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# Molecular characterisation of the *IRAK1* gene and its expression analysis in the Indian catfish *Clarias magur*, following *Aeromonas hydrophila* infection challenge

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## Abstract

Interleukin-1 receptor associated kinase 1 (*IRAK1*) is a crucial downstream signaling mediator of the Interleukin-1 (*IL-1*) and Toll-like receptor (*TLR*) signal transduction pathways, responsible for sensing invading pathogens or endogenous danger signals and initiating appropriate immune responses. In this study, the partial cDNA sequence of *IRAK1* gene was amplified, cloned, characterised and its modulation in response to *Aeromonas hydrophila* infection was studied in the Indian catfish *Clarias magur*, a promising fish species for aquaculture in the Indian subcontinent. The magur *IRAK1* (*mIRAK1*) cDNA sequence of 590 bp was obtained by cloning and was found to encode a putative protein of 196 amino acid residues. This protein contains a serine/threonine kinase catalytic domain (STKcD) with activation sites responsible for kinase activity having a calculated isoelectric point (*pI*) of 6.65 and molecular weight of 21.68 kDa. Phylogenetically, *mIRAK1* clustered together well with other members of the catfish family and showed the highest identity with pangasius catfish. Presence of highly conserved elements, including a conserved STKcD domain and cycline-dependent kinase 1 (CDK1) motif, were revealed by multiple sequence alignments in *mIRAK1*. Basal expression analysis indicated that *mIRAK1* was widely expressed in all examined tissues, with the lowest expression in muscle and the highest in liver. Following *A. hydrophila* infection challenge, *mIRAK1* expression was significantly up-regulated in vital immunological organs such as kidney, spleen, intestine and liver at 3 to 24 h post-infection. These results emphasise the critical role of *mIRAK1* in host defence mediated by *TLR* and *IL-1* signalling pathways during *A. hydrophila* infection.

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## Introduction

Toll like receptors (*TLRs*) are essential molecular receptors that sense the risk from pathogenic microorganisms or endogenous threats and transmit these signals to downstream signalling mediators, which in turn activate the innate and adaptive immune systems (El-Zayat *et al*., 2019). Among the downstream signalling mediators of the *TLR* signalling cascade, interleukin-1 receptor-associated kinases (*IRAK-1*) constitute a unique protein family with conserved domain architecture. *IRAKs* are composed of four typical domain structures: an N-terminal death domain (DD) that interacts with MyD88, Proline/serine/threonine-rich (ProSTD) domain, a highly conserved serine threonine kinase catalytic domain (STKcD) or pseudokinase domain, and a C-terminal protein domain for activation and recruitment of the downstream mediator TNF receptor-associated factor 6 (TRAF6) (Patra and Choi, 2016; Lange *et al*., 2021). In mammals, the IRAK family comprises four recognised members: *IRAK-1*, *IRAK-2* and *IRAK-4* which are active kinases; and *IRAK-3/M* which is an inactive pseudokinase (Ye *et al*., 2002; Wang *et al*., 2017; Lange *et al*., 2021). However, in fish only the *IRAK-1*, *IRAK-3* and *IRAK-4* orthologs have been reported. (Sadangi *et al*., 2020). The ATP-binding pocket with a conserved Lysine (Lys) amino acid residue in the STKcD domain

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is responsible for the active kinase activity (Gosu *et al*., 2012). Upon binding of a *TLR* to its cognate ligand, intracellular signaling cascade is initiated through *MyD88* adaptor protein which recruits *IRAKs* (*IRAK-4*, *IRAK-1* or *IRAK-2*) via their death domains to form the Myddosome complex, which triggers the activation of *IRAK* kinase (Wesche *et al*., 1997; Wang *et al*., 2017). Phosphorylation of *IRAK-1* is essential for the activation of downstream *NF-κB* signalling (Li *et al*., 2001). Subsequently, autophosphorylated *IRAK1* dissociates from the Myddosome complex and interact with *TRAF*6 and the *TAK1-TAB1-TAB2* kinase complex. This leads to ubiquitination of *IκBα*s, and subsequent activation of *p38* and *JNK MAPKs* cascades, as well as transcription factors such as *NF-κB*, or *AP-1*, ultimately resulting in the production of chemokines, cytokines, and immunomodulatory molecules (Gosu *et al*., 2012; Zhang *et al*., 2014; Wang *et al*., 2017).

*IRAK1* was the first member of *IRAKs* family to be recognised as a crucial downstream signal transducer in the *TLR* signaling pathway (Cao *et al*., 1996). Recent studies have characterised the structure and expression pattern of *IRAK1* in several important fish species, including mandarian fish (*Siniperca chuatsi*), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), orange spotted grouper (*Epinephalus cocoidus*), humphead snapper (*Lutjanus sanguineus*), Nile tilapia, (*Oreochromis niloticus*), rohu (*Labeo rohita*), and rainbow trout (*Onchorhynchus mykiss*) (Zhang *et al.*, 2009; Huang *et al.*, 2012; Shan *et al*., 2015; Li *et al*., 2016; Huang *et al*., 2017; Han *et al*., 2019; Sadangi *et al*., 2020; Xie *et al*., 2020; Yang *et al*., 2020). The genetic structure and functional characterisation of *IRAK1* have been contextualised through these investigations. Further, these investigations have also documented the expression profiles of *IRAK1* in response to various ligand stimulations and bacterial, viral, or parasite infections. Collectively, these studies highlight the essential role of *IRAK1* in enhancing innate immune potentiation in fish against diverse pathogens and stimuli. Nevertheless, little is known about *IRAK1* in the magur cat fish, and its role in triggering innate immune responses against bacterial infections is still unknown.

