To study the genetic etiology in Autism Spectrum Disorder (ASD).

A dissertation thesis submitted to

Nirma University

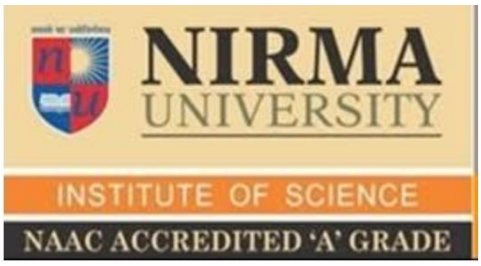
In partial fulfilment of the requirements for

The degree of

Master of Science In

Biotechnology

May 2019



Institute of Science, Nirma University

Ahmedabad 382481

Gujarat, India.

Submitted by

Shreya Shah (17MBT035)

Vidhi Sambhvani (17MBT041)

Under the Guidance of

Dr. Sonal R. Bakshi

Acknowledgement

We would like to thank each and every person who is associated with our project. We would like to thank almighty for giving us an opportunity to work with the most reputed and acknowledged faculties.

It is a genuine pleasure to express my deep sense of thanks and gratitude to my mentor, my guide and philosopher **Dr. Sonal R. Bakshi** for suggesting the project and helping to commence it. Her dedication, keen interest and above all her overwhelming attitude to help her students had been mainly responsible for completion of our work. Her timely advice, creative skills and scientific approach have helped us to a wide extent to accomplish our work.

We express our sincere gratitude to **Prof. Sarat Dalai**, Director, Institute of Science, Nirma University for giving us the invaluable opportunity of carrying out our dissertation project at Institute of science, Nirma University. We would also like to thank our teachers **Dr. Vijay Kothari, Dr. Shriram Seshadari, Dr. Shalini Rajkumar, Dr. Nasreen Munshi, Dr. Amee Nair, Dr. Kuldeep Verma** and **Dr. Dablulal Gupta** for guiding us and providing technical suggestions in every possible way.

We would also like to thank our mentors JRF **Abhipsha Debnath** and PhD student **Dr. Naziya Saiyed** for timely planning of our work. Their prompt suggestions and everlasting support has helped us a lot in our project work. We also thank our lab technicians **Mr. Sachin Prajapati** and **Shweta Patel** for providing us the technical and analytical assistance for our work.

We are extremely thankful to **Manisha Patel, Afsa Ansari** and **Mr.Vaibhav Mehta** who were our phlebotomist. We would also like to thank our dissertation group members **Bansi, Aanal, Pankaja, Radhika, Pratichi, Harsh, Vishal, Sunil** and **Sonam** for always being an helping hand and encouraging us throughout the dissertation period. They have always maintained the healthy and friendly environment while working.

We would also like to sincerely thank **Dr. Sarita Agarwal**, S.G.P.G.I, Lucknow for her collaborative work. We would also like to thank **Dr. Meenakshi Agrawal**, Tanay Foundation, Ahmedabad for her assistance in providing us the blood samples of ASD patients. Without which it would have been impossible to complete this project.

Lastly we would like to thank our parents and grand-parents for being the main pillars of our life and our friends for giving us emotional support and helping in every way they could. Their constant support and belief in us have been the foremost reason for accomplishment of our project. We are blessed to have friends like **Manisha, Dhruvi, Bansi, Yesha, Shikha, Heta, Krushali** and **Leen** who never lost hope in us and stayed with us in thick and thin.

Dr. Sonal R. Bakshi

(Dissertation Guide, ISNU)

CONTENTS

Sr No.	Title	Page No
1.	Abstract	6
2.	Introduction	8
3.	Review of Literature	15
4.	Materials	19
5.	Methods	25
6.	Results	30
7.	Discussion	38
8.	Conclusion	42
9.	References	43
10.	Annexure of SOP	47

ABSTRACT

ASD (Autism Spectrum Disorder) is a multifactorial disorder in which the genetic causes are unknown (Zeidan-Chulia F *et al*). The study of constitutional genetics in ASD patients is important for clinical assessments of ASD, for better treatment and early prognosis of the disorder. 14 clinically approved ASD patients were enrolled in the study and their karyotyping was performed. The peripheral blood lymphocytes were cultured and GTG banding was done. GTG banding is the foremost step to rule out chromosomal aberrations. The banding pattern of chromosomes is unique to every chromosomes hence any aberration in chromosome can be visible. The images were captured using ocular imaging software version 2.0. The DNA was also isolated from the ASD patients's blood samples and they were run on the agarose gel electrophoresis. The DNA was quantified by taking absorbance ratio A260/280. This absorbance ratio should be approx. 1.8 - 2, value beyond this could be as a result of RNA contamination and below 1.8 may be due to presence of phenol compounds.

In this study all the patients had normal karyotype and there were no chromosomal aberrations. Hence there is no abnormalities at cytogenetic level. Studies at molecular level could be performed to check the abnormalities at DNA level.

INTRODUCTION

Autism Spectrum Disorder

Autism Spectrum Disorder (ASD) is a neurological disorder. It is also called developmental disorder, which occur in the beginning of the childhood and persists throughout the person's life. It is called developmental disorder because its symptoms generally appear in first two years of life. Autism can be evaluated at any age as it affects both the communication and behaviour of a person. People diagnosed with ASD have wide range of symptoms and hence it is known as "spectrum" disorder. Autism is characterized by impairment in social interactions and communication and repetitive behaviour (Faras *et al*). According to DSM-V (Diagnostic and Statistic manual) developed by American psychiatric society ASD is classified into following 5 categories:

1. Asperger disorder
2. Rett's syndrome
3. Pervasive developmental disorder-not otherwise specified (PDD-NOS)
4. Childhood Disintegrative Disorder
5. Autistic disorder

2nd April is celebrated as world Autism Awareness Day in order to spread awareness about autism in public. The ribbon puzzle was adopted in 1999 as the universal sign of autism awareness. This reflects the complexity of the disorder and different shapes and colours represents the diversity of the people and families living with the conditions.

Autism occurs more often in male than in females with a 4:1 male to female ratios. The reported prevalence rates of ASD have been increasing over the past few decades. The reasons for this includes awareness amongst the people, broadening of diagnostic concepts, improved detection etc. The exact cause of autism and other ASD is still not known. Twin studies have shown that there is higher concordance rate in monozygotic twins than in dizygotic twins. Various environmental factors have been associated as possible agents to cause autism such as prenatal infections, pollution, insufficient folic acid during pregnancy etc. Whereas various mutations can also lead to autism.

Asperger Disorder

Asperger disorder shares similar features of social impairment and repetitive behaviour with autistic disorder but it usually is apparent at the age of pre-school while autistic disorder is evident by the age of 3 years. Unlike autistic disorder, most Asperger disorder patients have normal intellectual functioning and have various social activities(Khouzam *et al*).

Rett's syndrome

Rett's syndrome is includes intellectual disability in females. It is caused by mutations in MECP2 gene which plays a role in regulating the neuronal activity. Rett's disorder is a neuro developmental disorder which is characterized by the loss of intellectual functioning, deceleration of head growth and development of stereotypic hand movements. The diagnosis of Rett's disorder is based on proper diagnostic criteria which includes prenatal and perinatal tests during the age of first 5-6 months(Weaving L *et a*l).

Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS)

PDD-NOS is the fifth edition in DSM-V manual which is used to describe the people who do not fit into any of the ASD spectrum. It is also known as atypical autism which differs from childhood autism either in the age of onset or failing to fulfil other ASD characteristics.

