Amplification of transcript (GAPDH) responsible for carbohydrate metabolism in Snow trout (*Schizothorax richardsonii*)

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Abstract: *The Snow trout (Schizothorax richardsonii) is distributed in North Himalayan rivers, snow melt springs and land locked lakes of high altitude areas in Arunachal Pradesh, Himachal Pradesh, Jammu & Kashmir, Sikkim and Uttarakhand where water temperatures decrease to 4-5°C during winter months. Glyceraldehyde -3-phosphate dehydrogenase (GAPDH), the enzyme possibly plays a key role in gluconeogenesis for survivability of fishes during winter months. In the present study, Partial sequence of the GAPDH gene from snow trout was amplified. The isolation of GAPDH cDNA on cold treated Schizothorax richardsonii, suggested that GAPDH is stressed responsive gene. Cold shock may alter the expression of genes regulating under glycolysis system as revealed in present study.*

Keywords - Fish, Schizothorax richardsonii, Cold Stress, GAPDH

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I: Introdution:

Schizothorax richardsonii is the common cold water fish. The genus Schizothorax is also known as *snow trout* due to its similarity with trout that is having sharp teeth. These are very active species and forages for food and spawning during winter time (Sahoo et al., 2009 and Sharma et al., 1989). *Schizothorax* inhabit fast flowing torrential snow fed streams with water temp from 8°C to 22°C and lakes in the North east Himalayans region .It is a short migratory fish and migrates upward when water temp rises up and downwards when water temp decrease (Talwar and Jhingran, 1991).

The distinguish character of *Schizothorax richardsonii* is characterized by streamlined and compressed but, cylindrical body, very small scales, over 100 in the series next to the lateral line, scales in complete lateral line somewhat larger, the vent and anal fin base are sheathed in enlarged scales and there may be enlarged scales near the pectoral fin and edge of the gill opening, dorsal and anal fins and short, dorsal fin with a thickened last un branched ray bearing denticles, pharyngeal teeth in 3 rows and hooked at the tip, 4 barbells, mouth inferior or sub terminal, lower jaw may have a horny sheath, a papillated area on the chin may be present the lower labial fold may be interrupted in the middle, elongated gut and black peritoneum, and poisonous eggs (Camp, 1998).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, E 1.2.1.12), one of the enzymes studied most in the glycolyticpathway, reversibly catalyses the oxidative phosphorylationof D-glyceraldehyde-3-phosphate to form1,3-diphosphoglycerate in the presence of NADþ and inorganic phosphate (Harris JI *et al*., 1976). This enzyme is widely distributed in nature in a variety of species ranging from bacteria to humans (Fothergill-Gilmore. *et al.,* 1993). It is found mainly in the cytosol and in some organelles. Organellar GAPDHs are expressed as precursor polypeptides and then posttranslationally imported into the organelles (Cerff R., 1995). In addition to its well-characterized glycolytic activity, a housekeeping function essential for the normal metabolism of all cells, GAPDH also plays a pivotal role in the Embden-Meyerhoff pathway in gluconeogenesis (Iddar A, 2002). This enzyme has been well characterized not only because of its key role in the central metabolism, but also because of its abundance, easy preparation and remarkable conservation during evolution. It is a multimeric protein with a native molecular mass in the range of 140–200 kDa and composed of four identical subunits of approximately 35– 50 kDa (Fothergill-Gilmore. *et al.,* 1993 and Iddar A, 2002). The glycolytic pathway is particularly suitable for testing the processes of enzyme evolution and the involvement of possible gene/genome duplications

and/or horizontal gene transfer events. This central metabolic route is highly conserved and ancient; it is therefore possible to compare the enzymes included in this pathway from phylogenetically distant organisms. GAPDH is one of the most highly conserved glycolytic enzymes, for instance the rate of evolution of the catalytic domain is only 3% per 100 million years (Fothergill-Gilmore. *et al.,1993*).

In animal, chilling injury occurs at temperature between 0°C and 15°C. Although, chilling affects the growth of fish, however, only in extreme conditions it affects the animal. Chilling injuries can be categorized as primary injuries and secondary injuries (Raison and Lyons, 1986). Primary chilling injuries are described as temporary dis functionings of animal metabolism that are usually reversible in nature. The animals exposed to chilling may exhibit a loss of vigour and reduced growth rate in absence of other visual symptoms of injury.