*Aeromonas hydrophila* is a significant bacterial pathogen that affects finfish, particularly magur catfish raised in subtropical regions such as India. In this study, a virulent field isolate of *A. hydrophila* was used to evaluate the *IRAK1* mediated immune response in magur. The objectives of the study were to characterise *IRAK1* gene in magur catfish (*mIRAK1*) and evaluation of its molecular response following infection with *A. hydrophila,* a fish pathogen of significant concern. The findings would help to understand the innate immune regulatory role played by *IRAK1* in *C. magur*.

# Materials and methods

## Experimental fish and maintenance

Approximately healthy juveniles (80 Nos.) of *C. magur*, weighing 50±4.54 g and measuring 22.31±1.34 cm, were collected from adjacent farms in Lucknow and housed in a 2000 l fibre-reinforced plastic (FRP) tanks having a sandy-clay bed with hide-outs. Forty numbers of fish were stocked in each 1000 l tank. Commercial magur feed (Growel Feed Pvt. Ltd., India) was used to feed fishes twice a day @ 2% of their body weight. Prior to initiation of the experiment, the fish were acclimatised for a month. Half of the water was exchanged every week.

## Cloning of magur *IRAK1 (mIRAK1)*

To amplify cDNA sequence of *mIRAK1* gene, *IRAK1*-cDNA sequences from various fish species were first aligned in Clustal Omega to find conserved motifs. *IRAK1*-cDNA sequences of channel catfish, *Ictalurus punctatus* (XM_017468550), *C. idella* (JQ239168.1), *C. carpio* (KF660220.1) and *Carassius auratus* (XM_026269160.1) were retrieved from NCBI GeneBank. PCR primers (Table 1) were designed from conserved regions using GeneRunner software. Total RNA was extracted from the spleen tissue, and cDNA was prepared in accordance with the standard methodology which will be discussed later. PCR was performed under the following conditions: 95°C for 5 min of initial denaturation, 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min of. Subsequently, 20 µl of the PCR product was examined on a 2% agarose gel, and the predicted band size fragment was excised from the gel using Gel extraction kit (Thermo Scientific). Thermo Scientific's pTZ57R/T vector was used to clone the purified PCR fragment. Using M13 forward and reverse sequencing primers, the recombinant was sequenced in both directions.

## Bioinformatics analyses of *mIRAK1* sequence

The Chromas version 2.6.5 was utilised to read the amplicon sequences. In order to determine the sequence homology of *mIRAK1* with other known *IRAK1*, NCBI-BLAST search was conducted. The NCBI's ORF finder tool was used to identify the open reading frame (ORF). The SMART software (http://smart.embl-heidelberg.de/) and EXPASY (ScanProsite) tools were used to analyse the corresponding protein sequence. The protParamExpasy was used to calculate the molecular weight (*Mw*) and theoretical isoelectric point (*pI*) (http://web.expasy.org/protparam/). The identity percentage of *mIRAK1* with other

Table 1. Sequences of primers used for PCR and qPCR amplification of magur *IRAK1*

| Primer name | Primer sequence (5'-3') | Annealing temperature (ºC) | Amplicon size (bp) | Reference | Usage |
|---|---|---|---|---|---|
| *IRAK1*-F | TCGGAGAGGGAGGTTTCGG | 58 | 590 | This sudy | PCR |
| *IRAK1*-R | CTCCAGCAACACCACTCCAA | | | | |
| *qIRAK1*-F | TAGCCGAACTCCAGGGAAGA | 60 | 140 | | qPCR |
| *qIRAK1*-R | GCAACACCACTCCAAAGCTG | | | | |
| *qEF1α*-F | GCAGCTTATCGTTGGAGTCA | 60 | 144 | Muduli *et al.* (2022a, b) | qPCR |
| *qEF1α*-R | GAAATTGGGACGAAAGCAACG | | | | |

related fish species was ascertained by nucleotide and protein blast. Using Clustal Omega, multiple-sequence alignment was created. The phylogenetic tree of *mIRAK1* was constructed by bootstrapping 1000 times with default parameters using the neighbour joining method of the MEGA 7.0 programme.

## *In vivo* bacterial challenge and sample collection

Eighty (n = 80) seemingly healthy magur juveniles (~50 g) were randomly assigned into two groups: the *A. hydrophila* injected group and the control group (PBS, pH 7.2 injected) (40 fish each) in 1000 l FRP tanks.Before the experiment began, to confirm the absence of pre-existing bacterial or parasitic infections, a random subsample of apparently healthy *C. magur* was screened using microbiological media, which showed no bacterial growth after 24–48 h incubation, and wet mounts of internal organs such as, gills, and skin revealed no parasitic infestation. Using a multimode water quality parameter instrument (Hanna Instruments, Romania), the temperature, pH, and dissolved oxygen were measured and recorded as 30±2°C, 8.2±0.45, 6.8±0.78 mg $l^{-1}$ (Mean±SE), respectively during the experimental period.

