Genetic and Molecular Profiling of FIX (FACTOR 9) Gene of Hemophilia B in Karnataka

Thesis submitted to the BLDE (DU) University for the Partial Fulfilment for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

ALLIED HEALTH SCIENCE

(HUMAN GENETICS)

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ACKNOWLEDGEMENTS

First, I wish to express my gratitude to the Almighty God, who brought me out of my mother's womb. He is my strength, my refuge, my shelter and my ever present help, so I shall not be in want. The lifter of my head and the giver of all wisdom, He truly deserves all the glory and the honour, for He did not let my foot slip.

It is with great pleasure that I express my sincere thanks and profound gratitude to my research guide Professor and Head Dr. Rudragouda S Bulagouda, Department of Anatomy, BLDE University, Karnataka, India and co guide Dr Sanjeev I Kolagi, Department of Anatomy, S Nijalingappa Medical College and HSK Hospital, Navanagar, Bagalkote and another co guide, Dr Pramod B. Gai, Director, Karnataka Institute for DNA Research, Dharwad for their inspiring guidance. I consider it a great privilege to have their thoughtful suggestions and directions, which enabled me to write the thesis and complete it on time.

I am thankful to Dr. Ashok S Mallapur, Professor & Dean S Nijalingappa Medical College and HSK Hospital, Navanagar, Bagalkote, Prof. Kusal K. Das, Laboratory of Vascular Physiology & Medicine, Department of Physiology, Shri B.M.Patil Medical college, BLDE (Deemed to be University), Viajayapur, Dr. Yasmeen A Maniyar, Co-ordinator, CRL, S Nijalingappa Medical College and HSK Hospital, Navanagar, Bagalkote, Dr. G. S. Kadkol, Asst Professor, Dept of Anatomy, and other ministerial staff from anatomy department, Shri B.M.Patil Medical college, BLDE (DU), Vijayapura for their encouragement to carry out my research work.

I express my heartfelt thanks to Dr. Suyamindra S. Kulkarni, Scientist "C", KIDNAR, Dharwad, Mr. Rajat V Hegde, Mrs. Smita R Hegde, SRF, KIDNAR, Dharwad for their help where I carried out all my experimental research work.

I thank Dr. Suresh Hanagavadi, Prof, Dept of Pathology, JJM Medical College, Davangere and the president of Karnataka Hemophilia Society who has helped me in the data collection and sample preparation. I am thankful to all the patients and their family members who have willingly participated in the study and provided their blood samples for this study. An expression of endless gratitude to

Dr Neelima Dongre, Dr Akaram Naikwadi, Smt Ashwini H, for providing facilities, and all those who rendered support for the successful completion of the thesis.

I wish to acknowledge the help rendered by the lab members of KIDNAR, Dharwad and Ph.D colleagues BLDE and SNMC. Last but not the least; I remain indebted to my parents and my wife Apoorva Kulkarni, for their constant support and encouragement throughout the course of this work.

Sujayendra Kulkarni

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ABBREVIATIONS

%	-	Percentage
µg	-	Microgram
µl	-	Microlitre
µM	-	Micro molar
^{0}C	-	Degree centigrade
HB	-	Hemophilia B
HA	-	Hemophilia A
HC	-	Hemophilia C
bp	-	Base pairs
cDNA	-	Complimentary Deoxyribonucleic acid
CFP	-	Covaris Fragmentation Plate
cm	-	Centimeter
CPP	-	Clean Up PCR Plate
CSGE	-	Conformation sensitive gel electrophoresis
CTL	-	Ligase Control
dATP	-	2'-deoxyadenosine 5'-triphosphate
DCT	-	Diluted Cluster Template
dCTP	-	2'-deoxycytidine 5'-triphosphate
ddNTP	-	2',3'-dideoxynucleotide 5'-triphosphate
DDW	-	Double distilled water
DEPC	-	diethyl pyrocarbonate
dGTP	-	2'-deoxyguanosine 5'-triphosphate
DNA	-	Deoxyribonucleic acid
dNTP	-	2'-deoxynucleotide 5'-triphosphate
dsDNA	-	double stranded DNA
dTTP	-	2'-deoxythymidine 5'-triphosphate
EDTA	-	Ethylene diamine tetra acetic acid
Et.Br	-	Ethidium bromide
EUC	-	Experienced User Card
g	-	Gram
gDNA	-	genomic DNA
GOI	-	Gene of interest

HT	-	High Throughput
IMP	-	Insert Modification Plate
Indel	-	Insertion and deletion
ISP	-	Intermediate Source Plate
kb	-	kilo base
LIG	-	DNA Ligation Mix
LT	-	Low Throughput
LTF	-	Lab Tracking Form
M	-	Molarity
mA	-	Milli ampere
mg	-	Milligram
min.	-	Minutes
ml	-	Milliliter
mm	-	Millimeter
mM	-	Millimolar
N	-	Normality
NaOH	-	Sodium hydroxide
ng	-	Nanogram
nm	-	Nanometer
OD	-	Optical density
PCR	-	Polymerase chain reaction
pM	-	Picomole
PMM	-	PCR Master Mix
PPC	-	PCR Primer Cocktail
QA	-	Quality Assessment
QC	-	Quality control
qPCR	-	Quantitative Polymerase chain reaction
RBC	-	Red blood cells
RNA	-	Ribonucleic acid
rpm	-	Revolutions per minute
RSB	-	Resuspension Buffer
RT-PCR	-	Reverse transcription PCR
s	-	Seconds
SNP	-	Single nucleotide polymorphism

SSC	-	Sodium saline citrate
SSCP	-	Single-strand conformation polymorphism
SSP	-	Size Separate Plate
STL	-	Stop Ligase Mix
TAE	-	Tris-Acetate-EDTA
TE	-	Tris-EDTA
TSP	-	Target Sample Plate
UCSC	-	University of California, Santa Cruz
UV	-	Ultraviolet
V	-	Volts
v/v	-	Volume/Volume
W	-	Watts
w/v	-	Weight/Volume
WBC	-	White blood c

CHAPTER 1

INTRODUCTION

Bleeding disorders are one of the rare genetic disorders which are characterized by persistent or increasing bleeding. Hemophilia is one of many diseases in the category of hereditary bleeding disorders (1). Hemophilia is characterized by abnormal or excessive bleeding. Hemophilia has been a Royal disease for its effect on the Royal family of Europe. Two of Queen Victoria's daughters were carriers for the disease and passed the defective gene to other Royal families, including that of Leopald, who had haemophilia, and other members of the family (2).

1.1 Types of Hemophilia

Among different bleeding disorders such as factor deficiencies of FV, FVII, FIX, FX, FXI, FXIII, and fibrinogen; Hemophilia A (factor VIII deficiency), Hemophilia B (Factor IX deficiency), Hemophilia C (factor XI) are the most common types.

1.1.1 Hemophilia A

Hemophilia A is an X-linked recessive blood coagulopathy, arising out of the deficiency of clotting factor VIII. Factor VIII (FVIII) is a protein cofactor, which on activation in the presence of calcium ions and phospholipid surface, forms an active complex with factor IX called tenase complex, which further activates factor X to complete the coagulation (3). It can be inherited or acquired; inherited haemophilia A is brought on by a mutation in the FVIII gene, whereas acquired haemophilia A is brought on by an inhibitory autoantibody to the FVIII gene. A spontaneous mutation that is frequently passed down from parents to children will account for about one-third of the instances. 1 in 5000 male babies are born with haemophilia A (4).

1.1.2 Hemophilia B

Hemophilia B (HB), also known as Christmas disease, is the second most prevalent type of haemophilia. It is a genetic disorder caused on by a lack of or a fault in the factor IX gene, also known as factor IX deficiency, and affects roughly 1 in 20–30,000 live male births. In 1952, Stephen Christmas identified Hemophilia B. The factor IX gene is located on chromosome Xq27.1 (5).

The main sign of the condition is bleeding, which can occasionally but not usually happen after a baby is circumcised. When a baby gets older, further bleeding issues frequently emerge. People with haemophilia B are advised to get the hepatitis B vaccine because they have a higher risk of getting the disease from contact with blood products (4).

1.1.3 Hemophilia C

Factor XI (FXI) deficiency, commonly known as haemophilia C, is an autosomal recessive condition marked by the lack or insufficiency of FXI in plasma. According to existing studies, FXI deficiency affects between 1 in 100,000 and 1 in 1 million people. Clinical signs of the illness might range from mild bleeding to severe bleeding, including hemarthrosis and gastrointestinal bleeding (6).

The pattern of inheritance for FXI deficiency is often incompletely recessive or intermediate. Therefore, severe bleeding is a possibility for both heterozygotes and homozygotes. The FXI gene has 15 exons and is found on chromosome 4 long arm (4q35). Exons 3 to 10 code for the four apple domains, while exons 11 to 15 code for the carboxyterminal, which contains the active site. Exon 1 is non-coding. Exon 2 codes for the signal peptide (7).

1.2 Coagulation Pathway

The process of blood coagulation converts the circulating components of the blood into an insoluble gel. The gel plugs seal blood artery leaks and stops blood loss. Coagulation factors, calcium, and phospholipids are necessary for the procedure. The 11 proteins in blood known as coagulation factors operate in concert to cause blood to clot. A bleeding condition is brought on by a reduction in any one of these protein quantity or functionality (8).

- Factor I – fibrinogen
- Factor II – 2rothrombin
- Factor III – tissue thromboplastin (tissue factor)
- Factor IV – ionized calcium (Ca++)
- Factor V – labile factor or proaccelerin

- Factor VI – unassigned
- Factor VII – stable factor or proconvertin
- Factor VIII – antihemophilic factor
- Factor IX – plasma thromboplastin component, Christmas factor
- Factor X – Stuart-Prower factor
- Factor XI – plasma thromboplastin antecedent
- Factor XII – Hageman factor
- Factor XIII – fibrin-stabilizing factor

The normal hemostasis or coagulation process has three steps. To reduce bleeding, the wounded blood vessels contract. At the site of the injury, the blood platelets then adhere to the endothelial cells that line the blood channel walls. Platelets start to gather, which causes them to plug together and form the major clot. The fibrinogen is converted to fibrin by the plasma proteins, creating a stable fibrin network that clots the blood. Through both internal and extrinsic coagulation routes, this network is constructed. Together, these processes cause fibrin to be produced from fibrinogen. The 11 clotting factors circulate inactively until they are sequentially transformed into fully activated enzymes that trigger the activation of next factor in the sequence (8,9).

The intrinsic pathway is activated by the contact of blood with the damaged vessel wall and the conversion of factor XII into its active form. When factor V is present, the common pathway activates factor X, which in turn activates factor XI, factor IX, and factor VIII with von Willebrand factor, converting 3rothrombin into thrombin. Fibrinogen is then transformed into fibrin by thrombin (10).

In the extrinsic pathway, the subendothelial tissue factor that is released during tissue injury activates Factor VII, which in turn activates Factor X, which then through the common pathway activates FV and FII and finally converts fibrinogen to fibrin (11) (Fig.1).

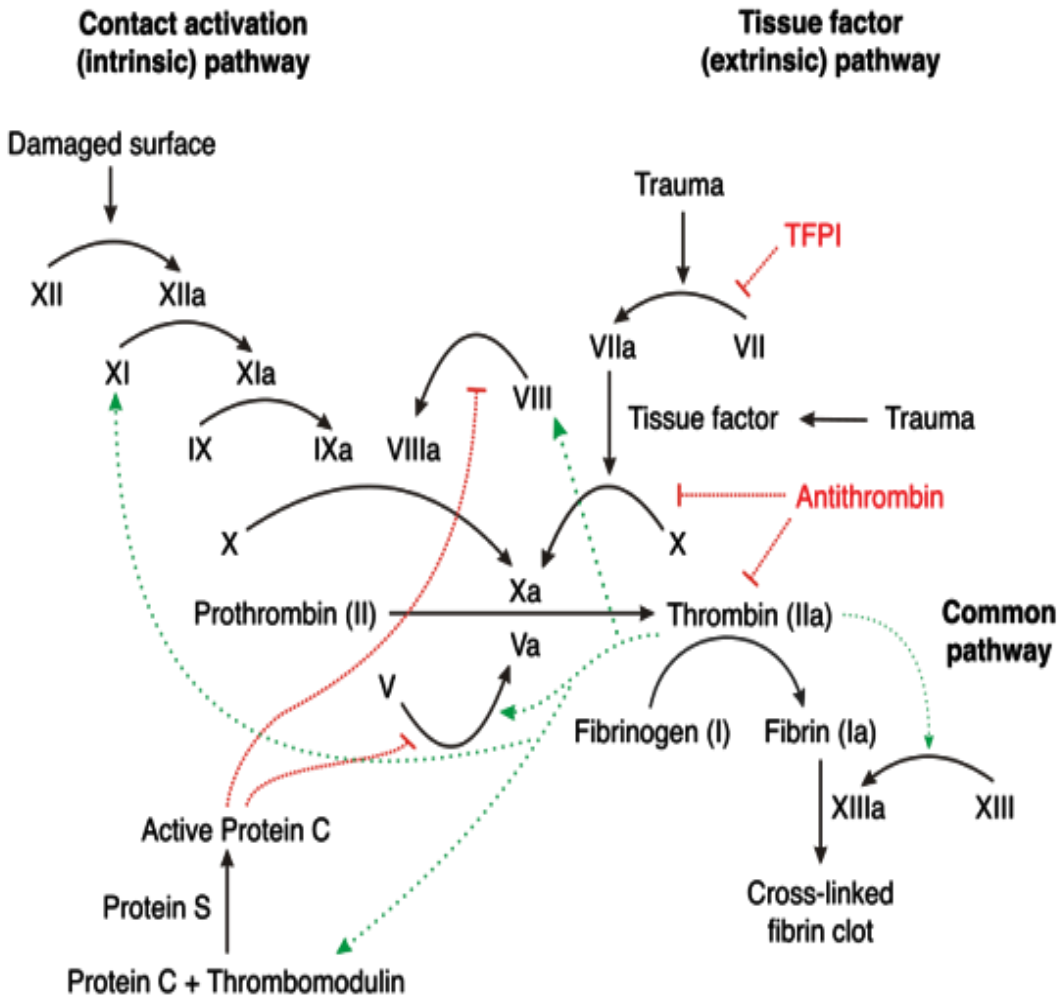


Fig 1: Blood coagulation pathway.

1.2 Inheritance of Hemophilia

Haemophilia is an X-linked inherited disorder. The genetic alteration causing hemophilia is passed down from parent to child through generations (4). Male with hemophilia will pass the altered gene on to their daughters but not their sons because of the presence of the defective gene on X-chromosomes. Women who carry the altered gene in heterozygous condition can pass it on to their sons and daughters. They are called as Carriers. Sons with the gene will have hemophilia (12) (Fig.2).

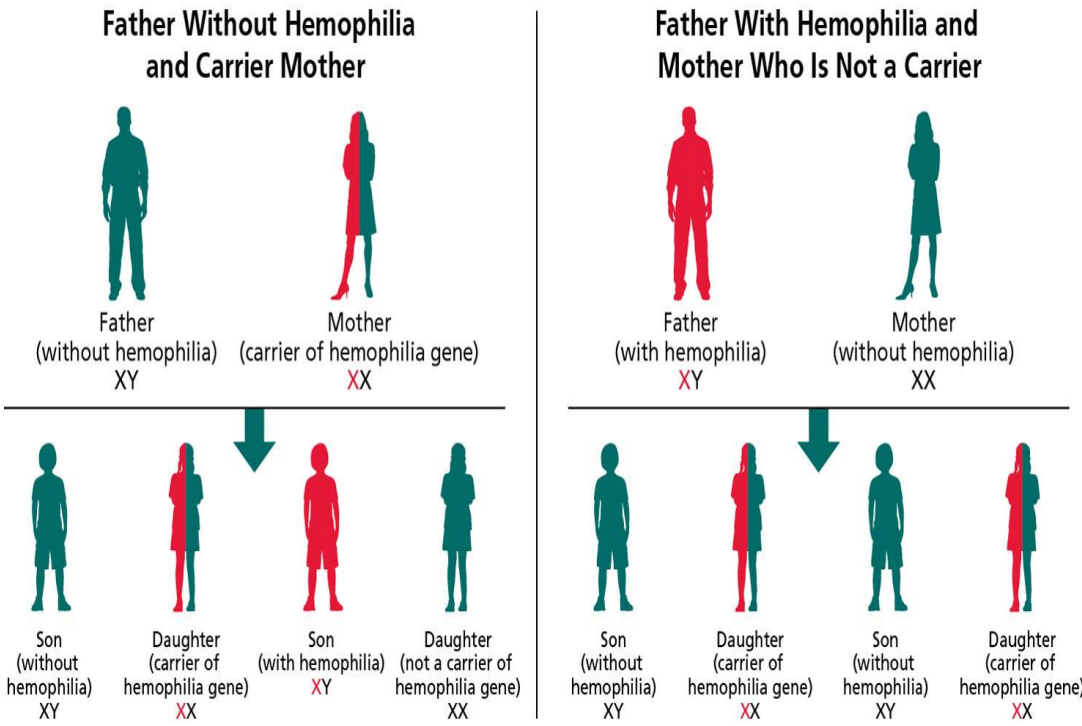


Fig 2: Pattern showing the genetic inheritance of Haemophilia.

1.4 Hemophilia B

Hemophilia B (Christmas disease) (HB) is a hereditary x-linked recessive bleeding disorder of coagulation caused by mutation in factor IX (FIX) gene which results in FIX deficiency (4 , 13).

The main treatment for hemophilia is called replacement therapy. Currently, the most difficult side effect of medication is inhibitor (alloantibody) formation. A hereditary propensity for producing inhibitors is related to the type of FIX gene mutation. These study's goals include locating the FIX gene mutations, understanding the molecular underpinnings of HB disease and identifying the mutations that cause various manifestations of the disease (14).

Hemophilia B results in deficiency of functional plasma coagulation factor IX. Spontaneous mutation and acquired immunologic processes can result in this disorder as well. Hemophilia B constitutes approximately 20% of hemophilia patients, and about 50% of HB patients have factor IX levels greater than 1%. The role of the coagulation system is to produce a stable fibrin clot at sites of injury (15). FIX activity can be classified into severe (< 1%), moderate (1–5%), and mild (5–30%) hemophila based on factor 9 concentration (16).

1.4.1 Signs and symptoms

The hallmark of hemophilia is hemorrhage in the joints. This bleeding is painful and leads to a long-term inflammation and deterioration of the joint (typically the ankles in children knees, elbows in adolescents and adults), resulting in permanent deformities, misalignment, loss of mobility, and extremities of unequal lengths. Hemorrhage in people with mild haemophilia is most likely to happen during trauma or during surgery. Before mild or severe haemophilia is suspected, a traumatic challenge that happens very late in childhood may take place (17,18) .