Childhood Disintegrative Disorder (CDD)

Childhood Disintegrative Disorder is a rare condition that is characterized by late onset of developmental delays in language, social impairment and motor skills. The cause of Childhood Disintegrative Disorder is still unknown however children with CDD have abnormal electroencephalogram which measures the electrical conductivity in the brain. Children with 2 years of age have normal development but they gradually start to lose the skills after the age of 3 years(Charan *et al*).

Autistic Disorder

Autism is said to be complex neuro-behavioural multifactorial disorder. According to DSM-V autistic people exhibit qualitative impairment in social interaction, facial expressions, body postures, delay in speech etc. The exact cause of autism is still not know but it is suggested that there might be role of environmental factors that interacts with genetic make up of the body.

Symptoms of ASD

- Avoid eye contact
- Delayed speech and other linguistic skills
- Repeat particular words or phrases over and over again (echolalia)
- Give unrelated answers to questions
- Get upset by minor changes
- Have obsessive interests
- Flap their hands, rock their body, or spin in circles
- Have unusual reactions to the way things sound, smell, taste, look, or feel
- The child is hyperactive, impulsive and aggressive
- Have Unusual mood or emotional reactions

Social Skills

- Does not share interests with others
- Only interacts to achieve a desired goal
- Has flat or inappropriate facial expressions
- Does not understand personal space boundaries

- Avoids or resists physical contact
- Is not comforted by others during distress
- Has trouble understanding other people's feelings or talking about own feelings

Communication

- Delayed speech and language skills
- Gives unrelated answers to questions
- Uses few or no gestures

Unusual Interests and Behaviors

- Plays with the same toys every time
- Gets upset by minor changes
- Has obsessive interests
- Has to follow certain routines
- Flaps hands, rocks body, or spins self in circles

Diagnostic tools for ASD:

Various diagnostic tools are used to assess autism such as:

- CARS (Childhood Autism Rating Scale)
- GDT (Gessel's Drawing Test)
- DST (Developmental Screening Test)
- VSMS (Vineland Social Maturity Scale)
- BKT (Binet Kamat Test)
- OT (Occupational Therapy indicators for identification of symptoms and its severity)

SHANK2 (SH3 and Multiple Ankyrin Repeat Domains 2)

SHANK2 is located on 11q13.3-q13.4. This gene encodes for the protein that is member of the Shank family of synaptic proteins. These proteins may function as molecular scaffolds in the postsynaptic density of excitatory synapses. Shank protein contain multiple domains for protein-protein interaction including ankyrin repeats and SH3 domains. This family of proteins contain a PDZ domain, a consensus sequence for SH3 domain and a sterile alpha motif. Alteration in this protein may associated with the susceptibility to ASD (Berkel *et al*).

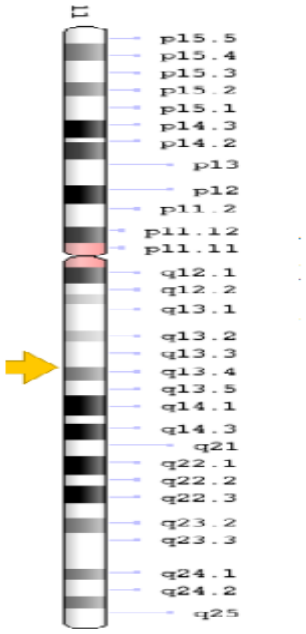


Fig (1): SHANK2 is located on q arm of chromosome 11

Cytogenetic Location: 11q13.3-q13.4, which is the long (q) arm of chromosome 11 between positions 13.3 and 13.4

Molecular Location: base pairs 70,467,856 to 71,252,724 on chromosome 11

Giemsa banding

The introduction of Giemsa banding (G-bands) by Sumner et al in 1971 was one of the major advancements in the field of cytogenetics. It eliminated the use of expensive fluorescence microscope and provided permanently stained slides with banded chromosomes. Sumner observed that reagents that attacked protein disulfide or sulfhydryl groups had a noticeable effect on the banding. He suggested that proteins in dark G-bands were relatively rich in disulfides whereas those weekly stained bands were rich in sulfhydryls. But later with the help of fluorochromes it was proved that there is no relationship between banding patterns and disulphide (-SS-) or sulfhydryl groups (-SH-) along the chromosomes According to Sumner, the initial stages involved the ionic binding of methylene blue molecules in the areas of DNA where the phosphate groups are in the correct position to bind two thiazin molecules. The next thiazin binds to the eosin to form the magenta compound which precipitates in the hydrophobic regions that results in the banding pattern of chromosomes. Duijin and colleagues discussed the involvement of nucleosomes in G banding of chromosomes. They concluded that the explanation of Sumner was not sufficient to explain the magenta color of the chromosomes. Their study provided a detailed model which concerns the effects of fixation, pre-treatment, collapse, swelling and staining of the chromosomes that ultimately results in the banding patterns of the chromosomes.

REVIEW OF LITERATURE

Research papers and review articles:

Sr.No	Author	Source	Title	Information
1.	Hyejung won and Won Mah	Front Mol Neurosci. 2013; 6: 19.	Autism spectrum disorders Causes, mechanisms and treatments: focus on neuronal synapses	Autism Spectrum Disorder (ASD) is a neurological developmental disorder Autism occurs more often in male than in females with a 4:1 male to female ratios.
2.	Khouza m HR	Comprehensiv e psychiatry 2004 May-June;45(3):184-91.	Asperger's Disorder: A review of its diagnosis and treatment	Asperger disorder shares similar features of social impairment and repetitive behaviour with autistic disorder
3.	Weaving , L. S	Journal of medical genetics 42.1 (2005): 1-7.	Rett syndrome: clinical review and genetic update.	Rett's disorder is a type of disorder that contributes in intellectual disability in females. It is caused by

				mutations in MECP2 gene which plays a role in regulating the neuronal activity.
4.	Charan, Sri Hari	Journal of pediatric neurosciences 7.1 (2012): 55	Childhood disintegrative disorder	Childhood Disintegrative Disorder is a rare condition that is characterized by late onset of developmental delays in language, social impairment and motor skills.
5.	Fakhoury	International Journal of Developmental Neuroscience 43 (2015): 70-77.	Autistic spectrum disorders: A review of clinical features, theories and diagnosis	The exact cause of autism is still not know but it is suggested that there might be role of environmental factors that interacts with genetic makeup of the body.
6.	Berkel	Nature Genet. 42: 489-491, 2010.	Mutations in the SHANK2 synaptic scaffolding gene in autism spectrum	SHANK2 gene function

			disorder and mental retardation.	
7.	Marshall	Molec. Cell. Biol. 18: 5838-5851, 1998.	Identification of a novel cortactin SH3 domain-binding protein and its localization to growth cones of cultured neurons.	SHANK2 structure.