Our previously characterised *A. hydrophila* 9C strain (Muduli *et al*., 2020, 2021) was used for this experiment. The bacterium was cultured in nutrient broth at 37ºC, overnight. The culture was centrifuged at 5,000 rpm for 5 min at 4°C in order to collect the bacterial cells. This was followed by two steps of washing in PBS. Fish were intraperitoneally (i.p.) injected with predetermined $LD_{50}$ dose of *A. hydrophila* 9C strain *i.e.* $1 \times 10^5$ CFU per fish suspended in 50 µl PBS (Muduli *et al*., 2020). The control group was injected with 100 µl of PBS. Three fish each from the *A. hydrophila*-injected and control groups were sacrificed at 3, 8, 24, 72, and 144 h post-infection (hpi). During subsequent samplings, moribund fish were preferentially selected from the treatment group. The tissues, including the gill, intestine, kidney, liver, and spleen, from the sampled fish were excised and transferred into *RNAlater* and preserved at -80ºC until the RNA was extracted. The Institutional Animal Ethics Committee of ICAR-NBFGR, Lucknow, approved the experimental challenge and animal care.

## Total RNA extraction and cDNA synthesis

The sampled tissues were subjected to total RNA isolation using Sigma-Aldrich's TRI Reagent in accordance with the manufacturer's guidelines. RNA purity and concentration were examined using Nanodrop spectrophotometer (Denovix Inc). Total RNA of 1 µg concentration was processed with 1 U of DNase I, (Thermo Scientific) to eliminate any residual genomic DNA. RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) with oligo-dT primer was used to reverse-transcribe RNA to cDNA.

## Evaluation of RT-qPCR primer efficiency

The RT-qPCR primers for *mIRAK1* gene were designed (Table 1) and their amplification efficiency was evaluated prior to qPCR. The cDNA synthesised from the spleen tissue samples of naive *C. magur* was subjected to four-fold serial dilution with concentrations ranging from 2000 ng to 7.8 ng. The qPCR was performed using serialy diluted cDNA as template and cycle threshold (Ct) values were plotted to generate standard curve. Based on the Ct value, the ABI StepOnePlus Real-time PCR system (Applied Biosystems, USA) automatically generated the standard curve, correlation coefficient ($R^2$), and also calculated the qPCR amplification efficiency (*E*). For relative quantification, primers with qPCR efficiencies between 90 and 105% were employed.

## Basal expression analysis of *mIRAK1*

Twelve distinct tissues were extracted independently from three native magur (~50 g), including the gills, liver, anterior and posterior kidneys, spleen, intestine, heart, muscle, stomach, skin, and accessory respiratory organ (ARO). Total RNA was extracted from each tissue sample and cDNA was synsthesised. The RT-qPCR, (as described in the subsequent section) was performed to evaluate tissue specific expression of *mIRAK1* gene in twelve different tissues. Our previously standardised reference gene *EF-1α* was used as internal control for gene expression normalisation (Muduli *et al*., 2022a). The tissue with the lowest expression level served as a calibrator. Basal expression of *IRAK1* was represented as relative fold changes from the lowest expressing tissue (calibrator). The relative fold-change in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

## RT-qPCR

The RT-qPCR amplification reactions were performed in triplicate wells of a 96-well plate, using a 13 µl of reaction mixture containing the following ingredients in each well: 1 µl of cDNA (100 ng $ul^{-1}$), 0.5 µl of forward and reverse primer (10 µM each), 0.25 µl of ROX dye, 6.5 µl TB green Premix Ex Taq II (TAKARA), and 4.25 µl of nuclease-free water under the subsequent cycling condition comprising 30 s of initial denaturation at 95°C, 40 cycles of 10 s at 94°C, 10 s at 60°C, and 10 s at 72°C. Melt-curve analysis was then subsequently performed for 15 s at 95°C and 1 min at 60°C. Negative control without any template was run in triplicate for each plate. The Ct value was normalised with *EF-1α* and fold-change in expression was calculated.

## Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the data from the three separate biological replicates in order to compare the groups' levels of significance. All the statistical analyses were performed in SPSS 16.0 software following Tukey *post-hoc* test at 95 or 99% significance level.

