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A GOLD STANDARD METHOD FOR EVALUATION OF CYTOGENETIC INVESTIGATIONS

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ABSTRACT

Karyotyping is a conventional Cytogenetic technique for identification of the stained metaphase spread, scanned under microscope and capture its images inorder to investigate the structure and aberrations of chromosomes. An understanding of key terms such as homologous chromosomes, Centromere, p arm and q arm, Metacentric, submetacentric and acrocentric chromosomes, chromosome banding, and Satellite bodies are essential terms required for Chromosomal analysis. Karyotype is the picture of chromosome by arranging of homologous pairs of chromosomes beginning with largest to shortest in a karyotyping work sheet for which (A-G) group of autosomes are first and sex chromosomes are placed last (X,Y). A diagram of karyotype based on chromosome measurement and banding pattern is called Ideogram which is used for the detection of aneuploidy and break point or chromatid breakage (CB). Other structural aberrations like acentric chromosome (AC), chromatid interconnection (CI), dicentric chromosomes (DC), and ring chromosome (RC) have been studied based on morphology. The protocol of Karyotyping includes Preparation of Culture medium, Incubation, Harvesting of Culture, Hypotonic treatment, Treatment with Fixative, Preparation of Slides, Staining of slides-GTG Banding, Microscopy and Automation of Karyotyping and Analysis and Recording of Data.

KEYWORDS: Cytogenetics, Chromosomes, Karyotyping, Aberrations.

INTRODUCTION

Cytogenetics or Chromosome analysis is the study of structure, function, and evolution of chromosomes which involves testing of samples of tissue, blood or bone marrow in laboratory. Flemming first drawn the illustrations of human chromosomes in 1882.[1] Waldeyer introduced the term Chromosome, Greek meaning stained body.[2&3] Von Winiwarter observed that male and female had different number of chromosomes n=47 and n=48 respectively.[4] Painter studied of meiotic chromosomes in several testis biopsies taken from incarcerated, castrated males who had been sentenced to death.[5] In 1950 two major breakthroughs led to rapid advances in human cytogenetics are the use of **Phytohemagglutinin** (PHA), a substance from plants, to stimulate cell division of lymphocytes so that the number of cells in metaphase can be obtained and Hsu et al described the so called hypotonic shock in which he found out that cells that had been kept for washing of cells fixation for several minutes in a **Hypotonic salt** solution to cause cells to absorb water and swell and gave a much better spreading of the chromosomes whilst dropping.[6] Another important finding was the addition of **Colchicine** to cell cultures, which destroys the mitotic spindle and captures the cells in their metaphase stage for which the cells are not able to finish mitosis and an increasing number of metaphases become available for microscopic studies. In the year 1955 Tjio and Levan performed several experiments with cultured embryonic lung cells and they made beautiful chromosome slides which unequivocally proved that the number of chromosomes in man being 46. Later at the same year, their observations had confirmed in studies of human spermatocytes by Ford and Hamerton which paved the way for the beginning of a new era of Clinical Cytogenetics.[7&8] The chromosomes could be arranged in eight different groups (A–G) and the sex chromosomes based on their size and location of the primary constriction or centromere which enabled an easy counting of the chromosomes. In 1966, Steele and Breg reported that cells cultured from amniotic fluid could be used to determine the chromosome content of the fetus which has been using for Prenatal chromosome studies.[9] Caspersson et al. was the first to introduce a banding pattern in every single chromosome which had been based on a fluorescent staining technique with quinacrine mustard (QM) which could clearly visible in fluorescent microscopy.[10] In routine clinical setting, the G-banding technique being based on the application of Trypsin followed by Giemsa staining which is globally accepted method and it enabled gene mapping and detection of various structural aberrations like translocations, inversions, deletions, and duplications.[11] The pattern is unique to each chromosome, and therefore serves as a landmark for chromosome identification. The G-light bands are thought to be relatively GC-rich (rich in the DNA bases guanine and cytosine), and the G-dark bands relatively AT-rich (rich in the DNA bases adenine and thymine). Furthermore, the light bands represent the regions which are

relatively open and which contain most of the genes, including housekeeping genes (genes active in every cell type). On the other hand, the G-dark bands represent regions which are relatively compact and contain few genes. The genes in the dark regions are mainly tissue-specific.

