

Full Length Research Paper

Development and application of a real-time quantitative PCR assay for determining expression of benzo-a-pyrene-Inducible cytochrome P450 1A in Nile tilapia (*OREOCHROMIS NILOTICUS*)

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Cytochrome P4501A's (CYP1A) constitute a ubiquitous family of proteins associated with the detoxification of organic compounds such as PCB (polychlorinated biphenyl), PAH (polyaromatic hydrocarbons) and dioxin. These compounds are documented to induce the CYP1A gene in a variety of tissues of many fish species. Consequently, changes in CYP1A gene expression have been used as a biomarker for contaminant exposure in fish populations using a variety of techniques. Of all of these methods, quantitative PCR appears to be the most sensitive. It has been used to assess impact of environmental pollution in marine ecosystems using different fish models. Subsequently, for measuring benzo-a-pyrene (BaP) induction of CYP1A mRNA in different organs of tilapia (*OREOCHROMIS NILOTICUS*), ribosomal protein large P0-like protein (RPLP0-like protein) and β -actin genes as internal controls were selected based on previous studies to assess their expression variability. Real-time polymerase chain reaction (real-time PCR) analysis of liver, intestine, gills and kidney revealed a distinct induced expression in liver and intestine (127.1 and 79.3 in liver, 26 and 56.1 in intestine using RPLP0 and β -actin genes respectively as internal controls) with no detectable expression in the other organs studied.

Key words: Benzo-a-pyrene, oreochromis, real-time PCR, internal control.

INTRODUCTION

Aquatic ecosystems serve as the ultimate sink for many environmental pollutants, which accumulate in fish species. Fish are among the most sensitive species to the toxicity of an AhR agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin, particularly at early developmental stages (Walker and Peterson, 1991; Walker et al., 1992; Dong et al., 2001, 2002; Carney et al., 2006). In addition, fish exposed to dioxin-like compounds show induction of CYP1A activity (Ethoxyresorufin-O-deethylase activity) and

its mRNA (Kreamer et al., 1991; Stegeman and Hahn, 1994; Bucheli and Fent, 1995; Goksoyr, 1995; Goksoyr and Husoy, 1998; Fenet et al., 1998; Levine and Oris, 1999; Wong et al., 2001).

Therefore, in fish, the CYP1A gene has been used as a useful biomarker of dioxin-like compounds for assessing the level of contamination in an aquatic environment (Williams et al., 1998; Moore et al., 2003; Fent, 2003). Real-time PCR (qRT-PCR) is a sensitive, reproducible and high-throughput method that can show subtle changes in relative quantities of a large number of genes, consuming small sample amounts (Bustin, 2002; Bustin and Nolan, 2004). Detection with SYBR Green has been recommended due to its adaptability to most quantification

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Table 1. Oligonucleotide primers used in PCR amplification of tilapia RPLP0 cDNA fragments.

Primer	Nucleotide Sequence
F	5'-CCCTCTCACCATCCCCACTCC
Long UPM	5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT
Short UPM	5'-CTAATACGACTCACTATAGGGC

systems (Rasmussen et al., 1998; Schmittgen and Zakrajsek, 2000; Vandesompele et al., 2002).

For accurate data interpretation a stable normalizer is mandatory. Housekeeping genes have been considered to be constitutively expressed and minimally regulated. Therefore, they have been widely used as internal RNA references for qualitative analysis and are used as reference genes (endogenous controls) for quantitative analysis in qRT-PCR assays (Carrillo-Casas et al., 2008). However, the mRNA expression of many internal controls varies with the experimental conditions and no single gene has been shown to be invariant *per se* (Thellin et al., 1999; Stürzenbaum and Kille, 2001; Radonic´ et al., 2004).

Therefore, in this study, we determined the expression pattern of CYP1A mRNA in liver, intestine, gills and kidneys of tilapia after intraperitoneal injection of benzo-a-pyrene (BaP), a CYP1A inducer (Hahn and Stegeman, 1994) using real-time PCR and RPLP0, β -actin mRNAs as internal controls.