# Results

## Partial characterisation of *IRAK1* gene in *C. magur (mIRAK1)*

The 590 bp *mIRAK1* cDNA was amplified using the designed primers. The resulting cDNA fragment was subsequently cloned, sequenced and analysed using NCBI-BLAST. BLAST analysis revealed that the sequence shared exclusive similarity with *IRAK1*homologs from other fish species. It encodes a polypeptide protein of 196-amino acid (Fig. 1). The deduced *mIRAK1* protein had a theoretical isoelectric point (*pI)* of 6.65 and a computed molecular weight of 21.68 kDa. SMART analysis revealed the presence of the

```
1 TCGGAGAGGGAGGTTTCGGCCACGTGTACCGCGCCACCATGAGGAACACCGACTTTGCAGTG 6
    G  E  G  G  F  G  H  V  Y  R  A  T  M  R  N  T  D  F  A  V
63 AAGAGACTCAAAGAGGATTCCCATTTGGGATGGAGTGTGGTGAAGGAAAGTTTCAGGACG 122
   K  R  L  K  E  D  S  H  L  G  W  S  V  V  K  E  S  F  R  T
93 GAGGTGGAAAAACTCTCACAATATCGACACCCAAACATCGTGGACTTGTGTGGTTACAGT 182
   E  V  E  K  L  S  Q  Y  R  H  P  N  I  V  D  L  C  G  Y  S
183GTGGAAGGTGAAATCTACTGCCTGATCTACGCGTACATGCCCAGTGGTTCTCTGGAGGAC 242
   V  E  G  E  I  Y  C  L  I  Y  A  Y  M  P  S  G  S  L  E  D
243CGGCTGCGCTGTGAGAAAGTCTCTGCCCTCTCCTGGTCGCAGCGGGTAAACGTGGTGCTG 302
   R  L  R  C  E  K  V  S  A  L  S  W  S  Q  R  V  N  V  V  L
303GGTTCGGCGAAAGCTCTTCAGTGCCTTCATTCCTGCTCTCCCGCTCTCATCCACGGAGAC 362
   G  S  A  K  A  L  Q  C  L  H  S  C  S  P  A  L  I  H  G  D
363GTCAAAAGCTCCAACATTCTCTTAGGGGAACATCTGGAGCCGAAGCTGGGTGACTTTGGT 422
   V  K  S  S  N  I  L  L  G  E  H  L  E  P  K  L  G  D  F  G
423CTGGCGCGGTTGTGTCGCAACCCTAGCCGAACTCCAGGGAAGACCAGCAGCGTGGCTCAG 482
   L  A  R  L  C  R  N  P  S  R  T  P  G  K  T  S  S  V  A  Q
483ACCTCTACAGTGCGCGGCACGCTGGCGTACCTGCCCGACGAGTACCTGAAGGACGGCCAG 542
   T  S  T  V  R  G  T  L  A  Y  L  P  D  E  Y  L  K  D  G  Q
543CTGGGCATGGAGATCGACGTTTACAGCTTTGGAGTGGTGTTGCTGGAG 590
   L  G  M  E  I  D  V  Y  S  F  G  V  V  L  L  E
```

Fig. 1. Nucleotide and deduced amino acid sequence of *C. magur IRAK1* cDNA

core serine-threonine kinase catalytic (STKc) domain in this partial *mIRAK1* sequence (Fig. 2). These STKc domains are highly conserved (Fig. 3). The *mIRAK1* has highly conserved areas, including a conserved STKcD domain containing cycline-dependent kinase 1 (CDK1) motif and activation sites responsible for kinase activity as demonstrated by the multiple sequence alignment using Clustal Omega software. The *mIRAK1* nucleotide sequence has been deposited to the NCBI GenBank database and is available to the public under the Accession No. MZ423198.

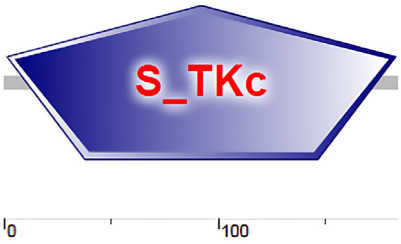


Fig. 2. *C. magur IRAK1* (*mIRAK1*) encoding for the central serine threonine kinase catalytic domain (STKc) containing activation sites which are responsible for kinase activity constructed by SMART/ScanProsite programme

## Phylogenetic relationship and structural identity of *mIRAK1* with other species

In order to comprehend the evolutionary link between *mIRAK1* and *IRAK1* reported from other teleosts, a phylogenetic tree was constructed. The high bootstrap support indicated the presence of three major clades of higher vertebrates, carps and catfishes. Phylogenetically, *IRAK1* representing catfish group *viz*., magur, pangas, channel catfish and yellow head catfish formed one cluster (cluster-I) based on their close evolutionary relationships, and were well separated from carps *viz*., zebra fish, rohu, gold fish, common