Freezing stress occurs when the temperature drops below 0°C. During freezing of animal, ice forms first in the extracellular compartment, reducing its water potential and leading to loss of water from cells by osmosis. Ice formation initiates in large vessels where solute concentration is at the lowest (Levitt, 1980). These ice crystals continue to grow until chemical potentials between ice and unfrozen water is reached to equilibrium (Mazur, 1970). Extent of freezing injury is related to the formation of ice crystals that occurs when ice nucleation can no longer be avoided (Burke *et al.,* 1976). Formation of ice could be avoided to a certain extent by the presence of solutes like glycerol and by supercooling, allowing the cell fluid to be cooled down to the temperature below freezing without ice nucleation. Glycerol is the most important metabolite which has frequently been used as reliable indicators for physiological response under stressful condition. It was confirmed by many worker that the accumulation of glycerol in winter serves as antifreeze when fish were held at high temperature and subjected to low temperature. Driedzic *et al.,* (1998) establish that glycerol appears to be synthesized by conversion of DHAP to glycerol phsphate (G3P) then converted to glycerol; the reactions are catalyzed by glycerol-3 phasphate dehydrogenase (GPDH). Another enzyme that is potentially critical to increase in glycerol production is glyceraldehydes phosphate dehydrogenase (GAPDH) as this enzyme is a necessary component of gluconeogenesis.

A number of studies have been carried out to amplify the gpdh gene in fishes which produce glycerol to adopt the cold temperature. Alternatively, limited studies are available on the GAPDH gene in fishes which also involved in glycerol production.In the present study; we amplify the partial cDNA sequences of GAPDH in liver of snow trout.

II: Material and Methods:

Collection of Fish

The Snow trout fishes, average weight 50-100g were collected from River Kosi, near Almora, during the month of December, 2010 the fishes were transported to wet laboratory and acclimatized at cold temperature (10-15°C).

Tissue collection

Samples of 100mg tissue were harvested from liver of freshly sacrificed fish. Tissue samples were immediately stored in 1ml. of RNAlater (Ambion, USA) solution. Initially, tissue samples were kept at 4^0C overnight for complete infiltration of RNAlater in the tissue and then kept at 80^0C.

RNA preperation

Total RNA was obtained from frozen tissue by ultra centrifugation in Tri Reagent solution which combines phenol and guanidine thiocynate (Ambion, USA). The sample is homogenate in Tri reagent solution and then homogenate is than seprate in to aqueous and organic phase by adding bromochloropropane and centrifuging.RNA partitions to the aqueous phase, DNA to the interphase, and proteins to the organic phase then RNA is precipitated from the aqueous phase with isopropanol, and finally it is washed with ethanol and solubilized. To remove endogenous DNA contamination the preparation of total RNA was digested with DNase. An aliquot of 5µg of total RNA was digested with 5U of DNase (Fermentus, USA) and 40U of ribonuclease inhibitor (Fermentus, USA), and then the solution was incubated at 37^0C for 1hr. The enzyme was inactivated at 65^0C for 10min. and the RNA solution was chilled on ice. RNA quality was assessed on 1.2% agarose gel containing 37% formaldehyde and showing clear 28S and 18S rRNA fragments and no smears, confirming integrities of all RNA. The quantity of the isolated RNA was assessed spectrophotometrically at 260 and 280 nm and obtained a good quantity of RNA.

cDNA preperation

Total RNA was treated with RNase free DNase (Fermentas) for 30 min at 37°C to eliminate genomic DNA contamination. For cDNA synthesis, some reagents were added 2µg total RNA, 1µl Oligo dT Primers, 1 µl Template (1µg), 1 µl Sterile, distilled water.

The tube was incubated at 65°C for 5 min to melt RNA secondary structures. Following reagents were added to the tube, mixed by pipetting and centrifuged briefly.

5X First Strand Buffer	4 µl
10mM DNTP	1 µl
RiboLock™ RNase Inhibitor™ (40 U/ µl)	1 µl
RevertAid™ M-MuLV Reverse Transcriptase (200 U/ µl)	1 µl
Nuclease free Water	to 20 µl

The tube was then centrifuged briefly and incubated at 50°C for 60 min. The enzyme was inactivated by

heating at 70°C for 5 min. For PCR amplification, 1µl aliquot from the cDNA was used as template with gene specific primers. The PCR amplified DNA fragments were resolved by agarose gel electrophoresis (1.2%) and documented. The reverse transcription can be used immediately in second strand cDNA synthesis reactions or stored at -20°C for less than a week. For longer storage, -70°C is recommended.

PCR for amplification of first stand cDNA

Dilute the cDNA generated with the first strand cDNA reaction 1:1000 in Water, nuclease-free.