MATERIALS AND METHODS

Treatment of fish

Nile tilapia (*Oreochromis niloticus*) with a mean weight of 500 g were obtained from a local fish farm and were treated with a single intraperitoneal injection of benzo-a-pyrene (100mg/ kg body weight) suspended in corn oil. Simultaneously with the treated fish, control fish of similar mean weight was intraperitoneally injected with an equivalent volume of the vehicle (corn oil). The treated and control fish were killed 24 h after the injection and samples of liver, kidney, gills and intestine were collected, immediately frozen in liquid nitrogen and stored at -80°C.

RNA isolation

Total RNA was isolated from 2 g of each of the samples of frozen liver, kidney, gills and intestine according to the Standard Acid Guanidinium Thiocyanate Phenol Chloroform (AGPC) extraction method (Chomczynski and Sacchi, 1978). Total RNA concentration and purity were determined spectrophotometrically as described by (Sambrook and Russel, 2001), and A_{260}/A_{280} ratio were between 1.7 and 1.9.

Reverse transcription

Reverse transcription (RT) was performed using Primescript™ RT reagent kit (Takara, Japan) according to the manufacturer's instructions. Reactions were incubated for 15 min at 37°C then 5 s at 85°C to inactivate the reverse transcriptase. RT products were stored at

4 °C for further PCRs.

Identification and cloning of *O. niloticus* ribosomal protein large P0-like protein (RPLP0) gene as an internal standard

We designed one sense (F) primer specific to Tilapia RPLP0 for 3' RACE PCR. The primer sequence is shown in Table 1. The sense gene specific primer was used in combination with the universal primer mix (UPM) included in a RACE PCR kit (Clontec Inc) to generate partial sequence of *O. niloticus* ribosomal protein large P0-like protein gene.

The cycle conditions for RACE PCR were as follows: 5 cycles of denaturation for 30 s at 94°C and annealing for 3 min at 58°C; 5 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension for 3 min at 72°C; and finally 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 s and extension at 72°C for 3 min. After purification using GFX PCR DNA and a gel band purification kit (GE Health Care, UK), the PCR products obtained were cloned into PT7BlueT-vector (Novagen, USA). Purified plasmids were directly sequenced by dye terminator cycle sequencing using an ABI PRISM dye terminator cycle sequencing kit (PE Biosystems, USA) and an applied Biosystems 3130 xl DNA sequencer.

Phylogenetic analysis of RPLP0-like protein gene

DNA sequences with the following Genbank accession numbers were retrieved from the database and used in the phylogenetic analysis: EU714144 {orange-spotted grouper (*Epinephelus coioides*) RPLP0}, EU081838 {yellow perch (*Perca flavescens*) RPLP0}, AY550965 {gilthead seabream (*Sparus aurata*) RPLP0}. In order to determine homology among RPLP0 family cDNAs or deduced amino acid sequences from various species, sequence alignment was performed by the CLUSTAL W method using Laser gene Megalign program (Ver 5.52,2003, DNASTAR Inc).

Primer design for real-time PCR reaction

Primers for *Oreochromis niloticus* CYP1A (accession no. FJ389 918), RPLP0 (accession no. FJ389919) and β -actin (accession no. EU887951) genes (Table 2) were designed using Laser gene primer select program (Ver5.52, 2003, DNASTAR Inc), with melting temperatures (T_m) ranging from 58 - 60°C, and amplicon lengths of 50-150 bp. Optimal programmed primer annealing temperatures were designed closely so that the optimal annealing temperatures were close enough to run all reactions under the same thermal parameters.

Real-time PCR conditions and analysis

Each PCR reaction consisted of 10 μ l of SYBR® Premix Ex Taq™ II (2X), 0.5 μ l of each primer (10 μ M), 2 μ l of cDNA template (500 ng/ μ l) and double distilled water to a final volume of 20 μ l. Reactions were then analyzed on an ABI 7300 Real-Time PCR System under

Table 2. Real-time PCR primers of *Oreochromis niloticus* CYP1A, RPLP0 and β -actin genes.