Websites

Sr. No	URL	Last accessed	Information
1.	https://www.nimh.nih.gov/health/topics/autism-spectrum-disorders-asd/index.shtml	March 2019	Diagnosis/Assessment/Evaluation of ASD.
2.	https://www.genecards.org/	April 2019	SHANK 2 gene structure, location and function
3.	https://medlineplus.gov/autismspectrumdisorder.html	April 2019	Shows the brief idea of Autism Spectrum Disorder

MATERIAL

Table 1: List of Reagents

Reagents	Company
Acetic acid	Merck
Agarose	HiMedia
Bromophenol Blue	HiMedia
Chromic acid	Merck
Colchicine	HiMedia
DPX	HiMedia
EDTA	HiMedia
Ethidium Bromide	HiMedia
Giemsa stain	HiMedia
KCl	Merck
KH_2PO_4	Merck
Methanol	Merck
Na_2HPO_4	Merck
NaCl	Merck
NaH_2PO_4	Merck
RPMI 1640	HiMedia
Tris Base	HiMedia
Trypsin	Sigma

Table 2: List of materials

Materials	Company
Aluminium foil	Fresh-wrap
Blood Collection Needles	BD Vacutainers
Coplin Jars	Tarsons
Cover slips	Blue Star
Glass Pipettes	Borosil
Glass wares	Borosil
Gloves	Tarsons
Micropipette	Eppendorf
Pasteur pipettes	Tarsons
Slides	Riviera TM
Vacuatte (EDTA)	BD Vacutainer
Vacuatte (Na-Heparin)	BD Vacutainer

Table 3: List of instruments

Instruments	Company
Biosafety Cabinet	ESCO
Cooling Centrifuge	Remi
Fluorescence Microscope	Olympus
Freezer	Blue star

Freezer (-20^{o}C)	Blue Star
Incubator	EIE instruments
Microscope	Labomed
pH Meter	E1
Water Bath	EIE serological water bath (Wiswo Instruments)

Mechanisms and Mode of Action:

1) RPMI 1640

Roswell Park Memorial Institute (RPMI-1640) is a growth media that consists of vitamins, amino acids (High amount of glutamine), inorganic salts, glucose, glutathione, phenol red indicator, HEPES buffer, penicillin, Streptomycin and 2% $NaHCO_{3.}$ It is supplemented with 15% fetal bovine serum.

2) Hypotonic KCl solution

0.56 gm of KCl powder was dissolved in 100ml of distilled water. Hypotonic solution results into swelling of cells which facilitates the proper spreading of the chromosomal metaphase plates. The pre-warming of KCl solution at 37^{o}c in water-bath for 2 hours increases the potency of water transport across the cell membrane thereby altering the permeability of the cell membrane.

3) Carnoy's Fixative

Carnoy's fixative is made by 3:1 ratio of methanol: glacial acetic acid. Fixation removes water from the cells and preserves them, hardens the cell membrane and chromatin and also prepares the chromosome for the banding procedure.

4) Giemsa Stain

Giemsa stain is the complex mixtures of dyes. The main components are the basic aminophenothiazin dye azure A, azure B,azure C,thionin and

methylene blue and acidic dye eosin. The thiazin dyes vary in number of methyl groups attached to a core of two benzene rings bound together by nitrogen and sulphur atom.

Sorenson's buffer: 0.345 gm of monobasic sodium phosphate (NaH_2PO_4) and 0.454 gm dibasic sodium phosphate (Na_2HPO_4) was added in 250ml of sterile distilled water for obtaining pH 7.0

Stock Giemsa Stain: 1g giemsa powder was added in 54ml glycerol, mixed and kept at 60^{o}c water bath overnight. 84ml of methanol was added and filtered and kept in dark bottle.

Working Giemsa Stain: 2ml of stock Giemsa stain was added in 30ml Sorenson's buffer having pH 7 and 30ml distilled water, mixed well and freshly prepared whenever required.

5) Trypsin-EDTA solution

PBS (Phosphate Buffer Saline): 0.05g KCl, 2g NaCl, 0.36g NaH_2PO_4 and 0.06g KH_2P0_4 were added in 250ml of sterile distilled water. (pH 7)

Stock Trypsin-EDTA solution: 0.01g of EDTA and 0.02g of trypsin were added to 50ml of PBS.

Working Trypsin-EDTA solution: 25ml solution was taken from stock and 25ml of PBS was added to it.

6) Colchicine (1mg/ml)

10mg colchicine was dissolved in 1000ml of sterile deionized water and aliquots were dispensed. Colchicine is a natural product obtained from the plant *Colchicum autumnale* and does not allow polymerization of tubulin monomers to form microtubules and thus prevent formation of mitotic spindle. It is thus used to arrest dividing lymphocytes at metaphase stage. It also increases chromosome spreading as it releases them from the mitotic apparatus.

7) Chromic acid

20gm of $K_2Cr_2O_7$ was added in 300ml of conc. H_2SO_4 for washing the slides.

8) **DPX :** DPX was used to mount slides permanently and it has a refractive index similar to that of glass hence it does not hinder the observation through the lens of microscope.

9) Sodium Hypochlorite

It was used to wash the glasswares and syringes used for blood before their disposal in order to inactivate microbes including virus.

10) Proteinase K

It is a proteolytic enzyme that helps in protein degradation.

11) Absolute Alcohol

It was used for DNA precipitation of the sample.

METHODS

Subject Selection

This study was approved by our institutional ethical committee (Institute of Science, Nirma University) and S.G.P.G.I (Sanjay Gandhi Post Graduate Institute of Medical Sciences), Lucknow. The institutional consent forms were signed by the parents and only then the blood samples were taken for the study purpose. Clinically approved ASD patients were chosen as subjects

The inclusion criteria were as follows:

Clinically approved ASD patients

The exclusion criteria were:

Any comorbid diseases like fragile X syndrome, epilepsy, Down's syndrome etc.

1) Sample collection (in EDTA Vacutainer and Na-Heparin Vacutainers)

I. From EDTA vacutainers

DNA Isolation

- 200µL of Proteinase K was added to the blood sample to degrade the protein.A brief vortex was done. 2400µL of AL lysis buffer was added for cell lysis to occur and vortex was done for 3 mins.
- The mixture was then maintained in water bath at $70^{O}C$ for 10 minutes to obtain the maximum yield of DNA. The mixture was allowed to cool for few minutes.
- 2000µL of absolute alcohol was added to the mixture for DNA precipitation to occur.
- The mixture was then loaded into the column of the collection tube and was centrifuged at 3000rpm for 2 minutes. The filtrate was discarded.

- The mixture was given subsequent washing steps. 2000µL of AW1 wash buffer was added to the column and then it centrifuged at 3000 rpm for 5 mins.
- Then another 2000µL wash of AW2 buffer was given and the column was again centrifuged at 4000 rpm for 20 minutes.
- The filtrate was discarded and the column was given a dry spin at 4000 rpm for 3 mins.
- The column was then transferred to fresh collection tube and 100µL of the AE elution buffer was carefully placed on the silica membrane of the column and was incubated at room temperature for few minutes. The column was centrifuged at 3000 rpm for 5 mins.
- Again 100µL of the AE elution buffer was carefully placed on the silica membrane of the column and was incubated at room temperature for few minutes. The column was centrifuged at 3000 rpm for 5 minutes. The DNA was collected in a separate centrifuge tubes and was stored at -20^{O}C.

The DNA sample was then allowed to run on the agarose gel electrophoresis and the bands were obtained and then the samples were quantified by taking the absorbance at A260/280.

