Transgenic zebrafish biosensor for the detection of cadmium and zinc toxicity

Nilambari Pawar[1], P. Gireesh-Babu[1], Sridhar Sivasubbu[2] and Aparna Chaudhari[1,*]

[1]Fish Genetics and Biotechnology Division, ICAR – Central Institute of Fisheries Education, Mumbai 400 061, India
[2]CSIR – Institute of Genomics and Integrative Biology, Mathura Road, New Delhi 110 025, India

A transgenic zebrafish (*Danio rerio*) biosensor for zinc and cadmium was developed. For this, zebrafish metallothionein promoter cloned upstream to the DsRed2 gene in *Tol2* transposon vector was microinjected into one/two-celled zebrafish embryos. The F_1 embryos (48 h post-fertilized) of confirmed transgenics were exposed to sub-lethal doses of Cd^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2+} for 8 h. Reporter expression was visualized as fluorescence signal and quantified using real-time PCR system. The reporter expression increased with increasing metal ion concentration. Cadmium is the most potent inducer with 4.6-fold induction followed by zinc (2.3-fold). These zebrafish biosensors could be used as a preliminary testing tool to detect heavy metals in water bodies.

Keywords. *Danio rerio*, DsRed2, fluorescence, metallothionein, promoter.

POLLUTION of water bodies with heavy metals is a global concern and is alarmingly shooting up each day. Cadmium, due to its general anthropogenic usage pattern is one of the most toxic heavy metal pollutants[1]. Although zinc is an essential element within all cells, it is also harmful at elevated concentrations[2]. Hence, it is a priority to monitor the levels of these metals in the effluents before discharging into the water bodies. Analytical methods and chemical analyses can be used for measuring total metal concentrations but biological systems need to be employed for evaluating toxic, genotoxic and bioavailable doses. Metal-specific recombinant bacterial sensors have been developed using various reporter genes[3–6]. However, these prokaryotic biosensors may not provide a response that is comparable to eukaryotic systems. Hence, eukaryotic biosensors were developed using yeast, microalgae, ciliated protozoa and *C. elegans* followed by zebrafish[7–9]. In this study, zebrafish, a tiny tropical fish was used to develop a heavy metal biosensor.

Zebrafish (*Danio rerio*) is used for toxicology, developmental biology and molecular genetics research[10–12]. Carvan *et al.*[13] have explained the use of transgenic zebrafish for detecting aquatic pollution. Several transgenic zebrafish biosensors employing estrogen response elements (ERE), aryl hydrocarbon response elements (AHREs), heat-shock protein promoter elements (HSPs), DNA-damage inducible promoter elements, etc. are reported for monitoring aquatic pollution[14–20].

Metallothioneins (MTs) are small, ubiquitous, cysteine-rich proteins that are triggered in response to the toxic levels of heavy metals and hence are regarded as effective markers of metal pollution in aquatic systems[21]. Using this quintessential property of metallothionein, we aimed to design a fish metal biosensor model to detect cadmium and zinc toxicity using zebrafish metallothionein (zMT) promoter and DsRed2 reporter gene.

Zebrafish metallothionein gene promoter comprises four putative metal responsive elements (MREs), three activator protein 1 (AP1) and one specific protein 1 (Sp1) binding sites. Cadmium and zinc were found to be excellent inducers of zMT promoter in the transient expression studies with zebrafish caudal fin cell line (SJD.1), zebrafish liver cell line (ZFL) and human hepatocellular carcinoma cell line (HepG2)[22–24]. In the present study, *Tol2* transposon system has been used for the generation of transgenic fish. *Tol2* has been reported to be highly active in zebrafish germline and is used for transgenesis studies[25–27].