First stand cDNA was amplified with the use of GAPDH primer forward primer 5'CAAGGTCATCCATGACAACTTTG–3'and reverse primer 5' GTCCACCACCCTGTTGCTGTAG - 3' (universal primer). Gently vortex and briefly centrifuge all PCR reagents after thawing. Place a thin-walled PCR tube on ice and add the following reagents:

cDNA from control RT reaction (1:1000 dilution)	2 µl
10X PCR buffer	5 µl
10 mM dNTP Mix	1 µl (0.2 mM each)
25 mM MgCl2	3 µl
Forward GAPDH Primer	1.5 µl
Reverse GAPDH Primer	1.5 µl
Taq DNA polymerase (5 u/µl)	0.5 µl
Water, nuclease-free	35.5 µl
Total volume	50 µl

Perform PCR in a thermal cycler with a heated lid or overlay with 25 µl of mineral oil. PCR was carried with an initial denaturation at 94°C for 3 min followed by 35 cycles of 94ºC for 30s, 58ºC for 30s, and 72ºC for 45 sec.

Products obtained by PCR were run on 1% agarose gels. DNA was visualized on a spectrophotometer under UV light (364 nm). The gels were photographed with Alphaimager gel documentation system (Alpha Innotech Corporations). Molecular size markers were used alongside the sample to determine the quantity and size of sample DNA.

III: Result:

RNA isolation

RNA was isolate by the use of trizole methord. Isolation of RNA was quantified with U.V visible spectrophotometer.

Concentration of RNA (µg/ml) = $OD_{260} \times 40 \times$ Dilution factor

	260	280	Ratio
O.D.	0.035	0.017	2.05

= 0.035× 40 ×250 = 350 (µg/ml) =0.35 ng/ml

$n_1 \times v1 = n2 \times v2$

0.35 s×? = 1ng/ml × 50 µl = 0.007 ng RNA and rest water.

The working RNA samples were further qualitatively analyzed by 1.2% formaldehyde agrose gel. Sharp clear 28s and 18s RNA band were visualized.

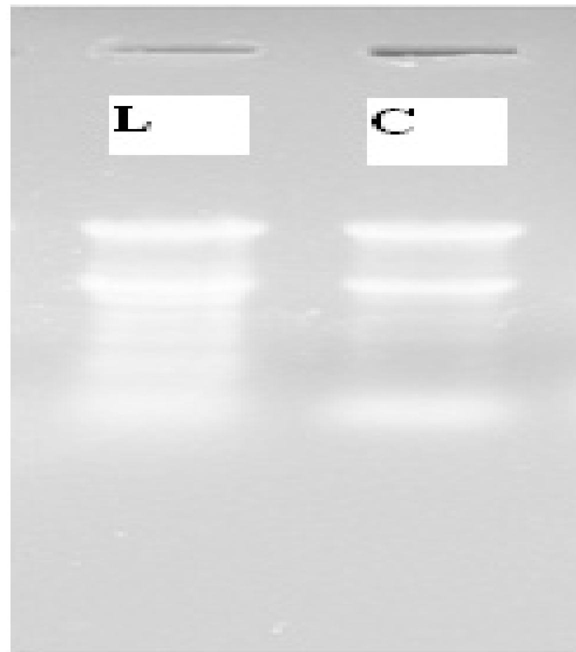

Fig.1. L – Liver RNA C- Control RNA

cDNA synthesis

cDNA was quantified with U.V visible spectrophotometer.

Concentration of cDNA (µg/ml) = $OD_{260} \times 50 \times$ Dilution factor

	260	280	Ratio
O.D.	0.037	0.020	1.85

First stand cDNA is synthesize by the ferments kit and further qualitatively analyzed by the 1.2% agrose gel. Gel analysis confirmed the clear band was visualized.

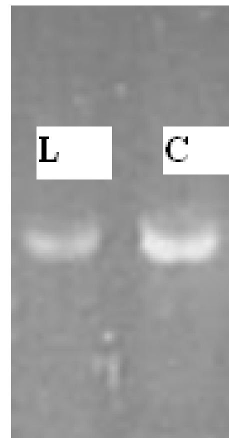

Fig 2. L- Liver cDNA C- Control cDNA

Reverse transcriptase Polymerase chain reaction

Dilute the cDNA generated with the first strand cDNA reaction 1:1000 in Water, nuclease-free. The RT-PCR using the primers obtained a GAPDH fragment of 508 bp fig.4 in the liver cDNA of *S. richardsonii.*