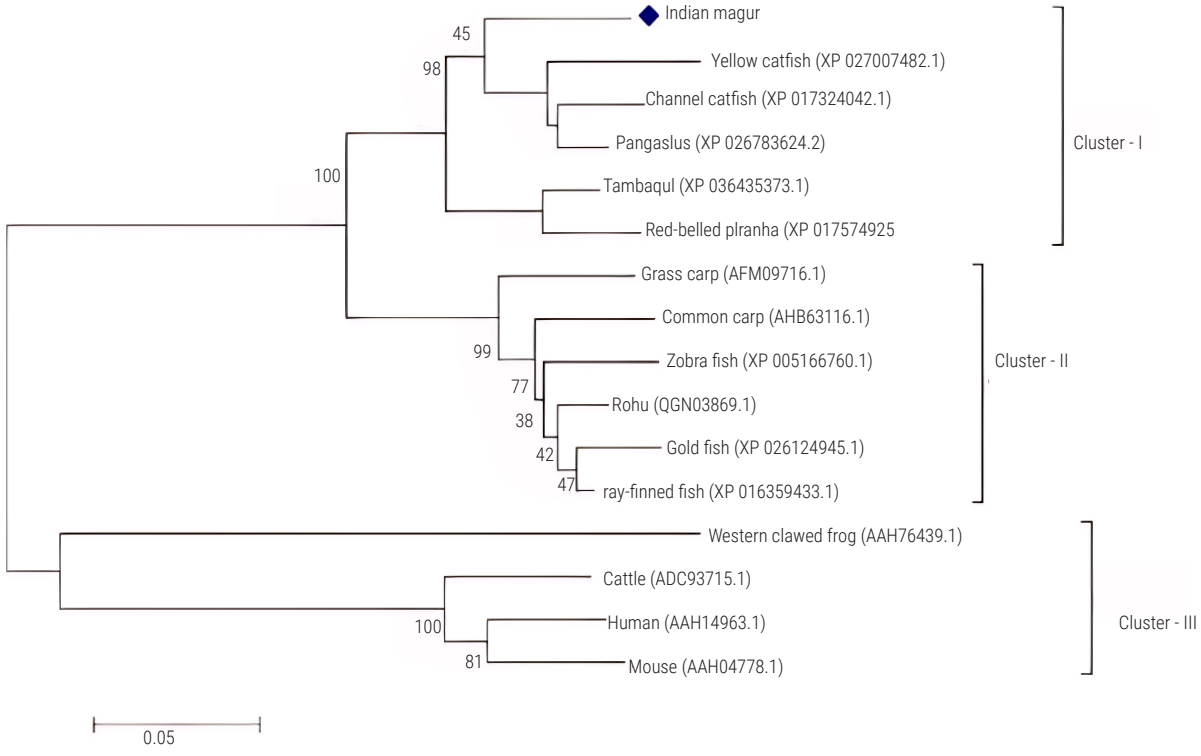


Fig. 4. Phylogenetic tree of *mIRAK1* constructed by neighbour joining method using MEGA 7.0 programme bootstrapped 1000 times using default parameters

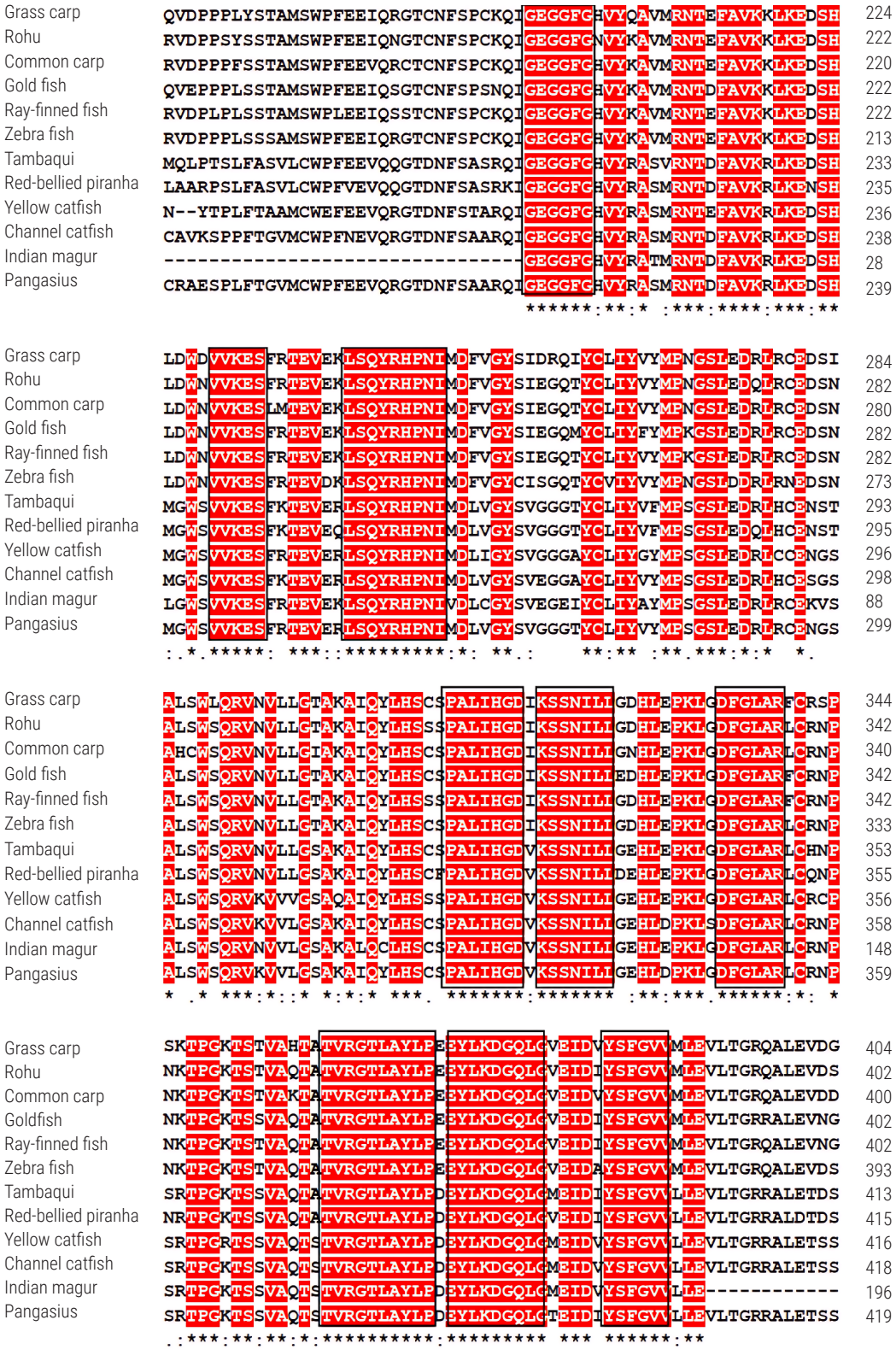


Fig. 3. Conserved motifs present in *IRAK*1 protein of different fishes. *IRAK1* amino acids sequences of various fish species were retrieved from the GenBank data base and were aligned through Clustal Omega. The red boxes represent strictly conserved regions and other boxes represent well conserved (>50%) regions

carp and grass carp (cluster-II). It is evident from the evolutionary tree that fish *IRAK1* diverges significantly from that of other higher vertebrates or animals (cluster-III) (Fig. 4). The *mIRAK1* shares a close phylogenetic relationship with pangasius.