Several numerical chromosome aberrations had been reported like Trisomy-21 in Down syndrome,[12] 45, X in Turner syndrome,[13] 47, XXY in Klinefelter syndrome.[14] Trisomy-13,[15] and Trisomy-18.[16] Infact the first acquired chromosome abnormality Philadelphia chromosome was recognized in bone marrow cells of a patient suffering from chronic myeloid leukemia.[17] Neverthless it took 13 years before it became apparent that this was not been a simple deletion but the result of a reciprocal translocation between the chromosomes 9 and 22,[18] and later discovered that the translocation resulted in a new fusion gene directly responsible for the leukemic development of the cells.[19] Micro-deletion of p arm of chromosome 15 of Paternal and Maternal results Prader Willi and Angelman syndrome respectively.[20] Smith-Magenis and Miller-Dieker syndrome with deletions in the short arm of chromosome 17, and DiGeorge /Velo Cardio Facial (VCF) syndrome with deletions in the long arm of chromosome.[21] Manoj et al documented the mean frequency of chromosomal aberration being 3.40+_0.28 in Perinatal Asphyxia.[22]

MATERIALS AND METHODS

The materials required for Karyotyping is been depicted in Table.No.1 and the Protocol for the preparation of stock solution and working solution of Colchicine, Hypotonic buffer, Fixatives, Sorensen's buffer, Giemsa stain, PBS, Proteolytic enzymes, Lymphocyte culture media are been available in Table No.2. In Cytogenetic investigation, Metaphase screening and scanning of chromosomes for chromosomal aberrations have done by using conventional short term lymphocyte cell culture based on recommendation of rules and guidelines of Rooney et al.[21]

Table 1: Showing Materials Required for Karyotyping.

Sl. No	Materials	Specification	Company/ Firm
1.	Colchicine	Approx. 95%(HPLC)	SIGMA-Aldrich
2.	RPMI-1640	With glutamine without NaCo3	HIMEDIA
3.	Fetal Bovine Serum-10%	RM 111-500 ml	HIMEDIA
4.	Phytohaemagglutinin (PHA)	1%	SIGMA
5.	Pencillin- Streptomycin	5000 IU	SIGMA
6.	Gentamicin	Lot 124K2325	SIGMA
7.	Potassium Chloride AR	M.W 74.55	
8.	Methanol	Prod. No.32407	Fisher scientific
9.	Glacial Acetic acid	99-100%	MERC
10	Centrifuge	Fixed Angle Rotator, Small bench	REMI
11.	Incubator	12A 230 H VAC, 50/60 HZ	HICOOL
12	Laminar Hood	Mod. No CAV 900	CLEANAIR
13.	Hot Air Oven	Auto clave	TECHNICO
14	Voltex Mixer	230V/50 HZ	KEMI
15	Slide Warmer	230V/50 HZ	KEMI
16.	Deep freezer		VEST FROST
17.	Epiflurescent microscope-CCD Camera	BX-51	OLYMPUS
18	IKROS Karyotyping software	Digital imaging	METASYSTEM
19.	Disod. Orthophosphate	M.W-358.14	LOBA
20.	Pot. hydrogen phosphate	M.W-136.09	LOBA
21	Analytical Balance	Digital 0.1 mg-200 gm	Shimadzu
22	Sodium Chloride	DNA grade M.W 58.45	MERC
23.	Culture Vials	5ml, 10ml	
24	Coplin jar	50 ml	
25	Ethidium bromide GR	Art 3715 m.w-394.32	PAXMY
26.	Trypsin 1:250	Tissue culture grade	HIMEDIA
27	Sterile hypodermic syringe	2ml	PRICON
28	Needle	24G, 0.55x38mm	Oyster
26	Glass Beaker	500ml and 200ml	
27	Microscope Plain Glass Slide	75x25mm	
28	Conical Flask	100,500 ml	
29	Glass Marking Pencil		Faber Castil
30	Slide Trey		
31	Slide Storage Box		
32	Culture vials		
33	Centrifuge Tube	15ml	
34	Pasteur Pipette	5.75 in	
35	Micropipette	10-100µl	
36	Transparent Staining Trough		
37	Squeeze Bottle		
38	Graduated cylinders	100 ml	
39	Heparinised PB	2ml	

Table 2: Depicting Preparation stock and working solution for Karyotyping.

