

IDENTIFICATION OF MOLECULAR MESSENGER GENES IN *LABEO ROHITA* RESPONSIBLE FOR DETECTION, CO-ORDINATION AND REGULATION OF ITS IMMUNE SYSTEM

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ABSTRACT : *Labeo rohita* (Rohu) is one of the most important fish with high potential for aquaculture. It is a major constituent of human diet. As the aqua-culturist goes for intensive or semi-intensive culture systems the incidence of disease shall exceed accordingly. To evaluate the impact of stress, it is important to study the molecular messengers in detail. Acquired immunity is not functional in fishes at the stage of hatching. Consequently, at the long run of life, these fishes are dependant on innate immunity for defence against the myriad of microorganisms found in the aquatic habitat. This work was carried out to understand the protective mechanism of rohu against these pathogens. The presence or absence of the molecular messengers found in higher vertebrates, which play a vital role in immune response and regulation was ascertained. Amplification of the genes through RT-PCR technology with novel primers indicated the presence of MHC2b, interleukin 1 beta chain, MHC 2a, MHC 1a, tumour necrosis factor (TNF) and macrophage colony stimulating factor (MCSF) gene. Whereas the presence of Natural Killer cell Enhancing Factor (NKEF), Inducible Nitrous Oxide Synthetase (iNOS), Interferon (INF), and β -actin genes could not be justified in this work as no amplification was observed in the RT-PCR experiment.

Key words: RT-PCR, Interleukin, Major histocompatibility complex, primer designing, Tumour Necrosis Factor.

INTRODUCTION

The organization of the teleost fish immune system is quite different from mammals. The major difference is that fish lack bone marrow and lymph nodes, instead they use kidney as a major lymphoid organ. Besides, they have thymus, spleen, and mucosa-associated lymphoid tissues (Press and Evensen, 1999). The cell population that is responsible for both innate and adaptive immune responses includes lymphocytes, monocytes, macrophages, granulocytes, and thrombocytes. Also, teleosts have mast cells, non-specific cytotoxic cells, and dendritic cells (Press and Evensen, 1999). Apparently, the innate immune system is of primary importance in combating infections in fish since the adaptive immune system is not that efficient due to its evolutionary status and poikilothermic nature. Therefore, the adaptive system has a limited antibody repertoire, affinity maturation and memory, and slow lymphocyte proliferation. Thus, the adaptive immune system of fish is sluggish compared to the instant and relatively temperature independent innate immune response (Alexander and Ingram 1992; Ellis, 2001; Du Pasquier, 1982). Over the past few years, many fish immune genes have been isolated and have shown compelling evidence indicating the homology between teleost and mammalian immune systems. The most important ones are the cloning of teleostean major histocompatibility (MH) genes along with immunoglobulin genes and T-cell receptors (TCR). Both MHC I & MHC II genes have been cloned in teleosts and they exhibit typical levels of polymorphism seen in mammals (Dixon et al., 1996). Moreover, some additional molecules involved in antigen presentation (invariant chain and calreticulin) have been cloned (Fujiki et al., 2003; Kales et al., 2004) in rainbow trout.

All four TCR genes have been isolated recently (Nam et al., 2003) along with accessory molecules including CD4 (Hansen, 2000) and CD8 (Dijkstra et al., 2006). Again, the complete sequence of the immunoglobulin heavy-chain gene has been cloned in teleost (Amemiya and Litman, 1990). Interestingly, fish possess a network of signalling molecules, such as, cytokines and chemokines, which control and coordinate the innate and adaptive immune systems (Secombes et al., 2001). Progress in the discovery of fish chemokines has accelerated in recent years, but still not too many fish chemokines have been discovered (Liu et al., 2002). All of the fish chemokines that have been reported to date belong to CXC and CC families, and equivalents of the C and CXXXC have not been discovered yet (Dixon et al., 1998; Long, 2000; Laing, 2002; Liu et al., 2002). The first reported chemokine belonged to CC family, and it was named CK-1 (Dixon et al., 1998). Recently, more CC chemokine genes have been discovered making the total number of CC chemokine in trout 18. Some of these recent chemokines (CK4, CK5, CK7, CK8, CK12) have been found to exist in pairs (a & b); therefore, still it is not certain if they are alleles or separate genes (Laing and Secombes, 2004). In addition, 26 CC chemokines have been identified in catfish (Peatman et al., 2005).

