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West Bengal Chapter

The Biochemistry Chronicles

From the Desk of the President of AMBI WB Chapter

A session of Hands-on **Workshop and Continuing Medical Education (CME)** was conducted by DBT NIDAN Kendra NRSMCH, Kolkata **in Association with AMBI, WB Chapter** on **03-04, February 2023** (Friday & Saturday) from 10:00 am onwards at **Department of Biochemistry, NRSMC**. The programme Schedule are mentioned below.

PROGRAMME SCHEDULE of 03.02.2023

Sl No.	Time	Session
1.	10:00 – 10:10	Welcome address & Objectives of the Workshop
2.	10.10 - 10.20	Estimation of G6PD from DBS card
3.	10:20 – 12:00	DNA isolation from human peripheral blood
4.	12:00 – 13:00	Restriction Fragment Length Polymorphism Analysis for "*Identification of common mutations in G6PD gene"*
5.	13.00 – 13.30	**LUNCH**
6.	13:30 – 13:45	Different types of Polymerase Chain Reaction (PCR)
7.	13:45 – 16:00	PCR analysis: "*Amplification of coding regions of G6PD gene"*

PROGRAMME SCHEDULE of 04.02.2023

1.	09:30 – 9:40	Opening Remarks
2.	09:40 – 09:55	Importance of NBS screening in Indian
3.	09:55 – 10:10	TEA BREAK
4.	10:10 – 10:30	Introduction to Inborn Error of metabolic Disorders: Clinical Perspective
5.	10:30 – 10:50	Biochemical Identification of Inborn Error of metabolic Disorders: with emphasize to G6PD screening among neonates
6.	10:50 – 12:45	Data analysis using Bioinformatic tools • Exon-Intron Map of a gene • Primer designing • RFLP designing • Database browsing (SNP & Mutation)
7.	12.45 – 13.15	**LUNCH**
8.	13:15 – 15:15	Workshop continues
9.	15:15 – 15:30	Closing Remarks & Vote of Thanks

Molecular Identification of Mediterranean Mutation in Patients with G6PD deficiency

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most common human enzyme defects, being present in more than 400 million people worldwide. It has been identified as one of the diseases to be screened during neonatal life. During glucose 6 phosphate dehydrogenase deficiency, the red cells are unable to regenerate reduced nicotinamide adenine dinucleotide phosphate (NADPH), a reaction that is normally catalyzed by the G6PD enzyme.

Since the X-chromosome carries the gene for G6PD enzyme, this deficiency mostly affects males. The two major conditions associated with G6PD deficiency are haemolytic anemia and neonatal jaundice, which may result in neurological complications and death.

G6PD deficiency has been reported from India more than 30 years ago and about 13 variants have been characterized biochemically. Molecular characterization revealed that G6PD Mediterranean (563 C-->T) was the commonest (60.4%) deficient variant followed by G6PD Kerala-Kalyan (949 G-->A; 24.5%) and G6PD Orissa (131 C-->G; 13.3%).

DNA of the blood of Screen positive patients suspected for G6PD deficiency was isolated and tested for Mediterranean Mutation by molecular techniques in the following way:

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique, which amplify a segment of DNA specified by two, forward and reverse, complementary primers. The repeated cycles of denaturation of dsDNA, followed by annealing of primers to ssDNA and then elongation at 72 ^{0}C using Taq polymerase and dNTPs generate a million copies of specified DNA fragment which makes DNA analysis very easy.

PCR was carried out in a total volume of 25.0 µl, containing 40 - 80 ng genomic DNA, 20 pM of each primer, and 1X PCR Master mix (SRL, Maharashtra, India) in a thermocycler (GeneAmp-2700, PE Applied Biosystems, Foster City, CA, USA). The conditions specific for different PCR reactions vary widely. The condition specific for the amplification of exon 6 of *G6PD* gene is used. The Mediterranean variant, Ser188Phe (rs5030868; c.563C>T) is in the exon 6 of *G6PD* gene.

Restriction enzyme digestion

Restriction fragment length polymorphism (RFLP) is a technique invented in 1984 by the English scientist Alec Jeffreys. It is used for the analysis of unique patterns in DNA fragments in order to genetically differentiate between organisms – these patterns are called Variable Number of Tandem Repeats (VNTRs).Genetic polymorphism is defined as the inherited genetic differences among individuals in over 1% of normal population. The RFLP technique exploits these differences in DNA sequences to recognize and study both intraspecies and interspecies variation.

Restriction enzymes bind specifically to and cleave DNA at specific sites within or adjacent to a particular sequence known as the recognition sequence. These enzymes have been classified into three groups. Type I and type III enzymes carry modification (methylation) and ATP-dependent restriction (cleavage) activities in the same protein. Type III enzymes cut the DNA at the recognition site and then dissociate from the substrate. However, type I enzymes bind to the recognition

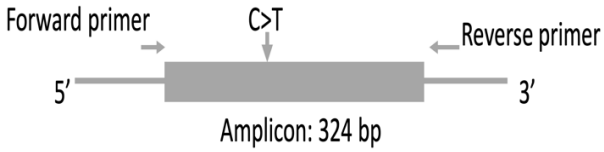
sequence but cleave at random sites when the DNA loops back to the bound enzyme.

