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PAVAN KUMAR

RAVINDRA KUMAR

NARESH SAHEBRAO NAGPURE

PRAKASH NAUTIYAL

BASDEO KUSHWAHA

See next page for additional authors

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Authors
PAVAN KUMAR, RAVINDRA KUMAR, NARESH SAHEBRAO NAGPURE, PRAKASH NAUTIYAL, BASDEO KUSHWAHA, CHRISTOPHER DIDIGWU NWANI, and WAZIR SINGH LAKRA

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In vivo assessment of DNA damage in *Cyprinus carpio* after exposure to potassium dichromate using RAPD

Pavan KUMAR[1], Ravindra KUMAR[1,*], Naresh Sahebrao NAGPURE[1], Prakash NAUTIYAL[2], Basdeo KUSHWAHA[1], Christopher Didigwu NWANI[3], Wazir Singh LAKRA[1]

[1]Molecular Biology and Biotechnology Division, ICAR - National Bureau of Fish Genetic Resources, Lucknow, India
[2]Department of Zoology and Biotechnology, HNB Garhwal University, Uttarakhand, India
[3]Molecular Biology and Biotechnology Division, Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Nigeria

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Abstract: The present study used the random amplification of polymorphism DNA (RAPD) technique to evaluate possible DNA damage in the blood cells of *Cyprinus carpio* exposed to potassium dichromate. Test specimens were exposed to 3 sublethal test concentrations (93.95, 187.9, and 281.85 mg L^{-1}) of potassium dichromate for a period of 96 h. The samplings were collected at 24, 48, 72, and 96 h after exposure. DNA was isolated from the blood samples of both the control and treated groups of *C. carpio*, and 3 selected decamer primers were used for RAPD-PCR. In comparison to the control, potassium dichromate-treated groups showed differences in RAPD profiles with respect to the disappearance of several bands and the appearance of new ones. There was a significant ($P < 0.05$) difference between 72 and 96 h after exposure with respect to genomic template stability. The variation in RAPD profiles indicated both time- and concentration-dependent relationships. The results suggested significant genomic template instability as a measurement of DNA damage and further demonstrated the potential of RAPD as a powerful tool for detecting genotoxicity induced by potassium dichromate in aquatic environments.

Key words: *Cyprinus carpio*, DNA damage, potassium dichromate, RAPD

1. Introduction

Heavy metals are toxic and nonbiodegradable, therefore persisting extensively in aquatic and terrestrial ecosystems. The bioaccumulation of these metals in the organisms inhabiting contaminated waters, especially fish, is additionally important because they may enter the food chain (1,2). Numerous types of chemicals and xenobiotics pollute the aquatic ecosystem daily through agricultural, industrial, and other human activities. Heavy metals entering the aquatic systems deteriorate the life-sustaining quality of water, damaging both flora and fauna (3,4).

Chromium (Cr) and its compounds are water pollutants that reach natural water resources through industrial discharges. Cr occurs predominantly in 2 oxidation states, namely trivalent, Cr(III), and hexavalent, Cr(VI). Hexavalent chromium is present in the water as $HCrO_4^-$, $HCr_2O_7^-$, $Cr_2O_7^{2-}$, and CrO_4^{2-}. It has been found to be connected with antagonistic biological effects at all levels of biological organization (5). These compounds accumulate in the environment and cause direct and indirect toxic effects to aquatic life, especially to fish (6).

Random amplification of polymorphism DNA (RAPD) is a powerful method that involves the amplification of arbitrary segments of genomic DNA using PCR (7). Molecular geneticists have reported that RAPD is a fast, simple, sensitive, and reliable method for genotoxicity assessment. It does not require sequence information for the object under study, and arbitrarily chosen short primers are used at low stringency to amplify multiple segments from genomic DNA. RAPD is presently used in many different fields such as phylogeny, taxonomy, genotoxicity, and epidemiology (8). Furthermore, it is used in surveying genomic DNA to detect various types of DNA damage and mutations (e.g., rearrangements, point mutations, small inserts or deletions of DNA, and ploidy changes). In fact, it may potentially form the basis of a novel biomarker assay for the detection of DNA damage and mutations in the cells of bacteria, plants, and animals (9). A comparison between 'untreated' and 'treated' genomes shows that RAPD analysis can be used to evaluate how environmental pollutants modify the structure of DNA in living organisms (10).

