCTATGGA 385
KF_R2Rc  CCCAGAAACCCCGACGGACAAACTCGTGAACGAGCGGCTGATAAGCATGGCCTATGGA 420
          *****

BTA      CAGATTGGTGAGCCAGCAGATTTCAAGTATCTGTAGGGGTGCTGTCTGCC - - - 465
KF_R2F   CAGATTGGTGAGCCAGCAGATTTCAAGTATCTGTAGGGGTGCTGTCTGCAAAG 438
KF_R2Rc  CAGATTGTTAGGCCCGCCCTTTT - - - - - 443
          ***** *      *** **      ***

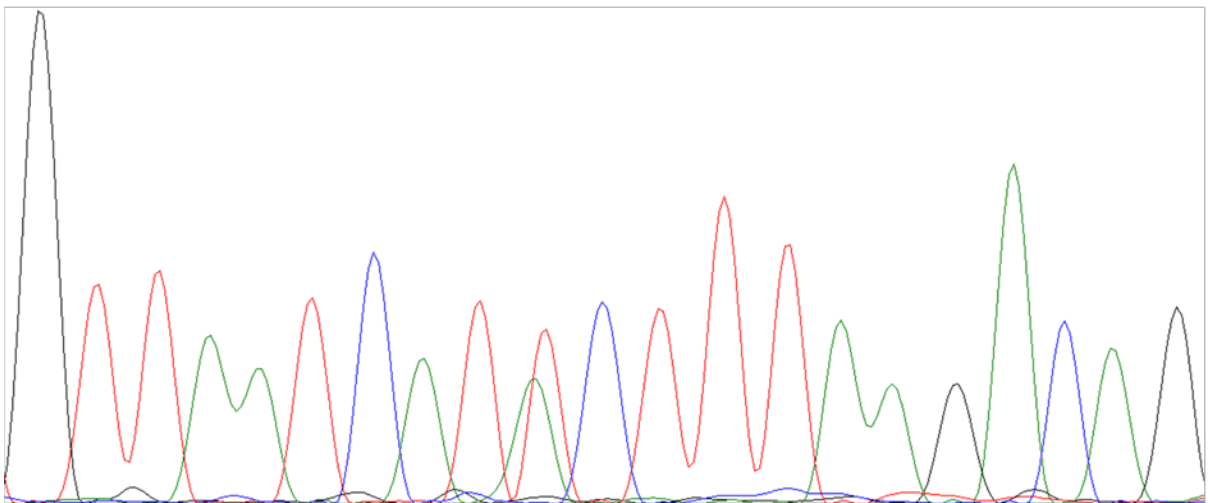
```

13 Chromatograph showing T27008097A SNP in Karan Fries cows

G T T A A T C A T T C T T T A A G A C A G

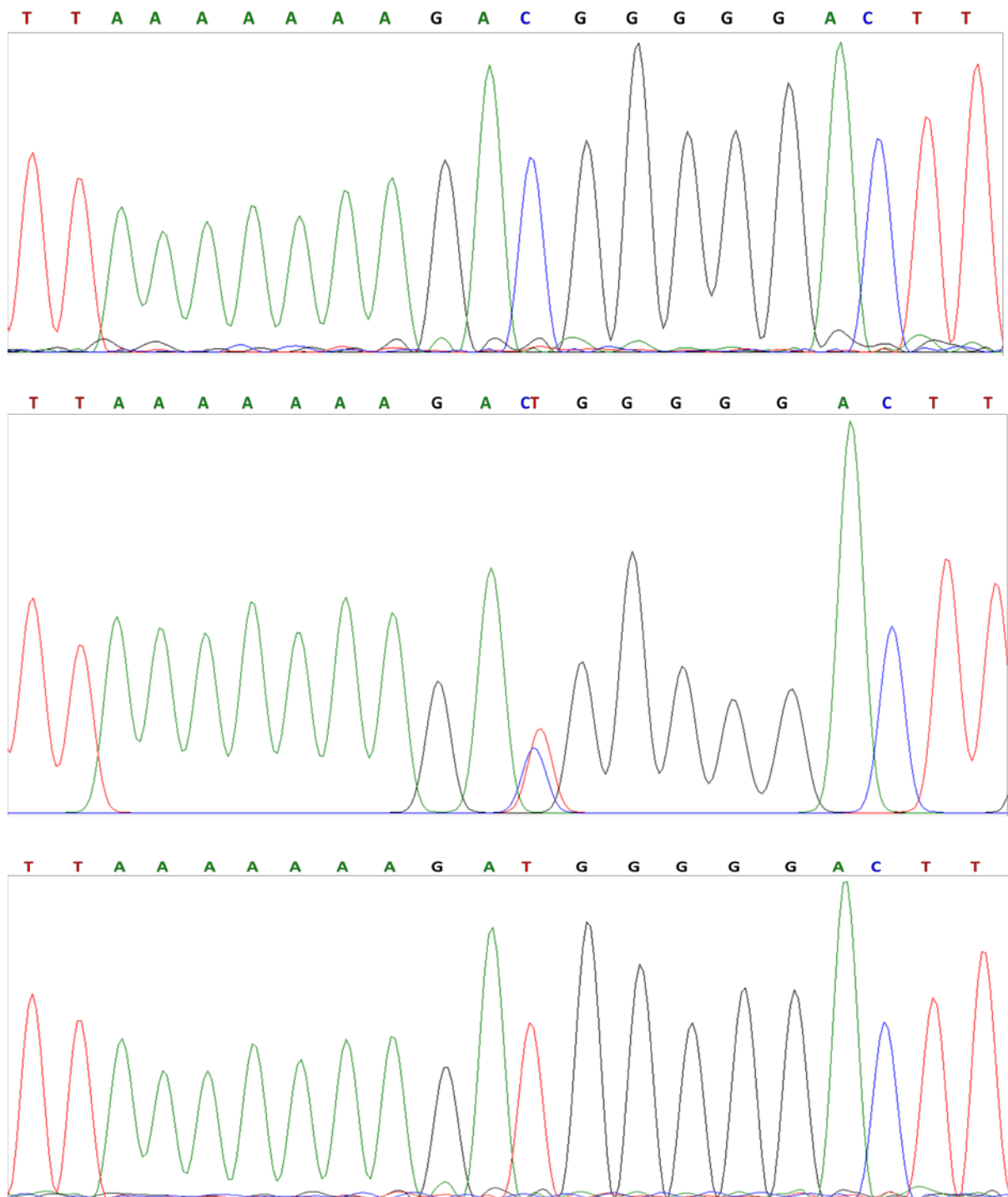


G T T A A T C A T TA C T T T A A G A C A G



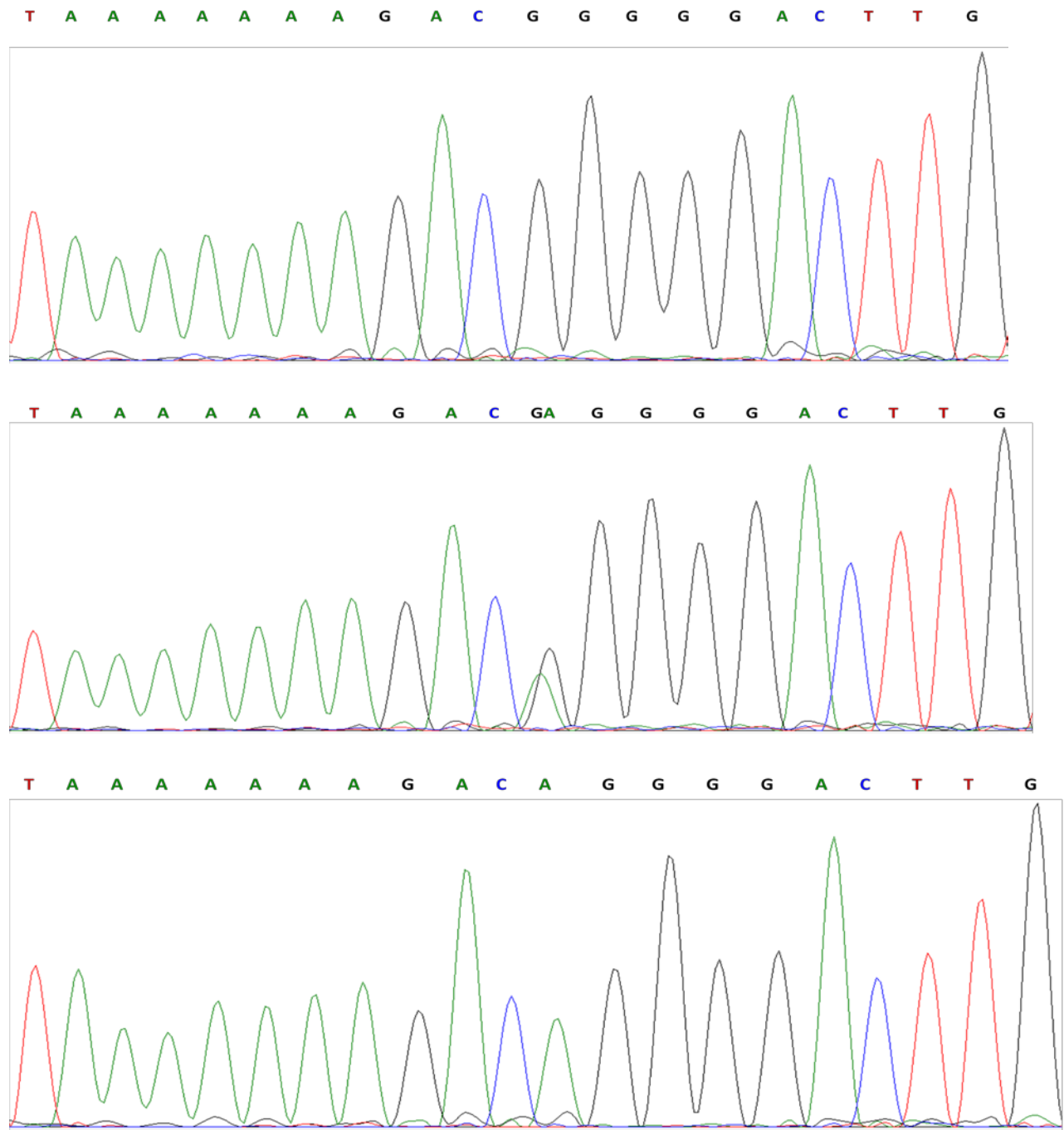
Reference AGTGTTAATCATTCTTTAAGACAGGTGTCA
T27008097A A.....

