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DETECTION OF DNA DAMAGE OF *Clarias gariepinus* EXPOSED TO 2, 4-D USING CHROMOSOMAL ABERRATIONS AND RAPD ASSAYS*

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ABSTRACT

The widely used herbicide 2, 4- dichorophenoxy acetic acid (2, 4-D) is evaluated for acute toxicity and stress factors on fresh water fish. In this study, induced chromosomal aberrations *in vivo* were studied using three concentrations of 2, 4 -D (10, 20 and 30 mg/ml) for 7 days, the percentage of chromosomal aberrations was found to be statistically highly significant after treatment with the different doses. RAPD (PCR- based diagnostic assay) was used also as a bioindicator of the pollutant's toxicity to assess the genetic damage applied on primary liver and spleen cell culture of *Clarias gariepinus* using different concentrations of the pollutant (0.05, 0.1 and 0.5 ug /ml). RAPD– PCR was applied using six primers.

The PCR results demonstrated genetic different damage in the RAPD fingerprinting as a result of toxicity; these differences may be due to selection pressure of pollutant on fish.

From these results, it could be concluded that chromosomal aberrations and RAPD- PCR are useful tools for estimating genotoxic effects of chemical pollutants and as a bioindicator for genetic damage and diseases.

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Key words: chromosomal aberrations, fish, genotoxicity, RAPD assay, 2,4 –D.

1. INTRODUCTION

The extensive usage of pesticides is considered as a chemical pollution, which can affect the fresh water funa particularly fish. This can lead to the induction of genotoxic effects giving rise to mutations (that are manifested as a disease), genetic damage, even it may not manifest for several generations. Previous studies have suggested that human activities such as pollution may influence the genetic composition of fish populations (Nevo *et al., 1984* and Gillespie and Guttman, *1993)*. For the last few decades assays, studying of genotoxicity depended on chromosomal aberrations induced by exposure to pollutants. Recently, a large number of genetic approaches has been developed to monitor the environmental effects of pollutants, one of these is DNA damage as indicator of genotoxicity. Genotoxic pollutants act directly on the DNA structure and function. DNA alterations can be detected by a number of techniques; one of these techniques is the random amplified polymorphic DNA (RAPD) (Peinado *et al.*, 1992).

One method of documenting the occurrence of contaminant selection and adaptation is by comparing the genotypes between contaminated and reference populations. *In vitro* systems based on cells derived from fish are accepted as an alternative to *in vivo* assays. Cells provide an excellent experimental system for studying toxic mechanisms at the molecular level (Castano *et al.*, 2003). Also they can contribute a better understanding of the genotoxic effects because cultured cells are genetically homogenous.

2, 4-D is still used as a common herbicide employed in weed control of wheat, rice, maize and aquatic weeds *(Hayes, 1982)*. Many authors reported the cytogenetic effects of 2, 4-D. It induces chromosome abnormalities in the meiosis of *Vicia faba* (*Amer and Ali, 1974*), wheat (El Najjar and Soliman, 1982) and barley plants (Khalatkar and Bharagava, 1985). It also induced mitotic chromosomal aberrations in the roots of some plants (Kumari and Vaidyanath, 1989 and Bobade, 1996).

2, 4-D induced chromosome aberrations and micronuclei in human lymphocyte cultures (Zeljezic and Garaj-Vrhovac, 2004). It was found to be genotoxic in rat bone-marrow (Adhikari and Grover,

1988), as well as, bone-marrow and germinal cells of the mouse *(Amer and Aly, 2001).*

2,4-D also has the ability to induce micronuclei in the catfish *Clarias batrachus* (Ateeq *et al.,2002*)and in fresh water fish *Channa punctatus*(Farah *et al.,2003*). 2, 4-D increased the oxidative enzyme stress and lipid peroxidation in *Tilapia niloticus* (Ozcan *et al., 2003*). Detection of 2, 4-D genotoxicity using comet assay was confirmed as DNA damage in a study carried out by Ateeq *et al.,* (2004).

