TRANSCRIPTIONAL ABUNDANCE OF MONOCARBOXYLATE TRANSPORTERS IN DIGESTIVE TRACT OF BLACK BENGAL GOAT AT DIFFERENT AMBIENT TEMPERATURE

Thesis

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science

(Veterinary Physiology)

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Dedicated to...

My Beloved Family

Friends

and

Guide

भारतीय पशु चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)
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Dated: 28/07/2016

Certificate

Certified that the research work embodied in this thesis entitled "Transcriptional Abundance of Monocarboxylate Transporters in Digestive Tract of Black Bengal Goat at Different Ambient Temperature" *submitted by* **Dr. Chandrabhan Singh Banjare, Roll No. 5507,** *for the award of* **Master of Veterinary Science** *Degree in* **Veterinary Physiology** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that **Dr. Chandrabhan Singh Banjare,** *has worked for more than 30 months in this Institute and has put in more than 300 days attendance under me from the date of registration for the degree of Master of Veterinary Science of the Deemed University, as required under the relevant ordinance.*

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Certificate

We the undersigned members of Advisory Committee of **Dr. Chandrabhan Singh Banjare, Roll No. 5507** *a candidate for the degree of Master of Veterinary Science with the major discipline* **Veterinary Physiology,** *agree that the thesis entitled,* ***"Transcriptional Abundance of Monocarboxylate Transporters in Digestive Tract of Black Bengal Goat at Different Ambient Temperature"*** *may be submitted in partial fulfilment of the requirement for the degree.*

We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of Master of Veterinary Science of this Institute.

It is further certified that the candidate has completed all the prescribed requirements governing the award of ***Master Degree*** *of the Deemed University, Indian Veterinary Research Institute, Izatnagar.*

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Date: 24/08/2016 **(Chandrabhan Singh Banjare)**

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ABBREVIATIONS

%	Percentage
<	Lesser than
>	Greater than
^{o}C	Degree Celsius
µl	Microliter
µm	Micrometre
µg	Microgram
ANOVA	Analysis of variance
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
CO_2	Carbon dioxide
Ct	Cycle Threshold
DEPC	Diethyl pyrocarbonate
DAB	Diaminobenzidine tetrahydrochlride
DAPI	4, 6-diamidino-2- phenylindoledihydrochloride
DNA	Deoxy ribonucleic acid
dNTP's	Deoxynucleoside triphosphate
DW	Distilled Water
EDTA	Ethylene diamine tetraacetic acid
HSP70	Heat Shock Protein 70
Fig.	Figure
GAPDH	Glyceraldehyde-3-phosphate dehydrogenage
GI	Gastrointestinal
h	Hour
H_2O_2	Hydrogen peroxide
HRP	Horse radish peroxidase
HSP	Heat Stress Protein
kDa	Kilo Dalton
M	Molar
MCT	Monocarboxylate transporter
Mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minutes
ml	Millilitre
MM	Master mix

mM	:	Millimolar
Mol.Wt.	:	Molecular weight
mRNA	:	Messenger ribonucleic acid
N	:	Normal
NFW	:	Nuclease free water
OD	:	Optical density
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PBST	:	Phosphate buffered saline Tween
PCR	:	Polymerase chain reaction
PE	:	Phycoerythrin
pH	:	Log Hydrogen ion concentration
PVDF	:	Polyvinylidene difluoride
qPCR	:	Quantitative polymerase chain reaction
RNA	:	Ribonucleic acid
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SDS	:	Sodium dodecyl sulphate
Sec	:	Seconds
SE	:	Standard error
TAE	:	Tris acetate EDTA buffer

LIST OF TABLES

LIST OF FIGURES

CONTENTS

Introduction

1

INTRODUCTION

Goats are an integral part of rural India's symbiotic system of crop and livestock production and make up a significant part of the livestock wealth of country. Goats are among the main meat-producing animals in India and goat meat (chevon) faces huge domestic demand, with no social, cultural, and religious restrictions, thus sharing the profit to the national GDP. Most goats are found in the drier areas of the developing world. The total world population of goats in 2012 was estimated about 996 millions, out of which 608 million were in low income food deficit countries and 160 millions in India (FAO, 2012). In global scenario, India ranks second (140.537 million goats) in terms of goat population and meat production and occupies top position in goat milk productions (DAHD, 2012-13; CIRG, 2011). Rajasthan (21.5 million), West Bengal (15 million), Uttar Pradesh (14.79 million), Maharashtra (10.3 million), Bihar (10.16 million), Andhra Pradesh (9.6 million), Tamil Nadu (9.27 million), Madhya Pradesh (9 million), Odisha (7.1 million), Jharkhand (6.59 million) states possess near about 81% of Indian goat population. About 90 percent of the world's goats are reared primarily for meat. In India about 42 percent of the total population of goats is slaughtered for meat every year. Goats provide not only livelihoods for much of the rural population, but also valuable animal protein to both rural and urban populations. They play an important role in income generation, capital storage, employment generation and household nutrition.

Among the various meat-producing indigenous breeds of goat in India owned by small farmers, peasants, and landless labourers, the Black Bengal goat (*Capra hircus bengalensis*) is the most common. This breed of goat is mostly found in the Eastern and North-Eastern region of India. A number of factors make the rearing of Black Bengal goats a preferred option among marginal and small farmers and even landless farmers, as this animal mainly depend on common grazing and forest lands for fodder (Biswas, 2010). Other factors for rearing Black Bengal goat includes low capital intensity, prolific breeding, superior chevon quality, early sexual maturity, high-quality skin, low kidding intervals, good adaptability, no religious taboo against consumption of chevon and steady returns. These factors have led to the realization that there is need to pay more attention to this goat breed, whose genetic material has been used since time immemorial. Thus there is need to have a study to understand how this breed of goat is adapting during different climatic season.

Black Bengal goats are generally reared under extensive farming system. While browsing during daytime, they are exposed to variable climatic conditions and thereby affected by both heat and cold stress. Since India is a tropical country the climatic variations are in broader range here. The lower critical temperature for goat is not specified but the limits of thermo neutrality for goats may be taken as a climatic environment having an air temperature of 13°C –27°C, relative humidity of 60-70 per cent and wind velocity of 5-8km/hr and a medium level of solar radiation (Mishra, 2009). In most domestic species including goat, hot as well as cold weather has deleterious effects on nutritional, physiological and reproductive functions. Exposure of goats to harsh environment cause depression of feed intake, reduction in production and increase susceptibility to wide range of microbes. This situation is often worsened by dry, poor quality forage. Adverse season is also detrimental to semen quality, embryo survival and foetal development. Goats are more susceptible to cold stress than hot weather. The grazing activity of domestic goats exposes them to a wide range of pathogenic organisms (cysts of helminths and nematodes) and pesticides (used by local farmers) as a part of environmental challenges. Moreover, incidents of infectious diseases such as enterotoxaemia, diarrhoea and various infections are more frequent during certain period of the year, i.e. rainy season. In tropical zone, rainy season provides high humidity and temperature for the growth of bacteria and several pathogens and facilitates their horizontal as well as vertical migration in domestic goat population. On the other hand, during rest of the year, the health of the domestic goats is optimum and produce maximum amount of milk and meat.

In ruminants, short-chain fatty acids (SCFAs) constitute the major source of energy, providing up to 80% of their maintenance energy requirements (Bergman, 1990). Propionate serves as the primary precursor of glucose, which must be synthesized *de novo* because little glucose is absorbed into the hepatic portal system (Baird *et al.,* 1980; Huntington *et al.,* 1981). In contrast, a high proportion of the acetate is not taken up by hepatocytes, but passes into the systemic circulation to be utilized for lipogenesis by peripheral tissues such as skeletal muscle, adipose tissue, and myocardium as well as mammary gland (Bergman, 1975). In addition to the energetic or nutritional contributions of SCFAs to the body, the SCFAs helps in regulation of endocrine (Bassett, 1975) and exocrine (Harada and Kato, 1983) secretions of the pancreas. Moreover, SCFAs production and absorption have a significant effects on epithelial cell growth, blood flow, and the normal secretory and absorptive functions of gastrointestinal tract (Bergman, 1990). Prior to use by the animal, these microbial products must be transferred and effectively absorbed across the gastrointestinal epithelium.

Despite the significance of SCFAs in maintaining the ruminant physiology, the mechanism of SCFA absorption is still not fully studied. SCFAs transfer across the apical membrane of ruminal epithelium is thought to occur by either passive permeation of undissociated acids, or exchange of dissociated SCFAs for anions like bicarbonate (Kramer *et al.,* 1996; Gabel and Sehested, 1997). However, no mechanism for the transport of SCFAs anions across the basolateral membrane into the bloodstream has so far been identified in the gastrointestinal tract of ruminants.

The SLC16 family of solute carriers, also known as the monocarboxylate transporter (MCT) family, has 14 members. MCT1–4 catalyze the proton-linked transport of monocarboxylates such as lactate, pyruvate, short-chain fatty acids, and the ketone bodies across the plasma membrane, whereas MCT8 transports thyroid hormone, and MCT10 transports the aromatic amino acid (Halestrap, 2012). MCT5–7, 9, and 11–14, however, are orphan members, and their substrates are as yet not characterized (Halestrap, 2012).

In a series of molecular screenings, together with *in vivo* and *in vitro* functional studies, it has been demonstrated that the absorption of SCFAs by the different parts of the gastrointestinal epithelium of various ruminant species is regulated by MCT1 and MCT4 (Kirat *et al.,* 2006b; Kirat and Kato 2006; Kirat *et al.,* 2007). Concerning the expression and cellular localization of MCT isoforms in the ruminant gastrointestinal tract, it has been identified the MCT1 in the bovine, caprine, and ovine gastrointestinal tract (Kirat *et al.,* 2006a, 2006b, 2007) and established that MCT1 plays a direct role in the efflux of SCFA across the cells of the stratum spinosum and stratum basale of the caprine rumen (Kirat *et al.,* 2006b) and across the basolateral membranes of the bovine caecal epithelium (Kirat and Kato 2006) toward the blood side. It has been also shown the precise cellular localization of the MCT4 protein, which might represent an important transporter for short-chain monocarboxylates, in the bovine gastrointestinal tract (Kirat *et al.,* 2007). In a study conducted by Graham *et al.* (2007), only mRNA transcripts for MCT1 and MCT2 have been detected by reverse transcription with the polymerase chain reaction (RT-PCR) among the evaluated MCT isoforms (MCT1- MCT8) in bovine rumen epithelium. In another study in reindeer, MCT1 and MCT4 proteins have been found to be expressed in the rumen, whereas MCT1, MCT2, and MCT4 are present in the small intestine (Koho *et al.,* 2005).

Thermal stress results in increased demand of net energy for maintenance and subsequent production in various livestock and leads to nutrient deficiency which affects animal's ability to counter the stress (Ames *et al.,* 1980). Thermal stress has negative impact on food consumption and metabolic activities. Westra and Christopherson (1976) demonstrated that heat stress leads to change in the activity of digestive system and exposure of heat stress has been shown to accelerate the digestibility of DM, crude protein and various feed fraction resulting in reduce passage rate in the GI tract (Warren *et al.,* 1974).

Heat shock proteins (HSP) are highly conserved proteins found in all prokaryotes and eukaryotes. Under normal physiological conditions HSP are expressed at low levels. The primary function ascribed to HSP is as intracellular molecular chaperones of native, aberrantly folded or mutated proteins as well as in cytoprotection following the kinds of stressful stimuli mentioned above. HSPs play crucial roles in cell-cycle control and signalling and protection of cells against apoptosis (Zihai *et al.,* 2003). HSPs were originally identiûed as proteins whose expression was markedly increased by heat shock (Lindquist, 1986). HSP70s are a family of ubiquitously expressed heat shock proteins. It is found in prokaryotes and eukaryotes (Tavaria *et al.,* 1996) and is mainly localized in the cytosol, mitochondria and endoplasmic reticulum and exhibit constitutive and inducible regulation. HSP 70 contains two distinct functional regions: a peptide binding domain (PBD) and the amino-terminal ATPase domain (ABD). Under normal conditions, HSP70 functions as ATP dependent molecular chapereon that assist the folding of newly synthesized polypetides, the assembly of multi protein complexes and the transport of proteins across cellular membranes. Under stressful conditions, elevated HSP70 levels allow cells to cope with increased concentrations of unfolded or denatured proteins (Panjwani *et al.,* 1999).

The mRNA expression pattern for HSP and MCT genes is breed-specific, most likely due to variations in thermal tolerance and adaptation to different climatic conditions (Parkunan *et al.,* 2015).

Therefore, the present study aimed to investigate the expression profile of MCTs isoforms (MCT1, MCT2 and MCT4) and HSP70 in rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, caecum, and colon of Black Bengal Goat at different ambient temperature.

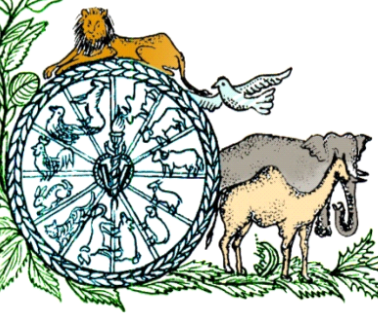
Objective

Keeping in view of these facts, the present research work is formulated to carry out with the following objective:-

1. **To determine the expression profile of MCT isoforms i.e. MCT1, MCT2, MCT4 and HSP70 in rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, caecum and colon of Black Bengal goat at different ambient temperature.**

* * *

Review of Literature



2

REVIEW OF LITERATURE

Monocarboxylate transporters

The MCT family comprises 14 members (MCT1-MCT14), only four (MCT1, MCT2, MCT3 and MCT4) of which have been experimentally demonstrated to catalyse the proton-linked transport of metabolically important monocarboxylates (Halestrap and Meredith, 2004). TAT1 (MCT10) has been shown to transport aromatic amino acids (Halestrap and Meredith, 2004) and MCT8 to be an active and specific thyroid hormone transporter (Friesema *et al.*, 2003). In addition, MCTs in the epithelium of the small intestine, colon and blood-brain barrier, provide routes for many carboxylated pharmaceutical agents (Enerson and Drewes, 2003). All MCTs are membrane proteins, with 12 transmembrane regions and cytoplasmic N- and C-terminal ends (Poole *et al.*, 1996; Halestrap and Price, 1999). They transport a monocarboxylate anion and a proton together through the cell membrane according to the electrochemical gradients of substrates. Lactate is quantitatively the most abundant monocarboxylate transported by MCTs, but also other monocarboxylates, such as butyrate, acetate, propionate and ketone bodies, are transported (Halestrap and Price, 1999). At least MCT1 and MCT4 need a chaperone protein, CD147 (also known as basigin, EMMPRIN, HT7 or OX-47), in muscle (Halestrap and Price, 1999; Juel and Halestrap, 1999), red blood cells (Koho *et al.*, 2002) and the intestines (Buyse *et al.*, 2002). The suggested model of the topology of 11 CD147 and MCT1 in the plasma membrane is a dimer of CD147 associated with two MCT1 molecules such that the C-terminus of CD147 in the cytosol is close to the C-terminus of its partner CD147 and to the C- and N-termini of an associated MCT1 molecule (Wilson *et al.*, 2002). Koho *et al.* (2002) have reported that lactate transport via MCT1 in

RBCs of horses that express small amounts of CD147 is insignificant compared with horses with larger amounts of CD147 even though the amount of MCT1 is similar in all horses. This result indicates that MCT1 does not function well without CD147. This has been confirmed later in studies with site-specific mutations (Wilson *et al*., 2005). CD147 belongs to the immunoglobulin superfamily, and in addition to its role as a chaperone of MCTs, it has several other functions, such as induction of matrix metalloproteases (Toole, 2003). A co-protein for MCT2 has recently been identified as gp70, a member of the same protein family as CD147 (Wilson *et al*., 2005).