Sl. No.	Reagent	Stock solution			Double distilled water	Working Solution
		Name of Chemical	Requirement	Mol.Wt		
1.	Mitotic inhibitor	Colchicine	10 mg		10 ml -	1ml stock+9 ml D .H2O
2.	Hypotonic solution	(0.075M) KCl	0.559 gm	74.55	100 ml	10-12 ml
3.	Fixatives	Methnol & Acetic acid	3:1	32.04 60.05	–	5-10 ml
4.	Sorensen's buffer	Na_2HPO_4 M KH_2PO_4	4.1740 4.1620	358.14 136.9	490 510	48 ml
5.	Giemsa Stain	Geimsa Glycerine	3.8 gm 250 ml	291.80 92.09	-	2 ml Giemsa stain
6.	Phosphate saline buffer (PBS) (pH-7.4)	NaCl KCl 0.2 M Na2HPO4 0.2 M KH2PO4	8 gm 0.2 gm 1.5gm 0.2 gm	58.45 74.55 358.14 136.09	1 litre	
7.	Proteolytic enzyme	Trypsin	32mg		12.5ml	1ml Trypsin+ 48 ml PBS
8.	Lymphocyte culture media	RPMI-1640 RPMI* PHA FBS Streptomycin & Pencillin Gentamycin	100 gm (80ml) + 3ml 20ml 0.1 ml 0.1 ml		100ml (autoclaved)	*10 ml

The methodology of Cytogenetic screening includes Preparation of Culture medium, Incubation, Harvesting of Culture, Hypotonic treatment, Treatment with Fixative, Preparation of metaphase spreading Slides, Staining of slides -GTG Banding, Microscopy and Automation of Karyotyping and Analysis and Recording of Data.

Preparation of culture media

Culture media was prepared under aseptic conditions by following conventional standard as described by Rooney et al.[21] 20ml stock solutions of RPMI 1640 with folic acid have taken separately in sterilised beakers and 180 ml of double distilled water was added and made up to 200ml. Of which only 160 ml was taken and mixed with 40ml of fetal bovine serum, 05 ml of Phytohemagglutinin.0.2ml of Penicillin-Streptomycin and 0.2 ml of Gentamycin added to the above and mixed thoroughly (Table:2) (5-Azacytidine can be used for detection Fragile sites). Culture media thus made is to be aliquoted into 5ml each in screw capped culture bottles. The bottles have then transferred into -20^0c refrigerator until further use.

Incubation

Required culture vials stored in the deep freezer have taken out and were allowed to go for thawing under UV light exposure in laminar flow cabinet. Once thawing was done 0.1 ml of heparinized whole blood has to be inoculated in the culture media and gently mixed to promote a uniform mixing of blood cells. Then bottles have been screw capped with flat bottom after exposing towards the spirit lamp flame to avoid contamination. Subsequently the vials were given code numbers and kept for incubation at 37ºC for 67-69 hours with an uninterrupted power supply (Fig.1:3&4).

Harvesting of culture

Between 67^{th} to 69^{th} hour of incubation, culture bottles have taken out from the incubator and placed in laminar flow cabinet. About 100 µl of working solution of colchicine and 50 µl of stock solution of Ethidium bromide has to be added to the culture bottles under aseptic conditions (Fig.1:5). Then bottles have been transferred back to incubator and re-incubated for a period of one hour. At the end of one hour the contents of the bottle have to be transferred into 15ml centrifuge tubes and centrifuged at 1000 rpm for 10 min (Fig.1:6). After centrifugation, supernatant have carefully aspirated using suction apparatus without disturbing the cell sediments in the bottom of the tubes (Fig.1:7).

Treatment with Hypotonic solution

Placing the centrifuge tube over a cyclomixer, 10ml of pre-warmed (37 °C) KCl hypotonic solution has to be added to the cell sediments, dispersed uniformly and transferred once again to incubator and incubated for 30 mins (Fig.1:8&9). Following KCl treatment, test tubes have been taken out and centrifuged at 700-800 rpm for 10 min and the supernatant has to be discarded once again using suction apparatus.