4.14 Chromatogram showing C27008016T SNP in Karan Fries cows



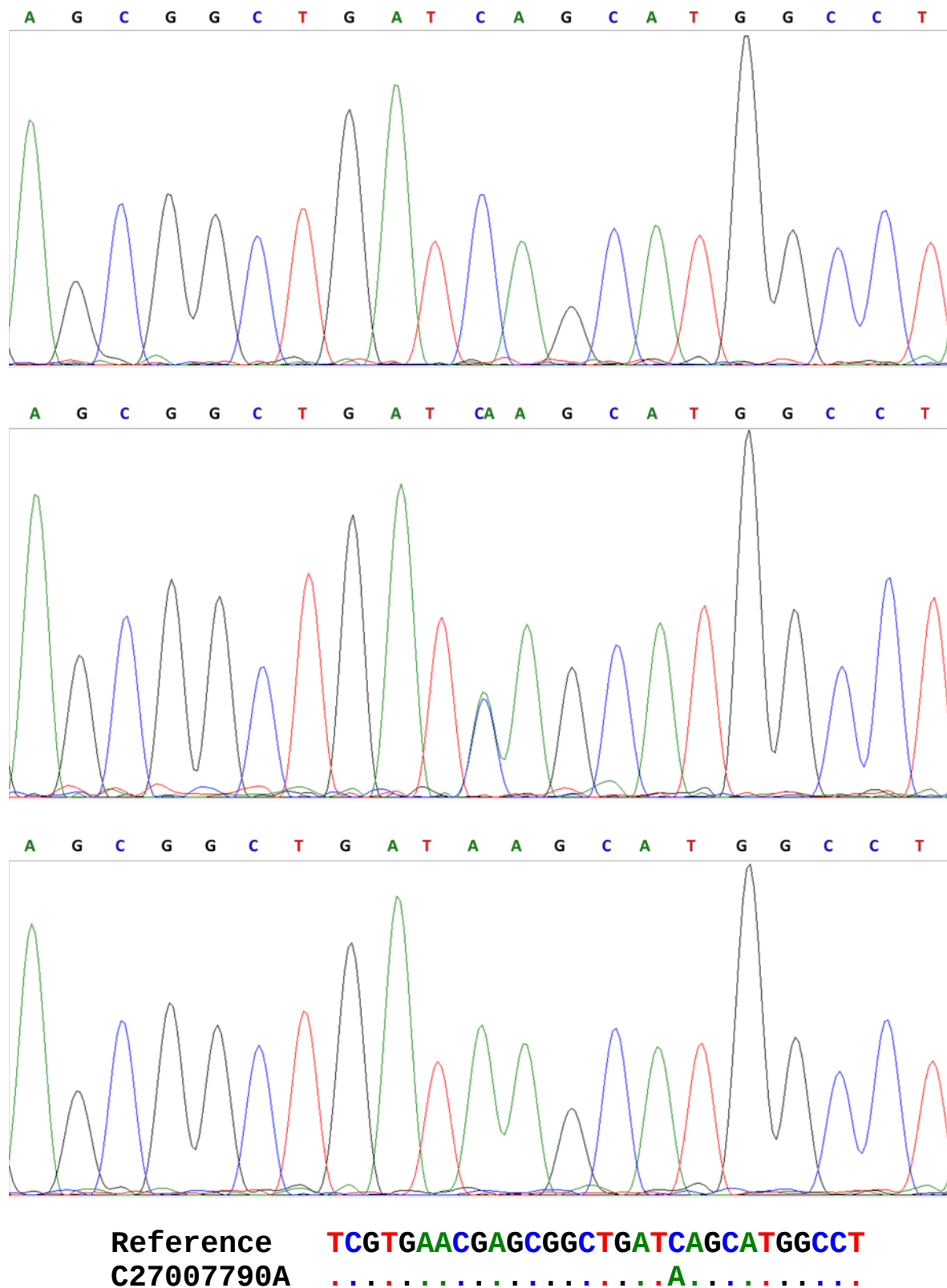
Reference TTTTAAAAAAGACGGGGGACTTGTAAATAT
 C27008016T T.....

4.15 Chromatogram showing G27008015A SNP in Karan Fries cows



Reference TTTTAAAAAAGACGGGGGACTTGTAAATAT
G27008015A A.....

Figure 4.16 Chromatograph showing C27007790A SNP in Karan Fries cows



4.17 and 4.19). The SNPs of ATP1A1 gene were identified and shown in respective chromatograms of Sahiwal (Figure 4.2, 4.3 and 4.5-4.8), Karan Fries (Figure 4.10, 4.11 and 4.13-4.16) and Jersey crossbred cattle (Figure 4.18 and 4.20-4.23). The positions of SNPs on ATP1A1 gene screened in primer fragment - 1 and primer fragment - 2 sequences in the present study are shown in Table 4.1 and 4.3, respectively. Details of SNPs and Genotypes for primer fragment – 1 and primer fragment - 2 in Sahiwal, Karan Fries and Jersey crossbred cattle are given in Table 4.2 and Table 4.4, respectively.

4.6 Allele and Genotype frequencies of ATP1A1 gene

The allele and genotype frequencies of ATP1A1 gene were calculated using POPGENE software package (Yeh *et al.*, 1999). Nucleotide changes at loci A27008442G, A27008436C and G27007963A were found to be monomorphic for genotype AA, AA and GG, respectively in all three breeds of dairy cattle (table 4.5 and 4.6). Remaining six SNPs were found to be polymorphic in Sahiwal and Karan Fries cows whereas, SNP at locus T27008243C was monomorphic for allele T with genotype TT for Jersey crossbred cows. SNP at locus T27008243C, it was observed that allelic frequency of allele 'C' was highest (0.61) in Sahiwal and frequency of allele 'T' was highest (0.81) in Karan Fries cows. Similar pattern was observed at SNP locus A27008223G where allelic frequency of allele 'G' was highest (0.88) in Sahiwal and Jersey crossbred (0.51) but frequency of allele 'A' was highest (0.78) for Karan Fries cows. At SNP loci T27008097A, C27008016T, G27008015A and C27007790A, it was found that allelic frequency of allele 'T', 'C', 'G' and 'C' were highest compared to their respective mutated alleles in the studied animals.

All possible three genotypes (homozygous and heterozygous) were present in Sahiwal cows at each SNP locus. But the availability of all possible three genotypes (homozygous and heterozygous) was variable in Karan Fries and Jersey crossbred cows (table 4.6).

4.7 Association analysis of SNPs with rectal temperature (RR), respiration rate (RT) and heat tolerance coefficient (HTC)

Identification of SNPs that are associated with variation in animal sensitivity to thermal stress will permit screening of animals having presence or absence of desirable or undesirable alleles (Collier *et al.*, 2008). Rectal temperature and

respiration rate are the most sensitive indices of heat tolerance among the physiological reactions studied (Verma *et al.*, 2000). Relationship of each SNP locus with rectal temperature, respiration rate and heat tolerance coefficient in Sahiwal, Karan fries and Jersey crossbred cows was analyzed using GLM procedure of SAS. For association study RR, RT and HTC were taken as dependent variables. Relationship of each dependent variable with polymorphic variants of ATP1A1 gene was analyzed separately. Parity, THI and each polymorphic SNP locus were taken into consideration as fixed effects for Sahiwal and Karan fries cows, however, for Jersey crossbred cows only THI and each polymorphic SNP locus were used as fixed effects because data on parity of Jersey crossbred cows was not available.