The distribution of MCT isoforms is species and tissue specific. MCT1 is found in the majority of tissues of all species studied (Halestrap and Meredith, 2004). In muscle tissue, it is present in all fibre types, although being more abundant in muscles that have a high percentage of oxidative fibres, and thus, has been suggested to have a major role in influx of lactate for oxidation (McGullagh *et al*., 1996; Halestrap and Price, 1999; Juel and Halestrap, 1999). The Km of MCT1 for lactate is 3.5-10 mM (Bröer *et al*., 1998; Halestrap and Price, 1999) and as mentioned above, it requires a co-protein, CD147, for its correct plasma membrane expression and function (Halestrap and Price, 1999; Juel and Halestrap, 1999). Recently, MCT1 has been reported to be a target for immunomodulatory compounds used in immunosuppressive therapy. These compounds block the activity of MCT1 and prevent the rapid phase of T cell division necessary for immune response (Murray *et al*., 2005). The reason for this has been suggested to be the accumulation of lactate in T cells (Murray *et al*., 2005). Mutations in the MCT1 gene are responsible for subnormal lactate transport in patients with myopathy on exertion (Merezhinskaya *et al*., 2000).

MCT2 is present in skeletal muscle of man, rats and hamsters (Garcia *et al*., 1995, Bonen *et al*., 2006), although not all studies agree with this (Jackson *et al*., 1997; Price *et al*., 1998). MCT2 has previously been proposed to be especially species and tissue specific (Jackson *et al*., 1997), but a recent study suggests that it is also widely distributed (Bonen *et al*., 2006). Besides skeletal muscle, MCT2 has been found in several tissues of rats, mice and hamsters (Garcia *et al*., 1995; Jackson *et al*., 1997, Gerhart *et al*., 1998). In addition, MCT2 is expressed in equine RBCs (Koho *et al*., 2002). It has a low Km for lactate (0.7 mM) and may be adapted to transport lactate more efficiently in an environment where pH is acidic and rapid transport is required (Garcia *et al*., 1995, Bröer *et al*., 1999).

The other major MCT isoform found in muscles, especially in white, type II muscle fibres, is MCT4 (Wilson *et al.*, 1998; Pilegaard *et al.*, 1999b) which has been speculated to have a primary role in the efflux of lactate from muscles during intense exercise (Wilson *et al.*, 1998; Juel and Halestrap, 1999; Manning Fox *et al.*, 2000). MCT4 is expressed widely also in other glycolytic tissues, such as astrocytes, white blood cells and chondrocytes, in addition to in glycolytic muscle fibres (Halestrap and Meredith, 2004). This isoform has a high Km (17-34 mM) for lactate and its Vmax is also high (Wilson *et al.*, 1998; Bonen, 2000, Dimmer *et al.*, 2000; Manning Fox *et al.*, 2000). MCT4 also needs CD147 for its proper function (Halestrap and Price 1999; Juel and Halestrap 1999). In human muscle tissues, interindividual variation in MCT4 content is larger than interindividual variation in MCT1 content (Pilegaard *et al.* 1999b).

The transport via MCT2 is saturable because its Vmax is low compared with that of MCT1 and MCT4 (Lin *et al.* 1998; Bröer *et al.*, 1999). Its biological significance has been proposed to be related to modulation of cell pH and volume (Okamura *et al.*, 2001). Recently, MCT7 was found to be expressed in human but not in rat skeletal muscle (Bonen *et al.*, 2006), but the physiological role of this transporter is presently unknown.

The expression of MCT proteins changes as a result of such physiological factors as exercise training (Bonen *et al.*, 1998; Pilegaard *et al.*, 1999a; Dubouchaud *et al.*, 2000; Green *et al.*, 2002), diet (Leino *et al.*, 2001), obesity (Py *et al.*, 2001), hormones (Buyse *et al.* 2002; Fanelli *et al.* 2003; Wang *et al.*, 2003) and various diseases (Knott *et al.*, 1999, Froberg *et al.*, 2001; Jóhannsson *et al.*, 2001), including bacterial infections (Borthakur *et al.*, 2006). Because of the exceptionally large role of anaerobic metabolism in porcine muscles, these muscles may prove useful in investigating MCTs.

The expression of MCTs have been investigated in the gastrointestinal tract of humans (Ritzhaupt *et al.*, 1998b; Hadjiagapiou *et al.*, 2000; Stein *et al.*, 2000; Buyse *et al.*, 2002; Cuff *et al.*, 2002; Fishbein *et al.*, 2002), rats (Takanaga *et al.*, 1995; Tamai *et al.*, 1995; Orsenigo *et al.*, 1999; Schröder *et al.*, 2000), pig (Ritzhaupt *et al.*, 1998a, 1998b), reindeer (Koho *et al.*, 2005), sheep (Müller *et al.*, 2002; Kirat *et al.*, 2006) and calves (Kirat *et al.*, 2005).

In the gastrointestinal tract, MCTs are important in the transport of short-chain fatty acids (SCFAs) and lactate (Ritzhaupt *et al.*, 1998a; 1998b, Müller *et al.*, 2002; Koho *et al.*, 2005). SCFAs, such as acetic, propionic, butyric, isobutyric, valeric, isovaleric, 2-methylbutyric, hexanoic and heptanoic acid are produced in several parts of the gastrointestinal tract by microbial fermentation of dietary fibre. They are weak acids, but because the pH of the gastrointestinal tract, with the exception of the stomach, is nearly neutral, 90-99% of SCFAs are present as anions rather than as free acids. In all mammals examined, acetate is the main SCFA produced. Propionate and butyrate are also present in large concentrations, although their amounts can vary considerably with diet. Commonly, molar ratios of acetate to propionate to butyrate in mammals vary from ~75:15:10 to 40:40:20 (Bergman, 1990). In goat, as in other ruminant species, the short-chain fatty acids (SCFA) are produced by the anaerobic microbial fermentation of carbohydrates. Acetate, propionate and butyrate are the predominant SCFA occurring in the rumen fluid; their concentration and relative proportions are related to the level of feed intake (Sutton, 1985) and the composition of the diet (Murphy *et al.,* 1982). Total SCFA concentrations in the rumen contents are found to range from 60 to 150mm, with proportions of 60–70% acetate, 15–20% propionate, and 10–15% butyrate (Titus and Ahearn, 1992). The net absorption of SCFA reaching the blood is dependent on its concentration in the rumen as well as the quantity used by the rumen wall. The rates of utilization by the rumen wall are butyrate>propionate>acetate (Stevens and Stettler, 1966); however, their respective concentrations in the blood were found to be in the reverse order (Masson and Phillipson, 1951).

The two functions of MCT proteins described above, namely the regulation of cell pH in skeletal muscles and facilitation of the absorption of SCFA in the gastrointestinal tract, can thus be assumed to be important for physiological harmony and also for animal welfare.

Sepponen *et al.* (2003) suggest that MCT2 function as housekeeping lactate transporter in porcine muscles that prevents acidification in highly glycolytic muscle whose oxidize capacity is too low. He proposed that size of MCT1 protein was about 55 kDa whereas MCT2 was 40 kDa and MCT4 was 50 kDa. MCT4 expression between the muscles showed no significant difference whereas MCT2 expressed higher in glycolytic longissimus, gluteus and semimembranosus than in more oxidative muscle such as masseter and infrasinatus

Sapponen *et al.* (2003) concluded that MCT2 efflux the lactate at low concentration during rest, whereas MCT4 would be more important during stressful conditions when lactate concentrations are high and they also found that MCT2 and MCT4 are the principal MCT isoforms present in domestic pig skeletal muscle irrespective of type of fiberes.

Welter and Claus (2008) stated that porcine MCT1 cDNA fragment was 94% homologous to the equine MCT1, 92 % homologous to the human, bovine and rat MCT1. MCT1 protein expressed only by immune cells present predominantly in the crypt and glandular region of the lamina propria of duodenum, jejunum and ileum, whereas in colon it strongly expressed in colonic epithelia towards lumen and weakest at the base of the colonic crypts and immune cells of the lamina propria. In large intestine MCT1 protein expressed in a larger amounts while less protein expressed in duodenum with nearly undetectable levels in jejunum and ileum.

Heat Shock Proteins

Heat shock proteins (HSP) are highly conserved proteins found in all prokaryotes and eukaryotes. Under normal physiological conditions HSP are expressed at low levels. However, a wide variety of stressful stimuli including environmental (UV radiation, heat shock, heavy metals, and amino acids), pathological (viral, bacterial, parasitic infections or fever, inflammation, malignancy, or autoimmunity), or physiological stimuli (growth factors, cell differentiation, hormonal stimulation, or tissue development), induce a marked increase in intracellular HSP synthesis (Lindquist and Craig, 1988), known as the stress response. The primary function ascribed to HSP is as intracellular molecular chaperones of native, aberrantly folded or mutated proteins as well as in cytoprotection following the kinds of stressful stimuli mentioned above. HSPs play crucial roles in cell-cycle control and signalling and protection of cells against apoptosis (Zihai, 2003). HSPs were originally identified as proteins whose expression was markedly increased by heat shock (Lindquist, 1986). HSP70 mRNA expression is higher during summer season as compared with winter season in tropical region goats (Dangi *et al.*, 2012).

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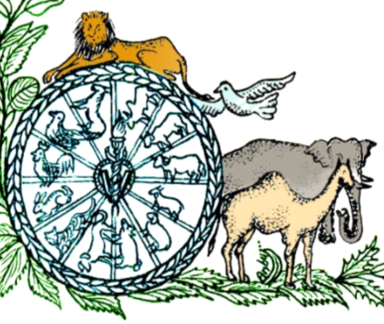
Materials and Methods



3

MATERIALS AND METHODS

The present study was accomplished by using a number of materials and techniques which are described in this section. The first part deals with the chemicals used, followed by the methodologies, applied to achieve the target.

3.1. Chemicals / Reagents

Chemicals for molecular biology were obtained from different companies like Thermo scientific, Bio-Rad, 3B Black Bio, Invitrogen, as per requirement. 50bp plus DNA ladder, Loading dye was from (MBI, Fermentas) were used. The chemicals like Tris, Ethanol, Agarose, Ethidium Bromide, were used. Trizol reagent (Invitrogen), SDS– 12.5% PAGE, polyvinylidene difluoride (PVDF) membrane, bovine serum albumin (BSA).

3.1.1 Kit

RevertAid First Strand cDNA synthesis kit (Thermo scientific), SsoFast TM Eva Green ®Supermix kit (Bio-Rad, USA) oligo primer (GCC Biotech). Ripa lysis Buffer, Protease inhibitor cocktail (Amresco).

3.2 Antibodies

3.2.1 Primary antibodies

Rabbit polyclonal MCT1 (sc-50325; Santa Cruz Biotechnology, Inc., Dallas, TX), rabbit polyclonal MCT2 (sc-50323; Santa Cruz Biotechnology, Inc., Dallas, TX) and rabbit polyclonal MCT4 (sc-50329; Santa Cruz Biotechnology, Inc., Dallas, TX).

3.2.2 Secondary antibodies

Goat anti rabbit IgG-PE (sc-7239; Santa Cruz Biotechnology, Inc., Dallas, TX) and goat anti rabbit IgG-HRP (sc-2004; Santa Cruz Biotechnology, Inc., Dallas, TX).

3.3 Primer sequences

The primers for MCT1, MCT2, MCT4, GAPDH and RPS15A were designed using the DNAstar, Genetools, Oligoanalyzer software. Published primers were used for HSP70 (Dangi et al., 2012). Details of the primers used are presented in Table 1.

3.4 Glasswares and plastic wares

For RNA work, RNase-free plastic wares and glassware were used, and they were thoroughly treated with 0.1% DEPC overnight at 37°C. It was further autoclaved to make it DNase and RNase free before use. For PCR and other DNA related work plastic wares were autoclaved (121°C for 15 min at 101.3 kpa or 1 atmospheric pressure) and then used.

3.5 Equipments

Major equipments used were as follows:

1. -20°C Deep freezer (Vestfrost)
2. -80°C Sanyo Biomedical freezer
3. Agarose gel electrophoresis apparatus (Biorad)
4. Air displacement pipettes viz. P10, P100, P1000 (Gilsogen,France)
5. Blotting apparatus (IBI scientific USA)
6. Centrifuge
7. Dry bath, Bangalore Genei
8. Gel documentation analysis system, DNR-Bio Imaging System (Minibus Pro)
9. Hot air oven (Yorco instrument, Bombay)
10. Ice flaking machine (Harrison Scientific Instrument Co. Delhi)
11. Incubator (humid CO_2 incubator recommended)
12. Liquid nitrogen (LN_2) freezer or cryostorage container
13. Nanodrop spectrophotometer

Table 1. Target gene, primer sequences and resulting fragment size used in qPCR study

Gene	Sequence of nucleotide (5'-3')	Fragment size (bp)	Annealing Temp (^{0}C)	NCBI/ reference
MCT1	F : CCTCAACCGGGCTTTCTTTATG R : TCCCTTTCTCTACAGTGGTTG	115	58	XM_013962525.1
MCT2	F : GCCTGGTGGTGTATGCTGTATTT R :ACCAAGGAGGACAGGACAACACT	152	58	NC_022297.1
MCT4	F : TTCCCCAAGGCGGTCAGTGTCT R :GCAGCCAAAGCGATTCACACA	150	58	NC_022311.1
HSP70	F: GACGACGGCATCTTCGAG R :GTTCTGGCTGATGTCCTTC	132	58	Dangi et al. (2012)
RPS15a	F:AATGGTGCGCATGAATGTC R :GACTTTGGAGCACGGCCTAA	125	60	XM_005679050.1
GAPDH	F : GCGATACTCACTCTTCTACTTTCGA R : TCGTACCAGGAAATGAGCTTGAC	82	60	U85042.1

NCBI accession number or reference of published sequence

14. Non refrigerated Centrifuge (Remi, India)
15. PowerPac-200 Bio-Rad
16. Refrigerated Microcentrifuge (Hettich, Germany)
17. Refrigerator and freezer (-20^{o}C)
18. Refrigerator (4^{o}C)
19. RT-PCR Quantam Biotech s-96
20. Sanyo, microwave oven
21. Scientronic Double distillation apparatus
22. Spinix vortex machine
23. Sterilizer (i.e., autoclave)
24. Thermal cycler, Eppendorf tubes
25. Water bath
26. Weighing balance, Sartorius, Germany

3.6 Methods

Standard protocols have been followed everywhere with slight modifications.

3.6.1 Animals and sampling

The present study was conducted in 12 animals each from Black Bengal goats. The animals were divided equally into three groups and the experiment was carried out in three different ambient temperature of the year, viz, thermo-neutral (mid-January to mid-February), winter (December to January), and summer (mid-April to mid-June). The goats were raised at a Goat farm, Kalyani, West Bengal, India, latitude 22°562 N and longitude 88°322 E. The ambient temperature at the day of slaughter in thermo-neutral zone was 22^{0}C, in winter was 16^{0}C and in summer 34^{0}C. On the day of slaughter, goats were transported from the farm to the slaughter house located nearby and were allowed to rest 3 h before exsanguination.

