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GENETIC VARIATION AND POLYMORPHISM IDENTIFICATION IN MICE TREATED WITH TRAMADOL AND TRAMACET USING AFLP FINGERPRINTING "COMPARATIVE STUDY"

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ABSTRACT

Tramadol and tramadol/ acetaminophen (tramacet) as a centrally acting analgesic are commonly prescribed as effective in treatment of acute and chronic moderate to severe pain. The present study was established to investigate genotoxic effect and genetic variation level induced by tramadol and tramacet in mice "comparative study". Sixty male albino mice were divided into five groups. Group one received vehicle (Saline), group two and three received orally 45 and 90 mg/kg body weight of tramadol respectively. However, group four and five received 450 and 900 mg/kg body weight of tramacet respectively for 20 days followed by 10 days for drug withdrawal. AFLP-PCR analysis, chromosomal aberration and sperm shape abnormalities were investigated on brain tissue, bone marrow cells and sperm. Tramadol and / or tramacet significantly increased frequencies of chromosomal aberration and sperm shape alteration in dose dependent pattern with slight improvement after recovery period. Moreover, result of AFLP analysis indicated genetic variation evident by percent of polymorphism (mean: 72.7%) and disappearance of some loci of DNA due to drug treatment. Conclusion: The current results showed that both tramadol and tramacet at therapeutic doses produced genotoxicity and high level of genetic variation.

KEYWORDS: Tramadol, Tramadol/Acetaminophen, Polymorphism, AFLP, Chromosomal aberration, Genotoxicity, Genetic variation.

1. INTRODUCTION

Tramadol is a synthetic centrally acting analgesic widely prescribed for treatment of moderate to severe pain. It is related to opiates like morphine and codeine, chemically derived from amino cyclohexanol.[1-2] The maximum allowed dose is 400mg per day. It has high bioavailability ranged from 70 to 80% and reached mean peak plasma concentration in about 2 hours after oral treatment.[3-4] Three mechanisms were reported to control tramadol analgesic action: μ-opioid binding through O-dimethyl tramadol metabolite, inhibition of serotonin reuptake through (+)-tramadol and inhibition of norepinephrine reuptake through (-)-tramadol.[5-7] O-desmethyltramadol (ODT) is the major active metabolite of tramadol formed in liver through O-demethylation, catalyzed by CYP2D6 (isoenzyme cytochrome p450) and is 2 to 4 times the analgesic potency of the parent drug.[8-9] Tramadol and its metabolites are mainly excreted via kidneys.[10] However, long term use of tramadol probably induces accumulation of toxic metabolites in cells and lead to high risk of pharmacokinetic interactions.[11-13]

Tramadol abuse is popular among teens all over the world. Tramadol like other opiates carries their risks and side effects including central nervous stimulation, sweating, dizziness, headache, pruritus and nausea.[14,15] Cerebral dysfunction and degeneration in neuron cells of rat brain were resulted in chronic use of tramadol.[16] Over doses of tramadol were reported to cause death duo to respiratory depression through mechanism of GABA (A)alpha1 and GABA(B)1 genes over-expression in medulla oblongata[17,18] Neurologic and cardiovascular effects were occurred in children intoxicated with tramadol.[19,20] Rhabdomyolysis and skeletal muscle injury were also occurred due to tramadol toxicity.[21] The Vtg gene, a biomarker of reproduction was significantly down regulated due to tramadol administration (34mg/kg) for 21-day.[22] Exposer to 200 mg/ kg tramadol for 45 days exhibited significant hepatic DNA damage using comet assay and alteration of protein pattern through protein electrophoresis, even after 9 week of drug withdrawal.[23] Recent study reported that tramadol and dactinomycin treatment induced significant genotoxic effects on mice stem cells. It showed a significant increase in chromosomal aberrations and micronuclei but significant reduction in mitotic index caused by tramadol.[24]

Liver and kidney played an important role in tramadol metabolism and excretion, so it may responsible for hepatotoxicity and nephrotoxicity resulted in tramadol use. Several studies reported that, long term of tramadol treatment induced acute renal failure (increase of

creatinine phospho kinase (CPK), high white blood cell count, bleeding risk (in case of interface with oral anticoagulants) and overproduction of nitric oxide and oxidative.[25-28] **El-Gaafarawi 2006.[29]** demonstrated significant hepatotoxicity, nephrotoxicity and sexual dysfunction after long term treatment of 40 and 80 mg/kg tramadol for month among treated rats. They found significant increase in serum aminotransferase (ALT, AST) and lactate dehydrogenase (LDH) levels and significant decrease in luteinizing and follicle stimulating hormones. Also, chronic use and withdrawal periods of tramadol (8 and 12 mg/100g) were found to decrease cellularity of spleen and IL-6 levels in rats in dose dependent manner.[30] Hepatic lesions and liver impairment function were demonstrated in rats treated with therapeutic doses of tramadol and tramadol/APAP for month.[31]

Sexual dysfunction as adverse effect of drug use has been documented with a range of drugs. About 15% of studied drugs have risk on male reproduction.[32] Opiate use was known to decrease the levels of sex hormones which responsible for dimension of fertility of both male and female opiate users**.** Significant decrease in luteinizing and follicle stimulating hormones was observed after administration of 80 mg /Kg tramadol for a month in male rats.[29]

Paracetamol (acetaminophen, APAP), non-steroidal anti-inflammatory drugs (NSAIDs) is widely used as antipyretic and analgesic agents.[33] It is metabolized in liver by cytochrome P450 forming N-acetyl-p-benzoquinone imine a toxic metabolite, which reduce glutathione.[34] Thus, an overdose of APAP was reported to lead to sever liver injury and adverse gastrointestinal effects.[35,36] However, tramadol un like NSAIDs were found to did not cause adverse effects on gut, platelet and urinary system after use.[37,38]

Tramacet (tramadol/APAP) is an effective analgesic prescribed for moderate to severe pain. It is related to opiates, chemically formed of combination of tramadol (opioid) and paracetamol (APAP: acetaminophen), non-steroidal anti-inflammatory). It performs complementary constituent analgesic action supported from APAP, rapid onset and tramadol, sustained analgesic effect.[39,40] Its bioavailability and that of its metabolite (o-desmethyl-tramadol) was found to be lower than that of tramadol alone.[41,42] The side effects of tramacet overdose may be like that of tramadol and APAP toxicity. Previous study demonstrated that tramadol and /or APAP induced DNA alteration in liver cells in dose dependent manner and histopathological changes in addition to impairment of liver function.[31]

From all previous studies, there are a few studies on genetic and molecular effects of tramadol and tramacet. Therefore, the present study was conducted to assess the molecular markers and genotoxic effects of tramadol and tramacet in comparative study after 20 days of treatment followed by extra 10 days as withdrawal period in male albino mice.