II .From Na-Heparin Vacutainers

a) Karyotyping

❖ Peripheral blood Lymphocyte culture:

- 1ml of blood sample of the ASD patients were short term cultured into 10ml RPMI 1640 media into falcon tubes
- The culture tubes were to incubated for 72hrs at 37^{o}C in the CO_2 incubator

❖ Colchicine addition and harvesting the cultured cells :

- The cultured cells were treated with colchicine at the 70th hour to arrest the cells at metaphase stage and were incubated for 2 hours in CO_2 incubator

- The cells were then harvested at 72th hour. The cells were first centrifuged at 3000 rpm for 10 mins and the supernatant was discarded
- The pellet was then mixed with 9ml of pre-warmed KCl (0.56%) and was incubated kept at 37^{0}C for 20 minutes in the water bath
- After the incubation, 2 ml of chilled fixative was added to each tube and was centrifuged at 3000 rpm for 10 mins. The fixative was maintained at 4^{o}C
- The supernatant was then removed and 7ml of chilled fixative was added and mixed properly and centrifuged at 3000 rpm for 10 minutes
- The pellet was then washed with 5ml of fixative and centrifuged at 3000 rpm for 10 mins. These washing steps were repeated until clear pellets were obtained.
- Once a clear pellet is obtained, excess of supernatant was removed and the pellet was mixed gently with the remaining fixative
- Using this suspension of cells, the slides were prepared

❖ Slide preparation

- Slides were cleaned with concentrated chromic acid. The clean slides stored in chilled water and were used for slide preparation
- Few drops of the cell suspension were dropped from a certain height onto the slide which was held in slanting position with forceps. This ensures proper spreading of chromosomes onto the slide
- These slides were then given a steam treatment. The steam allows the mitotic plate to spread properly as well as accelerates drying time of the slide

❖ Slide ageing

- The slides were allowed to age for a week at room temperature followed by baking for two hours at 60^{o}C in hot air oven
- Ageing provides the chromosomes with better contrast and crispness as it drives off the water content and makes the chromosomes more resistant to Trypsin.

- The major changes seen in chromosome may be due to oxidation of protein sulfhydral groups and degradation of the chromosome DNA. The crisp chromosomes show distinct band patterns after staining

b.) GTG banding

- Giemsa stain is used which has affinity for the AT rich regions on the chromosomes
- G-banding is the most frequently used techniques in clinical cytogenetics laboratories because of the clear and sharp bands produced and the ease with which they can be photographed
- GTG-banding (G-banding by Trypsin with Giemsa) involves use of the proteolytic enzyme trypsin for pre-treatment followed by staining with Giemsa

❖ Trypsin treatment :

- The slides were dipped in the coplin jar filled with Trypsin-EDTA solution for 15 seconds. Trypsin is a serine protease which hydrolyzes the protein component of chromatin, thereby allowing the Giemsa dye to react with exposed DNA

- It also degrades the Histones which are basic proteins with strong positive charge at neutral pH and are present at extremely high concentration in the cell nucleus. The high pH is because of the presence of greater proportion of the positively charged amino acid lysine and arginine. Histones bind tightly to negatively charged DNA

❖ Brief washes in Phosphate buffer saline :

- The slides were then given two consecutive washes of chilled PBS to arrest the trypsin activity

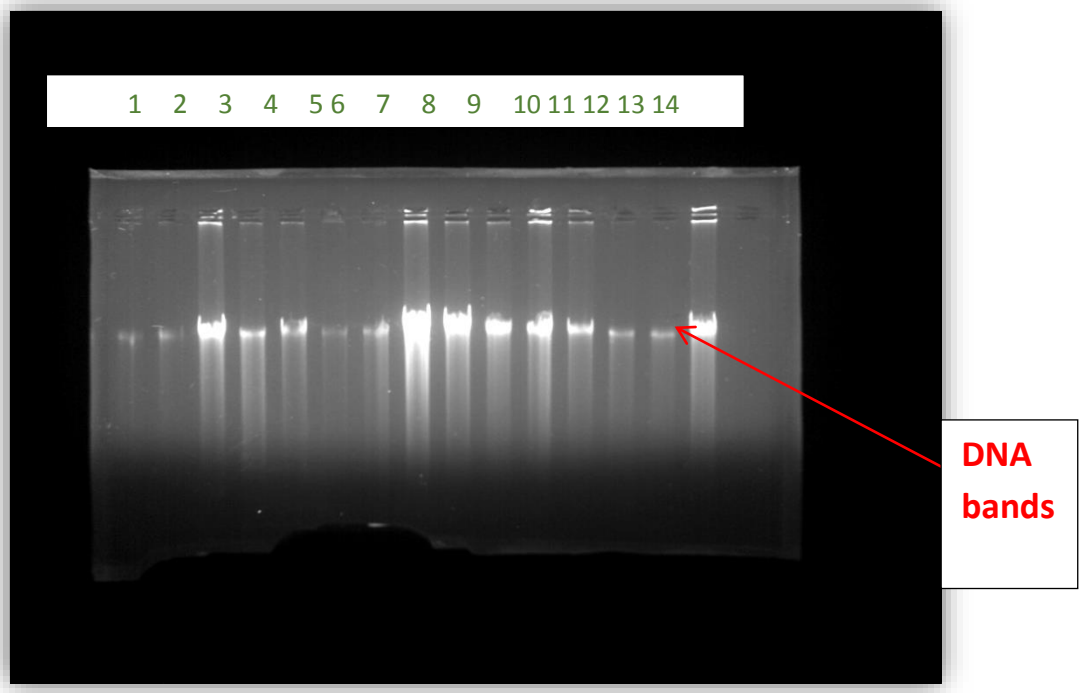
❖ Giemsa Staining :

- The slides were then dipped in coplin jar filled with Giemsa stain for 8-10 minutes, followed by washes with distilled water to remove excess of stain

❖ Slides observation and scoring :

- The GTG banded slides were observed under microscope and mitotic plates showing proper banding and spreading were scored
- Per sample approx 2-5 metaphase plates were scored under oil immersion in bright field microscopy

RESULTS



The sample in well numbers 2, 7, 8, 10, 11 and 14 have distinct bands while the samples in other wells have light bands.

Also the absorbance ratio in wells- 2, 7, 8, 10, 11 and 14 is nearly 1.8 which indicates the DNA is present in the detectable amount in the sample as per the standards.

Also there is no RNA contamination in the samples because if there would be RNA contamination then 2 or more bands have appeared below the DNA bands.

The DNA can be further quantified by taking the absorbance ratio in spectrophotometer.

Absorbance ratio of DNA sample:

Sample	Absorbance at 260nm	Absorbance at 280nm	A260/280
TF01	0.0642	0.0368	1.7444
TF02	0.1034	0.0561	1.8445
TF03	0.0784	0.456	1.7214
TF04	0.1367	0.0796	1.7182
TF05	0.0617	0.0335	1.8454
TF06	0.1341	0.0839	1.5986
TF07	0.1759	0.0976	1.8016
TF08	0.0966	0.0510	1.8955
TF09	0.0944	0.0553	1.7057
TF10	0.1437	0.0764	1.8806
TF11	0.0438	0.0236	1.8501
TF12	0.0654	0.0478	1.3676
TF13	0.0525	0.0339	1.5507
TF14	0.0975	0.0541	1.8034

