



## Poly I: C stimulates, Mx transcript and promotes an antiviral state in blood of *Labeo catla* (Hamilton 1822)

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### ABSTRACT

In the current investigation, the kinetics of Mx transcripts expression in the blood of *Labeo catla* (Hamilton 1822) inoculated with poly I: C, was studied for 14 days. The level of Mx transcript in the blood cells increased as compared to control fish inoculated with phosphate buffered saline. The level of Mx transcript was quantified along with semi-quantitative expression for  $\beta$ -actin. The level of Mx transcript was maximum on day 2 and 3 ( $\beta$ -actin: Mx ratio of about 0.8) which reduced after day 4 and the response disappeared on day 7. Western blots of the blood showed an immune-reactive band of 72 kDa, which is typical for *L. catla* Mx protein. Results suggest an increase in levels of Mx transcript after poly I: C injection which can stimulate an antiviral phase and the appropriateness of Mx expression investigation as an indicator to assess protective action.

Keywords: Immuno-histochemistry, Myxovirus resistance, Poly I: C, Reverse transcriptase, Western blotting

### Introduction

Indian aquaculture is growing day by day and different culture practices with species diversification are emerging in the country. Various diseases of known and unknown etiology are stumbling blocks for successful farming. The diseases are caused by various factors including bacteria, parasites, fungi and viruses. The conventional approaches towards control of diseases is use of antibiotics and other chemotherapeutants. The indiscriminate use of these measures renders many unwanted risks in terms of development of antibiotic resistance as well as loss of aquatic microflora. Viruses can effectively be controlled by the application of vaccines, which is not economically feasible in Indian aquaculture scenario. Indian aquaculture revolves around the Indian major carps (IMCs) particularly *Labeo rohita* and *Labeo catla*. *L. catla* is considered as a priced fish which has better consumer preferences and it has good tolerance towards varying environmental conditions. The intensification in culture practices leads to development of disease havoc in culture systems and viruses are becoming increasingly reported in Indian aquaculture. Therefore there is a need to establish and explore new prophylactic measures and health management strategies for successful

fish farming in Indian conditions. Antiviral defense is a vital component in the success of aquaculture operations. The Mx protein is an interferon-induced protein that defends against viral infections, which is widespread in eukaryotes. The Mx protein has antiviral actions and slow down the multiplication of viruses by interfering with viral replication in the early stage. However, their antiviral action is still not clear and the findings vary amongst various Indian major carp species. Therefore, the present study was undertaken to evaluate the assumed Mx antiviral action in *L. catla* which would give an insight into its role in protection against viral infection.

The most important anti-viral activity is understood to be governed by interferons. The interferon-stimulated Mx protein is one of the best-studied determinants of immunity to viral infection. Mx protein exhibited to have anti-viral activity against a variety of viruses and should be liable for most of the antiviral effects of type-I interferons. IFNs are molecules that are quickly broken down once they are activated. Mx proteins, on the opposite, can continue for weeks following a single virus infection. Hence, it has been used as a molecular indicator for IFN secretion and viral infection in mammals (Roers *et al.*, 1994; Trobridge *et al.*, 1997; Nygaard *et al.*,

2000; Plant *et al.*, 2005). Recognition of Mx proteins in the blood leukocytes could be employed as a method for non-lethal investigation for virus infection. The present study intended to monitor the kinetics of expression of Mx mRNA and Mx protein in blood cells, after induction of an IFN reaction by inoculating poly I: C (a synthetic analogue of ds RNA) in *L. catla*.

The Mx proteins are often initiated in diverse nature of organisms, including invertebrates to higher animals where they exhibit their vital role against viral infections (Leong *et al.*, 1998; De Zoysa *et al.*, 2007). In most of the species, an erratic existence of Mx genes have been recognised. In addition, allelic polymorphisms at Mx locus have also been described in mice and chickens, which influence the probability of survival after virus infections (Lindenmann, 1962; Ko *et al.*, 2002). In addition, several studies have shown that fish viruses, such as channel catfish virus (CCV) in channel catfish, infectious pancreatic necrosis virus (IPNV) in salmonids, the infectious salmon anaemia virus (ISAV) in Atlantic salmon, infectious haematopoietic necrosis virus (IHNV) in salmon and trout, can induce Mx expression (Jensen and Robertsen, 2000).