2. MATERIALS AND METHODS

2.1. Experimental design

2.1.1. Animal and experimental design

Sixty male Swiss albino mice weighing 18-22 g were used. The animals were housed in standard cages fed on a rodent basal diet and water *ad libituim*. After a week of acclimatization mice were randomly divided into five groups (12 mice per each). The first group served as control (Co), administered 0.1 ml saline orally for 20 days. The 2nd and 3rd groups (T and TD), administered oral doses of 45 or 90 mg/kg b. wt./ day of tramadol HCL (Global Napi. Pharma. Co., Cairo, Egypt) suspension in saline solution for 20 days respectively. The 4th and 5th groups (TC and TCD), administered oral doses of 450 or 900 mg/kg b. wt./ day of tramadol/APAP (Delta Pharma, Co., Tenth of Ramadan city, Egypt) suspension in saline solution for 20 days respectively. These doses were equivalent to therapeutic doses and were calculated for mice using Paget and Barnes (1964) tables. Twenty-four hours after last treatment, 6 animals from each group were randomly selected and sacrificed by cervical dislocation. The remaining animals were kept ten days later without any additional treatments and served as drug withdrawal groups (TR, TDR, TCR and TCDR respectively). Brain and testes tissues were obtained for molecular and genetic variation analysis, also epididymis and bone marrow cells were obtained for sperm and chromosomal aberration analysis.

2.2. Molecular analysis

2.2.1. DNA Extractions

Extractions of DNA were performed using GenElute ^{TM} Animal Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer′s manual. DNA quality and quantity were checked using an Eppendorf Spectrophotometer, 1.6% agarose gel in 1X Tris-Boric EDTA (TBE) buffer, stained with 0.01 mg/ml Ethidium Bromide.

2.2.2 Amplified Fragment Length Polymorphism analysis (AFLP)

The AFLP protocol was carried out following Vos *et al.* (1995)[43] with small modification. All fluorescent, radioactive labelled primers and adapters were purchased from Eurofins,

Hamburg, Germany (Table1). Extracted DNA was digested with *Eco*RI and *Mse*I and ligated to double stranded adapters. Next preamplification were achieved using *Eco*RI and *Mse*I primers and PCR condition; 94ºC for 30 Sec, 56ºC for 60 Sec and 72ºC for 60 Sec, 25 cycles in a final volume 20µL. The amplified products quality was verified by electrophoresis on 1.5% agarose gel. Selective amplifications were performed using six primers designed for *Eco*RI and *Mse*I digestion sites and expanded with three additional nucleotides (3 *Eco*RI+NNN fluorescently labelled forward primers x 2 *Mse*I+NNN revers primers) selected from Magdy *et al*. (2016).[44] The *Eco*RI digestion enzyme was labeled with 5- carboxy-fluorescein (5-FAM) or 5- hexachloro-fluorescein(5-HEX) or 5- cyanine- fluorescein (5-CY3) for fluorescence diagnosis, while *Mse*I primers were not labeled. The original PCR programme were carried out duo to Vos *et al*. (1995).[43] The size of amplified fragment was estimated by RoxGene scan 500 size standard addition, then screened and analyzed using Secugen, S.L. sequencing service (Madrid, Spain), with DNA analyzer (ABI3730, Applied Biosystems, Carlsbad, CA, USA).

2.2.3. Loci scoring

Analysis of AFLP data is very sensitive to factors such as, dimers, small and stutter bands. To avoid these factors loci fragments generated by AFLP were analyzed using computer program Rawgeno version 2 for band scoring and quality test. The analysis was based on band -binary criterion, refer to locus as (+) when the band is present and (-) when it is absent. The percentage of polymorphism (%P), polymorphic information content (PIC) and Marker index (MI) were calculated according to **Powell *et al*. 1996[45] and Ghislaine *et al*. 1999[46]** using the formula.

$PIC = 1 - [(p)^2 + (q)^2]$

Where "*p*" is frequency of present alleles and "*q*" is frequency of absent alleles across tested groups.

$MI = PIC \times n\beta$

Where "n" represents bands number and "β" represents the percent of polymorphism. Jaccard's coefficient (Jaccard, 1908) was used to constructed genetic dissimilarity coefficient and to asses principle components analyses (PCA), applying the default settings. Dendrogram was calculated according to unweighted pair group method arithmetic mean algorithm (UPGMA) using XLSTAT software 2016.[47]

Table 1: Primers and fluorophores used in AFLP analysis.

Pre-selective amplification primers:
*Eco*RI specific primer core sequence-0:
5′-GACTGCGTACCAATTC-3′
*Mse*I specific primer core sequence-0:
5′-GATGAGTCCTGAGTAA-3′
Selective amplification primers:
*Eco*RI specific primer core sequence-**3nucleotides**:
EcoRI-**ACA**
EcoRI-**AGG**
EcoRI-**ATA**
*Mse*I specific primer core sequence-**3nucleotides**:
MseI-**CAA**
MseI-**CTA**
Six primer combinations:
1-*EcoRI*-ACA/ *MseI*-CAA 4-*EcoRI*-ACA/ *MseI*-CTA
2-*EcoRI*-AGG/ *MseI*-CAA 5- *EcoRI*-ACA/ *MseI*-CTA
3-*EcoRI*-ATA/ *MsIe*-CAA 6-*EcoRI*-ACA/ *MseI*-CTA
Fluorescent tags of selective primers:
EcoRI-ACA: **FAM** (blue); 5′-GACTGCGTACCAATTC**ACA**-3′
EcoRI-AGG: **HEX** (green);5′-GACTGCGTACCAATTC**AGG**-3′
EcoRI-ATA: **CY3**(yellow);5′-GACTGCGTACCAATTC**ATA**-3′

2.3. Sperm analysis

Sperm analysis was performed using conventional methods of Morakinyo *et al*. 2009.[48] Cauda epididymides were minced with anatomical scissors in 1 ml saline. Sperm morphological abnormalities were examined in sperm smear (500 sperm cell/ sample) stained with hematoxylin and eosin at 100X magnification and expressed in percentages as normal or abnormal sperm.