* Correspondence: ravindra.scientist@gmail.com

The cyprinid common carp fish, *Cyprinus carpio*, a widely used model in aquatic toxicology, was used as a test specimen to evaluate the genotoxicity of waterborne hexavalent chromium at its environmental concentrations. To the best of our knowledge, several bioassays have been recommended for assessing its toxicological effects at cellular level, but no work has been reported to date on DNA damage in *C. carpio* using RAPD. Investigations on the genotoxicity of potassium dichromate in *C. carpio* are, therefore, necessary to gain a better understanding of water quality monitoring, and to provide data to policy makers for planning biological diversity management in aquatic ecosystems. For this purpose, the present study assessed the potential of RAPD as a biomarker for detecting potassium dichromate-induced DNA damage in *C. carpio.*

2. Materials and methods

2.1. Experimental animals and acclimatization

The specimens of *C. carpio* were procured from the Aquaculture Research and Training Unit, Chinhat Farm, National Bureau of Fish Genetic Resources, India. The specimens had a mean ± SE length and wet weight of 10.8 ± 0.47 cm and 47.8 ± 2.5 g, respectively. The specimens were acclimatized to the laboratory conditions for 2 weeks under a 12-h photoperiod. The specimens were fed with aquarium flake feed twice daily and every effort was made to maintain optimal conditions during acclimatization, as suggested by Bennett and Dooley (11). No fish mortality was observed during acclimatization.

2.2. Determination of median lethal concentration (LC_{50})

The acute toxicity bioassay was conducted in a semistatic system to determine the 96-h LC_{50} value of the test chemical following standard methods (12). First, a range-finding assay was conducted to select the 7 concentrations (300, 325, 350, 375, 400, 425, and 400 mg L^{-1}) of the test chemicals for the definite test. The stock solution for the definite test was prepared in double-distilled water. Test concentrations were prepared by diluting the test chamber tap water with the appropriate quantity of stock solution and reaching the final volume of 20 L. The fish did not feed for 24 h before and during the experiment, as recommended by Ward and Parrish (13) and Reish and Oshida (14). Proper oxygenation was provided to the test solution with showers fixed above the test chamber. No distinction was made between sexes in the experiment. Ten acclimatized specimens were randomly selected and exposed individually to the above 7 test concentrations, along with a control in tap water. The experiment was repeated thrice under normal day/night illumination to obtain the 96-h LC_{50} value of the test chemical for the test species. Fish mortality was recorded as 0%, 10%, 30%, 50%, 70%, 80%, and 100% at 96 h after exposure for respective concentrations of the test chemical. No mortality was observed in the control experiment. The 96-h LC_{50} value of the test chemical was determined using probit analysis, as described by Finney (15). Water quality of the test solution was determined using standard procedures (12).

2.3. Estimation of sublethal concentrations and in vivo exposure

Using the 96-h LC_{50}, 3 sublethal test concentrations of the chemical, namely SL-I (1/4 of LC_{50}), SL-II (1/2 of LC_{50}), and SL-III (3/4 of LC_{50}) were calculated as 93.9, 187.9, and 281.8 mg L^{-1}, respectively, for the in vivo experiment. This study was conducted under static test conditions following OECD Guideline No. 203 (16). The specimens were exposed to the test concentrations continuously for 96 h. A set of test specimens maintained in tap water was considered as the control. The blood was sampled from the control at 24, 48, 72, and 96 h after exposure. Similarly, blood samples were collected at the same time intervals at a rate of 5 specimens per sampling per group by puncturing the caudal vein with a heparinized syringe and processed for DNA extraction. The physiochemical properties of the test water, namely temperature, pH, conductivity, dissolved oxygen, chloride, and total hardness, were analyzed by standard methods (12).

2.4. Genomic DNA isolation and RAPD

DNA extraction from the blood samples was carried out following the standard phenol:chloroform:isoamyl alcohol protocol of Sambrook and Russell (17). The concentration of DNA was estimated using a NanoDrop 2000 spectrophotometer (Thermal Scientific, USA). DNA amplification conditions were optimized following the procedure of Chen et al. (18). Initially, 20 random decamer primers were screened in order to test amplification profiles for polymorphism and reproducibility. Finally, the present study utilized 3 primers for RAPD-PCR analyses that gave good results, as shown in Table 1. All PCR amplifications were repeated twice in order to evaluate the reproducibility of the polymorphic bands. The control was run in parallel with every set of treated samples. The amplicons mixed with loading buffer were resolved electrophoretically on 2% agarose gel stained with ethidium bromide (0.5 µg/mL) at 100 V for 1 h and were visualized using the Genegenius gel documentation (Syngene Bio Imaging System, UK).