3.6.2 Collection of samples

Apparently normal GI tract of healthy goats were collected at a local slaughterhouse within 10 to 20 min after exsanguinations and were transported on ice to the laboratory. The forestomach (rumen, reticulum, omasum and abomasum), small intestine (duodenum, jejunum and ileum), large intestine (caecum and colon) were identified and collected separately for RNA extraction, immunoblotting, and immunohistochemistry studies. All tissue samples were frozen in liquid nitrogen and stored at -80°C until RNA and protein isolation (Kumar et al., 2012).

3.6.2.1 Quantitative RT-PCR analysis

Total RNA was isolated from tissue by Trizol reagent (Invitrogen, USA) according to manufacturer instructions. RNA quality, quantity and integrity were verified by agarose gel electrophoresis and spectrophotometric readings. Constant amounts of 1 µg of total RNA were reverse transcribed using RevertAid First Strand cDNA synthesis kit (Thermo scientific) at 65^{0}C for 5 min Followed by 42^{0}C for 60 min and at last 70^{0}C for 5 min. The resulting complimentary DNAs (cDNAs) were used in qRT-PCR reactions.

3.6.2.2 Total RNA extraction from Tissue

Total RNA extraction from different parts of gastrointestinal tract such as rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, caecum and colon were done manually using TRIzol reagent (INVITROGEN, UK) described as below:

1. Approximately 100mg of the tissue sample (stored at -80°C) was crushed/grinded in pre-chilled mortar and pestle with LN_2.
2. The thoroughly grinded tissue were taken separately in 0.1% DEPC treated eppendorf tubes containing 1ml TRIzol reagent, homogenised properly in a homogenizer and kept in a fresh eppendorf tube at room temperature for 10min.
3. The tube was centrifuged at 12000Xg at 4°C for 10mins and to the supernatant 200 µl of Chloroform per 1ml of TRIzol reagent was added, mixed by inverting and kept at room temperature for 10 min.
4. The tube was again centrifuged at 4°C for 10min at 12000Xg and the upper aqueous phase was taken in a new 0.1% DEPC treated eppendorf tube.
5. To the aqueous phase 500µl of isopropanol per 1ml of TRIzol reagent was added and kept at room temperature for 10-15 min after proper mixing.

6. It was centrifuged at 12000Xg for 15 min at 4°C to precipitate the RNA as pellet

7. The RNA pellet was washed with 70% ethanol by centrifuging again at 4°C for 5 min at 12000Xg.

8. The pellet was air dried and dissolved in 30 µl of 0.1% DEPC water at 65°C for 5 min.

3.6.2.3 DNase Treatment of the isolated RNA

Contaminating DNA was removed from isolated RNA by treating the RNA with RNase free DNase I as described below:

1. To the sample 0.5µl of RNasin and 1.5µl of RNase free DNase I was added and incubate at 37°C for 45 min in hot water bath.

2. To the sample 68µl of 0.1% DEPC water was added to make up the volume to 100µl and then an equal volume of TRIzol reagent was added, mixed gently and kept at room temperature for 5 min.

3. The tube was centrifuged at 12000Xg for 10 min at 4°C and 20µl of Chloroform was added to the supernatant, mixed slowly and incubated at room temperature for 10 min.

4. The tube was then centrifuged at 12000Xg at 4°C for 10 min and the upper aqueous phase was taken in a new 0.1% DEPC treated eppendorf tube.

5. To the aqueous phase 50µl of isopropanol was added and kept for 10min at room temperature.

6. Then the tube was centrifuged at 12000Xg for 15 min at 4°C to precipitate the RNA as pellet.

7. The RNA pellet was washed with 100 µl of 70% ethanol made with 0.1% DEPC water by centrifuging at 12000Xg for 10min at 4°C and pellet was semi-dried at room temperature and was dissolved in 20µl of 0.1% DEPC water by incubating at 65°C for 5 min in a water bath.

3.6.2.4 RNA Integrity and Purity

The integrity of total RNA was checked on 1.0% agarose gel using 1X TBE as electrophoresis buffer. Total RNA was in good yield in all the samples. The bands of 28sRNA and 18sRNA reflected the high quality of extracted total RNA. The purity and concentration of total RNA was checked using nanodrop. Isolated RNA samples were free from the protein contamination as the OD 260: OD 280 values were more than 1.8. The concentrations of the RNA samples were in the range of 500-2000 ng/µl.

3.6.2.5 Synthesis of cDNA

The first strand cDNA was synthesized from the isolated total RNA. RT-PCR was done using reverse transcription system RevertAid First Strand cDNA synthesis kit (Thermo scientific) following manufacturers instruction. Reverse transcription was carried out in 20µl reaction mixtures. To take 1µg of total RNA per reaction, calculation was done using concentration of total RNA from nanodrop (ng/µl).

Components of reaction mixture	**Quantity**
RNA template	1µg/reaction (vol. variable)
Nuclease free water	variable (x µl)
Primer	2 µl
5X Reaction Buffer	4 µl
RiboLock RNase Inhibitor (20U/µl)	1 µl
10 mM dNTP Mix	2 µl
RevertAid M-MuLV RT (200U/il)	1 µl
Total	20 µl

Reaction mixture was mixed to RNA-primer complex and spinned, followed by incubation at 65°C for 5 min and 42°C for 60 min. Reaction was stopped by incubating at 70°C for 5 min and finally at 4°C forever. The cDNA was stored at -20°C for long term use.

3.6.2.6 End point PCR optimization

End point PCR was standardized to amplify gene sequence using PCR system (Quantam Biotech s-96). Factor specific primers were used for the amplification genes. The annealing temperature was standardized using cDNA prepared from mRNA of tissue samples by PCR. The reaction was carried out at different gradients of annealing temperatures (58°C/ 60°C). The optimum temperatures were found to be most suitable for annealing for primers and were used in subsequent polymerase chain reaction.

The reaction mixture was put according to standardized PCR reaction mixture composition. cDNA was diluted to ten times with nuclease free water. Following reactant were added to a nuclease free thin walled 0.2ml micro centrifuge tube pre- chilled on ice in same order.

Components	**Volume**
Platinum ® PCR Super Mix	5 µl
Forward primer (10µM)	0.25 µl
Reverse primer (10µM)	0.25 µl
cDNA template	0.5 µl
Nuclease free water	4µl
Total	10.00 µl

The contents were gently vortexed and then spun down to collect at the bottom of tube by brief centrifugation. The reaction was carried out in a thermal cycler using the following cycling parameters that was found optimum for amplification of gene fragments.

Cycling steps	**Temp.**	**Time**
Initial denaturation	95°C	5 min
40 cycles of Denaturation	95°C	30 sec
Annealing	58°C /60°C	30 sec
Extension	72°C	45 sec
Final extension	72°C	5 min
Hold	4°C	

The primers specific annealing temperature employed for various examined factors were as follows **58°C** for MCT1, MCT2 and MCT4, **60°C** for HSP70,GAPDH and RPS15A.

3.6.2.7 Agarose gel electrophoresis

The confirmation of amplification of specific qPCR amplicon was done by agarose gel electrophoresis (Appendix)**.** 2.0% agarose was mixed with 1X TAE buffer and melted in a microwave oven. When the molten gel had cooled to about 42°C, ethidium bromide was added to make final concentration 0.5 µg/ml. The gel was mixed thoroughly by gentle swirling and then poured into the gel casting tray fitted with the comb. The gel was allowed to solidify and the comb was removed. The PCR product were mixed with 1x gel loading dye (Final concentration) and loaded into the wells. For the comparison a 50bp molecular weight markers were gel electrophoresed in parallel to the qPCR amplicons. The gel was run at a voltage of 10 V/cm till the running dye crossed at least two third of the gel. The bands were visualized under UV light and recorded on a gel documentation system {DNR-BioImaging system (Minibus Pro)}.

3.6.2.8 Determination of efficiency of primers

Efficiencies were determined by running a standard curve for each assay prior to processing experimental samples. A standard curve was obtained by serial dilution of the cDNA containing the template and a regression line equation in relation to the threshold values ("Ct") was formulated. To obtain best accuracy level 6 serial dilutions of cDNA were taken in triplicate. Slopes were calculated using Bio-Red CFX Manager (version 3.0).

3.6.2.9 Real time PCR

Quantitative Real-time PCR was performed with SsoFast TM Eva Green ®Supermix kit (Bio-Rad, USA), Bio-Red CFX-96 Real Time PCR operated by Biored CFX Manager (version 2.1). Reaction setup was performed in area separate from nucleic acid preparation or PCR product analysis. Pipetting was done with sterile filter tips. Exposure of light to the qPCR mastermix was minimised. Careful pipetting was done without creating bubbles to avoid interference in reading of fluorescence by the instrument. No template control (NTC) was put for either gene quantification for checking the contamination in the reaction components other than the cDNA. To ensure the cDNA samples were not contaminated with genomic DNA, reactions were set up using 10ng of non-reverse transcribed RNA in place of cDNA in NTC and it was failed to generate a detectable signal signified the samples as DNA free. For reaction set up optically clear caps were used 0.5 µl of cDNA was taken. Following master mix was prepared:

The qPCR for each cDNA and the housekeeping gene GAPDH and RPS15A was performed in duplicate

Components	Volume
Nuclease free water	4 µl
Forward primer (10 pm/µl)	0.25 µl
Reverse primer (10 pm/µl)	0.25 µl
cDNA	0.5 µl
Eva Green®	5 µl
Total volume	10 µl

Touching of the optical surface of the caps without gloves was avoided. Strips were centrifuged before starting the cycling programme to force the solution to the bottom of the tubes and to remove any possible bubbles.

3.6.2.10 Gene Expression Analysis

The thermo-nutral season sample was used as calibrator for obtaining relative mRNA expression in winter and summer season samples. GAPDH and RPS15A were used as housekeeping gene. Efficiency corrected relative quantification of mRNA was obtained as described earlier (Pfaffl, 2001). For this, efficiency of primers was determined by serial dilution of template cDNA sample and running in triplicate.

The qPCR for each cDNA and the housekeeping gene GAPDH and RPS15A were performed in duplicate using SsoFast ™ Eva Green ®Supermix kit (Bio-Rad, USA) in Bio-Red CFX-96 (Real Time) system instrument as per manufacturers' instructions. Briefly, PCR templates containing 0.5µl reverse transcribed total RNA was added to 0.25 µl forward primer (0.2mM), 0.25 µl reverse primer (0.2mM) and 5 µl of SsoFast ™ Eva Green ®Supermix to a final volume of 10 µl and were subjected to general real-time PCR protocol for all investigated factors. The following general real-time PCR protocol was employed for all investigated factors: denaturation for 30sec at 95^{o}C, 40 cycles of a three segmented amplification and quantification program [denaturation for 10sec at 95^{o}C, annealing for 10sec at the primer specific temperature (58^{o}C for MCT1, MCT2 and MCT4,60^{o}C for HSP70,GAPDH and RPS15A elongation for 15 sec at 72^{o}C), a melting step by slow heating from 60 to 95^{o}C with a rate of 0.58^{o}C/sec and continuous fluorescence measurement, and a final cooling down to 4^{o}C. After the run ended, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the "EVA green (with dissociation curve)" method of the real time machine (Biored CFX-96).

Real-time PCR efficiencies were determined by amplification of a standardized dilution series, and slopes were obtained. The specificity of desired products was documented using analysis of melting temperature, which is product specific and a high resolution gel electrophoresis to verify that transcripts were of exact molecular size and further confirmed by sequence analysis. Negative control PCR containing all components except template were included for each sample to check out the formation of primer dimer. For qPCR analysis of each gene including the house keeping gene, three replicates of each season sample from each group of animals were used. The amplification plot and dissociation curve of all the genes are shown in Fig. 5-11 (A, B) and Fig. 19-28 (A, B) respectively.

3.6.2.11 Calculation of Relative Expression

Optical data were collected at end of each extension step, and relative expression of PCR product was determined by the equation (Pfaffl, 2001) given below:

$$\text{Ratio} = \frac{\mathbf{(Etarget)}^{\text{ÄCt target (control - sample)}}}{\mathbf{(Eref)}^{\text{ÄCt ref (control - sample)}}}$$

Where, **ratio** is the relative expression, **Etarget** is the real time efficiency of target gene transcript, **Eref** is the real time efficiency of housekeeping gene transcript.

3.6.3 Immunoblotting

To obtain total proteins, liquid nitrogen triturated tissues were suspended in RIPA lysis (Ameresco) buffer and Halt protease inhibitor cocktail (Thermo Scientific), homogenized, and centrifuged at 12,000g. The supernatant containing mostly the total soluble protein was estimated using Bradford protein assay. Furthermore, the supernatant was diluted in sodium dodecyl sulfate (SDS) sample buffer (final concentration to 60 mMTris, pH6.8, 2% SDS, 100mM dithiothreitol, and 10% glycerol), followed by boiling for 5 min. The protein samples (100mg from each sample) were subjected to SDS 12.5% polyacrylamide gel electrophoresis, electro transferred onto polyvinylidene difluoride membrane (PVDF), and blocked with 5% bovine serum albumin (BSA) before incubation with primary antibodies, namely, rabbit polyclonal MCTs (MCT1, MCT2 and MCT4) at a 1:200 dilution for overnight at 4°C. After incubation, the membrane was washed thrice with PBS-T (PBS 0.01% Tween 20) for 5 min each and the respective secondary antibody conjugated with horseradish peroxidase was added at 1:5000 dilutions and incubated at 37°C for 1 h. After washing 3 to 4 times in PBS-T solution, the positive signals were detected by incubating the membrane using 0.06% 3, 3'-diaminobenzidine tetrahydrochloride (Genei) in 1xPBS (pH 7.4) containing 0.06% H_2O_2 for 10 to 15 min. The bands were visualized under white light and recorded on a digital camera.

Detailed procedure:-

3.6.3.1 To obtain total proteins

1. Different season tissue samples were triturated in liquid nitrogen in a pestle mortar.
2. The triturate was suspended in Tissue-PE LBTM (G Biosciences, USA) buffer with Halt TM protease inhibitor cocktail (Thermo Scientific) mixture, homozenized and kept in ice for 30 min.
3. The homogenate was centrifuged at 12000Xg for 30min. The supernatant containing the total soluble protein was taken in a eppendorf tube and kept in - 20°C for future use.

3.6.3.2 SDS-PAGE and immunoblotting

SDS-PAGE was carried out in a vertical maxigel electrophoresis apparatus. Glass and notch plates were cleaned and set in a gel caster. Running gel was prepared by accurate combination of given chemicals, poured between the two plates. 0.2-0.3ml butanol was poured over the gel to prevent oxidation. After polymerization, butanol was thoroughly removed with distilled water (DW). Stacking gel was layered over the running gel with appropriate comb. The polymerized gel was set onto the electrophoresis unit along with running buffer. The sample stored in -20^{o}C freezer was thawed, diluted in sodium dodecyl sulphate (SDS) sample buffer (final concentration to 60 mM Tris, pH 6.8, 2% SDS, 100 mM dithiothreitol, and 10% glycerol), boiled for 5min and immediate cooling in ice for 5min. 10 µl of each sample (100 mg) were loaded along with a standard protein ladder (MBI- Fermentas). Electrophoresis was carried out at a constant voltage of 60V for a period of 15min (sample crossing the stacking gel) and then with constant voltage of 150 V, until the tracking dye reaches the bottom of the gel. After the run, gel was removed from the unit and stained with Coomassie brilliant blue for 1h and then destained with de-staining solution. Proteins were characterized by Western blot analysis in order to confirm specificity.

