Study of arsenic genotoxicity in a freshwater fish (*Channa punctatus*) using RAPD as molecular marker

Deepak K Jha

Patna University

Niti Yashvardhini

Patna Women's College

Saurav Bhattacharya

Techno India University

Kumar Sayrav

VKSU: Veer Kunwar Singh University

Amod Kumar

Kirori Mal College

Parimal Khan (✉ parimal_khan@yahoo.co.in)

Patna University <https://orcid.org/0000-0002-6936-414X>

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Abstract

Arsenic, the proven genotoxic carcinogen in humans, is a groundwater contaminant of global concern. The present work investigates the applicability of randomly amplified polymorphic DNA (RAPD) as molecular marker to demonstrate arsenic genotoxicity in the freshwater fish, *Channa punctatus*. Fish specimens, segregated into three groups, were exposed to 10, 50 and 500 μgL^{-1} of arsenic respectively for 20 consecutive days. DNA extracted from same specimens of each group before and after exposure was amplified by polymerase chain reaction (PCR) using single arbitrary primers. Marked changes in RAPD profiles of fish DNA were observed upon exposure to arsenic compare to RAPD profiles of their pre-exposure state, resulting from loss or gain of certain bands. Altogether a total of 41 loci were amplified with 37–41 bands in each group. Band 1 of pre-exposure state was lost in all post-exposure samples while bands 4, 6 and 7 appeared as new bands after exposure to arsenic. The changes in the RAPD banding patterns upon exposure to arsenic reflect alterations in fish DNA. The RAPD bands, therefore, appeared as potential markers, capable of revealing the genotoxicity induced by arsenic in this piscine model.

Introduction

Arsenic is a toxic environmental contaminant and group-I human carcinogen [1] which ranks first in the Priority List of Hazardous Substances [2]. According to a recent estimate, over 94 million to 220 million people in 70 countries are exposed to harmful levels of inorganic arsenic through the uses of groundwater as drinking water that affects the human population with adverse outcomes including cancers [3–5]. Moreover, inorganic arsenicals are proven genotoxic carcinogens [6] and their genotoxic effects have been reported in both humans and experimental animals using cytogenetic markers [7–11].

Advances in toxicogenetics have led to the application of molecular markers, including those based on polymerase chain reaction (PCR), in the assessment of genotoxicity. In PCR based techniques, multiple amplicons of variable length are produced when a single oligonucleotide primer anneals to several regions of genomic DNA [12]. Such amplicons appear as fingerprints of randomly amplified polymorphic DNA (RAPD) upon agarose gel electrophoresis which are now used extensively as molecular marker to demonstrate damage/alterations in DNA induced by environmental contaminants [13–19]

Atienzar et al. [14] observed that changes in the patterns of RAPD reflected toxicological insults to DNA that ranged from single base substitutions to complex chromosomal aberrations. In many other observations, RAPD assay successfully manifests DNA alterations in fish induced by DNA damaging agents [20, 16, 21]. It appears to be a rapid screening assay at molecular level that also complements other well established techniques of genotoxicity assessment including micronucleus and comet assay [15, 22, 16].

For the monitoring of genotoxic agents, *Channa punctatus* (among the non-mammalian organisms) has emerged as reliable and excellent piscine model which appears to be more sensitive to contaminants in

water because of its various ecotoxicological characteristics [23–29]. The present work was an attempt to demonstrate arsenic induced DNA damage/alterations by comparing the RAPD profiles of the specimens of freshwater fish, *Channa punctatus*, obtained before and after exposure to arsenic.