In this study, zMT promoter (986 bp) was amplified from zebrafish genomic DNA using zMT-F (AAA AGATCTTTCCAGAGAGACACTGCACACG) and zMT-R (AAAGATATCGCACTTGCAGGTAGCACCACAG) primers with linkers for *Bgl*II and *Eco*RV restriction enzyme (RE) sites respectively. Specific primers (DsRed2-F: AAA GATATCAGTTCAGCCGGAATTCACC and DsRed2-R: AAAAAGCTTACAGAGTGAGCCGATCCGAG) with linkers for *Eco*RV and *Hin*dIII RE sites, respectively were used to amplify *DsRed2* gene from pFRM-DsRed2 plasmid (kind gift from Dr S. C. Ekker, Mayo Clinic, USA). DsRed2 amplicon and pTol2 vector were RE-digested with *Eco*RV and *Hin*dIII to produce sticky ends and ligated to form pTol2-DsRed2 plasmid. The zMT amplicon was digested using *Bgl*II and *Eco*RV enzymes, ligated into similarly digested pTol2-DsRed2 plasmid and transformed into *E. coli* DH5 α-cells[28]. Positive clones were confirmed by sequencing of insert DNA. The recombinant clone was named pTol2-zMT-DsRed2 (Figure 1).

Tol2 transposase mRNA was produced *in vitro* from pDB600 plasmid (kind gift from Dr S. C. Ekker, Mayo

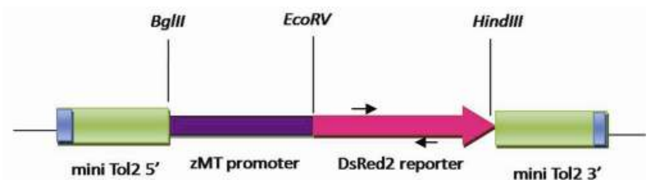

Figure 1. pTol2-zMT-DsRed2 construct map showing promoter-reporter cassette microinjected into zebrafish embryos. Primer positions within the reporter gene (*DsRed2*) used for quantitative RT-PCR analysis are marked with arrows.

*For correspondence. (e-mail: achaudhari67@gmail.com)