2.4. Chromosomal aberration analysis

Analysis chromosomal aberrations on bone marrow cells were done using following Preston *et al*. 1987.[49] Fifty metaphases, Giemsa stained were analyzed for structural and numerical chromosomal abnormalities. Structural chromosomal aberrations were represented as deletion, chromatid gaps, centric fusion, a centric fragment and ring chromosomes and expressed in percentage. Numerical chromosomal aberrations were classified into chromosomal counts less or more than 40 chromosomes as hypoploidy and polyploidy metaphase and expressed in percentage.

2.5. Statistical analysis

The data of chromosomal and sperm analysis were analyzed using one-way analysis of variance (ANOVA) and represented as means ± SE. Least significant difference (LSD) at 5

% comparisons were performed to assess the significance of differences among groups. Statistical Processor System Support "SPSS" for Windows software, Release 16.0 (SPSS, Chicago, IL) was used.

3. RESULTS

3.1. Effect of tramadol and tramacet on chromosome changes

Table (2) represented the results of chromosomal aberrations analysis. Administration of tramadol and tramacet for 20 days significantly ($P < 0.05$) increased frequencies of structural chromosomal aberrations between tested groups (49.3%, 76.3%, 63.3% and 88%; in groups T, TD, TC and TCD respectively). These increases were dose dependent with both drugs and the highest value was found after tramacet administration. Centric separation (C.S), deletion (del.), centric fusion (C.F) and gap were the most frequent types of structural chromosomal aberration resulted in tested drugs. However, the values of acentric fragment (A.F) and ring chromosome were rare. Moreover, tramadol and/or tramacet treatments produced significant ($P < 0.05$) increase in percent of numerical chromosomal aberrations in dose dependent manner. Hypoploidy was found to be more abundant than polyploidy. The referred data documented that percent of structural and numerical chromosomal aberration under the effect of tramacet were higher than that of tramadol (Fig. 1).

On the other hand, slight improvement was observed in value of chromosomal aberration after drug withdrawal periods. The percent of structural chromosomal aberration were significantly increased ($P < 0.05$) between groups (36.7%, 54.3%, 47.6% and 61%; in groups TR, TDR, TCR and TCDR respectively). With regards to value of numerical chromosomal aberration, was found to be significantly increased in TR, TDR, TCR and TCDR groups as compared to control (5%, 8.3%, 7.6%, 9.67% and 0.67% respectively).

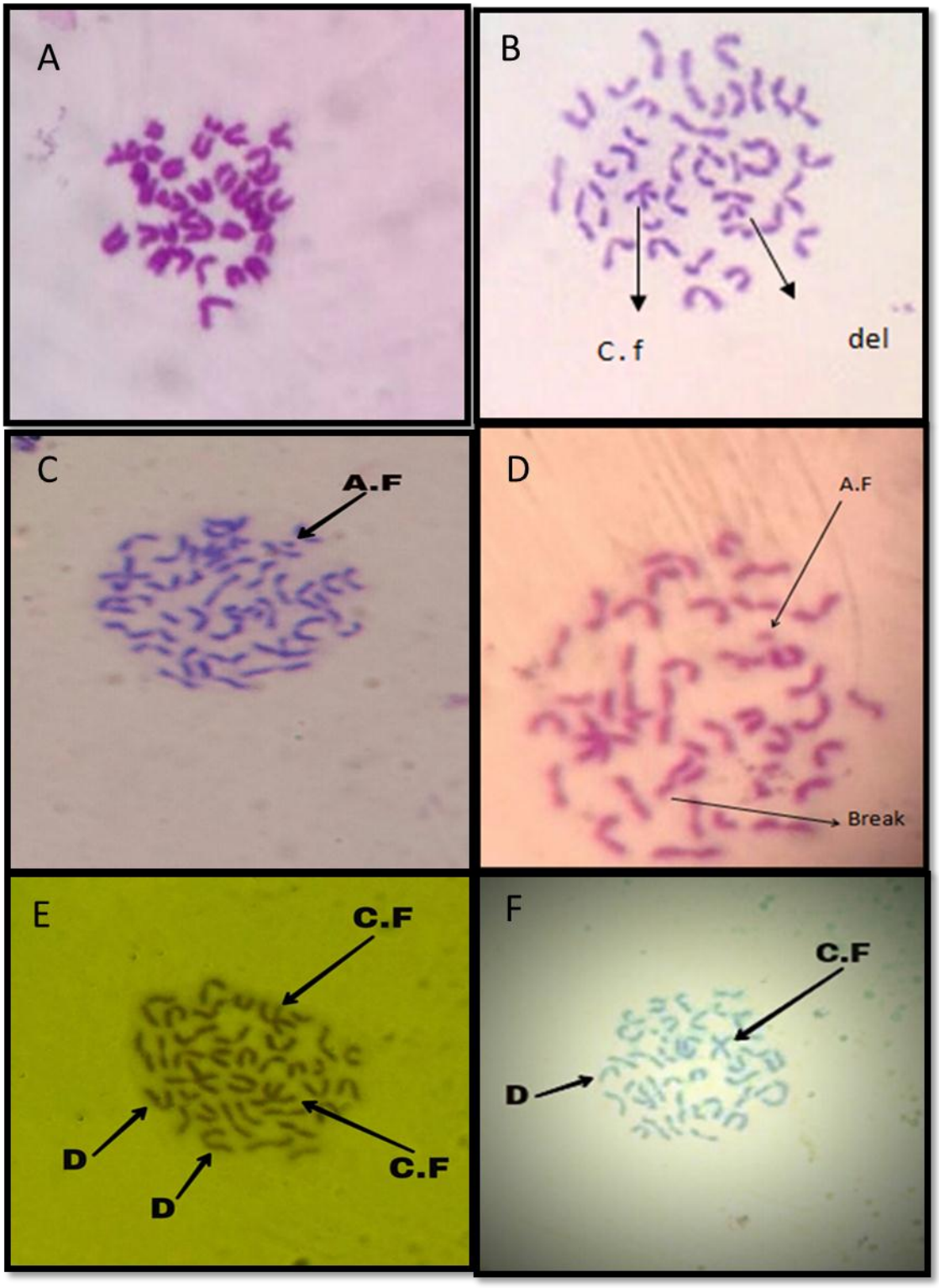

Fig. 1: Metaphase chromosomal spread of control and treated groups showing: A: Normal metaphase; B-F: metaphases spread of treated groups showing, Deletion (D), Centric Fusion(C. f), Break(B) and A-centric Fragment(A.F).