2.5. Estimate of genomic DNA template stability

In the present study, a comparison of polymorphism, as observed in the RAPD profile, with the control showed disappearance of a normal band, appearance of a new band, and variation in band intensities (19). Changes in these values, as a percentage to their control, were calculated in order to compare the sensitivity of the parameters (genomic template stability, GTS). Each obvious difference observed in the RAPD pattern was given the arbitrary score of +1. The average for each experimental fish group was calculated by considering 3 primers that showed clear

Table 1. Details of primers used in the present study. Tm: Melting temperature.

Primer code	Sequence (5'–3')	Molecular weight (g/mol)	Concentration (pmol)	Tm (°C)
OPA-04	AATCGGGCTG	3068	95	32
OPB-05	AGGGGTCTTG	3099	110	32
OPB-18	CCACAGCAGT	2997	80	32

variations in their RAPD profiles. However, the primers with no specific changes in their RAPD profiles or too difficult to be scored were not considered in the final calculation of the GTS. The GTS (%) was calculated as $(1 - a/n) \times 100$ (20), where a is the average number of the polymorphic bands detected in each treated sample and n is the total number of bands found in the control.

2.6. Data analysis

All gels were visualized and documented using GeneSnap 4.00 software (Genegenius, Syngene Bioimaging System, UK). Each RAPD-PCR-amplified DNA fragment was considered to represent a single locus. The gel image files were analyzed using Syngene GeneTool software. Data from each treatment were subjected to repeated analyses of variance (rANOVA) using SPSS 16.0 for Windows (SPSS Inc., USA). Statistical significance was determined at $P < 0.05$ and the results were presented as mean ± SE. When overall differences were significant, the Mann–Whitney test was used to compare the means between individual treatments.

3. Results

3.1. Physiochemical parameters of test water

During experimentation, test water temperature varied from 31.3 to 33.6 °C, whereas pH ranged from 7.5 to 8.0. The dissolved oxygen varied from 7.0 to 8.5 mg L^{-1}. Chloride content, total hardness, and total alkalinity ranged from 42 to 46, 170 to 180, and 270 to 295 mg L^{-1} as $CaCO_3$, respectively.

3.2. Estimation of median lethal and sublethal concentrations

No mortality or visible behavioral changes were observed in the exposed specimens of the control group. In the treated groups, fish mortality heightened with the increase in test concentrations of the chemical, and the specimens swarmed to the surface more often than the control group. The 96-h median lethal concentration (96-h LC_{50} value) of the test chemical was estimated by using 375.8 mg L^{-1} acute toxicity bioassay for *C. carpio*. The 3 calculated sublethal test concentrations of the chemical were 93.9 (SL-I), 187.9 (SL-II), and 281.8 mg L^{-1} (SL-III).

3.3. RAPD-based DNA fingerprinting

Figure 1 shows the RAPD profiles generated by using the genomic DNA of *C. carpio* exposed to different test concentrations of potassium dichromate. The 3 primers used yielded specific and stable results. In all cases, the RAPD profiles of the exposed specimens are clearly different from the control group and exhibit a distinct change with increasing test concentration and exposure time. The differences in RAPD patterns reflect the loss of normal bands and the appearance of new bands with different band intensity as compared to the control.

A total of 16 bands with molecular size ranging from 100 to 3000 bp were amplified using 3 primers of the *C. carpio* control group. At 24 h after exposure, the number of bands that disappeared were 1, 1, and 5, and this increased to 3, 4, and 5 at 96 h after exposure at test concentrations of 93.95, 187.9, and 281.85 mg L^{-1}, respectively, as compared to the 16 bands of control specimens. This indicated that the number of bands increased with heightened test concentration. At 24, 48, 72, and 96 h after exposure, 1, 1, 3, and 4 new bands appeared at the highest test concentration in treated specimens, which indicated an increase in the number of new bands that appeared with increasing exposure duration. At the lowest test concentration, 1 and 2 new bands appeared in the treated groups at 48 and 96 h after exposure, respectively, as compared to the control.

3.4. Genomic DNA template stability

Changes were observed in total amplified polymorphic bands (a + b) for each primer, with change in both test concentration and exposure duration (Table 2). As test concentration increased, GTS (%) decreased. Similarly, as exposure time increased, GTS decreased (Figure 2). Lowest GTS was recorded at 96 h after exposure. There was significant ($P < 0.05$) difference between 72 and 96 h after exposure with respect to genomic template stability; however, there was no significant difference in genomic template stability at lower test concentrations.