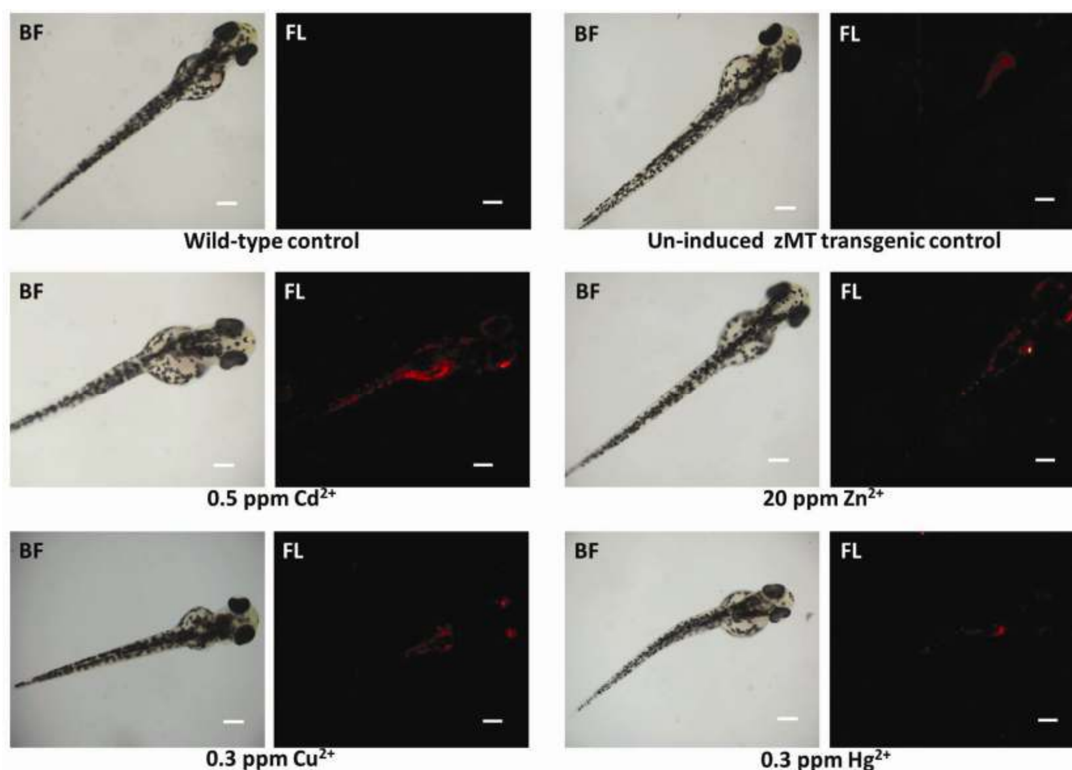

Figure 2. Red fluorescence in F_1 transgenic zebrafish (*Daniorerio*) larvae (48 h post fertilization) at 8 h post-exposure to Cd^{2+}, Zn^{2+}, Cu^{2+} and Hg^{2+} at 0.5, 20, 0.3, 0.3 ppm respectively. Red fluorescence is observed in various body parts of the metal-induced transgenic larvae while un-induced transgenic control had fluorescence in the yolk sac only. BL, Bright field; FL, Fluorescence. Scale bar: 200 μm.

Clinic, USA) using mMESSAGE mMACHINE transcription kit (Ambion, USA). One/two-celled zebrafish embryos were co-injected with 8.3 ng/μl of pTol2-zMT-DsRed2 plasmid and 100 ng/μl of *in vitro* transcribed transposase mRNA at the blastoderm/yolk interface in a total volume of 3 nl using Picolitre injector (Harvard Apparatus, USA). The guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment, Forests & Climate Change (Animal Welfare Division), Govt of India were followed for handling of fish and work plan was approved by appropriate committees. We injected a total of 1199 zebrafish embryos with the biosensor construct. Injected embryos were reared to adults. Fish were kept at 28°C in de-chlorinated system water. Photoperiod cycle of 14 h light and 10 h dark was maintained. One week-old fish larvae were fed paramecia while mature fish were given artemia twice daily. The embryo survival was found to be approx. 50% at 72 h post-injection. Only 75 fish matured (6.3%), of which 8 bred on mating with its wild-type counterpart. F_1 (48 h old) embryos were pooled, genomic DNA isolated and presence of transgene was confirmed by PCR. The microinjected parent of the embryo pool that tested positive was marked as transgenic and was raised separately. Two males were screened to be positive for the transgene resulting in an integration rate of 25%. These transgenic males were mated with wild-type females and the embryos were used for further experimental studies.

Dechorionated embryos (48 h post-fertilization; hpf) were exposed to different heavy metals to study the induction efficacy of the biosensor. Dechorionated embryos of 48-h-old zebrafish were preferred over 24 h to reduce the phenotypic abnormality on metal exposure. Four doses for each of the metal ions (0.05, 0.1, 0.3 and 0.5 ppm for copper, cadmium and mercury, and 5, 10, 15 and 20 ppm for zinc) were selected for induction studies. These doses were selected as they represent low to high dose ranges across the LC_{50} value of each metal ion (unpublished data). Two groups of control were set, one transgene injected but unexposed control and the other wild-type control. The experiment was conducted in triplicates and repeated twice for the consistency of the data. Due to increased mortality observed at 24 h post-exposure, the induction time was reduced to 8 h to allow maximal DsRed2 reporter expression with minimal mortality. Mercury and copper at 0.5 ppm dose resulted in 100% mortality. DsRed2 fluorescence 8 h post-exposure in the experimental transgenic zebrafish larvae was imaged using a digital camera attached to the Zeiss Axioscope 2 microscope with a rhodamine filter set. The imaging conditions were set with constant exposure time