The following are signs and symptoms of moderate and severe haemophilia:

- Neonates: Intracranial haemorrhage, prolonged bleeding, and/or severe hematoma after procedures including circumcision, phlebotomy, and/or vaccinations.
- Toddler: Teething-associated oral bleeding and soft tissue bleeding related to trauma.
- Children: Traumatic intracranial haemorrhage (life threatening), persistent arthropathy, and hematomas with increased physical activity

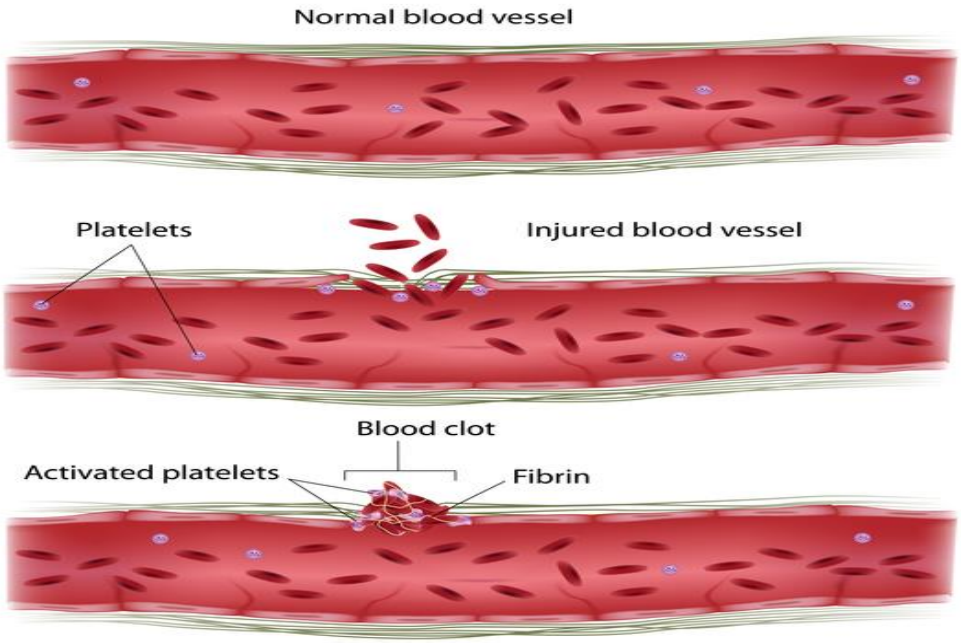


Fig 3: Normal blood clotting mechanism

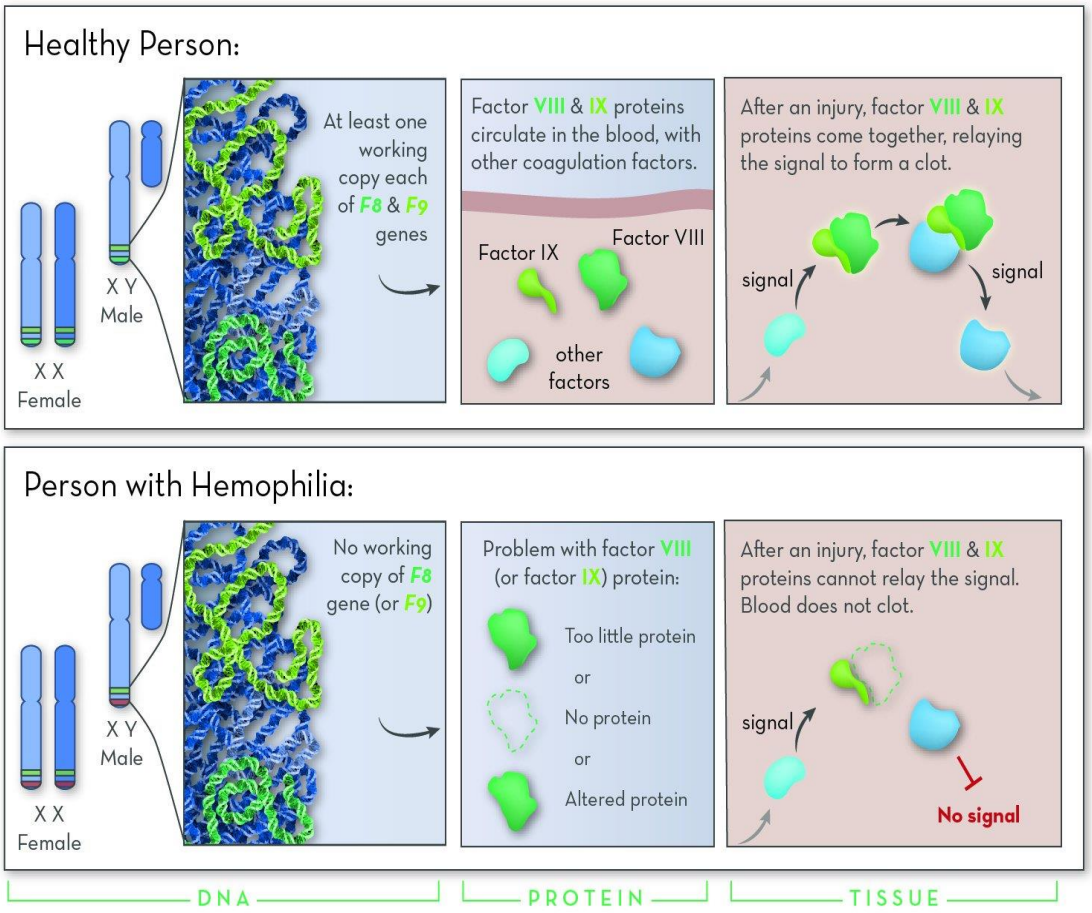


Fig 4: Blood coagulation in healthy and hemophilic person

1.4.2 Clinical features of Heamophilia B

Immediate or delayed bleeding and prolonged oozing after injuries, tooth extraction, surgery or bleeding after the initial bleeding stoped (19,20). Hemophilia B disease is diagnosed by using clinical features such as swelling, hemarthrosis, deep muscle hematomas, intracranial bleeding, prolonged or delayed bleeding, poor wound healing, hematuria (21).

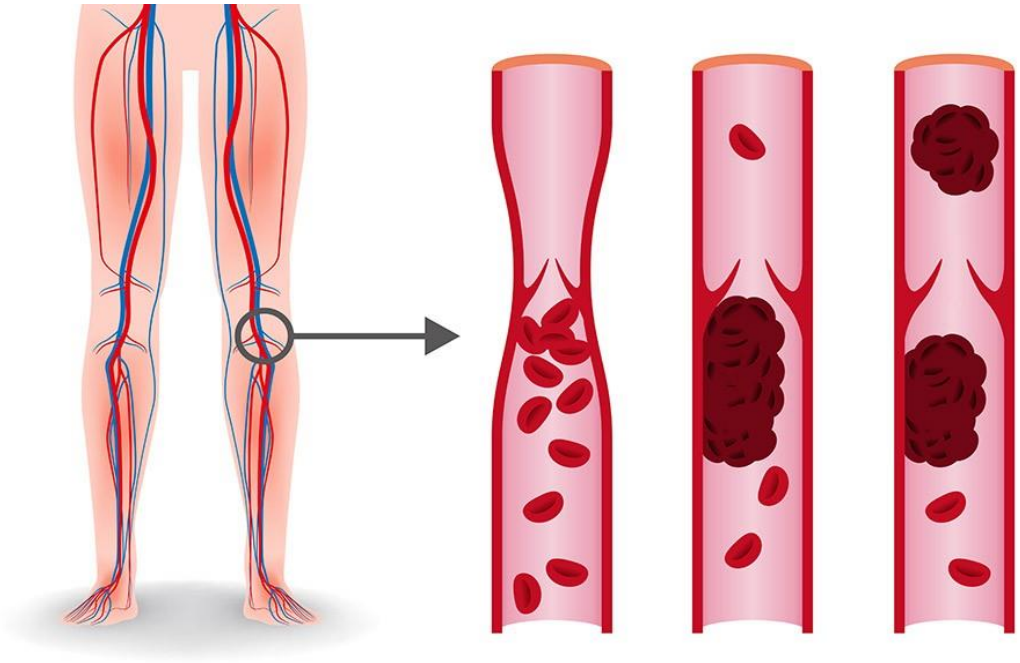


Fig.5: Clinical features of hemophilia B

1.4.3 Treatment

- Intravenous infusion of plasma derived or recombinant factor 9 - for bleeding that should be initiated within the hour of noticing symptoms.
- Pediatric issues - Special consideration for care of infants and children.
- Immunization should be administered subcutaneously;

  Note: Immunization should be administered subcutaneously; Intramuscular injection should be avoided unless under factor coverage (22).

1.4.4 Genetics of Hemophilia B

Factor IX protein consist of 415 amino acids, its molecular weight is 57,000 D (23). It is approximately 34kb in length and and is located on the long arm of the X chromosome at Xq27.1 (24). It contains eight exons, the exon 8 is largest & it contain 1935bp and FIX mRNA comprises of 2.8 kb. The haemophilia B is classified on the basis of level of FIX cotting factor activity in the plasma, with<1%of normal levels causing severe,1-5% activity causing moderate and 5-30% activity causing mild symptoms (25).

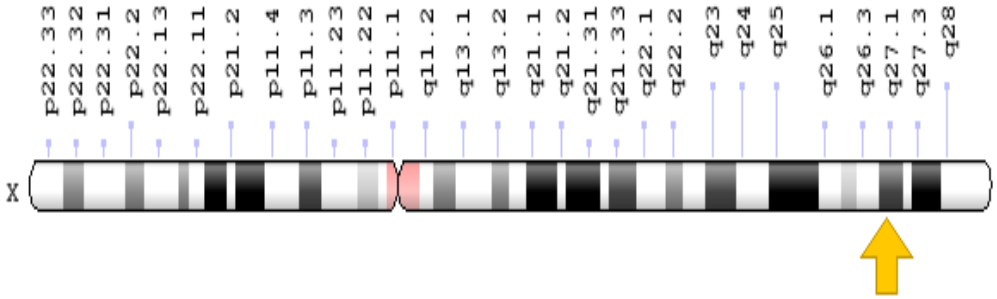


Fig.6: Chromosomal location of X-linked *F9* gene

Hemophilia B contain 8 exons (a-h) encoding 6 major domains of Factor IX These are (26, 27).

- Exon-a (117bp) – Hydrophobic signal peptide which targets the protein for secretion from the hepatocyte into the blood stream.
- Exon b (164bp) and Exon c (25bp)– Propeptide & gladomain – the latter containing 12 gamma Residues. This post translational modification is required for the correct folding & calcium binding of Factor IX.
- Exon d (114bp) - Its type B or 1st epidermal growth factor, like domain, which shows homology to epidermal growth factor (E & F) in addition , contains conserved carboxylate residues including a beta-hydroxyasperate at amino acid 64. This domain binds additional calcium with high affinity.
- Exon e(129bp) – It's type A or 2nd EGF domain which lacks the conserved carboxylate residues of the E &F type B domain.
- Exon f (203bp)– An activation domain, within which Factor Xia cleaves twice, converting Factor IX to IXa.
- Exon g(115bp) and Exon h(1935bp) – The serine protease or catalytic domain responsible for proteolysis of Factor X to Factor Xa (26,29).

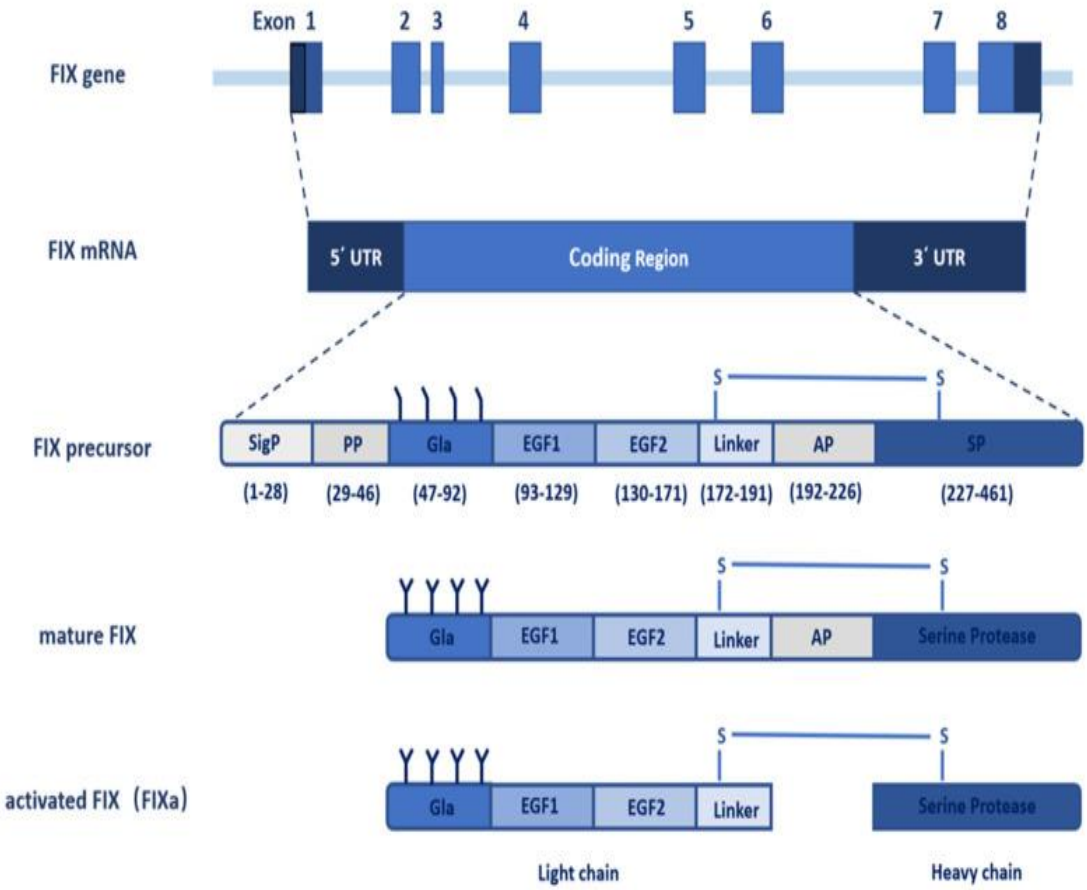


Fig.7: Schematic diagram of the *F9* gene structure and FIX protein processing.

A mutation in *F9* gene has been mostly expressed in males. Low activity of plasma FIX is necessary to confirm a diagnosis of HB. Severity of HB cases is determined by plasma FIX levels and is closely correlated with the kind and location of FIX gene mutations. Currently, 1095 mutations in the FIX gene have been found globally, with point mutations making up 73%, deletions 16.3%, and insertions, duplications, or combinations of insertions and deletions making up the rest (29).

In this study, we performed genetic analysis of *F9* genes of 150 Hemophilia B patients from the hemophilia society of Karnataka, India, to look for novel molecular alteration using sequencing technology.

CHAPTER 2

OBJECTIVES AND HYPOTHESIS OF THE STUDY

2.1 Objectives:

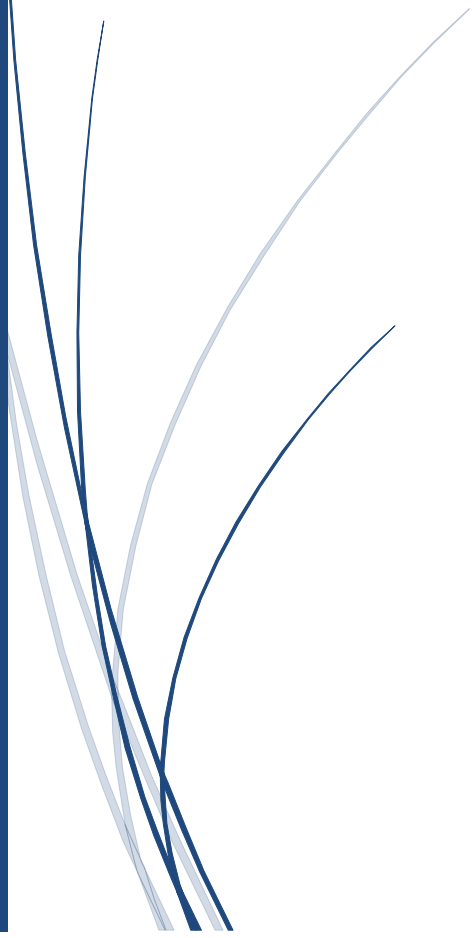
1. Collecting data relating to Hemophilia B and finding out the prevalence and incidence of Hemophilia B in this region.
2. Finding out the possible role of factor IX (FIX) cellular path way genes in Hemophilia B.
3. Defining the candidate genes and their possible role in Hemophilia B.
4. Mutation analysis of selected genes among targeted genes using automated DNA sequencing methods.
5. Identification of novel mutations.

2.2 Hypothesis:

Hemophilia B is a bleeding disorder caused by the genetic deffect in F9 gene and it has high mortality and disability. Even though hemophilia B is single gene disorder but it is heterogenic in nature interms of genetic/DNA mutations. India is known for multiethnic population with 1.4 bilions of people and previous studies are recorded different results with respect to types of mutations. At present, there are no enough effective markers for carrier detection and prenatal diagnosis of hemophilia B in our population, with on this hypothesis, the present study “Molecular Genetic study of Hemophilia B in Karnataka Population” was undertaken to evaluate the role of F9 gene mutation.

CHAPTER 3

REVIEW OF LITERATURE



Hemophilia B, a hereditary blood coagulation disorder, is caused by a factor IX deficiency. Females who have one affected factor IX gene are carriers but show no symptoms. Male offspring of carriers have a 50% chance of inheriting the disease, while female offspring have a 50% chance of becoming carriers. It is brought on by a lack of factor IX, a blood plasma protein. The blood cannot properly clot to control bleeding if there are not enough factors IX present. Seven times more people have hemophilia A than hemophilia B. A lack of clotting factor IX causes hemophilia B (4).

In India, hemophilia treatment is currently evolving and developing and is about to become a mainstream healthcare consideration (18). Kawankar N et al. (2016) conducted a clinical and molecular epidemiology study on 10 unrelated Indian patients with FXI deficiency. The diagnosis of FXI deficiency was made in 2 patients after surgery when they presented with excessive bleeding among 10 patients (age range 4-66 years, median age 28 years; 6 men and 4 women). Four patients had no history of bleeding, while two had joint bleeds when they were examined. The final two patients only experienced brief episodes of bleeding, such as epistaxis. Eight mutations were found in ten patients, with one common novel splice site mutation, IVS3 + 4A N G, found in three unrelated patients. FXI deficiency is an autosomal recessive disease that is mostly found in the Ashkenazi Jewish population, though it is occasionally reported in non-Jewish populations. In some studies, the heterozygote frequencies of the F11 mutation in the Ashkenazi Jewish population were found to be as high as 11%; as a result, routine testing for FXI deficiency prior to surgery is required in these populations. However, in the Indian population, neither the type of mutations nor the FXI levels were associated with the bleeding symptoms (2).

Rare X-linked bleeding disorders hemophilia A and hemophilia B are brought on by mutations in the genes for coagulation factor VIII (FVIII) and factor IX (FIX). With a prevalence of one in every 5,000 male live births compared to one in every 30,000, hemophilia A (HA) is more prevalent than hemophilia B (HB). The plasma level of FVIII or FIX activity is used to classify the severity of hemophilia. A factor level of 1% or less of normal is considered severe, a level of 1% to 5% is considered moderate, and a level of >5% and 40% is considered mild. Both the F8 and F9 genes are found on the X chromosome, with the F8 gene being at the end of the long arm at Xq285, and the F9 IX gene on the long arm, more toward the centromere, at Xq27.6.

The F8 gene is extremely large (approximately 180 kb) and structurally complex (26 exons), whereas the F9 gene is significantly smaller (approximately 34 kb in length). Numerous thousands of patients have helped to characterize the mutations that cause hemophilia A and B. The broad range of mutations that have been identified highlights the fact that the molecular causes of hemophilia are extremely diverse (13).

A national strategy for the prevention and management of genetic disorders is inadequate in India. There is little information on the epidemiology of other genetic illnesses in India, despite the fact that hemoglobinopathies have gotten some attention. When untreated or receiving inadequate care, haemophilia, an inherited single gene illness with a prevalence of 1 per 10,000 births, presents in individuals as spontaneous or trauma-induced hemorrhagic episodes and progresses to lifelong impairment and premature mortality.Although the genetics of this disorder has been extensively investigated in India, there is no information available on the prevalence, trends, social costs, or opportunities for providing genetic counselling as part of a public health initiative. India is home to the second-highest number of haemophilia A patients worldwide. 11,586 cases with haemophilia A have been reported, however there may be as many as 50,000 people who are affected. This evaluation also highlights the urgent need to launch a nationwide haemophilia programme with elements of prevention, patient care, surveillance, education, and support for families (34).

Ghosh K et.al (2009) studied spectrum of FIX gene mutations causing haemophilia B from India. In 93 patients 45 single-base substitutions, one donar splice, 2 small delection, 9 nonsense mutations, 17 novel mutations and 2 common mutations were detected. They concluded, in 6 out of 93 patients having double mutation were severe with less than 1% FIX levels, this is because defect in an A-G transition at genomic nucleotide position 31142. Majority of mutation located in the exon 8 which code for the catalytic domain (35).

A. Mahajan et.al (2004) studied Novel Missense mutation in the coagulation factor IX catalytic domain associated with severe haemophilia B. FIX is a vitamin K dependent multidomain protein which exists as a zymogen in the blood. Zymogen gets activated through proteolytic cleavage by FIXa and tissue factor. Activated factor IXa in presence of calcium and FVIIIa, enzymatically converts FX to FXa to bring about

the coagulation cascade, this causes abnormal protein named as factor IXDehli. Hence, they concluded that this abnormal protein affected it's surface loop and altered the Ca2+ accommodation, there by contributing to severe bleeding disorder (36).