Aquatic birnaviruses are the most important and diverse group of viruses within the family of the Birnaviridae (Blake *et al.*, 2001), which are morphologically similar and genome is ds RNA (Dobos *et al.*, 1979; Wu and Chi, 2007; Shen *et al.*, 2018;). IPNV is the only important diverse group antigenically within the family Birnaviridae. It is the etiological agent of an acute disease of salmonids in the juvenile stage (Wolf *et al.*, 1960) and have also been reported from several species of fish (Castric *et al.*, 1987; Mortensen *et al.*, 1990). In the recent past, tilapia lake virus, has been found to exhibit significant mortality in fish species including tilapia and rohu in South-east Asia (Pradhan *et al.*, 2020). This shows cosmopolitan nature and etiology of the tilapia lake virus. The spread of virus is triggered due to fluctuating environmental conditions.

Mx is not only an antiviral interferon-inducible gene but also induced by several biotic and abiotic stressors including disease pathogenesis. The induction of Mx gene expression by poly I: C in *L. rohita* and *C. mrigala* was recently reported (Das *et al.*, 2019; Roy *et al.*, 2019). In this study, the *L. catla* Mx gene expression after stimulation with poly I:C is reported. There is no such literature available on Mx expression in blood cells of fish particularly, *L. catla*. In this backdrop, our study is an attempt to investigate the expression kinetics of Mx in blood cells of *L. catla*.

## Materials and methods

### *Fish maintenance*

One hundred numbers of adult *L. catla* (average weight 100±12.5 g) were collected from the fish farm of ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA), Kausalyaganga, Bhubaneswar, India. Fish were acclimatised in a circular 500 l FRP (fibreglass reinforced plastic) tank with sufficient aeration. During the acclimatisation period, the fish were fed with artificial feed and water was changed (30-50%) routinely to remove the excretory wastes and unutilised feed. After acclimatisation, before starting the experiment, ten fish were randomly selected and checked for the presence of any parasite or any clinical signs of diseases.

### *Poly I: C treatment and blood sample collection*

A set of 56 nos. of healthy catla fishes were selected for the experiment and were distributed into two groups of 28 nos. each, out of which fishes of one group (treatment group) were intraperitoneally injected with 0.1 ml Poly I:C solution, as prescribed previously (Das *et al.*, 2019; Roy *et al.*, 2019). The second set of 28 fishes were maintained as control group. Whole blood and serum samples were collected from four fishes each sampled on day 1, 2, 3, 4, 5, 7 and 14 following poly I:C injection. Control fishes were also sampled in a similar manner. Fishes were anaesthetised using tricaine methane sulfonate (0.1 ppm) and whole blood was drawn from the caudal vein from each fish with a heparinised 2 ml syringe and smears were prepared on clean glass slides. The slides were dried and immunostained for Mx protein as per the protocol of Das *et al.* (2007) and the remaining blood samples were transferred to separate sterile nuclease free plastic vials and frozen quickly in liquid nitrogen and stored at -80°C, until used for extraction of RNA and in PBS for protein profiling.

### *RNA extraction and cDNA synthesis*

Total RNA was isolated from blood tissue samples, following the Guanidium Thiocyanate method as per Chomczynski and Sacchi (1987). cDNA was synthesised using oligo d(T)<sub>18</sub> primers and M-MLV polymerase (New England Biolabs, UK), as per manufacturer's instructions. The reverse transcription was confirmed by PCR using  $\beta$ -actin primers as positive control.

### *PCR amplification of catla Mx cDNA fragment*

To amplify the partial cDNA sequence of catla Mx gene, primers were designed using fast PCR software from the conserved region of reported fish Mx cDNA sequence of catla (Gene bank accession No KP282448). PCR was run using Mx primer pair

(6F-5'-GTCCAGTACCACATGCTGGACC-3' and 7R-5'-TTTGCCAGCACTCCTCAGGCGT-3') of product size 165 bp.  $\beta$ -actin primer pair 5'-TTTCGAGCAGGAGATGGGCACTG-3' and 5'-GCATCCTGTCAGCAATGCCA-3') of product size 254 bp was used as a reference housekeeping gene. PCR products were purified using gel purification kit (Sigma) and sub-cloned into pGEMT easy vector (Promega). Multiple clones were sequenced using automatic sequencer ABI 3700 (Applied Biosystems). The Mx and  $\beta$ -actin amplified products were run on a 1.5% agarose gel and visualised by ethidium bromide staining and observed in an image analyser (Gene Genius Bio Imaging System, SynGene). The results of the expression study were quantified by calculating Mx:  $\beta$ -actin ratio.

#### Real-time PCR for Mx expression

For quantification of Mx mRNA transcript expression, qPCR was performed.

