MATERIALS AND METHODS:

The study will be carried out in Central Research Laboratory, Rama Medical College, Rama University, Kanpur, U.P, India. The ethical clearance will be obtained from the Institute Ethical Committee (IEC).

Patient Selection
Total 200 cases of CHD will be taken from Rama Medical College, Rama University, Kanpur, U.P, during the period of 2018 to 2019. The consent of the patients and their parents will be taken.

Data Collection
The common parameters like age, sex and stage of heart disease will be taken. The age at the time of diagnosis of heart defects will be recorded. All cases will be thoroughly examined by chest x-ray, electrocardiogram, and 2D echocardiography. Family history of any heart abnormality, history of multiple abortions, nutrition and drug intake, any other patho-physiological conditions, and parity status of mother will be recorded for analysis. Age of below 5 years is included in this study. The normal children of equivalent age group will be taken as control. Parents are also to be investigated wherever possible.

Inclusion criteria.
1. Below 5 years of age groups are included in this study.
2. Only Kanpur and peripheral region CHD cases are included in this study.

Exclusion criteria
1. Age more than 5 years is excluded.
2. If there is no data on history of mother it will be excluded.
Investigations: Echocardiogram, Electrocardiogram, Chest X-ray and BMI

Blood investigations: GTT

Genetic findings: Molecular NKX2-5 Gene studies in Congenital Heart Diseases by Conventional PCR.

Molecular Analysis:

Sampling: 5ml of venous blood will be drawn from peripheral vein using Di sodium EDTA vaccutainers. All the samples will be aliquoted and stored at -80\(^{\circ}\)C until tested. The laboratory work will be carried out in the central research laboratory, Rama Medical College, Hospital & Research Centre. All the groups have their NKX2-5 gene will be analyzed. The sequence of chromosome located in 5q34.

Methodology

1) Isolation of DNA:
DNA will be extracted from whole blood containing EDTA by Ponez et al., standard salting out procedure.

Protocol for DNA isolation (salting out Method)
1. Thawed blood samples (5ml) from EDTA vaccutainers will be transferred into clean and sterile centrifuge tubes.
2. 10 ml of RBC lysis buffer will be added and the volume will make upto 15ml.
3. 0.1% of triton X will be added.
4. The centrifuge tubes will be incubated at 37\(^{\circ}\)c for 5 mins
5. The samples will be spun at 2000rpm for 15 mins in a cold centrifuge at 40\(^{\circ}\)c.
6. After centrifugation the supernatant will be discarded off taking care of the pellet.
7. 10 ml of RBC lysis buffer will be again added and vortex mixed.
8. The samples will be spun at 2000 rpm for 15mins.
9. The supernatant will be discarded off and a white pellet will be obtained.
10. 1 ml of nucleus lysis buffer will be added then vortex mixed.
11. About 20\(\mu\)l of 10%SDS will be added and mixed properly.
12. The mixture will be incubated in a water bath at 55\(^{\circ}\)c for 1 hour.
13. After incubation, the contents will be transferred into 2ml eppendorf tubes.
14. 400 \(\mu\)l of 5M Nacl will be added.
15. The contents will be spun at 10,000 rpm for 15mins.
16. After spinning the supernatant will be transferred into a new centrifuge tube leaving behind the pellet in the eppendorf tubes.
17. About double the volume if cold absolute ethanol will be added.
18. Large clumps of DNA will be obtained by gentle swirling of the tubes.
19. The DNA will be scooped with the help of a sterile spatula or a sterile loop from the centrifuge tubes and transferred into eppendorf tubes.
20. 200μl of 70% ethanol will be added.
21. The contents will be centrifuged and decanted into the eppendorf tubes and keep for air drying.
22. After drying, about150μl of TE buffer will be added and the DNA stored at -20⁰c for later use.

**Concentration and quality assurance of DNA:**
The quality of the DNA will be analyzed by 0.8% agarose gel electrophoresis and the quality assessed by standard spectrophotometer at 260 nm and 280 nm.

Primers will be obtained from Banglore Genei and will be reconstituted with sterile double distilled water based on the manufacturer’s instruction.

**PCR reaction mix (working concentration)**
The working conditions for PCR reaction mixture are consumed from Banglore Genei, India:
1. 10 x PCR Buffer: 1X
2. dNTPs :200μM
3. Forward primerr: 30pM
4. Reverse primer: 30pM
5. Taq polymerase: 1.5U
6. Template DNA: 50μg
7. Distilled water: to make up the volume

**PCR conditions:**
1. Initial denaturation: 94⁰c-5mins
2. Denaturation: 94⁰c-30secs
3. Annealing: 58⁰c-45secs
4. Extension : 72⁰c-45secs
5. Cycling condition: 30 cycles
6. Final extension: 72°C-7mins
7. Hold at 4°C

Agarose Gel Electrophoresis

Once the amplification will be obtained, they will be subjected to 2% agarose gel electrophoresis with Ethidium bromide and the bands will be visualized under UV light in gel documentation system (Biorad).

Procedure:
1. 2% agarose gel Himedia will be prepared, by mixing 2 g of agarose in 100ml of 1% buffer.
2. The contents will be boiled completely till a clear solution obtained.
3. About 100 μg of Ethidium bromide will be added and mixed properly.
4. Agarose will be casted into specific trays and allowed to solidify.
5. Specific combs will be used to make sample wells on the gel.
6. The PCR products will be mixed with Bromophenol blue.
7. 2μl of DNA ladder will be loaded into the first well.
8. Then, samples will be loaded into the gels and run at 100v.
9. After electrophoresis, The PCR products will be visualized under UV light in gel documentation system (Biorad).