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Cytochrome P450 3A and its role in metabolism of erythromycin by hepatic microsomes of Indian major carps, *Labeo rohita* (Ham.), *Catla catla* (Ham.) and *Cirrhinus mrigala* (Ham.)

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Abstract
Indian major carps, *Labeo rohita, Catla catla* and *Cirrhinus mrigala* were challenged intraperitoneally with a dose of erythromycin (50 mg kg^{-1} body weight) for 24 hrs, 48 hrs, 72 hrs and 96 hrs and hepatic microsomes were isolated. Cytochrome P450 3A (CYP3A) specific erythromycin N-demethylation activity (ERND) in the hepatic microsome was assayed. All three species showed positive reactions for CYP3A. In addition, CYP1A catalyzed ethoxyresorufin-O-deethylase activity (EROD) and CYP2E catalyzed aniline-p-hydroxylation activity (APH) were also studied. It was concluded that the activity of CYP3A was induced in all the fishes while CYP1A and CYP2E activities were not significantly altered.

Keywords: Microsomes, Erythromycin, CYP1A, CYP2E**,** Indian major carps.

1. Introduction
Erythromycin is a macrolide antibiotic isolated from *Streptomyces erythreus* [1]. Since no registered forms of erythromycin are available for use in fish culture, present applications utilize drug formulated for other veterinary usages. The vast majority of bacteria that cause disease in fish are gram-negative thus, erythromycin finds great use in the fish farming sector regardless the fact that it is not Food and Drug Administration (FDA) approved for use in fish food [2]. Also erythromycin is not very effective in bath treatment, so, it is routinely administered by injection or mixed with food. The aquadrug, althrocin (AI, erythromycin) is a common antibiotic used in Indian major carp and catfish hatcheries of west Bengal against bacterial diseases [3]. After a survey of almost 50 farms in two districts of West Bengal (north and south 24 Parganas), India reveals that most of the *Macrobrachium rosenbergii* and ornamental fish farms use erythromycin (20%) along with other antimicrobials to reduce the attack of the different pathogens, to improve the survival rate and to increase the intensity of feeding and achieve better growth rate [4, 5]. Erythromycin can be also included in almost all formulations for treatment of epizootic ulcerative syndrome, (EUS) affected fish [6, 7].
Cytochrome P450 (P450) is a representative enzyme involved in phase I metabolic reactions of drugs. It can be regarded as the rate-limiting enzyme for the elimination of drugs as it catalyzes the initial reaction resulting in an increased polarity of the substrate molecule [8]. Among P450 CYP3A4 is the most abundant P450 and is involved in the metabolism of about half of all prescribed drugs in use today [9]. Erythromycin assimilated by tissues is metabolized by CYP3A4 to N-demethylated metabolites and formalin. The latter is then predominantly converted to formate and carbon dioxide. Thus, erythromycin is mainly eliminated by the hepatobiliary system with only a small fraction that is eliminated unchanged in the urine [10].
Carps had been used extensively in South-East Asia due to their importance in culture fisheries. Indian major carps, (*L. Rohita, C. catla and C. mrigala*) are among the 15 carp species of major aquacultural importance in Asia. They have continuously dominated global carp production worldwide along with the grass carp, bighead carp and common carp [11]. By using drugs commonly used in fish farming, the effect of induction or inhibition profiles can

be studied for a better understanding of detoxification of drugs *in vivo*. The present study was aimed to identify the CYP-isoforms involved in the metabolism of erythromycin in Indian major carps.

2. Materials and Methods

2.1 Chemicals

Erythromycin (97.00%) was provided from HIMEDIA, India. EDTA, 2-amino-2-(hydroxymethyl) propane-1-3-diol (TRIS), ammonium acetate, glacial acetic acid, glycerol, acetylacetone, potassium chloride, formaldehyde and sulphuric acid (98%) were provided from Merck, India. Sodium hydrosulphite, technical grade (85%) was provided from Sigma Aldrich, USA. All chemicals were of analytical grade.