The structural identity of *mIRAK1* nucleotide sequence, when compared with pangas, channel catfish, and yellow catfish, showed sequence identities of 92.37, 90.51 and 88.31% respectively. At the deduced amino acid level, *mIRAK1* shared 91.33, 91.33, and 89.29%, identities respectively with pangas, channel catfish, and yellow catfish. . In comparison, the amino acid identity of *mIRAK1* was approximately 76% with carps and 61% with higher vertebrates (Table 2).

## RT qPCR primer amplification efficiencies

PCR amplification efficiencies (E) of *mIRAK1* and *EF-1α* primer sets were 101.11 and 99.25%; while correlation coefficient ($R^2$) was 0.995 and 0.998, respectively (Table 3; Fig. 5)

## Basal expression of *mIRAK1*

Liver had the highest basal expression of *mIRAK1*, while muscle exhibited the lowest. Although the expression levels varied among tissues, *mIRAK1* expression was discernible in all tissues examined (Fig. 6).

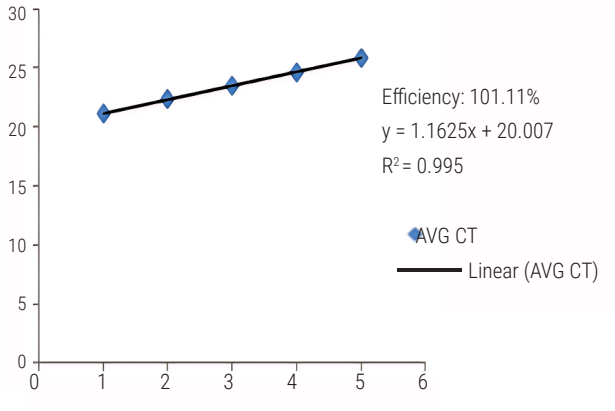


Fig. 5. Standard curve showing qPCR amplification efficiencies (E) of *mIRAK1* primer sets

Table 3. Amplification efficiencies (E) and correlation coefficient ($R^2$) of qPCR primers

| Symbol | Amplification Efficiencies (%) | Correlation coefficient ($R^2$) |
|---|---|---|
| IRAK1 | 101.11 | 0.995 |
| EF-1α | 99.25 | 0.998 |

## Expression of *mIRAK1* in response to *A. hydrophila* infection

The expression of *mIRAK1*gene was upregulated following infection with *A. hydrophila* as compared to the control. Significant induction of *mIRAK1* was observed in gill at 8 hpi and liver at 3 and 8 hpi. In the gill of infected magur, *mIRAK1* gene expression was significantly increased ~7.16 fold at 8 hpi. In liver tissue also, *mIRAK1* expression was upregulated significantly to 9.4 and 4.5 fold at 3 and 8 hpi respectively. Significant upregulation was also observed in intestine, kidney and spleen at 3, 8 and 24 hpi. In the kidney, *mIRAK1* expression increased significantly to 22.34 fold at 3 h, peaked to 44.41 fold at 8 h and then decreased to 10.18 fold at 24 h. In spleen, expression enhanced significantly to 14.7 fold at 3 hpi, 23.7 fold at 8 hpi, and then lowered to ~19.1 fold at 24 hpi. In intestine, *mIRAK1* expression was significantly enhanced to 32.75, 66.49 and 43.35 fold at 3, 8 and 24 hpi respectively (Fig. 7).

# Discussion

*IRAK1*, a protein kinase is an essential downstream effector molecule of the TLR/IL-1R signalling pathway responsible for the initiation of inflammatory and innate immune response against pathogen invasion or endogenous danger signal (Flannery *et al*., 2014). In this study, we cloned partial cDNA sequence of *IRAK1* gene from *C. magur* catfish *(mIRAK1)* and investigated their expression profiles in healthy and *A. hydrophila* infected fish. Protein domain prediction revealed that *mIRAK1* encodes a central serine threonine kinase catalytic (STKc) domain containing several activation sites to promote kinase activity, as well as a cycline-dependent kinase 1 (CDK1) motif. The

Table 2. Identity of magur *IRAK1* at nucleotide and amino acid level with other fish and animal species

| Species | Common name | *mTLR2* nucleotide identity (%) | *mTLR2* deduced aminoacid identity (%) |
|---|---|---|---|
| *Pangasionodon hypophthalmus* | Pangasius | 92.37 | 91.33 |
| *Ictalurus punctatus* | Channel catfish | 90.51 | 91.33 |
| *Tachysurus fulvidraco* | yellow catfish | 88.31 | 89.29 |
| *Colossoma macropomum* | Tambaqui | 82.51 | 88.78 |
| *Pygocentrus nattereri* | Red-bellied piranha | 80.54 | 86.22 |
| *Labeo rohita* | rohu | 76.23 | 82.65 |
| *Cyprinus carpio* | Common carp | 75.21 | 82.14 |
| *Sinocyclocheilus anshuiensis* | Ray-finned fish | 77.25 | 83.16 |
| *Carassius auratus* | Gold fish | 77.08 | 84.18 |
| *Ctenopharyngodon idella* | Grass carp | 76.74 | 82.65 |
| *Danio rerio* | Zebra fish | 75.85 | 81.12 |
| *Homo sapiens* | Human | 53.43 | 61.11 |