Gene	Direction	Sequence (5'-3')	Location	Product size
CYP1A gene	F	5'-AGTTTGTGCTCCTGCTGTATTGTT	1950 - 1974	124 bp
	R	5'-GGTTGGATAGTTTAGGATGCTGCA	2051 - 2074	
RPLP0 gene	F	5'-CCCTCTCACCATCCCCCACTCC	1-22	92 bp
	R	5'-TTGTCTGCCAGGAGGAAGGA	74 - 93	
β-actin gene	F	5'-GGGTCAGAAAGACAGCTACGTT	42 - 63	143 bp
	R	5'-CTCAGCTCGTTGTAGAAGGTGT	164 - 185	

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CCTCTCACCATCCCCCACTCCGTCATCAATGGCTACAAGAGAGTCTGGCTGTTGCTGTGGAGACAGACTACTCCTTCCCCCTGGCA 87

PLTIPHSVINGYKRVLAVAVETDYSFPLA 29

GACAAAGTCAAAGCCTTCTGGCTGACCCATCTGCATTTGCTGTGCGAGCACCTGCAGCAGCTGCTGAGACTGCCGAGCTCCAGCT 174

DKVKAFLADPSAFAVAAPAAAAETA AAPA 58

GCTGCTGCTAAGGAGGAGGCCAAGGAGGAGTCTGAGGAATCAGATGGCGACATGGGCTTCGGTTTGTTCGACTAATAAAATACAGCG 261

AAAKEEAKKEESEESDGMGFGLFD* 83

AACAGAACCACATCTAATAAGAACATGATTC**AATAAA**TAAACGTAAGATACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 342

Figure 1. Nucleotide sequence (343 bp) of cytochrome RPLP0-like protein cDNA and its deduced amino acids (83) residues. Consensus sequence for polyadenylation signal is in bold. The stop codon, TAA, is marked with an asterisk.

the following conditions: 95 °C for 10 s and 40 cycles of 95 °C for 5 s followed by 60 °C for 1 min. All standard dilutions, no template controls, and induced samples were run in triplicates. The fluorescence signals were measured at the end of each extension step. The threshold cycle (Ct) was determined for each sample using the exponential growth phase and the baseline signal from the fluorescence versus cycle number plots. To ensure that a single product was amplified, melt curve analysis was performed on the PCR products at the end of each PCR run.

The amount of CYP1A mRNA, normalized to RPLP0-like protein and β -actin mRNAs, was given by the formula: 2^{-C_T} ; where C_T is the threshold cycle indicating the fractional cycle number at which the amount of amplified CYP1A reached threshold. The C_T value is determined by subtracting the average RPLP0-like protein C_T value from the average CYP1A C_T value. Then, the calculation of

C_T involves subtraction of the C_T value of the calibrator (in our case the calibrator was average C_T value of control fish response in the BaP studies) from C_T value of each sample. Accordingly, CYP1A mRNA levels were reported as fold change in abundance relative to the average calibrator response.

Statistical analysis

The statistical differences between the groups were determined, and the data expressed as mean \pm standard deviation. Excel (Microsoft, NY) were used to analyze the data.

RESULTS

Partial nucleotide sequence of RPLP0 gene

The partial nucleotide sequence (Figure 1) was about 343 bp contained 249 bp of the open reading frame, and a 3' noncoding region of 93 bp. The sequence has one polyadenylation signal (AATAAA) and a poly A tail of 31 nucleotides. This sequence was aligned with *Epinephelus coioides* ribosomal protein LP0-like protein, *Perca flavescens* ribosomal protein LP0-like protein and *Sparus*

Table 3. Percent identities of deduced amino acid sequences of fish RPLP0 gene.

	Orange-spotted grouper	Yellow perch	Gilthead seabream
Nile tilapia	84.3	18.1	18.1
Orange-spotted grouper		12.8	12.8
Yellow perch			72.2

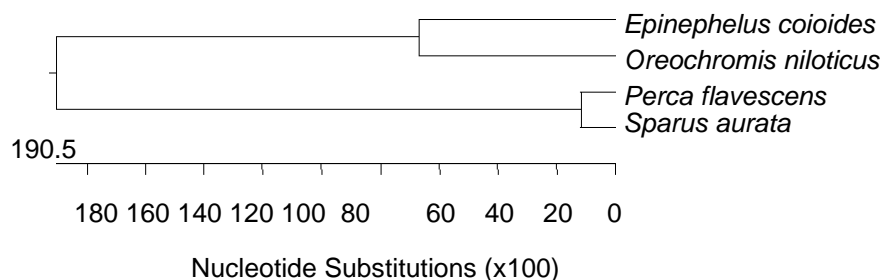


Figure 2. Phylogenetic tree of RPLP0 genes in fishes.