Also, a highly inducible CC chemokine has been isolated in lipopolysaccharide stimulated rainbow trout macrophages, which is similar to human CCL4 (MacKenzie et al., 2004). Several CXC, chemokines have been identified in fish, including a CXCL8 (IL-8) like molecule in lamprey (a jawless fish or aganatha) (Najakshin et al., 1999), which is one of the earliest chordates. CXC chemokines, similar to the IL-8 gene, have also been found in flounder (Lee et al., 2001), in rainbow trout (Laing et al., 2002; Sangrador-Vegas et al., 2002), in carp (Savan et al., 2003), zebrafish (Long et al., 2000) as well as, three catfish CXC chemokines (Baoprasertkul et al., 2005). To date only one functional study has been published on this subject. There are many chemokines identified in mammals; however, the discovery of fish chemokines is promising and the presence of fish chemokines representing several structural groups suggests that the chemokine network in fish may be as extensive as the one found in mammals.

Variation within class II major histocompatibility α genes had been compared between domesticated Australian Atlantic salmon and their ancestral Canadian population. (Wynne et al., 2006). First non-mammalian interleukin-11 gene was identified, cloned and expressed in rainbow trout (Wang et al., 2005). Immunostimulatory activity of trout interleukin (IL)-1 α -derived peptides had been studied in vivo (Scott et al., 2003). Schwartz et al., (1992) had identified an interleukin-2 like substance in *Cyprinus carpio*. INOS gene had been identified cloned and expressed in Rainbow trout (WANG, et al., 2001). Molecular and Functional Analysis of an Interferon Gene from the Zebrafish had been accomplished (Altmann, et al., 2003). Endotoxin recognition had also been identified through the presence of toll-like receptor (TLR) family, which recognizes specific pathogen-associated molecular patterns (Iliev et al., 2005). Macrophage Colony Stimulating Factor genes had been identified and sequenced in rainbow trout (Wang and Secombes, 2005). Natural killer cell enhancing factor has also been verified and sequenced in *Paralichthys olivaceus* (Wang, et al., 2005). Messenger RNA for putative tumour necrosis factor alpha gene has been identified, cloned and expressed in *Oncorhynchus mykiss* (Laing, et al., 2001). Unfortunately, the immune system of the Indian fish is yet to be characterized. One of the most important fish in India, which is being extensively used for aquaculture as a potential source of food is rohu, *Labeo rohita*. In the current paper we have tried to detect the genes like INOS, Interleukin, MHC, NK cell enhancing factor, Macrophage Colony Stimulating Factor, β -actin, Interferon & Tumour Necrosis Factor in the *Labeo rohita*.

MATERIALS AND METHODS

Sample preparation:

For the present work rohu fish were taken from the local market. They were then acclimatized in 6*3*2 feet³ plastic pool for a minimum period of one week and were fed @2% of the body weight. After acclimatization the fish were transferred to wet laboratory.

The fish of 200-250gm weight were then sacrificed to obtain Liver, heart, kidney, gill and spleen. The tissues were then preserved in 90% ethanol until the tissues were used for DNA and total RNA extraction.

Extraction of total RNA and DNA

Various tissues were selected i.e. kidney, liver, heart, spleen, gill and thymus from *Labeo rohita* for total RNA isolation. The tissues were preserved in 90% DPEC treated 90% ethanol until they were used for total RNA isolation. Guanidinium thiocyanate –phenol-chloroform isolation method, with some modifications was employed for total RNA isolation. Guanidinium thiocyanate is RNASE inhibitor and hence is widely used for undegraded RNA isolation. Hundred milligrams of tissues were first homogenized in Solution D with homogenizing sticks. The homogenization was finally done by squeezing and releasing the mixture through an 18 gauge needle for 5 to ten times.

After homogenization the homogenized tissues were successively treated with 0.1 volume of Sodium acetate, 1 volume of water saturated phenol and 0.2 volume of fresh chloroform/isoamyl alcohol (24:1). The contents were properly mixed after the addition of each compound. The contents were finally mixed through vortex for 10 seconds. The eppendorf tube containing the reaction mixture was then placed on ice for 15 minutes. The tube was then centrifuged at 10,000*g for 30 minutes at 4°C. The aqueous phase was separated and the steps after homogenization were repeated. The aqueous phase again collected was treated with equal volume of Isopropanol and was incubated at -20°C for precipitation of RNA. The tube was centrifuged at 10,000*g for 30 minutes at 4°C. The pellet was dissolved in 600 μ l of solution D and was then successively treated with 0.1 volume of Sodium acetate, 1 volume of water saturated phenol and 0.2 volume of fresh chloroform/isoamyl alcohol (24:1). The contents were properly mixed after the addition of each compound. The contents were finally mixed through vortex for 10 seconds. The eppendorf tube containing the reaction mixture was then placed on ice for 15 minutes. The tube was then centrifuged at 14,000*g for 15 minutes at 4°C. The aqueous phase was collected and treated with equal volume of Isopropanol and was incubated at -20°C for precipitation of RNA. The tube was then centrifuged at 14,000*g for 5 minutes at 4°C. The pellet obtained at this step was washed with 70% ethanol by brief centrifugation at 10,000*g for 2 minutes. The pellet was air dried and then dissolved in 200 μ l, 0.5% SDS and was stored at -20°C until it was used for cDNA synthesis.

