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MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF METALLOTHIONEIN GENE IN *CATLA CATLA*

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ABSTRACT

Metallothionein gene (MT) has been used commonly as a potential molecular marker to detect the harmful effects of heavy metals in aquatic environment. Fish(*Catla catla*) was exposed to sliver nanoparticles for induction of MT gene. Gene MT was successfully characterized and 179bp amplicon product was amplified. MT-2 gene, isolated from the liver, was highly similar to the *Cyprinus carpio* metallothionein gene. The particle size of the sliver nanoparticles were found to be in the range of 70nm to 585nm with an average size of 192 nm. All the above results revealed thatfish MT gene would be a useful biomarker for metal pollution.

INTRODUCTION

Aquatic pollution arises when a water body is harmfully affected due to the accumulation of large amounts of materials to the water(rivers, ocean, lakes and groundwater). Aquatic pollution occurs when pollutants are discharged into water bodies without adequate treatment to remove harmful compounds. Aquatic environment is considered as a main factor for controlling both health and disease in cultured and wild fishes. The pollution of the aquatic environment caused by inorganic and organic chemicals is a major factors posing serious threat to the survival of aquatic organisms including fish. Heavy metal like, Sliver, Copper, Gold, Lead. Mercury etc may cause serious consequences to fish species in relations of retarded growth, decreased reproduction rate and increased sensitivity to diseases (Langston *et al*., 2002). The pollution level at the sub-lethal dose is difficult to detect. In such case, Metallothionein (MT) gene is commonly used as a molecular marker to identify the harmful effects of heavy metals in aquatic environment .

Metallothioneins (MTs) are group of cysteine-rich intracellular metal binding proteins (Kagi and Schaffer, 1986). MTs play multifunctional roles, including homeostasis of trace elements and detoxification of poisonous heavy metals. MTs involve in a number of biochemical processes by providing a pool of Cu^{2+} and Zn^{2+} in the biosynthesis of metalloenzymes and metalloproteins within the cells (Kelly *et al* ., 1996; Jiang *et al*., 1998).

In metal detoxifications, MTs may reduce the toxic effects of metals by degrading the ratio of the uptake of heavy metal into cells (Kagi and Schaffer, 1986; Kagi *et al*., 1991; Roesijadi *et al*., 2000). In fish and shellfish, exposure to sub-lethal levels of heavy metals results

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in the production of MTs Klaverkamp *et al.* (1984). It has already been reported in many fishes such as winter flounder (*Pleuronectesa mericanus*) (Jessen-Eller and Crivello, 1998), gibelcarp (*Carassius auratusgibelio*) De Boeck *et al.*, (2003) and gudgeons (*Gobiogobio*) Knapen *et al.* (2007). Since fish are vulnerable to metal contamination in water, MT gene expression can be used as a biomarker of metal ion contamination. In mammals, 4 MT isoforms have been found and MT-1 and MT-2 are the major isoforms (Thirumoorthy *et al.*, 2011). However, only 2 MT isoforms (MT-1 and MT-2) have been found in fishes such as, common carp (*C. carpio*) Hermesz *et al.*, (2001); icefish (*Chionodra cohamatus*) Scudiero *et al.*, (2001) and pike (*Esoxlucius*) Kille *et al.*,(1993).

Silver is an infrequently occurring metal in the earth's crust but is dumped at much higher concentrations in ores in association with other elements (Renner, 2009). In aquatic environment, silver originates from anthropogenic sources, leaching or mining (Purcell and Peters, 1998). During the current development era of nanotechnology, silver nanoparticles (Ag-NPs) have been used for various types of consumer products, such as health care products, cosmetics and textiles and are easily enter into aquatic environment through waste product of above products . Since silver ion is toxic to fish (Lima *et al.*, 1982 ; Morgan and Wood, 2004) it is vital to establish the toxicity of silvernanopartilces. During last few years, an increasing number of recent reports have provided evidence of the cytotoxicity of Ag-NPs at doses of low exposure(Kawata *et al.*, 2009; Kim *et al.*, 2009). Ag-NPs have harmful impact on aquatic organisms, such as zebrafish and medaka fish, and cause oxidative stress, cellular apoptosis, chromosomal aberrations .Wise *et al.*, (2009), and other developmental toxicity effects during early life stages and adulthood Wu *et al.*, (2010). However there is less information on the development of biomarker in response to the Ag-NPs on commercially important fish species.Therefore, the present study was aimed to synthesis sliver nanoparticles, molecular characterization and phylogenetic analysis of MT gene in one of the commercially important Indian major carps (*Catlacatla*) using silver nano particles (Ag-NPs) induction.

MATERIALS AND METHODS

Synthesis of Silver nanoparticles: Silver nanoparticles were synthesized following the procedure given by Dragieva *et al.* (1999) with slight modification. Briefly, an aqueous solution of 200 mM AgNO3 was prepared and kept in an ice bath under continuous stirring using a magnetic stirrer to cool for 20 minutes. After that 2mL aqueous solution of 200mM Sodium Borohydride (NaBH4) solution was added drop- by- drop to this cool solution under continuous stirring. Stirring was stopped as soon as the solution became yellow in colour, which indicated the formation of silver nanoparticle. After 30 seconds, a drop of an aqueous solution of 0.3% polyvinyl pyrrolidone (PVP) was added to prevent aggregation.