Treatment with Fixative

Then, the cells have to be re-suspended using freshly prepared fixative and kept undisturbed overnight for effective fixation in refrigerator (Fig.1:10). On the following day, test tubes have been taken out from the refrigerator and brought to room temperature for re-centrifugation at 700-800 rpm for 10 mins and for a further wash with fixative. The same procedure has to be repeated thrice or until clear visible cell pellet collection / formation at the bottom of the test tube (Fig.1:11).

Preparation of slides (Splash Technique)

Cell pellets obtained after fixation alone has to be aspirated using the pasteur pipette from the bottom of test tubes and dropped onto a clean cold slide from a height of about two feet (Fig.1:12). Two to four slides have to be made for each case and the details of the case in the form of number code has to be marked at the edge of the slide using a diamond pencil for the purpose of identity. The slides have to be warmed on a slide warmer at 45-55ºC for 1-2 minutes or kept at 37º for 72 hours and later collected and stored in slide rack and subjected to staining after 2-3 days.

Staining by Giemsa: (Giemsa Trypsin G Banding)

1. Trypsin (working solution), phosphate buffer saline, Giemsa (working solution) (Table: 2) and tap water were taken in clean, grease free coupling jars each and were arranged in series.
2. Then slides to be stained were immersed in Trypsin solution for 15 seconds, followed by a rinsed in the phosphate buffer saline (PBS).
3. Later the slides has to be placed in Giemsa stain for 3-5 minutes followed by a wash in tap water (Fig.1:13).
4. Later the slides have to be kept over a slide warmer for drying or air dried.

Microscopy and Automation of Karyotyping

- All Slides have to be screened with bright field microscope Olympus BX 51, Japan using low power objective (10 X) until good metaphase spreads has to be found (Fig.2A&B).
- Then over the area of good metaphase spread a drop of immersion oil has to be added and the spread is to be analysed under high magnification objective (100X) (Fig.2A&B).
- The image observed has to be captured using – Ikaros-Metasystems, Germany and later analysed for chromosomal aberrations (Fig.1:15&16) (Fig.4A&B).
- Chromosomes have to be arranged based on their banding pattern according to Denver classification of chromosomes (Fig.1:15) and (Fig.3).
- Chromosomal aberrations have to be identified and named using ***International System for Human Cytogenetics Nomenclature (ISCN)***[21] (Fig.4B**).**
- All the chromosomal abnormalities have to be analysed and determined in accordance with the International system for human cytogenetics Nomenclature (ISCN).[21]
- Analyse whether structural abberation like chromatic breakage (CB), a centric chromosome (AC), Chromatid interconnection (CI), Dicentric chromosome (DC), and Ring chromosome have observed or not (Fig.5).
- Ideogram can be used for detection of break point in chromosome. However other aberrations like interchromatid connection, Dicentric chromosome, Ring and Acentric chromosomes can be identified based on their morphology.
- The scoring criteria of chromosomal aberration has been based on the standard protocol ISCN.[21]

Analysis and Recording of Data

Data have been recorded on a score sheet. All types of aberrations have to be included in the data sheet. From each sample the structural aberrations have to be counted and the percentage of aberration and mean ± SD has to be calculated.