In this study, two approaches were used to investigate the genotoxicity of 2, 4 dichlorophenoxy acetic acid (2, 4-D). (1), the chromosomal aberration analysis in kidney cells of *Clarias gariepinus* fish; and (2), the RAPD assay, a polymerase chain reaction, PCR – based DNA fingerprinting technique.

2. MATERIALS AND METHODS

2.1. Experimental organisms

A total of 100 fish of *Clarias gariepinus* 100-150 g were brought to the laboratory. Samples were acclimatized to laboratory conditions for 10 days before the beginning of the experiment. The fish were held in aquarium (70 × 50 × 50 cm) and supplied with dechlorinated tap water and aerated at 30° ±2 °C and pH =7.3 ±.0.2, fed on balanced ratio of 32% protein content used in the form of artificial pellets, provided as 2-3 % of the body weight. The fish were divided into three pools treated with three concentrations of 2, 4-D (10, 20 and 30 mg/l), a fourth pool contained water without 2 , 4-D and a final one was treated with DMSO and used as control. Another group was brought to the lab. to be used in RAPD technique.

2.2 Chemical dosage and treatment for chromosomal analysis

2, 4-Dichlorophenoxy acetic acid was used as a genotoxic pollutant (2, 4-D, 98%, Merck), it was dissolved in DMSO solvent. Fish were treated with three concentrations of 2, 4-D (10, 20, and 30 mg/l) for 7 days.

2.3 Chromosome metaphase spread and analysis

After 7 day treatment, fish were injected intraperitoneally (IP) with 0.01% cholchicine (1 ml/100 gm b.wt.). Four hours after injection, the fish were sacrificed and chromosome preparations were carried out according to Danial and Andre, (1986). Slides were

stained with 10% Giemsa and analyzed. Twenty five fish metaphases were analyzed in five fishes per group. Only well-spread chromosomes were selected for scoring. One hundred and twenty five metaphases were investigated microscopically for the control and each tested dose. Statistical analysis was carried out using Student's t-test.

2.4. Cell culture and chemical exposure

Cells of liver and spleen tissues of *Clarias gariepinus* fish were grown on RPMI 1640 medium supplemented with 10% Foetal bovine serum (FBS), ampicillin (0.05 1U/ml), streptomycin (0.05 mg/ml),con A(2µg /ml) at it's optimum growing temperature 20 ± 1C°. 2, 4-D was added in concentrations of 0.05, 0.1 and 0.5µg/ml for 24 hr. At the end of the exposure period, the medium was removed. Cells were collected and washed in phosphate buffered saline for subsequent analysis.

2.5. DNA extraction and RAPD analysis

DNA was extracted separately from each of the replicates by chloroform- isoamyl extraction method after treating the cells with the digestion buffer (5M.NaCl; 1M.Tris HCl, pH 8; 0.5M. EDTA, pH 8; 10% SDS; 0.1mg/ml protienase K). DNA was then precipitated using cold ethanol, dried and resuspended in Tris EDTA buffer (All buffers used according to Sambrook *et al.* (1989).

RAPD reaction previously described (Ferrero *et al.*, 1998 and Becerril *et al.*, 1999) was performed using100 ng DNA in a total volume of PCR mixture 25 µl. Six 10-mer primers obtained from Metabion technologies were employed , the nucleotide sequences of the primers are:

Primer 1 (Z - 17): CCTTCCCACT, Primer 2 (A - 06): GGTCCCTGAC, Primer 3 (Z - 04): AGGCTGTGCT, Primer 4 (F - 14): TGCTGCAGGT, Primer 5 (A– 09): ACCCGGTTCT and Primer 6 (Z - 02): AGGTTTCAAG.

The amplificd PCR products were analyzed electrophoretically on 1.5% agarose gel stained with ethidium bromide, and photographed by a Polaroid camera. A hundred base pair ladder was used in the same gel. The products of amplification were analyzed qualitatively by detecting the appearance or absence of the bands in the RAPD profiles which were tested statistically by performing one-way analysis of variance (ANOVA).

Quantitative analysis was perfcrmed taking as a comparative parameter the percentage of amplification of each band relative to the total band intensity obtained with each primer. Comparison was made after the densitometry analysis of the control (unexposed) and exposed DNA. To compare the sensitivity of genomic DNA template stability, changes in their values were calculated as a percentage of their control value.