Table 2: Effect of tramadol and tramacet on chromosomal abnormalities.

CA	Co	T	TD	TC	TCD	T-R	TD-R	TC-R	TCD-R
Structural CA									
gap	1	7	25	17	21	7	12	10	8
del.	1	28	62	35	70	25	43	28	44
C.S	2	55	79	77	91	45	52	51	69
C.F	2	25	31	23	33	17	26	23	26
Break	1	8	4	7	13	5	8	7	4
Ch.ex	1	2	3	5	5	3	4	3	5
Ring	0	4	5	3	5	0	3	5	5
A.F	1	11	15	14	16	3	9	11	16
Iso-g	0	8	5	9	10	5	6	5	6
Total	9	148	229	190	264	110	163	143	183
Percent%	3.00	49.33	76.34	63.30	88	36.67	54.33	47.67	61.00
Mean ± SE	**1.5±0.22^{b}**	**24.7±0.61^{a}**	**38.2±0.87^{ab}**	**31.7±0.88^{a}**	**44±1.18^{ab}**	**18.3±0.61^{ab}**	**27.2±0.94^{ab}**	**23.8±0.40^{a}**	**30.7±0.80^{a}**
Numerical CA									
Polyploidy	1	14	19	11	15	9	10	8	12
Hypoploidy	1	12	16	19	28	6	15	15	17
Total	2	26	35	30	43	15	25	23	29
Percent%	0.67	8.67	11.66	10.00	14.33	5.00	8.33	7.67	9.67
Mean ± SE	**0.3±0.02**	**4.3±0.33^{ab}**	**5.8±0.30^{a}**	**5.0±0.36^{a}**	**7.2±0.74^{ab}**	**2.5±0.22^{ab}**	**4.2±0.40^{a}**	**3.8±0.30^{a}**	**4.8±0.31^{a}**

Values represent mean ± SE; n = 6 per group. Values with superscript **a** are significantly different from control; values with superscript **b** are significantly different from each other ($P < 0.05$).

3.2. Effect of tramadol and tramacet on sperm analysis

Data of sperm analysis was deposited in table 3. The value of abnormal sperm shape was significantly ($P < 0.05$) increased between groups in treated animals (11.7%, 17.9%, 16.8% and 28.46%; in groups T, TD, TC, and TCD respectively), even after period of drugs withdrawal (9.7%, 14.8%, 14.23% and 20.93%; in groups TR, TDR, TC and TCDR respectively).

Abnormal head sperm was found to be the most frequent type of sperm abnormalities followed by abnormal tail and broken sperm (fig. 2).

Table 3: Effect of tramadol and tramacet on sperm abnormalities.

Sperm abnormalities	Co	T	TD	TC	TCD	T-R	TD-R	TC-R	TCD-R
Abnormal head	35	90	160	156	280	75	142	127	199
Abnormal tail	27	83	142	116	198	66	115	108	135
Broken sperm	43	178	235	233	376	150	189	192	294
Total	105	351	537	505	854	291	446	427	628
Per Cent%	3.50	11.70	17.90	16.83	28.46	9.70	14.86	14.23	20.93
Mean ± SE	**17.5±0.42^{b}**	**58.5±0.8^{ab}**	**89.5±1.05^{ab}**	**84.2±0.7^{ab}**	**142.3±1.2^{ab}**	**48.5±0.56^{ab}**	**74.3±0.5^{ab}**	**71.2±1.35^{ab}**	**104.7±0.6^{ab}**

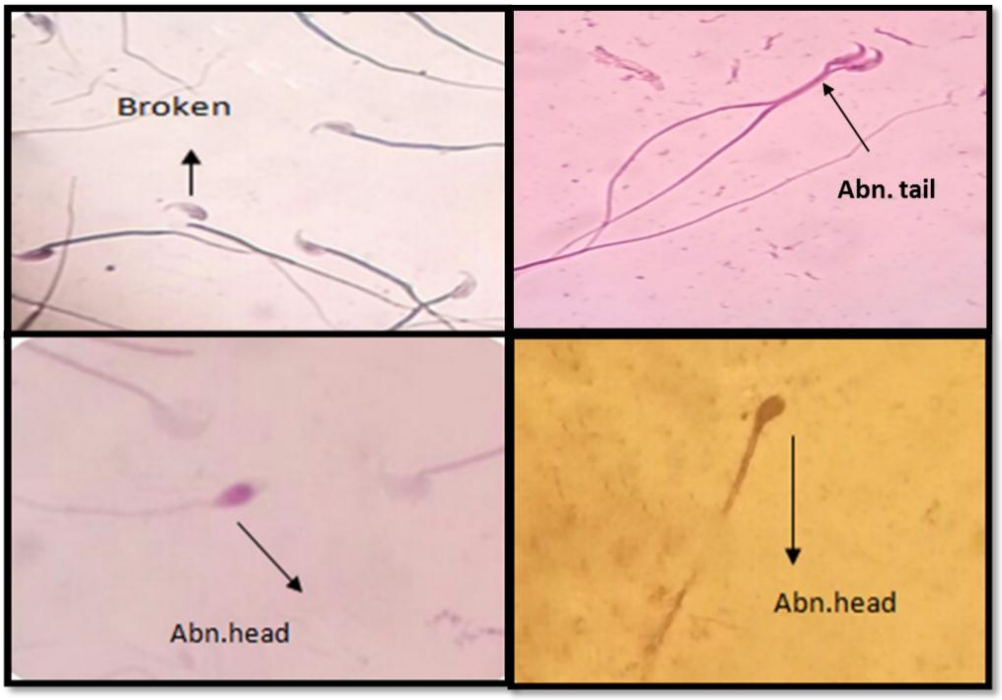

Fig. 2: Sperm morphology of treated animals shows broken sperm, abnormal head and abnormal tail.