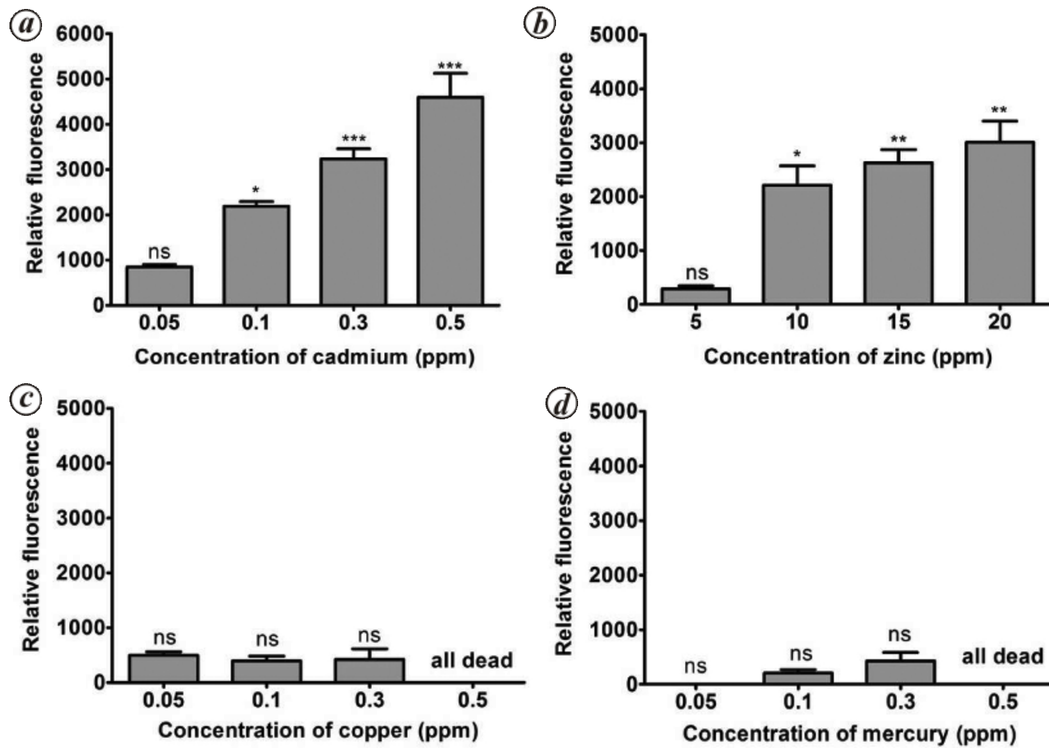

Figure 3. Quantification of fluorescence intensity in F_1 transgenic zebrafish larvae exposed to Cd^{2+}, Zn^{2+}, Cu^{2+} and Hg^{2+} (***a–d***) at different doses. Mean fluorescence intensity values normalized to background fluorescence of the un-induced control from each experimental group were plotted. Analysis was done using one-way ANOVA followed by Tukey's test. Data represents mean ± SEM (ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