Table 4.5 Allelic frequency at each SNP locus of ATP1A1 gene in Sahiwal, Karan Fries and Jersey crossbred cows

SNP	Allele	Allelic frequency		
		Sahiwal	KF	Jersey crossbred
A27008442G	A	1.00	1.00	1.00
	G	-	-	-
A27008436C	A	1.00	1.00	1.00
	C	-	-	-
T27008243C	T	0.39	0.81	1.00
	C	0.61	0.19	- -
A27008223G	A	0.12	0.78	0.49
	G	0.88	0.22	0.51
T27008097A	T	0.70	0.97	0.99
	A	0.30	0.03	0.01
C27008016T	C	0.55	0.62	0.60
	T	0.45	0.38	0.40
G27008015A	G	0.62	0.85	0.97
	A	0.38	0.15	0.03
G27007963A	G	1.00	1.00	1.00
	A	-	-	-
C27007790A	C	0.88	0.64	0.67
	A	0.12	0.36	0.33

(Shaded rows indicate monomorphic nature in all the breeds)

Figure 4.17 Multiple Sequence Alignment of PCR product of ATP1A1 Gene obtained by Primer 1 in Jersey crossbred cows

```

BTA      -----AATGACTCCCCGGCTTTGAAGAAGGCAGACATTGGGGTTGCCATGGGGATTGCAG 55
J_R1F    -----TGGGGGTGC-ATGGGGATTGCAG 22
J_R1Rc    TTTTAATGACTTCCCCGGCTTTGAAGAAGGCAGACATTGGGGTTGCCATGGGGATTGCAG 60
          *****

BTA      GCTCAGACGTGTCTAAGCAAGCTGCTGACATGATTCTCCTAGATGACAACTTTGCCTCAA 115
J_R1F    GCTCAGACGTGTCTAAGCAAGCTGCTGACATGATTCTCCTAGATGACAACTTTGCCTCAA 82
J_R1Rc    GCTCAGACGTGTCTAAGCAAGCTGCTGACATGATTCTCCTAGATGACAACTTTGCCTCAA 120
          *****

BTA      TTGTGACTGGAGTAGAGGAAGGTGAGAGTGCTGCGTGAAACCTGGTCCAGATGCTGCTGA 175
J_R1F    TTGTGACTGGAGTAGAGGAAGGTGAGAGTGCTGCGTGAAACCTGGTCCAGATGCTGCTGA 142
J_R1Rc    TTGTGACTGGAGTAGAGGAAGGTGAGAGTGCTGCGTGAAACCTGGTCCAGATGCTGCTGA 180
          *****

BTA      GCAGTATCCATTTCTGCACGTCATTTCCGGTCCTTTTCATTAAGCTCTACATTA AAAACA 235
J_R1F    GCAGTATCCATTTCTGCACGTCATTTCCGGTCCTTTTCATTAAGCTCTACATTA AAAACA 202
J_R1Rc    GCAGTATCCATTTCTGCACGTCATTTCCGGTCCTTTTCATTAAGCTCTACATTA AAAACA 240
          *****

BTA      ATCTTGCTTTCCAGGTCGTCTGATCTTTGATAACTTGAAGAAATCCATTGCTTATACCC 295
J_R1F    ATCTTGCTTTCCAGGTCGTCTGATCTTTGATAACTTGAAGAAATCCATTGCTTATACCC 262
J_R1Rc    ATCTTGCTTTCCAGGTCGTCTGATCTTTGATAACTTGAAGAAATCCATTGCTTATACCC 300
          *****

BTA      TCACCAGTAACATTCTGAGATTACCCCTTCCTGATATTTATTATTGCAAACATTCCAC 355
J_R1F    TCACCAGTAACATTCTGAGATTACCCCTTCCTGATATTTATTATTGCAAACATTCCAC 322
J_R1Rc    TCACCAGTAACATTCTGAGATTACCCCTTCCTGATATTTATTATTGCAAACATTCCAC 360
          *****

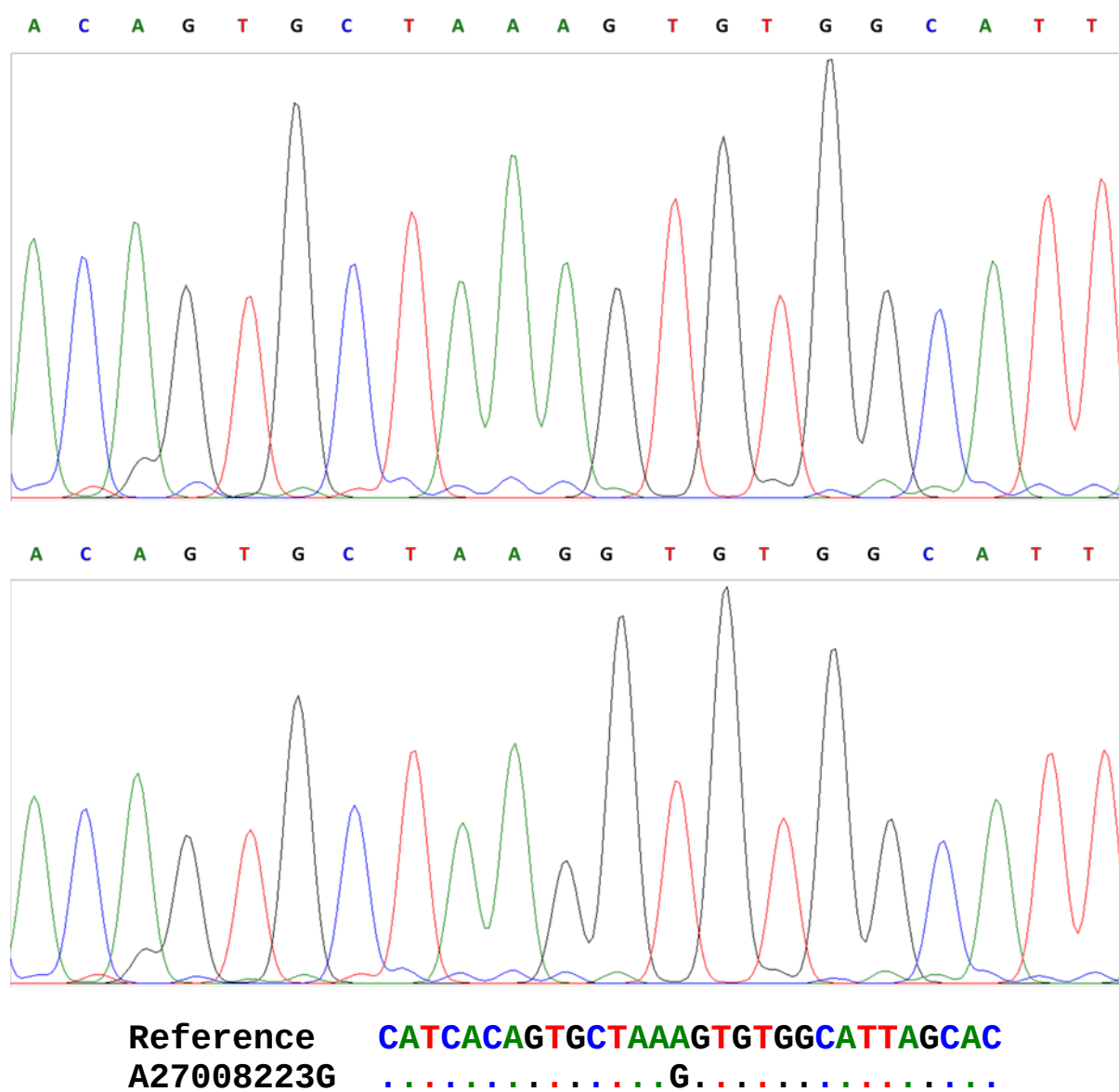
BTA      TCCCTCTGGGGACCGTCACCATCCTCTGCATCGACTTGGGAACGGACATGGTAAGTGACT 415
J_R1F    TCCCTCTGGGGACCGTCACCATCCTCTGCATCGACTTGGGAACGGACATGGTAAGTGACT 382
J_R1Rc    TCCCTCTGGGGACCGTCACCATCCTCTGCATCGACTTGGGAACGGACATGGTAAGTGACT 420
          *****

BTA      GGCACACATTCCACACATCACAGTGCTAAAGGTGTGGCATTAGCACTCACTACTGTGTTCT 475
J_R1F    GGCACACACTCCACACATCACAGTGCTAAAGGTGTGGCATTAGCACTCACTACTGTGTTCT 442
J_R1Rc    GGCACACACTCCACACATCACAGTGCTAAAGGTGTG-----CATTGCT---CCT 465
          *****

BTA      CTGGGTCCCCGTGGCC--- 491
J_R1F    CTGGGTCAACCGTGGCCAAA 461
J_R1Rc    TT----- 467
          *

```

Figure 4.18 Chromatograph showing A27008223G SNP in Jersey crossbred cows



BTA	-----GGTCCCCGCTGGCCAGCATTTTGGAAATATCACTACCGAGAATTTCTTGGCCTGTGTG	56
J_R2F	-----AACGAAA---TCTTGGCCTGTGTG	21
J_R2Rc	TTTTGGTCCCGGCTGCCAGCATTTGGAATATCACTACCGAGAATTTCTTGGCCTGTGTG	60
	* * * * *	
BTA	TGGCAGCCTCAGCATCAAAGATTCAGTGTTAATCATTTCTTAAGACAGGTGTCAATACCT	116