Type II restriction/modification systems are binary systems consisting of a restriction endonuclease that cleaves a specific sequence of nucleotides and a separate methylase that modifies the same recognition sequence. Most of the type II restriction enzymes recognize specific sequences that are usually four, five or six nucleotides in length and display two-fold symmetry. A few enzymes, however, recognize longer sequences or sequences that are degenerate. The location of cleavage sites within the axis of dyad symmetry differs from enzyme to enzyme: some cleave both strands exactly at the axis of symmetry generating fragments with blunt ends; others cleave each strand at similar locations on opposite sides of the axis of symmetry, creating fragments of DNA that carry protruding single-stranded termini.

The nucleotide variations (mutations, single nucleotide variants, etc.) identified by DNA sequencing and/or reported in any databases will be screened by RFLP analysis. Generally, two web tools, NEBCUTTER 2 (https:// nc2.neb.com/NEBcutter2/) and WEBCUTTER (http://www.firstmarket.com/cutter/cut2.html) were used to design the RFLP pattern. The changes were examined for any alteration of Restriction enzyme site. If the nucleotide variations caused alteration of any restriction enzyme site, scoring of alleles bearing those changes was performed with appropriate restriction enzyme. The PCR products (50–70 ng) were digested with appropriate restriction enzymes (1-2 units per reaction) and the compatible buffer that distinguished between the mutant and normal alleles under the condition described by the manufacturer in a total reaction volume of 20 µl. The DNA fragments in the digest were separated by PAGE as described earlier. Alleles were scored based on the DNA band patterns in the gel.

Detection of Ser188Phe mutation in *G6PD* gene

Nucleotide change	Name of the RE	Fragment size (bp)	Buffer	Incubation Temp	Incubation Time
C (Wild type)	MboII	287 + 37	rCutsmart	37 ^{0}C	180 mins
T (Mutant)		189 + 98 + 37			



Five µl of the PCR products will be then analysed by electrophoresis in 6% polyacrylamide gels / 1% Agarose gel (depending on the size of the amplicon) and visualized under UV light after staining the gel in ethidium bromide solution.

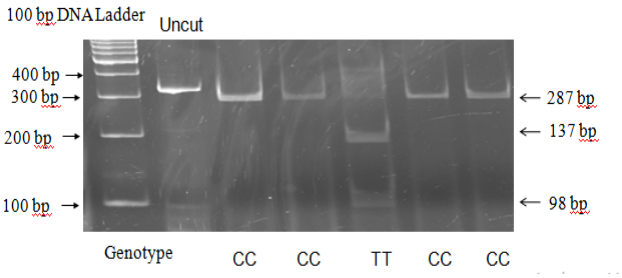
Gel electrophoresis

Gel Electrophoresis is used to separate macromolecules - especially proteins and nucleic acids - that differ in size, charge, or conformation. In gel electrophoresis, the charged molecule is placed in an electric field which migrates toward the positive or negative pole based on its charge. In contrast to proteins, which can have either a net positive or net negative charge, nucleic acids have a consistent negative charge imparted by their phosphate backbone, and migrate toward the anode. Mainly two types of electrophoresis were performed; i) *agarose gel electrophoresis in*

submerged gel and ii) polyacrylamide gel electrophoresis (PAGE) in vertical gel.

Agarose gel electrophoresis

The Agarose gel has a large range of separation, but relatively low resolving power. By varying the concentration of agarose (0.5 – 2%), fragments of DNA from about 200 to 50,000 bp can be separated using standard electrophoretic techniques. The agarose gel is prepared by melting agarose in 1X buffer in a microwave oven, then cooled at 55 ^{0}C and then poured to cast a submerge gel with a comb in it. Gels was prepared using TBE buffer [Table X]. Slab gels of different sizes was used for different experiments. Samples to be electrophoresed was mixed with 1/6th volume of 6x Gel Loading dye. Electrophoresis was carried out at 2-3 volts per cm of gel for 1-6 hours at room temperature depending on the size of the gel and run length. To visualize the DNA fragments, the gel was stained in ethidium bromide solution (0.5 µg/ml) for 10-15 minutes and de-stained in water for 2-3 minutes and documented in a gel documentation system (Gel Doc 1000, BioRad, Hercules, CA, USA).In this study, 1.5% Agarose gel was prepared to check the amplification of PCR products.



Polyacrylamide gel electrophoresis (page)

Polyacrylamide is a cross-linked polymer of acrylamide and bis-acrylamide. The Polyacrylamide gel has a very high resolving power; however, its separation range is small (less than 1 Kb DNA fragments). For PAGE the vertical gel apparatus was used. In this study, the samples were electrophorized in 6% polyacrylamide gel. Five µl of the samples was mixed with 1/6th volume of gel loading dye. Electrophoresis was carried out at 6-8 volts/cm of the gel for 1.5 to 2.5 hours at room temperature depending on the size of the gel and the fragment size. To visualize the DNA fragments, the gels were stained in ethidium bromide solution (0.5 µg/ml) for 20 minutes and then de-stained in water for 5 minutes. Then the gels were visualized and documented as mentioned above. PAGE was prepared to check the RFLP pattern of Ser188Phe mutation in *G6PD* gene.

Only those PCR products that had a single amplification product with no evidence of non-specific amplification will be used for Restriction Fragment Length Polymorphism (RFLP) analysis.

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