The protein was run in 12.5% SDS-PAGE gel along with pre-stained protein marker. After electrophoresis the gel was taken out from the plates and kept in transfer buffer for atleast 5min prior to transfer. PVDF membrane (milipore, USA) was soaked in methanol for 5min followed by transfer buffer. Four Whatman filter papers (pre-soaked with transfer buffer), PVDF membrane, gel and another set of pre-soaked Whatman filter papers were stacked one by one in respective order on the anode plate of blotting apparatus (IBI SCIENTIFIC USA). Care was taken for avoiding air bubbles in between gel and PVDF membrane. The complete stack was saturated with ice cold transfer buffer before the cathode plate was placed in position over the stack and a constant current of 0.8 mA/cm^2 was applied for 1h. After transfer, the gel was stained to check the efficiency of transfer of protein from gel to the membrane and the membrane was subjected to immunological detection. The membrane, after transfer, was incubated 2h at 37^{o}C in 2% bovine serum albumin diluted with PBS in rotary shaker. After blocking, the membrane was washed thrice with PBS-T (PBS+0.01% Tween 20) for 5 min each and incubated with primary antibodies i.e. MCT1, MCT2 and MCT4 at 1:200 dilution for overnight at 4^{o}C.

After incubation, membrane was washed thrice with PBS-T (PBS+0.01% Tween 20) for 5 min each then respective secondary antibody conjugated with horse radish peroxidase was added and incubated at 37°C for 1h. After washing 3 to 4 times in PBS-Tween 20 solution. The positive signals were detected by incubating the membrane using 0.06% 3,30-diaminobenzidine tetrahydrochloride (Genei) in 1X PBS (pH 7.4) containing 0.06% H_2O_2 for 10 to 15 min. The bands were visualized under white light and recorded on a digital camera.

3.6.4 Immunohistochemistry

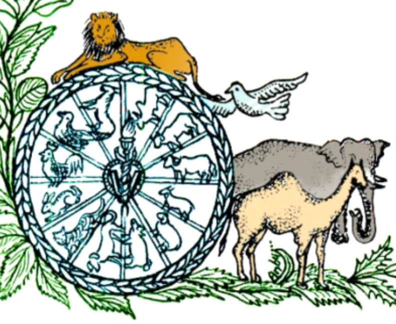
Freshly collected tissue samples were fixed with 10% neutral buffer formalin, dehydrated through a series of graded alcohols, paraffin-embedded, serial-sectioned (5 µm), mounted on 3-Aminopropyl triethoxysilane (Thermo Scientific) coated slides, and dried at 37°C over night. Deparaffinization was carried out in xylene followed by rehydration in a series of graded alcohols at room temperature, epitope retrieval in sodium citrate buffer (10 mM sodium citrate, pH 6.0, 0.05% Tween- 20), rinsing, and blocking with 5% BSA for 2 h at 37°C. Subsequently, sections were probed with rabbit polyclonal MCTs (MCT1, MCT2 and MCT4) antibodies, at 1:200 dilutions. Primary antibodies were detected by fluorescent conjugated goat anti rabbit IgG-PE secondary antibody at 1:400 dilutions. The slides were rinsed and 0.4 mg/mL of 4, 6-diamidino-2- phenylindoledihydrochloride (DAPI) in PBS was applied to stain the nuclei of the cells in the tissue sections. The control slides were processed under similar conditions except for the addition of isotype IgG and omission of the primary antibody. Fluorescently stained sections were mounted with antifade mounting media (MP Biomedicals) and images were captured using AxioObserver.Z1 (Carl Zeiss Micro Imaging GmbH, Germany) microscope.

3.6.5 Statistical Analyses

All experimental data are shown as Mean ±SEM. The statistical significance of difference in mRNA expression of the examined factors across different parts of GI tracts was assessed using the software SPSS.22 by two-way analysis of variance followed by Duncan's honestly significant differences (HSD) test. Differences were considered significant at $P<0.05$.

* * *

Results



4

RESULTS

4.1. Gene expression analysis

The moderate season was used as calibrator for obtaining relative mRNA expression. RPS15A (Ribosomal protein 15a) and GAPDH were used as housekeeping gene (Fig. 1-2). Efficiency corrected relative quantification of mRNA was obtained by Pfaffl (2001). For this, efficiencies of primers were determined by serial dilution of template cDNA sample and running in triplicate. The relative change in the copy number of MCTs and HSP70 during different season in the Black Bengal goat is presented in Table 2 and Fig. 3-10. Transcripts of MCT1 was significantly upregulated in forestomach and large intestine during both summer and winter as compare to moderate season ($p<0.05$; Fig. 3-4). However, the relative fold change of MCT2 mRNA was significantly upregulated in rumen, reticulum and caecum during summer whereas in caecum and colon in winter season as compared to callibrator ($p<0.05$; Fig. 5-6). The messages of MCT4 (Fig. 7-8) during the different season was comparable with that of MCT1. In contrast, HSP70 showed a higher expression in duodenum and colon in summer than other season ($p<0.05$; Fig. 9-10). Invariably, none of the MCTs showed significant upregulation in small intestine in all the season ($p<0.05$).

4.2. Immunoblot analysis

Immunoblot study in various part of GI tract was done irrespective of any season. The MCT1, MCT2 and MCT4 proteins were visualized on Immunoblotting as bands of molecular weight ~55 kDa, ~43 kDa and ~43 respectively (Fig. 4d, 6d and 8d). Higher expression of proteins MCTs were detected in fore gut (rumen, reticulum and omasum) and hind gut (caecum and colon) whereas less expression in abomasum and small intestine (duodenum, jejunum and ileum) of the Black Bengal goat.

4.3. Immunohistochemistry of GI tract tissue

Although in gene expression study we found a differential pattern of mRNA expression of MCTs but the immunohistochemistry study in various part of GI tract was done irrespective of any season (Fig. 11-14). Immunohistochemistry of the different part of GI tract for MCTs revealed that the peak intensity of fluoroscence is appeared in epithelial linning of rumen, reticulum and omasum as compare to small and large intestine. MCT expression in abomasum, caecum and colon is evidenced by mild fluorescence. However, in jejunum and duodenum the staining showed little fluorescence which was comparable with that of the negative control.

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Table 2. Relative Expression (mean±SEM) of monocarboxylate transporters (MCTs) and HSP70 in various organs of gastrointestinal tract in Black Bengal goat during different seasons. Different superscripts denote statistically different values ($p < 0.05$)

Gene	Season	Rumen	Reticulum	Omasum	Abomasum	Duodenum	Jejunum	Ileum	Caecum	Colon
MCT1	Winter	2.824 ± 0.712^{bc}	$2.826\pm0.839^{bc*}$	1.059 ± 0.359^{a}	0.449 ± 0.250^{a}	1.915 ± 0.411^{ab}	1.419 ± 0.365^{ab}	0.870 ± 0.344^{a}	4.167 ± 0.644^{c}	3.800 ± 0.793^{c}
	Summer	4.955 ± 0.755^{C}	$3.196\pm0.739^{C*}$	$2.906\pm0.704^{BC*}$	0.635 ± 0.291^{A}	1.039 ± 0.277^{AB}	$2.176\pm0.478^{ABC*}$	0.799 ± 0.313^{A}	$3.987\pm1.149^{C*}$	$3.062\pm0.394^{C*}$
MCT2	Winter	0.666 ± 0.125^{a}	0.303 ± 0.101^{a}	0.534 ± 0.112^{a}	0.830 ± 0.243^{a}	0.766 ± 0.308^{a}	0.862 ± 0.309^{a}	1.263 ± 0.553^{a}	$2.731\pm0.619^{b*}$	$2.711\pm0.747^{b*}$
	Summer	$4.212\pm0.731^{A*}$	$3.513\pm0.538^{A*}$	1.050 ± 0.423^{B}	1.368 ± 0.355^{B}	1.027 ± 0.224^{B}	1.714 ± 0.285^{B}	1.125 ± 0.339^{B}	$3.201\pm0.734^{A*}$	1.154 ± 0.396^{B}
MCT4	Winter	$3.746\pm0.661^{c*}$	$4.423\pm0.502^{c*}$	0.411 ± 0.216^{a}	2.281 ± 0.426^{b}	0.513 ± 0.381^{a}	0.925 ± 0.531^{ab}	1.416 ± 0.684^{ab}	$3.735\pm0.858^{c*}$	1.269 ± 0.394^{ab}
	Summer	$4.909\pm0.492^{C*}$	$4.401\pm0.952^{BC*}$	1.700 ± 0.712^{AB}	$3.134\pm0.905^{B*}$	1.447 ± 0.405^{A}	0.332 ± 0.225^{A}	1.612 ± 0.697^{AB}	$2.612\pm0.758^{AB*}$	$2.482\pm0.378^{AB*}$
HSP70	Winter	0.726 ± 0.357^{a}	0.596 ± 0.244^{a}	0.247 ± 0.089^{a}	0.332 ± 0.221^{a}	0.736 ± 0.227^{a}	1.463 ± 0.425^{a}	0.427 ± 0.152^{a}	0.594 ± 0.330^{a}	0.486 ± 0.338^{a}
	Summer	0.642 ± 0.360^{A}	0.631 ± 0.348^{A}	0.296 ± 0.117^{A}	0.514 ± 0.159^{A}	$3.825\pm0.467^{C*}$	0.858 ± 0.257^{A}	0.809 ± 0.347^{A}	1.163 ± 0.416^{AB}	$2.058\pm0.413^{B*}$

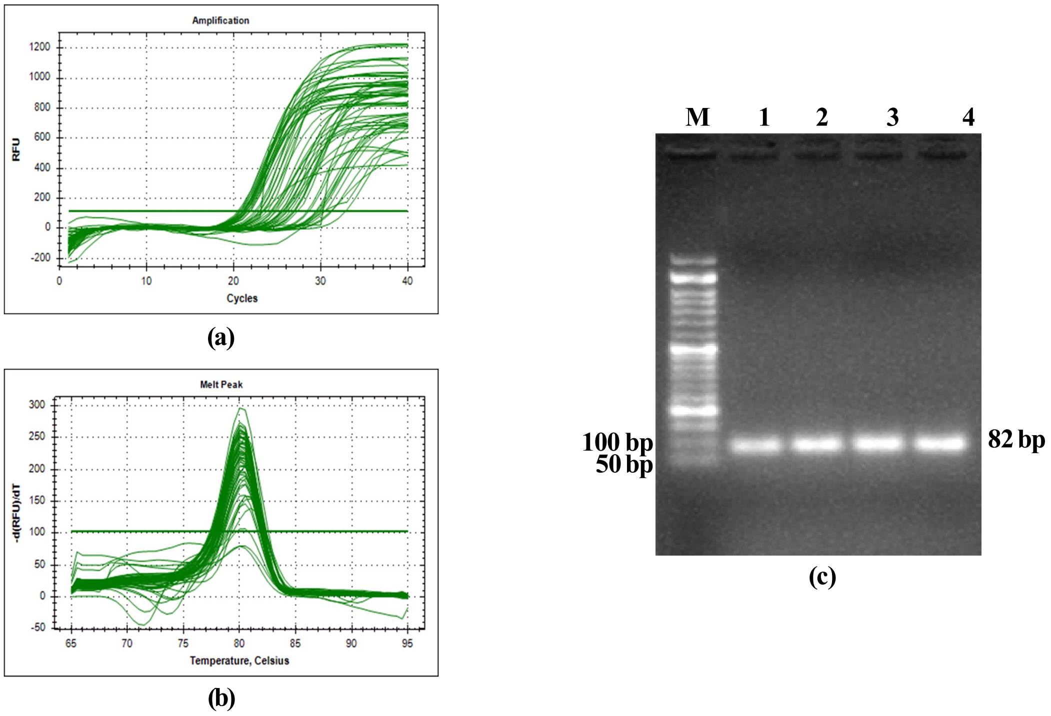


Fig. 1: GAPDH a) Amplification plot; b) Melt peak; c) 2% agarose gel showing 82 bp PCR products

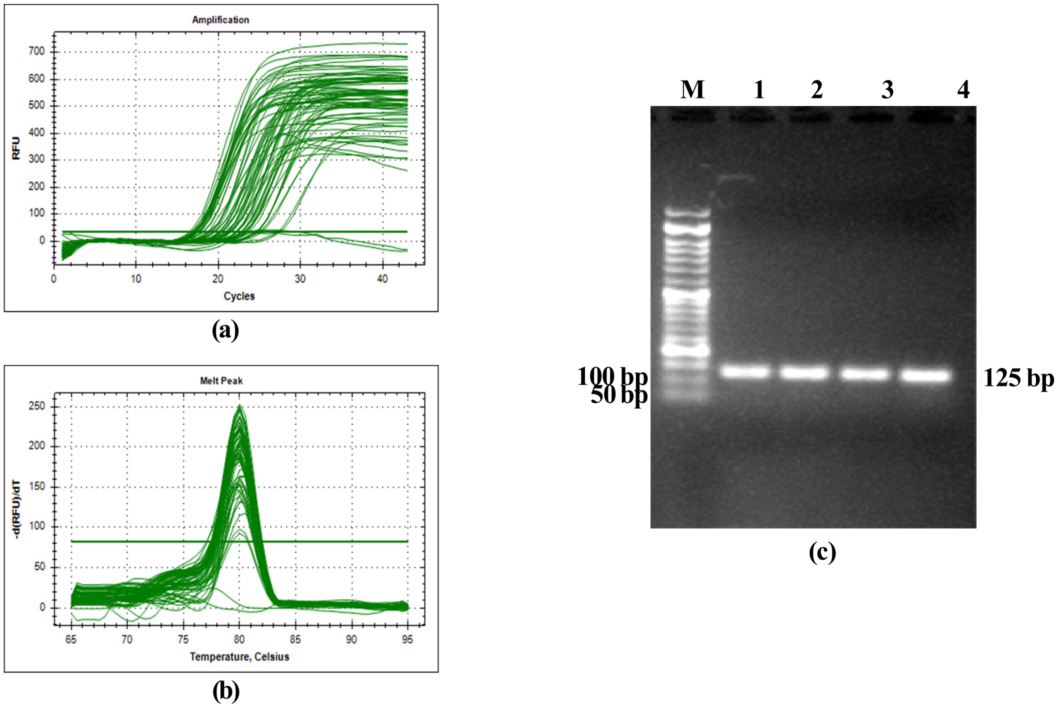
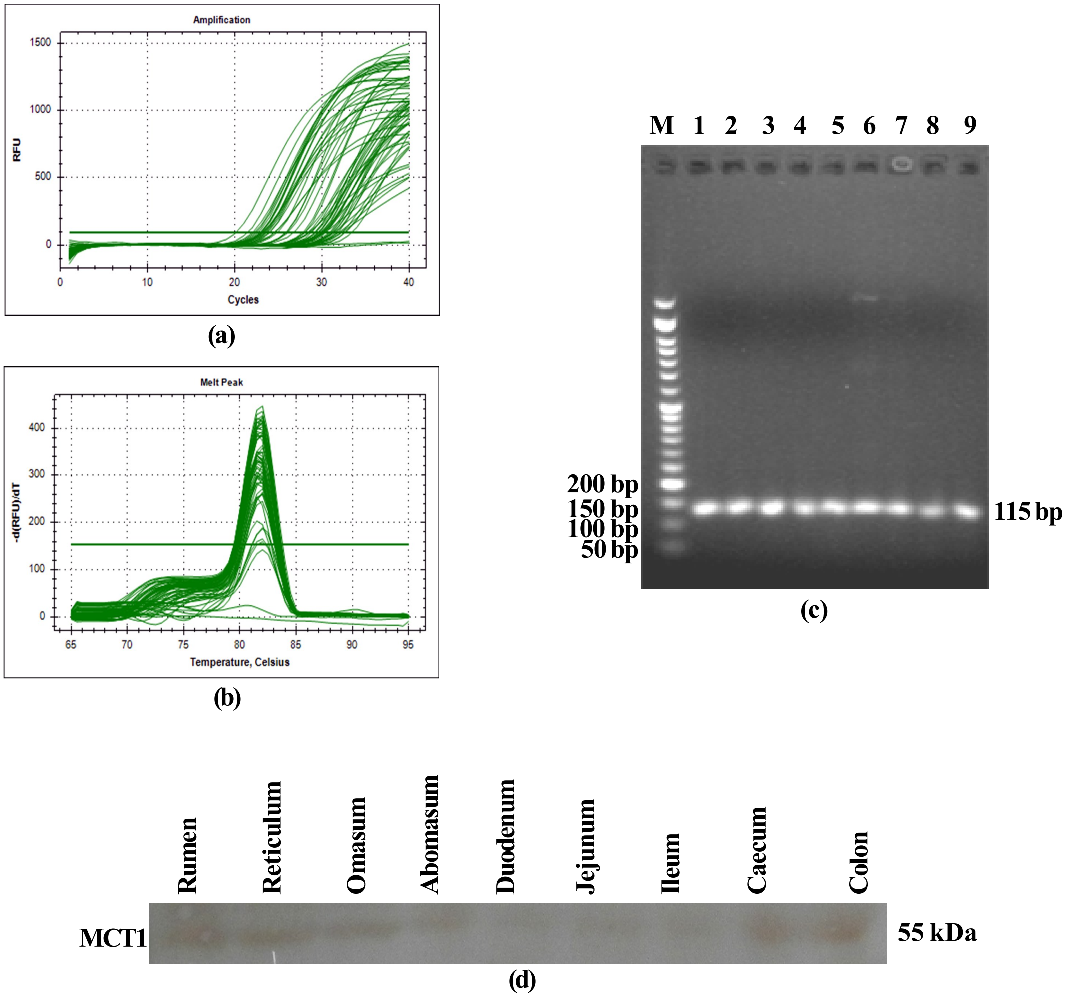