#### Protein profiling

Protein was extracted from blood tissue samples (Chomczynski and Sacchi, 1987) and electrophoresed on Sodium dodecyl Sulphate Polyacrylamide Gel (SDS-PAGE) in discontinuous buffer system according to the method described by Laemmli (1970) in a BIORAD mini protein-II electrophoresis unit.

#### Western blot analysis of Mx antisera

For *in vitro* analysis of Mx protein, blood tissue samples were processed at 48 and 72 h post-induction, blood samples were lysed in 200  $\mu$ l of SDS sample buffer and boiled for 3 min and then 15  $\mu$ l of each sample was loaded per well. Samples were electrophoresed in an SDS-10% polyacrylamide gel and the separated proteins were transferred on to a nitrocellulose membrane. Primary antibody developed in rabbit (diluted 1:500 using blocking solution) was added and incubated overnight at 4°C. The

membrane was washed thrice in PBST (phosphate buffered saline with 0.1% Tween 20) and incubated with the secondary antibody (horse anti-rabbit alkaline phosphatase conjugate) (Vecta Stain, USA) at 1:200 dilution for 60 min at room temperature. The membrane was washed in PBST thrice. In order to identify biotinylated standards, Avidin-AP conjugate (AB reagent, Sigma) was added and incubated for 30 min. The membrane was washed two times in PBST and finally in PBS for 15 min each and then developed using AEC (3-Amino-9-ethylcarbazole) reagent (Sigma, USA).

#### Data analysis

SPSS software (version 16.0 SPSS Inc., USA) was applied for estimating the differences between means. Dunnett's *post-hoc* test was performed for comparing the differences between treatments ( $p < 0.05$ ).

## Results

#### Kinetics of Mx in blood tissue

The expression of Mx transcript in blood tissue was noticed from day 1 to 14. Control catla showed faint expression during the same time (Fig. 1). In the qPCR assay, Mx was highly expressed on the second day (21 fold) and third day (25 fold) of post-induction which gradually decreased after the fourth (15 fold) day and became almost similar to control after seven days (Fig. 2).

#### Mx protein in blood

The Mx protein band was observed at 72 kDa on 3<sup>rd</sup> and 4<sup>th</sup> days after poly I: C inoculation. No band was observed in untreated fish. A faint band of 72 kDa was observed on 1<sup>st</sup> day of induction. A number of putative bands of different molecular weights were observed in control fish. Stable Mx expression in catla was observed up to four days and then a steady decline in expression was observed for up to 14 days (Fig. 3). The same trend was observed in the western blotting analysis of Mx protein (Fig. 4).

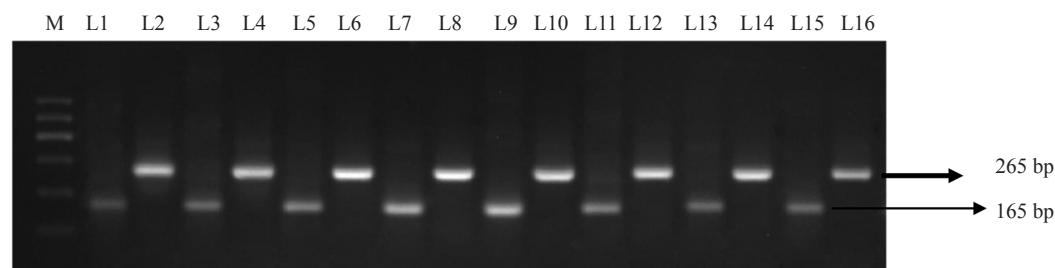


Fig. 1. Expression of Mx transcript in catla blood tissue after induction of poly I:C

M - 100 bp DNA ladder; Lane 1: Mx in Control; Lane 2:  $\beta$ -actin in Control; Lane 3: Mx on day1; Lane 4:  $\beta$ -actin on day1; Lane 5: Mx on day2; Lane 6:  $\beta$ -actin on day2; Lane 7: Mx on day3; Lane 8:  $\beta$ -actin on day3; Lane 9: Mx on day4; Lane10:  $\beta$ -actin on day4; Lane 11: Mx on day5; Lane 12:  $\beta$ -actin on day5; Lane 13: Mx on day7; Lane 14:  $\beta$ -actin on day7; Lane 15: Mx on day14; Lane 16:  $\beta$ -actin on day14