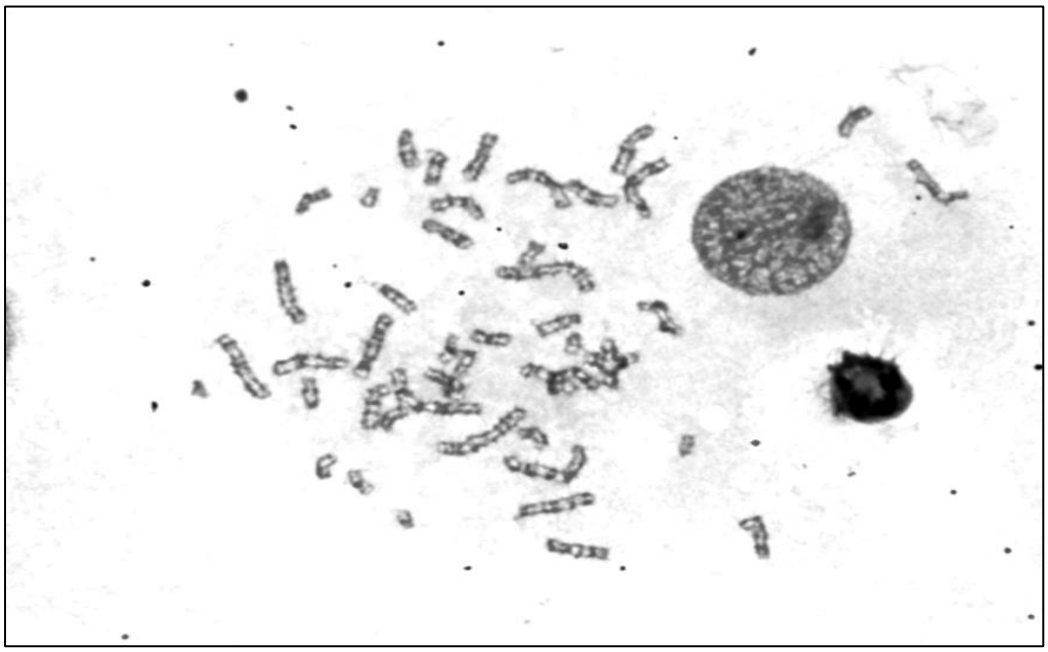
Samples having absorbance below 1.8 indicates the presence of phenol compounds.

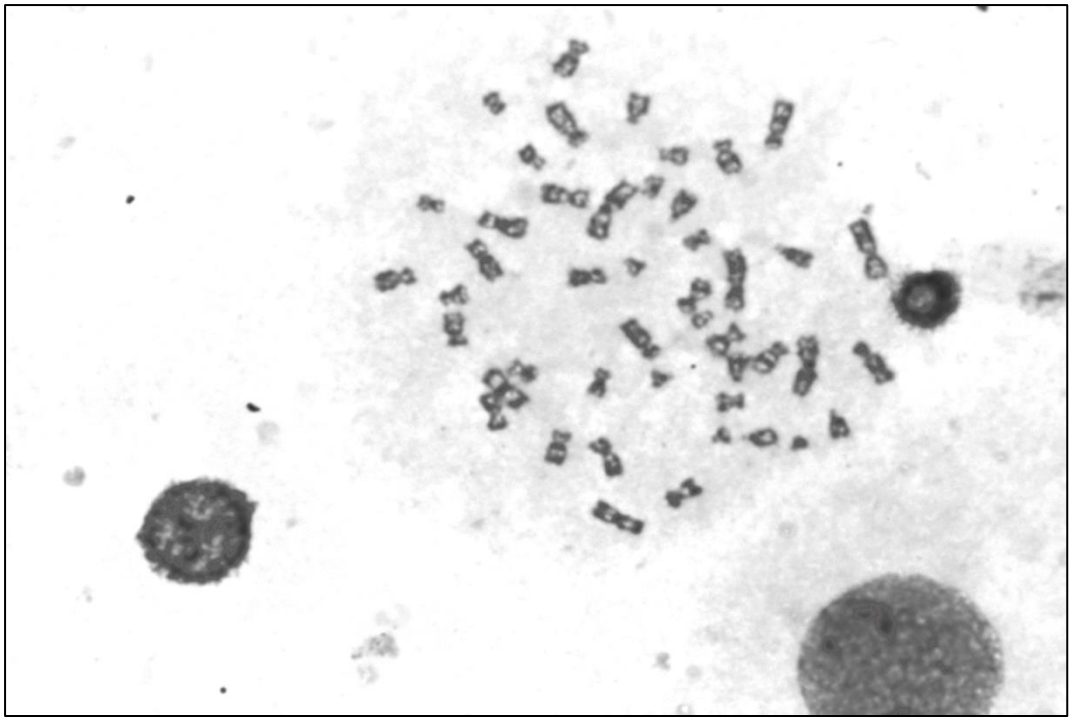
Samples having absorbance between 1.8-2 shows pure amount of DNA.

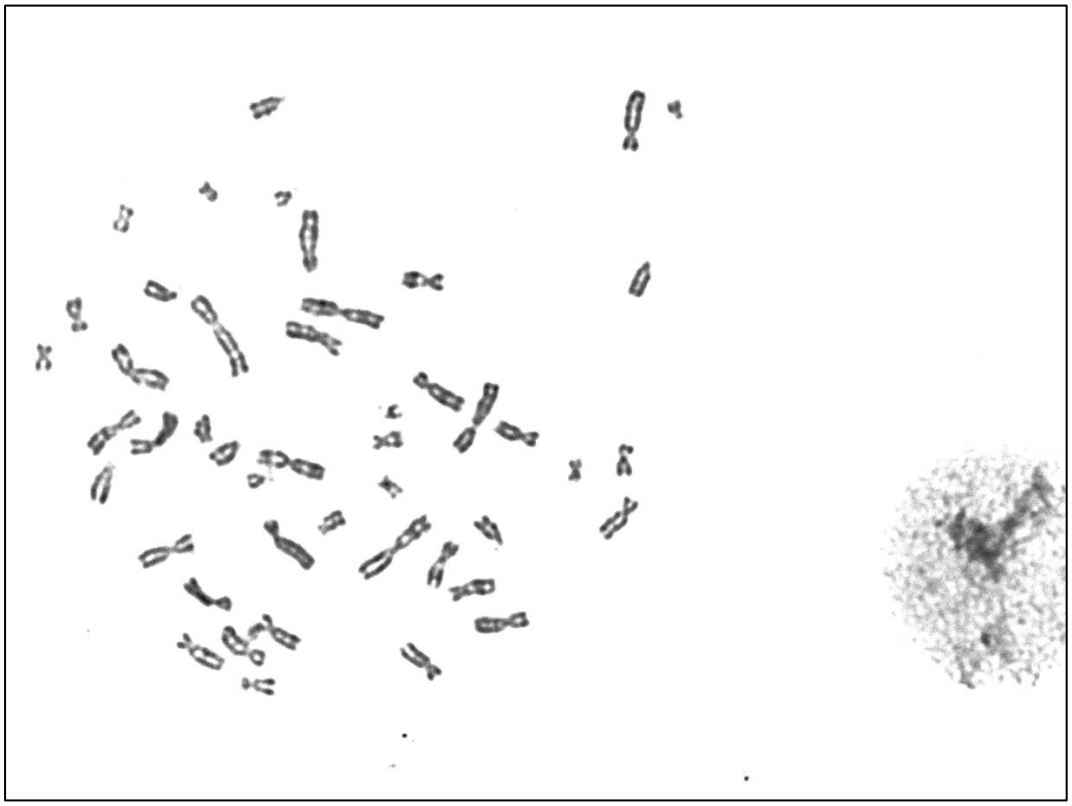
Samples having absorbance above 2 indicates the presence of RNA contamination.