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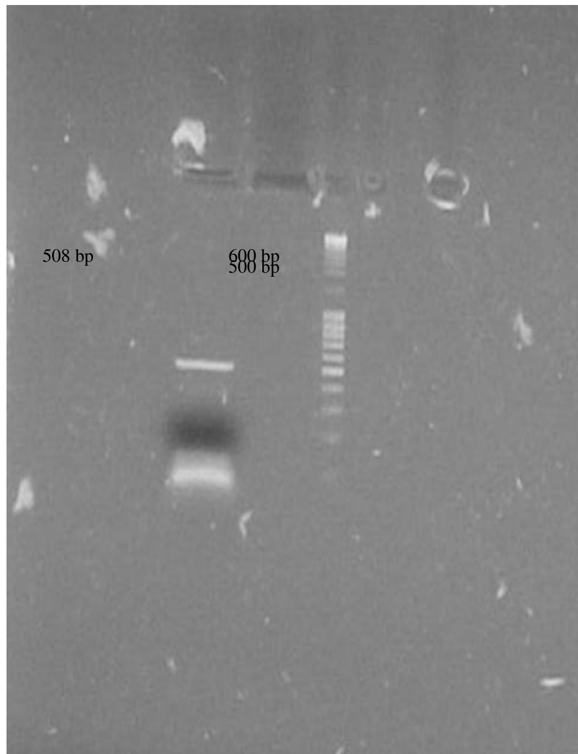

Fig.3. Expression of GAPDH gene in the liver cDNA of *S. richardsonii.*

VI: Disscusion:

Many genes involved in biosynthesis of compatible solutes have been identified and cloned to improve cold stress tolerance in animal. In this study we isolated glycaldehyde-3-phosphate dehydrogenase gene in Indian Snow trout, *Schizothorax richardsonii*. GAPDH is a potentially critical enzyme to increase in glycerol production; it is a necessary component of gluconeogenesis. Stress tolerance can never be explained by the action of only compatible solutes, many other physiological adaptations have to take place to allow animal to survive low temperature. Temperature has an effective role in glycerol accumulation (Lewis et al., 2004; Raymond et al., 1996). Low temperature accumulates a very high concentration of glycerol in fishes. (Drirdzic and Ewart, 2004, Fletcher *et al.,* 2001). Driedzic *et al.,* (1998) studied that the primary site of Glycerol production occurs in liver via two metabolic pathways that involves the conversion of dihydroxyacetone phosphate (DHAP) to glycerol -3-phosphate then glycerol produce, which are reactions catalyzed by glycerol-3-phasphate dehydrogenase (GPDH) and glycerol-3-phosphat, respectively. Initially, glucose/glycogen and amino acids are the major carbon source for glycerol accumulation and later on protein synthesis and other enzyme activities Glycerol-3-phosphate dehydrogenase (GPDH), phosphoenolpyruvate carboxykinase (PEPCK) etc. are being associated. Another enzyme that is potentially critical to increase in glycerol production is glyceraldehydes phosphate dehydrogenase (GAPDH) as this enzyme is a necessary component of gluconeogenesis. Glyceraldehyde 3-phosphate dehydrogenase is an enzyme of ~37kDa that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. The triose substrate of GAPDH is actually a product of several important metabolic pathways: stage one of glycolysis, fructose catabolism, pentose phosphate pathway and glycerol metabolism. The GAPDH reaction is reversible, hence, necessary for hepatic gluconeogenesis. In the glycolysis GAPDH enzyme plays key role and an interesting enzyme to study in the context of environmentally regulated glycerol elevation (Ewart *et al.,* 2001). More specifically, enzymes associated with the production of glycerol from glyceraldehyde-3-P were assessed. The isolation of GAPDH cDNA on cold treated *Schizothorax richardsonii*, suggested that GAPDH is stressed responsive gene. Cold shock may alter the expression of genes regulating under glycolysis system as revealed in present study. This enzyme may function as a metabolic mediator during abiotic stress.The observed intolerance to genetic mutation suggested that the genetic changes (i.e. those seen across species) may provide a treasure of information regarding the limits of genetic variability that can be tolerated and still allow for the protein to conduct essential glycolytic – as well as non-glycolytic – functions. Although in Fig 4 is now understand of the metabolic pathway leading to glycerol production and role of GAPDH in glycerol production.

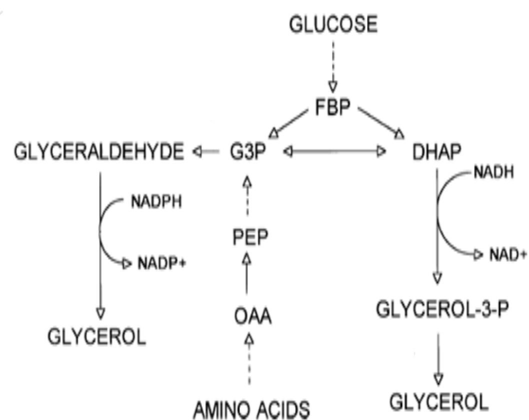

Fig 4. Metabolic pathway for the production of glycerol from carbohydrates and amino acids. (Driedzic et al., 1998)

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