Table 4. RT-PCR results for tilapia CYP1A, RPLP0 and β -actin genes.

Sample name	CYP1A		RPLP0		β -Actin	
	Mean Ct	Std Dev Ct	Mean Ct	Std Dev Ct	Mean Ct	Std Dev Ct
L.cont	25.06	0.02	23.42	0.62	21.09	0.09
L.ind	15.49	0.09	20.85	0.21	17.83	0.13
I.cont	30.75	0.52	22.13	0.34	23.02	0.14
I.ind	25.24	0.59	21.33	0.17	23.32	0.44

L.cont = Liver control; L.ind = liver induced; I.cont = intestine control; I.ind = intestine induced.

aurata ribosomal protein LP0-like protein sequences by CLUSTAL W (Thompson et al., 1994) using Lasergene Megalign program, version 5.52, 2003 (DNASTAR Inc) and has been deposited in the GenBank/NCBI data bank with an accession number FJ389919.

Comparison of amino acid sequences

Table 3 showed the percent identities of deduced amino acid sequences of Nile tilapia (*O. niloticus*) RPLP0-like protein gene with the other fish RPLP0-like protein genes. The highest identity was 84.3% with orange-spotted grouper (*Epinephelus coioides*), followed by 18.1% with both yellow perch (*Perca flavescens*) and gilthead seabream (*Sparus aurata*).

Phylogenetic analysis

The phylogenetic tree based on the amino acid sequences were used to assess the relationship of RPLP0-like protein gene of *O. niloticus* with those of other fish species. Figure 2 clearly shows tilapia RPLP0-like protein

and *E. coioides* RPLP0-like protein to be more closely related to each other than to *P. flavescens* and *S. aurata* RPLP0-like protein gene.

CYP1A mRNA level in different tissues of BaP treated fish

RT-PCR results revealed that there was a large increase in CYP1A mRNA in liver and intestine (127.1 and 79.3 in liver, 26 and 56.1 in intestine using RPLP0 and β -actin genes respectively as internal controls) (Tables 4, 5 and 6), (Figures 3 and 4) but no significant signals in gills and kidney were detected.

DISCUSSION

RT-PCR results revealed that there was a large increase in CYP1A mRNA in liver and intestine with no detectable expression in gills and kidney. The induction of CYP1A in liver and intestine provided a defensive mechanism against the pollutants entering from the external environment.

Table 5. Amount of CYP1A mRNA, normalized to RPLP0-like protein mRNA.

Sample name	Tilapia CYP1A average Ct	RPLP0 gene average Ct	Ct	Ct	2 ^{-Ct}
L.cont	25.06	23.42	1.63	-6.99	127.1
L.ind	15.49	20.85	-5.36		
I.cont	30.75	22.13	8.62	-4.7	26
I.ind	25.24	21.33	3.92		

L.cont = Liver control; L.ind = liver induced; I.cont = intestine control; I.ind = intestine induced.

Table 6. Amount of CYP1A mRNA, normalized to β -actin mRNA.

Sample name	Tilapia CYP1A average Ct	B-actin gene average Ct	Ct	Ct	2 ^{-Ct}
L.cont	25.06	21.09	3.97	-6.31	79.3
L.ind	15.49	17.83	-2.34		
I.cont	30.75	23.02	7.73	-5.81	56.1
I.ind	25.24	23.32	1.92		

L.cont = Liver control; L.ind = liver induced; I.cont = intestine control; I.ind = intestine induced.