Fig. 2: RPS15A a) Amplification plot; b) Melt peak; c) 2% agarose gel showing 125 bp PCR products



(a) (b) (c) (d)

Fig. 3: MCT1 a) Amplification plot; b) Melt peak; c) 2% agarose gel showing 115 bp PCR products; d) Western blotting showing 55 kDa protein

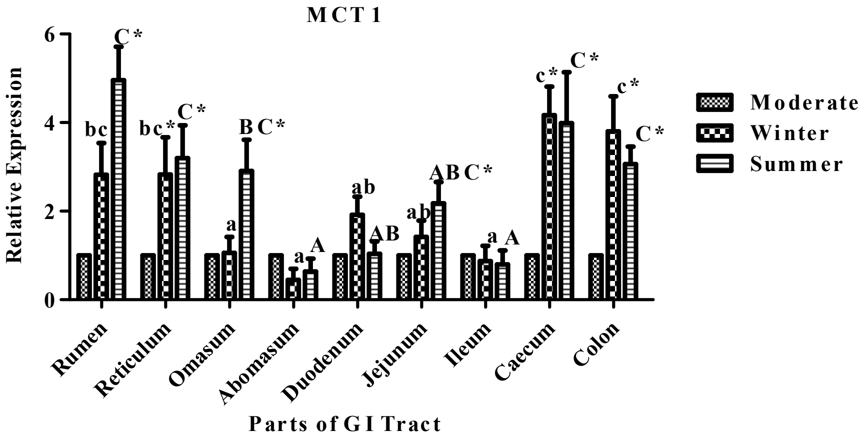
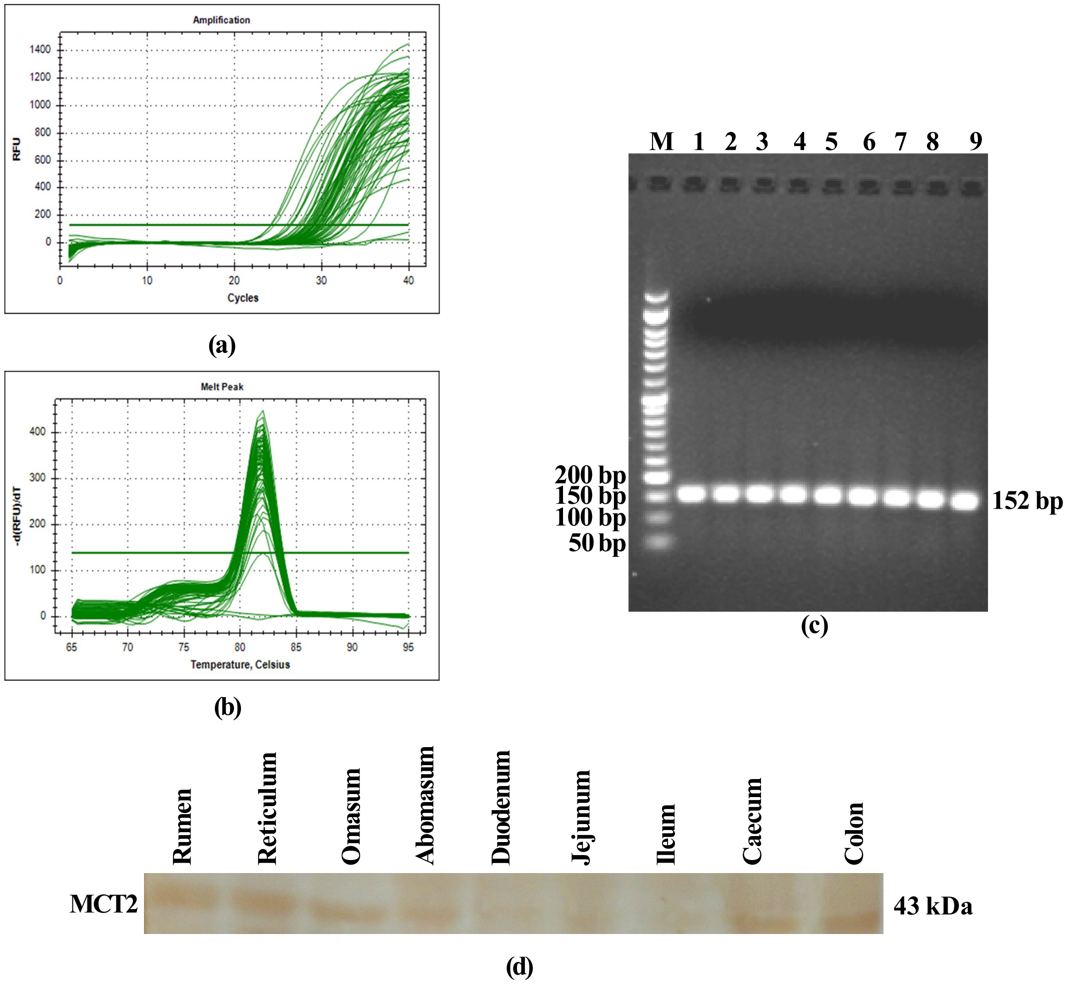


Fig. 4: Transcriptional profile of monocarboxylate transporter 1 (MCT1) in different parts of gastrointestinal tract of Black Bengal goat during different seasons. moderate season served as calibrator to calculate the fold change. RPS15A and GAPDH were used as reference gene to calculate ΔCt (delta C_t). Two way ANOVA followed by Duncan's *post-hoc* test was done to find the between as well as within group difference. Data represents the mean±SEM (n=4). Different superscripts denote statistically significant difference (p<0.05).



(a)

(b)

(c)

(d)

Fig. 5: MCT2 a) Amplification plot; b) Melt peak; c) 2% agarose gel showing 152 bp PCR products; d) Western blotting showing 43kDa protein

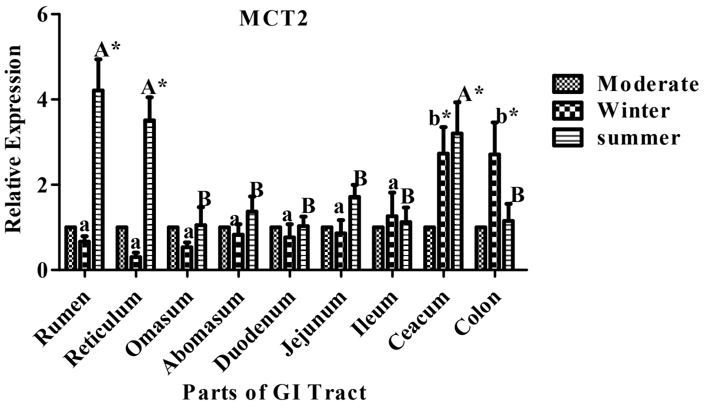


Fig. 6: Transcriptional profile of monocarboxylate transporter 2 (MCT2) in different parts of gastrointestinal tract of Black Bengal goat during different seasons. moderate season served as calibrator to calculate the fold change. RPS15A and GAPDH were used as reference gene to calculate ΔCt (delta C_t). Two way ANOVA followed by Duncan's *post-hoc* test was done to find the between as well as within group difference. Data represents the mean±SEM (n=4). Different superscripts denote statistically significant difference ($p<0.05$).

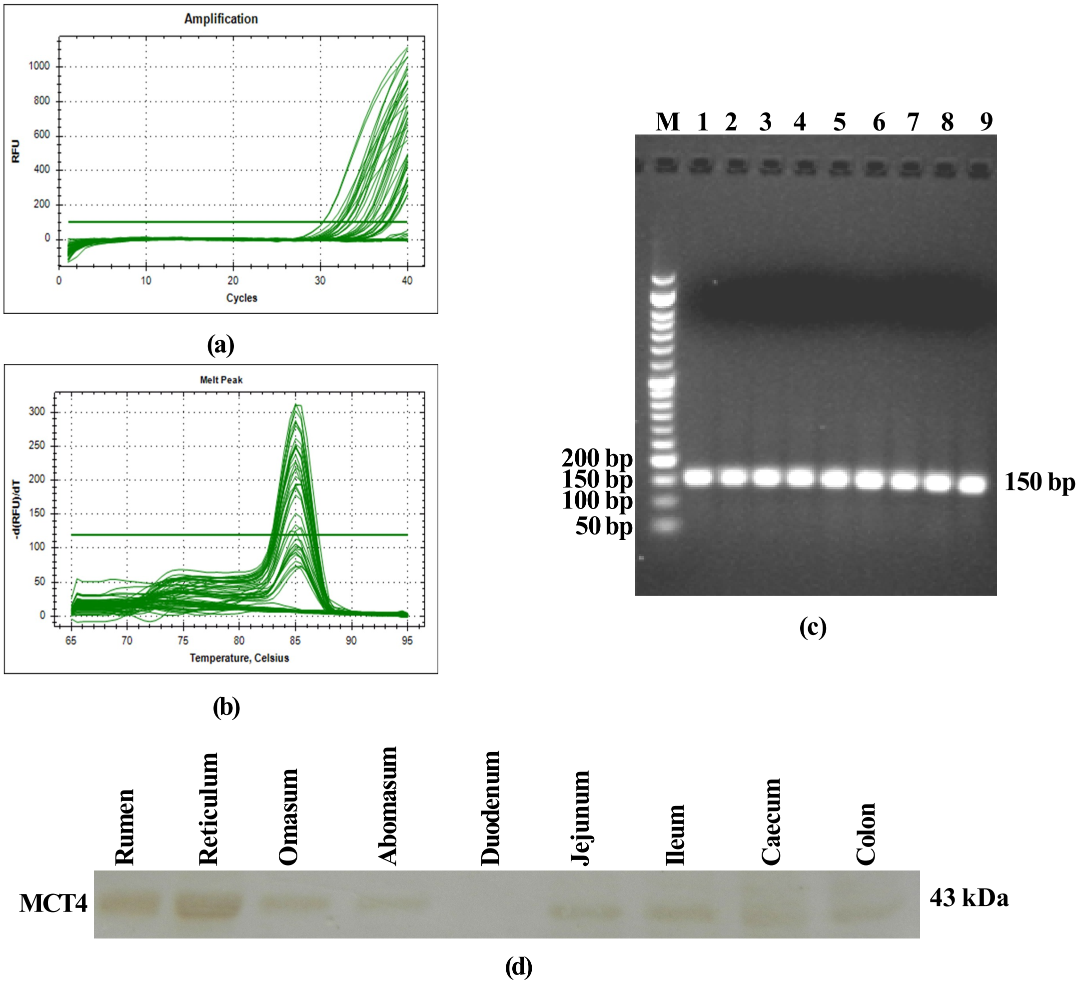


Fig. 7: MCT4 a) Amplification plot; b) Melt peak; c) 2% agarose gel showing 150 bp PCR products; d) Western blotting showing 43 kDa protein