For DNA extraction 100 mg of tissue was collected in a 1.5 ml microfuge tube with 600 μ l of lysis solution (100 mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA [ethylene diamine tetra-acetic acid], 0.5% SLS [sodium N-laurylsarcosinate] or 2% SDS [sodium dodecyl sulphate], and 0.5 mg/ml proteinase K added just before use). The tissue was homogenised in the tube thoroughly using a micropestle and incubated at 65°C for 1 hour. Thereafter, 5 M NaCl was added to a final concentration of 0.7 M. followed by slow addition of 1/10 volume of N-cetyl N,N,N-trimethyl ammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl). The samples were incubated at 65°C for 10 minutes, and then, at room temperature, an equal volume of chloroform/isoamyl alcohol (24/1) was added and mixed gently. The tubes were centrifuged at 13,000 g for 5 minutes and aqueous solution was transferred to a fresh 1.5 ml. An equal volume of phenol was added and mixed gently. The tubes were again centrifuged at 13,000 g for 5 minutes. The upper layer was collected and phenol extraction process was repeated. The upper layer was mixed gently with two volumes of chloroform/isoamyl alcohol (24/1) and centrifuged at 13,000 g for 5 minutes. The upper layer was transferred to a new tube and DNA was precipitated by adding two volumes of absolute ethanol followed by standing at -20°C for 30 minutes and later, centrifuged at 13,000 g for 30 minutes. The DNA pellet was washed with 70% ethanol, dried and resuspended in 50 μ l sterilised double-distilled water at 65°C for 15 minutes. One μ l of genomic DNA was loaded on 1% agarose gel electrophoresis.

Estimation of total RNA:

The concentration of total RNA isolated at the previous step was estimated through UV Spectrophotometer. The spectrophotometer was used with multiple wavelength range of 260 nm, 280 nm, and 320 nm. The 260 nm wavelength was used for measuring the optical density of RNA solution. RNA was estimated in the solution of 0.5%, which was used as blank solution. The 280 nm and 320 nm wavelength are used for DNA and protein estimation respectively. The ration between optical density at 260 nm and 280 nm gives the purity of the RNA. Purity of RNA sample = Optical density at 260 nm/ Optical density at 280 nm. The value of purity should always be greater than 2. From the optical density obtained at 260 nm total RNA yield was calculated by the following formulae: Total RNA yield = Optical density at 260 nm * 40(μ g/ μ l) * dilution factor

Generation of cDNA library:

Synthesis of cDNA was done using the Invitrogen kit for cDNA synthesis. We followed the single stranded cDNA synthesis specification provided by the manufacturer for total cDNA synthesis. 250ng Random hexamers were used for the total cDNA synthesis included with the kit by using 2 μ g of total RNA in a 12 μ l reaction volume containing 5 μ g of total RNA and 1 μ l of 10mM dNTP mix which were then heated at 65°C for 5 minutes. Immediately after 5 minutes the tubes was quickly chilled on ice and the contents were collected through brief centrifugation. 4 μ l of 5* first strand buffer, 2 μ l 0.1DTT and 1 μ l of RNASE OUT was added and the tubes were incubated at 37°C for 2 minutes. Finally 1 μ l of MMLV reverse transcriptase enzyme was added to make the final reaction mixture equal to 20 μ l. the tube was then incubated successively at 25°C for 10 minutes, 37°C for 50 minutes and 70°C for 15 minutes. The samples were then stored at -20°C until PCR was done.

Primer designing:

We retrieved the gene sequences from fishes like *Cyprinus carpio*, *Danio rario*, *Oncorhynchus mykiss*, *Salmo salar*, *Takifugu rubripes*, *Oreochromis niloticus*, *Sparus aurata* etc. for designing the primers for Rohu fish. The gene sequences were retrieved through NCBI. Homologous sequences were retrieved through BLAST N from NCBI web site. The homologous sequences of particular genes were used for generation of multiple alignment. We aimed at designing a universal primer for the genes, which may work for the amplification of immune response genes in *Labeo rohita*. The program used for multiple alignments was ClustalW.