4. Discussion

In the present study, the results of RAPD assay showed significant differences in RAPD patterns in potassium dichromate-treated *C. carpio* and in the control groups. These differences occurred with respect to variation in band intensity, the disappearance of existing bands, and the appearance of new bands. Our findings were in agreement with those of Savva et al. (20), who generated RAPD profiles in rats exposed to benzo(a)pyrene. The disappearance and appearance of DNA bands was achieved through DNA

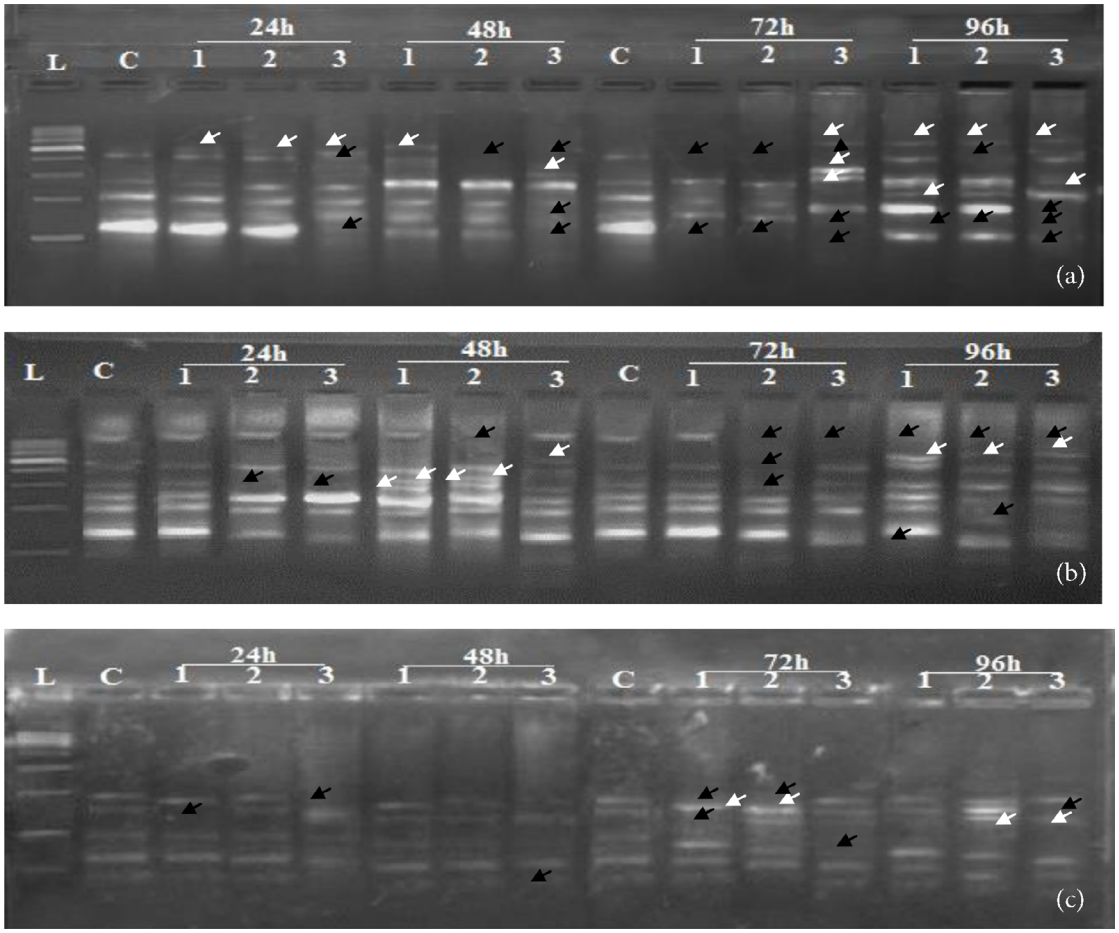

Figure 1. Gel image showing RAPD profiles generated with random decamer primers OPA-4 **(a)**, OPB-5 **(b)**, and OPB-18 **(c)** using genomic DNA of *C. carpio* exposed to various concentrations of potassium dichromate. Lane L: DNA ladder; C: control; Lane 1: 93.95 mg L^{-1}; Lane 2: 187.9 mg L^{-1}; Lane 3: 281.85 mg L^{-1} concentrations of potassium dichromate. DNA ladder size (top to bottom): 3000, 2000, 1500, 1000, 700, 400, 300, 200, and 100 bp. The black arrow indicates disappearance of bands and the white arrow indicates appearance of bands.

fingerprinting, which offers a useful biomarker assay in ecotoxicology (9). DNA fingerprints are the results of DNA genomic alterations that range from single base change to complex chromosomal changes (19,21). White et al. (22) observed variations in band patterns in prostatic adenocarcinoma and benign prostatic hyperplasia through DNA fingerprint analyses. Band disappearance is induced mainly by genomic rearrangements, decreased point mutation, DNA damage in primer binding sites, and interaction of DNA polymerase in the test organism with damaged DNA, as suggested by Liu et al. (23). Several researchers reported that RAPD was more sensitive than the comet and micronucleus assays, because RAPD analysis was capable of detecting DNA changes at a lower concentration of pollutants (20,24).