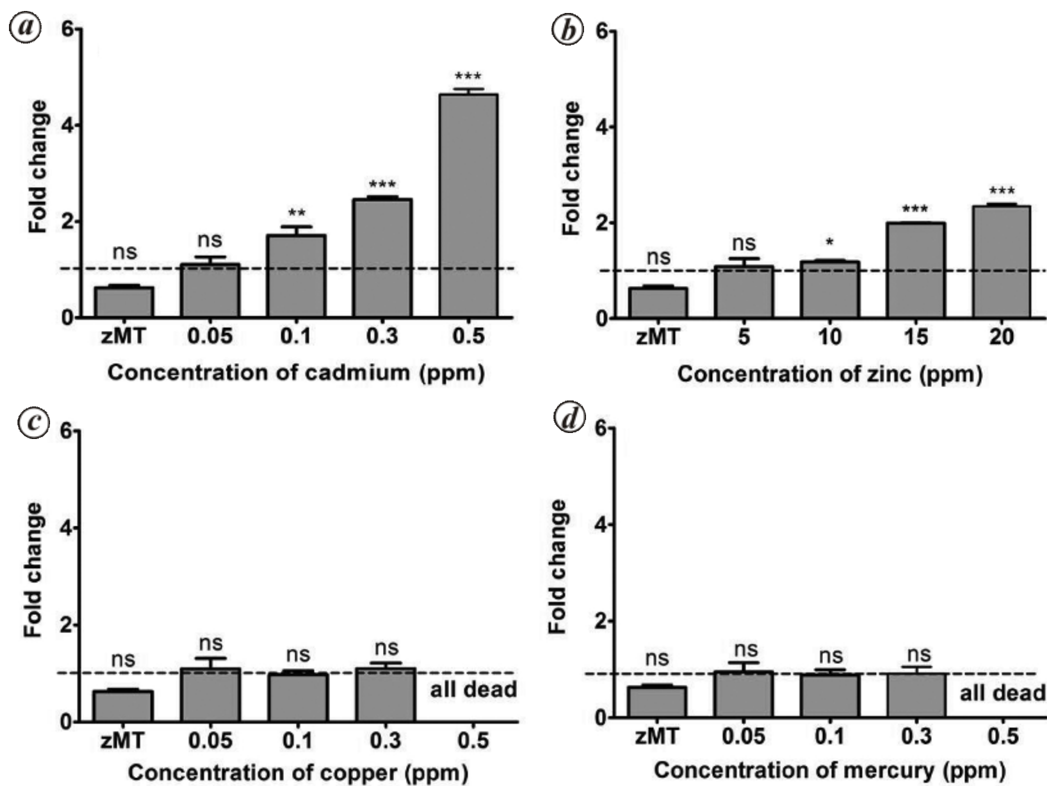

Figure 4. Real-time PCR analysis of the DsRed2 mRNA expression in F_1 transgenic zebrafish larvae exposed to Cd^{2+}, Zn^{2+}, $Cu^{2+}$and Hg^{2+} (***a–d***) at different doses. Analysis was done using one-way ANOVA followed by Tukey's test. Data represents mean ± SEM (ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

for imaging larvae of all the experimental groups. On average, 80–90% of F_1 progeny exhibited red fluorescence indicating presence of transgene. Cadmium showed highest promoter induction followed by zinc. The red fluorescent protein expression was detected in the exposed larval body in a mosaic pattern (Figure 2). Unexposed transgenic larvae showed fluorescence in yolk sac and eyes while it was absent in the wild-type control indicating some leaky expression from the promoter (Figure 2). To quantify the fluorescence, 18 larvae (6 from each experimental replicate) from each experimental group were rapidly imaged and analysed using ImageJ software. The fluorescence intensity values were normalized with the background fluorescence obtained from the un-induced transgenic fish larvae. The data was subjected to one-way ANOVA followed by Tukey's test. Cadmium induction revealed highest RFP expression ($P < 0.001$) followed by zinc ($P < 0.01$). Reporter expression in the transgenic fish increased with increasing concentration of cadmium and zinc suggesting dose dependency (Figure 3). Several researchers reported similar dose dependent pattern in case of human and zebrafish hsp70 promoters in detecting Cd^{2+} (0.2 to 125 μM), As^{3+} (10 to 300 μM) and $CuSO_4$ (1 to 1.5 μM)[16,17,29].