Selection and Analysis of primer:

Selection of primer pattern was manually done through the consensus of the multiple alignment obtained through JALVIEW of ClustalW program. The alignment residues were coloured on behalf of conservation. We selected the pattern for forward and reverse primers with length of primer to be about 17 to 24 and the length of gene to be in the interval of 250 to 500. Primers were analysed through Oligo 4.0 software. The software was used to screen the primer pool and the best primer combination was selected on the basis of T_m values, self-complementary regions, dimer formation, AT/CG ratio, and stability curve of 5' and 3' end. Based on these values the primer pool was screened and the best primer combination was selected.

Amplification of genes through RT PCR:

Amplification of genes was done using the designed gene specific primers through RT-PCR. The PCR conditions were manually evaluated for each specific gene using the primer specifications. Amplification of all the genes were done in all tissues isolated from the subject fish. Primers specific to rainbow trout β actin forward (5'-ACACCTTCTACAATGAGCTG-3') and reverse (5'-CTGCTTGCTGATCCACATCT-3') were used as internal PCR control. 18S-RNA forward and reverse primers were used as housekeeping genes to predict whether the PCR proceeded successfully.

Amplification was performed in 25 μ l reaction volumes containing the Taq polymerase (Bangalore genei) with 2.5 μ l of 10*PCR buffer (FERMENTAS), 2.5 μ l of 25mM MgCl₂, 1 μ l of 10mM dNTP mix, 2 μ l of template cDNA and forward and reverse primers 10 pmoles. Briefly, PCR's were performed at 94°C for 5 minute and then at 94°C for 1 minute 50°C for 1 minute and 72°C for 1 minute for 30 cycles, with a final extension at 72°C for 10 minutes. The amplification of genes were analysed through agarose gel electrophoresis.

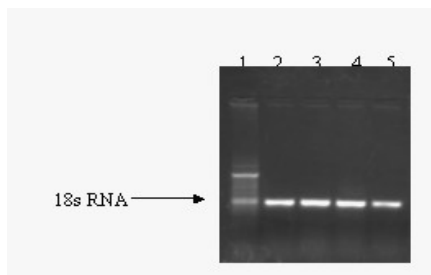
RESULT AND DISCUSSION

The primers designed for amplification of specific genes are listed in table 1. T_m Values of the primers is also given in table 1. 18SRNA has been used as a housekeeping gene in eukaryotes as the sequence of 18S RNA is highly conserved in all eukaryotes. The efficiency of PCR reaction and presence of c-DNA was confirmed by the PCR amplification of 18SRNA (figure1). Sharp band in agarose gel confirmed the presence of c-DNA as well as the successful PCR reaction amplification. The 18SRNA was amplified in c-DNA preparation of kidney, liver, gill and heart.

Table 1: List of primers designed for detection of immune response and regulation in rohu fish along with their T_m Values.

S.No.	Designed Primer Sequence	Annealing temperature
	INOS F(5'-TGGTACATGGGCACTGAG-3')	56
	INOS R (5'-AGTCTCCATTCCCAAAGG-3')	54
	INFg F (5'-GGTGGAGGACCAGGTGAA-3')	58
	INFg R (5'-CGATGATGTCCATCCTCTG-3')	57
	IL1b F (5'-AAGGCTGTGACGCTGAGT-3')	52
	IL1b R (5'-CTGAAGAAGAGGAGGCTGT-3')	53
	MHC1a F (5'-GGTTATGATGGAGAAGAC-3')	59
	MHC1a R (5'-TAGAAACCTGTAGCGTG-3')	57
	NKEF F (5'-CTGGACTTCACCTTGT-3')	49
	NKEF R (GATGGTGATCTGCCTCA-3')	47
	MCSF F (5'-TATGAGATCCGCTGGAAGATC-3')	58
	MCSF R (5'-CATCAGAGCCTCTCTCATC-3')	56
	TNF F (5'-CTTCGTTTACAGCCAGC-3')	48
	TNF R (5'-GTGCAAACACACCAAAG-3')	49
	MHC2a F (5'-GTGACTGGTTTCTATCCTGCT-3')	51
	MHC2a R (5'-CTTTGATGAGGAAGAAGGTTC-3')	52
	MHC2b F (5'-CAGGTTCAGCAGCAGTGTG-3')	54
	MHC2b R (5'-AGACCTGGGCGTGTACTG-3')	55
	bACT F (5'-ATGGAAGATGAAATCGCC-3')	55
	bACT R (5'-TGCCAGATCTTCTCCATG-3')	57

Figure 1: Agarose gel photograph of 18sRNA amplification in: lane1, positive control, lane2 kidney, lane 2, liver, lane 3 gill, lane4, heart. A single band in all the four lanes is the amplification of 18sRNA.