3.3. AFLP analysis

3.3.1. Band scoring and Level of DNA polymorphism

PCR amplification were successful with six primer pairs. In total, 213 loci were scored from all primer pairs for tested samples. Only, 37 bands were rare, characterized by band frequencies <5% among all tested groups. Thus, 176 reproducible loci with good resolutions were used for further analysis. The number of these bands were varied from 17 to 53 bands and their size ranged from 30 to 447 bp (Table, 4). Among these, 142 loci were polymorphic; 33 loci were monomorphic. The average value of percent of polymorphism among control, tramadol, tramacet treated groups and after recovery period were 77.5% ranged from 75%

(FAM: EcoRI-ACA/ MseI-CAA) to 86.5% (FAM: EcoRI-ACA/ MseI-CTA). Moreover, the mean value of polymorphism information content (PIC) was 0.44 among tested groups varied between 0.33 (FAM: EcoRI-ACA/ MseI-CAA) and 0.49 (HEX: EcoRI-AGG/ MseI-CAA and FAM: EcoRI-ACA/ MseI-CTA). As shown in table (6), only six (%P; 4.4) and fifty-two (%P; 39%) loci with variable size were disappeared after tramadol treatment (T and TD groups respectively) as compared to control. While only eight (%P; 6%) and fort six (%P; 34.6%) loci were disappeared compared to control as a result of tramacet treatment (TC and TCD groups respectively). Among these, twelve loci from 30 bp to 323bp were identified as unique fragments for control samples as completely disappeared in all treated samples and after recovery period (seen as shaded rows).

Table (4): Genetic polymorphism parameters based on AFLP analysis based on tested groups.

	Primer name & sequence(5'-----3')		Amplified bands		%P	PIC	MI	Fragment size (bp)	
		Band number	Mono-morphic bands	Poly-morphic bands				larger	smaller
1	*FAM: EcoRI*-ACA/ *MseI*-CAA	20	5	15	75	0.33	4.95	162	30
2	*HEX: EcoRI*-AGG/ *MseI*-CAA	24	5	19	79.2	0.49	9.3	301	31
3	*CY3: EcoRI*-ATA/ *MsIe*-CAA	23	8	15	65.2	0.39	5.9	371	23
4	*FAM: EcoRI*-ACA/ *MseI*-CTA	53	7	46	86.5	0.49	22	444	103
5	*HEX: EcoRI*-ACA/ *MseI*-CTA	39	9	30	76.9	0.48	14.4	399	101
6	*CY3: EcoRI*-ACA/ *MseI*-CTA	17	3	14	82.4	0.48	5.5	447	132
Total		175	33	142	72.7	0.44	10.34		
Mean		29.2	5.5	23.7					

%P, percent of polymorphism; PIC, polymorphism information content; MI, marker index and bp, base Pair.

Cluster analysis of AFLP data from all tested groups showed dissimilarity coefficient value varied from 0.53 in animals received low dose of tramacet (TC) treatment to 0.74 in animals received high dose of tramadol (TD) treatment (table, 5). The UPGMA dendrogram clearly showed a distribution of all tested groups into two main clusters. Cluster I included TD group, whereas cluster II represented the remaining groups. Cluster II divided into two sub-

clusters, one of them (cluster IIA) comprised animals received high dose of tramacet. However, the second sub-cluster IIB had five groups including control and the two recovery groups (Fig. 3).

Table 5: Jaccard's dissimilarity coefficient calculated from AFLP data among tested groups.

	C	T	TD	TC	TCD	TD-R	TCD-R
C	0	0.540	0.742	0.500	0.633	0.671	0.565
T		0	0.722	0.628	0.599	0.711	0.615
TD			0	0.717	0.731	0.691	0.623
TC				0	0.685	0.669	0.529
TCD					0	0.641	0.624
TD-R						0	0.565
TCD-R							0

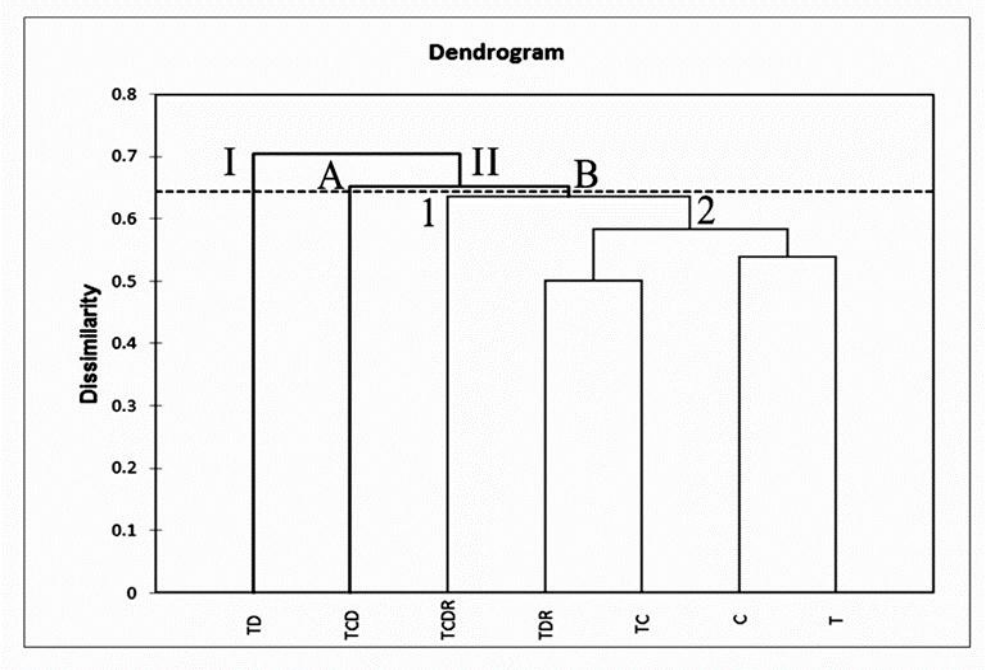

Fig. 3: Dendrogram of relationship among tested groups based on UPGMA cluster analysis of dissimilarity coefficient based on AFLP data. C, control; T, low dose tramadol group; TD, high dose tramadol group; TC, low dose tramacet group; TCD, high dose tramacet group, TDR and TCDR recovery groups.