3. RESULTS

3.1. Cytogenetic results

The ability of 2, 4-D to induce chromosomal aberrations was detected (Plate 1). Chromosomal aberrations are currently recognized as being a sensitive indicator of agents which damage the fish DNA. In this study, all the tested doses of 2, 4-D induced a significant increase in the frequency of the different structural chromosomal aberrations (Plate1) exemplified by chromatid type (gaps, breaks, deletions and fragments), also centromeric attenuations and centric fusions frequently appeared. The percentage and different types of

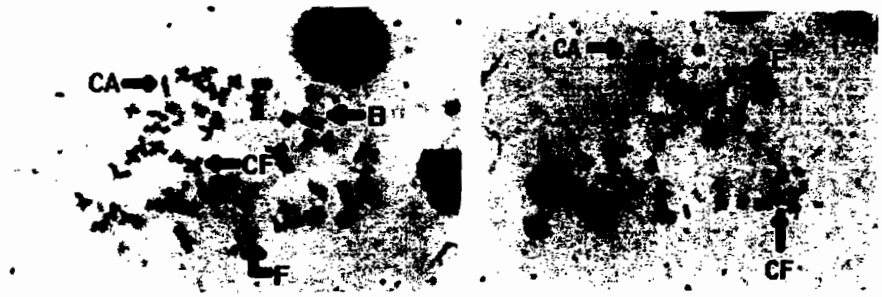

Plate (1): Chromosomal aberrations induced by 2, 4-D toxification of *Clarias gariepinus* (CA, centromeric attenuations; CF, centric fusion; B, break; F,fragment)

aberrations induced by *in vivo* treatment of *Clarias gariepinus* with different doses of 2, 4-D for 7 days are presented in Table (1). The percentage of aberrations was dose dependent with a maximum value of 30.4 ± 0.97 ($P<0.01$) after treatment reached with the highest dose of 2, 4-D compared with 3.20 ± 0.61 for control (non- treated). The

percentage of chromosomal aberrations detected in DMSO control was 4.0%.

3.2. The RAPD-PCR

Out of six 10-mer primers used, four primers were reproducible. Both the quantitative and qualitative analyses showed an increase in the instability of the DNA from the exposed cells. The results of RAPD amplification using the four primers are shown in Plate 2. The principal events noticed in the exposed DNA were a variation in the band intensity, as well as the disappearance or appearance of new bands. The primers used in this study gave a total of 25 bands whose molecular weights ranged from 231 to 1352 bp for the control samples. Table (2) illustrates the products of RAPD amplification using 4 reproducible primers, applied on the control (non treated) and spleen and liver cells exposed to 2, 4-D. Amplification profiles in the treated cells in comparison to the control profiles are indicated in Plate (2) and Table (2) which show different changes in the profiles exemplified by the appearance and the disappearance of some RAPD bands.

The number of disappearing bands which occurred in this investigation was greater at higher concentrations of the treatments. It was noticed that the bands of high molecular weight especially greater than 1 kb were shown to disappear frequently. Also it was noticed that extra bands appeared at the highest concentration of the treatment.

In fact, the sensitivity in the detection of bands (disappearance / appearance) is dependent on the sequence of the primer used. Perhaps the most obvious result was the appearance of new bands with different consistency among replicates as a function of treatment concentration. For example, three new bands were generated using primer 2, two of them of molecular size of 141 and 198bp were present in the spleen cells, the third band was present in liver cells 175bp. In primer 4, three new bands were generated, two of them 212 and 241 bp appeared with liver cells but one appeared in spleen cells (289 bp). In primer 3 the spleen DNA showed greater alternation, where two new bands appeared 231 bp and 265bp in contrast to liver DNA which showed no new band appearance. DNA alternations exemplified by band appearance / disappearance and alternation in band intensity was found to be less frequent in low concentrations in contrast to high concentration treatment.

Table (1): Number and mean percentage of metaphases with aberrations in kidney cells of *Clarias gariepinus* treated with 2,4-D.