S. Mukherjee et.al (2008) worked on structural analysis of FIX protein variants to predict functional aberration causing haemophilia B. They include out of total 16 severe mutations, 14 showed changes of hydrogen bonding pattern variable extent. Among the 9 mild haemophilia B mutation, 6 showed no change in hydrogen bonding pattern .By this they reported the effect of point mutation on the crystal structure of the native FIX by measuring the change in hydrogen- bonding pattern and electrostatic potential. The effect of missense mutations in FIX gene on the interaction of mutant proteins with other factors of the coagulation pathway was altered (37).

Sexton et.al (2009) studied two mutations in the FIX gene in a family with haemophilia B. Genetics testing had identified two mutation in FIX gene in the proband known as -23C>T and 30802insA. Hence, they concluded that haemophilia B Leyden phenotype was associated with the -23C>T promoter mutation of the FIX gene and also demonstrated that the 30802insA mutation of the FIX gene wasS not associated with lowered FIX levels or haemophilia B and should be regadred as a non-pathogenic variant (30).

Leera Quadeos et al., (2007) worked on a common G10430A mutation in haemophilia B in the majority of the Gujarati population. They included 22 patients in which 19 were unrelated, 3 were related. They concluded that a common G10430A (Gly60Ser) mutation, could be used for accurate carrier detection and prenatal diagnosis in mild to moderate factor IX deficient patients belonging to Gujarat state of western India (31).

S. Saini et al. (2014) investigated the genetic influences on FIX immunogenicity during the management of haemophilia B. The successful management of haemophilia B is restricted by inhibitors. The nature of the FIX gene mutation may be an important risk factor for the development of inhibitors. They concluded that, The Human Leucocyte Antigen alleles of individual patients, in conjuction with the mutation type, could be predictor for the development of inhibitors as the response to immune tolerance induction (32).

Lv X et al., in 2019 performed the genetic and prenatal diagnosis of hemopjilia b family using Sanger sequencing technology. All the exonic region and flanking regions were analysed for molecular alterations. Novel and previously recorded pathogenmic mutations were recorded and concluded that mutations of F9 gene in hemophilia B patients shows high polymorphism (67).

Kadhim K et.al (2019) prospectively described the epidemiological and clinical characteristics of patients with hemophilia. This cross-sectional study was carried out in Baghdad's four hemophilia treatment facilities. Between 2007 and 2016, the records of all hemophilia patients as well as the clinics' registry books were examined to compile the data. In Baghdad, hemophilia incidence and prevalence doubled over a ten-year period. Different complications were more common than they were in the neighboring nations, almost on par (11).

CHAPTER 4

MATERIALS AND METHODS

4.1 Study design: Cross-sectional study.

4.2 Study duration: Four years (March 2017- December 2021)

4.3 Source of data: Karnataka Hemophilia society.

4.4 Sample size: The sample size estimated for the proposed study was 150, which is calculated in OpenEpi, Version 3, using formula. Sample size *n* = [DEFF*Np (1-p)]/ [($d^2/Z^2_{1-\alpha/2}$*(N-1)+ p*(1-p)] and adding an 10% sample loss.

4.4.1 Calculation of sample size

$$n_1 = \frac{(\sigma_1^2 + \sigma_2^2 / \kappa)(z_{1-\alpha/2} + z_{1-\beta})^2}{\Delta^2}$$

$$n_2 = \frac{(\kappa * \sigma_1^2 + \sigma_2^2)(z_{1-\alpha/2} + z_{1-\beta})^2}{\Delta^2}$$

The notation for the formulae are:

n_1 = sample size of Group 1

n_2 = sample size of Group 2

σ_1 = standard deviation of Group 1

σ_2 = standard deviation of Group 2

Δ = difference in group means

κ = ratio = n_2/n_1

$Z_{1-\alpha/2}$ = two-sided Z value (eg. Z=1.96 for 95% confidence interval).

$Z_{1-\beta}$ = power

4.5 Ethical clearance

Institutional Ethics committee clearance was obtained from Shri B. M. Patil Medical College, BLDE (Deemed to be University) (BLDE (DU)/IEC/340/2018-19 dated 21-12-2018) and S. Nijalingappa Medical College, Bagalkot (SNMC/IECHSR/2018-19/A-82/1.1 dated 15-02-2019).

4.5.1 Consent: Written Informed consent was obtained from all the participants before enrollment.

4.6 Inclusion criteria

1. All haemophilia B patients who have been registered and in the centers from 2018 to December 2020 were included in the study.
2. Computerized treatment records were started at the center in 2011. Data of patients with inhibitors have been collected and include the following information:
   - ✓ Age of the patient at diagnosis of hemophilia B and detection of the inhibitor to F VIII and F IX
   - ✓ Ethnic origin
   - ✓ Severity of the hemophilia
   - ✓ Family history of hemophilia
   - ✓ Nature of the inhibitors (persistent or transient)
   - ✓ Types of products used and exposure days prior to inhibitor development. Age and cause of death for the diseased patients.

4.7 Exclusion criteria

1. Severally ill patients
2. Patients with other comorbidities
3. Those who don't give consent.

4.8 Sample collection

Selected Hemophilia B affected patients of Karnataka origin were enrolled in this study. Written informed consent was obtained from each participant and all the participants included in this study were male. The participants were from different part of Karnataka, cared from the association of Hemophilia Society, Karnataka.

The blood sample was taken from venipuncture into vacutanier tubes containing trisodium citrate (0.109M) and ethylenediaminetetraacetic acid as an anticoagulant. Following tests were performed; activated partial thromboplastin time (aPTT), FIX activity and FVIII activity. The severity of the disease was classified based on FIX activity. Clotting factor inhibitor screening was performed by activated partial thromboplastin time mixing studies using normal pool plasma.

Sample collection Site

Fig.8 Sample collection sites

Table.1 List of Hemophilia Societies in Karnataka where samples were collected.

Sl No	Hemophilia Society	District	In Charge
1	Hemophilia Society	Bangalore Chapter	Dr. Premroop Alva
2	Hemophilia Society	Davangere Chapter	Dr. Suresh Hanagavadi
3	Hemophilia Society	Hassan Chapter	Dr. Palasha H. K.
4	Hemophilia Society	Gangavathi Chapter	Mr. Ramakrishana Ch. Dr. V. L Patel
5	Hemophilia Society	Hubli-Dharwad Chapter	Mr. Raju Kundanhalli (President)
6	Hemophilia Society	Manipal Chapter	Dr. Annamma Kurien
7	Hemophilia Society	Mysuru Chapter	Dr. S.K. Mittal
8	Hemophilia Society	Bagalkot Chapter	Dr.B. C.Yelameli
9	Hemophilia Society	Gulburga Chapter	Dr. Asif Attar

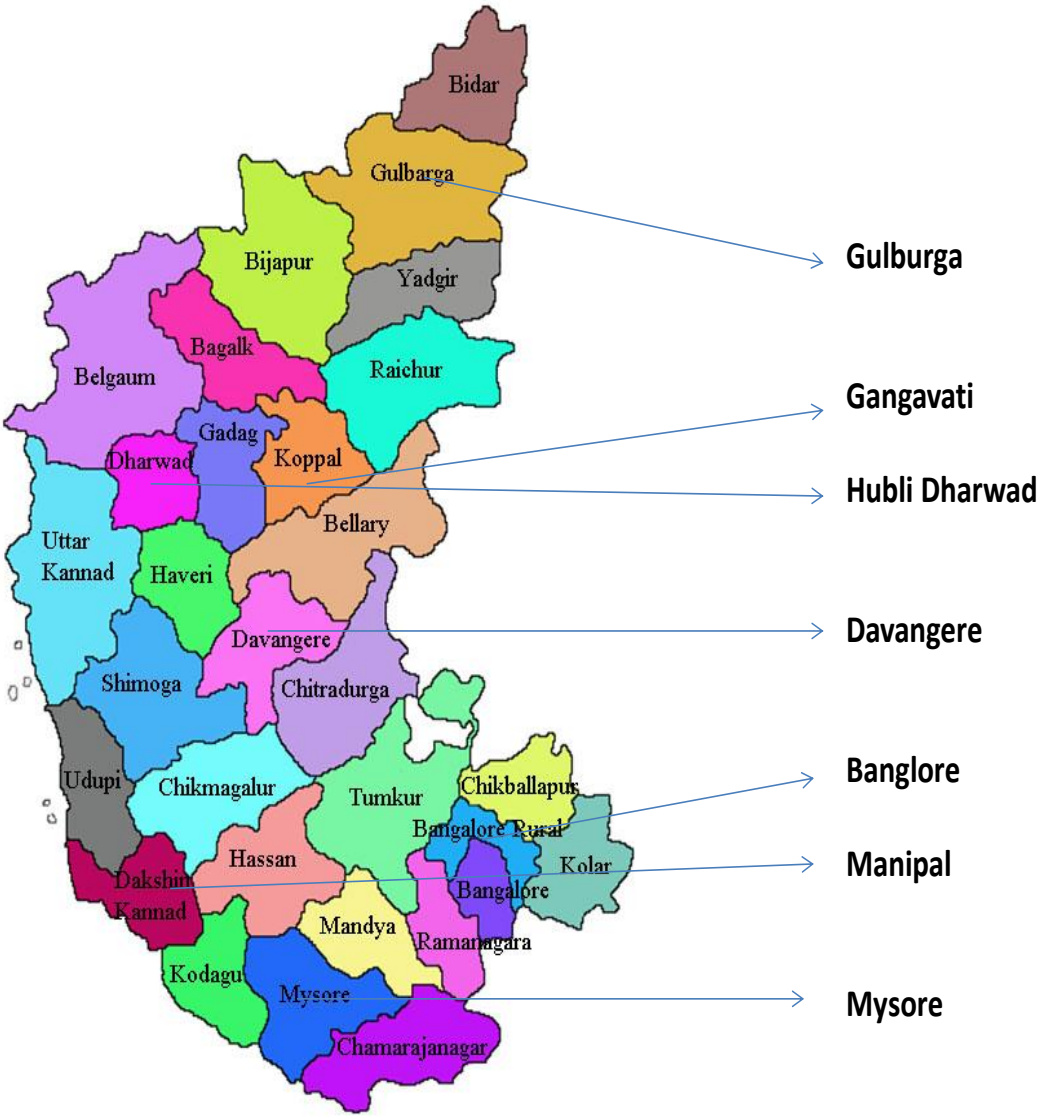


Fig.9: Geographical representation of location of Hemophilia chapters

4.9 DNA extraction

DNA Extraction was performed through spin column based Qiagen DNA extraction kit (Catlog No 51104).

About 2 to 3 ml of peripheral blood was collected into tubes containing ethylenediaminetetraacetic acid (EDTA); the DNA was extracted by kit method and stored at -20°C. This method comprises the following principles (a) lyses of cells with detergents that will disperse the lipid bilayers of membranes and denature proteins, especially those associated with DNA in chromatin. (b) Elimination of proteins by using a supersaturated NaCl solution. The denatured proteins form a precipitate, while the DNA remains in solution in the aqueous phase which was recovered after centrifugation. (c) Concentration of DNA by precipitation with alcohol (isopropanol) are added to the aqueous phase and redissolved in TE (Tris-EDTA) buffer.

Fig.10: DNA Isolation Kit

4.9.1 Isolation of genomic DNA:

Isolation of genomic DNA from blood was carried using Blood and Tissue DNA extraction Kit (QIAGEN, Germany).

- 20µl proteinase k was pipetted out into a 1.5ml micro centrifuge tube. 100µl blood sample was added and adjusted the final volume up to 220µl with an addition of potassium buffer saline (PBS).
- 200µl AL buffer, mix was added thoroughly by vortexing and incubate blood sample at 56°C for 10min.
- 200µl ethanol (96-100%) was added and Mixed thoroughly by vortexing.
- This mixture was pipetted out into a DNeasy Mini spin column placed in a 2ml collection tube. Centrifuged at 8000 rpm for 1 minute. Discarded the flow through and collection tube.
- Spin column was placed in a new 2ml collection tube and 500µl of AW1 buffer was added. Centrifuged for 1min at 8000rpm. The flow through was discarded
- Spin column was palced in a new 2ml collection tube and added 500µl AW2 buffer and centrifuged for 3 min at 14000 rpm. Flow-through and collection tube was diacrded.
- Again spin column was palced in to a new 2ml micro centrifuge tube.
- 200µl AE buffer was added to the centrifuged for the spin column membrane. Incubated for 1 min at 25°C. Centrifuged for 1min at 8000rpm.
- Eluted DNA was stored at -20°C until further analysis.

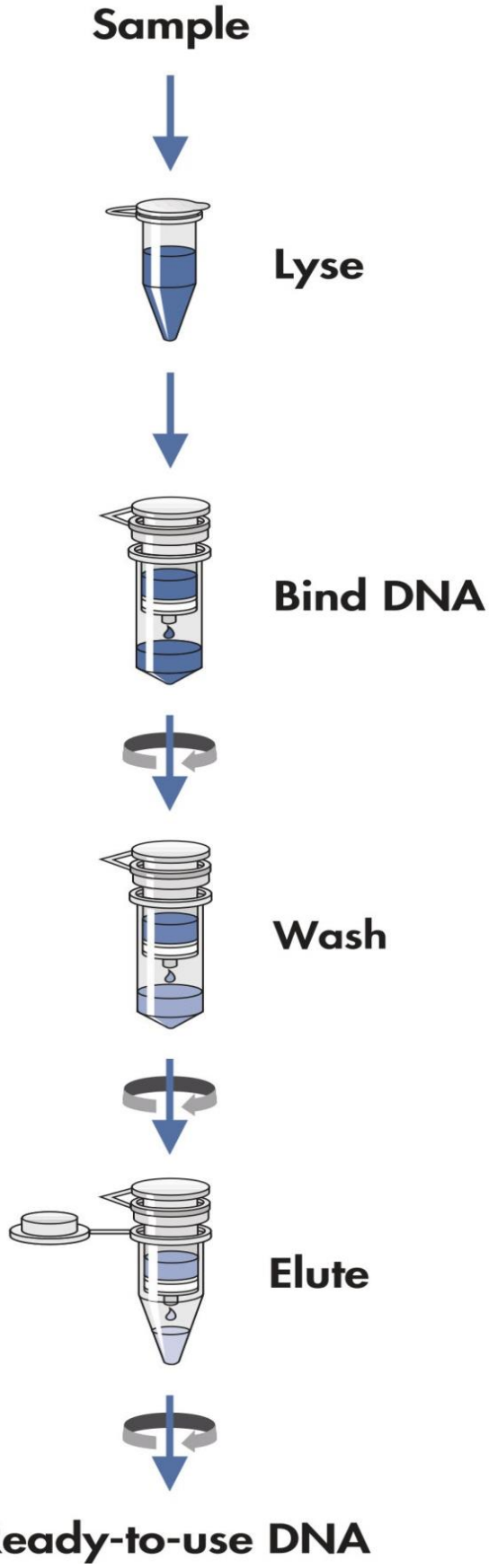


Fig.11: Schematic representation of DNA Isolation Protocol

4.9.2 Quality analysis of isolated genomic DNA (Gel electrophoresis)

Agarose gel electrophoresis is widely used to resolve or separate nucleic acid on the basis of molecular weight. Smaller fragments migrate faster than the larger fragments, and the size of the fragments can thus be determined by comparing the distance travelled by known size standard marker. Hence it helps in the quantification of DNA.

- 0.8% of agarose gel was prepared in 100ml of 1X TAE buffer by adding 0.8g of agarose. Dissolve the agarose by heating, and cool to 55˚C.
- 0.8µl of Ethidium bromide was mixed with the melted agarose and poured into the gel casting tray and the comb was inserted, making sure that no air bubbles were trapped in the gel. After solidifying, the comb was removed without causing any damage to the wells.
- The tray was placed in the electrophoretic tank containing 1X TAE buffer until the gel was completely submerged.
- 1µl of 1X loading dye was mixed with G.DNA. Samples were carefully loaded into the wells using a micropipette.
- After all the setup, the gel was allowed to run at 100 volts for 2 hours. The gel was visualized and photographed in the gel documentation unit.
- The intact double-stranded DNA forming a thick single band of high molecular weight confirmed the presence of good quality of extracted genomic DNA.

4.9.3 Quantification of DNA from nanodrop UV spectrophotometer

The NanoDrop Spectrophotometer from NanoDrop Technologies is designed for measuring nucleic acid concentrations in sample volumes of one microliter. The key to this advanced spectrophotometer is its unique sample retention technology that overcomes the need for cuvettes when taking measurements. This is accomplished by placing the sample directly on top of the detection surface and using the surface tension to create a column between the ends of optical fibers. Thus the measurement optical path is formed. The sensitivity range for DNA detection is between 2 and 3700 ng/ul. The spectral range of the device is 220 to 750nm and it is possible to scan all of the wavelengths.

DNA posseses maximum absorbance at around 260 nm based on existence coefficient and optical density at,

Absorbance = 260/280 = 1.8 = pure DNA

Absorbance = 260/280 = < 1.8 = protein contamination

Absorbance = 260/280 = > 1.8 = RNA contamination

4.10 Primer designing

Primers are short single stranded sequence of nucleic acid that serves as a starting point for DNA or RNA synthesis. While designing the primers, small intronic portions were added on both side of the coding region in order to avoid any kind of error or deletion of the coding region later in the process. Usually, the length of the primer is 18-24 nucleotides which are complementary to the target DNA fragment in our gene of interest.

4.10.1 The flow chart of primer designing

The sequence of the primer was generated using the "Bioinformatics Primer Designing Tool" (primer 3) for the *F9* gene of Hemophilia B gene as given below. These designed primers were confirmed through Insilico PCR method.

Go to http://www.ncbi.nlm.nih.gov.

↓

Search nucleotide reference sequence of the HB gene

↓

Select FASTA and convert to FASTA format.

↓

Mark all the exonic regions in the nucleotide sequence using GenBank format.

↓

Google search for primer 3 input version.

↓

Enter the exon regions and enter the product size depending upon the length of the sequence.

↓

The select the pick primer and the primer output is obtained.

↓

Mark the forward and reverse primers in the nucleotide sequence from primer 3 output.