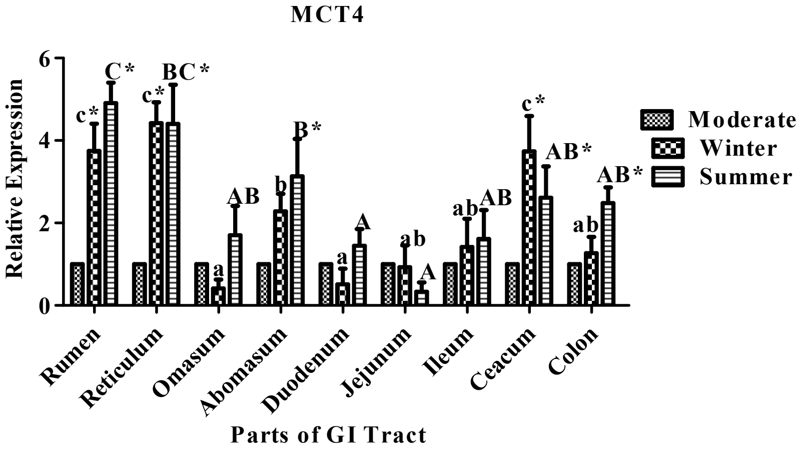
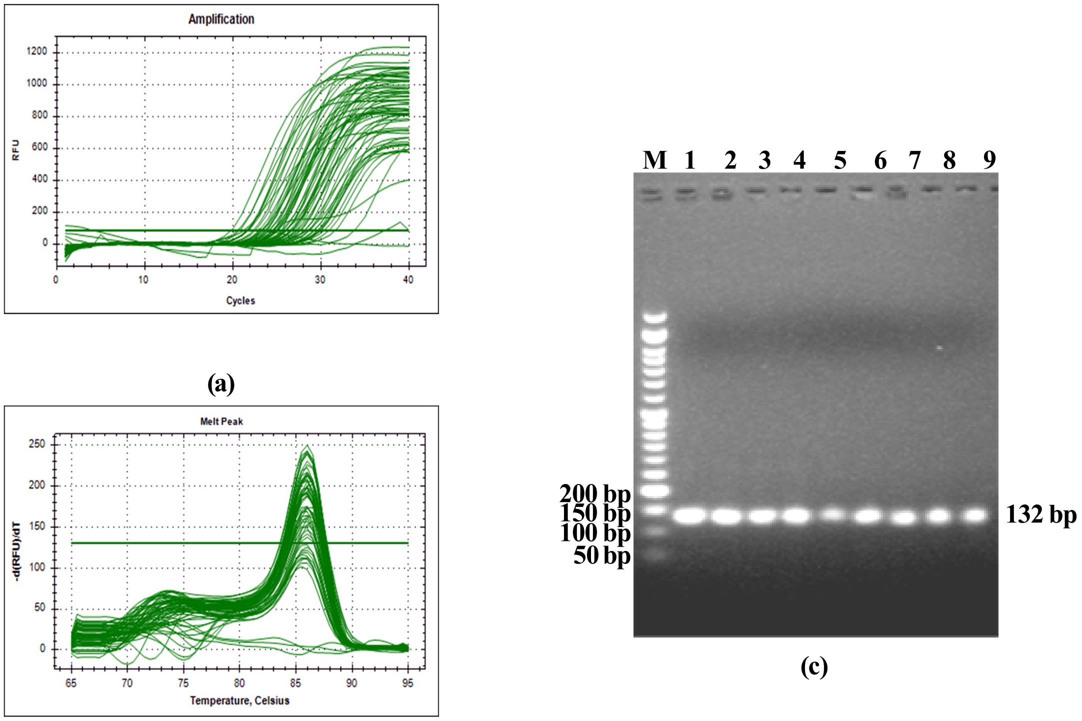


Fig. 8: Transcriptional profile of monocarboxylate transporter 4 (MCT4) in different parts of gastrointestinal tract of Black Bengal goat during different seasons. moderate season served as calibrator to calculate the fold change. RPS15A and GAPDH were used as reference gene to calculate ΔCt (delta C_t). Two way ANOVA followed by Duncan's *post-hoc* test was done to find the between as well as within group difference. Data represents the mean±SEM (n=4). Different superscripts denote statistically significant difference (p<0.05).



(a)

(b)

(c)

Fig. 9: HSP70 a) Amplification plot; b) Melt peak; c) 2% agarose gel showing 132 bp PCR products

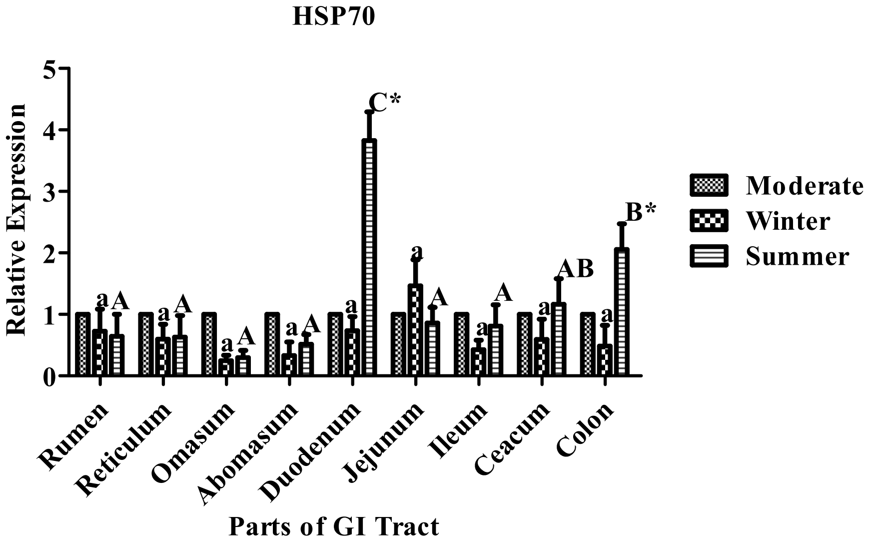


Fig. 10: Transcriptional profile of Heat shock protein 70 (HSP70) in different parts of gastrointestinal tract of Black Bengal goat during different seasons. moderate season served as calibrator to calculate the fold change. RPS15A and GAPDH were used as reference gene to calculate ΔCt (delta C_t). Two way ANOVA followed by Duncan's *post-hoc* test was done to find the between as well as within group difference. Data represents the mean±SEM (n=4). Different superscripts denote statistically significant difference ($p<0.05$).

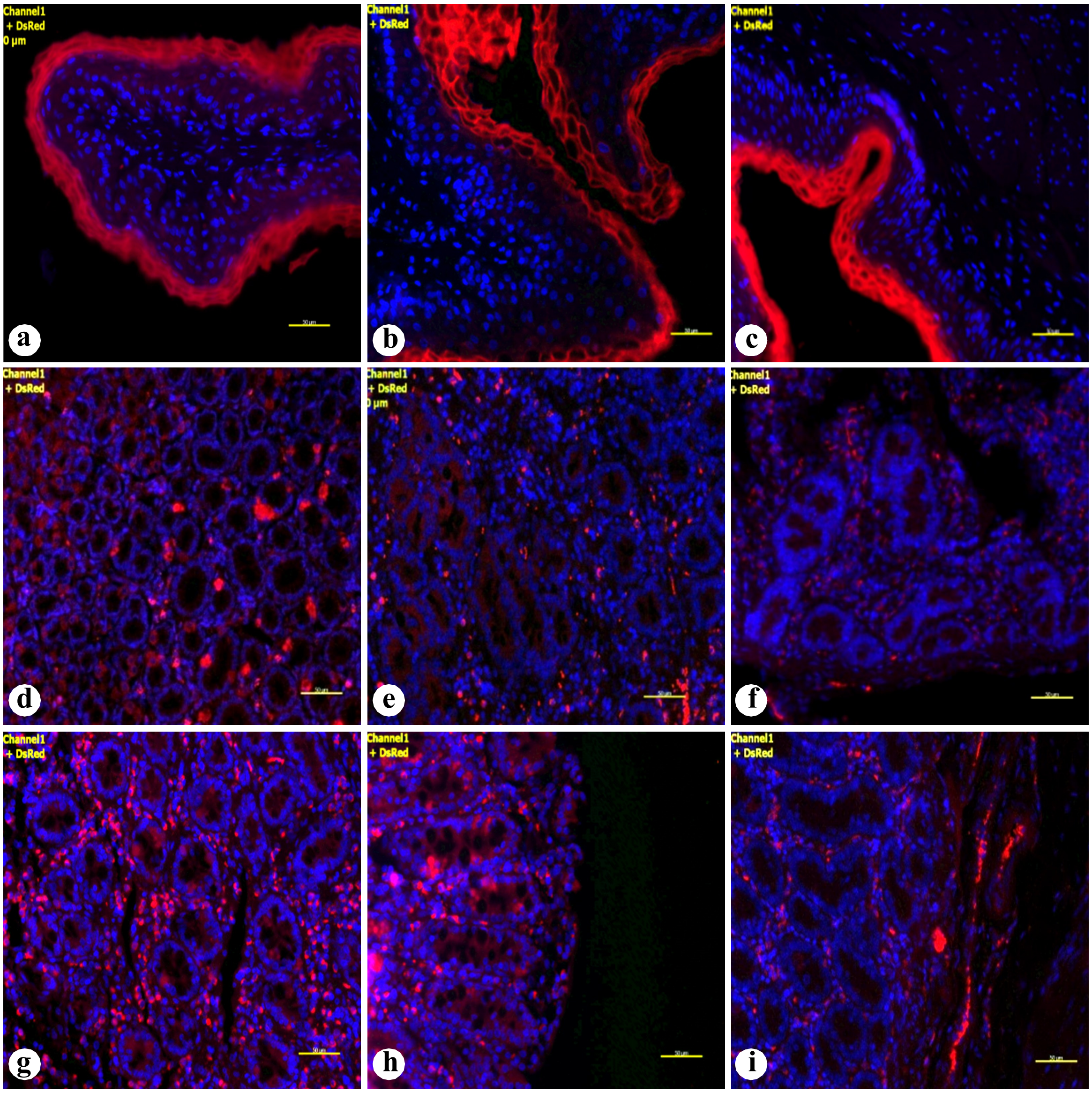


Fig. 11: Fluorescent immunohistochemical localization of MCT1 at different parts of GI tract of Black Bengal goat. Briefly, antigen retrieval was done using sodium citrate buffer method on 5µm thick paraffin embedded sections of GI tract tissue. BSA 5% was used to minimize the non-specific binding. Primary MCT1 antibody was used at 1:200 while the secondary antibody coated with PE was used at 1:400. Nucleus was counterstained with DAPI. Red filter was used while examination of the stained sections under the fluorescent microscope (Carl Zeiss Micro Imaging GmbH).Representative images a) rumen, b) reticulum, c) omasum, d) abomasum, e) duodenum, f) jejunum, g) ileum, h) caecum, i) colon. Scale bar =50 µm. Abbreviations: MCT, monocarboxylate transporter; GI, gastrointestinal; PE, phycoerythrin; DAPI, 4',6-diamidino-2- phenylindoledihydrochloride.

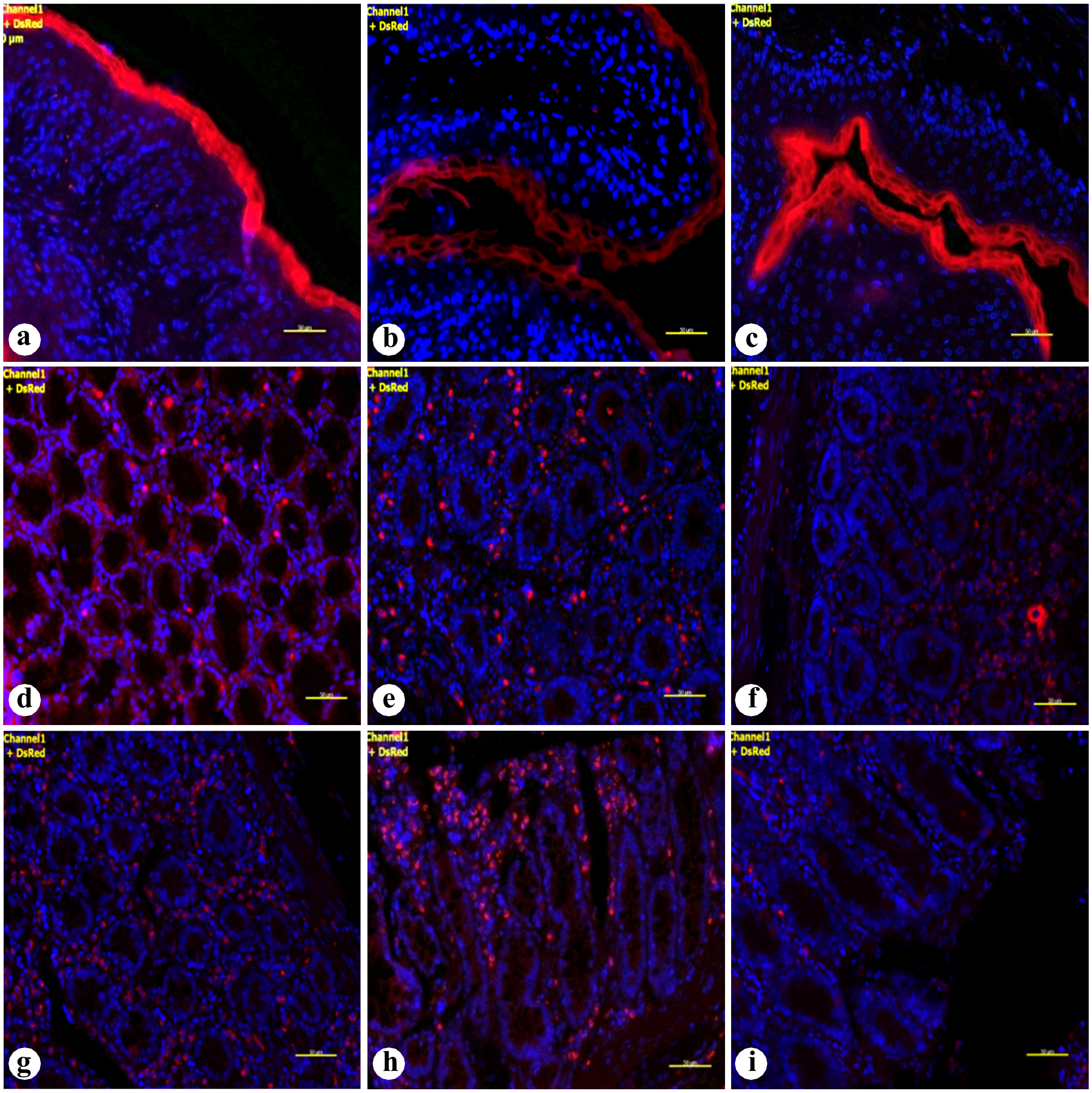


Fig. 12: Fluorescent immunohistochemical localization of MCT2 at different parts of GI tract of Black Bengal goat. Briefly, antigen retrieval was done using sodium citrate buffer method on 5µm thick paraffin embedded sections of GI tract tissue. BSA 5% was used to minimize the non-specific binding. Primary MCT2 antibody was used at 1:200 while the secondary antibody coated with PE was used at 1:400. Nucleus was counterstained with DAPI. Red filter was used while examination of the stained sections under the fluorescent microscope (Carl Zeiss Micro Imaging GmbH). Representative images a) rumen, b) reticulum, c) omasum, d) abomasum, e) duodenum, f) jejunum, g) ileum, h) caecum, i) colon. Scale bar =50 µm. Abbreviations: MCT, monocarboxylate transporter; GI, gastrointestinal; PE, phycoerythrin; DAPI, 4',6-diamidino-2- phenylindoledihydrochloride.