J_R2F	TGGCAGCCTCAGCATCAAAGATTCAGTGTTAATCATTTCTTAAGACAGGTGTCAATACCT	81
J_R2Rc	TGGCAGCCTCAGCATCAAAGATTCAGTGTTAATCATTTCTTAAGACAGGTGTCAATACCT	120

BTA	ATTTATCCGATAGAAATCTTCCCTTTTGTATCATTTTGCCTTTTTTTAAAAAAGACGG	176
J_R2F	ATTTATCCGATAGAAATCTTCCCTTTTGTATCATTTTGCCTTTTTTTAAAAAAGATAG	141
J_R2Rc	ATTTATCCGATAGAAATCTTCCCTTTTGTATCATTTTGCCTTTTTTTAAAAAAGATAG	180

BTA	GGGACTTGTAATATCTCATTATGGAAAACAGTTTTTTTAAAGGGAGAGCAAGAATTTGAGA	236
J_R2F	GGGACTTGTAATATCTCATTATGGAAAACAGTTTTTTTAAAGGGAGAGCAAGAATTTGAGA	201
J_R2Rc	GGGACTTGTAATATCTCATTATGGAAAACAGTTTTTTTAAAGGGAGAGCAAGAATTTGAGA	240

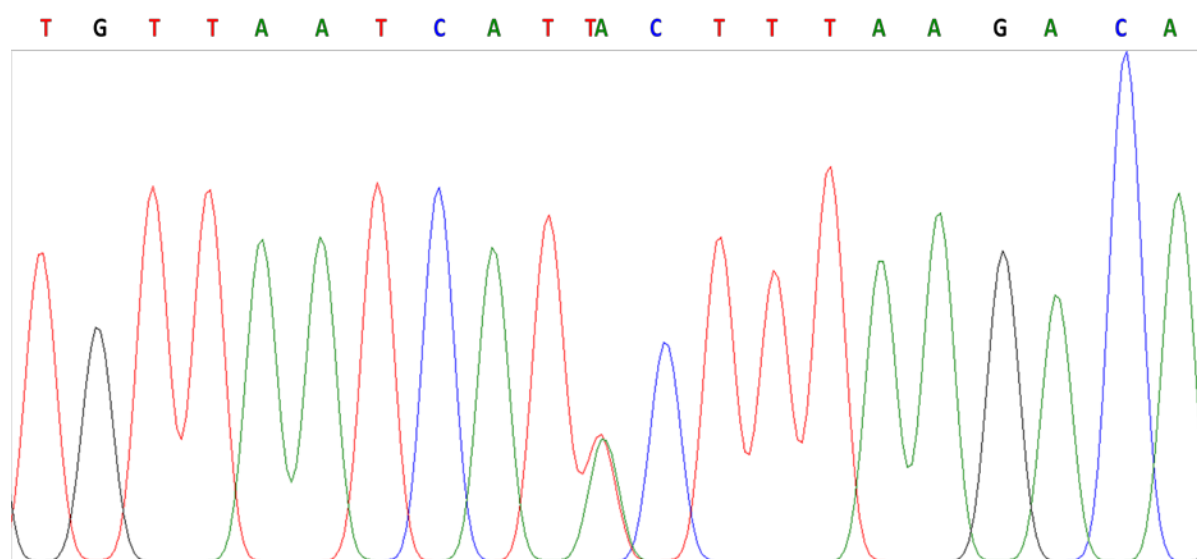
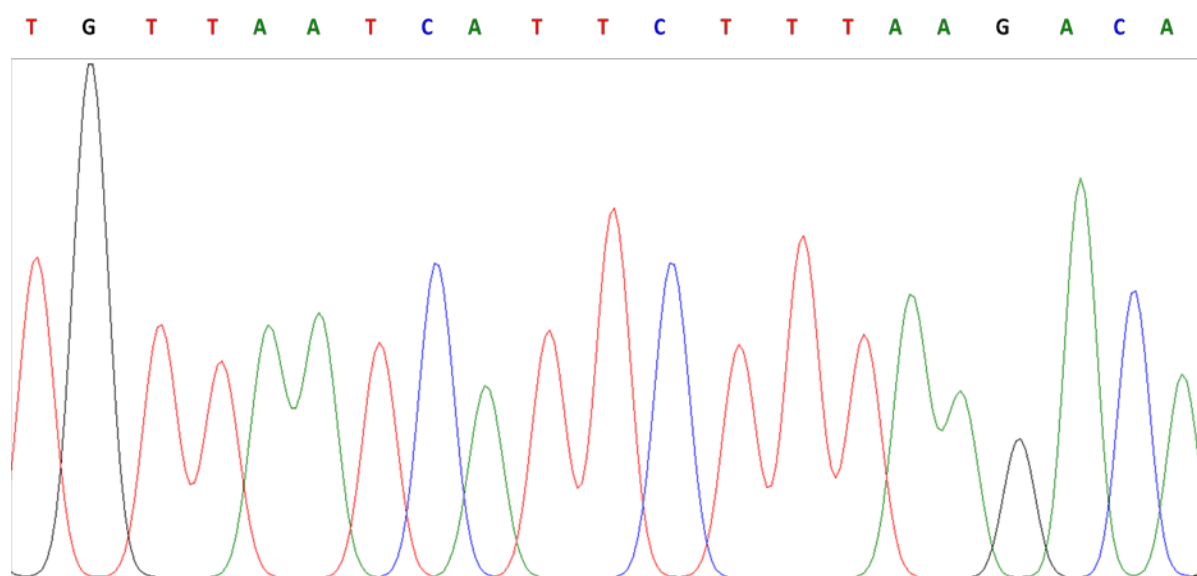
BTA	ACAAACAAAAGGGTCACAACATGAGCTTGCCAGTTTCTGATGACTTCTCGCCCCCTCCTTC	296
J_R2F	ACAAACAAAAGGGTCACAACATGAGCTTGCCAGTTTCTGATGACTTCTCGCCCCCTCCTTC	261
J_R2Rc	ACAAACAAAAGGGTCACAACATGAGCTTGCCAGTTTCTGATGACTTCTCGCCCCCTCCTTC	300

BTA	AGGTCCCTGCTATCTCCTTGGCTTATGAGCAGGCTGAGAGCGACATCATGAAGAGGCAGC	356
J_R2F	AGGTCCCTGCTATCTCCTTGGCTTATGAGCAGGCTGAGAGCGACATCATGAAGAGGCAGC	321
J_R2Rc	AGGTCCCTGCTATCTCCTTGGCTTATGAGCAGGCTGAGAGCGACATCATGAAGAGGCAGC	360

BTA	CCAGAAACCCCGAGACGGACAAACTCGTGAACGAGCGGCTGATCAGCATGGCCTATGGAC	416
J_R2F	CCAGAAACCCCGAGACGGACAAACTCGTGAACGAGCGGCTGATAAGCATGGCCTATGGAC	381
J_R2Rc	CCAGAAACCCCGAGACGGACAAACTCGTGAACGAGCGGCTGATAAGCATGGCCTATG-AC	419

BTA	AGATTGGTGAGCCAGCAGATTTCAAGTATCTGTAGGGGTGCTGTCTGCC--	465
J_R2F	AGATTGGTGAGCCAGCAGATTTCAAGTATCTGTAGGGGTGNTGTCTGCCAA	433
J_R2Rc	AGAT--GGAGCCAGC--ATTCC-----	437

Figure 4.20 Chromatograph showing T27008097A SNP in Jersey crossbred cows



Reference **AGTGTTAATCATTCTTTAAGACAGGTGTCA**
T27008097A **.....A.....**

Figure 4.21 Chromatograph showing C27008016T SNP in Jersey crossbred cows

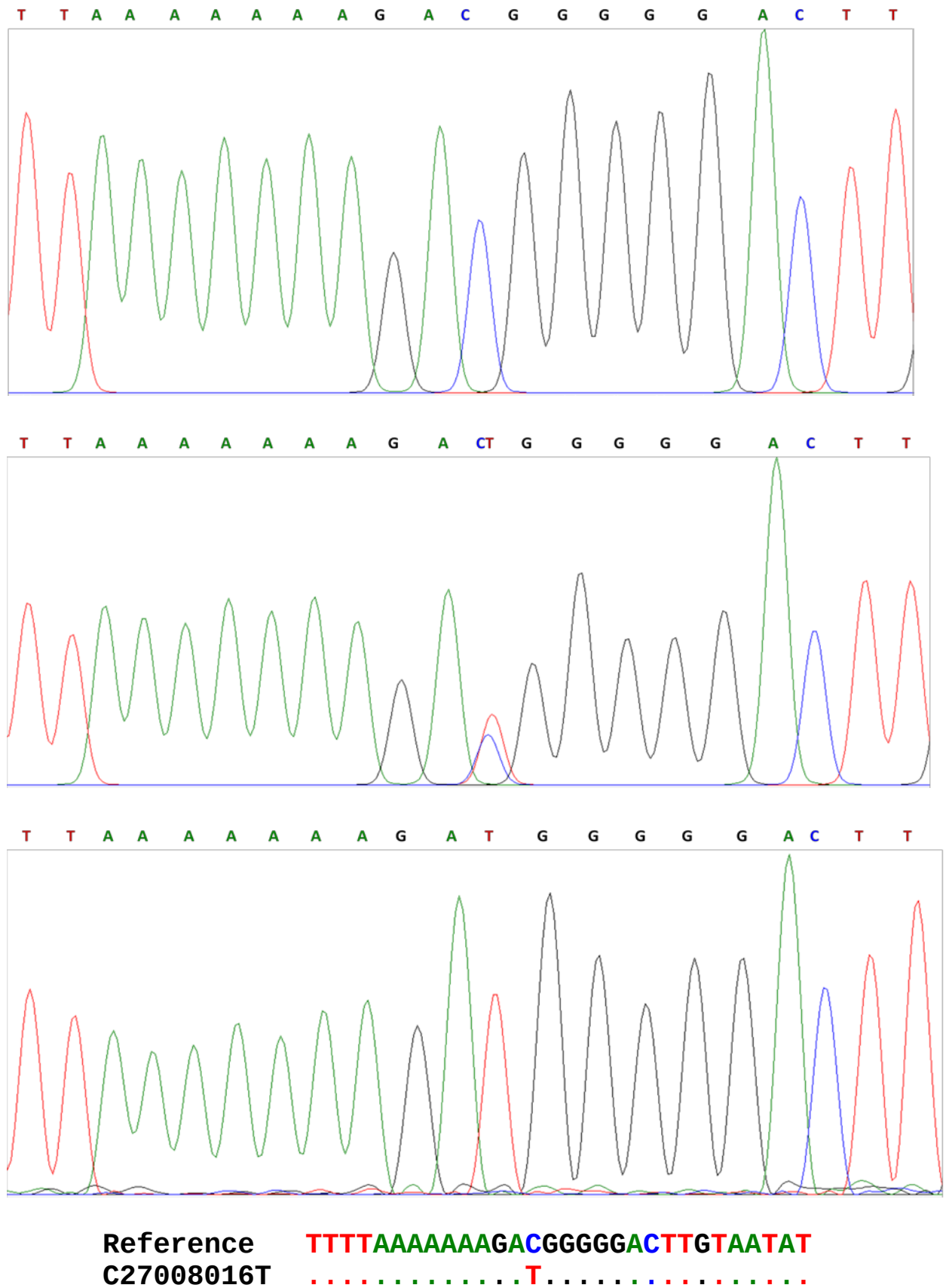


Figure 4.22 Chromatograph showing G27008015A SNP in Jersey crossbred cows

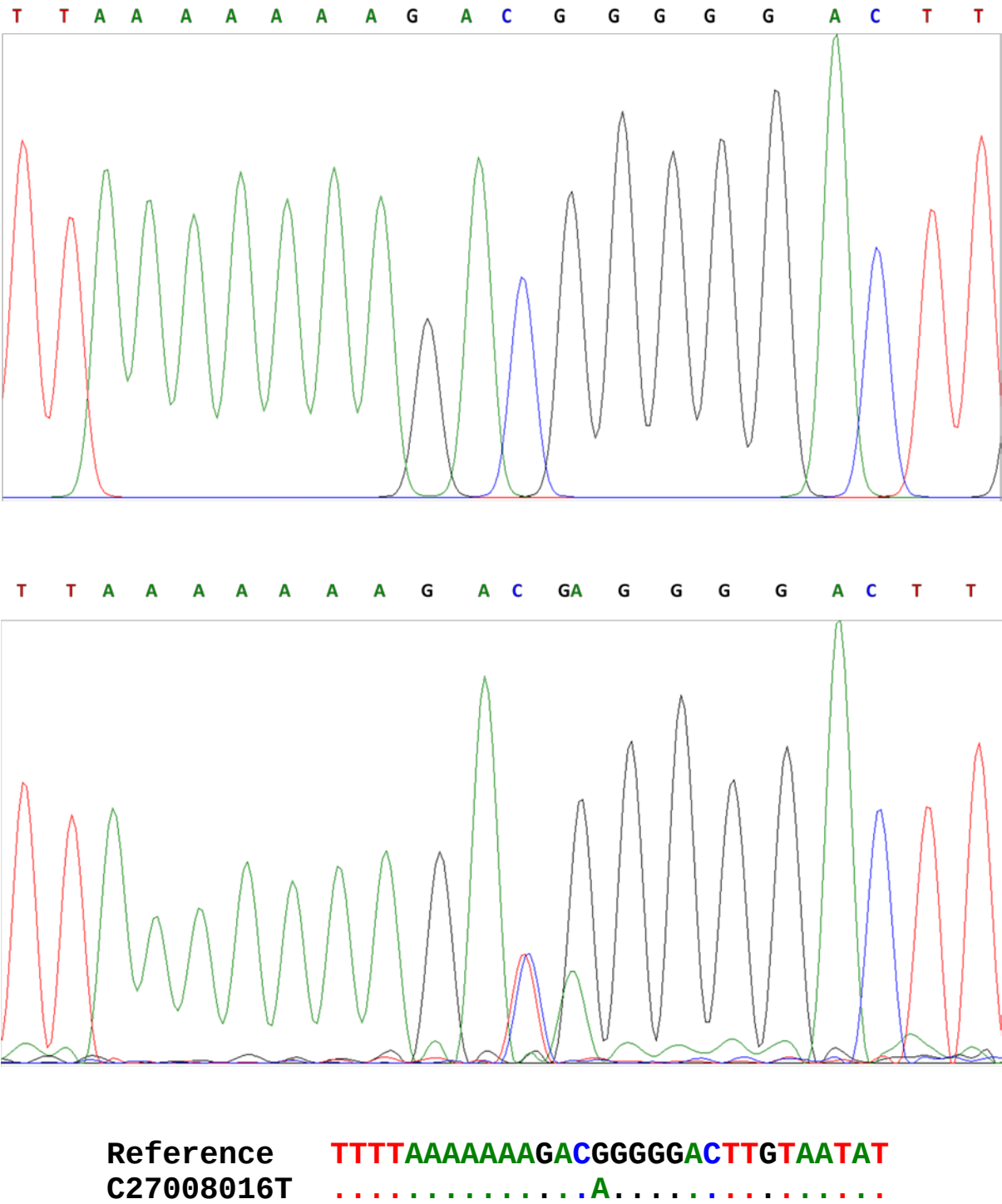
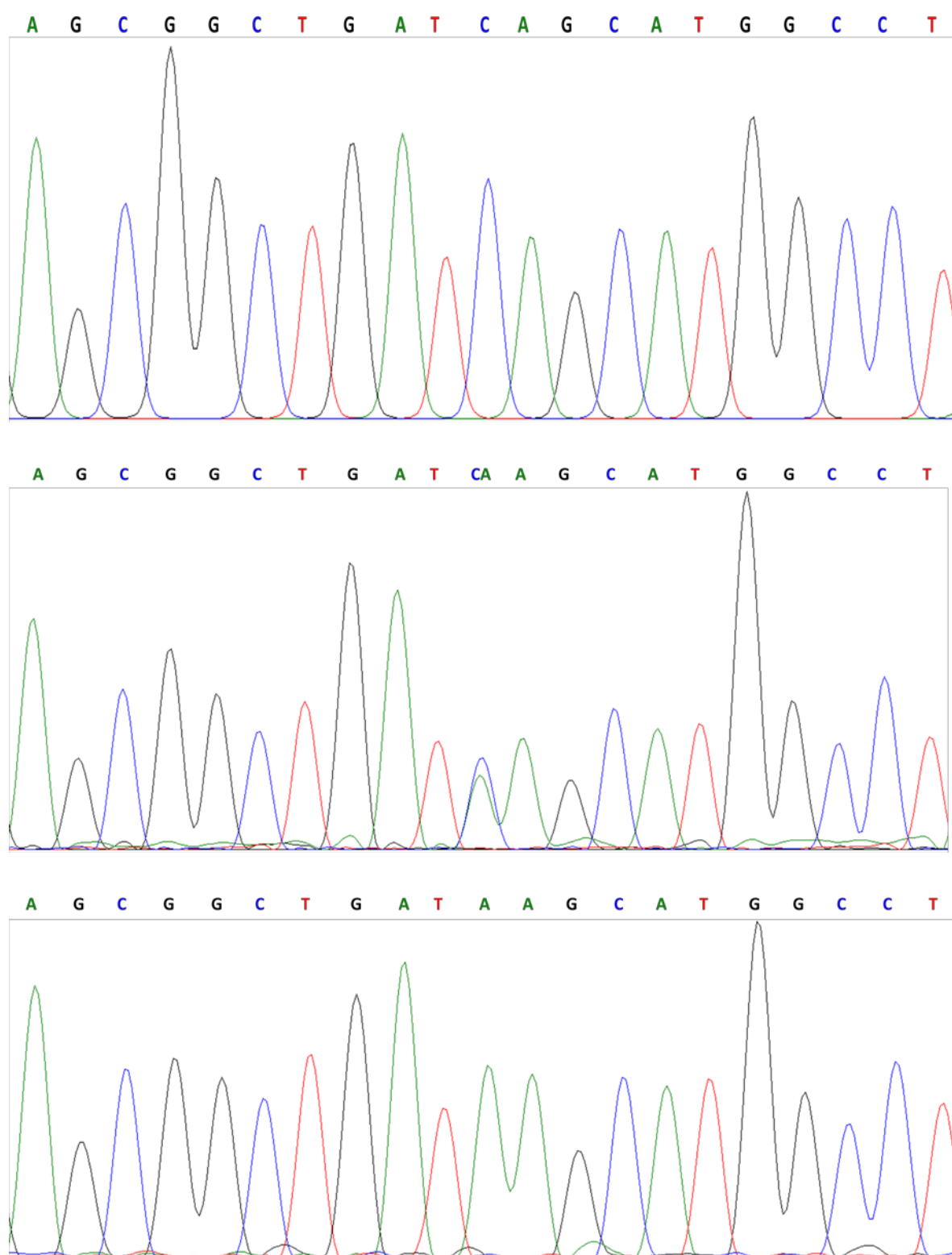


Figure 4.23 Chromatograph showing C27007790A SNP in Jersey crossbred cows



Reference **TCGTGAACGAGCGGCTGATCAGCATGGCCT**
C27007790A A.....

Table 4.1 Sequence of primer fragment - 1 of ATP1A1 gene and SNPs screened in Sahiwal, Karan Fries and Jersey crossbred cattle

27008667	AATGACTCCC	CGGCTTTGAA	GAAGGCAGAC	ATTGGGGTTG	CCATGGGGAT
27008617	TGCAGGCTCA	GACGTGTCTA	AGCAAGCTGC	TGACATGATT	CTCCTAGATG
27008567	ACAACTTTGC	CTCAATTGTG	ACTGGAGTAG	AGGAAGGTGA	GAGTGCTGCG
27008517	TGAAACCTGG	TCCAGATGCT	GCTGAGCAGT	ATCCATTCT	GCACGTCATT
27008467	TCCGGTCCTT	TTCATTAAGC	TCTACATTAA	AAACAATCTT	GCTTTCCAG
27008417	GTCGTCTGAT	CTTTGATAAC	TTGAAGAAAT	CCATTGCTTA	TACCCTCACC
27008367	AGTAACATTC	CTGAGATTAC	CCCCTTCCTG	ATATTTATTA	TTGCAAACAT
27008317	TCCACTCCCT	CTGGGGACCG	TCACCATCCT	CTGCATCGAC	TTGGGAACGG
27008267	ACATGTAAG	TGACTGGCAC	ACATCTCCACA	CATCACAGTG	CTAAAGGTGTG
27008217	GCATTAGCAC	TCACTACTGT	GTTCTCTGGG	TCCCCGTGGCC	



Reference SNPs observed in present study (1)



Reference SNPs not observed in present study (2)



SNPs observed in present study but not in reference (1)

Table 4.2 SNPs and Genotypes for primer fragment – 1 in Sahiwal, Karan Fries and Jersey crossbred cattle

Primer Fragment-1 (Size; Position)		491bp; (27008667.. 27008177)			
SNP position on gene		A27008442G	A27008436C	T27008243C	A27008223G
Region of gene		Intron 16	Intron 16	Intron 17	Intron 17