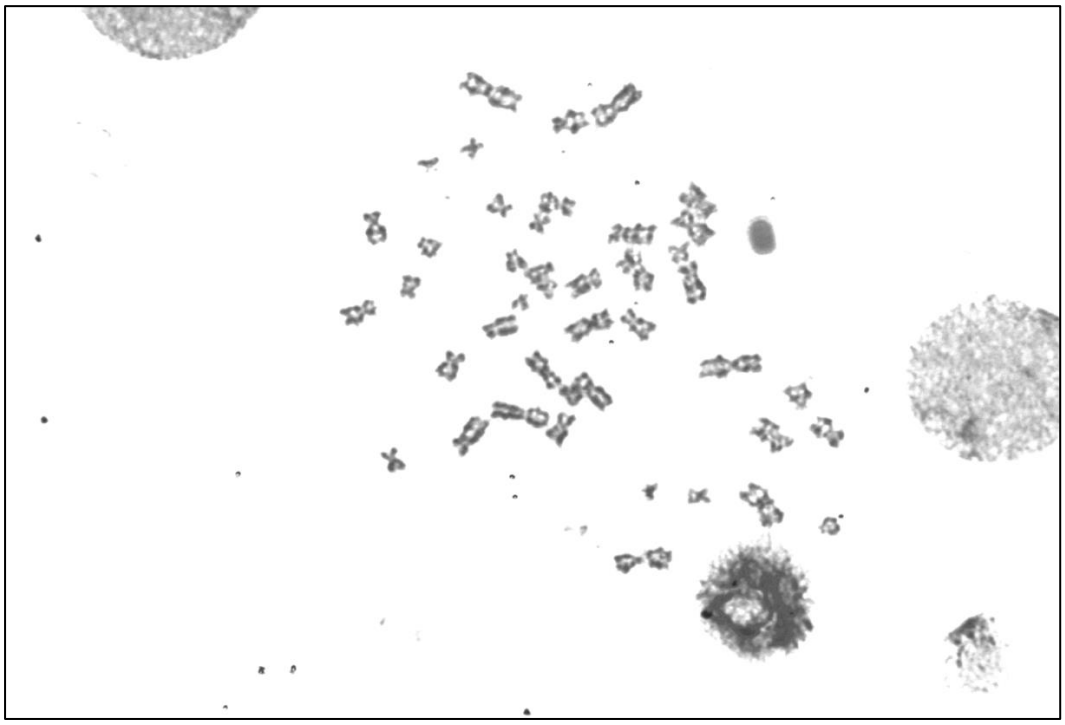
These DNA samples are suitable for further cytogenetics studies at molecular level as they show absence of contamination by RNA

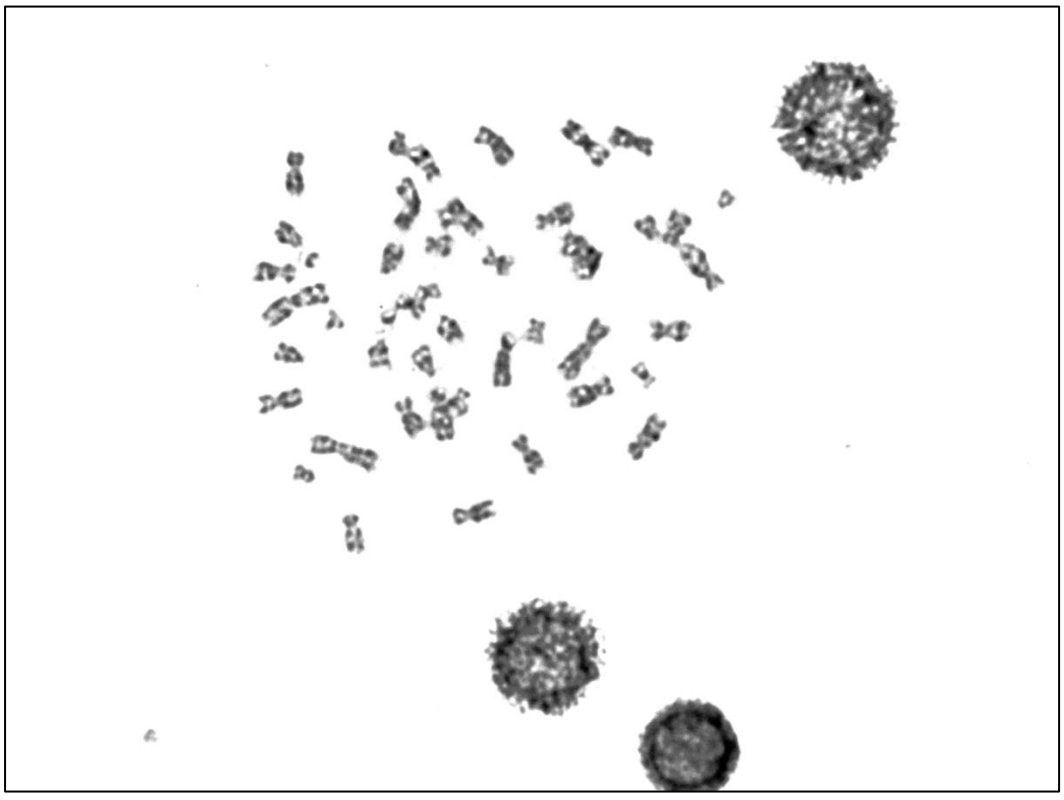
Representative photomicrographs depicting the G banded metaphase chromosome at in Bright Field Microscope (magnification 1000x with digital zoom)











Karyogram arrangement and analysis

I. Group A

The largest chromosomes are in group A i.e. chromosomes 1, 2 and 3. 1 is the largest chromosome. 1 and 3 are metacentric and 2 is sub-metacentric

II. Group B

It consists of chromosomes 4 and 5 that can be distinguished only by banding techniques. They both are sub-metacentric

III.Group C

Group C chromosomes are all medium sized and sub-metacentric. Chromosomes 6 to 12 and X includes in group C. Chromosome 12 is the most sub-metacentric of the group. X and 7 are of same size and have their centromeres little close to middle of the chromosome

IV. Group D

This group consists of medium sized acrocentric chromosomes. It includes chromosomes 13, 14 and 15

V. Group E

Chromosomes 16, 17 and 18 includes in group E. Chromosome 16 is metacentric and 17 and 18 are sub-metacentric