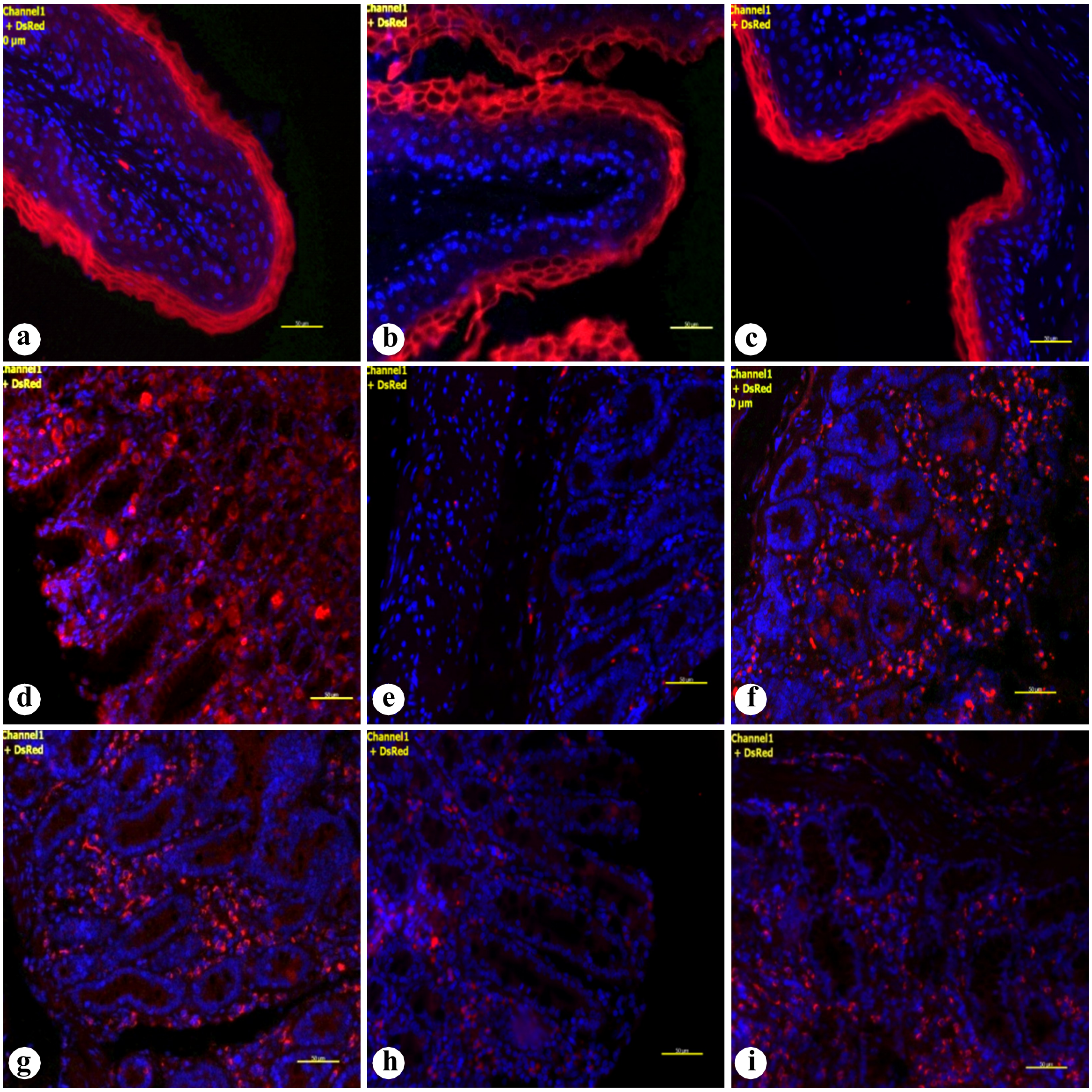


Fig. 13: Fluorescent immunohistochemical localization of MCT4 at different parts of GI tract of Black Bengal goat. Briefly, antigen retrieval was done using sodium citrate buffer method on 5µm thick paraffin embedded sections of GI tract tissue. BSA 5% was used to minimize the non-specific binding. Primary MCT4 antibody was used at 1:200 while the secondary antibody coated with PE was used at 1:400. Nucleus was counterstained with DAPI. Red filter was used while examination of the stained sections under the fluorescent microscope (Carl Zeiss Micro Imaging GmbH).Representative images a) rumen, b) reticulum, c) omasum, d) abomasum, e) duodenum, f) jejunum, g) ileum, h) caecum, i) colon. Scale bar =50 µm. Abbreviations: MCT, monocarboxylate transporter; GI,gastrointestinal; PE,phycoerythrin; DAPI, 4',6-diamidino-2- phenylindoledihydrochloride.

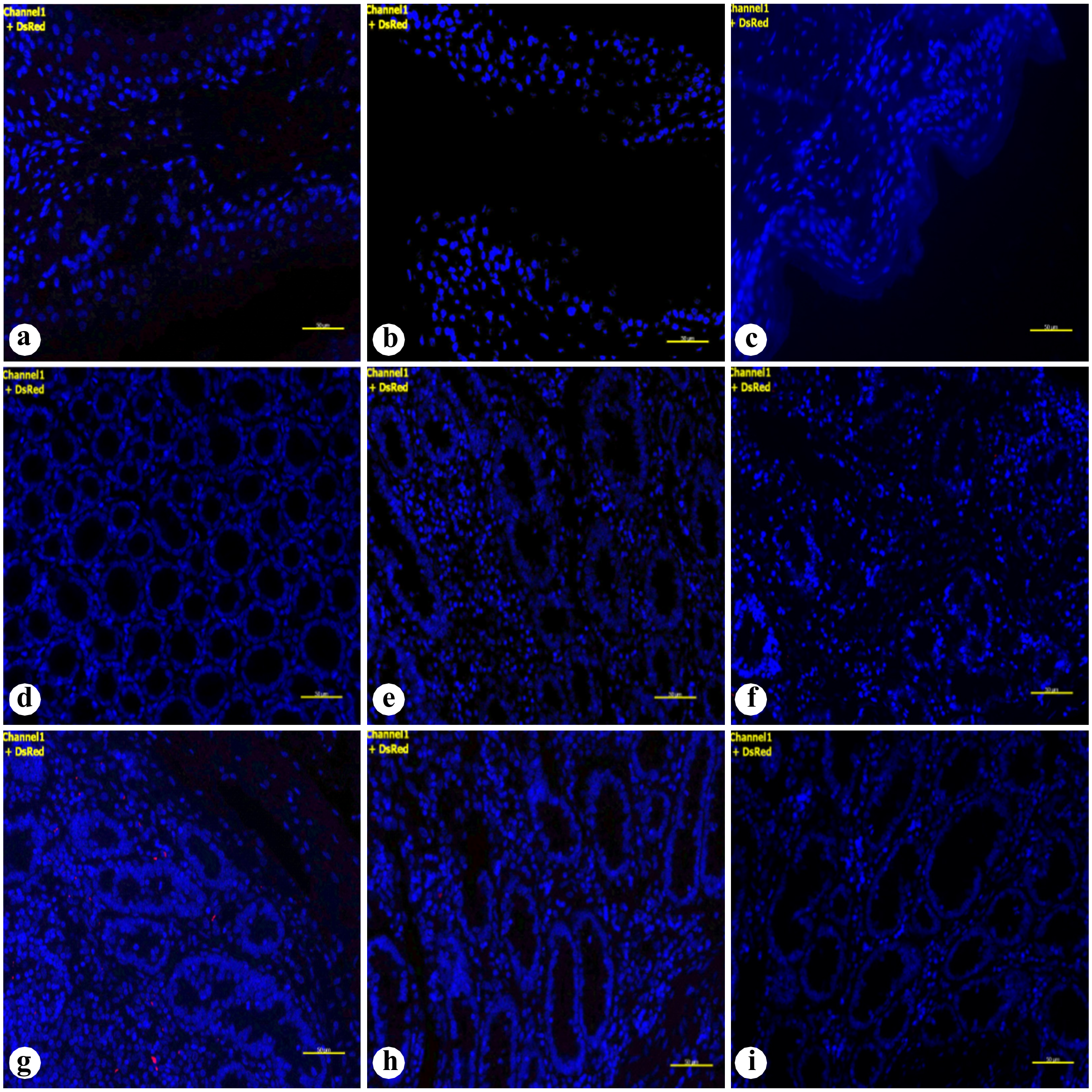


Fig. 14: Fluorescent immunohistochemical staining of MCTs (Negative control) at different parts of GI tract of Black Bengal goat. Briefly, antigen retrieval was done using sodium citrate buffer method on 5µm thick paraffin embedded sections of GI tract tissue. BSA 5% was used to minimize the non-specific binding. Slides were incubated with the omission of primary antibody while the secondary antibody coated with PE was used at 1:400. Nucleus was counterstained with DAPI. Red filter was used while examination of the stained sections under the fluorescent microscope (Carl Zeiss Micro Imaging GmbH).Representative images a) rumen, b) reticulum, c) omasum, d) abomasum, e) duodenum, f) jejunum, g) ileum, h) caecum, i) colon Scale bar =50 µm. Abbreviations: MCT, monocarboxylate transporter; GI,gastrointestinal; PE,phycoerythrin; DAPI, 4',6-diamidino-2-phenylindoledihydrochloride.

Discussion

5

DISCUSSION

Monocarboxylates such as SCFA, lactate and ketone bodies that are being produced in ruminant by anaerobic fermentation of feedstuffs play a central role in cellular metabolism and metabolic communication between tissues and understanding the mechanism is of major concern. The intraruminal synthesis of SCFAs and their metabolites are thought to be extruded out of the ruminal epithelium into the blood circulation via transepithelial transport mainly by MCTs. Since the heat stress adversely affects the physiology, hormone balance and growth performance of animal, there have been a number of studies reporting the response of animals to heat under conditions of various nutritional management as well as other environmental factors (Tajima *et al.,* 2007). Goats are well adapted to different environmental and geographical conditions and they must have developed some signalling pathways that allow their survival in very high as well as cold temperatures via efficient energy metablosim. To the best of our knowledge, no seasonal study have been so far conducted to understand the expression pattern of monocarboxylate transporters in various ambient temperature in goat. During summer and winter the animals were exposed to an ambient temperature beyond their comfort zone so they were under thermal stress in both the seasons.

In our present study we observed that there was significant upregulation ($p<0.05$) of MCT1 in rumen, reticlulum, omasum, caecum and colon during summer season and in rumio-reticulum and large intestine during winter as compare to moderate season. Similarly, the transcipt of MCT4 and MCT2 also showed higher expression in forestomach and large intetstine during summer season than moderate season. In winter season, the MCT2 showed no significant upregulation in forestomach and small intestine except in caecum and colon where expression was high ($p<0.05$). Our findings are in conformity with the other findings reporting that higher amount of MCT1 and MCT4 in the forestomach and large intestine was found in GI tract of cattle and sheep by immunehistochemistry and western blot study (Kirat *et al.,* 2005, 2006). In a study conducted by Graham *et al.,* (2007), only mRNA transcripts for MCT1 and MCT2 have been detected by reverse transcription with the polymerase chain reaction (RT-PCR) among the evaluated MCT isoforms (MCT1- MCT8) in bovine rumen epithelium.

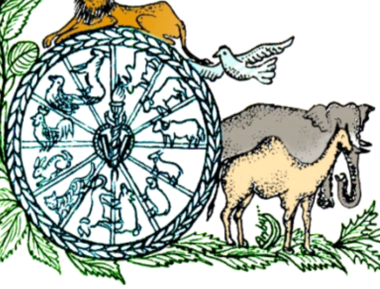
Intertingly in our study we observed no significant expression patern of MCTs in small intestine irrespective of any season which is in accordance with the fact that the absorption of SCFAs occurs mainly in rumen.

It is well known that high environmental temperatures increase the dry matter (DM) digestibility, because the residence time of feeds is prolonged in the rumen by the inhibition of ruminal mobility from heat stress (Warren *et al.*, 1974; Chnstopherson and Kennedy, 1983). In another study conducted by Tajima *et al.* (2007) reported that the level of acetic and butyric acids in ruminal fluid demonstrated the reverse tendencies, with the former decreasing and the latter increasing with the environmental temperatures rising. The caecum is functionally similar to the rumen, with relatively dense microbial population, rapid fermentation and SCFA production (Bugaut, 1987; Bergman, 1990) and SCFA butyric acid, main energy source of large intestine is taken up into colonic enterocytes via monocarboxylate transporter 1 (Goncalves *et al.,* 2012). Therefore in our observation, the higher expression of MCTs in summer season may expalin its role of transport of SCFAs especially butyrates across the rumino-reticlum and large intestine for efficient energy metabolism and thermoregulation as well. In winter season, upregulation of MCTs might explain its role in transport of huge quantity of SCFAs that are being produced in forstomach and large intestine because of high drymatter intake for the theromoregulation and protection from cold stress.

HSP70 family of proteins are most temperature sensitive and highly conserved among heat shock proteins and increased expression of HSP70 mRNA upon exposure to heat stress has been reported in caprine PBMCs (Dangi *et al.,* 2014; Paul *et al.,* 2015). In our present study we have seen significant upregulation of HSP70 transcript in duodenem and colon in summer only as compare to other seasons. Our findings are also in accordance with the previous studies of Parkunan *et al*., (2015) that HSP70 mRNA expression was found to be significantly higher in colon tissue during summer season in pig and hyperthermia resulted in tissue specific increase in HSP72 in liver, small intestine and kidney in rat (Flangan *et al.,* 1995). So, higher HSP70 expression in intestinal tissue during summer season may identify a target for its susceptibility to thermal damage. Observing the expression patter of both MCTs and HSP70, we could not draw a crosstalk between them during any of the season.

✍✍✍

Summary
and
Conclusions



6 SUMMARY AND CONCLUSIONS

Goats have fulfilled agricultural, economic, culture and even religious roles from very early times in human civilization. There are certain advantages for rearing the goats as they are considered as ‘poor man’s cow’ particularly the Black Bengal goats are preferred option among marginal and small farmers and even landless farmers, as they can be maintained with low input cost. Since the goats are reared under extensive farming condition thus while browsing, they get exposed to wide range of environmental stress during both summer as well as winter season. The ruminants have unique digestive system which enables them for a relatively efficient breakdown of feedstuffs in the digestive tract and subsequent microbial fermentation that results in the production of short chain fatty acids (SCFAs) such as acetic, propionic, butyric, isobutyric, valeric acid mainly in forestomach and large intestine. The SCFAs thus produced are readily absorbed and assimilated constitute 80% of their total maintenance energy requirements of the ruminants. The transport of SCFAs across the apical membrane of ruminal epithelium occurs by either passive permeation of undissociated acids or exchange of dissociated SCFAs for anions like bicarbonate.

The SLC16 family of solute carriers (SLC), also known as the monocarboxylate transporter (MCT) is proton-linked transporters that play a crucial role in cellular metabolism. To date, fourteen MCT related sequences have been identified in mammals and MCT1–4 catalyze the proton-linked transport of monocarboxylates such as lactate, pyruvate, short-chain fatty acids and the ketone bodies across the plasma membrane. The two major role of MCT proteins are the facilitation of the absorption of SCFAs in the gastrointestinal tract and the regulation of cell pH in skeletal muscles. Previous workers have already established that

MCT1 and MCT4 facilitate the absorption of SCFAs in different parts of the gastrointestinal epithelium of ruminants like MCT1 plays a pivotal role in the efflux of SCFA across the apical cells of rumen in ovine, caprine and bovine gastrointestinal tract and across the basolateral membranes of the bovine caecal epithelium towards the blood stream.

Thermal stress results in increased demand of net energy for maintenance and subsequent production in various livestock and leads to nutrient deficiency which affects animal's ability to counter the stress. From the previous findings it has been observed that heat stress leads to change in the activity of digestive system and exposure of heat stress has been shown to accelerate the digestibility of DM, crude protein and various feed fraction resulting in reduce passage rate in the GI tract. Heat shock proteins (HSP) are highly conserved proteins found in all prokaryotes and eukaryotes act as ATP dependent molecular chaperone that assists the folding of newly synthesized polypeptides, the assembly of multi protein complexes and the transport of proteins across cellular membranes. Given their role in transport of SCFAs, the present study was done with an objective to investigate the expression and localization of MCTs isoforms (MCT1, MCT2 and MCT4) and HSP70 in various part of gastrointestinal tract of Black Bengal Goat during different seasons.