Cattle Breed	Sahiwal (51)	AA=51	AA=51	TT= 13 TC=14 CC=24	AA=04 AG=04 GG=43
	Karan Fries (50)	AA=50	AA=50	TT=31 TC=19	AA=39 GG=11
	Jersey-cross (35)	AA=35	AA=35	TT=35	AA=17 GG=18

Table 4.3 Sequence of primer fragment - 2 of ATP1A1 gene and SNPs screened in Sahiwal, Karan Fries and Jersey crossbred cattle

27008189	GGTCCCCGTG	GCCAGCATTT	GGAAATATCA	CTACCGAGAA	TTTCTTGGCC
27008139	TGTGTGTGGC	AGCCTCAGCA	TCAAAGATTC	AGTGTTAATC	ATT/ACTTTAAG
27008089	ACAGGTGTCA	ATACCTATTT	ATCCGATAGA	AATCTTCCCT	TTTGTATCAT
27008039	TTTGCCCTTT	TTTTAAAAA	AGAC/TG/AGGGGA	CTTGTAATAT	CTCATTATGG
27007989	AAAACAGTTT	TTTTAAGGGA	GAGCAAGAAAT	TTGAGAACAA	ACAAAAGGGT
27007939	CACAACATGA	GCTTGCCAGT	TTCTGATGAC	TTCTCGCCCC	TCCTTCAGGT
27007889	CCCTGCTATC	TCCTTGGCTT	ATGAGCAGGC	TGAGAGCGAC	ATCATGAAGA
27007839	GGCAGCCCAG	AAACCCCAG	ACGGACAAAC	TCGTGAACGA	GCGGCTGATC/A
27007789	AGCATGGCCT	ATGGACAGAT	TGGTGAGCCA	GCAGATTTC	AGTATCTGTA
27007739	GGGGTGCTGT	CTGCC			



Reference SNPs observed in present study (2)



Reference SNPs not observed in present study (1)



SNPs observed in present study but not in reference (2)

Table 4.4 SNPs and Genotypes for primer fragment – 2 in Sahiwal, Karan Fries and Jersey crossbred cattle

Primer Fragment -2 (Size; Position)		465 bp; (27008189 .. 27007725)				
SNP position on gene		T27008097A	C27008016T	G27008015A	G27007963A	C27007790A
Region of gene		Intron 17	Intron 17	Intron 17	Intron 17	Exon 18
Cattle Breed	Sahiwal (51)	TT=27 TA=17 AA=07	CC=17 CT=22 TT=12	GG=19 GA=25 AA=07	GG=51	CC=41 CA=08 AA=02
	Karan Fries (50)	TT=47 TA=03	CC=19 CT=24 TT=07	GG=36 GA=13 AA=01	GG=50	CC=22 CA=20 AA=08
	Jersey-cross (35)	TT=34 TA=01	CC=13 CT=16 TT=06	GG=33 GA=02	GG=35	CC=17 CA=13 AA=05

Table 4.6 Genotypic frequency at each SNP locus of ATP1A1 gene in Sahiwal, Karan Fries and Jersey crossbred cows

SNP	Genotype	Breed		
		Sahiwal	Karan Fries	Jersey crossbred
A27008442G	AA	1.00	1.00	1.00
	AG	-		
	GG			
A27008436C	AA	1.00	1.00	1.00
	AC	-		
	CC			
T27008243C	TT	0.26	0.62	1.00
	TC	0.27	0.38	-
	CC	0.47	-	-
A27008223G	AA	0.08	0.78	0.49
	AG	0.08	-	-
	GG	0.84	0.22	0.51
T27008097A	TT	0.53	0.94	0.97
	TA	0.3	0.06	0.03
	AA	0.14	-	-
C27008016T	CC	0.33	0.38	0.37
	CT	0.43	0.48	0.46
	TT	0.24	0.14	0.17
G27005015A	GG	0.37	0.72	0.94
	GA	0.49	0.26	0.06
	AA	0.14	0.02	-
G27007963A	GG	1.00	1.00	1.00
	GA	-		
	AA			
C27007790A	CC	0.80	0.44	0.49
	CA	0.16	0.4	0.37
	AA	0.04	0.16	0.14

(Shaded rows indicate monomorphic nature in all the breeds)

4.7.1 Association analysis of SNPs with rectal temperature, respiration rate and heat tolerance coefficient in Sahiwal cows

Analysis of variance (ANOVA) of linear model for dependent variable RR, RT and HTC in Sahiwal cows were found to be highly significant at 1% level of significance. Coefficient of determination (R^2 value) for model of RR, RT and HTC were 65.58%, 66.77% and 76.68%, respectively (Table 4.7). Coefficient of variance (CV %) of RR, RT and HTC were 23.32, 1.25 and 9.25, respectively (Table 4.7). Further, ANOVA revealed that effect of THI was highly significant on RR, RT and HTC (Table 4.8). Effect of SNP T27008243C was significant on RR and that of SNP T27008097A was highly significant on HTC, whereas, effect of remaining all SNP was not significant on RR and HTC (Table 4.8). Parity was not significant for RR, RT and HTC. RT was not significantly affected by any SNP locus in Sahiwal cows.