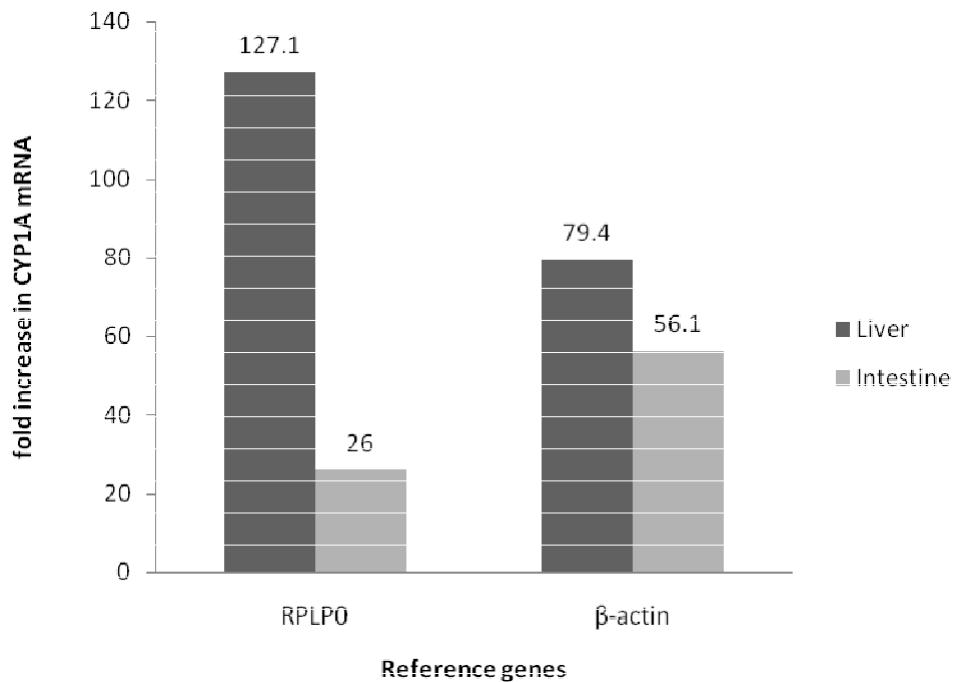


Figure 3. Tilapia CYP1A mRNA normalized to RPLP0- like protein mRNA.

Among different biochemical responses, CYP1A mRNA induction was considered to be a sensitive biomarker in response to persistent organic pollutants (POPs) contamination (Wong et al., 2000). The induction of CYP1A mRNA is of toxicological significance due to involvement in the activation and detoxification of procarcinogens and other toxicants (Nebert, 1989). Our results agreed with that of Chris et al. (2001), who studied the specific expression of cytochrome P4501A gene in gill,

intestine and liver of tilapia exposed to coastal sediments using the primer dropping polymerase chain reaction technique and found that following sediment exposure, there was a large increase in CYP1A mRNA in intestine and liver but no significant changes in gill tissues (In the first 3 days exposure, there was from 34-96 and 69-156% induction of the CYP1A1 transcripts in intestine and liver, respectively. Following 7 days of exposure, a continued induction of high level of CYP1A1 mRNA in