2.2 Experimental animal and design of the experiment

Sixty fish each of Indian major carps; *L. rohita, C. catla and C. mrigala* weighing 20±4g were obtained from fish farm in Gossainpur, West Bengal. Fish were transported during the early hours of the day in water holding jars with continuous oxygenation using battery operated air pumps to avoid stress and mortalities. They were then transferred to underground cemented tanks, (4 x 4 x 5.5) ft^3 in the premises of the department of Zoology, University of North Bengal, West Bengal, India for acclimation. Fish were fed with a mixture of rice bran and mustard oil cake. After two weeks of acclimation, the fish were transferred to glass aquaria, (90 x 35 x 35) cm^3 and feeding was stopped 24 hrs prior to the start of the experiment. Each fish species were divided into five groups of 6 fish each. One group of fish was considered as control while the other groups were administered a single intra-peritoneal dose of erythromycin (50 mg/kg body weight). The fishes were then sacrificed at the end of 24 hrs, 48 hrs, 72 hrs and 96 hrs and hepatic microsomes were isolated. The experiments were conducted in duplicates and repeated twice.

2.3 Microsome isolation

Microsomes were isolated using the procedure described by Chang and Waxman [12]. Livers of five fishes were dissected, pooled (2-3 g) and perfused with a large volume of perfusion buffer (1.15% KCl, 1mM EDTA, pH 7.4). The tissues were cut into small pieces and homogenized in four volumes of homogenization buffer (1.15% KCl, 1mM EDTA and 50 mM Tris, pH 7.4) using a teflon homogenizer. The homogenate was centrifuged at 12000 x g for 20 min. The supernatant was subjected to centrifugation at 100000 x g for 60 minutes. The pellet was resuspended in two volumes (tissue weight) of resuspension buffer containing 50 mM Tris, 1mM EDTA and 20% Glycerol v/v, pH 7.4 to obtain the hepatic microsomal fraction. All the above steps were carried out at 4 ^{0}C.

2.4 Protein Estimation

Protein in the microsomal fraction was estimated as described by Lowry *et al*. [13] using bovine serum albumin as standard. The values were used to construct a standard curve using Microsoft Excel and the protein concentration determined.

2.5 Total Cytochrome P450

Detection and estimation of CYP was done following the method described by Johannesen and De Pierre [14]. This method allows measurement of total CYP 450 in presence of contaminating hemoglobin and methemoglobin. Microsomes were appropriately diluted with 100mM potassium phosphate buffer (pH 7.4) and CO was bubbled for 30 seconds. The sample was equally divided between two cuvettes and the baseline (500 nm to 400 nm) was corrected. Sodium dithionite was then added to only one of the cuvettes and the absorbance was measured after 2 minutes.

2.6 Enzyme assays

CYP1A specific EROD activity was determined spectrophotometrically based on the method of Klotz *et al*. [15]. APH activity for determining CYP2E was done by measuring the amount of p-aminophenol formed at 630 nm. The method was modified from Imai *et al*. [16] by using aniline (10 mM) as substrate and NADPH (10mM) instead of an NADPH generating system. The method of Werringloer [17] was followed for the determination of CYP3A catalyzed ERND activity. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.2), 10 mM erythromycin, 150 mM $MgCl_2$. Reactions were initiated by the addition of cofactor (NADPH), and incubated for 10 min at 30 ^{0}C. Formaldehyde formed during the assay was measured by the method of Nash [18].

2.7 Calculation and Statistical analysis

Results are reported as mean ± standard deviation. Data sets were analyzed using one-way ANOVA test in SPSS version 16.0. Statistical significance was set at $\alpha = 0.05$.

3. Results

There was a significant correlation between total CYP450 content in the liver microsomes and ERND activity ($p < 0.01$). Tukey HSD showed homogeneity between mrigal and catla. The highest CYP450 level was scored at 96hrs of exposure to erythromycin (Table 1).