↓

Go to Insilico PCR

↓

Enter left and right primer sequence

↓

Select BLAST and enter

↓

Compare the product size with that of primer 3 output

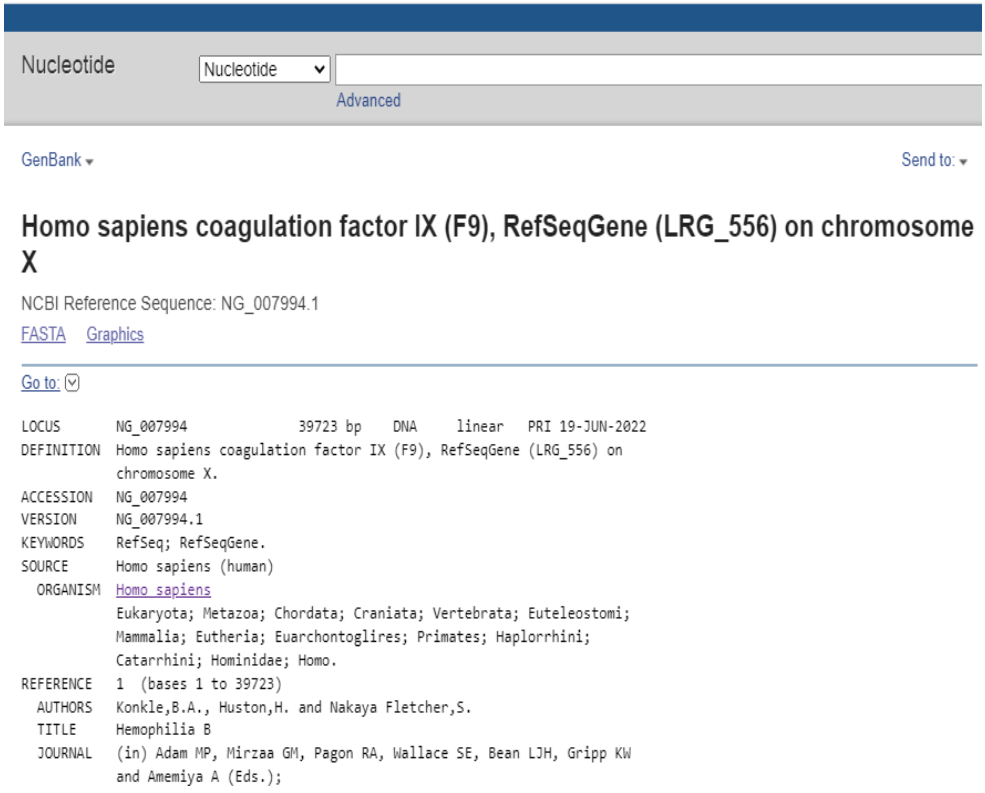


Fig. 12: Reference sequence of *F9* gene from NCBI

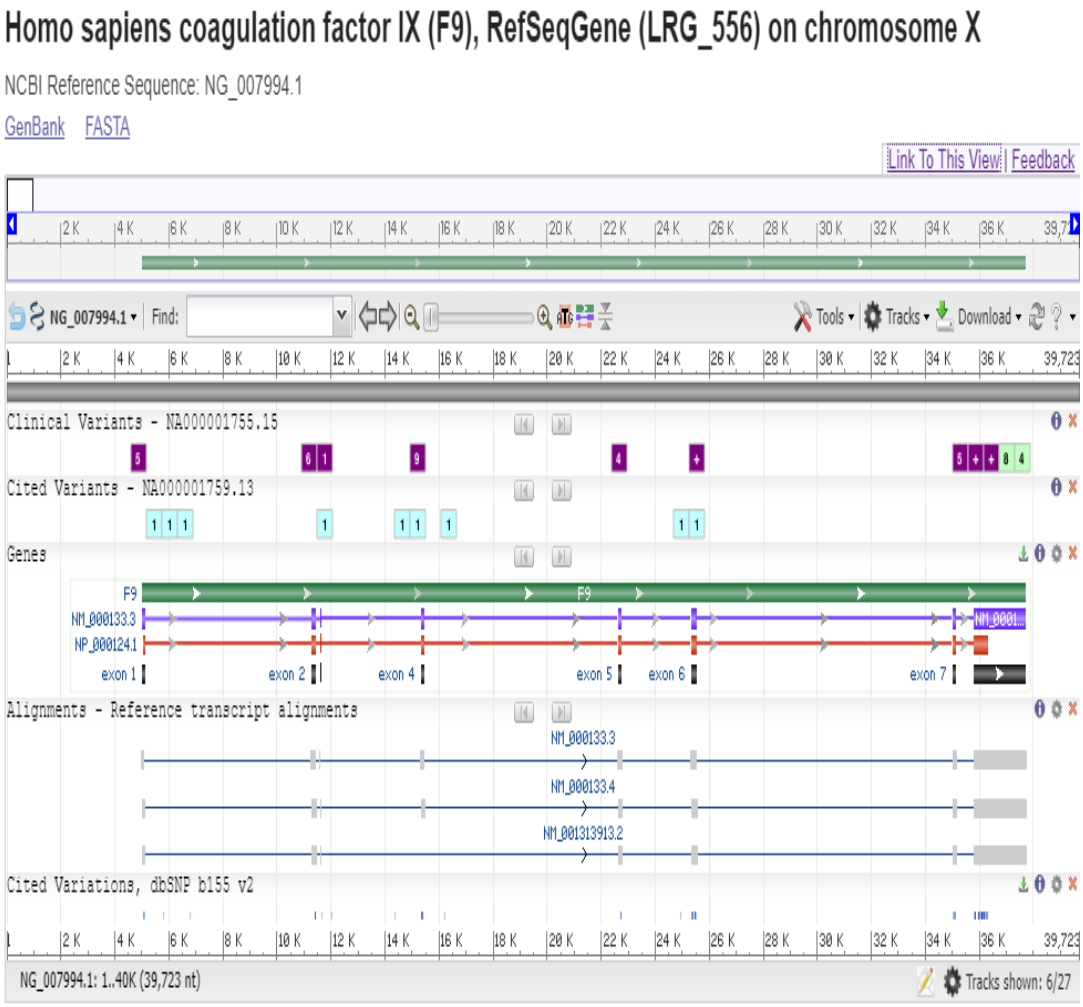


Fig.13: Graphical representation of *F9* gene from NCBI

4.11 PCR technique

PCR is a widely used method for amplification of a specific DNA sample and to rapidly make millions to billions of copies of a specific DNA sample. It works on the principle of thermal cycling which consists of cycles of repeated heating and cooling during the reaction of DNA denaturation and enzymatic replication of DNA. Being heat stable almost all PCR employs the enzyme called Taq polymerase which is originally isolated from the bacteria *thermusaquaticus.* The DNA polymerase enzymatically assembles a new DNA strand using single stranded DNA as template and primers.

PCR was first developed in 1924 by Kary Mullis and in 1993 who was awarded Nobel prize for chemistry for his work on PCR. PCR stands as an indespensible technique in medical and biological research labs for variety of application and also has become quite a common technique in today's world.

Most of the exons in the HB gene are relatively short. Direct sequencing of a complete exon is possible once it has been amplified from genomic DNA with a PCR technique therefore; all exons of the FIX gene were amplified by PCR

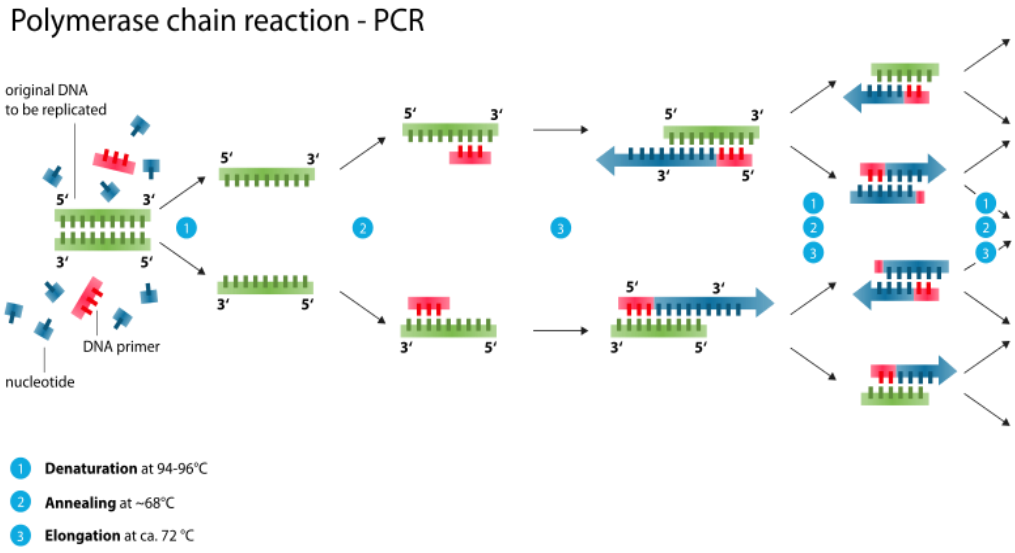


Fig.14: Schematic representation of Polymerase chain reaction

F9 gene was amplified using the following PCR conditions for all the isolated Genomic DNA from HB patients.

PCR master mixture composition*:* [For 10µl]

- Molecular Biology grade water - 7.35µl
- Taq buffer - 1.0µl
- dNTP"s – 0.2µl
- Forward primer – 0.2µl
- Reverse primer - 0.2µl
- Template - 1.0µl
- Taq polymerase – 0.05µl

Thermo cycling condition for PCR*:*

- Initial Denaturation of genomic DNA at 95^0C for 30 sec.
- Denaturation at 95^0C for 30sec.
- Primer annealing for (gene specific) 60sec.
- Primer extension at 72.0^0C for 60 sec.
- Repeated for 35 cycles.
- Final extension 72.0^0C for 7min.
- Hold at 4^0C.

4.11.1 Analysis of the amplified product

- The quality of the amplified product was checked on 2.0% agarose gel.
- 3g of agarose powder was added to 120ml of 1x of TAE buffer and agarose was dissolved by heating.
- The mixture was allowed to cool at 55 degree celcius afterwhich 3 microlitre of EtBr was added.
- The melted agarose was poured carefully into the electrophoresis gel tray and left to solidify.
- After solidification, the gel tray was very carefully taken out and placed in the electrophoretic tank containing 1X TAE buffer until the gel was completely submerged.
- 4 microlitre of PCR product was mixed with Bromophenol blue and loaded into wells.
- A 50bp/100bp ladder was used as molecular size marker. Initially gel was run at 50 V till bands comes out from wells and then it was changed to 80V and run for 1 hour.
- The bands were observed under UV documentation and compared with standard ladder.

4.12 DNA Sequencing

DNA sequencing is a process of determining the precise order of neucleotides within a DNA molecule. It works on the principle of the PCR product when subjected to cycle sequencing assay of sequence fragment generated as a result of dideoxy change termination process, this DNTPs further generate sequence assay and is separated in the capillary electrophoresis; the sequence is generated by laser UV excitation of the labeled dideoxy nucleotide present at the end of the terminated sequencing.

The foundation for sequencing proteins was first laid by the work of Fredrick Sanger who by 1955 had completed the sequence of all amino acids in insulin, a small protein secreted by the pancreas. The first method for determining DNA sequences involved a location specific primer extension strategy was established by Ray Wu at Cornell University in 1970.

The HB mutations were identifing by automated sequence analysis performing on a Genetic Analyzer using the Capillary electrophoresis method. The migration support is a liquid polymer (POP7) containing the separation polymer, salts required for migration and urea as a denaturing agent.

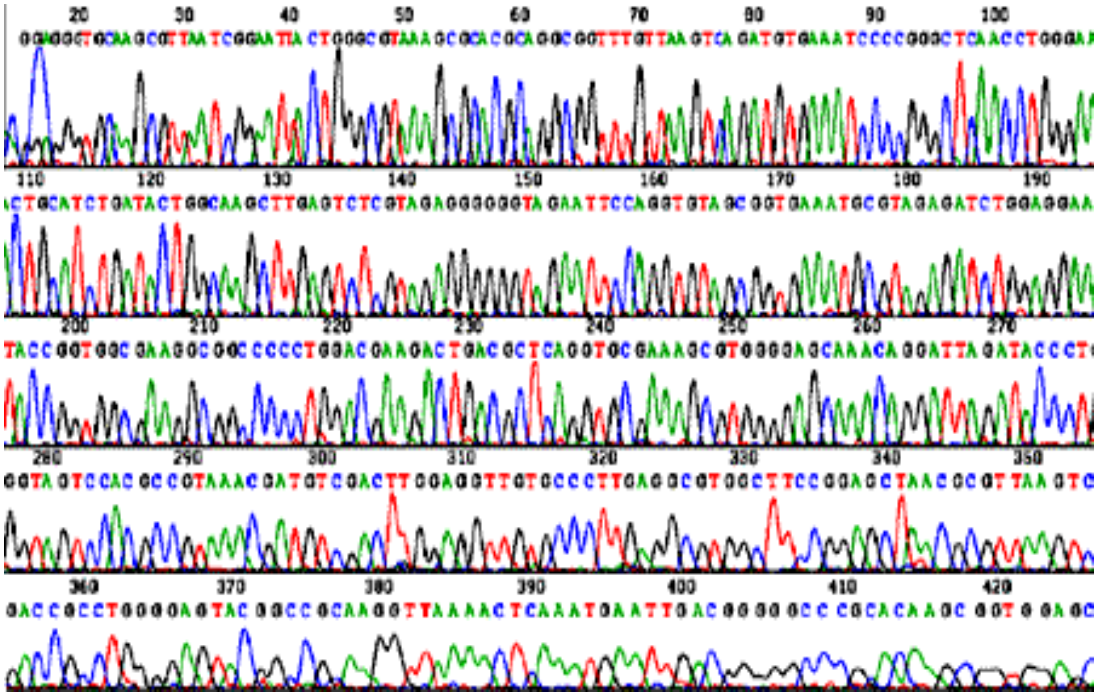


Fig.15: Sequencing machine and electropherogram.

4.12.1 DNA sequencing (Capillary based)

PCR products were subjected for capillary based Big-Dye terminator sequencing. Prior to sequencing, the PCR products were subjected to cycle sequencing and plate processing**.**

4.12.2 Cycle sequencing

As per the Sanger Sequencing protocol, Big-Dye labeling and chain termination were carried out by the cycle sequencing method. To label each base, the PCR amplicon was subjected to a cycle sequencing reaction with a single primer. Big-Dye TM terminator v3.1was used for cycle sequencing (Applied Biosystems, USA) with following manufacturer's guidelines. Cycle sequencing of the PCR products was carried out according to the annealing temperature of the primers.

Table 2. Standardised master mix conditions for sequencing

SL.No.	Constituents	Quantity
1	Molecular Biology grade water	6.3 µL
2	Big Dye Buffer (5X)	1.3 µL
3	Big Dye	1.0 µL
4	Template (PCR product)	1.0 µL
5	Forward Primer	0.2 µL
6	Reverse Primer	0.2 µL
Total		**10 µL**

Note: Only one of the primers i.e either forward or reverse primer was used during cycle sequencing

Table 3. The cycle sequencing conditions

Process	Temperature (°C)	Time
Initial. Denaturation	98	10sec
Denaturation	98	10sec
Annealing	Primer Dependent	10sec
Elongation	72	5min
Hold	4	

Note: The annealing temperature is primer dependant and varies for each primer

4.12.3 Sequencing clean-up

To remove the unbounded florescent DNTPs from the terminator sequencing reaction, 2µl of 3M sodium acetate, and 50µl of 100% ethyl alcohol were added to each sample and incubated at room temperature for 15 minutes to precipitate the DNA. The samples were centrifuged at 4000 rpm for 30 minutes at 4°C. The supernatant was discarded and the reaction plate was centrifuged in a reverse manner at 300 rpm for 20 seconds. 100µl of 75% alcohol was added to each sample and centrifuged at 4000rpm for15 minutes at 25°C. The supernatant was discarded and the plate was centrifuged in a reverse manner at 300 rpm for 20 seconds to remove the alcohol completely. The plate was dried at room temperature until the last drop of alcohol dripped off.

10µl of Hi-Di Formamide was added to each well of the sample plate. The samples were heated to 96°C for 5 minutes and immediately cooled to 4°C to denature and linearise the cycle sequencing products. The processed products were loaded in the sequencer for sequencing.

4.12.4 Sequencing run

Sample information sheets which contain analysis protocols along with the sample details were prepared and imported into the data collection software. Prepared samples were analyzed on ABI 3730 genetic analyzer (Applied Biosystems, USA) to generate DNA sequences or electropherograms. After completion of the sequencing reaction, the quality of generated sequence was checked by using Sequencing Analysis v5.4software (Applied Biosystems, USA)

4.12.5 Sequence alignment

The generated sequences were aligned to their respective reference sequences with the use of Variant reporter software (ABI v1.1). The variant reporter is one of the compatible software of Applied Biosystems designed for automated sequence data analysis. It performs sequence comparisons for novel mutations, known variants, insertions, and deletions. It allows analysis of the resequenced data, comparing the consensus sequences to a known reference sequence. The results of the variant reporter were tabulated in PDF format as the default program of the software.

4.12.6 Compilation of mutations

Compiling all the mutations which were identified, mutations which were common for all the patients were identified using sequence analysis tool and bioinformatics software.

4.12.7 Identification of novel mutations

Data base was created for all mutations which were found during the time of research work. This data base contained all types of mutations and was available for public for further use for research. From all the mutations entered in database very common mutations which were specific to region were identified.

4.13 BIOINFORMATICS ANALYSIS

Pathogenic effects of the missense variants were analysed by using following bioinformatics tools;

4.13.1 PROVEAN- Protein Variation Effect Analyser

PROVEAN is used to predict an effect of an amino acid substitution or indel on the biological functions of protein.

Link: http://provean.jcvi.org/seqsubmit.php

Procedure

- PROVEAN was accessed at http://provean.jcvi.org/seqsubmit.php
- The protein sequence in FASTA format was entered in text box
- The position of the mutation in the protein sequence was assigned
- Submit query button was clicked
- The view link was clicked to browse the PROVEAN prediction report for query of interest

4.13.2 POLYPHEN2

PolyPhen2 tool predict the effect of an amino acid substitution on the function of protein especially human protein using physical and comparative considerations.

Link: http://genetics.bwh.harvard.edu/pph2/index.shtml

Procedure

- The PolyPhen-2 was accessed at http://genetics.bwh.harvard.edu/pph2/.
- The protein sequence was entered in the text box
- The position of the substitution mutation in the protein sequence was assigned.
- Submit Query was clicked
- The view link was clicked to browse the PolyPhen-2 prediction report

4.13.3 PANTHER

PANTHER web tool estimates the effect of a coding single nucleotide polymorphism particularly non-synonymous variant's functional impact on the protein. Longer the preservation time, greater the likelihood of functional impact of missense mnutation on the protein.

Link: http://www.pantherdb.org/

Procedure

- PANTHER Web interface was accessed at http://www.pantherdb.org/
- Protein sequence in FASTA format was entered in text box
- Nucleotide substitution was entered
- Select the organism, to which protein of interest belongs to
- Run prediction was clicked
- Prediction results for mutations of interest were checked

4.13.4 SNAP2

SNAP2 tool used to predict functional effects of mutations. It utilizes a number of sequence and variant properties to identify between effect variant and neutral variants/non-synonymous SNPs.

Link: https://www.rostlab.org/services/snap/

Procedure

- SNAP2 was accessed at https://www.rostlab.org/services/snap/
- Protein sequence in FASTA format was entered
- Run prediction was clicked
- Prediction results for mutations of interest were checked

4.13.5 SNP & GO

SNP & GO determines if a particular variation is disease-related or neutral by combining several pieces of data, such as those derived from the Gene Ontology annotation.

Link: https://snps.biofold.org/snps-and-go/snps-and-go.html

Procedure

- The SNP & GO was accessed at https://snps.biofold.org/snps-and-go/snps-and-go.html
- Protein sequence in FASTA format was entered in a text box
- The nucleotide substitution was entered
- Clicked on run prediction
- Prediction results for mutations of interest was checked

4.13.6 PHD SNP

PHD-SNP tool used to predict effect of single nucleotide polymorphism on human protein. PHD SNP Support Vector Machines based Predictor of human Deleterious Single Nucleotide Polymorphisms.

Link: https://snps.biofold.org/phd-snp/phd-snp.html

Procedure

- The PHD SNP was accessed at https://snps.biofold.org/phd-snp/phd-snp.html
- Protein sequence in FASTA format was entered in a text box
- The nucleotide substitution was entered
- Suitable prediction was selected viz sequence based or sequence and profile based
- Then select multi SVM option
- Clicked on run prediction
- Prediction results for mutations of interest were checked

4.14 CONSERVATION ANALYSIS

The evolutionary conservation of variant residue over different species was investigated by using Clustal Omega web server

4.14.1 Clustal omega

Clustal Omega is a multiple sequence alignment program for aligning three or more nucleic acid or protein sequences together in a computationally efficient and accurate manner. Evolutionary relationships can be seen via viewing Cladograms or Phylograms. Note:

An * (asterisk) single and fully conserved residue

A: (colon) strongly similar properties

A. (period) weakly similar properties

Web link: https://www.ebi.ac.uk/Tools/msa/clustalo/

Procedure

- Clustal omega tool was opened using following web link

  https://www.ebi.ac.uk/Tools/msa/clustalo/

- Input sequences were selected as Protein.
- Sequence of interest was uploaded in any supported format like FASTA, Genbank, and GCG etc…
- Parameters of interest were selected as Clustal with character counts
- Clicked on submit

4.15 Protein structure prediction

Homology modelling of wild type and mutant protein was developed using the Swiss model (https://swissmodel.expasy.org/). SWISS model is web based server used for automated comparative modelling of three dimensional (3D) structures of proteins. The server handles template selection, alignment, and model construction entirely automatically. In "alignment mode" the modelling is based on a user-defined target-template alignment. In "project mode" the integrated sequence-to-structure workbench DeepView (Swiss-PdbViewer) can handle complex modelling jobs. Each model is returned with a thorough modelling report through email.