Treatment and doses	No. metaphases Examined	No.Ab.	No. of different types of aberrations						Mean % ± S.E
			C.A.	C.F.	G	B	D	F	
1-control (non treated)	125	4	-	-	-	1	1	2	3.2±0.61
2- 2,4-D									
a) 10 mg/l	125	15	4	5	1	2	2	1	12.0±0.92*
b) 20 mg/l	125	26	6	8	1	3	3	5	20.8±1.32*
c) 30 mg/l	125	38	8	10	3	5	4	8	30.4±0.97*
3- DMSO	125	5	-	-	-	2	2	1	4.0±0.64*

* = highly significant (t –test). Ab. = aberrations; C.A. = centromeric attenuations; C.F. = centric fusion; G. =gap;B. = break; D. = deletion; F. = fragment.

Table (2): The products of amplifications using different primers (numbers in bp).

Primer 1			Primer 2			Primer 3			Primer 4		
C	exposed liver cells	exposed spleen cells	C	exposed liver cells	exposed spleen cells	C	exposed liver cells	exposed spleen cells	C	exposed liver cells	exposed spleen cells
1163	-	-	1352	1352	1352	1007	1007	1007	1012	1012	1012
937	937	937	1296	1296	1296	749	-	749	792	792	792
792	792	792	831	831	831						
605	605	605	807	807	807	547	547	547	685	685	685
496	496	496	703	703	703	409	409	409	594	594	594
339	339	339	586	-	586			231	396	396	396
231	231	231	481	481	481			265	307	-	307
-	186	162	378	378	378					212	289
			311	311	-					241	
			236	236	236						
					141						
					198						

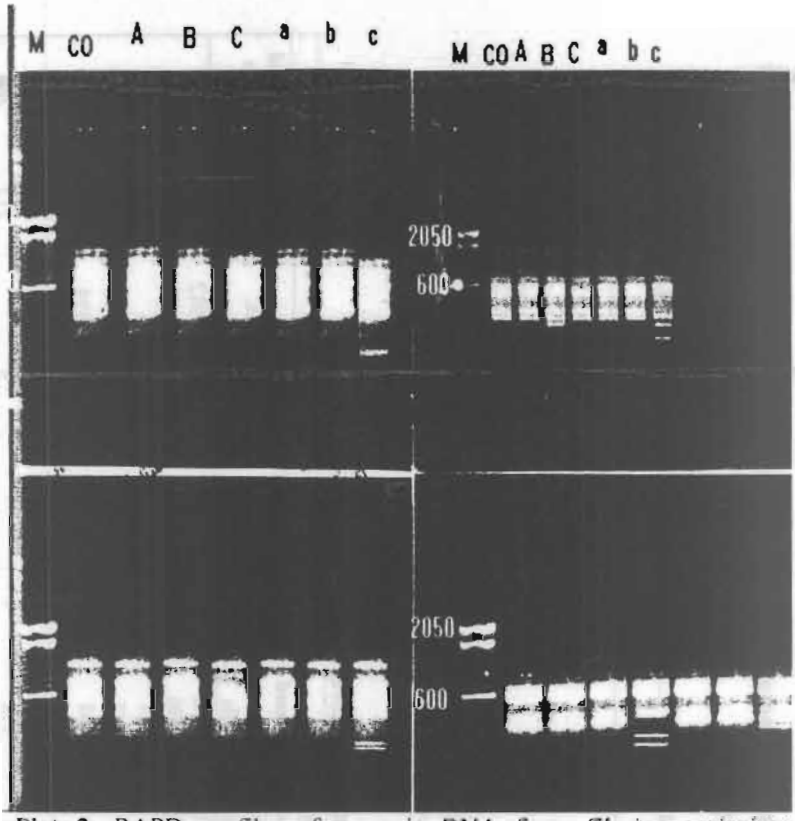

Plate.2. RAPD profiles of genomic DNA from *Clarias gariepinus* exposed to varying concentrations of 2, 4-D using four primers. M, DNA marker (100bp.). Co. control DNA without treatment. A, B and C, liver cells exposed to concentrations 0.05, 0.1 and 0.5 μg/ml respectively. a, b and c, spleen cells exposed to concentrations 0.05,0.1 and 0.5 μg/ml , respectively.