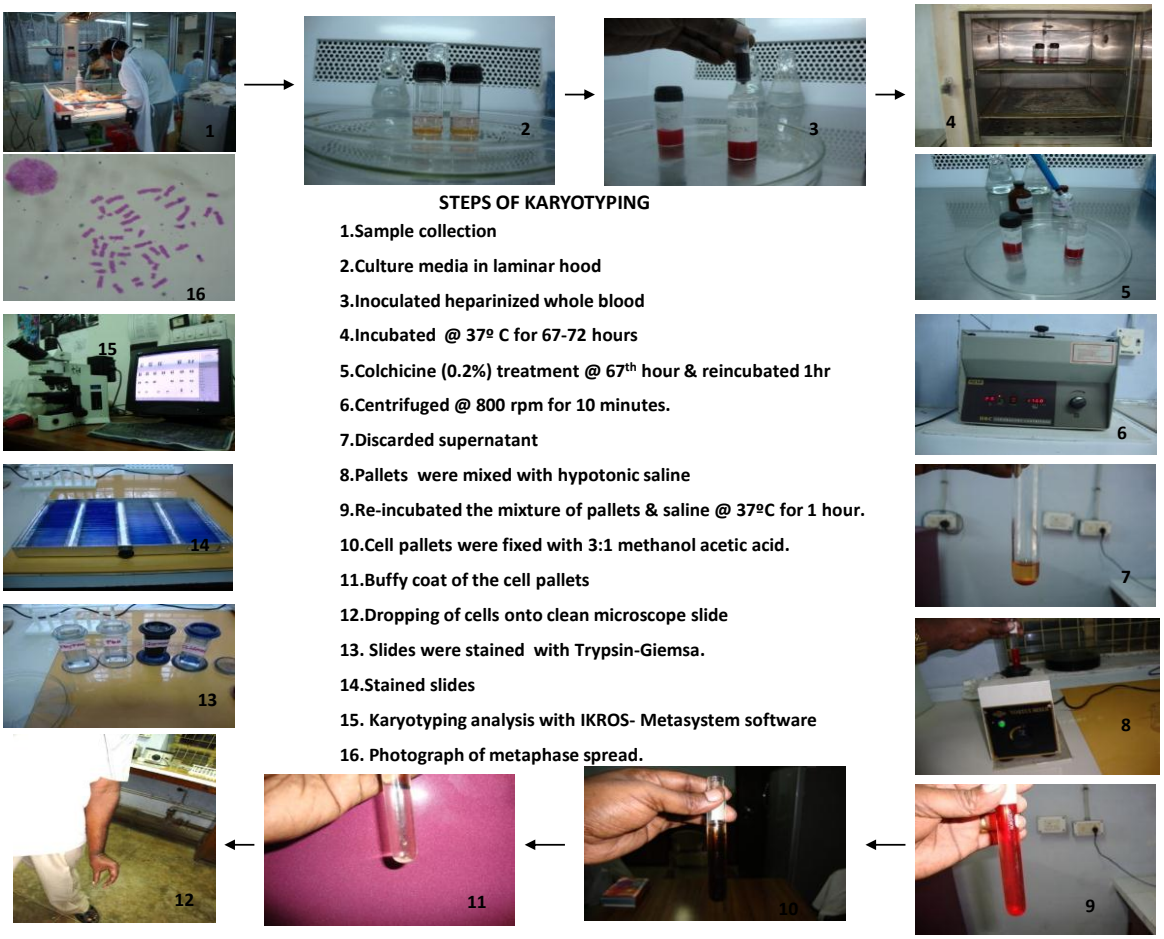

Figure 1: Delineating Techniques of Karyotyping in clockwise direction in which each capture with No.1 to 16.

OBSERVATIONS

Karyotype refers to a display of the chromosomes of a cell by lining them up, beginning with the largest and with the short arm oriented toward the top of the karyotyping sheet (Fig.3). According to Denver classification the chromosomes has to be arranged in seven (A–G) groups of autosomes and one group of Sex chromosomes (X,Y) which is placed in last or eighth group. The formula for a normal male is 46XY (Fig.4B) and for a normal female 46XX (Fig.3) and the diagram of the karyotype based on chromosome measurements and its bands called an Idiogram.[21] (Fig.4B).

The morphology of a typical metaphase chromosome consists of two arms separated by a primary constriction or centromere. A chromosome may be characterized by its total length and the position of its centromere. A chromosome with the centromere at or near the middle is called Metacentric. A Submetacentric chromosome has a centromere somewhat displaced from the middle point. Acrocentric chromosomes have their centromeres very near one end. The short chromosome arm is designated petite (p) and the long arm q which is one letter after p. Chromosomes may also contain a secondary constriction, which appears as an unstained gap or satellite stalk near the tip. The chromosomal segment distal to this gap appears as a satellite. Chromosome numbers 13–15 (D group) and 21–22 (G group) have satellites.[21] (Table:3). Based on the length and size the morphology of chromosomes are designated as large or small Metacentric, large or medium sized Submetacentric, medium and small Acrocentric chromosomes (Table: 3).