The present study was conducted in 12 animals each from Black Bengal goats. The animals were divided equally into three groups and the experiment was carried out in three different ambient temperature of the year, viz, thermo-neutral (mid-January to mid-February), winter (December to January), and summer (mid-April to mid-June). The forestomach (rumen, reticulum, omasum and abomasum), small intestine (duodenum, jejunum and ileum), large intestine (caecum and colon) were identified and collected separately for RNA extraction, immunoblotting, and immunohistochemistry studies. Total RNA isolation was done by Trizol reagent. The purity and concentration of total RNA was checked using nanodrop reading. RNA samples showing the OD 260: OD 280 values more than 1.8 were used further. The integrity of the total RNA was checked using 1.0 % agarose gel electrophoresis and bands of 28s and 18s with smearing indicated good quality and intactness of RNA. The RNA was reverse transcribed into cDNA by taking 1µg of total RNA and stored at -20°C for further use. The integrity of the cDNA was checked by PCR with RPS15 and GAPDH primers and desired band is obtained. qPCR was performed for the targeted genes with Ssofast™ Eva Green® qPCR kit following standard procedure in PCR system (Biorad CFX-96 Manager) real time machine. Efficiencies of primers were determined by amplification of a standardized dilution series of cDNA. Finally relative mRNA expression was calculated by Pfaffl equation by two way ANOVA using SPSS software version 22 followed by Duncan's honestly significant differences (HSD) test and using Graph Pad-5.0 software. Differences were considered significant if $p<0.05$. Immunoblot and immunohistochemistry study in various part of GI tract was done irrespective of any season as per lab established protocol. The MCT1, MCT2 and MCT4 proteins were visualized on Immunoblotting as bands of molecular weight ~55 kDa, ~43 kDa and ~43 respectively.

In our present study we observed that there was significant up regulation ($p<0.05$) of MCT1 in rumen, reticlulum, omasum, caecum and colon during summer season and in rumio-reticulum and large intestine during winter as compare to moderate season. Similarly, the transcipt of MCT4 and MCT2 also showed higher expression in forestomach and large intetstine during summer season than moderate season. In winter season, the MCT2 showed no significant upregulation in forestomach and small intestine except in caecum and colon where expression was high ($p<0.05$). However, we observed no significant expression patern of MCTs in small intestine irrespective of any season which is in accordance with the fact that the absorption of SCFAs occurs mainly in rumen. In our present study we also observed significant up-regulation of HSP70 transcript in duodenem and colon in summer only as compare to other seasons. Higher HSP70 expression in intestinal tissue during summer season may identify a target for its susceptibility to thermal damage. Observing the expression patter of both MCTs and HSP70, we could not draw a crosstalk between them during any of the season. Although in gene expression study we found a differential pattern of mRNA expression of MCTs but the immunohistochemistry study in various part of GI tract was done irrespective of any season. Immunohistochemistry of the different part of GI tract for MCTs revealed that the peak intensity of fluoroscence is appeared in epithelial linning of rumen, reticulum and omasum as compare to small and large intestine. MCT expression in abomasum, caecum and colon is evidenced by mild fluorescence. However, in jejunum and duodenum the staining showed little fluorescence which was comparable with that of the negative control.

Conclusion

The results of the present study reports for the first time that MCTs are expressed in regulated manner in different parts of GI tract and higher expression in fore and hind guts during summer and winter season may be involved in handling of SCFA and their intracellular metabolites for efficient energy metabolism.

* * *

Mini Abstract

MINI ABSTRACT

The present study was aimed to demonstrate the expression and immunolocalization of Monocarboxylate transporter (MCT) genes and heat shock protein 70 (HSP70) in different part of gastrointestinal tract during different seasons in Black Bengal goats. Real-time polymerase chain reaction was applied to investigate mRNA expression of examined factors. The study shows a wide variation in the expression of different MCT genes during different seasons in various part of GI tract. We observed that there was a significant upregulation (p<0.05) of MCT1 in rumen, reticlulum, omasum, caecum and colon during summer season and in rumino-reticulum and large intestine during winter than moderate season. Similarly, the transcripts of MCT2 and MCT4 also showed higher expression in forestomach and large intetstine during summer season than moderate season. In winter season, the MCT2 showed no significant upregulation in forestomach and small intestine except in caecum and colon where expression was high (p<0.05). Significant upregulation of HSP70 transcripts in duodenum and colon in summer as compare to other seasons was observed. In conclusion, the results of the present study reports for the first time that MCTs are expressed in regulated manner in different parts of gastrointestinal tract and higher expression in fore and hind guts during both summer and winter season. Thus they may be involved in transport of short chain fatty acids (SCFAs) and their intracellular metabolites for efficient energy metabolism. Higher HSP70 expression in intestinal tissue during summer season may identify a target for its susceptibility to thermal damage.

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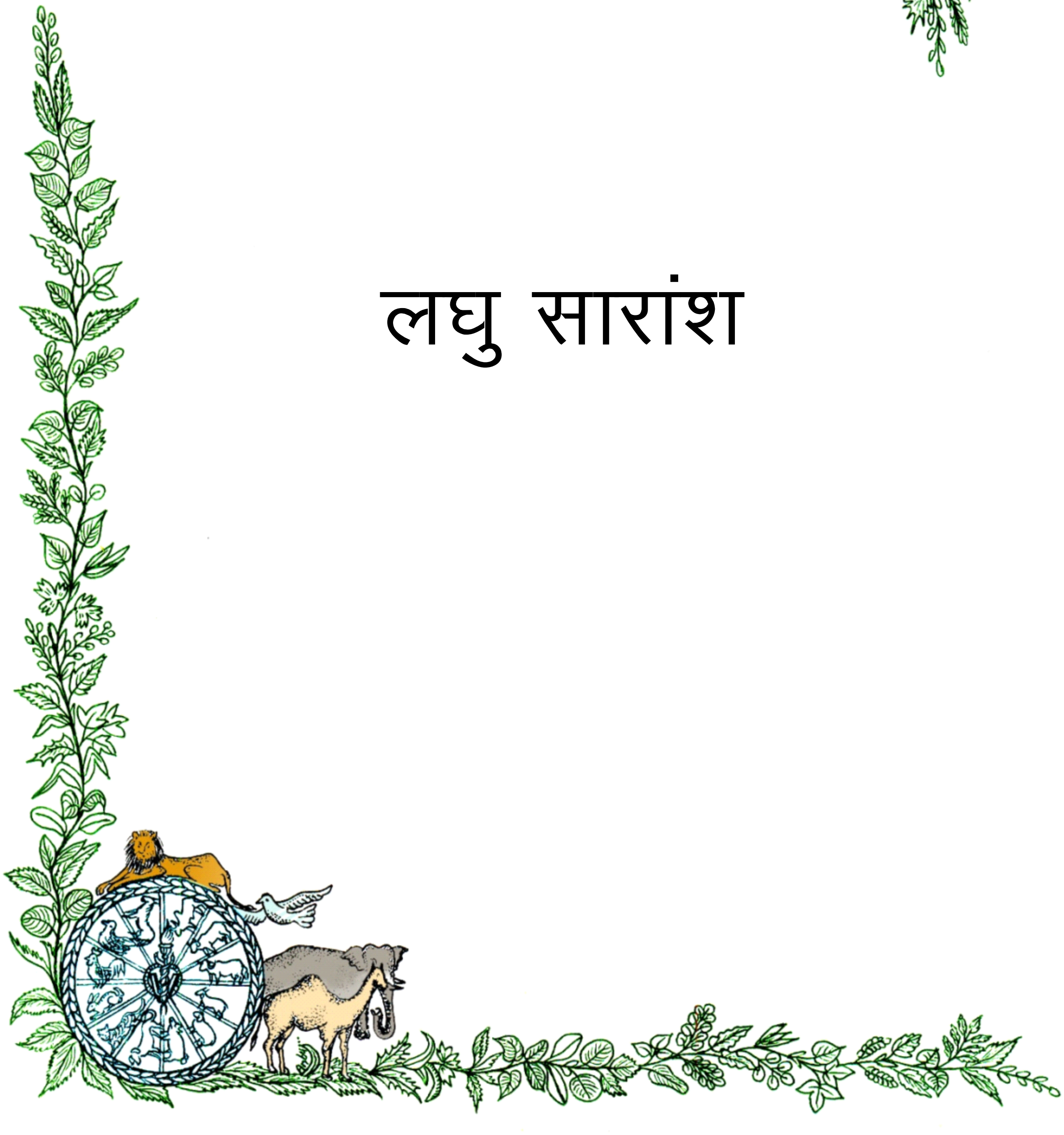

8

लघु सारांश

वर्तमान अध्ययन विभिन्न मौसमों के दौरान ब्लैक बंगाल बकरियों के आहार नाल के विभिन्न भागों में मोनोकार्बोक्सिलेट ट्रान्सपोर्टर (एम.सी.टी.) जीन तथा ऊष्ण आघात प्रोटीन 70 (एच.एस.पी.70) के अभिव्यक्ति और प्रतिरक्षा स्थानीयकरण को प्रदर्शित करने के उद्‌देश्य से किया गया था। एम.आर. एन.ए. के अभिव्यक्ति के परीक्षित कारकों की जांच के लिए रियल–टाइम पॉलीमरेस श्रृंखला अभिक्रिया का उपयोग किया गया। अध्ययन द्वारा आहारनाल के विभिन्न भागों में, भिन्न–भिन्न मौसमों में विभिन्न एम.सी.टी. जीनों के अभिव्यक्ति में विस्तृत विभिन्नता प्रदर्शित हुई। हमने देखा कि सामान्य मौसम की तुलना में, गर्मी के मौसम में रूमेन, रेटिकुलम, ओमेसम, सीकम और कोलोन में तथा ठण्ड के मौसम में रूमिनो–रेटिकुलम और बड़ी आंत में एम.सी.टी.1 का उल्लेखनीय उर्ध्वविनिमयन (पी<0.05) था। इसी प्रकार, एम.सी.टी.2 और एम.सी.टी.4 के प्रतिलिपियों ने भी सामान्य मौसम की तुलना में गर्मी के मौसम में अग्रआमाशय और बड़ी आंत में उच्च अभिव्यक्ति प्रदर्शित की। ठण्ड के मौसम में एम.सी.टी.2 ने सीकम और कोलोन जहाँ अभिव्यक्ति उच्च (पी<0.05) था को छोड़कर अग्रआमाशय और छोटी आँत में कोई उल्लेखनीय उर्ध्वविनियमन प्रदर्शित नहीं हुआ। अन्य मौसमों की तुलना में एच.एस.पी.70 के प्रतिलिपियों का गर्मी के मौसम में, ग्रहणी तथा कोलोन में उल्लेखनीय उर्ध्वविनियमन देखा गया। निष्कर्ष रूप में, वर्तमान अध्ययन का परिणाम पहली बार यह प्रतिवेदित करता है कि एमसीटी की अभिव्यक्ति विनियमित रूप से आहार जाल के विभिन्न भागों में होती है तथा गर्मी और ठण्डी दोनों मौसमों में अग्र और पश्च आंतों में उच्च अभिव्यक्ति होती है। इस प्रकार वे दक्ष ऊर्जा उपापचय हेतु लघु श्रृंखला वसीय अम्ल (एस.सी.एफ.ए.) और उनके अंतः कोशिकीय चयापचयों के परिवहन में संलग्न हो सकते हैं। गर्मी के मौसम के दौरान आंत्रीय ऊतकों में एच.एस.पी.70 की उच्च अभिव्यक्ति, ऊष्णीय क्षति हेतु इसकी संवेदनशीलता के लक्ष्य को चिन्हित कर सकती है।

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APPENDIX

I. REAGENTS USED IN AGAROSE GEL ELECTROPHORESIS

1. Tris-acetate-EDTA (TAE) buffer 50 X

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 ml EDTA (pH 8.0)	100 ml

Distilled water was added to make up to a final volume of 100 ml. A working solution of 1X was used.

II. SOLUTIONS USED FOR SDS-PAGE AND WESTERN BLOTTING

1. Separation gel buffer (pH 8.8)

Tris	18.17 gm
SDS	0.40 gm

Distilled water was added to make the final volume to 100 ml.

2. Stacking gel buffer (pH 6.8)

Tris	3.025 gm

Distilled water was added to make the final volume to 100 ml.

3. SDS-PAGE Running Gel 12.5%

30% acryl-0.8% bis	2.5 ml
1.5 M Tris (pH 8.8)	1.5 ml
Water	1.9 ml
10% SDS	60 µl
10% APS	60 µl
TEMED	2.4 µl
Total	6.0 ml

4. Stacking Gel

30% acryl-0.8% bis	0.33 ml
1 M Tris (pH 6.8)	0.25 ml
Water	1.4 0ml
10% SDS	20 µl
10% APS	20 µl
TEMED	20 µl
Total	2 ml

5. Tris Glycine electrophoresis buffer (5X)

Tris base	15.1 gm
Glycine (pH 8.3)	93.8 gm

Dissolve in 900 ml deionized water. 50 ml of 10% SDS solution was added and the volume was adjusted to 1 litre with deinozed water.

6. **Sample buffer (2X)**

Stacking gel buffer	1.7 ml
10% SDS	4.5 ml
Glycerol	1.0 ml
Bromophenol	2.0 ml
Distilled water	2.8 ml

7. **10% Ammonium persulphate**

APS	1.0 gm
Distilled water	10 ml

Stored at 4°C.

8. **30% acrylamide**

Acrylamide	29.2 gm
N-N-methylene bis acrylamide	0.80 gm

Dissolve in 60 ml distilled water by heating at 37oC and adjust the volume to 100ml. Filtered and stored in amber colored bottle at 4oC.

9. **Gel staining solution**

Coomassie brilliant blue	0.25 gm
Methanol : deionized water (1:1)	90.0 ml
Glacial acetic acid	10 ml

The solution was filtered through Whatman filter paper no. 1.

10. **Gel destaining solution**

Methanol : deinoized water (1:1)	90 ml
Glacial acetic acid	10 ml

11. **Phosphate buffered saline (PBS)**

NaCl	8.00 gm
KCl	0.20 gm
Na_2HPO_4(anhydrous)	1.44 gm
KH_2PO_4	0.24 gm

Distilled water was added to make the final volume up to 1litre. The pH was adjusted to 7.4 with HCl. The resulting solution was autoclaved and stored.

12. **Transfer buffer**

Tris base	2.90 gm
Glycine	11.95 gm
SDS-PAGE	0.185 gm
Methanol	100 ml

Distilled water was added to make the final volume up to 50 ml.

13. **Blocking buffer**

PBS	1000 ml
Tween-20	0.05%
BSA	1%

14. Dilution buffer

PBS	1000 ml
Tween-20	0.05%

III. SOLUTIONS USED FOR IMMUNOHISTOCHEMISTRY

1. PBS 0.01M, 7.4 pH

$Na_2HPO_4{:}2H_2O$	1.86 gram/litre
KH_2PO_4	0.43 gram/litre
NaCl	7.20 gram/litre

2. 5% BSA in PBS

3. 3% H_2O_2 (Freshly prepared, protected from light)

Dilute 10 times of 30%

4. 0.001 M Citrate buffer (MW 294.1)

For 10 ml take 29.41 mg

5. 0.025% Triton X

Triton X	250 µl
Distilled water	1000 ml

6. 4% Paraformaldehyde in PBS (pH 7.4)

Paraformaldehyde	4 gm
PBS	1000 ml

VITAE

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