Characterization of sliver nanoparticles: Mean particle size and size distribution of sliver nanoparticles was determined by photon correlation spectroscopy with Beckman Coulter DELSA Nano Particle Size analyser .

Animal procurement, rearing and experimental conditions: The animals (*Catlacatla*) were procured from Aquaculture Farm, Pen, RaigadDist, Maharashtra (India) and were stocked in a circular tank (1000 L) after giving a prophylactic dip treatment in KMnO4 solution (50 mg/L) for 2 min. The sliver nanoparticles (100µg/L) treatment was given for a period of 36 hrs and after that sample was collected for molecular characterization of metallothioneins (MTs) gene.

RNA isolation and cDNA synthesis: Total RNA from the target tissues (liver) was isolated using Trizol Reagent ((Invitrogen). RNA was quantified by measuring absorbance at 260nm in a nanodrop and purity of the sample was checked on a 1% agarosegel.First-strand cDNA synthesis was carried out using 2 µg of DNase-treated total RNA as template. Reverse transcription was performed using Moloneyleukemia virus reverse transcriptase (M/s Fermentas) following manufacturer's instructions. Briefly, 2 µg of total RNA was mixed with 1 µg of oligo(dT)18 primer and incubated at 65 °C for 5 min. After incubation, 10X RT reaction buffer, 1 mM each of dNTPs and 1 µg reverse transcriptase enzyme were added and reaction volume made up to 20 µL with DEPC treated water. Following incubation at 55°C for 30 min, and 85°C 5 min to complete the cDNA synthesis reaction. cDNA was directly used in PCR to amplify the target cDNA using specific primers

Polymerase Chain Reaction (PCR): PCR (Takara, USA) was performed to amplify the desired DNA fragments from the template. PCR was performed in 25 µL

reaction volume containing 1 µL template DNA, 100 pmol of each specific primer, 200 µM of each dNTPs, 0.75 units of Taq DNA polymerase and 10x Taq buffer containing 25mM MgCl2. PCR condition for 35 cycle was initial denaturation 95 °C for 4 min, denaturation, 93 °C for 0.30 sec, annealing 55 °C for 0.45 sec, Extension 72 °C for 0.30, and Final extension 72 °C for 8 min. Primer used for amplifying MT genewas, Forward 5'- ATGGAYCCYTGYGADTGCKCYAA-3'5'- and Reverse TTRCACACRCAGCCWCARGCRCA-3'

Sequence and Phylogenetic Analysis: The sequences was analysed using software Gene Runner V. 3.0.Homology searches of nucleotide sequence were performed using BLAST n algorithm of the National Centre for Biotechnological Information (http://www.ncbi.nlm.-nih.gov/blast). Phylogenetic tree was constructed by Maximum parsimony method using the software MEGA 5.05.

RESULTS AND DISCUSSION

The present study was carried out to study is the molecular characterization of MT gene using AgNPs for induced expression of MT gene in Catlacatla fish.

Characterization of sliver nanoparticles: The particle size of the sliver nanoparticles were found to be in the range of 70nm to 585nm with an average size of 192 nm (Figure 1). The zeta potential of the particle was recorded as +30 mv which indicates that nanoparticles are highly stable.

Molecular characterization of MT-2 gene: A partial sequence of 179 bpamplicon product was amplified from the liver tissue of *Catlacatla*. Gel picture is shown in Figure 2. The homology sequence of the MT-2 of *Catlacatla* is 91% identical to *Ctenopharyn godonidella* metallothionein-2 (KC256783.1), 91% with *Hemibarbusmylodon* metallothionein (MT) (EF689139.1), 92% with *Zacco platypus* metallothionein (KC952875), 92% with *Cyprinus carpio* metallothionein II mRNA, (AF249875.1) and 92% with *C. Auratus* metallothionein (X97271.1). Phylogenetic relationship between the *Catlacatla* fish MT-2 and MTs from other fishes are given in Figure 3.

Metallothionein gene is a biomarker frequently used as an indicator of metal exposure since it is extremely specific and sensitive to metals and is induced in response to elevated metal concentrations in living cells (Olafson *et al.*, 1988). Similar conclusion can be made from the present study also. Metallothionein (MT), Transferrin and Glutathione peroxidise has been found to be expressed in gill and liver tissue of zebra fish, rainbow trout, medaka, etc. leading to oxidative stress and apoptisis due to nanoparticles exposure (Picard *et al.*, 2008; Yun *et al.*, 2009). Chae *et al.*, 2009 reported that induction of the MT mRNA levels in the