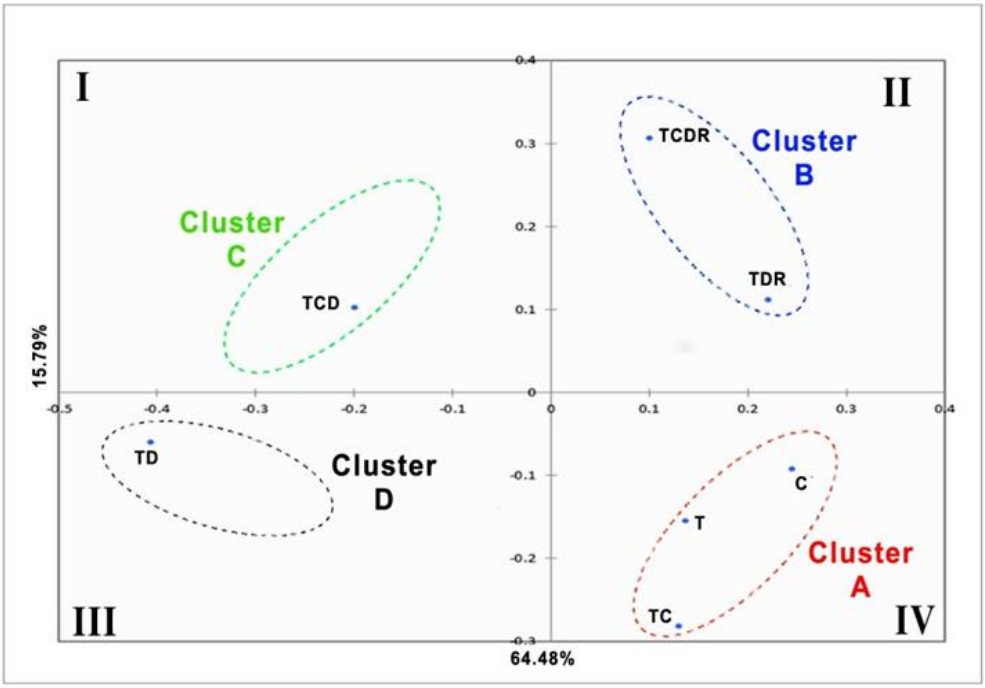

Fig. 4: Principal coordinate analysis of tested groups using Jaccard's coefficient dissimilarity based on AFLP data. C, control; T, low dose tramadol group; TD, high dose tramadol group; TC, low dose tramacet group; TCD, high dose tramacet group, TDR and TCDR recovery group.

4. DISCUSSION

The present study investigated the genotoxic effect and genetic variation level induced after repeated tramadol administration in male mice. These effects were compared with that induced by tramacet administration for the same period. Our data indicated that, opioid (tramadol and tramacet) administration induced high level of genetic variation based on cluster analysis and dissimilarity index of AFLP data. Also, results and observations of this study confirmed significant high frequencies of chromosomal abnormalities and sperm shape changes. Prolonged administration of these opioid may lead to accumulation of its toxic metabolites and increase risk of pharmacokinetic interactions causing toxicity. Therapeutic (low and high) doses of tramadol and/ or tramacet were administrated once daily for 20 days followed by 10 days for drug withdrawal study. The obtained data showed significant increase of chromosome aberrations in dose dependent manner. These results are in line with previous studies regarding opioid toxicity. Chromosome, chromatid breaks and sister chromatid exchange were reported in an *in vitro* study on hamster cells treated with actinomycin.[50] Moreover Darwish 2012[51] reported genotoxic effect of opioid (actinomycin

D); increased micro-nucleated erythrocytes occurrence and induced genotoxicity and alteration in serum protein electrophoresis in mice. Recent study showed an increase in chromosomal aberration and micronuclei induction with reduction in mitotic index in mice after tramadol and Dactinomycin treatment.[24]

About 15% of drugs induced sexual dysfunction.[32] The present study indicated that both tramadol and/or tramacet induced significant high frequencies of sperm shape abnormalities recorded as broken sperm and abnormal head and tail. These effects were irreversible after recovery period; so, may interfere with sexual function causing infertility. These findings agree with some previous studies regarding reproductive dysfunction resulted in opioid treatment. Mckim 2003[52] found that opiates treatment diminish fertility through decreasing levels of sex hormones in both male and female patient. Also, reduction in serum level of luteinizing hormone LH, follicle stimulating hormone FSH, Testosterone after paroxetine treatment.[53] Deleterious effects on reproductive function were deposited in male rat treated with tramadol, including reduction in sex hormones levels (LH, FSH and testosterone) and these effects remained after drug cessation.[29] Another recent study found that, opioids administration suppressed testosterone secretion in chronic pain patient[54] and damaged testicular structure in tramadol treated rat.[55] These results are consisted with present data. Such observations may be explained based on release of free radicals as a result of repeat opioid drug treatment causing accumulation of toxic metabolites and increase risk of pharmacokinetic interaction releasing free radical. These free radicals attack biological membranes rich in polyunsaturated fatty acids causing cell damage and attack DNA forming chromosomal aberration. This explanation supported by observation of several studies that reported significant increase of serum lipid peroxide resulted in opioid administration.[29,56]

Results presented in this study confirmed high frequencies of polymorphism among studied group with average of 72.7%. These, alteration provided an evidence of genetic variation between groups resulted in tramadol and/ or tramacet administration. Moreover, the highest value of dissimilarity index (0.74) was detected in high dose tramadol treated group. Meanwhile, low dose tramadol treated group showed the lest value of dissimilarity index. These polymorphisms may be due to induction of peroxide radical generation resulted in tramadol treatment. These free radicals may induce DNA single strand breaks elucidated by high frequencies of polymorphism.