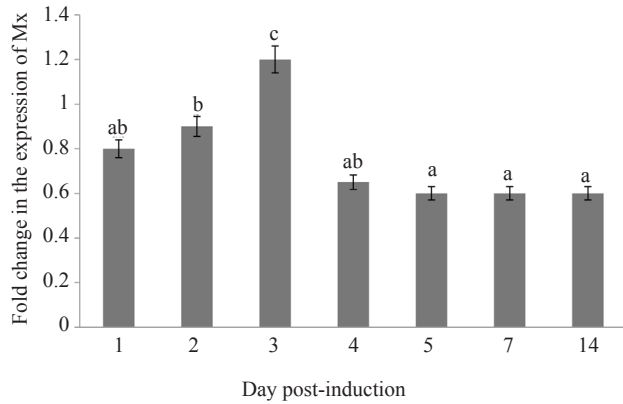


Fig. 2. Analysis by real-time quantitative RT-PCR expression of Mx transcript after poly I:C induction in catla blood tissues, over a period of 14-days. The results were expressed as mean±standard error. Bars show standard error

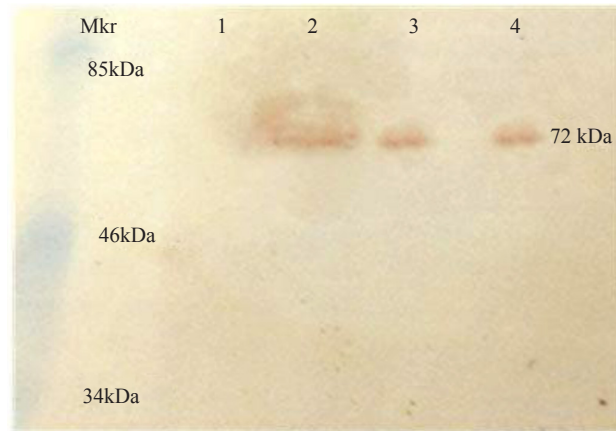


Fig. 4. Western blots analysis of Mx (72kDa) protein

*Immunostaining of blood*

Upon immunostaining of the blood smear, immature RBCs were found to express Mx protein (Fig. 5b). Besides immature RBCs, monocytes, lymphocytes and neutrophils too expressed Mx protein (Fig. 5a). The immunostaining was intense on days 2 and 3 which gradually reduced afterwards. In control samples, faint staining was noticed in blood cells indicating its basal expression.

**Discussion**

The Mx proteins are considered as vital components in IFN stimulated anti-viral phase of RNA viruses. The availability of information on the role of interferons in fishes and conserved nature of Mx, offer Mx, a great role

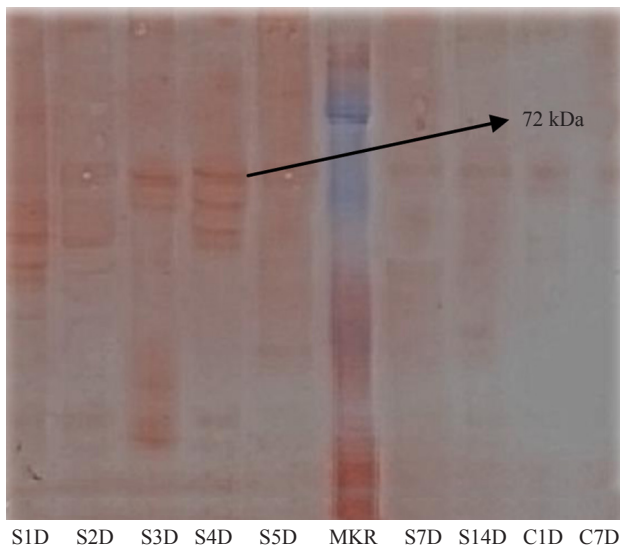
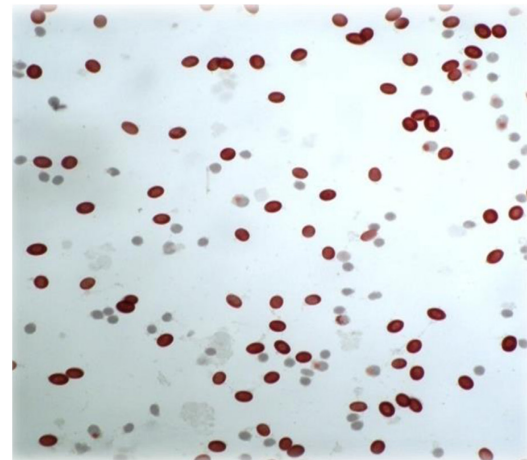
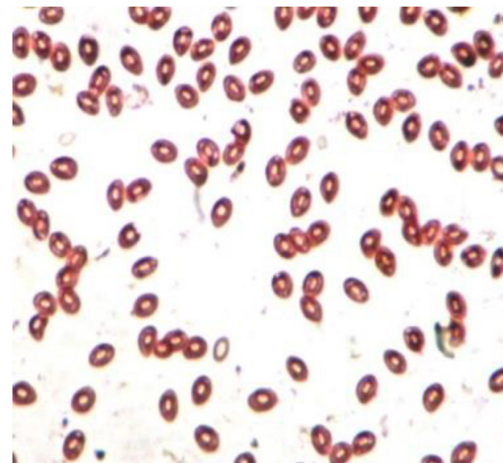