Results were visualized and analysed using downloaded version of UCSF ChimeraX program. UCSF Chimera is an extensible molecular modelling program for the interactive visualization and analysis of molecular structures and related data, including density maps, trajectories, and sequence alignments. (https://www.cgl.ucsf.edu/chimera/)

Procedure

- The Swiss model was opened using https://swissmodel.expasy.org/ web link
- Clicked on start modelling
- Target protein sequence in FASTA format was uploaded
- Project title and email address was entered in a text box
- Clicked on target-template alignment for proper template selection
- Clicked on start structure for model building of protein of interest
- Predicted protein model was evaluated for given protein of interest

4.16 Statistical analysis

Obtained data was tabulised and analysed via SPSS 15.0 (SPSS Inc., Chicago, USA). Data are presented as mean ± SD. Student t-test, one way ANOVA was used to compare the significant difference in mean. For categorical variables, the chi-square test was used. P-value <0.05 is considered statistically significant.

CHAPTER 5

RESULTS

5.1. Demographic data analysis

5.1.1. Sample collection

150 patients suffering from Hemophilia B were included in the present study. Our Study cohort included 148 male and 02 female HB patients. The age of the patients ranged from 05 to 35 years and the mean age was 25 years. In our study cohort majority of the 58 (38.7%) cases belonged to 21-30 years of age followed by 11-20 years 41 (27.3%). The mean age of onset of symptoms was 2.0±1.0 years in severe patients, 7.5±2.8 years in moderate patients and 10.0±3.5 years in mild HB patients. The same are represented in table 4.

Table. 4 Age distribution of HB patients in our study group

Sl No	Age group	No of HB patients	Percentage
1	1-10	18	12%
2	11-20	41	27.3%
3	21-30	58	38.7%
4	31-40	33	22%

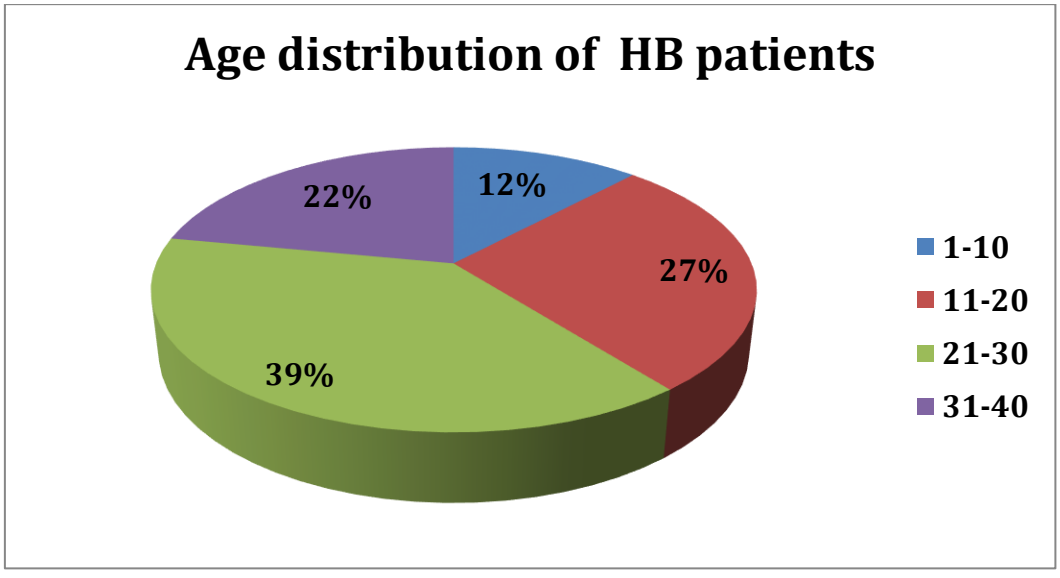


Fig 16. Age wise distribution of HB patients in our study

Out of 150 HB cases, 102 (68%) cases were diagnosed as severe, 30 (20%) cases were diagnosed as moderate and 18 (12%) cases were diagnosed as mild. 2 female HB patients were diagnosed as severe. Mean factor IX levels were 0.6±0.2, 2.5±1.3 and 8.0±2.6 in the severe, moderate and mild group respectively.

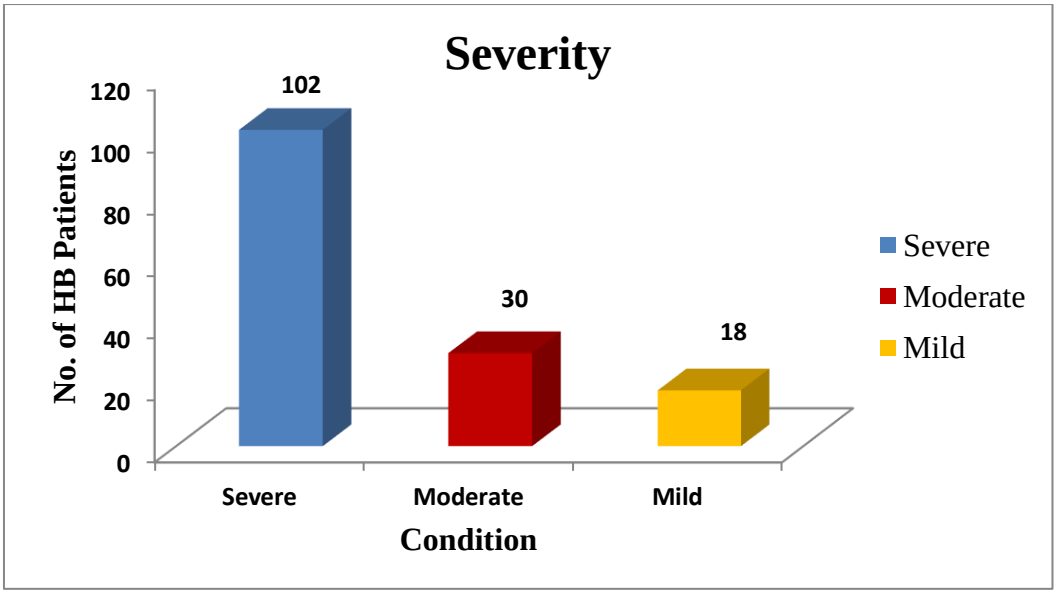


Fig 17: Distribution of HB patients according to severity

In our study, family history of bleeding was observed in 97 [64.7%] HB patients. In that, 80 [82.5%] cases belonged to a severe group and 10 [10.3%], 7 [7.2%] HB cases belonged to moderate and mild group respectively. 47 (32.3%) HB patients had a history of consanguinity.

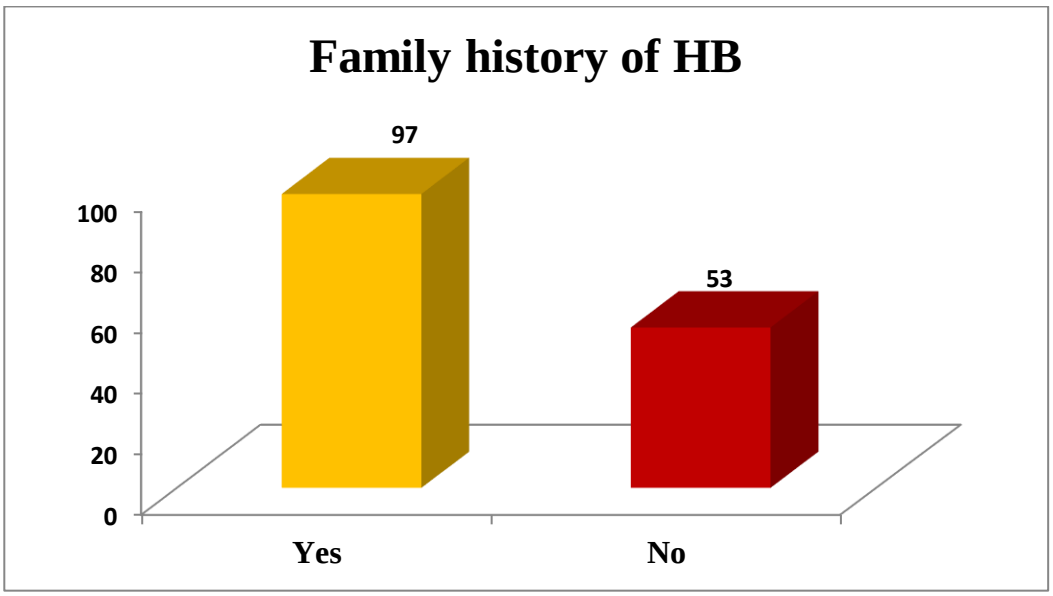


Fig 18: Distribution of HB patients according to family history

The most common initial site of bleed was in joints in 86 [57.3%] % of HB patients followed by the skin in 27 [18%], muscle in 16 [10.7%], epistaxis in 11[7.3%] and Petechiae in 10 [6.7%] of haemophilia B patients respectively.

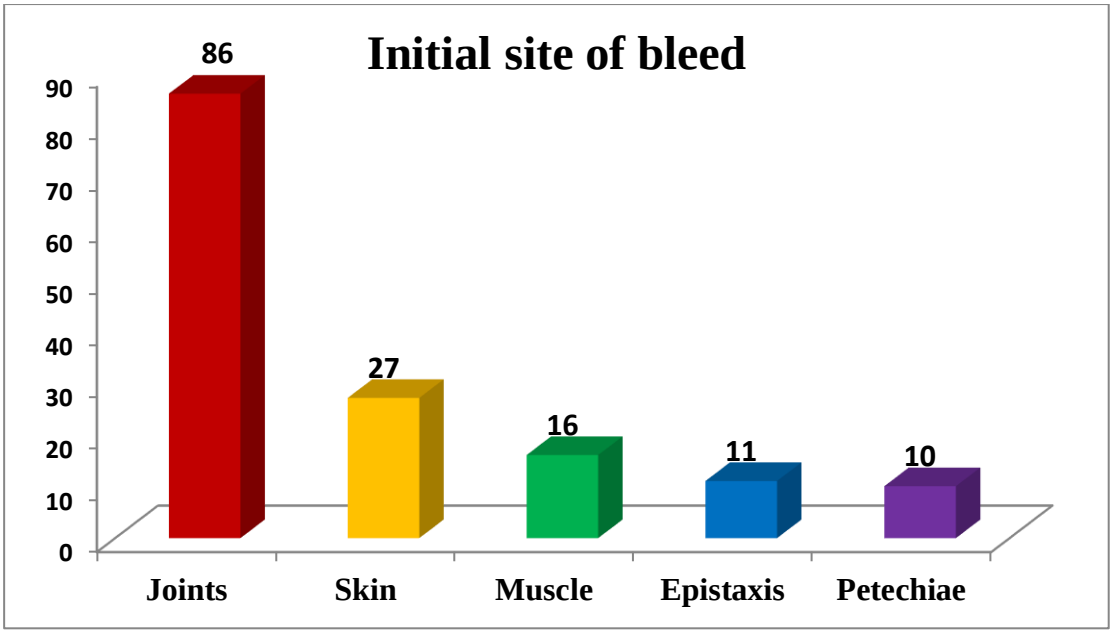


Fig 19: Distribution of HB patients according to initial site of bleed

Table 5 showes the detailed clinicopathological characters of Hemophilia B patients in the present study.

Table 5: Clinicopathological characters of Hemophila B patients

Clinicopathological Parameters	Severity of disease			P value
	Severe (Factor IX <1%) n=102 (68%)	Moderate (Factor IX 1-5%) n=30 (20%)	Mild (Factor IX>5%) n=18 (12%)	
Mean factor IX levels (%)	0.6±0.2	2.5+-1.3	8.0±2.6	0.001*
Family history of bleeding (n)	80	10	7	
Mean age of onset (years)	2.0±1.0	7.5±2.8	10.0+-3.5	0.001*
Inhibitor positive (n)	23	11	04	
Inhibitor negative (n)	79	19	14	
Bleeding sites • Joints	55 (54%)	19 (63.3%)	12 (66.7%)	
• Skin	17 (16.7%)	6 (20%)	4 (22.2%)	
• Muscle	13 (12.7%)	1(3.3%)	2 (11.1%)	
• Epistaxis	7 (6.9%)	4 (13.3%)	0	
• Petechiae	10 (9.8%)	0	0	

5.2. Mutation Analysis

5.2.1. Agarose gel electrophoresis

Genomic DNA was successfully isolated from all 150 hemophila B samples using a blood and tissue kit [QIAgen, Germany] as per manufactures instruction. The quality of the genomic DNA was analysed on 0.8% agarose gel electrophoresis**.**

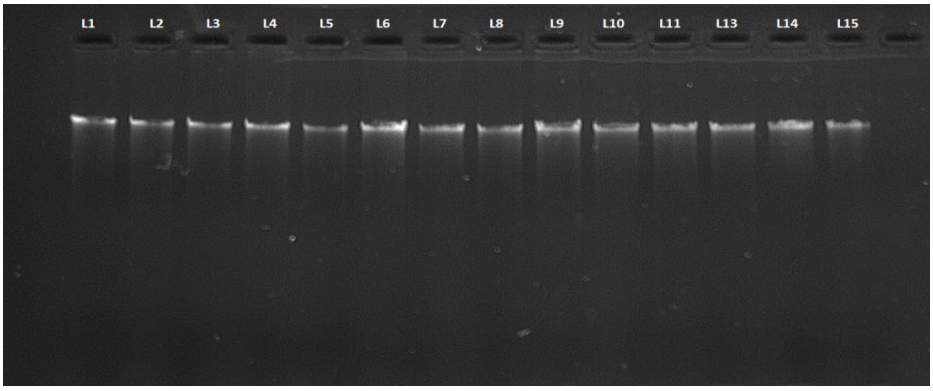


Fig.20: Agarose gel electrophoresis image of genomic DNA

5.2.2 Quantity analysis of isolated genomic DNA by nanodrop method

The concentration and purity of the isolated DNA samples were measured using a Nanodrop spectrophotometer. It functions by combined fibre optic technology and natural surface tension properties. The optical density reading was taken at 260 nm against blank i.e. T10 E1 buffer without DNA. The same are represented in table.6

Table 6. Nanodrop Readings of Hemophilia B Genomic DNA Samples

Sl. No	Sample Type	Sample ID	A260	A280	260/280	ng/µl
1	dsDNA	HB1	0.359	0.220	1.63	68.0
2	dsDNA	HB2	0.250	0.155	1.62	32.5
3	dsDNA	HB3	0.264	0.178	1.78	43.2
4	dsDNA	HB4	0.264	0.192	1.78	33.2
5	dsDNA	HB5	0.264	0.145	1.81	43.2
6	dsDNA	HB6	0.400	0.206	1.95	70.0
7	dsDNA	HB7	0.277	0.178	1.56	53.9
8	dsDNA	HB8	0.318	0.173	1.84	65.9
9	dsDNA	HB9	0.359	0.182	1.97	48.0
10	dsDNA	HB10	0.886	0.449	1.97	44.3
11	dsDNA	HB11	0.183	0.100	1.84	69.2
12	dsDNA	HB12	1.320	0.683	1.93	66.0
13	dsDNA	HB13	0.634	0.352	1.80	31.7
14	dsDNA	HB14	2.270	1.150	1.97	113.5

5.3. PCR amplification

Polymerase Chain Reaction (PCR) amplification for *F9* was carried out using a previously standardised protocol. Primer used for the present study was shown in Table.7 Table.7 List of primers used for amplification of F9 gene

Sl No	Exons	Primer sequence	Product size
Exon1	AGTCCAAAGACCCATTGAGG	GACTCTTCAATATTGCTGTCAAATC	550
Exons 2	TGCCCTAAAGAGAAATTGGC	TGGGTTAGAGGGTTGGACTG	290
Exons 3	AGCCCTCTGGAGATGTTAAC	TGCCTTTGAGAGTTCGAGA	260
Exon 4	5GAGGACCGGGCATTCTAAG	CCAGTTTCAACTTGTTTCAGAGG	286
Exon 5	CAAAATTTCTCTCCCCAACG	GGTCTAATTCAAGCTACTGATATTTT	321
Exon 6	CAAAATTTCTCTCCCCAACG-	TAGCCTCAGTCTCCCACCTG	392
Exon 7	TTTCTGCCAGCACCTAGAAG	ACCCTTCTGCCTTTAGCCC	383
Exon8a	GCCAATTAGGTCAGTGGTCC	CTTCATGGAAGCCAGCAC	538
Exon8b	TGTAAGTGGCTGGGGAAGAG	TGAGAGGCCCTGTTAATTTTC	388

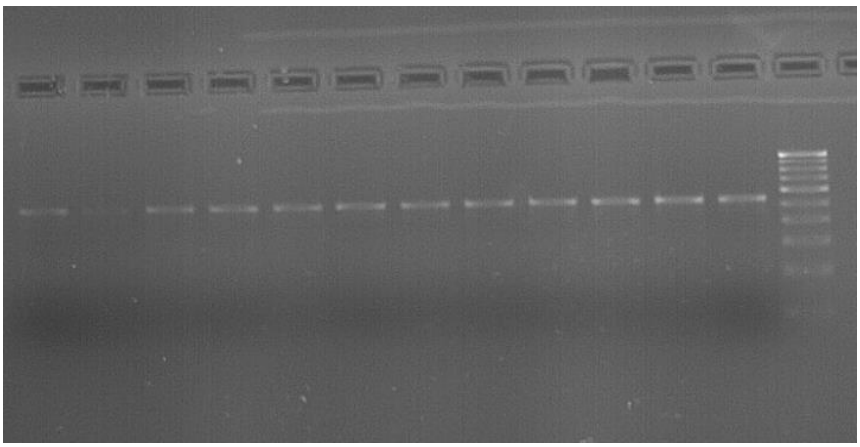


Fig.21: Agarose gel electrophoresis results of gradient PCR of EXON-1

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=61.0, G= ±4.0

Lane No.1- 59.0, Lane No.2-59.1, Lane No.3-59.3, Lane No.4- 59.7,
Lane No.5- 60.2, Lane No.6-60.8, Lane No.7-61.2, Lane No.8- 61.8,
Lane No.9- 62.3, Lane No.10-62.7, **Lane No.11- 62.9**, Lane No.12- 63.0

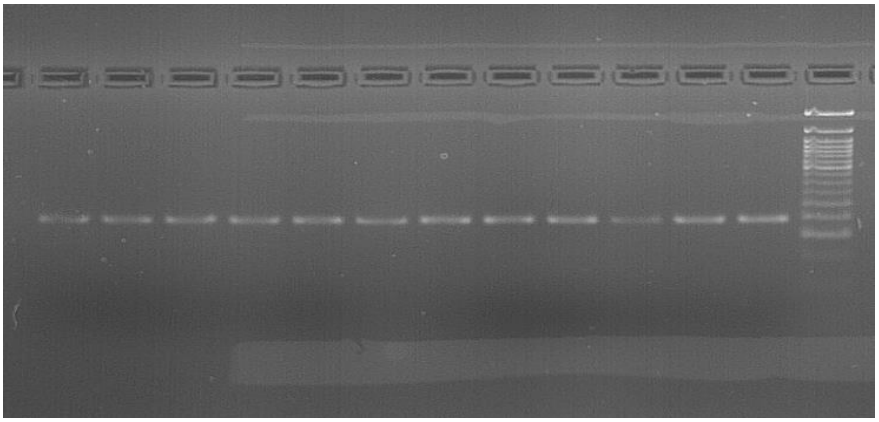


Fig.22: Agarose gel electrophoresis results of gradient PCR of EXON-2

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=61.0, G= ±4.0

Lane No.1- 59.0,	Lane No.2-59.1,	Lane No.3-59.3,	Lane No.4- 59.7,
Lane No.5- 60.2,	Lane No.6-60.8,	Lane No.7-61.2,	**Lane No.8- 61.8,**
Lane No.9- 62.3,	Lane No.10-62.7,	Lane No.11- 62.9,	Lane No.12- 63.0