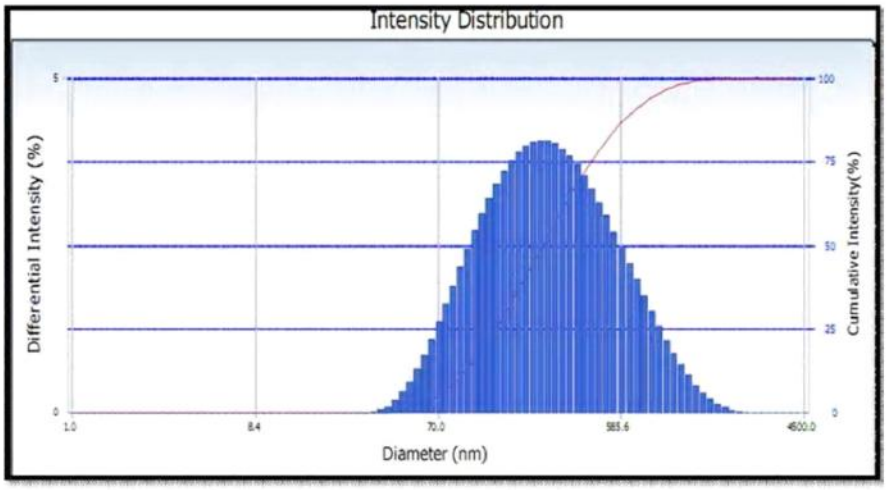

Fig. 1 Mean particle size of the sliver nanoparticles by using particle size analyser

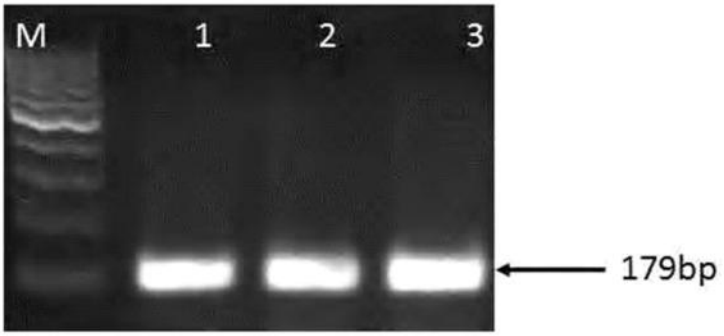

Fig. 2 Amplification of Metallothionein (MT) gene from *Catla catla*
Lane M : Generuler 100 bp plus ladder (MBI Fermentas) 1,2, 3 are three different individuals of *Catla catla*

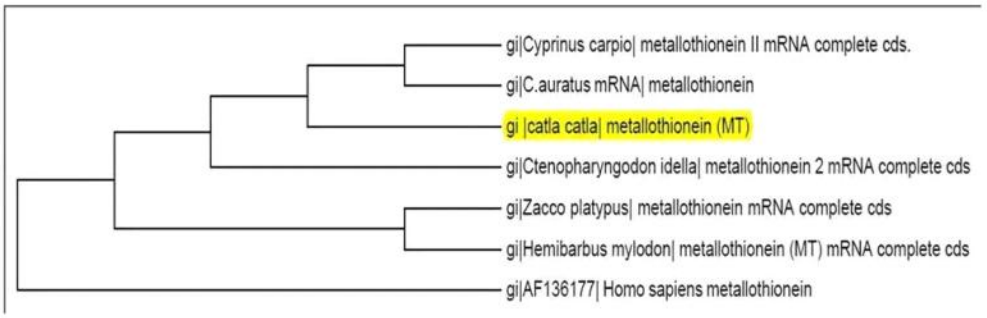

Fig. 3 Phylogenetic relationship between the *Catla catla* fish MT gene with other animals

liver is sudden but not prolonged, showing an initial increase during the first couple of days and then a decrease back to a normal level expression with longer exposure times (>4 days). An assessment between the MT expression levels when exposed to equivalent elemental silver amounts of Ag-NPs and AgNO3 showed noteworthy difference between the toxicants during short exposure times but reduced as the exposure time is extended(Chae *et al.*, 2009; Choi *et al.*, 2010).

Metallothionein gene expression is prompted by a high variety of stimuli, as metal exposure like Cu^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, Hg^{2+} and Zn^{2+}Ag+ Au+, oxidative stress etc. Chan *et al.*, 2004 found the Common carp Metallothionein mRNA levels in the intestine and kidney following exposure to Cd^{2+} and Zn^{2+} showed high fold induction. Toxicity effect of silver nanoparticles in aquatic midge, Chironomusripariuswas reported Chan *et al.*, (2011), where alteration in gene expression was observed and have significant implications in different developmental stages. Cheung *et al.*, (2005) reports Cu^{2+} and Ni^{2+} induced the production of hepatic MT mRNA in vivo and MT gene expression study shows highest fold induction in liver following the administration of heavy metal ions. *Oncorhynchus mykiss* fishes were exposed to colloidal AgNPs Johari *et al.*, (2011) at nominal concentrations of 100, 32, 10, 3.2, 1, 0.32, 0.1, and 0.032 mg/L and study revealed that the fish were more sensitive to colloidal silver nanoparticles during the early life stage than in later stages. Similar result has also been observed in present study. All above studies clearly indicates that MT gene can be used as biomarker and can provide valuable information on the aquatic environment pollutions.

CONCLUSION

The present study showed that metallothionein (MT) gene expression can be used as a biomarkers for nanotoxicity.

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