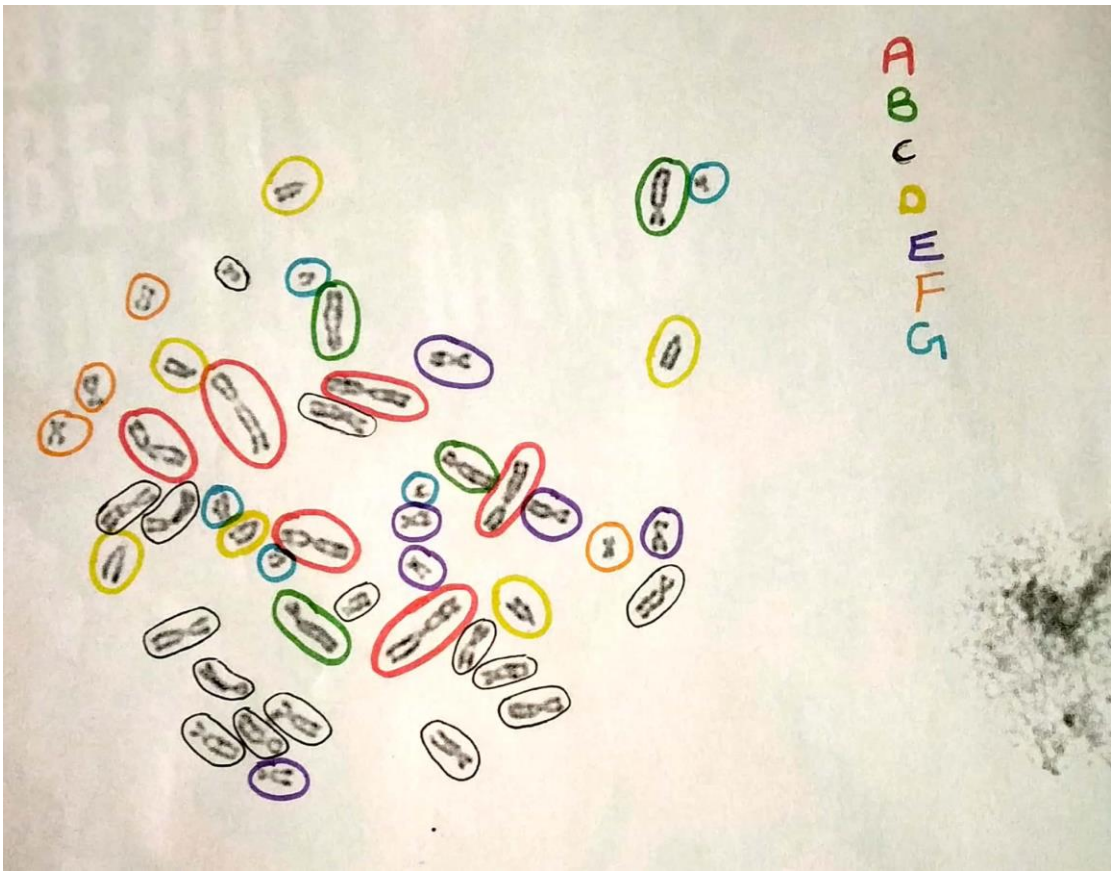
VI .Group F

It contains chromosomes 19 and 20. They both are metacentric

VII. Group G

Contains chromosomes 21, 22 and Y sex chromosomes. 21 and 22 are small acrocentrics which have satellites on their short arm. The size of Y chromosomes vary, although it does not have satellite on their short arm and are more defined than acrocentric chromosomes

Scoring and Grouping of G banded Chromosomes



DISCUSSION

Autism Spectrum Disorder is neuro-developmental disorder which hinders the way the brain cells communicate with each other. It is caused by various factors and the debate is still going on whether autism is caused by environmental factors or genetic factors or it is the interplay between the both. Environmental factors such as exposure to chemicals during pregnancy, convulsions after birth, diabetes etc. and genetic factors such as de novo mutations may result to Autism.

Various candidate genes are associated with ASD and mutation in such genes may result in ASD. To study the aberrations at chromosome level the foremost step is to rule of numerical and structural anomalies of the chromosome. Once we rule out only then we can say that there is no karyotypic aberrations.

The G-bands cytogenetic analysis often leads to the discovery of unbalanced karyotypes which require further characterization at molecular level. The peripheral blood lymphocytes are cultured with RPMI 1640 media and colchicine is added at 70^{th} hour to arrest the cells at metaphase stage. The harvesting was done at 72^{th} hour and cells were given the hypotonic treatment. At last the cells were fixed with 3:1 of methanol: glacial acetic acid. GTG banding is then performed to get light and dark bands. Adenine pairs with Thymine with two hydrogen bonds and Cytosine pairs with Guanine with three hydrogen bonds. Hence it is easy to break two H- bonds than three H-bonds. Thus AT rich region (heterochromatin) takes up high amount of dye and is stained dark while CG rich (euchromatin) region takes up less dye because three H-bonds are not easily broken and hence they are stained light.

This light and dark bands are unique to every chromosomes which makes it easy to identify every single chromosome. 14 samples were taken to study their karyotype. The metaphase plates were scored under oil immersion in bright field microscopy. All the patients showed normal karyotype and there were no structural and numerical abnormalities. The DNA of the patients was also isolated and was run on agarose gel electrophoresis. The samples were also quantified and the absorbance was taken at A260/280 which was approx. 1.8 to 2. Hence there was no RNA contamination.

One of the way to detect mutations in DNA is by using MLPA probemix. MLPA (Multiplex Ligation Probe dependent Amplification) is the gold

standard to for DNA number quantification. It is used to study various disorders. MRC-Holland is the inventor and manufacturer of MLPA. It is the method that detects the aberrant copy numbers upto 60 nucleotides in simple PCR reaction. This method is used to detect known point mutations such as deletion or duplication in the DNA sample. The SALSA MLPA P396 SHANK2 probemix contains probes for SHANK2 exons. This assay is used for the detection of deletion/duplication in SHANK2 gene which is associated in Autism Spectrum Disorder. Hence by this technique the de novo mutations in SHANK2 gene can be detected at molecular level. Various other analysis can also be done to detect such kind of mutations. And these further molecular studies will be done at S.G.P.G.I(Sanjay Gandhi Postgraduate Institute Of Medical Sciences, Lucknow).

CONCLUSION

From this study we can conclude that in order to study any genetic disorder we must first rule out the numerical and structural aberrations in the chromosomes. The karyotype of the ASD patients are normal and no chromosomal anomalies are reported. So the further studies could be conducted at molecular level.

The DNA isolated from the samples is also pure as most of the samples have absorbance ratio of A260/280 between 1.8-2. Further there is no RNA contamination and so the samples can be used of molecular cytogenetic analysis.

REFERENCES

“Encyclopedia of Autism Spectrum Disorders" Springer Nature, 2013

American Psychiatric Association (APA) 1994 : Diagnostic and statistic manual of mental disorders (DSM- IV), 4th edition.

Bakshi, Sonal R. and Kunwar F. “Phenotypic clues and karyotype: A combined study of Mental Retardation”. 1st edition Ahmedabad : Lambert Academic Publishing,2012

Charan, Sri Hari. "Childhood disintegrative disorder." *Journal of pediatric neurosciences* 7.1 (2012): 55

Chlebowski, C., Green, J. A., Barton, M. L., & Fein, D. (2010). Using the childhood autism rating scale to diagnose autism spectrum disorders. *Journal of autism and developmental disorders*, *40*(7), 787-799.

chromosomes. Journal of microscopy, 119(3), 397-406.

Fakhoury, M. (2015). Autistic spectrum disorders: A review of clinical features, theories and diagnosis. *International Journal of Developmental Neuroscience*, *43*, 70-77.

Hani Raoul Khouzam, Fayez El-Gabalawi, Nabeel Pirwani, Fiona Priest. "Asperger’s disorder: A review of its diagnosis and treatment", Comprehensive Psychiatry, 2004

Helen J. Lawce. "Chromosome stains", Wiley- Blackwell, 2017

http://www.autism-society.org/about-the-autism-society/history/autism-awareness-ribbon/

http://www.researchautism.net/autism/types-of-autism/pervasive-developmental-disorder-not-otherwise-specified

https://ghr.nlm.nih.gov/gene/SHANK2#location

https://medlineplus.gov/autismspectrumdisorder.html

https://www.cdc.gov/ncbddd/autism/signs.html

https://www.mlpa.com/WebForms/WebFormProductDetails.aspx?Tag=_tz2fAPIAupKyMjaDF-E-t9bmuxqlhe_Lgqfk8Hkjuss.&ProductOID=_5og0ZHOOEzw.

https://www.ncbi.nlm.nih.gov/gene/22941

https://www.nhp.gov.in/world-autism-awareness-day_pg

https://www.nimh.nih.gov/health/topics/autism-spectrum-disorders-asd/index.shtml

https://www.omim.org/entry/209850

Khouzam, H. R., El-Gabalawi, F., Pirwani, N., & Priest, F. (2004). Asperger's disorder: A review of its diagnosis and treatment. *Comprehensive Psychiatry*, *45*(3), 184-191.

Lawce, H. J., & Brown, M. G. (2017). Cytogenetics: an overview. *The AGT Cytogenetics Laboratory Manual*, 25-85.

Lundberg, A. (1979). "The Drawing Test"–A Tool for Assessment of Arm-Hand Function in Children of 1–3 Years of Age. *Neuropädiatrie*, *10*(01), 29-34.