Fig. 3. Expression of Mx protein in blood serum during the experimental period



(a)



(b)

Fig. 5a & b. Immunostained erythrocytes of poly I: C treated fish on day 2

to play in evolutionary genetics and biology (Roers *et al.*, 1994; Trobridge *et al.*, 1997; Nygaard *et al.*, 2000; Plant *et al.*, 2005). During the present investigation, absolute amounts of Mx mRNA molecules were measured in catla blood cells following poly I:C induction. Furthermore, greater poly I:C induced mx transcript was seen at 12 h and with these evidences it can be concluded that Mx has an anti-viral effect. Though, the Mx responses seem to be similar in fish, intrinsic factors such as stage, age and sex of the species impart a great role in response modulation (Salinas *et al.*, 2004). A previous study in Senegalese sole showed that, following poly I:C induction, greatest peak of Mx response was seen in spleen, kidney, liver, gill and intestine at 24 h as measured by relative quantification (Fernandez-Trujillo *et al.*, 2006).

In the present study, the expression of Mx mRNA in catla blood was measured following poly-I:C stimulation for a period of 14 days. The expression of Mx was found to be upregulated from the second to the fourth day post-induction, as compared with the control fish. The role of poly I:C induced Mx in viral defence of bony fishes has already been established. Cunningham (1973) reported Mx expression of various isoforms in trout and eel following poly I:C stimulation. Study revealed that Mx isomers elevated considerably to their respective transcript level across the organs with maximum expression in intestine as 510 folds and in muscle 211 folds at 6 h post-challenge. In seabream, the expression of Mx within vital organs was significantly upregulated (Lee *et al.*, 2000). It has been reported that in human beings, the peripheral blood cells were found to express basal level of Mx transcript (Ronni *et al.*, 1993). After inoculation, the human blood cells including monocytes, granulocytes and lymphocytes were observed to positively stain for Mx protein at 4, 6 and 24 h, correspondingly and thereafter it appeared more intense (Towbin *et al.*, 1992; Al-Masri *et al.*, 1997). In the current experiment, blood cells were found to stain positively for Mx expression. Das *et al.* (2009) have mentioned an elevated level of Mx expression at 4 weeks following administration of poly I:C in Atlantic salmon parr.

The existence of Mx protein in calta was witnessed with qPCR and immunostaining of the blood cells of catla. The Mx transcript was observed by western blotting of poly I:C stimulated blood cells with a band at 72 kDa in *L. catla* and *C. mrigala* (Roy *et al.*, 2017). Similarly, an assumed Mx protein of approximately 76 kDa was reported across the organs of poly I: C treated Atlantic halibut and Atlantic salmon parr (Das *et al.*, 2007; Lockhart *et al.*, 2007). The difference could be attributed to different fish species and climatic conditions that are triggering the expression of Mx induced protein.

The present study investigated the expression kinetics of induced Mx mRNA in *L. catla*. A dose of 500 µg poly I: C was inoculated to arouse IFNs in salmonids (Eaton, 1990; Nygaard *et al.*, 2000). The poly I:C induced a strong expression of Mx mRNA in diverse groups of fish but more percentage occurred in viral infected 3<sup>rd</sup> day induced catla. This is in agreement with the present study in which, Mx ascertained a peak by day 2 (mean Mx:β-actin, 0.75) and was considerably greater than the control group on day 4 and 5 and afterwards a decline was observed. The present study showed a decline in the level of expression of Mx and its protein, which is in accordance to the study of Jensen *et al.* (2002) who reported a low level of Mx in Atlantic salmon parr.

Although, the present study covered the expression kinetics of Mx, transcript, Mx protein and immunostaining of blood cells, further studies to elucidate the steadiness of Mx protein and its organisation with the mRNA levels are compulsory to build up a complete visualisation of the defensive reaction initiated by poly I:C and the precise role of Mx protein against viruses in catla. The Poly I: C is an artificial double-stranded polyribonucleotide capable of stimulating type I interferons. The current investigation provides proof of subsequent initiation of interferons by inoculation of poly I: C in *L. catla* and also furnishes baseline information for further downline applications of the findings in fish disease management.

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