Our data are like previous study reported that, tramadol and tramacet exerted DNA alteration in dose dependent pattern on rat liver cells supported by DNA fragmentation analysis.[31] Another study demonstrated that, treatment for 20 days with tramadol therapeutic dose significantly increased expression levels of pro-apoptotic Bax gene and caspase- 3 gene but suppressed level of anti-apoptotic Bcl-2 gene expression in brain tissue of rat.[57]

The current data of cluster and PCoA analysis confirmed high level of genetic variation among tested groups. Also, several loci were disappeared due to tramadol and/ or tramacet treatment. Cluster analysis grouped tested groups in two main major cluster, one of them include samples received high doses of both drugs but the second cluster divided into several sub- clusters including the remained groups. These observations confirmed polymorphism and alteration occurred in DNA caused by treatment. These findings are similar with previous study, reported an increase in DNA break level using comet assay and exhibited changes in serum protein electrophoresis resulted in tramadol treatment and after drug withdrawal period for 15 days.[23] Therefore, it may be concluded that the continuous use of tramadol and / or tramacet in chronic treatment exerted toxic effect reflected through high level of genetic variation and polymorphism confirmed in brain tissues. In addition of increased chromosomal aberration frequencies in bone marrow cells and sperms alterations, which reflect sexual dysfunction. All these alterations remained higher than control even after drug cessation.

Table 6: Genetic polymorphism parameters based on AFLP analysis based on tested groups.

primer name	No. of bands	Groups								Primer name	No. of bands	Groups							
		MW (bp)	C	T	TD	TC	TCD	R (T-TD)	R (TC-TCD)			MW (bp)	C	T	TD	TC	TCD	R (T-TD)	R (TC-TCD)
FAM: EcoRI-ACA/ MseI-CAA	1	162	+	+	+	+	+	+	+	HEX: EcoRI-AGG/ MseI-CAA	25	115	+	+	+	+	+	+	+
	2	108	-	+	-	-	-	-	-		26	100	-	+	-	+	-	-	-
	3	95	-	+	-	-	-	-	-		27	91	+	+	-	+	-	+	-
	4	92	-	+	+	+	+	-	+		28	83	-	-	-	+	+	-	-
	5	84	+	-	-	-	-	+	+		29	71	+	+	-	+	-	+	+
	6	55	+	-	-	-	-	-	-		30	55	+	+	+	+	+	+	+
	7	53	+	+	-	+	+	+	+		31	52	-	+	-	+	+	-	+
	8	51	+	+	+	+	+	+	+		32	49	+	+	-	+	-	-	-
	9	44	+	-	-	-	-	+	-		33	47	-	+	+	-	+	+	+
	10	43	+	-	-	-	-	+	+		34	44	+	+	-	+	+	+	-
	11	41	+	+	+	+	+	+	+		35	43	-	-	+	-	+	-	-
	12	40	+	+	-	+	+	+	-		36	42	+	+	+	+	+	+	+
	13	39	-	+	+	-	-	-	+		37	40	+	+	-	+	+	+	+
	14	38	+	+	-	+	+	-	-		38	38	+	+	+	+	+	+	+
	15	37	+	+	-	+	+	-	-		39	36	-	-	+	+	+	+	+
	16	35	+	+	+	+	+	+	+		40	35	+	+	-	+	-	-	-
	17	33	+	+	-	+	+	+	-		41	34	-	+	-	+	-	+	-
	18	32	+	+	+	+	+	+	+		42	33	+	-	-	-	-	-	-
	19	31	-	+	+	+	+	+	+		43	32	+	+	+	+	+	+	+
	20	30	+	-	-	-	-	-	-		44	31	-	-	-	+	+	-	-
	21	301	-	+	+	-	-	-	-		45	371	+	+	-	-	+	+	+
	22	163	-	+	+	-	-	-	-		46	231	+	+	+	+	-	+	-
	23	159	+	-	-	-	-	-	-		47	163	+	+	+	+	-	-	-
	24	132	-	+	+	-	-	-	+		48	159	+	+	+	-	-	+	-

primer name	No. of bands	Groups								primer name	No. of bands	Groups							
		MW (bp)	C	T	TD	TC	TCD	R (T-TD)	R (TC-TCD)			MW (bp)	C	T	TD	TC	TCD	R (T-TD)	R (TC-TCD)
CY3: EcoRI-ATA/ MsIe-CAA	49	115	+	+	+	+	+	+	+	FAM: EcoRI-ACA/ MseI-CTA	74	299	+	-	-	+	-	-	-
	50	94	+	+	+	+	+	+	+		75	296	+	+	-	+	-	+	+
	51	91	+	+	+	+	+	+	+		76	280	+	+	+	+	-	-	-
	52	88	+	+	+	+	+	+	+		77	272	+	-	-	+	-	-	-
	53	82	-	-	+	+	+	+	-		78	271	+	-	-	-	-	-	-
	54	71	+	+	+	+	+	+	+		79	270	+	-	-	-	-	-	-
	55	57	+	+	+	+	+	+	+		80	268	+	+	+	+	+	+	+
	56	55	+	-	+	+	+	+	+		81	251	+	-	-	+	-	-	-
	57	51	+	+	-	-	-	+	-		82	236	-	+	-	-	+	-	-
	58	49	+	+	+	+	+	+	+		83	220	+	-	-	+	+	+	+
	59	44	+	+	+	+	+	+	+		84	219	+	+	+	+	-	-	-
	60	43	+	+	-	-	+	-	-		85	217	+	-	-	+	-	-	-
	61	42	+	+	+	+	+	+	+		86	213	-	-	-	+	+	-	-
	62	38	-	+	+	+	+	-	+		87	190	+	+	-	+	+	+	+
	63	37	+	+	-	-	+	-	-		88	189	+	+	-	+	-	+	+
	64	35	+	+	-	-	+	-	-		89	184	+	+	-	+	-	+	+
	65	33	+	+	-	+	-	-	+		90	183	+	+	-	-	-	+	-
	66	32	+	+	+	-	-	+	+		91	182	+	+	-	+	-	+	+
	67	30	-	+	+	+	+	+	+		92	181	+	+	-	+	-	+	+
	68	444	-	+	-	+	-	-	-		93	180	+	+	-	+	-	-	-
	69	388	+	+	-	+	-	-	-		94	178	+	+	+	+	-	+	+
	70	342	-	+	+	+	+	-	-		95	177	+	-	-	-	-	-	-
	71	323	+	-	-	-	-	-	-		96	175	+	+	+	+	+	+	+
	72	319	+	-	-	-	-	-	-		97	174	+	+	+	+	+	+	+
	73	313	+	-	-	+	-	-	-		98	170	+	-	-	-	-	-	-