In Sahiwal cows overall means of RR per minute, RT in °C and HTC were found to be 20.02 ± 0.60 , 38.08 ± 0.06 and 1.90 ± 0.03 , respectively (Table 4.9). RR, RT and HTC differed significantly in all three THI subclasses (Table 4.9). Respiration per minute during winter (THI = 49.70), spring (THI = 64.65) and summer (THI = 86.44) were 12.78 ± 1.64^c , 15.09 ± 1.64^b and 25.55 ± 1.64^a , respectively. Rectal temperature in °C during winter (THI = 49.70), spring (THI = 64.65) and summer (THI = 86.44) were 37.30 ± 0.17^c , 38.17 ± 0.17^b and 38.79 ± 0.17^a , respectively. This observed pattern in RR and RT revealed that with increase in THI there was increase in both RR and RT in Sahiwal cows.

An increased RR is an important thermoregulatory response to heat stress. It aids in heat dissipation via evaporative cooling (Hammond *et al.*, 1996; Beatty *et al.*, 2006). Thus, a low RR may indicate an improved thermotolerance (Charoensook *et al.*, 2012).

RR at locus T27008243C for genotype CC was highest (19.24 ± 1.52^a) compared to TT (15.91 ± 1.89^b) and TC (18.25 ± 1.77^{ab}) while HTC at locus T27008097A for genotype TT was highest (1.95 ± 0.05^a) compared to TA (1.79 ± 0.06^b) and AA (1.65 ± 0.11^c). Benezra (1954) suggested that lower the value of HTC, higher the degree of adaptability for thermal stress. Thus for Sahiwal cows it could be inferred that cows with TT genotype at T27008243C locus recorded least number of respirations per minute and cows of genotype AA at T27008097A locus with least HTC are better thermotolerant compared to remaining cows.

Table 4.7 ANOVA of linear model for dependent variable RR, RT and HTC in Sahiwal cows

	RR		RT		HTC	
Source of Variance	Sum of Squares	Mean Square	Sum of Squares	Mean Square	Sum of Squares	Mean Square
Model	5440.0712	259.0510** (21)	59.9905	2.8567** (21)	13.3404	0.6353** (21)
Error	2854.8700	21.7929 (131)	29.8439	0.2278 (131)	4.0568	0.03097 (131)
Corrected Total	8294.9412		89.8345		17.39707165	
R²(%)	65.58		66.77		76.68	
CV(%)	23.32		1.25		9.25	

Figures in parenthesis are degree of freedom; ** significant at 1% level of significance

Table 4.8 ANOVA of fixed effects for dependent variable RR, RT and HTC in Sahiwal cows

Source of Variance	DF	RR		RT		HTC	
		Sum of Squares	Mean Square	Sum of Squares	Mean Square	Sum of Squares	Mean Square
Seasons (THI)	2	4723.1765	2361.5882**	57.5743	28.7872**	12.5303	6.2652**
Parity	7	277.7267	39.6752	0.5357	0.0765	0.2879	0.0411
T27008243C	2	158.3804	79.1902*	0.5074	0.2537	0.1432	0.0716
A27008223G	2	11.1928	5.5964	0.1922	0.0961	0.0428	0.0214
T27008097A	2	86.0045	43.0022	0.2875	0.1438	0.3526	0.1763**
C27008016T	2	27.9848	13.9924	0.1707	0.0853	0.1694	0.0847
G27008015A	2	44.7435	22.3717	0.7354	0.3677	0.0444	0.0222
C27007790A	2	14.5588	7.2794	0.2377	0.1188	0.0834	0.0417

* Significant at 5% level of significance; ** Significant at 1% level of significance

Table 4.9 Least squares means of subclasses of different fixed effects for RR, RT and HTC in Sahiwal cows

Effect	Subclass	RR	RT	HTC
Overall Mean		20.02±0.60	38.08±0.06	1.90±0.03
Seasons (THI)	Winter (THI=49.70)	12.78±1.64 ^c	37.30±0.17 ^c	1.53±0.06 ^c
	Spring (THI=64.65)	15.09±1.64 ^b	38.17±0.17 ^b	1.64±0.06 ^b
	Summer (THI=86.44)	25.55±1.64 ^a	38.79±0.17 ^a	2.20±0.06 ^a
Parity	1 (01)	25.70±3.32	38.23±0.34	2.10±0.13
	2 (09)	17.15±2.22	38.00±0.23	1.71±0.08
	3 (10)	18.13±1.91	38.12±0.20	1.73±0.07
	4 (07)	17.86±1.97	38.08±0.20	1.71±0.07
	5 (11)	18.75±1.82	38.19±0.19	1.74±0.07
	6 (05)	17.53±2.06	38.05±0.21	1.81±0.08
	7 (07)	16.81±1.80	37.97±0.18	1.79±0.07
	9 (01)	10.50±3.35	38.05±0.34	1.79±0.13
T27008243C	TT (13)	15.91±1.89 ^b	38.08±0.19	1.74±0.07
	TC (14)	18.26±1.77 ^{ab}	37.99±0.18	1.82±0.07
	CC (24)	19.24±1.52 ^a	38.18±0.16	1.84±0.06
A27008223G	AA (04)	16.90±2.01	38.08±0.21	1.74±0.08
	AG (04)	18.29±2.23	38.16±0.23	1.83±0.08
	GG (43)	18.22±1.51	38.01±0.16	1.82±0.06
T27008097A	TT (27)	20.23±1.27	38.06±0.13	1.95±0.05 ^a
	TA (17)	17.86±1.58	38.18±0.16	1.79±0.06 ^b
	AA (07)	15.32±2.83	38.02±0.29	1.66±0.11 ^c
C27008016T	CC (17)	16.62±2.42	37.98±0.25	1.69±0.09
	CT (22)	18.58±1.79	38.09±0.18	1.81±0.07
	TT (12)	18.22±1.52	38.19±0.16	1.89±0.06
G27008015A	GG (19)	18.64±1.55	37.92±0.16	1.84±0.06
	GA (25)	16.68±1.63	38.19±0.17	1.80±0.06
	AA (07)	18.10±2.19	38.15±0.22	1.76±0.08
C27007790A	CC (41)	18.73±0.91	38.16±0.09	1.88±0.03
	CA (08)	18.36±1.91	37.98±0.20	1.80±0.07
	AA (02)	16.32±3.06	38.12±0.31	1.71±0.12

Figures in parenthesis are number of animals;
Figures with dissimilar superscript differ significantly

4.7.2 Association analysis of SNPs with rectal temperature, respiration rate and heat tolerance coefficient in Karan Fries cows

ANOVA of linear model for dependent variable RR, RT and HTC in Karan Fries cows were found to be highly significant at 1% level of significance. R^2 value for model of RR, RT and HTC were 86.28%, 64.52% and 75.87%, respectively (Table 4.10). CV (%) of RR, RT and HTC were 20.00, 1.47 and 9.08, respectively (Table 4.10). Further ANOVA revealed that effect of THI was highly significant on RR, RT and HTC (Table 4.11). Parity was found to be highly significant on RR whereas not significant on RT and HTC. Effect of SNP locus A27008223G was significant on RR and that of SNP locus T27008097A was highly significant on RT, whereas, effect of remaining all SNP loci was not significant on RR, RT and HTC (Table 4.11).

Overall means of respiration per minute, rectal temperature in °C and HTC were found to be 29.13 ± 1.21 , 38.21 ± 0.07 and 1.90 ± 0.03 in Karan Fries cows (Table 4.12). RR, RT and HTC differed significantly in all three THI subclasses (Table 4.12). Respiration per minute during winter (THI = 49.70), spring (THI = 64.65) and summer (THI = 86.44) were 17.70 ± 2.23^c , 24.90 ± 2.23^b and 49.22 ± 2.23^a , respectively. Rectal temperature in °C during winter (THI = 49.70), spring (THI = 64.65) and summer (THI = 86.44) were 37.07 ± 0.22^c , 37.98 ± 0.22^b and 38.77 ± 0.22^a , respectively. This observed pattern in RR and RT revealed that with increase in THI there was increase in both RR and RT in Karan Fries cows.

According to Srikanthakumar and Johnson (2004) higher RR suggests greater metabolic heat production. Therefore, in Karan Fries population studied it could be inferred that cows of genotype AA at SNP locus A27008223G with least number of respirations per minute (28.85 ± 1.96^b) and cows of genotype TA at SNP locus T27008097A with least rectal temperature in °C (37.56 ± 0.32^b) are better thermotolerant compared to remaining cows (Table 4.12).