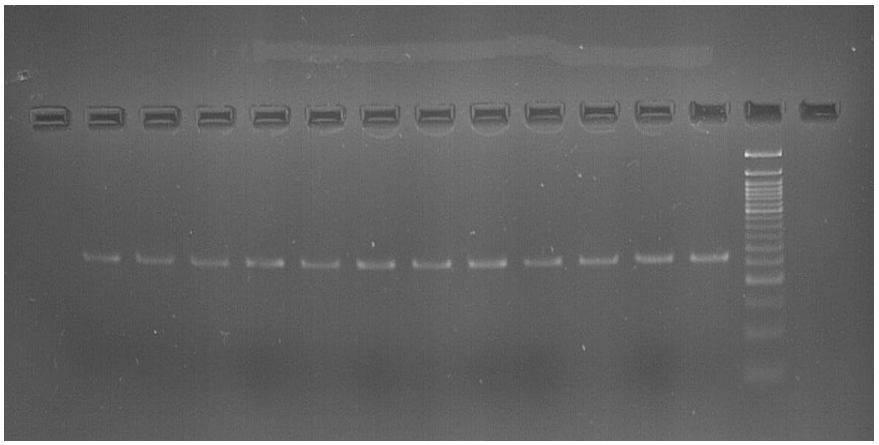


Fig.23: Agarose gel electrophoresis results of gradient PCR of EXON-3

L- 50bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=60.0, G= ±1.0

Lane No.1- 59.0,	Lane No.2-59.1,	Lane No.3-59.3,	Lane No.4- 59.5,
Lane No.5- 59.9,	Lane No.6-60.2,	Lane No.7-60.4,	Lane No.8- 60.7,
Lane No.9- 60.9,	**Lane No.10-61.2**,	Lane No.11- 61.5,	Lane No.12- 61.5

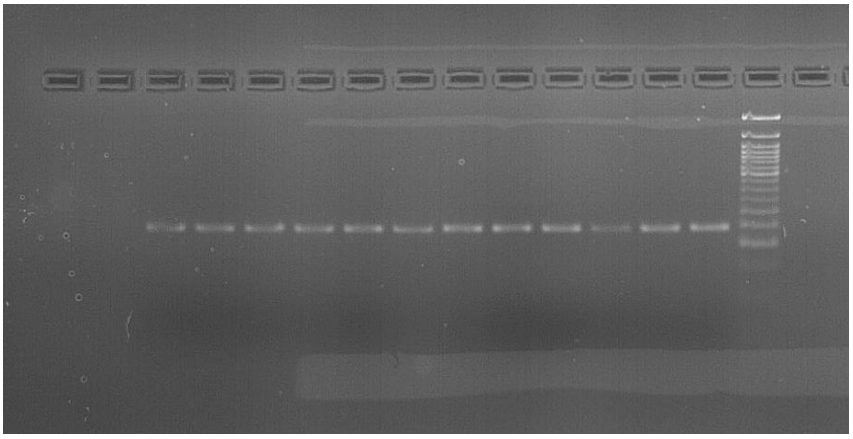


Fig.24: Agarose gel electrophoresis results of gradient PCR of EXON-4

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=51.0, G= ±4.0

Lane No.1- 49.0,	Lane No.2-49.1,	Lane No.3-9.3,	Lane No.4- 49.7,
Lane No.5- 50.2,	Lane No.6-50.8,	Lane No.7-51.2,	**Lane No.8- 51.8,**
Lane No.9- 52.3,	Lane No.10-52.7,	Lane No.11- 52.9,	Lane No.12-53.0

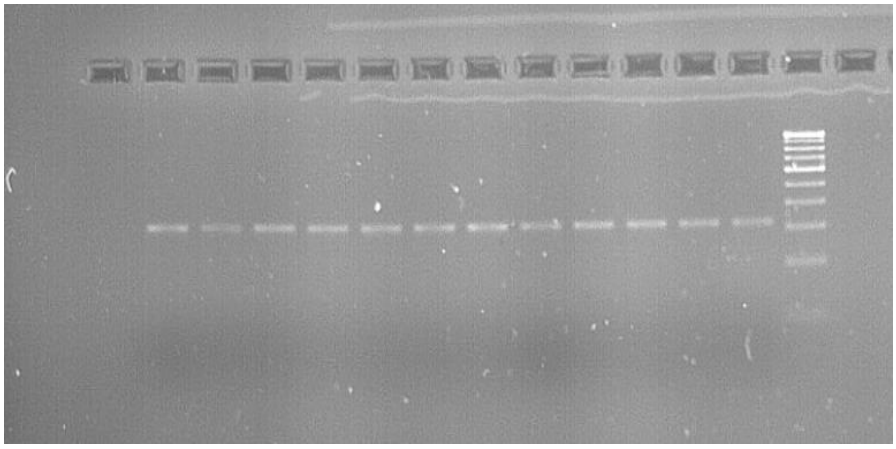


Fig.25: Agarose gel electrophoresis results of gradient PCR of EXON-5

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.7, G= ±1.3

Lane No.1- 54.4,	Lane No.2-54.5,	Lane No.3-54.6,	Lane No.4- 54.9,
Lane No.5- 55.2,	Lane No.6-55.5,	Lane No.7-55.9,	Lane No.8- 56.2,
Lane No.9- 56.4,	**Lane No.10-56.8,**	Lane No.11- 56.9,	Lane No.12- 57.2

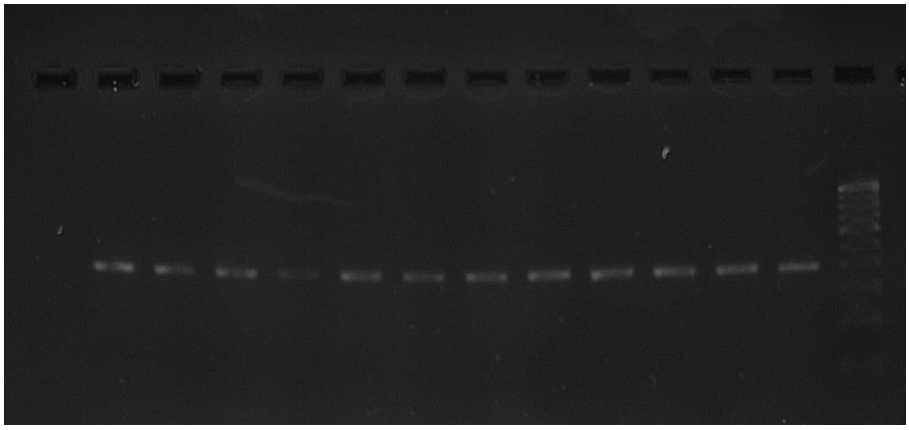


Fig.26: Agarose gel electrophoresis results of gradient PCR of EXON-6

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.0, G= ±1.0

Lane No.1- 54.0,	Lane No.2-54.2,	Lane No.3-54.6,	Lane No.4- 54.8,
Lane No.5- 55.0,	Lane No.6-55.2,	Lane No.7-55.5,	Lane No.8- 56.7,
Lane No.9- 55.9,	**Lane No.10-56.0,**	Lane No.11- 56.2,	Lane No.12- 56.3

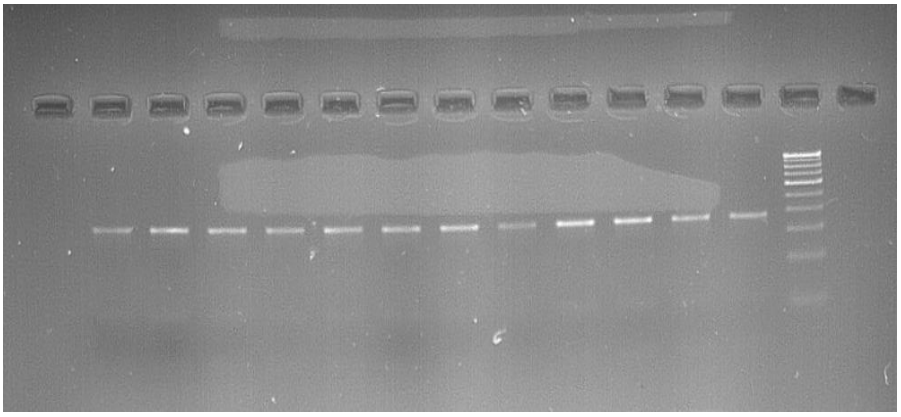


Fig.27: Agarose gel electrophoresis results of gradient PCR of EXON-7

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=61.0, G= ±4.0

Lane No.1- 59.0,	Lane No.2-59.1,	Lane No.3-59.3,	Lane No.4- 59.7,
Lane No.5- 60.2,	Lane No.6-60.8,	Lane No.7-61.2,	Lane No.8- 61.8,
Lane No.9- 62.3,	**Lane No.10-62.7**,	Lane No.11- 62.9,	Lane No.12- 63.0

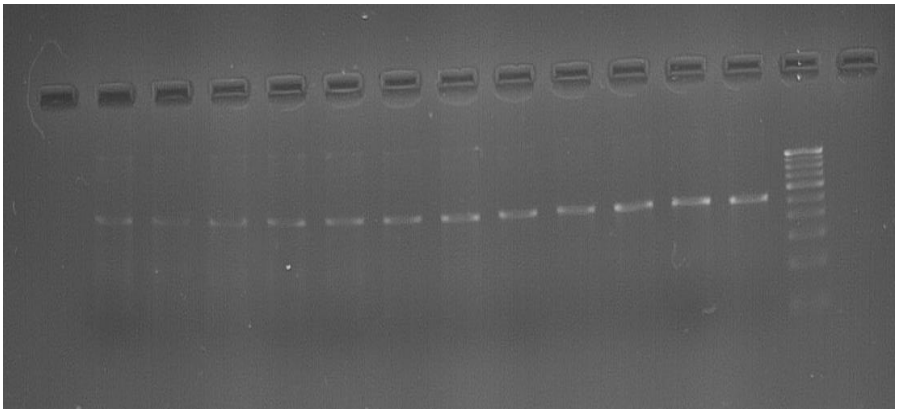


Fig.28: Agarose gel electrophoresis results of gradient PCR of EXON-8A

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.0, G= ±1.0

Lane No.1- 54.0,	Lane No.2-54.2,	Lane No.3-54.6,	Lane No.4- 54.8,
Lane No.5- 55.0,	Lane No.6-55.2,	Lane No.7-55.5,	Lane No.8- 56.7,
Lane No.9- 55.9,	**Lane No.10-56.0,**	Lane No.11- 56.2,	Lane No.12- 56.3

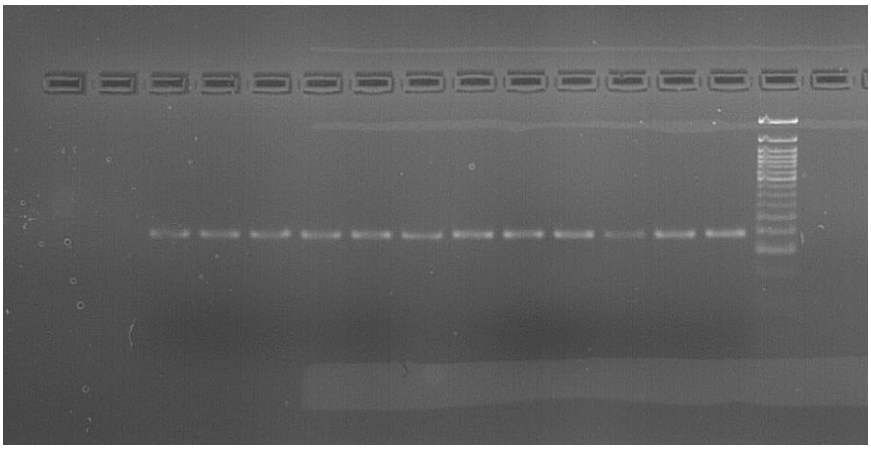


Fig.29: Agarose gel electrophoresis results of gradient PCR of EXON-8B

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.0, G= ±1.0

Lane No.1- 54.0,	Lane No.2-54.2,	Lane No.3-54.6,	Lane No.4- 54.8,
Lane No.5- 55.0,	Lane No.6-55.2,	Lane No.7-55.5,	**Lane No.8- 56.7,**
Lane No.9- 55.9,	Lane No.10-56.0,	Lane No.11- 56.2,	Lane No.12- 56.3

5.4. Molecular analysis of *F9* gene

In our study, we recorded 16 mutations in that one was synonymous mutation, twelve were missense mutation, and two were stop gained and one 3' UTR variants. 13 (81.25%) mutations were previously recorded and 3(18.75%) were novel mutations which were not recorded previously in any of the human SNP databases. Majority of the mutations which were found in exon 8 of the gene were missense mutations and exon 8 showed the high number of mutations compared to other exons of *F9* gene.

Novel missense mutation c.198A>T recorded in 4 moderate HB patients with mean FIX concentration 2.0±0.5 & mean age of onset was 7±1.0 and in 07 severe HB patients with mean FIX concentration 0.3±0.5 & mean age of onset was 1.5±0.5. This mutation was observed at a high rate among moderate HB patients. Novel stop gained mutation c.1094C>A recorded in 3 moderate HB patients with mean FIX concentration 2.6±0.8 & mean age of onset was 6.5±0.5and in 12 severe HB patients with mean FIX concentration 0.05±0.15 & mean age of onset was 2±1.0. This mutation was observed at high rate among severe HB patients.

Previously recorded pathogenic mutations c.127C>T was observed in 3 mild (FIX concentration 8.1±1.9), 4 moderate patients (FIX concentration 2.1±0.2) and 2 severe patients (0.65±0.15),

c.470G>A mutation was observed in 3 mild patients (FIX concentration 7.6±2.7), 5 moderate patients (FIX concentration 2.9±0.9) and 9 severe patients (0.1±0.7),

c.676C>T mutation was observed in 1 moderate patients and 5 severe patients (0.1±0.7),

c.1070G>A mutation was observed in 1 mild patient, 3 moderate patients (FIX concentration 2.8±0.8) and 4 severe patients (0.05±0.1),

c.223C>T patient observed in 2 severe patients and c.314A>G observed in 1 moderate patient and 3 severe patients (0.1±0.09). Remaining mutations were recorded in low frequency. all the mutations are presented in table.8

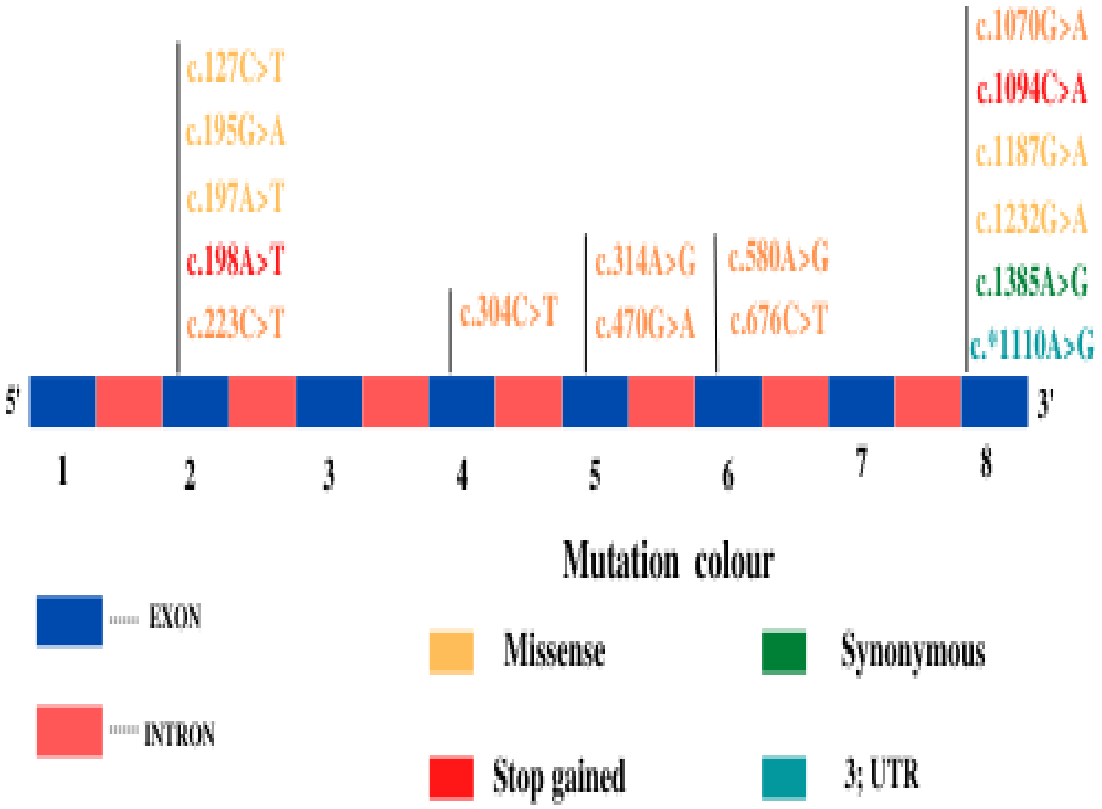


Fig. 30: Graphical representation of the distribution of the mutations in the Factor 9 (*F9*) gene

Table.8 List of Mutations recorded in present study cohort

Mutation type	Nucleotide change	cDNA position	Amino acid change	Exon	Status	Frequency n (%)
Missense	g.11313C>T	c.127C>T	p.R43W	2	reported rs1603264205	9 (6.0%)
Missense	g.11381G>A	c.195G>A	p.M65I	2	Reported rs763568424	1 (0.66%)
Missense	g.11383A>T	c.197A>T	p.E66V	2	Reported CM940423	3 (2.0%)
Missense	g.11384A>T	c.198A>T	p.E66D	2	Not reported	12 (8.0%)
Stop gained	g. 11409 C>T	c.223C>T	p.R75*	2	Reported rs137852227	2 (1.33%)
Missense	g.15369 T>C	c.304C>T	p.C102R	4	Reported CM960574	2 (1.33%)
Missense	g. 22664 T>C	c.314A>G	p.G143R	5	Reported CM940499	4 (2.66%)

Missense	g. 22706 G>A	c.470G>A	p.C157Y	5	Reported rs1367198680	17(11.33%)
Missense	g. 25386 A>G	c.580A>G	p.T194A	6	Reported rs6048	3 (2.0%)
Missense	g. 25482C>T	c.676C>T	p.R226W	6	Reported rs137852240	6 (4.0%)
Missense	g.36020 G>A	c.1070G>A	p.G357E	8	Reported rs137852275	8 (5.33%)
Missense	g. 36137G>A	c.1187G>A	p.C396Y	8	Reported rs137852273	1 (0.66%)
Missense	g. 36182 G>A	c.1232G>A	p.S411N	8	Reported rs137852276	3 (2.0%)
Stop gained	g. 36044C>A	c.1094C>A	p.S365*	8	not reported	15 (10.0%)
synonymous	g. 36335 A>G	c.1385A>G	p.Ter462=	8	Reported rs561793582	1 (0.66%)
3’ UTR	g.37446A>G	c.*1110A>G	…….	8	Not reported	3 (2.0%)

Table 9. Genotypic -phenotypic association of mutation recorded in study population

Mutation type	**cDNA position**	Seviarity			**FIX concentration level (%)**	**age of onset**	**Inhibitor positive (n)**	**Inhibitor negative (n)**	**Clinical significance**
		m	**M**	**S**					
Missense	c.127C>T	03	04	02	6.5, 8.0, 10 (m) 2.0, 2.3. 1.8, 2.0 (M) 0.8, 0.5 (S)	10, 12,8 (m) 7, 6, 9, 9 (M) 1,1.5 (S)	2 (m) 2 (M) ---	1 (m) 2 (M) 2(S)	Pathogenic
Missense	c.195G>A	00	01	00	3.0 (M)	7.5 (M)	1 (M)	---	Likely pathogenic
Missense	c.197A>T	02	00	01	6.0, 9.0 (m) 0.3 (S)	13, 11 (m) 1 (S)	--	2 (m) 1(S)	Likely Benign
Missense	c.198A>T	00	04	07	1.5, 2.0. 2.5, 2.0 (M) 0.8, 0.5, 0.1, 0.6, 0.6, <0.01,0.01 (S)	6, 7, 7, 8 (M) 1, 2, 1, 1.5, 2, 2, 1.6 (S)	4(M) 4(S)	--- 3 (S)	Pathogenic
Stop gained	c.223C>T	00	00	02	<0.01, <0.01 (S)	1, 1 (S)	1 (S)	1(S)	Pathogenic
Missense	c.304C>T	02	00	00	12.0, 10.0 (m)	13, 10 (m)	2 (m)	---	Likely pathogenic
Missense	c.314A>G	00	01	03	2.0 (M) 0.2, 0.1, 0.01 (S)	8 (M) 1, 2, 1(S)	---	1(M) 3 (S)	Pathogenic
Missense	c.470G>A	03	05	09	5.0, 7.5, 10.3 (m) 2.0, 3.5. 2.5, 1.8, 4.0 (M) 0.8, 0.5, 0.01, 0.2, <0.01, <0.01, 0.4, 0.2, <0.01, (S)	6.5, 10, 11 (m) 5, 7, 7, 9, 7 (M) 1,2,2,3, 2,2,2, 1, 2 (S)	3(m) 1 (M) 2(S)	--- 4(M) 7(S)	Pathogenic
Missense	c.580A>G	03	00	00	10.7, 9.0, 9.0 (m)	10, 8, 8 (m)	---	3(m)	Likely Benign

Missense	c.676C>T	00	01	05	4.0 (M) 0.5,0.1,<0.01, 0.01, <0.01 (S)	5.5 (M) 3, 1, 2, 2, 1 (S)	1 (M) 1(S)	--- 3(S)	Pathogenic
Missense	c.1070G>A	01	03	04	6.5 (m) 2.0, 3.8. 2.5 (M) 0.01,0.1,<0.01,<0.01 (S)	12 (m) 6, 7,7 (M) 2,2,2,1 (S)	-- 2(M) 2(S)	1(m) 1(M) 1(S)	Pathogenic
Stop gained	c.1094C>A	00	03	12	2.6, 3.5, 1.8 (M) 0.01, 0.01, 0.1, 0.2, <0.01,<0.01, 0.1, 0.01, <0.01, 0.01, <0.01, <0.01 (S)	7,7,6 (M) 1,2,2,2,2,3,1.5,3,2,2,2.5,1 (S)	-- 10(S)	3(M) 2(S)	Pathogenic
Missense	c.1187G>A	00	00	01	0.6 (S)	2 (S)	---	1(S)	Benign
Missense	c.1232G>A	00	00	03	0.6, 0.3. 0.1 (S)	2,1, 1.5 (S)	1(S)	2(S)	Pathogenic
Synonymous	c.1385A>G	00	01	00	4.0 (M)	10 (M)	1(M)	---	Benign
3’ UTR	c.*1110A>G	01	03	00	12.0 (m) 5.0, 4.0, 3.8 (M)	13 (m) 7,7.5,9 (M)	1(m) 1(M)	-- 2(M)	------

5.5. Pathogenicity of the novel missense variants

Pathogenecity prediction of novel missense mutation p.E66D showed harmful effect on the function of F9 protein by PROVEAN, SNAP2, PholyPhen2, SNP&GO, PHD-SNP and PANTHER tools. The second novel mutation, p.S365* also showed deleterious effect on the function of *F9* protein.