Materials And Methods

Collection of fish specimens

The healthy and adult specimens of fish *Channa punctatus* Bloh (Family; Channidae; order: Perciformes) were collected from local ponds having negligible arsenic content (below detection level) in their waters. The arsenic content of pond waters was estimated by the technique of flow injection hydride generation atomic absorption spectrometry (FI- Hg-AAS) using an atomic absorption spectrometer (AAS-Varian AA-140; Agilent, USA) [30]. This technique was preferred since hydride generation mode can detect the level of arsenic up to as low as $1 \mu\text{g L}^{-1}$. For quality control, the standard reference material and reagents blank supplied from National Institute of Standards and Technology (NIST, USA) was used. Fish specimens, each weighing 25-30 g and measuring 12-15 cm in length, were given prophylactic treatment of 0.05% KMnO_4 solution for two min to prevent dermal infections, and were acclimatized for 15 days under controlled laboratory conditions. They were fed with fish feed and small pieces of boiled egg at 5% of their body weight.

Exposure level of test substance

Fish specimens were segregated into three groups (T_1 , T_2 and T_3 ; Table 1) where each group had 18 specimens. Experiment was performed in three replicates with 6 specimens in each replicate per group. These were exposed to three environmentally relevant levels of arsenic in separate aquaria (40-L capacity) which were $10 \mu\text{g L}^{-1}$ (permissible level in drinking water), $50 \mu\text{g L}^{-1}$ (acceptable level in India) and $500 \mu\text{g L}^{-1}$ (frequently reported high level arsenic content in groundwater) [31]. Sodium arsenite (NaAsO_2 , CAS No. 7784-46-5; Loba-Chemie, Mumbai, India) was used for arsenic exposure as it is considered more toxic, soluble and mobile in water than arsenate [32]. The exposures were continued for 20 consecutive days. Arsenic contents of aquaria waters, determined by the process described above were regularly verified during the period of exposure to confirm the nominal levels (10 , 50 and $500 \mu\text{g L}^{-1}$) with less than 10% difference. Further, aquaria waters, under static removal system, were changed every day on regular basis to prevent accumulation of nitrogenous waste.

Sampling of blood

Blood sampling from fish specimens was done before and after exposure using heparinized tuberculin syringes fitted with 26 gauge needles after anaesthetizing them with benzocaine (0.1g L^{-1}). Every time about $100 \mu\text{L}$ blood was derived from each fish specimen through caudal vein puncture. The blood samples were then added to 95% ethanol in 1:5 ratio using Eppendorf tubes and kept at 4°C until used for DNA extraction.

DNA extraction and amplification

DNA was extracted from the blood samples of pre- and post-exposure fish specimens of each experimental group separately following the technique adopted by Kumar et al. [16]. It was followed by polymerase chain reaction (PCR) using Taq DNA polymerase in a thermal cycler (Eppendorf, USA) for random amplification of polymorphic DNA (RAPD) following the method established by Atienzar et al. [33] with minor modifications. Briefly, PCR reactions were carried out for 40 cycles, each involving denaturation of template DNA for 1min (94°C), annealing of primers for 1 min (48±2°C) and extension of new strand for 1 min (72 °C) except initial denaturation for 3 min and final extension for 10 min. Each reaction mixture (25µL) had 2.5 µL polymerase buffer (1X), 2.0 µL MgCl₂ (2mM), 0.5 µL dNTPs (0.2 mM), 1.0 µLTaq polymerase (1U), 1.0 µL RAPD primer (10 pM) and 1.0 µL genomic DNA (20 ng). PCR ingredients and RAPD primers were procured from Fermentas Life Sciences (USA) and Integrated DNA Technology (USA) respectively. 10 µL sample of each amplified RAPD product was loaded in the sample well along with loading dye before electrophoresis.

A total of nineteen decamers (designed as OPA1 to OPA19; Table 2) were used as primers to amplify different loci and the polymorphism among amplified loci was detected upon electrophoretic mobility of the bands in 1.5% agarose gel. Electrophoresis was carried out for 5 hour at 40V for proper development of bands followed by image capturing under UV light and its analysis using a gel documentation system (Vilber, E-BOXCX5).