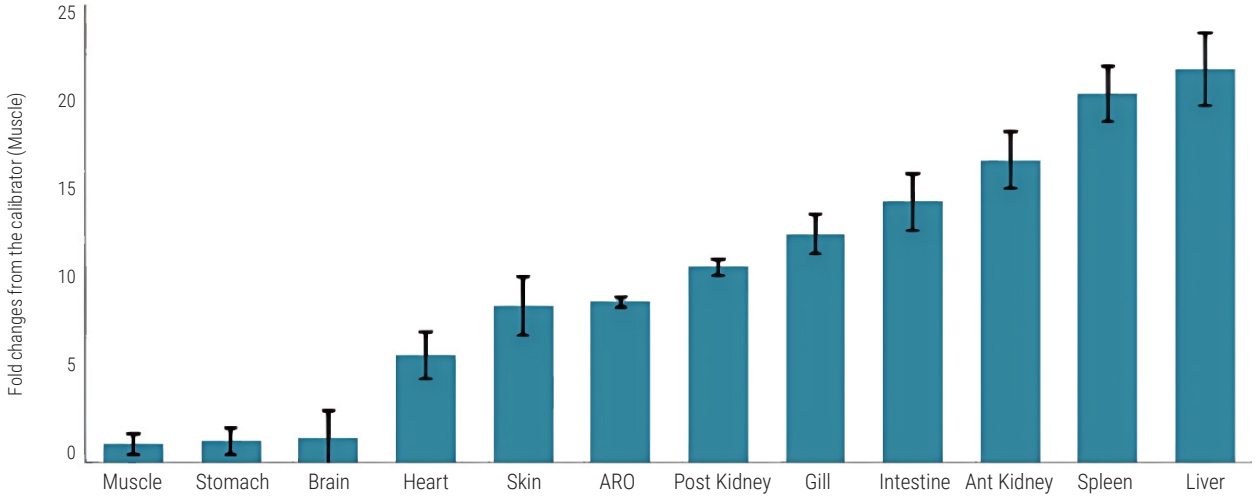


Fig. 6. Basal expression of *mIRAK1* in various tissues [gill, liver, spleen, intestine, anterior kidney, posterior kidney, stomach, skin, muscle, heart, brain and accessory respiratory organ (ARO)]. mRNA transcript levels were normalised to *EF-1α* (internal control). Muscle, which showed the lowest *mIRAK1* expression, was used as the calibrator. Expression in all other tissues is presented as fold change relative to this calibrator. Values represent Mean±SE (bars) from three fish (n=3)

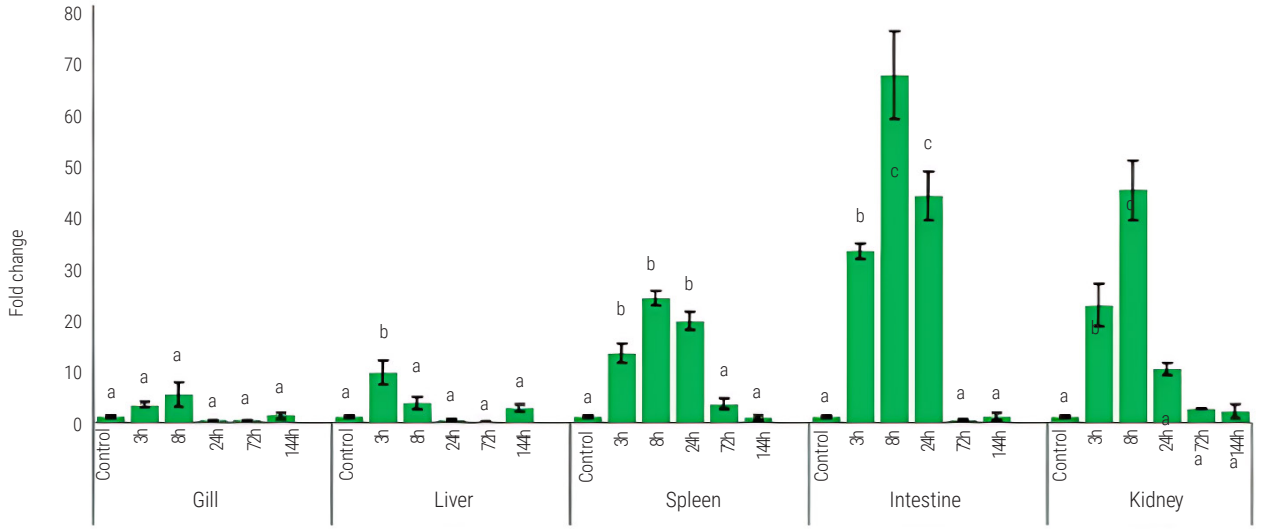


Fig. 7. Modulation of *mIRAK1* expression following *A. hydrophila* infection. Bars bearing different superscript letters indicate statistically significant differences in *mIRAK1* expression at the corresponding time point (p≤0.01), whereas bars sharing the same superscript denote no significant difference (p≥0.01)

presence of STKc domain and CDK1 motif has also been reported in the *IRAK1* gene of mandarin fish, rohu, rainbow trout and other fish species (Zhang *et al.*, 2009; Sadangi *et al*., 2020; Yang *et al*., 2021). Multiple sequence alignment of *mIRAK1* with *IRAK1* amino acid sequences from other fish species revealed a highly conserved STKc region, indicating potential functional similarities. IRAK family members are highly conserved in vertebrates (Gosu *et al*., 2012).