CYP3A catalyzed erythromycin N-demethylase activity was positively detected in all the fishes. Naive mrigal and rohu scored a lower value and were homogenous with respect to the activity in contrast to catla which had a relatively high CYP3A enzyme activity. All the fishes exposed to erythromycin showed a steady increase in the CYP3A enzyme activity achieving the maximum value at the 96 hrs of exposure that was significantly different from the control. The response in terms of induction of basal activity upon exposure of 96hrs was lowest in rohu (4 fold increase) followed by catla (5 fold increase) and mrigal (10 fold increase) (Table 1).

EROD activity was lowered in the erythromycin exposed groups in comparison to the control within 24 hrs of exposure. The activity of this isoform was suppressed throughout the exposure period in all three fishes (Figure 1a). CYP2E activity also showed a similar trend. A deviation from this could be observed in catla where the activity was marginally but not significantly elevated upon exposure (Figure 1b).

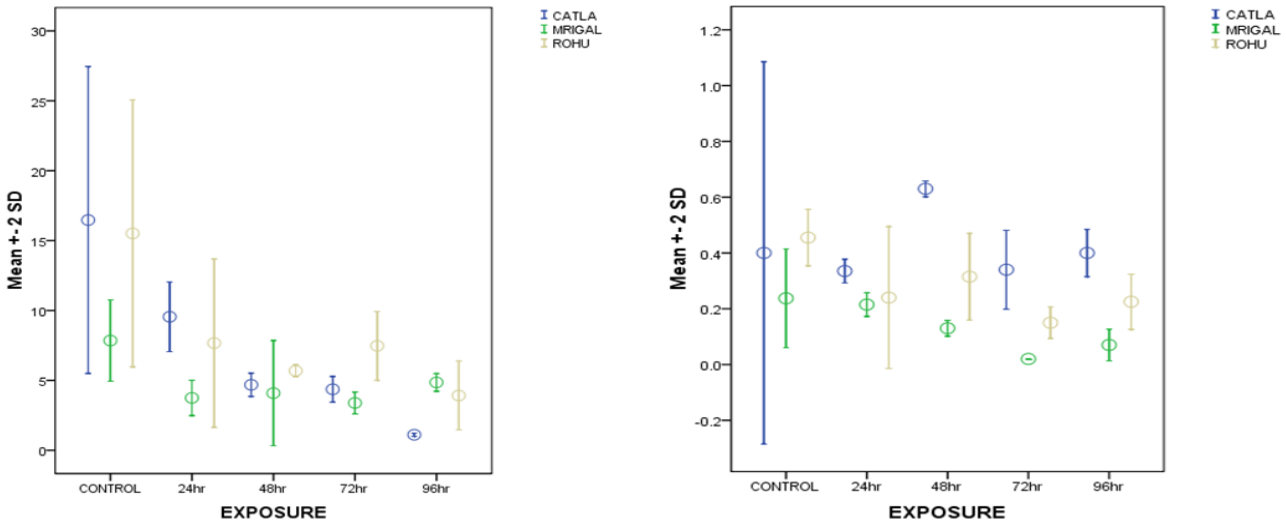
Table 1: Microsomal erythromycin n demethylase activity and total CYP450 in Indian major carps following exposure of erythromycin.

Parameters	Fish	Exposure				
		Control	24 hrs	48 hrs	72 hrs	96 hrs
Erythromycin-N-Demethylation (nmoleformaldehyde formed/min/ mg Pr)	Mrigal	0.64±0.13[a]	3.26±0.63*	5.11±0.00*	3.22±0.07*	6.53±0.45*
	Rohu	0.92±0.14[a]	3.46±0.70*	2.70±0.80*	3.31±0.11*	3.46±0.05*
	Catla	2.69±1.16	9.88±2.11*	12.50±1.24*	12.45±0.38*	12.97±0.58*
Total CYP450 (nmole/mgPr)	Mrigal	0.483±0.204[a]	0.768±0.017	1.197±0.431*	0.814±0.076	1.521±0.165*
	Rohu	0.884±0.021	1.664±0.301	0.797±0.243	1.676±0.884	2.409±0.541*
	Catla	0.438±0.189[a]	1.304±0.129*	1.887±0.187*	2.436±0.538*	2.114±0.183*

Data represented as mean ± stdev. (n=4). Values marked by '*' are significantly different ($p < 0.05$) from control according to Dunett's multiple comparisons test. The superscript 'a' signifies homogeneity between species according to Tukey HSD.