Table 4.10 ANOVA of linear model for dependent variable RR, RT and HTC in Karan Fries cows

	RR		RT		HTC	
Source of Variance	Sum of Squares	Mean Square	Sum of Squares	Mean Square	Sum of Squares	Mean Square
Model	28386.6980	1774.1686** (16)	76.6343	4.7896** (16)	12.4024	0.7752** (16)
Error	4513.8953	33.9391 (133)	42.1378	0.3168 (133)	3.9447	0.0297 (133)
Corrected Total	32900.5933		118.7722		16.3471	
R ² (%)	86.28		64.52		75.87	
CV(%)	20.00		1.47		9.08	

Figures in parenthesis are degree of freedom; ** significant at 1% level of significance

Table 4.11 ANOVA of fixed effects for dependent variable RR, RT and HTC in Karan Fries cows

Source of Variance	DF	RR		RT		HTC	
		Sum of Squares	Mean Square	Sum of Squares	Mean Square	Sum of Squares	Mean Square
Seasons (THI)	2	27280.2133	13640.1067**	71.9066	35.9533**	11.9160	5.9580**
Parity	5	756.8039	151.3608**	0.7386	0.1477	0.0975	0.0195
T27008243C	1	7.6453	7.6453	0.1369	0.1369	0.0601	0.0601
A27008223G	1	202.2335	202.2335*	0.2150	0.2150	0.0495	0.0495
T27008097A	1	17.2675	17.2675	2.3624	2.3624*	0.0000	0.0000
C27008016T	2	128.6765	64.3382	0.4354	0.2177	0.1263	0.0631
G27008015A	2	25.0859	12.5430	0.2999	0.1500	0.0268	0.0134
C27007790A	2	117.3478	58.6739	0.5294	0.2647	0.0682	0.0341

* Significant at 5% level of significance; ** Significant at 1% level of significance

Table 4.12 Least squares means of subclasses of different fixed effects for RR, RT and HTC in Karan Fries cows

Effect	Subclass	RR	RT	HTC
Overall Mean		29.13±1.21	38.21±0.07	1.90±0.03
Seasons (THI)	Winter (THI=49.70)	17.70±2.23 ^c	37.07±0.22 ^c	1.70±0.07 ^c
	Spring (THI=64.65)	24.90±2.23 ^b	37.98±0.22 ^b	1.82±0.07 ^b
	Summer (THI=86.44)	49.22±2.23 ^a	38.77±0.22 ^a	2.35±0.07 ^a
Parity	2 (07)	33.59±2.14 ^a	37.96±0.21	1.91±0.06
	3 (17)	32.95±2.49 ^a	37.83±0.24	1.92±0.07
	4 (12)	30.27±2.07 ^a	37.85±0.20	1.98±0.06
	5 (08)	32.74±2.48 ^a	37.86±0.24	1.96±0.07
	6 (04)	32.26±2.78 ^a	37.98±0.27	1.99±0.08
	7 (02)	21.82±3.28 ^b	38.17±0.32	1.97±0.10
T27008243C	TT (31)	30.89±2.39	37.90±0.23	1.93±0.07
	TC (19)	30.32±2.03	37.98±0.20	1.98±0.06
A27008223G	AA (39)	28.85±1.96 ^b	37.88±0.19	1.93±0.06
	GG (11)	32.37 ±2.51 ^a	38.00±0.24	1.98±0.07
T27008097A	TT (47)	29.58±1.41	38.32±0.14 ^a	1.96±0.04
	TA (03)	31.63±3.35	37.56±0.32 ^b	1.95±0.10
C27008016T	CC (19)	28.91±2.65	37.78±0.26	2.04±0.08
	CT (24)	26.96±2.33	37.93±0.23	1.94±0.07
	TT (07)	35.95±3.90	38.12±0.38	1.88±0.12
G27008015A	GG (36)	29.79±1.64	37.96±0.16	1.92±0.05
	GA (13)	30.72±1.83	38.05±0.18	1.93±0.054
	AA (01)	31.30±4.26	37.81±0.41	2.02±0.13
C27007790A	CC (22)	31.75±2.48	38.11±0.24	1.89±0.07
	CA (20)	34.28±3.44	37.90±0.33	1.97±0.10
	AA (08)	25.79±3.06	37.81±0.30	2.00±0.09

Figures in parenthesis are number of animals;
Figures with dissimilar superscript differ significantly

4.7.3 Association analysis of SNPs with rectal temperature, respiration rate and heat tolerance coefficient in Jersey crossbred cows

ANOVA of linear model for dependent variable RR and HTC in Jersey crossbred cows were found to be highly significant at 1% level of significance, whereas, RT was found to be significant at 5% level of significance. R^2 value for model of RR, RT and HTC were 39.30%, 17% and 40.48%, respectively. CV (%) of RR, RT and HTC were 12.5, 0.85 and 5.98%, respectively (Table 4.13). Further, ANOVA revealed that effect of THI was highly significant on RR and HTC, whereas, on RT it was found significant at 5% level of significance (Table 4.14).

Overall means of respiration per minute, rectal temperature in °C and HTC were found to be 20.85 ± 0.31 , 38.12 ± 0.03 and 1.90 ± 0.01 in Jersey crossbred cows (Table 4.15). Respiration per minute during winter (THI = 69.92) and spring (THI = 71.15) were 17.97 ± 1.20^b and 18.34 ± 1.20^b , respectively, which were not significantly different but differed from summer (THI = 69.80) RR which was 21.60 ± 1.20^a (Table 4.15). Whereas, Rectal temperature in °C during winter (THI = 69.92), spring (THI = 71.15) and summer (THI = 69.80) were 37.81 ± 0.15^b , 37.97 ± 0.15^{ab} and 38.02 ± 0.15^a , respectively (Table 4.15).

Effect of SNP locus A27008223G was significant on RT but RT for genotype AA (38.01 ± 0.16^a) and GG (37.86 ± 0.14^a) were not significantly different from each other (Table 4.15). Effect of SNP locus C27007790A was highly significant on RR and HTC. Both RR and HTC for genotype CC (19.25 ± 1.16^b and 1.83 ± 0.05^b) and CA (21.76 ± 1.68^a and 1.94 ± 0.07^a) differed from each other. However, RR and HTC of genotype AA (16.91 ± 1.57^{ab} and 1.72 ± 0.07^{ab}) were not differ significantly from both genotype CC and CA. Barani (2013) also reported that C>A transversion in Deoni cattle. However, at the same SNP locus Chinese Holstein cows with CC genotypes showed a higher ability to adapt heat stressed environment (Liu *et al.*, 2011).

Table 4.13 ANOVA of linear model for dependent variable RR, RT and HTC in Jersey crossbred cows

	RR		RT		HTC	
Source of Variance	Sum of Squares	Mean Square	Sum of Squares	Mean Square	Sum of Squares	Mean Square
Model	418.0109	46.4457**(9)	2.0236	0.2248*(9)	0.8352	0.0928**(9)
Error	645.5510	6.7953(95)	9.9989	0.1053(95)	1.2281	0.0129(95)
Corrected Total	1063.5619		12.0225		2.0633	
R ² (%)	39.30		17.00		40.48	
CV(%)	12.50		0.85		5.98	

Figures in parenthesis are degree of freedom; ** significant at 1% level of significance; * Significant at 5% level of significance

Table 4.14 ANOVA of fixed effects for dependent variable RR, RT and HTC in Jersey crossbred cows

Source of Variance	DF	RR		RT		HTC	
		Sum of Squares	Mean Square	Sum of Squares	Mean Square	Sum of Squares	Mean Square
Seasons (THI)	2	278.9905	139.4952**	0.8585	0.4292*	0.5541	0.2770**
A27008223G	1	0.0086	0.0086	0.4490	0.4490*	0.0005	0.0005
T27008097A	1	3.4171	3.4171	0.1670	0.1670	0.0083	0.0083
C27008016T	2	31.5524	15.7762	0.5065	0.2533	0.0663	0.0331
G27008015A	1	22.2700	22.2700	0.0425	0.0425	0.0443	0.0443
C27007790A	2	45.7349	22.8675*	0.1598	0.0799	0.0878	0.0439*

* Significant at 5% level of significance; ** Significant at 1% level of significance

Table 4.15 Least squares means of subclasses of different fixed effects for RR, RT and HTC in Jersey crossbred cows

Effect	Subclass	RR	RT	HTC
Overall Mean		20.85±0.31	38.12±0.03	1.90±0.01
Seasons (THI)	Winter (THI=69.92)	17.97±1.20 ^b	37.81±0.15 ^b	1.77±0.05 ^b
	Spring (THI=71.15)	18.34±1.20 ^b	37.97±0.15 ^{ab}	1.79±0.05 ^b
	Summer (THI=69.80)	21.60±1.20 ^a	38.02±0.15 ^a	1.93±0.05 ^a
A27008223G	AA (17)	19.32±1.25	38.01±0.16 ^a	1.83±0.06
	GG(18)	19.30±1.10	37.86±0.14 ^a	1.83±0.05
T27008097A	TT (34)	19.97±0.62	38.08±0.08	1.86±0.03
	TA (01)	18.64±1.99	37.79±0.25	1.80±0.09
C27008016T	CC (13)	18.28±1.46	37.74±0.18	1.78±0.06
	CT (16)	17.98±1.12	37.94±0.14	1.77±0.05
	TT (06)	21.66±1.73	38.11±0.22	1.94±0.08
G27008015A	GG (33)	20.39±0.98	37.98±0.12	1.88±0.04
	GA (02)	18.22±1.54	37.88±0.19	1.78±0.07
C27007790A	CC (17)	19.25±1.16 ^b	38.03±0.14	1.83±0.05 ^b
	CA (13)	21.76±1.68 ^a	37.97±0.21	1.94±0.07 ^a
	AA (05)	16.91±1.57 ^{ab}	37.80±0.20	1.72±0.07 ^{ab}

Figures in parenthesis are number of animals;
Figures with dissimilar superscript differ significantly

CHAPTER- 5

Summary and conclusions

5. Summary and Conclusions

Environmental, psychological, nutritional, pathological and managerial stressors cause significant heat stress to livestock resulting in reduced feed consumption rate, decreased milk production and a lower reproductive success rate. Genetic selection for core body temperature regulation is one potential strategy to mitigate effects of heat stress on dairy cows so that an animal with genetic adaptations that make for more effective regulation of core body temperature will experience a less severe reduction in production. Heat Shock proteins (HSPs) are the best-suited examples of genes whose expression is effected by heat stress. The ATP1A1 gene encodes the $\alpha 1$ isoform, the major isoforms of α subunit of $\text{Na}^+ - \text{K}^+$ -ATPase pump. ATP1A1 gene is responsible for establishing the electrochemical gradient of Na^+ and K^+ across the plasma membrane. These gradients are essential for osmoregulation, for sodium-coupled transport of a variety of organic and inorganic molecules and for electrical excitability of nerve and muscle. ATP1A1 gene has been mapped on BTA 3 and spans about 22.77 kb and includes 23 exons and coding sequence of 3066 nucleotides.

The present study was carried out in Sahiwal (51), Karan Fries (50) and Jersey crossbred cows (35) with the objectives of identification of SNPs in the targeted regions of ATP1A1 gene and to analyze their association with respiration rate and rectal temperature. Genomic DNA was isolated from whole blood and quantitative and qualitative analysis of extracted DNA was carried out. Two sets of primers were designed using DNASTAR software by referring bovine ATP1A1 gene sequence number ENSBTAG00000001246. The PCR parameters were standardized. The PCR amplification was performed using Thermal cycler (MJ research and BioRad T100) in a final volume of 25 μl containing 3 μl genomic DNA, 12.5 μl 2X PCR Master Mix (Fermentas), 0.5 μl of each primer and 8.5 μl nuclease free water. The PCR reaction mixture was incubated in thermal cycler initially at 94°C for 3 minutes followed by 34 cycles of 94°C for 30 seconds, 56.5°C for 30 seconds, 72°C for 25 seconds and a final extension of 72°C for 8 minutes to obtain PCR product of 491 and 465 bp sizes for primer set 1 and 2, respectively. The PCR products were stored at -20°C . PCR products were sent for sequencing to find out nucleotide sequence of targeted regions of ATP1A1 gene in Sahiwal, Karan Fries and Jersey crossbred cattle.