In this study, the phylogenetic analysis showed that *mIRAK1* clustered closely with channel catfish and pangasius catfish, suggesting a close functional association within the catfish family. Also *mIRAK1* shares 92.37 and 90.51% nucleotide sequence identity as well as 91.33%, and 91.33% amino acid sequence identity with pangasius and channel catfish, respectively. Nonetheless, the amino acid identity of *mIRAK1* was approximately 76% with carps and 61% with higher vertebrates, indicating a functional divergence of catfish *IRAK1* from carps and higher vertebrates. A similar observation has been reported for rohu *IRAK1* (Sadangi *et al*., 2020).

Basal expression analysis showed that, despite considerable variation in expression among the twelve organs examined, *mIRAK1* is constitutively expressed in all of them. The liver had the highest

basal expression of *mIRAK1*, followed by the spleen and anterior kidney, whereas muscle had the lowest basal expression. Previous studies on basal expression of *IRAK1* gene in other fish species indicate that, highest basal expression of *IRAK1* reported in blood of mandarin fish and tilapia, liver in grass carp, humphead snapper and rohu, gills in common carp and orange-spotted grouper, and muscle and liver in rainbow trout, which are all important immune-related organs. Lowest basal expression of *IRAK1* reported in muscle of mandarin fish, common carp and orange-spotted grouper, skin in tilapia, head kidney in grass carp, blood of rohu, and stomach in rainbow trout. Our observation on basal expression of *mIRAK1* shows similarity with observation reported in other fish species (Zhang *et al*., 2009; Huang *et al*., 2012; Shan *et al*., 2015; Li *et al*., 2016; Huang *et al*., 2017; Sadangi *et al*., 2020; Han *et al*., 2020; Yang *et al*., 2021).

Significant upregulation of *mIRAK1* expression was observed in prominent immune organs such as spleen, intestine and kidney from 3 to 24 hpi, in liver from 3 to 8 hpi and in the gill at 3 hpi, following infection with *A. hydrophila*. The sharp decline in *mIRAK1* expression after 24 hpi may reflect the natural resolution of early innate immune signaling through feedback inhibition, post-transcriptional regulation, or pathogen-mediated suppression of the TLR pathway. Such transient expression is common for key signaling mediators that are rapidly induced and subsequently downregulated to prevent excessive inflammation. In our previous reported study, *A. hydrophila* infection significantly induced *TLR2* and *TLR22* signaling cascade inducing synthesis of effector cytokine *IL-1β* (Muduli *et al*., 2021, Paria *et al*., 2023). Considering the downstream signaling adaptor in the TLR-signalling pathway, *IRAK1* is thought to play a crucial role in the production of cytokines after infection with the Gram-negative bacterium *A. hydrophila*. In line with our findings, *IRAK1* expression was markedly elevated in the kidney, liver, spleen, and gills of rohu infected with *A. hydrophila* (Sadangi *et al*., 2020). In rainbow trout infected with *Aeromonas salmonicida*, *IRAK1* expression was considerably increased in the spleen, head-kidney, and liver, which concurs with our observations (Brietzke *et al*., 2015). Previous studies have demonstrated that various pathogens, including viral agents like grass carp reovirus (GCRV), infectious spleen and kidney necrosis virus (ISKNV), the parasitic protozoan *Cryptocaryon irritans*, and bacterial pathogens like *A. salmonicida*, *A. hydrophila*, *Edwardsiella tarda*, *Vibrio harveyi*, *Vibrio anguillarum*, *Flavobacterium columnare*, *Bacillus subtilis* and *Streptococcus agalactiae*, as well as the bacterial analogue LPS, significantly induced the *IRAK1* expression in key immune organs across different fish species (Zhang *et al*., 2009; Huang *et al*., 2012, 2017; Brietzke *et al*., 2015; Shan *et al*., 2015; Li *et al*., 2016; Han *et al*., 2019; Sadangi *et al*., 2020; Yang *et al*., 2021). Overall, these findings suggest that *IRAK1* is involved in immune defence against a wide range of diseases in fish. Recent findings further highlight the conserved role of *IRAK1* in teleost innate immunity. A zebrafish study by Weng *et al.* (2024) demonstrated that *IRAK1*, together with *IRAK3*, activates the MyD88–TRAF6 pathway, confirming its function in TLR/IL-1R–mediated NF-κB signaling. These results, along with earlier reports in *L. rohita*, *O. niloticus*, and *S. chuatsi*, emphasise the evolutionary conservation of *IRAK1* as a key adaptor in pathogen recognition. The present findings in *C. magur* further support this, indicating that *IRAK1* plays a crucial role during *A. hydrophila* infection and may serve as a potential biomarker of innate immune activation in catfish.

In summary, the partial cDNA sequence of magur *IRAK1* gene were cloned and characterised. Important immunological organs such as the kidney, intestine, and spleen showed upregulated expression of *mIRAK1* during the early phase (3 to 24 hpi) following *A. hydrophila* infection in magur cat fish. The findings provide vauable insights into the critical role of *IRAK1* in the TLR-signaling pathway in *C. magur,* as a component of the innate immune response to *A. hydrophila* infection. Future research should advance beyond expression profiling to functional and applied investigations of *IRAK1* in *C. magur*, focusing on its molecular regulation, protein activation, functional validation, genetic variation, and potential applications in disease resistance breeding and vaccine development to enhance health management in magur aquaculture.

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