For reporter gene expression analysis, the zebrafish larvae were sampled from the experimental groups as described for fluorescence imaging and stored in RNA *later* solution (Qiagen, NL) at –20°C. Total RNA was isolated from pools of six larvae from each experimental replicate using RNeasy mini kit (Qiagen, NL). First strand cDNA was synthesized from total RNA following standard protocol[28]. Real-time PCR was performed on ABI7500 PCR system using SYBR Green PCR master mix (Thermo Scientific, USA). Primers qDsR-F (CTACCTGGTGGAGTT CAAGTCC) and qDsR-R (CGCTACAGGAACAGGT GGTG) amplified 165 bp of *DsRed2* gene, while qGapdh-F (GTGGAGTCTACTGGTGTCTTC) and qGapdh-R primers (GTGCAGGAGGCATTGCTTACA) amplified 173 bp fragment of the reference control, *GAPDH* gene. Comparative C_t method was used to estimate the relative expression of DsRed2 mRNA. Fold change in reporter expression was calculated by $2^{-\Delta\Delta C_t}$ method. Statistical significance of reporter expression on promoter induction was studied using one-way ANOVA followed by Tukey's test. Real-time PCR studies confirmed higher expression of DsRed2 reporter transcript with increasing metal concentration of cadmium followed by zinc, while it was absent in the control. To eliminate background fluorescence (leaky expression of the promoter), the expression values were normalized with un-induced biosensor control. A reporter response of 4.6-fold on exposure to 0.5 ppm cadmium ($P < 0.001$) was recorded followed by 2.3-fold for zinc at 20 ppm ($P < 0.001$). However, copper and mercury did not significantly induce reporter expression (Figure 4). Transient transfection assays performed on SJD.1, ZFL and HepG2 cell lines showed that Zn^{2+} and Cd^{2+} are excellent inducers of zMT promoter irrespective of types of cell lines[22,24,30]. However, minor differences were observed between different cell lines in terms of moderate induction by other metal ions. Cu^{2+} and Hg^{2+} caused moderate induction of zMT promoter in SJD.1 and HepG2 cell lines while only As^{3+} caused moderate zMT promoter activity in ZFL cell line. In the present study too, Zn^{2+} and Cd^{2+} were found to be excellent inducers of zMT promoter. No significant induction by Cu^{2+} and Hg^{2+} in this study could be attributed to the type of system and reporter gene used.

Several transgenic zebrafish metal biosensor models are reported using stress promoters such as heat shock protein (hsp)[16,18,29]. Tissue-specific expression of GFP was reported in a transgenic zebrafish biosensor harbouring upstream open reading frame of the human *chop* gene on exposure to sub-lethal doses of heavy metals and endocrine-disrupting chemicals[9]. However, in the present study, the transgenic zebrafish larvae were mosaic to transgene expression and hence no tissue-specific expression pattern could be ascertained. Also, since the metallothionein proteins are ubiquitously expressed in all tissue types, it is unlikely that the zMT promoter would show any tissue-specific activity.

The general standard limit of 1 to 2 ppm for cadmium and 5 to 15 ppm for zinc for effluent discharge of environmental pollutants is higher than the maximum permissible limit of 0.005 ppm (WHO) and 0.01 ppm (ISI) for cadmium with 5 ppm (WHO and ISI) for zinc in drinking water[31,32]. Thus, this fish metal biosensor provides a detection range (Cd: 0.1–0.5 ppm; Zn: 10–20 ppm) that can be used to screen effluent water samples prior to their discharge into the water bodies. These fish can be used as a simple first-level screening tool for confirming toxic bioavailable concentrations of cadmium and zinc, specifically.

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Food habits of dhole *Cuon alpinus* in tropical forests of southern India

Shahid A. Dar* and Jamal A. Khan

Department of Wildlife Sciences, Aligarh Muslim University, Aligarh 202 002, India

The food habits of dhole were evaluated in the tropical forests of Silent Valley National Park (SVNP) from December 2011 to May 2012 by analysing their scats. Eleven prey species were identified. Sambar was found to be the principal prey species for dhole as inferred from the relative biomass consumption of prey remains in dhole scats. Regarding prey biomass contribution, sambar was highest (66.74%) while grey jungle fowl was the lowest (0.32%). The aim of this study was to assess the food habits of dhole co-existing with large predators, tiger and leopard in the tropical forests of SVNP.

Keywords: Dhole, food habit, prey species, scat analysis, SVNP.

DHOLE is a terrestrial, 'pack-living' cursorial hunter, known as a voracious feeder which disembowels the prey[1]. Its distribution ranged from Siberia in the north,

*For correspondence. (e-mail: shahidahmaddar@gmail.com)