For determining SNPs, reference sequences of *Bos taurus* were aligned with sequencing results of each animal for each target region using ClustalW multiple alignment programme. A total of six SNPs (T27008243C, A27008223G, T27008097A, C27008016T, G27008015A and C27007790A) were found in the studied breeds, allele and genotype frequencies of ATP1A1 gene were calculated using POPGENE software package. Nucleotide variations at A27008442G, A27008436C and G27007963A loci were found to be monomorphic for allele A with genotype AA, A with genotype AA and G with genotype GG, respectively in all three breeds of dairy cattle. Remaining six SNPs were found to be polymorphic in Sahiwal and Karan Fries cows whereas, SNP locus T27008243C was monomorphic for allele T with genotype TT for Jersey crossbred cows. It was observed that in Sahiwal cows at each SNP locus all possible three genotypes (homozygous and heterozygous) were present. Similar pattern of all possible three genotypes was observed at SNP loci C27008016T and C27007790A in Karan Fries and Jersey crossbred cows, whereas, SNP locus G27005015A revealed all possible three genotypes in Karan Fries cows along with Sahiwal cows. Respiration rate and rectal temperature were recorded once during probable extreme hours in winter, spring and summer season in all studied animals.

Association of each SNP genotype with rectal temperature (RR), respiration rate (RT) and heat tolerance coefficient (HTC) in Sahiwal, Karan fries and Jersey crossbred cows was analyzed using GLM procedure of Statistical Analysis System (SAS) software. RR and HTC were found to be significantly associated with T27008243C and T27008097A loci, respectively in Sahiwal cows. RR at locus T27008243C for genotype CC was highest (19.24 ± 1.52^a) compared to genotype TT (15.91 ± 1.89^b) and TC (18.25 ± 1.77^{ab}). HTC at locus T27008097A for genotype TT was highest (1.95 ± 0.05^a) compared to genotype TA (1.79 ± 0.06^b) and AA (1.65 ± 0.11^c). RR and RT were found to be significantly associated with A27008223G and T27008097A loci, respectively in Karan Fries cows. RR at locus A27008223G for genotype GG was highest (32.37 ± 2.51^a) compared to AA (28.85 ± 1.96^b). RT at locus T27008097A for genotype TT was highest (38.32 ± 0.14^a) compared to AA (37.56 ± 0.32^b). RT was found to be significantly associated with SNP locus A27008223G, whereas, RR and HTC were found to be significantly associated with C27007790A in Jersey crossbred cows. RT at locus A27008223G for genotype AA (38.01 ± 0.16^a) and GG (37.86 ± 0.14^a) did not differ significantly. RR at locus C27007790A for genotype CA was highest (21.76 ± 1.68^a) as compared to CC

(19.25 ± 1.16^b) and AA (16.91 ± 1.57^{ab}). HTC at locus C27007790A for genotype CA was highest (1.94 ± 0.07^a) as compared to CC (1.83 ± 0.05^b) and AA (1.72 ± 0.07^{ab}).

Conclusions

- Genomic DNA samples of 136 cows from three cattle breeds i.e. Sahiwal (51), Karan Fries (50) and Jersey crossbred (35) were analysed.
- Two primer sets were designed to amplify target region of bovine ATP1A1 gene.
- Targeted regions were comprised of part of exon 16 + Intron 17 + Exon 17 (Primer set 1) and Part of Intron 17 + exon 18 + Intron 18 (Primer set 2).
- A total of six SNPs (T27008243C, A27008223G, T27008097A, C27008016T, G27008015A and C27007790A) were found in all studied breeds.
- Among six SNPs 4 were transition type and 2 were transversion.
- All six SNPs and possible three genotypes (two homozygous & a heterozygous) at each SNP locus were observed in Sahiwal cows.
- Respiration rate (RR) and Heat Tolerance Coefficient (HTC) were found to be significantly associated with TT and AA genotypes at T27008243C and T27008097A loci, respectively in Sahiwal cows.
- All six SNPs were also observed in Karan Fries cows.
- RR and Rectal temperature (RT) were found to be significantly associated with AA and TA genotypes at A27008223G and T27008097A loci, respectively in Karan Fries cows.
- Five SNPs were observed in Jersey crossbred cows and SNP T27008243C was absent.
- RT was found to be significantly associated with SNP locus A27008223G in Jersey crossbred cows.
- RR and HTC were found to be significantly associated with AA genotypes at C27007790 locus in Jersey crossbred cows.
- With increase in Temperature Humidity Index there was increase in both RR and RT in Sahiwal and Karan Fries cows.

The AA and TA genotype at 27008097 position in ATP1A1 gene of Sahiwal and Karan Fries cows were desirable for heat tolerance coefficient and rectal temperature respectively, whereas, AA and GG genotype at 27008223 position in Karan Fries and Jersey crossbred cows were desirable for respiration rate and rectal temperature respectively. The findings may be validated and used as an aid to selection.

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APPENDIX

Appendix

Most of the chemicals used in this study were of molecular biology grade. The list of chemicals and the preparation of reagents are as follows:

Preparation of Reagents

Tris (1 M) pH 8.0

Tris base (121.10 gm) was dissolved in 800 ml of double distilled water and the pH was adjusted to 8.0 by adding concentrated HCl (40 ml). The solution was allowed to cool to room temperature before making final volume to 1 liter and stored at 4 °C.

Sodium chloride (5 M)

Sodium chloride (29.40 gm) was dissolved in 80 ml of double distilled water and the final volume made up to 100 ml. Sterilized by autoclaving and stored at 4 °C.

Sodium acetate (3 M)

24.60 gm of Sodium acetate (anhydrous) was dissolved in 80 ml of double distilled water. pH was adjusted to 5.2 with glacial acetic acid and the final volume was adjusted to 100 ml. Sterilized by autoclaving and stored at room temperature.

EDTA (0.5 M)

Disodium salt of EDTA (18.60 gm) was dissolved in 80 ml of double distilled water by adding NaOH pellets. pH adjusted to 8.0 and final volume made up to 100 ml. Sterilized by autoclaving and stored at 4 °C.

Ammonium chloride (1 M)

Ammonium chloride (5.35 gm) was dissolved in 80 ml of double distilled water and final volume was made up to 100 ml. Sterilized by autoclaving and stored at 4 °C.

Sodium bicarbonate (1 M)

10.00 gm of sodium bicarbonate was dissolved in 80 ml of double distilled water and made to a final volume of 100 ml. Sterilized by autoclaving.

Sodium dodecyl sulphate (SDS) 10 %

Dissolved 10.00 gm of ammonium chloride in 80 ml of autoclaved double distilled water and made up to a final volume of 100 ml.

RBC Lysis buffer (1 X prepared freshly every time)

NH ₄ Cl (155 mM)	:	155.0 ml
KHCO ₃ (10 mM)	:	10.0 ml
EDTA (0.1 mM)	:	2.0 ml

Final volume was made up to 1000 ml by double distilled water. Sterilized by autoclaving and stored at 4 °C.

Digestion buffer (1 X prepared freshly every time)

NaCl (75 mM)	:	75.0 ml
Tris (10 mM)	:	10.0 ml
EDTA (2 mM)	:	4.0 ml

Final volume was made up to 1000 ml by double distilled water and sterilized by autoclaving and kept at room temperature.

Phenol equilibration (Tris saturation)

Phenol crystals (500 gm) stored at -20 °C was liquefied by keeping in a water bath maintained at 65 °C for 1 hour. 8-hydroxyquinoline was added to a liquefied phenol at a final concentration of 0.1 %. Then an equal volume (500 ml) of 0.5 M Tris (pH 8.0) was added and stirred for 4 hours on magnetic stirrer and pH repeatedly checked till it reached 8.0. Finally, 0.1 M Tris was added to an equilibrated phenol and stirred well and stored in amber colored bottles at (4 °C).

Proteinase-K

Proteinase-k (20 mg) was dissolved in 1ml of double distilled water. Aliquots of 400µl per vial made and stored at -20 °C.

Ethanol 70 %

Ethanol 99.9 %	:	70 ml
Distilled water	:	30 ml

Phosphate Buffer Saline (1X) Ca⁺⁺ and Mg⁺⁺ free

NaCl	:	4.0 gm
KCl	:	0.1 gm
Na ₂ HPO ₄	:	0.7 gm
KH ₂ PO ₄	:	0.1 gm

The pH was adjusted to 7.4 and final volume made up to 500 ml with double distilled water. Autoclaved and stored the buffer at room temperature.

TE buffer (10 mM)

Tris (1M, pH 8.0)	:	1.00 ml
EDTA (0.5M, pH 8.0)	:	200 µl
Final volume made up to	:	100 ml

Sterilized by autoclaving and stored the buffer at room temperature.

Agarose gel:**Agarose 0.6 %**

Agarose	:	0.60 gm
TBE (10 X)	:	10 ml
Double distilled water	:	90 ml

Agarose 1.7 %

Agarose	:	1.70 gm
TBE (10X)	:	10 ml
Double distilled water	:	90 ml

Agarose 2.0 %

Agarose	:	2 gm
TBE (10X)	:	10 ml
Distilled water	:	90 ml

Ethidium bromide

Ethidium bromide (0.25 gm) was dissolved in 25 ml of double distilled water. Stirred on a magnetic stirrer for several hours to ensure that the dye dissolved. Wrapped the container with aluminum foil and stored at 4 °C.

Preparation of 6X gel loading dye

Bromo phenol blue	:	0.25 % (w/v)
Xylene cyanol FF	:	0.25 % (w/v)
Glycerol in water	:	30 % (v/v)

TBE buffer (10X)

Tris base	:	108.0 gm
Boric acid	:	55.0 gm
EDTA (0.5 M, pH 8.0)	:	40 ml

Double distilled water was added to make the final volume 1000ml, filtered and autoclaved.

TAE buffer (50X)

Tris base	:	242.0 gm
Glacial acetic acid	:	57.1 ml
EDTA (0.5 M, pH 8.0)	:	100 ml

Double distilled water was added to make the final volume 1000ml, filtered and autoclaved.