Amplification of target genes through RT-PCR

To test the presence of the genes under study, sequence specific amplification of the genes through the selected primers was done. The genes were amplified at their respective annealing temperature and were run on agarose gel to detect the band of selected genes with appropriate size. Presence of weak band was seen in the agarose gel (figure 2a & 2b).

From RT-PCR six out of ten genes showed different degree of amplification with total RNA amplification was light. The light amplification of the six genes (MHC1a, MHC2a, MHC2b, Interleukin beta chain, TNF, MCSF) may be due to unoptimized PCR conditions. The PCR condition is subject to optimization for sharp amplification. Genes like NKEF, INOS, INF, and BACT didn't show any amplification in the current study.

Figure 2a: band pattern in agarose gel photograph of gene amplification of selected genes under study. Lane1, 18sRNA, lane2, mhc1a; lane3, mhc2b; lane4, MCSF; lane5, TNF.

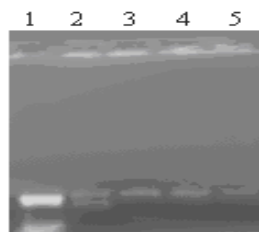
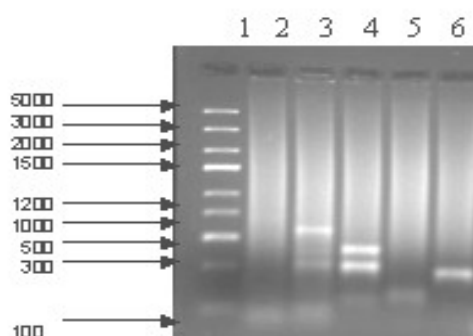


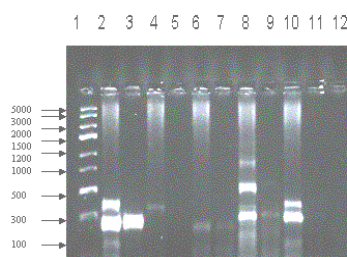
Figure 2b: band pattern of agarose gel photograph for remaining genes under study i.e Lane1, 5kb generuler express ladder, lane2, mhc 1a, lane3 mhc2a, lane4, interleukin, lane5, inos, lane6, 18s RNA



Detection of amplification in both DNA and RNA

As we got some weak amplification of gene through amplification from c-DNA therefore we did PCR reaction from both genomic DNA and c-DNA sample. The result is shown in figure 3.

Figure 3: Agarose gel photograph for detecting amplification through PCR reaction in both c-DNA and genomic DNA sample. Lane 2,4,6,8,10 contain sample through amplification of genomic DNA whereas lane3,5,7,9,11 contain amplification of gene same genes through c-DNA samples.



Through RT-PCR we found amplification in six out of the ten genes we used in our study. Initially very light amplification was detected through RT-PCR but with the modification of PCR condition we finally obtained sharp bands in some genes like MHC2b, interleukin 1 beta chain gene and MHC 2a, and light bands in MHC 1a, tumour necrosis factor gene, macrophage colony stimulating factor gene. Also a light band in BACT gene was detected from the DNA sample of rohu.

The presence of light bands may be an indicative for lesser expression levels of the genes but with accurately optimizing the RT-PCR condition we can get sharp amplification of the genes. Later on we modified RT-PCR conditions and amplification was done with both DNA and RNA sample. As the amplification of the genes was more prominent and sharp amplification was observed with DNA sample concludes that at the stage of evolution the genes of innate immunity are present in the genome of rohu but the expression level is lesser as evident from the amplification of the same genes in RNA sample. One interesting finding is presence of multiple bands of same length in both DNA and RNA sample indicates presence of isoforms of the same gene. Two sharp bands are visible in interleukin gene in both DNA and RNA sample indicating that there are two isoforms of the interleukin genes.

Also similar pattern is seen in MHC2b where 3 consecutive bands are visible indicating the presence of three isoforms of the gene. Presence of MHC gene is what is responsible for allograft rejections as rohu is able to distinguish between self and non-self discrimination. Though amplification was not observed in NKEF, BACT, INOS, and interferon genes but that doesn't apply that the genes are not present. There may be possibility of the absence of the genes but PCR optimization is necessary before final conclusion.

In future we will assess the functionality and expression of the genes through lymphocyte and cell culture.

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