primer name	No. of bands	Groups								Primer name	No. of bands	Groups							
		MW (bp)	C	T	TD	TC	TCD	R (T-TD)	R (TC-TCD)			MW (bp)	C	T	TD	TC	TCD	R (T-TD)	R (TC-TCD)
	99	169	+	+	-	+	-	+	+	HEX: EcoRI-ACA/ MseI-CTA	**123**	350	-	-	+	-	-	-	-
	100	164	-	+	+	+	+	-	-		**124**	343	-	+	+	-	-	-	-
	101	163	+	+	+	+	+	+	+		**125**	281	+	+	-	-	-	-	-
	102	153	+	-	-	+	-	+	+		**126**	279	+	+	-	-	-	-	-
	103	149	+	+	-	-	-	-	-		**127**	274	+	-	-	+	-	-	-
	104	143	-	+	+	+	+	+	+		**128**	269	+	-	-	+	-	+	+
	105	137	+	-	-	+	-	+	+		**129**	268	+	+	-	+	-	+	+
	106	134	-	-	+	-	+	-	-		**130**	254	+	-	-	-	-	-	-
	107	133	+	+	-	+	+	+	+		**131**	249	+	-	-	-	-	-	-
	108	132	+	+	+	+	+	+	+		**132**	237	+	-	-	+	-	-	+
	109	129	+	+	-	+	+	+	+		**133**	232	-	+	-	+	+	+	+
	110	127	-	+	+	-	+	-	+		**134**	222	+	+	-	+	-	+	+
	111	120	+	+	-	-	-	+	+		**135**	185	+	+	-	+	-	+	+
	112	118	+	-	-	+	-	+	+		**136**	182	+	-	-	+	-	-	-
	113	117	+	+	+	+	+	+	+		**137**	180	+	+	+	+	+	+	+
	114	111	+	+	+	+	+	+	+		**138**	178	-	-	+	-	+	-	+
	115	110	+	+	-	+	+	+	+		**139**	176	+	+	+	+	+	+	+
	116	108	+	+	-	-	-	+	-		**140**	169	-	-	+	-	-	-	-
	117	107	+	+	-	+	+	+	-		**141**	167	+	+	+	+	+	+	+
	118	105	+	+	-	+	-	+	-		**142**	155	+	+	-	+	+	+	+
	119	104	-	+	+	+	+	-	-		**143**	140	+	-	-	+	-	+	+
	120	103	+	+	-	+	+	+	+		**144**	137	-	+	+	-	+	+	-
	121	399	+	-	-	+	-	-	-		**145**	136	+	+	+	+	-	+	+
	122	372	-	-	-	-	+	-	-		**146**	135	+	+	+	+	+	+	+

primer name	No. of bands	Groups								primer name	No. of bands	Groups							
		MW (bp)	C	T	TD	TC	TCD	R (T-TD)	R (TC-TCD)			MW (bp)	C	T	TD	TC	TCD	R (T-TD)	R (TC-TCD)
	147	134	-	+	+	+	+	-	-		**159**	101	+	+	+	+	+	+	+
	148	133	-	+	-	+	-	-	-	CY3: EcoRI-ACA/ MseI-CTA	**160**	447	-	+	+	-	-	-	-
	149	132	+	+	+	+	+	+	+		**161**	343	-	+	+	+	-	-	-
	150	128	+	+	+	+	+	+	+		**162**	297	+	+	+	+	+	+	+
	151	123	+	+	-	+	-	-	-		**163**	281	+	+	-	+	-	-	-
	152	120	+	+	+	+	-	-	-		**164**	274	+	-	-	+	-	-	-
	153	119	+	+	-	+	-	-	+		**165**	269	+	+	-	+	+	+	+
	154	116	+	+	+	+	+	+	+		**166**	261	-	+	+	-	-	-	-
	155	113	+	+	+	+	-	+	+		**167**	228	+	+	+	+	-	+	+
	156	111	+	+	+	-	-	-	-		**168**	222	+	-	-	+	+	+	+
	157	107	+	+	+	+	+	+	+		**169**	188	-	-	+	-	-	-	+
	158	104	+	+	-	+	-	+	+		**170**	185	+	+	-	+	-	+	+
	159	101	+	+	+	+	+	+	+		**171**	178	-	-	+	-	+	+	-
	147	134	-	+	+	+	+	-	-		**172**	176	+	+	+	+	-	+	+
	148	133	-	+	-	+	-	-	-		**173**	155	+	+	-	+	+	+	+
	149	132	+	+	+	+	+	+	+		**174**	137	-	-	-	-	+	-	-
	150	128	+	+	+	+	+	+	+		**175**	136	+	+	+	+	+	+	+
	151	123	+	+	-	+	-	-	-		**176**	132	+	+	+	+	+	+	+
	152	120	+	+	+	+	-	-	-	**Total Bands present %**			**133 75.6**	**127 72.2**	**81 46**	**125 71**	**87 49.4**	**100 56.8**	**93 52.8**
	153	119	+	+	-	+	-	-	+										
	154	116	+	+	+	+	+	+	+	**Total Bands absent %**			**43 24.4**	**49 27.8**	**95 54**	**51 29**	**89 50.6**	**76 43.2**	**83 47.2**
	155	113	+	+	+	+	-	+	+										
	156	111	+	+	+	-	-	-	-										
	157	107	+	+	+	+	+	+	+										
	158	104	+	+	-	+	-	+	+										

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