O'Connor, Eoin C., Sebastiano Bariselli, and Camilla Bellone. "Synaptic basis of social dysfunction: a focus on postsynaptic proteins linking group-I mGluRs with AMPARs and NMDARs" , European Journal of Neuroscience,2014
on Comorbidities" , ACS Chemical Neuroscience, 2017
Published by John Wiley & Sons,Inc.,Hoboken,New Jersey.

Rhona R. Schreck. "Chromosome Banding Techniques" , Current Protocols in Human Genetics, 05/2001

Seabright, M. (1971). A rapid banding technique for human chromosomes. *The Lancet*, *298*(7731), 971-972.

Simons, A., Shaffer, L. G., & Hastings, R. J. (2013). Cytogenetic nomenclature: changes in the **ISCN** 2013 compared to the 2009 edition. *Cytogenetic and genome research*, *141*(1), 1-6.

Simonoff, E., Pickles, A., Charman, T., Chandler, S., Loucas, T., & Baird, G. (2008). Psychiatric disorders in children with autism spectrum disorders: prevalence, comorbidity, and associated factors in a population-derived sample. *Journal of the American Academy of Child & Adolescent Psychiatry*, *47*(8), 921-929.

Sumner, A. T. (1980). Dye binding mechanisms in G- banding of

The AGT cytogenetics Laboratory Manual,4th edition, Marilyn S. Arsham,Margaret J. Barch, Helen J. Lawce

Weaving, L. S., Ellaway, C. J., Gecz, J., & Christodoulou, J. (2005). Rett syndrome: clinical review and genetic update. *Journal of medical genetics*, *42*(1), 1-7.

Wendy A Gold, Rahul Krishnarajy, Carolyn Ellaway, John Christodoulou. "Rett Syndrome: A Genetic Update and Clinical Review Focusing

Won, H., Mah, W., & Kim, E. (2013). Autism spectrum disorder causes, mechanisms, and treatments: focus on neuronal synapses. *Frontiers in molecular neuroscience*, *6*, 19.

ANNEXURE

Standard Operating Practice

Standard Operating Practice

High speed cooling centrifuge

- ✓ Switch on the main button
- ✓ Switch on power button of the instrument
- ✓ Press SET to set the programme
- ✓ Press set again, set the rotor number
- ✓ Press set again, set the required RPM
- ✓ Press set again, it will blink on the timer and set the required timing by pressing up and down button
- ✓ Press set again, Blinking number shows acceleration. Set zero pressing up and down button
- ✓ Press set again, Blinking number shows deceleration. Set zero pressing up and down button
- ✓ Press set again, Blinking number shows temperature. Set required temperature by pressing up and down button
- ✓ Press set again. It will save the programme
- ✓ Put your tube with proper balance
- ✓ Close the lid. Press START to start your run

Bio safety cabinet

- ✓ Ensure that the bio safety cabinet is properly connected to the power supply
- ✓ Switch ‘ON’ the main switch and then the cabinet switch
- ✓ Work in the bio safety cabinet for the required time and clean the equipment after work

- ✓ Work in the bio safety cabinet for the required time and clean the equipment after work
- ✓ Clean the equipment with IPA 70%
- ✓ Switch ON the UV light for 30 minutes
- ✓ After UV light exposure switch OFF the main switch and then the cabinet switch

Microscope

- ✓ Remove the dust cover
- ✓ Switch on the microscope
- ✓ Clean the slide dry if wet, clean the stage
- ✓ Check the optical lens, clean if required with tissue paper only
- ✓ Start with 4X, move to higher magnification clockwise only
- ✓ When using higher magnification adjust stage height to avoid impact on objective lenses
- ✓ If using 100X objective, use very little amount of oil and avoid 40X touching the oil
- ✓ Clean the stage and optical lenses with tissue paper
- ✓ Put the dust cover back

Micropipette

- ✓ Volume setting

The volume can be changed continuously by rotating the setting of the ring

- ✓ Aspirating liquid

Attach the tips to the pipette firmly

Immerse the pipette the tips approximately 3mm to 5mminto the liquid or suitable depth into liquid

Allow the control button to slide back slowly

Pull the micropipette tips out of the liquid slowly

- ✓ Dispensing liquid

Press down the control button slowly to the first stop and wait until the liquid stops following

Press down the control button to the second stroke until the tip is completely empty
Allow the control button to slide back slowly

Weighing balance

- ✓ Switch off fan and A.C. before use.
- ✓ Press the power key located at the back of the instrument.
- ✓ Always check the bubble position. Keep it in the center.
- ✓ The balance will be calibrated, and then it will show OFF.
- ✓ Press the power button, it will show zero and press UNIT to select g/mg mode.
- ✓ When using a tare, load the tare on the pan and press the TARE key after the stability mark is lit. Check that the display shows zero.
- ✓ Weigh your sample(s).
- ✓ Clean the pan with a brush once weighing is done.
- ✓ Press TARE after completion of work.
- ✓ Switch off the power.

Deep Freezer/-80^{0}c freezer

- ✓ **Operation of the deep freezer**

Plug in the deep freezer

Switch on the mains. The green lamp lights up. The red lamp lights up whenever the compressor is on

Adjust the thermostat knob to maintain the desired temperature minimum, middle and maximum

- ✓ **Cleaning of the deep freezer**

Thin frost shall be removed with a brush and plastic scraper

Switch ‘off’ the freezer and disconnect from the power supply to remove the heavy ice deposit

Clean the inside chamber of the freezer with warm water

- ✓ **Shutting down procedure**

Switch off the main switch

Disconnect all cables when the equipment is not in use.

CO_2 incubators

- ✓ **Filling the Humidity Reservoir or pan.**

The Humidity pan may be autoclaved prior to use.

The Humidity pan should be filled with 5 Liters of Water for Production (WFP).

The Humidity pan should be placed on the floor of the incubator.

The water level in the humidity pan should be checked frequently, and water should be changed weekly when incubator is in use.

- ✓ **Set the Chamber temperature**

Before the initial temperature setting is made, push in the “PUSH TO SET” button on the alarm monitor module, and using the screwdriver on the control panel, turn the over temperature set screw until the display shows a temperature that is 2 to 3 degrees above the desired operating set point. The overtemperature safety may be reset after the chamber temperature has stabilized at set point.

If a chamber temperature of 37 degrees C is desired, set the variable/37° C switch to the 37°C position.

If a value other than 37°C is desired refer to Operators Manual.

- ✓ **Setting the over temperature safety thermostat**

Once the chamber temperature has stabilized (as indicated by the digital display), the overtemperature safety should be set as follows:

Push in on the Push to Set button on the alarm monitor module.

Using the screwdriver mounted on the control panel, turn the set screw until the desired overtemperature alarm point is shown on the digital display. The over temperature set point can be set within 0.1 degree of operating set point, but it is recommended that it not be set within 0.5 degrees of set point.

Water Bath

- ✓ Firstly check the water level in Water bath.
- ✓ Switch on Main power and ON knob of the Instrument.
- ✓ Set the thermostat knob to the required temperature.
- ✓ Rising in the temperature is indicated by red indicator lamp.
- ✓ Put your solution/material, When desire temperature is achieved.
- ✓ Check temperature of water by thermometer also.
- ✓ Close the lid of water bath during incubation.
- ✓ It is required to maintain water level throughout incubation.
- ✓ After completion of heating process, reduce the temperature to zero.
- ✓ Switch of power supply and allow the water bath to cool.
- ✓ USE ONLY DISTILLED WATER/R.O. TO AVOID CORROSSION