DNA analysis

Alterations in DNA were identified according to the scored RAPD profiling data where the presence or absence of amplified fragments were marked as '1' or '0' and were scored as dominant or recessive alleles in a two allele system. The bands migrated to the same position with the same intensity were deemed to be homologous bands from the same allele as suggested by Lynch and Milligan [34]. Based on binary matrix, the total number of RAPD bands in all the experimental groups was calculated including the polymorphic ones. Using their allele frequencies and percentage of polymorphic loci (%P), Nei's genetic distance and genetic variability between pairs of groups (population) were calculated with the help of POPGENE version 1.32 [35]. Finally, Un-weighted Pair Group Method with Arithmetic Means (UPGMA), as suggested by Sokal and Sneath [36], was used for clustering analysis, generating dendrogram. Genetic similarity (S) was calculated by the method suggested by Nei and Li [37] using the formula, $S = 1/4 \cdot 2NAB / (NA + NB)$ where NAB is the number of bands common in both the individuals A and B, NA is the number of bands in individual A and NB is the number of bands in individual B.

Results

RAPD bands were compared between pre- and post- exposure to arsenic for the same individual. Seven out of nineteen (about 37%) decamer primers (OPA1, OPA3, OPA4, OPA5, OPA6, OPA8 and OPA9) were capable of producing strong and reproducible banding patterns in all samples tested, and the patterns

were most distinguishable between the pre- and post- exposure samples. Altogether a total of 41 individual loci were amplified in pre- and post- exposure samples. The number of loci amplified in the pre-exposure specimens was 40 while specimens post- exposure to arsenic exhibited 39, 37, and 40 loci in T₁, T₂, and T₃ groups respectively. The variation in the number of bands in different exposure levels appeared to be resulting from loss of few normal bands and/or gain of new bands upon arsenic exposure compared to pre- exposure state (Fig. 1). Some bands were polymorphic (marked in red arrows), showing their presence at different loci in various experimental groups. The band 1, generated by the primers OPA1 and OPA8, was found only in the pre- exposure sample but failed to appear after arsenic exposure. In contrast, bands 4, 6 and 7, generated by the primers OPA6, OPA1 and OPA4 or OPA9 respectively were observed only in post- exposure samples to high level of arsenic (500µg L⁻¹). These loci, however, did not amplify in the pre- exposure as well as in the post- exposure to low levels of arsenic (10 and 50 µg L⁻¹), indicating that high level exposure to arsenic had comparatively more severe effect than its low level exposures. Moreover, emergence of new bands exhibited a weak dose response relationship.

The UPGMA classification showed no intra-group variation but the banding patterns were found quite different between pre- and the post- exposure specimens. Moreover, the difference was more pronounced upon high level arsenic exposure (T₃) than upon low level arsenic exposure (T₁ and T₂). The dendrogram generated by Nei and Li's formula reflects the results of UPGMA cluster analysis (Fig. 2).

Discussion

The blood cell formation in fishes was extensively studied by Catton [38]. Although cell cycle dynamics and erythropoiesis have been well documented in mammalian system, less information is available with respect to fishes because of variation in their cell dynamics with temperature [39–40]. In *Channa*, maturation of erythrocytes takes around 20 days [41], as occurs in many other fishes [42–43]. The design of present study with an exposure period of 20 days at an almost constant temperature followed by blood sampling on the next day, therefore, is in accordance with the duration of blood cell formation in *Channa*.

The changes in the RAPD profile upon exposure to arsenic as occurred in the present work also reported with earlier studies in animals and plants [44–45], might have resulted from alterations in genomic DNA of fish. Arsenic has been shown to induce a variety of DNA alterations including DNA strand breaks, oxidation of bases, adduct formation and even DNA- protein cross links [46–48]. Since a single lesion in DNA can affect its amplification efficiency [49], arsenic induced alterations in DNA are likely to affect the kinetics of PCR events, leading to changes in the RAPD patterns. Alterations in the base sequences may lead to the accessibility of oligonucleotide primers to new priming sites in DNA, resulting in the emergence of new PCR products. As the probability of mutation in primer binding sites is unlikely, creation of new priming sites may results mainly from genomic rearrangements induced by arsenic. When new priming sites are used for amplification, it leads to appearance of new bands. In contrast,

interaction between DNA polymerase and altered DNA may either cause complete cessation of DNA polymerization or slowing down of PCR reaction; consequently, few of the normal bands may disappear.