4. DISCUSSION

Chemical environmental contamination is suspected to affect natural populations. Environmental studies have shown that continuous exposure to certain contaminants produces a selective pressure on populations. It can alter the allele frequency, resulting in a decrease in the reproduction of affected individuals, and Alteration of population genetic structure by two mechanisms: First,

contamination may reduce population size with a resultant decrease in genetic diversity (Bickham and Smolen, 1994). Secondly, contaminant selection and subsequent adaptation to toxicants may lead to shifts in genotype frequencies.

Individuals within natural wildlife populations are subdivided into more or less distinctive groups that differ genetically from each other. Variation in response of organisms to toxic stress can be attributed to their genetic variations (Nevo *et al.*, 1984 and Kopp *et al., 1994)*. Genotoxic effects of environmental pollutants can threaten the survival of wild populations by modifying their genetic ability for adaptation to variable environmental conditions (Krane *et al.*, 1998). In fish, populations living in polluted areas were found to be altered when compared with fish in unpolluted areas (Theodorakis and Shugart, 1997). In studying natural populations, they will be associated with inherent methodological difficulties, particularly with chronic exposures. This problem was solved by the use of cell cultures from fish with a measurement of DNA damage, because cell cultures are genetically homogenous and the presence of polymorphisms will be avoided.

In this study, chromosomal analysis showed that the total number of *Clarias gariepinus* chromosomes is (2n=54). Good preparation of chromosome metaphase helps to avoid the presence of abnormalities in the control. 2, 4-D induced significant increase in the percentage of chromosomal aberrations in kidney cells. The mean percentage of chromosomal aberrations was statistically highly significant and increased with the increase of 2, 4-D concentration. Studies on 2,4-D has already confirmed it's genotoxic potential with regard to micronuclei induction and cytotoxic effects on two fish species, heteropneustes fossils, *C. punctatus* and *C. batrachus* .The percentage of micronuclei induction was dose dependent (Farah *et al.*,2003, Ateeq *et al.,2004,*).

Many insecticides such as dichlorovos, methyl parathion and pyrethroid lambda-cyhalothrin were found to have the ability to induce chromosomal aberration, sister chromatid exchange (SCE) and micronuclei (MN) on fish (Rishi and Grewal, 1995; Cavas and Ergene-Gozukara, 2003).

However, this study indicated the presence of different types of chromosomal aberrations induced by treatment with the herbicide controller 2, 4-D. These aberrations may lead to alternations in the genetic material; this was studied here also using the RAPD

technique, which offers a constant DNA fingerprint. Alternations in the DNA can be detected when comparing the fingerprints of control and treated cells. These alternations are visualized as loss and or gain of bands and for changes in their amplification intensity (Kubota *et al.*, 1992). Both alternations are detected in this study. Although the generation of RAPD profiles has often been criticized as unreliable (El Sworth *et al., 1993),* reproducible DNA profiles have been generated from a range of aquatic invertebrates, plants and bacterial species (Grayson *et al., 1999;* Atienzar *et al., 2000)* and used successfully to detect genotoxins induced DNA damage (Becerril *et al.*, 1999). Significant alternations in band absence, presence and intensity using RAPD assay were indicated in RTG-2 fish cells exposed to benzo(a)pyrene (Argelia and Concepcion, 2004). Studies of 2,4-D have already confirmed it's genotoxic potential with regard to micronuclei and comet assays(Ateeq *et al.*, 2004). Based on their results ,they suggested that micronuclei to some extent were also induced by strand break in DNA .In agreement with them and from combined results we recorded in this study ,it can be confirmed that 2,4-D induces DNA damage and lesions .

Previous studies have shown that changes in band patterns observed in DNA RAPD profiles reflect DNA alternations from single base changes (point mutations) to complex chromosomal rearrangements (White *et al.*, 1990; Welsh *et al.*, 1991). This is in agreement with our study, where DNA damage induced by 2, 4-D exposure showed changes in RAPD profiles; changes in band intensity and appearance and disappearance of PCR products in exposed DNA.