Table 3: Depicting the Classification of Chromosome and its Morphology.

Group	Chromosome Number	Total No. Of Pairs	Morphology Of Chrmosomes
A	1-3	3	Largest Metacentric
B	4-5	2	Largest Sub-metacentric
C	6-12	7	Medium-sized Sub-metacentric
D	13-15	3	Medium-sized acrocentric
E	16-18	3	Small-sized sub-metacentric and metacentric(16)
F	19-20	2	Small metacentric
G	21-22	2	Small-sized acrocentrics
Sex chromosomes	23	2	X-chromosome (sex- chromosomes) Similar to C group chromosomes Y chromosome (Similar to G group chromosomes)

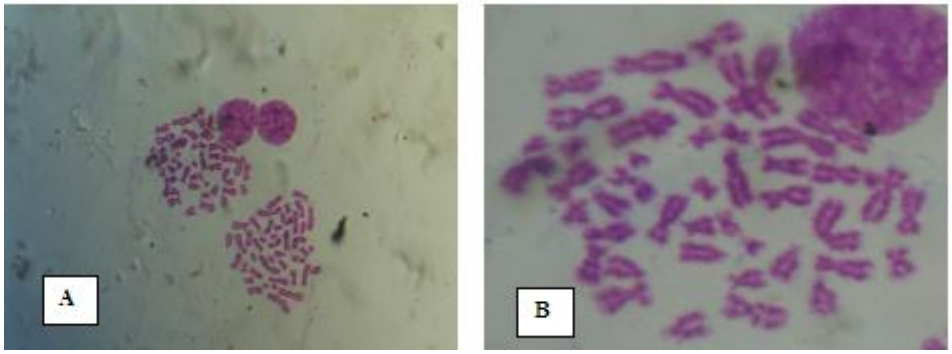

Figure 2: Delineating the Geisma-Trypsin stained Photographic Captures of Metaphase spread A.10X and B.100X.

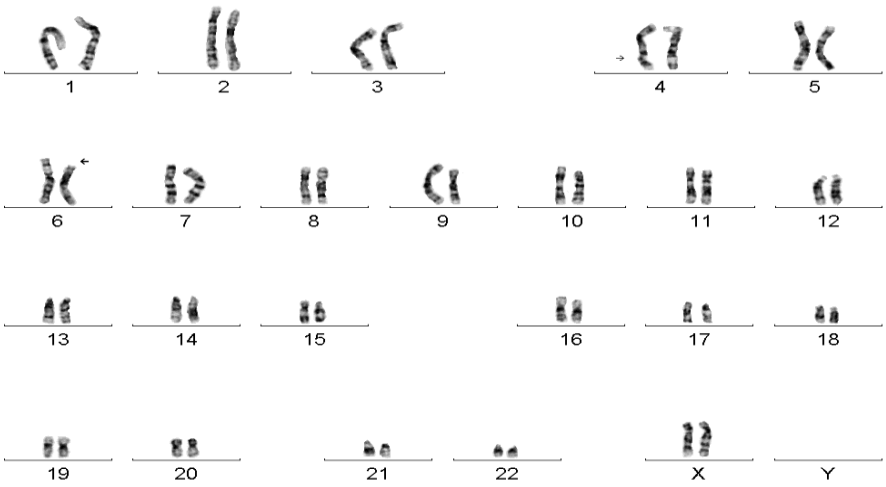

Figure 3: Images of Geisma-Trypsin stained Karyotype of Female by using –software, Carl Zeiss, Ikaros-Metasystems, Germany.