The present experiment, showing changes in the RAPD profiles of fish DNA upon exposure to arsenic, therefore, confirms the occurrence of DNA alterations induced by arsenic and also indicates gradual genomic instability with increase in the level of arsenic exposure. This finding in *Channa punctatus* exposed to sodium arsenite is consistent with similar observation in another *Carassius auratus* exposed to arsenic trioxide [16], suggesting RAPD-PCR assay is a reliable and sensitive method to detect arsenic induced genotoxicity at molecular level.

Conclusion

In the present study, RAPD-PCR method was found as a potential tool to show DNA alterations induced upon arsenic exposure and proved to be sensitive enough to unmask genotoxicity of arsenic at its various exposure levels in the freshwater fish *Channa punctatus*. In our results exposure to arsenic seems to induce changes in the oligonucleotide priming sites in DNA that produces new band(s) or results in loss of pre-existing band(s).

Declarations

Ethical approval

All procedures performed in this study approved by institutional ethical committee and each step was kept within the ethical norm suggested by CPCSEA (2006).

Consent to participate: not applicable

Consent for publication: not applicable

Availability of data and materials: The data generated and analyzed during this study are included in this article.

Competing interests: The authors declare that they have no competing interest.

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Author's contribution: P. K. Khan contributed to the study conception and supervision. Material preparation, experimentation and analysis of results were performed by D. K. Jha, N. Yashvardhini and Saurav Bhattacharya. Kumar Sayrav estimated arsenic content in sample and aquaria water. Amod Kumar interpreted the results of RAPD analysis. The editing of the original draft, first prepared by D. K. Jha, was done by P. K. Khan. All authors read and approved the final manuscript.

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Tables

Table 1 Exposures levels of arsenic in the fish *Channa punctatus*

Experimental Groups	Exposure Level ($\mu\text{g L}^{-1}$)	No of fish Specimens
T1	10	6x3
T2	50	6x3
T3	500	6x3

Table 2 List of random oligonucleotide decamer primers used during RAPD assay

Primer name	Nucleotide sequence
1. OPA1	5'- TGC CGA GCT G- 3'
2. OPA2	5'- AGG GGT CTT G- 3'
3. OPA3	5'- GAA ACG GGT G- 3'
4. OPA4	5'- GTG ACG TAG G- 3'
5. OPA5	5'- GGG TAA CGC C- 3'

6. OPA6	5'- AGC TCA CTG A- 3'
7. OPA7	5'- AGT CGG GCT G- 3'
8. OPA8	5'- AAT CGG GCT G- 3'
9. OPG-09	5'- TCA CGT CCA C- 3'
10. OPG-10	5'- CTG ACG TCA C- 3'
11. OPG-11	5'- AGG GCC GTC T- 3'
12. OPG-12	5'- TGC CCG TCG T- 3'
13. OPG-13	5'- CAG CTC ACG A- 3'
14. OPG-14	5'- GGA TGA GAC C- 3'
15. OPG-15	5'- ACT GGG ACT C- 3'
16. OPG-16	5'- AGC GTC CTC C- 3'
17. OPG-17	5'- ACG ACC GAC A- 3'
18. OPG-18	5'- GGC TCA TGT G- 3'
19. OPG-19	5'- GTC AGG GCA A- 3'

Figures

Figure 1

RAPD profiles of genomic DNA from the same specimen of *Channa punctatus* before (C) and after exposure to arsenic at three different levels (T1: 10 $\mu\text{g/L}$, T2 :50 $\mu\text{g/L}$, T1: 500 $\mu\text{g/L}$). The polymorphic bands are shown with red arrow.

Figure 2

UPGMA dendrogram for cluster analysis of RAPD band profiles of the fish specimen of *Channa punctatus* before (C) and after exposure to arsenic at three different levels (T1: 10 µg/L, T2: 50 µg/L and T3: 500 µg/L) using Jacard's coefficient of similarity.