The resulted DNA alternations may induce changes in the DNA sequence at specific sites generating different annealing primer – template sites (Jones and Kortenkamp, 2000). This illustrated why in this study, the disappeared bands are frequently the same in liver and spleen cells. The same authors also demonstrated that RAPD assay is able to detect mutations only if they occur in 2% of the DNA.

In this study, the variation in band intensity, appearance and or disappearance of bands may be attributed to the presence of DNA nitrogenous base dimmers (*e.g.* pyrimidin dimmers), which can block or reduce (bypass events) the polymerization of DNA in PCR reaction (Donahue *et al.*, 1994; Nelson *et al.*, 1996) where the bypass event is a process depending on the polymerase enzyme, the structure of lesion and the sequence of it's location.

Extra bands detected in this study mean new PCR amplifications which reveal a change in the DNA sequence due to mutations resulting in new annealing events.

However, the use of *in vitro* assays permits to detect alternations in the DNA induced by genotoxic pollutants. In this study, RAPD technique(*in vitro*) in conjunction with chromosomal aberrations(*in vivo*) has been successfully used to detect DNA damage and show reproducible assays for genotoxicity induced by chemical pollutants in fish.

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اكتشاف تلف الحمض النووى " دنا الناتج عن استخدام مبيد التوفوردى 2,4-D " باستخدام تقنية الشذوذات الكروموسومية والتضخيم العشوائى لقطع الدنا (الرابيد) فى اسماك قراميط المياة العذبه

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ملخص

يعتبر الاستخدام المفرط للمبيدات الكيميائية تلوثاً كيميائيا ذا تأثير سلبي شديد على جميع الكائنات بما في ذلك الكائنات الحيوانية المائية ومن أهمها الأسماك. من تلك التأثيرات الضارة عطب فى المادة الوراثية والتي يترتب عليها حدوث طفرات جينية مما يؤدي غالباً إلى ظهور أمراض متعددة.

يعتبر 2,4-D من أكثر المبيدات الكيميائيا شيوعاً واستخداما في مكافحة الحشائش. تم في هذه الدراسة تقييم سمية هذا المبيد في أسماك القراميط في المياه العذبة من خلال استخدام نوعين من التقنيات هما: دراسة التغيرات الكروموسومية و دراسة تغيرات المادة الوراثية باستخدام تقنية الرابيد RAPD .

١- دراسة التغيرات الكروموسومية الناجمة عن استخدامه بتركيزات مختلفة (١٠، ٢٠، ٣٠ ملجم/ ل) لمدة ٧ أيام وكانت نتائج التشوهات هي على التوالي (١٢%، ٢٠,٨%، ٣٠,٤%). ووجدت علاقة وثيقة بين نسب التغيرات الكروموسومية والتركيزات المستخدمة من المبيد.

٢- دراسة تشوهات الدنا من خلال إستخدام تقنية تفاعل البلمرة المتسلسل (PCR) بتطبيق تقنية التضخيم العشوائي لجزيئات الدنا RAPD- PCR وذلك باستخدام ٦ بادئات عشوائية كل " يتركب من عشر قواعد نيتروجينيه (تم تطبيق تلك التقنية على خلايا الكبد والطحال المستزرعة). أظهرت نتائج هذه التجربة اختلافات في قطع الدنا الناتجة باستخدام ٤ بادئات و تميز هذا الاختلاف في ظهور الحزم الجديدة واختفاء البعض الآخر وكذلك في اختلاف كثافة بعض الحزم الناتجة من التضخيم لجزيئات DNA من خلايا الكبد والطحال المعاملة بالمبيد بتركيزات مختلفة (٠,٠٥ ، ٠,١ ، ٠,٥ ميكروجرام/ مل).

أثبتت هذه الدراسة كفاءة استخدام تقنيات التغيرات الكرموسومية والتضخيم العشوائي لجزيئات الدنا في تقييم سمية المبيد الكيميائي وتأثيره على المادة الوراثية للخلايا في أسماك قرموط المياه العذبة.

المجلة العلمية لكلية الزراعة – جامعة القاهرة – المجلد (٥٧) العدد الرابع (أكتوبر ٢٠٠٦):٦٨٥-٧٠٠.