Our results indicated increased polymorphism in band profile with increasing concentration and exposure duration of potassium dichromate in the test specimen. It is possible that the relationships between strand breakage and genotype, as well as the differences in band patterns before and after exposure, are the result of induced mutation, DNA damage, or DNA alteration. The presence and absence of bands in the products amplified with 3 primers indicated a clear ability of the test chemical to induce DNA damage in test species. Our result was consistent with the observations of Zhou et al. (24), who validated DNA damage with RAPD analyses in marine ciliate *Euplotes vannus* (Protozoa: Ciliophora) exposed to nitrofurazone and found that the damage was dose- and exposure time-dependent.

The appearance of new DNA bands in the present study may also be due to the fact that some oligonucleotide priming sites might have become available to primers after structural change or changes in DNA sequence

Table 2. RAPD band profiles generated from genomic DNA of control and potassium dichromate-treated specimens of *C. carpio*.

Exposure duration (h)	Test concentration (mg L^{-1})	Band profile	Primers			Total band	a + b
			OPA-4	OPB-5	OPB-18		
	Control	–	5	6	5	16	–
24	93.95	a	0	0	1	1	2
		b	1	0	0	1	
	187.9	a	0	1	0	1	2
		b	1	0	0	1	
	281.85	a	2	1	2	5	5
		b	0	0	0	1	
48	93.95	a	0	0	0	0	3
		b	1	2	0	3	
	187.9	a	1	1	0	2	4
		b	0	2	0	2	
	281.85	a	3	1	0	4	5
		b	1	0	0	1	
72	93.95	a	2	0	1	3	3
		b	0	0	0	0	
	187.9	a	2	3	1	6	6
		b	0	0	0	0	
	281.85	a	3	1	0	4	7
		b	3	0	0	3	
96	93.95	a	1	2	0	3	6
		b	2	1	0	3	
	187.9	a	2	2	0	4	7
		b	1	1	1	3	
	281.85	a	3	1	1	5	9
		b	2	1	1	4	

a: disappearance of normal bands, b: appearance of new bands, a + b: polymorphic bands.

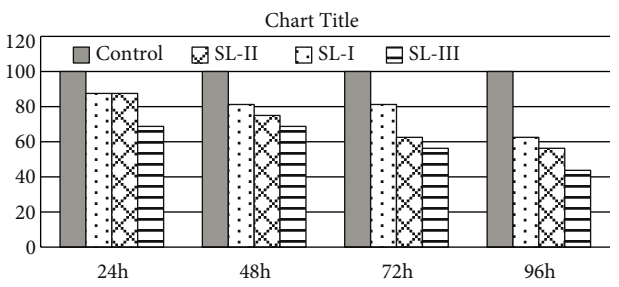

Figure 2. Genomic DNA template stability of *C. carpio* exposed to various concentrations and exposure durations of potassium dichromate.

due to mutations, large deletions, and/or homologous recombination and may be the result of GTS due to DNA damage (19). GTS in test specimens decreased with increasing test concentrations and exposure time, which indicated that GTS was significantly affected by potassium dichromate stress. The decrease in genomic DNA stability may be the result of band disappearance and appearance of new bands (19). The data obtained in the present study on genomic stability were in agreement with the findings of Mohanty et al. (25), who examined *Labeo rohita* fingerlings exposed to furadan at 24, 48, 72, and 96 h after exposure. A similar study on DNA damage due to UV radiation was

conducted on marine alga *Palmaria palmata* by Atienzar et al. (19). They demonstrated that changes in RAPD profiles induced by pollutants can be viewed as changes in genomic DNA template stability. This genotoxic assessment can be directly compared with modifications in other parameters.

Potassium dichromate and its metabolites may cause oxidative DNA damage by generating reactive oxygen species (ROS) through interaction with reduced glutathione (26). In addition to an ROS attack, DNA strand breaks and mismatch errors also occurred due to incomplete excision repair of DNA adducts (27).

In conclusion, the findings of the present study confirmed that the heavy metal potassium dichromate is genotoxic to *C. carpio*, as was made evident from the DNA damage induced in blood tissues. The RAPD-PCR technique of detecting DNA damage appears promising, owing to gain or loss in the number of bands and the effect of genomic template stability.

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