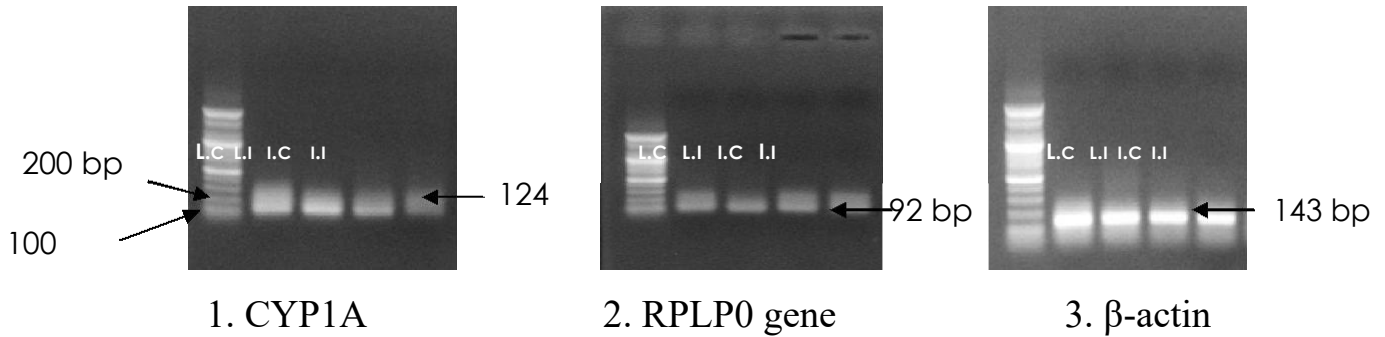


Figure 4. Real-time PCR results for tilapia CYP1A (1), RPLP0 (2), β -actin (3) genes. Where L.cont = Liver control; L.ind = liver induced; I.cont = intestine control; and I.ind = intestine induced.

intestine (73-157%) was observed). The relatively low inducibility in gill tissue was supported by Leguen et al. (2000), demonstrating that gill cells had a significantly lower capacity (1.5-14%) of the biotransformation systems compared with hepatocytes. Liver CYP1A regulation and enzymatic activity shows species-specific responses to organic contaminants (Stein et al., 1993; Flammarion et al., 1998; Arinc et al., 2000; Whyte et al., 2000). It was reported that a single intra peritoneal injection of BaP induced liver CYP1A expression in various fish species such as *Fundulus heteroclitus* (>5 $\mu\text{g/g-BW}$, Willett et al., 2001), *Turbot* (>5 $\mu\text{g/g-BW}$, *Scophthalmus maximus*, Telli-Karakoc et al., 2002), Japanese medaka (*Oryzias latipes*, >20 $\mu\text{g/g BW}$, Carlson et al., 2002).

Oh et al. (2009) determined the expression pattern of CYP1A gene in brain, heart, liver, kidney, gonad, intestine, gill, spleen, and muscle of goldfish (*Carassius auratus*) after intraperitoneal exposure to benzo-a-pyrene (BaP) using quantitative RT-PCR and found that BaP-IP exposure significantly increased CYP1A gene expression in the intestine and gill as well as liver, which are known as major target tissue of xenobiotics.

Neilson (1994) and Wong et al. (1996) owing the high level of expression of CYP1A in intestine with no detectable expression in gills to the fact that polycyclic aromatic hydrocarbons (PAHs) have very low water solubility, therefore ingestion of sediments and bottom detritus suggested being the major route of sediment-associated contaminants intake by tilapia. Van Veld et al. (1990) reported that fish collected from PAHs contaminated sites could show CYP1A induction in the intestine, which is a major target tissue of xenobiotics uptake for highly lipophilic persistent organic pollutants (POPs) such as many AhR ligands (Van Veld et al., 1990; Wong et al., 2001).

Analysis of gene expression requires sensitive, precise, and reproducible measurements for specific mRNA sequences. To avoid bias, real-time PCR is referred to one or several internal control genes. In our experiment we used RPLP0-like protein and β -actin genes as internal

standards as both show nearly similar expression levels in both control and BaP treated tissues (Table 4). Hongbao Ma et al. (2006) mentioned that a gene that is to be used as a loading control (or internal standard) should have various features; the standard gene should have the same copy number in all cells, it should be expressed in all cells and a medium copy number is advantageous since the correction should be more accurate. They also suggested the use of mRNA for RPLP0 protein and β -actin as commonly used internal standards.

A reliable internal control should show minimal changes, whereas a gene of interest might change over the course of an experiment (Bustin et al., 2005; Dheda et al., 2005; Huggett et al., 2005). Thus, choosing an internal control is critical for gene expression quantification. Consequently, it is necessary to validate the expression stability of a control gene under specific experimental conditions prior to its use for normalization.

In conclusion, we have developed a real-time quantitative PCR assay for analysis of CYP1A expression in different organs of Nile tilapia after intraperitoneal injection of benzo-a-pyrene and found distinct induced expression in liver and intestine with no detectable expression in gills and kidneys. The real-time assay has a high degree of sensitivity detecting down to 1000 molecules CYP1A μl^{-1} total RNA. This validation study for real-time assay will enable more accurate and reliable normalization of real-time PCR results to encourage further studies directed toward a better understanding of the function of specific genes in *Oreochromis niloticus*.

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