Table 10. Pathogenicity predictions of p.E66D and p.S365* mutation.

Mutation	**PROVEAN**	**SNAP2**	**PolyPhen2**	**PHD-SNP**	**SNP&GO**	**PANTHER**
p.E66D	Deleterious Score: -2.540	Effect Score: 37	Probably damaging Score: 0.999	Disease Score:4	Disease Probability: 0.705	probably damaging
p.S365*	Deleterious Score: -3.440	Effect Score: 27	Probably damaging Score: 0.999	Disease Score:5	Disease Probability: 0.605	probably damaging

Note: PROVEAN- “Deleterious” if the prediction score was </ -2.5, “Neutral” if the prediction score was >/-2.5. PHD-SNP tool- if the probability is >0.5 mutations are predicted “Disease” and if less than <0.5 mutations are predicted to be “Neutral”. SNP&GO prediction tool- Probability is >0.5 then it is predicted to be Disease causing nsSNPs. Polyphen-2 predicted -“Probably damaging” is the most disease causing ability with a score near 1. “Possibly damaging” is less disease causing ability with a score of 0.5–0.8. “Benign” which does not alter protein functions with a score closer to zero. SNAP2 prediction tool –“Neutral” if the score lays 0 to - 100. “Effect” if the score lays 0 to 100.

5.6. Multiple sequence analysis

Multiple sequence alignment analysis of novel missense mutation using Clustal omega indicates that, this mutation residue (p.E66) was present at the highly conserved residue of F9 protein.

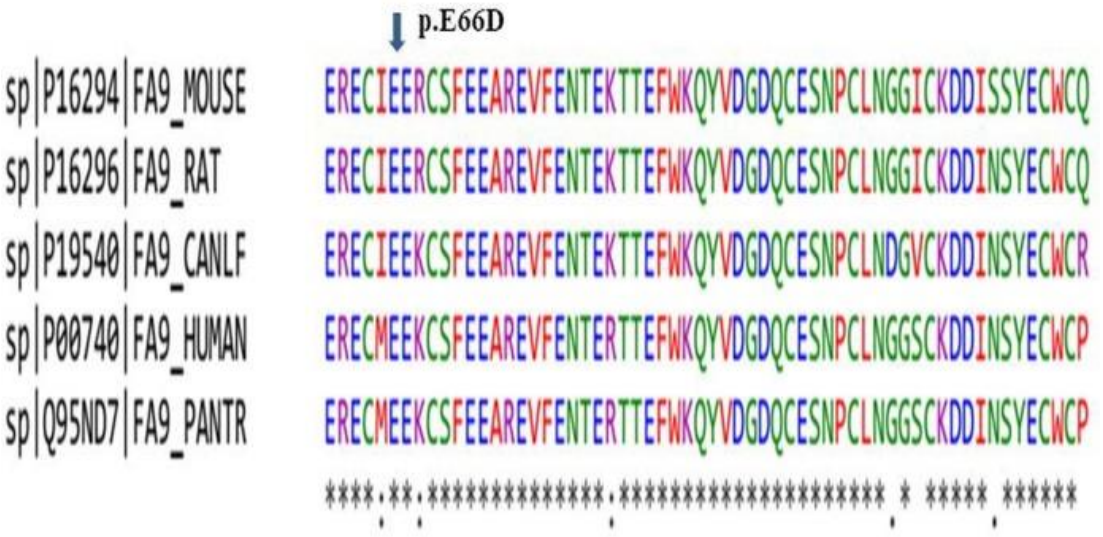


Fig.31: Multiple sequence alignment of FIX protein over different species showing conservation status of missense variant residue 66E.

5.7. Protein Structure prediction:

3D Homology modelling of the wild type and mutant FIX protein structure was developed using Swiss model server and it was visualized and analysed on chimera program.

In p.E66D, the mutant residue was smaller than wild type residue and this might lead to loss of interactions with the metal-ion: "Calcium 4 or magnesium 1; via 4-carboxyglutamate". This might produce abnormnal FIX protein during blood clotting.

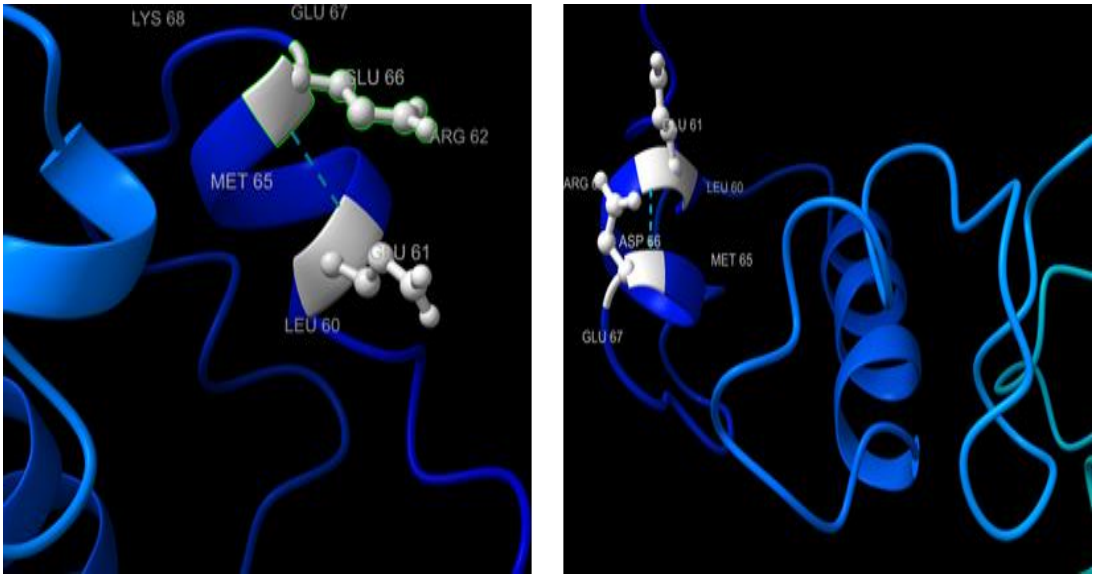


Fig.32: 3D model of wild type (left) and mutant protein; E66D (right)

Novel Stop gained mutation, p.S365* resulted in premature stop codon that caused truncated F9 protein with 365amino acid and leads to loss of approximately 21% of original F9 protein, which leads to loss of interaction with other coagulation protein.

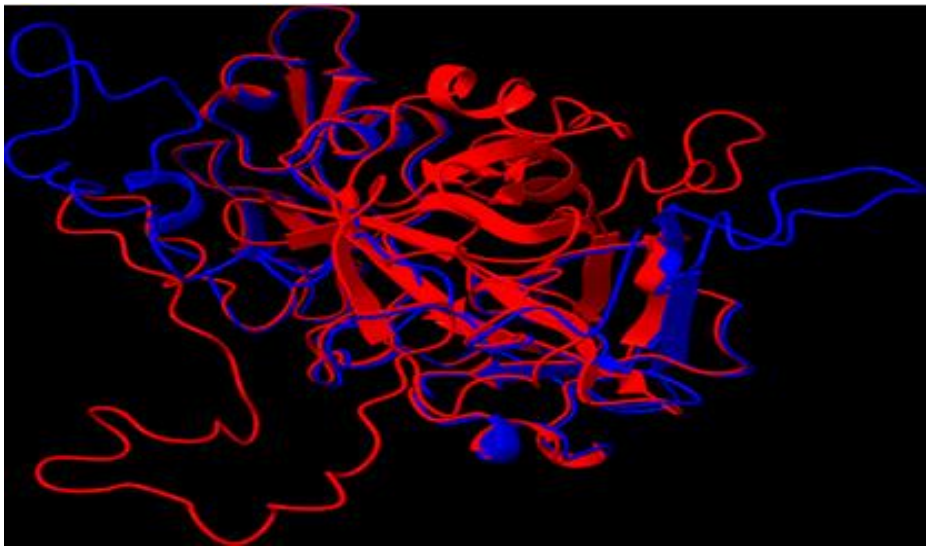


Fig.33: 3D model of p.S365*, mutant protein (blue colour) shown below.

CHAPTER 6

DISCUSSION

Hemophila is a bleeding disorder that causes abnormal or poor blood clotting. Its pathogenic gene is *F9* gene [38]. *F9* is an essential element of the intermediate stage in the blood coagulation cascade. Nearly one third of haemophilia cases occur without any previous family history, mainly due to new genetic variations [39]. 1.3 to 7.8 percent of haemophilia cases were reported to have more than one mutation in *F9* gene [40] and as per other study reports in the literature most of the mutations were single nucleotide variants [41]. Missense mutations were the most common single nucleotide variant in HB which accounts for more than 58.4% and 15.4% were frameshift mutation due to deletion, insertion or duplication. Nonsense and splice mutations account for 8.3% and 9.4% respectively [42].

Bleeding disorders like hemophilia A [classic hemophilia] and Hemophilia B [Christmasdisease] are the commonest inherited disorders worldwide and in India. HA is more common than HB. Being an inherited disorder, family history has been observed in 45-47% of cases of Hemophilia B patients [43, 44]. In our study, it was 97 [64.7%] HB patients had a family history of bleeding disorder. Rest of the cases may be due to spontaneous mutations or transferred by mildly affected or carrier parents who were asymptomatic and undiagnosed [44].

The majority of cases (96%) had bleeding manifestation before the age of 5 years of age [45] with the mean age of onset ranging from 9-11 months depending upon severity [46]. In our study, the mean age of onset of symptoms was 2.0±1.0 years in severe patients, 7.5±2.8 0 years in moderate patients and 10.0±3.5 years in mild HB patients. Munira et al., 2013 reported mean age onset to be 15.8 years [45]. All the patients in the present study were male except 2 females. Hemophilia A and Hemophilia B are X-linked disorder; it most commonly affects the male and females act as a carrier.

In the present study 102 (68%) HB patients having <1% of factor IX concentration were designated as severe, 30 (20%) cases having 1-5% of factor IX concentration were designated as moderate and 18 (12%) cases having >5-<40% of factor IX concentration were designated as mild. In the study, Ahmed et al, 2008 hemophilia B patients had 69.6% severe, 19.2% had moderate and 11.2% had a mild condition of hemophilia B [47]. The study by Sahoo et al.,

2020 and Pawan et al 2021 showed a higher percentage of severe hemophilia cases i.e 87% and 84% respectively [44].

The initial bleeding site depends upon FIX level factors. In a place where circumcision is a routine practice, post circumcision bleed is the very common initial bleed [51%-62%]. Joint bleed [hemarthrosis] is the most common [82%-100%]. Ahmed et al reported the most common presenting features in hemophilia as hemarthrosis in 82%. Hemarthrosis is most common in 72.85 % of HB cases followed by skin bleed [77-90%] [48-51]. In the present study, joint bleeds [hemarthrosis] was the most common clinical manifestation 57.3% followed by skin bleed 18%, muscle in 10.7%, Epistaxis in 7.3% and Petechiae in 6.7%. The study by Pawan et al., 2021 shows 88% of hemarthrosis and 96% of hematoma in severe HB patients but our study recorded a low rate of hemarthrosis [54%] and hematoma [16.7%] in severe HB. This may be due to differences in the geographical region and population. In our study family history of bleeding was observed in 97 [64.7%] HB patients and it is high compared to the study conducted by Pawan et al., 2021 and Sahoo et al., 2020 which showed positive family history in 45% and 47% respectively [45, 52].

We recorded stop gained mutation; p.R75W in 2 severe HB patients which was earlierrecorded by Parrado Jara, YA et al.,2020, Radic, C et al.,2013, KWON, M.J et al.,2008, Saini, S et al.,2015, Koeberl, D.D et al.,1990 [53, 54-57]. This mutation results in the loss of >80% of the total F9 protein and increased the probability of an immune response. p.R226W mutation affected the activation of zymogen by altering the cleavage of factor XIa [58,59] which was recorded in 1 moderate and 5 severe patients (FIX Concentration 0.1±0.7) in our study group. Mutation p.C396Y interrupted the environment of the active site that involved in the conversion of the zymogen to an enzyme which was observed only in one severe HB patient [60]. We also recorded p.T194A in 3mild HB patients (FIX concentration 9.8±0.8) and p.C357Y missense mutation which were predicted to be pathogenic on the function of F9 protein in the blood coagulation process byprevious studies. We recorded this mutation in 1 mild, 3 moderate patients (FIX concentration 2.8±0.8) and 4 severe patients (0.05±0.1 [61-64].

R43 is a mutation hotspot that accounts for 73.5% of hemophilia B cases [65] in the present study, we recorded R43W mutation in c.127C>T observed in 3 mild patients (FIX concentration 8.1±1.9), 4 moderate (FIX concentration 2.1±0.2) and 2 severe patients (0.65±0.15). Novel missense mutation p.E66D was recorded in 4 moderate HB patients with mean FIX concentration 2.0±0.5 and in 07 severe HB patients with mean FIX concentration 0.3±0.5. It was found to be harmful on the function of F9 by insilico pathogenicity prediction tools. Moreover this novel mutation was present in highly conserved regions of F9 protein. p.E66D mutant residue was smaller than wild type residue this might lead to loss of interactions with the metal-ion: "Calcium 4 or magnesium 1; via 4-carboxyglutamate hence this missense variant has a pathogenic effect over normal F9 activation and blood coagulation process. As per the ACMG-AMP guidelines p.E66D is classified as "Pathogenic" [66]. Along with missense variants we also recorded one novel stop gained mutation; p.S365* in 3 moderate HB patients with mean FIX concentration 2.6±0.8 and in 12 severe HB patients with mean FIX concentration 0.05±0.15.This leads to the loss of 21% of the F9 protein that result in loss of the majority of the functions of peptidase S1 of F9 protein. As per the ACMG-AMP guidelines p.S365* is classified as "Pathogenic". Only one 3'UTR variant was recorded in our study cohort. Functional analysis of the mutations was the major limitation of our study.

Out of 150 HB patients, 90(60%) HB patients recorded the mutations in *F9* gene including mild, moderate and severe condition. In mild 15 (10%) patients, in moderate 26 (17%) patients and severe 49 (32%) patients showed the mutations in their *F9* gene. Among 3 groups, severe group showed high rate of mutations (32%) in the HB patients compared to other 2 groups and in our study cohort, number of severe patients were also high, followed by moderate and mild groups. In 16 mutations, 9 mutations were recorded as pathogenic and all the 49 severe mutated haemophilic patients were coming under these 9 pathogenic variants viz c.127C>T, c.198A>T, c.223C>T, c.314A>G, c.470G>A, c.676C>T, c.1070G>A, c.1094C>A and c.1232G>A. These are the location of mutations which are involved in causing the pathogenic effects which may lead to severe

symptoms in haemophilic patients of our population. c.1187G>A and c.1385A>G mutations were recorded as benign and one moderately affected patient is diagnosed by c.1385A>G variant and c.1187G>A mutation was found in severe HB patient. 2 mutations c.195G>A and c.304C>T were likely pathogenic and c.195G>A variant found in one moderate Haemophilic patient. In two mild patients c.304C>T mutation was found. Likely benign mutations c.197A>T and c.580A>G recorded in 2 mild, 1 severe and 3 mild haemophilic patients study.

2 stop gained mutations and 7 missense mutations were major pathogenic variations or disease-causing mutations recorded in our study, patients who were recorded with these mutations showed severe condition compared to other 2 groups (mild and moderate). Likely pathogenic and benign mutations were also missense mutation and all the patients who recorded thesemutations were mild to moderately affected by HB except 2 patients. Only 3 mutations (c.127C>T, c.470G>A, c.1070G>A) were associated with all different severity. 2 mutations were only associated with mild HB condition (c.304C>T, c.580A>G), 2 mutations were with moderate HB condition (c.195G>A, c.1385A>G) and 3 mutations were only associated with severe HB (c.223C>T, c.1187G>A, c.1232G>A). One mutation was associated with mild-moderate HB (c.*1110A>G) and Mild-severe HB (c.197A>T). 4 mutations were associated with moderate- severe condition of Hemophilia B (c.314A>G, c.198A>T, c.676C>T, c.1094C>A). 48% (49/102) of the severe HB patients showed mutations in *F9* gene followed by 86.6% (26/30) of moderate HB and 83.3% (15/18) of mild HB patients.

Patients with haemophilia-B may have a wide range of clinical symptoms and require proper management for the rest of their life. Primary care physicians are the first point of contact for diagnosis and treatment. Our study provides knowledge about hemophilia B prevalence, clinical features and laboratory diagnosis which will help the physicians in better diagnosis and early treatment. Only a few clinics offer the testing such as factor concentration and mutation analysis needed to diagnose inherited bleeding problems in our population. Several previous studies from India carried on Hemophilia B were referred to hospitals and those were not properly determining the severity of the disease in our population. So present study provides

better understanding of concentration of factor levels with respect to severity of the Hemophilia B and it also provides demographic and clinicopathological aspects of Hemophilia B in the present study population.

Hemophilia is common worldwide and shows heterogenous presentation upon the disease severity over different populations. The present study is one of the fewer studies from the Karnataka region of India studying the demographic and clinicopathological features of hemophilia B. In India, hemophilia inhibitor screening and other genetic tests are not commonly available for the identification and so management of the patient is difficult. Care of hemophilia is complex, often requiring health management beyond the prevention and treatment of bleeding. Early diagnosis might be helpful with the knowledge of spectra presentation of Hemophilia B in a local population.

CHAPTER 7

SUMMARY AND CONCLUSION

Hemophilia B is a bleeding disorder caused by the genetic deffect in F9 gene and it has high mortality and disability. Even thopugh hemophilia B is single gene disorder, but it has heterogenic in nature interms of genetic mutations.

Present study was performd in 150 hemophilia B patients from Karnataka, India. Among 150 HB patients, 102 (68%), 30 (20%), and 18 (12%) suffered from severe, moderate, and mild Hemophilia, respectively.