The chromosomal aberrations are numerical and structural aberrations. Numerical aberrations refers to gain/ extra or loss/ missing of chromosome called aneuploidy which have to be designated with a + or − sign, respectively, before the number of chromosome. Thus, a male with trisomy (three chromosomes) for chromosome 18 is 47XY+18, and a female with a monosomy (one chromosome) for 22 is 45 XX−22. Addition or deletion of a chromosome segment is denoted with a plus + or minus − sign after the symbol of the chromosome arm, respectively. For example, the formula for a female with the cri du chat syndrome is 46,XX,5p− (missing a piece of the short arm of chromosome5). The formula for a male with a translocation (exchange of chromosome segments) between chromosome 14 and 21 is 46,XY,t (14;21).[21]

Structural aberrations are those that involve a change in the chromosome structure. These include, chromatid breakage (CB), acentric chromosomes (AC), chromatid interconnection (CI) or Sister chromatid Exchange (SCE), dicentric chromosomes (DC), and ring chromosome (RC).[21,22&23] (Fig.5). Translocations, Insertion, Inversion and Isochromosomes are other types of structural aberrations.[21] The structural aberrations of chromosomes are detected by using Morphology of chromosomes and Idiogram which has to be recorded on data sheet for autosomes and sex chromosomes (Fig.4B).

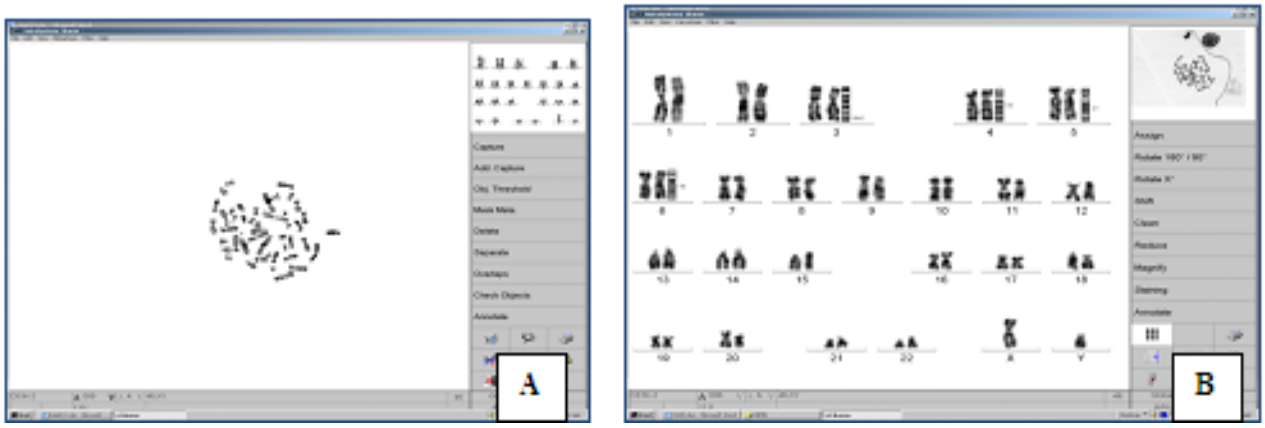

Figure 4: Showing Metaphase spread and Karyotype matched with Ideogram showing

Breaks in 4q of 3,4,5 and 6 chromosomes captures of male by using IKaros Metasystems software, Carl Zeiss, Germany.

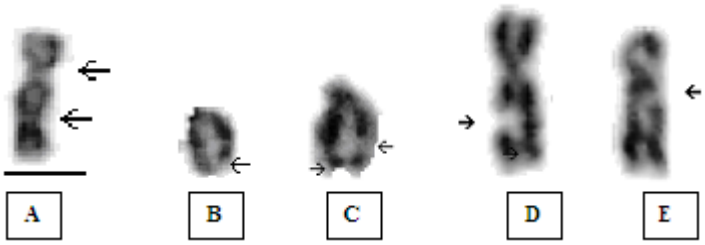

Fig. 5: Illustrating Images of Chromosomal Aberrations, Dicentric chromosome (A), Acentric Fragments (B), Ring Chromolsome (C) Chromatid breakage (D) Chromatid interconnection /Sister chromatid exchange (E).

Chromosomal analysis is a powerful and useful technique in human genetics which have been revealing the structural features of each chromosome, helps for studying banding pattern for detecting small changes by Ideogram and aids in studying evolutionary changes.

CONCLUSION

Cytogenetic screening is a Gold standard method to Identify and detect Genomic Instability such as Numerical and Structural chromosomes disorders and diagnosis of Prenatal genetic defects.

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