4. Discussion

The effect of induction is simply to increase the amount of P450 present and speed up the oxidation and clearance of a drug, leading to decreased plasma drug concentrations [19, 20]. However, the induction, in natural environment can lead to unexpected drug-drug interactions which may lead to the formation of drug residues and thus threaten the safety of fishery products. Although it has been exhaustively established that CYP3A is involved in the metabolism of erythromycin in humans [21, 22, 23, 24, 25], little information is available about CYP3A induction in fish particularly carps [26, 27, 28]. The induction of CYP3A in erythromycin treated fishes as indicated by its activity of erythromycin demethylation (ERND) in the hepatic microsomes is a direct indication of its role in the metabolism of the substrate. Such a case of auto induction, where a drug induces its own metabolism, can also be seen in the case of carbamazepine [29].



1(a) 1(b)

Fig 1: (a) EROD activity (pmol resorufin formed/min/mg Pr) and **(b)** APH activity (nmol p-aminophenol formed/min/mg Pr) in Indian major carps following exposure of erythromycin (50 mg/kg body weight).

CYP-enzyme induction usually results from an increased amount of enzyme protein, and a period of time is needed before enzyme induction becomes clinically significant [30]. The induction in activity of CYP3A in the present study was evident within 24 hrs of the exposure and the elevation in activity was progressive and highest during the longest period of exposure, i.e. 96 hrs. Among the several factors that determine the time-course of induction, the important ones are the drug half-life and the enzyme turnover. It has been observed that a short half-life results in an induction within 24hrs of exposure as in case of rifampicin while those with a longer half-life like phenobarbital (half-life 3-5 days) require at least a week for the induction to become apparent [31]. Both the drugs induce CYP3A in the time indicated. The reason for the highest activity at 96hrs of exposure can be accounted for the fact that many drugs, including the macrolide antibiotic erythromycin, undergo metabolic activation by CYP3A4 to form inhibitory metabolites [32]. These metabolites can form complexes with the enzyme called metabolic intermediate (MI) complexes, resulting in a functionally inactive CYP3A4 enzyme. Thus, CYP enzymes are unavailable for further oxidation and synthesis of new enzymes is therefore the only means by which activity can be restored and this may take several days [33]. Drugs may be metabolized by only one CYP450 enzyme or by multiple enzymes [34, 35]. The bulk of drug metabolism is catalyzed by a relatively small number of CYP450 enzymes found in families 1, 2 and 3. Thus, CYP2E and CYP1A were chosen to determine if these isoforms had any significant role in the metabolism of erythromycin. The CYP2E family contains

only one inducible enzyme, which is responsible for the metabolism of low molecular weight toxins and partly responsible for the metabolism of acetaminophen [36] and isoniazid [37]. On the other hand CYP1A although is highly induced by polycyclic aromatic hydrocarbons (PAHs), also has a role in the metabolism of drugs such as omeprazole [38] and haloperidol [39]. Apparently, their activity remained at par with the control without any significant increase or decrease suggesting little or no role in the metabolism of the macrolide antibiotic.

5. Conclusions

The knowledge of the isoenzymes involved in metabolism of drugs will allow a prediction of interactions of these drugs with other drugs and pollutants in the environment. Simultaneously, the risk of development of bacterial resistance to therapeutic antimicrobials and their rate of subsequent transfer is high. Although their adverse effect on human health is not well documented, still the issue cannot be ignored.

Although, the Indian major carps can bio transform erythromycin through CYP3A activity, a distinct difference in the pattern of induction was evident. Several factors directly or indirectly influence the CYP activity. This study requires additional research on the dose response relationship and metabolic clearance of the drug. Differences between species are not only numerous but also often unpredictable as such generalizations in medication should not be practiced. The drug must be investigated on a species-by-species basis to guarantee its effective and safe use, thus ensuring the well-being of animals and of humans consuming them.

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