Genetic analysis of the *F9* gene in 150 hemophiloia B patients identified 16 mutations, including 3 novel mutations which were not recorded previously in any inhouse mutation databses. Nine mutations (7 missense and 2 stop-gained) were found to be pathogenic.

Only 3 mutations (c.127C>T, c.470G>A, and c.1070G>A) were associated with different severities. While 2 mutations were associated with mild HB cases (c.304C>T and c.580A>G), 2 (c.195G>A and c.1385A>G) and 3 mutations (c.223C>T, c.1187G>A, and c.1232G>A) resulted in moderate and severe disease, respectively.

Additionally, one mutation each was associated with mild-moderate (c.*1110A>G) and mild-severe HB disease (c.197A>T), 4 mutations were associated with moderate-severe HB cases (c.314A>G, c.198A>T, c.676C>T, and c.1094C>A). FIX concentrations were lower in the mutated group (5.5±2.5% vs. 8.0±2.5%). Novel p.E66D and p.S365* mutations were predicted to be pathogenic based on changes in FIX structure and function.

Clinical Significanes:

Novel single nucleotide polymorphisms (SNPs) of *F9* gene is an important pathophysiologic and prognostic insight showed novel p.E66D and p.S365 mutations. Novel p.E66D and p.S365 mutations were predicted to be pathogenic based on changes in FIX structure and function and failed in proper production of FIX.

Management Strategies:

Novel single nucleotide polymorphisms (SNPs) largely contributed to the pathogenesis of HB. Our study strongly suggests that population-based genetic screening will be particularly helpful to identify risk prediction and carrier detection tools for Indian HB patients. Probably, the mutations detectedint his study may be used as biomarkers in this specific populationafter validating in large number of HB samples.

REFERENCES

1. Kadhim KAR, Al-Lami FH, Baldawi KH. Epidemiological Profile of Hemophilia in Baghdad-Iraq. Inquiry. 2019 Jan-Dec; 56:46958019845280.
2. Kawankar N, Rathi J, Ghosh K, Shetty S. Clinical and molecular epidemiology of factor XI deficiency in India. Thromb Res. 2016 Nov; 147:85-87.
3. Singh M, Singh P. Factor VIII gene haplotypes and linkage disequilibrium for the indirect genetic analysis of hemophilia A in India. Clin Appl Thromb Hemost. 2009 May-Jun;15(3):334-9.
4. Col J. Philip, Brig R.S. Sarkar, Lt Col S. Kumar, B.R. Prathip, Amardeep Pathak. Factor IX deficiency (Christmas disease). Medical Journal Armed Forces India 2012; 68:379-380.
5. Philip J, Sarkar RS, Kumar S, Prathip BR, Pathak A. Factor IX deficiency (Christmas disease). Med J Armed Forces India. 2012 Oct;68(4):379-80.
6. Gomez K, Bolton-Maggs P. Factor XI deficiency. Haemophilia. 2008 Nov;14(6):1183-9.
7. Mannucci PM, Tuddenham EG. The hemophilias--from royal genes to gene therapy. N Engl J Med. 2001 Jun 7;344(23):1773-9.
8. Palta S, Saroa R, Palta A. Overview of the coagulation system. Indian J Anaesth. 2014 Sep;58(5):515-23.
9. Ogston D. The physiology of hemostasis. Cambridge, MA: Harvard University Press; 1983.
10. Chaudhry R, Usama SM, Babiker HM. Physiology, Coagulation Pathways. 2022 Aug 29. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan–. PMID: 29489185.
11. Kadhim KAR, Al-Lami FH, Baldawi KH. Epidemiological Profile of Hemophilia in Baghdad-Iraq. Inquiry. 2019 Jan-Dec; 56:46958019845280.
12. How hemophilia is inherited [Internet]. Centers for Disease Control and Prevention. Centers for Disease Control and Prevention; 2022 [cited 2022Dec31]. Available from: https://www.cdc.gov/ncbddd/hemophilia/inheritance-pattern.html

13. Castaman G, Matino D. Hemophilia A and B: molecular and clinical similarities and differences. Haematologica. 2019 Sep;104(9):1702-1709.

14. Arruda VR, Samelson-Jones BJ. Gene therapy for immune tolerance induction in hemophilia with inhibitors. J Thromb Haemost. 2016 Jun;14(6):1121-34.

15. Alshaikhli A, Rokkam VR. Hemophilia B. [Updated 2022 Oct 19]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan.

16. Morfini M, Coppola A, Franchini M, Di Minno G. Clinical use of factor VIII and factor IX concentrates. Blood Transfus. 2013 Sep;11 Suppl 4(Suppl 4):s55-63

17. Knobe K, Berntorp E. Haemophilia and joint disease: pathophysiology, evaluation, and management. J Comorb. 2011 Dec 27; 1:51-59.

18. Phadke S. Hemophilia care in India: a review and experience from a tertiary care centre in uttar pradesh. Indian J Hematol Blood Transfus. 2011 Sep;27(3):121-6.

19. Josephson N. The hemophilias and their clinical management. Hematology Am Soc Hematol Educ Program. 2013; 2013:261-7.

20. Flaherty LM, Josephson NC. Screening for fall risk in patients with haemophilia. Haemophilia. 2013 May;19(3): e103-9.

21. Srivastava A, Brewer AK, Mauser-Bunschoten EP, Key NS, Kitchen S, Llinas A, Ludlam CA, Mahlangu JN, Mulder K, Poon MC, Street A; Treatment Guidelines Working Group on Behalf of The World Federation Of Hemophilia. Guidelines for the management of hemophilia. Haemophilia. 2013 Jan;19(1): e1-47.

22. Bertamino M, Riccardi F, Banov L, Svahn J, Molinari AC. Hemophilia Care in the Pediatric Age. J Clin Med. 2017 May 19;6(5):54.

23. Miller CH. The Clinical Genetics of Hemophilia B (Factor IX Deficiency). Appl Clin Genet. 2021 Nov 23; 14:445-454.

24. Al-Allaf FA, Taher MM, Abduljaleel Z, Bouazzaoui A, Athar M, Bogari NM, Abalkhail HA, Owaidah TM. Molecular Analysis of Factor VIII and Factor IX Genes in Hemophilia Patients: Identification of Novel Mutations and Molecular Dynamics Studies. J Clin Med Res. 2017 Apr;9(4):317-331.

25. Sexton A, Furmedge J, Barnes C, Cheetham G, Wallace J, Savarirayan R. Clinical significance of two mutations in the factor IX gene in a family with haemophilia B. Haemophilia. 2010 Mar;16(2):374-6.

26. Orlova NA, Kovnir SV, Vorobiev II, Gabibov AG. Coagulation Factor IX for Hemophilia B Therapy. Acta Naturae. 2012 Apr;4(2):62-73.

27. Goodeve AC. Hemophilia B: molecular pathogenesis and mutation analysis. J Thromb Haemost. 2015 Jul;13(7):1184-95.

28. Kulkarni S, Hegde R, Hegde S, Kulkarni SS, Hanagvadi S, Das KK, Kolagi S, Gai PB, Bulagouda R. Mutation analysis and characterisation of F9 gene in haemophilia- B population of India. Blood Res. 2021 Dec 31;56(4):252-258.

29. High KA, Anguela XM. Adeno-associated viral vectors for the treatment of hemophilia. Hum Mol Genet. 2016 Apr 15;25(R1): R36-41.

30. Dargaud Y, Negrier C. Thrombin generation testing in haemophilia comprehensive care centres. Haemophilia. 2010 Mar;16(2):223-30.

31. Quadros L, Ghosh K, Shetty S. A common G10430A mutation (Gly 60 Ser) in the factor IX gene describes the presence of moderate and mild hemophilia B in the majority of the Gujarati population. Ann Hematol. 2007 May;86(5): 377-9.

32. Saini S, Hamasaki-Katagiri N, Pandey GS, Yanover C, Guelcher C, Simhadri VL, Dandekar S, Guerrera MF, Kimchi-Sarfaty C, Sauna ZE. Genetic determinants of immunogenicity to factor IX during the treatment of haemophilia B. Haemophilia. 2015 Mar;21(2):210-218.

33. Chan JKY, Gil-Farina I, Johana N, Rosales C, Tan YW, Ceiler J, Mcintosh J, Ogden B, Waddington SN, Schmidt M, Biswas A, Choolani M, Nathwani AC, Mattar CNZ. Therapeutic expression of human clotting factors IX and X following adeno-associated viral vector-mediated intrauterine gene transfer in early-gestation fetal macaques. FASEB J. 2019 Mar;33(3):3954-3967.

34. Kar A, Phadnis S, Dharmarajan S, Nakade J. Epidemiology & social costs of haemophilia in India. Indian J Med Res. 2014 Jul;140(1):19-31.

35. Ghosh K, Shetty S, Kulkarni B, Nair S, Pawar A, Khare A, Baindur S, Mohanty D. Development of inhibitors in patients with haemophilia from India. Haemophilia. 2001 May;7(3):273-8.

36. Mahajan A, Chavali S, Kabra M, Chowdhury MR, Bharadwaj D. Molecular characterization of hemophilia B in North Indian families: identification of novel and recurrent molecular events in the factor IX gene. Haematologica. 2004 Dec;89(12):1498-503.

37. Mukherjee S, Mukhopadhyay A, Banerjee D, Chandak GR, Ray K. Molecular pathology of haemophilia B: identification of five novel mutations including a LINE 1 insertion in Indian patients. Haemophilia. 2004 May;10(3):259-63.

38. Zahari M, Sulaiman SA, Othman Z, Ayob Y, Karim FA, Jamal R. Mutational Profiles of F8 and F9 in a Cohort of Haemophilia A and Haemophilia B Patients in the Multi-ethnic Malaysian Population. Mediterr J Hematol Infect Dis. 2018 Sep 1;10(1): e2018056.

39. Kumar S, Sinha S, Bharti A, Meena LP, Gupta V, Shukla J. A study to determine the prevalence, clinical profile and incidence of formation of inhibitors in patients of hemophilia in North Eastern part of India. J Family Med Prim Care. 2019 Jul;8(7):2463-2467.

40. Huang L, Li L, Lin S, Chen J, Li K, Fan D, Jin W, Li Y, Yang X, Xiong Y, Li F, Yang X, Li M, Li Q. Molecular analysis of 76 Chinese hemophilia B pedigrees and the identification of 10 novel mutations. Mol Genet Genomic Med. 2020 Nov;8(11): e1482.

41. Abla Z, Mouloud Y, Hejer EM, et al. Mutations causing hemophilia B in Algeria: identification of two novel mutations of the factor 9 gene. Biodiversitas 2018;19:52-8

42. Bhattacharya DK. Haemophilia in the Indian Scenario. Int J Hum Genet 2006;6:33-9

43. Mannucci PM. Hemophilia therapy: the future has begun. Haematologica. 2020 Mar;105(3):545-553.

44. Benson G, Auerswald G, Dolan G, Duffy A, Hermans C, Ljung R, Morfini M, Šalek SZ. Diagnosis and care of patients with mild haemophilia: practical recommendations for clinical management. Blood Transfus. 2018 Nov;16(6):535-544.

45. Sahoo T, Naseem S, Ahluwalia J, Marwaha RK, Trehan A, Bansal D. Inherited Bleeding Disorders in North Indian Children: 14 years' Experience from a Tertiary Care Center. Indian J Hematol Blood Transfus. 2020 Apr;36(2):330-336.

46. Karim MA, Siddique R, Jamal CY, Islam A. Clinical profile of haemophilia in children in a tertiary care hospital. Bangladesh J Child Health 2013; 37:90-6.

47. Hazewinkel MH, Hoogerwerf JJ, Hesseling PB, Hartley P, MacLean PE, Peters M, et al. Haemophilia patients aged 0-18 years in the Western Cape. S Afr Med J 2003; 93:793-6.

48. Ahmad F, Kannan M, Ranjan R, Bajaj J, Choudhary P, Saxena R. Inherited platelet function disorders versus other inherited bleeding disorders: An Indian overview. Thromb Res 2008; 121:835-41.

49. Uddin MM, Rahman MJ, Rahman MM, Sultana SA, Shah MS. Clinico-pathological study on haemophilia: An analysis of 50 cases. J Bangladesh Coll Phys Surg 2006; 24:50-3.

50. Kim KY, Yang CH, Cho MJ, Lee M. Comprehensive clinical and statistical analysis of hemophilia in Korea. J Korean Med Sci 1988; 3:107-15.

51. Qasim Z, Naseem L, Asif N, Hassan K. Haemophilia; pattern of clinical presentation and disease severity. Int J Pathol 2013;11:58-63

52. Pawan PK, Mahima Y, Vijai T, Manjula L. Clinicopathological features of hemophilia in a tertiary care centre of India. J Family Med Prim Care. 2021 Jan;10(1):295-299.

53. Parrado Jara YA, Yunis Hazbun LK, Linares A, Yunis Londoño JJ. Molecular characterization of hemophilia B patients in Colombia. Mol Genet Genomic Med. 2020 May;8(5): e1210.

54. Radic CP, Rossetti LC, Abelleyro MM, et al. Assessment of the F9 genotype-specific FIX inhibitor risks and characterisation of 10 novels severe F9 defects in the first molecular series of Argentinian patients with haemophilia B. Thromb Haemost 2013;109:24-33.

55. Kwon MJ, Yoo KY, Kim HJ, Kim SH. Identification of mutations in the F9 gene including exon deletion by multiplex ligationdependent probe amplification in 33 unrelated Korean patients with haemophilia B. Haemophilia 2008;14:1069-75.

56. Saini S, Hamasaki-Katagiri N, Pandey GS, et al. Genetic determinants of immunogenicity to factor IX during the treatment of haemophilia B. Haemophilia 2015;21:210-8.

57. Koeberl DD, Bottema CD, Sarkar G, Ketterling RP, Chen SH, Sommer SS. Recurrent nonsense mutations at arginine residues cause severe hemophilia B in unrelated hemophiliacs. Hum Genet 1990; 84:387-90.

58. Suehiro K, Kawabata S, Miyata T, et al. Blood clotting factor IX BM Nagoya. Substitution of arginine 180 by tryptophan and its activation by alpha-chymotrypsin and rat mast cell chymase. J Biol Chem 1989; 264:21257-65.

59. Bertina RM, van der Linden IK, Mannucci PM, et al. Mutations in hemophilia Bm occur at the Arg180-Val activation site or in the catalytic domain of factor IX. J Biol Chem 1990; 265:10876-83.

60. Taylor SA, Deugau KV, Lillicrap DP. Somatic mosaicism and female-to-female transmission in kindred with hemophilia B (factor IX deficiency). Proc Natl Acad Sci U S A 1991;88:39-42

61. Li Y, Bezemer ID, Rowland CM, et al. Genetic variants associated with deep vein thrombosis: the F11 locus. J Thromb Haemost 2009; 7:1802-8.

62. Graham JB, Lubahn DB, Lord ST, et al. The Malmö polymorphism of coagulation factor IX, an immunologic polymorphism due to dimorphism of residue 148 that is in linkage disequilibrium with two other F.IX polymorphisms. Am J Hum Genet 1988; 42:573-80.

63. McGraw RA, Davis LM, Noyes CM, et al. Evidence for a prevalent dimorphism in the activation peptide of human coagulation factor IX. Proc Natl Acad Sci U S A 1985;82:2847-51

64. Miyata T, Sakai T, Sugimoto M, et al. Factor IX Amagasaki: a new mutation in the catalytic domain resulting in the loss of both coagulant and esterase activities. Biochemistry 1991; 30:11286-91.

65. Gao W, Xu Y, Liu H, et al. Characterization of missense mutations in the signal peptide and propeptide of FIX in hemophilia B by a cell-based assay. Blood Adv 2020; 4:3659-67.

66. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-24

67. Lv X, Li T, Li H, Liu HY, Wang Z, Guo ZP. Genetic analysis of a hemophilia B family with a novel F9 gene mutation: A STROBE-compliant article. Medicine (Baltimore). 2019 May;98(21): e15688.

CONSENT FORMS

CONSENT FORM I

INFORMATION FOR PARTICIPANTS OF THE STUDY

01. **Title of the project:** Genetic and Molecular Profiling of FIX (FACTOR 9) Gene of Hemophilia B in a Karnataka

02. Name of the PI/Ph.D Student and Department:

Sujayendra S Kulkarni,
Lecturer,
Division of Human Genetics (CRL),
S. Nijalingappa Medical College,
Bagalkot
And
Ph.D Scholar,
Dept of Anatomy (Allied Health Science),
BLDE University, Vijayapura.

03. Name, Designation, Address, Phone No. and Email ID of the Guide:

Dr R.S. Bulagouda,
Professor and Head,
Departmentt of Anatomy,
Shri B M Patil Medical College,
Vijayapura.
Ph : 9845130231

04. Name of Co-guide/Co-investigator with designation, Department, Phone No. and Email ID:

1) Dr. P.B.Gai
Professor and Vice Chancellor,
Karnataka University, Dharwad.
And
Director,
Karnataka Institute for DNA Research,
Dharwad
Email. pramodbgai@gmail.com
Phone No: 9591171725

2) Dr Sanjeev Kolagi
Professor and HOD,
Dept of Anatomy, S N Medical College, Bagalkot.
Email. drsanjeevkolagi@hotmail.com
Phone No: 9731798355

05. **Purpose/ Objectives of this project /study:**

Identification of novel mutations leading to future discovery of specific biomarker will not only help the patients suffering from Hemophilia B but, also

usefull the global research community. The data generated by the research will enable the clinicians to offer effective treatment with more specificity. By identification of novel mutations present in targeted FIX genes specific to Indians, it may be possible to develop probes and to detect risk factor of developing hemophilia B in the early time. In this way "Molecular Genetic study of Hemophilia B in a Karnataka population" will definitely improve the quality of diagnosis and treatment of Hemophilia B in the populations.

1. Collecting data relating to Hemophilia B and finding out the prevalence and incidence of Hemophilia B in this region.
2. Finding out the possible role of factor IX (FIX) cellular path way genes in Hemophilia B.
3. Gene expression profiling of specific targeted genes using super array technology.
4. Validation of the results in more number of patients through Q-PCR methods.
5. Defining the candidate genes and their possible role in Hemophilia B.
6. Mutated analysis of selected genes among targeted genes using automated DNA sequencing methods.
7. Identification of novel mutations.

06. **Procedure/Methods of the study:**

Selected families including Hemophilia B affected patients of Karnataka origin were enrolled in this study. Informed consent was obtaining from each family and all the patients were male. The participants were from any region of Karnataka, cared from the association of hemophilia Society, Karnataka. The blood sample is taking from venipuncture into evacuated tubes containing trisodium citrate (0,109 M) as an anticoagulant. These tests perform activated partial thromboplastin time (aPTT), FIX activity and FVIII activity. The severity of the disease was classified based on FIX activity. Clotting factor inhibitor screening was performed by activated partial thromboplastin time mixing studies using normal pool plasma.

07. Expected duration of the subject participation: 30 minutes

08. Expected benefits from the research to the participant:

You are being chosen for this study because you are normal and healthy. Once you consent to participate in this study, 5ml blood sample will collected from you. By participating in this study, you will be providing us with valuable information regarding variations in the biochemical parameters between healthy subjects such as yourself and patients with Hemophilia B. This knowledge will ultimately lead to improvements in care of patients with Hemophilia B. Results of the tests will be informed to you. If any abnormality is detected, it will be managed appropriately. The data generated from the study may be used for scientific publications.

09. Any risks expected from the study to the participants:

You may feel slight discomfort/ pain during withdrawal of blood.

10. Maintenance of confidentiality of records:

The study records will be kept confidential. Your personal identity will not be revealed in any publication or release of results. Study record will be kept indefinitely for analysis.

11. Provision of free treatment for research related injury:

Although the study procedure itself carries more then minimal risk, treatment of any unforeseeable event will be provided free of cost by the Institute to you

12. Compensation of the participants for disability or death resulting from such injury:

Compensation for any unforeseeable research related injury or death resulting from such injury will be duly given to you through hospital insurance policy number 68040236180200000009

13. Freedom to withdraw from the study at any time during the study period without the loss of benefits that the participant would otherwise be entitled:

It is entirely your decision to participate in the study. If you want to discontinue from the study at any point of time, you are free to leave without stating any reason. Your withdrawal will not affect your treatment at your sight. Irrespective of your decision to participate in the study, you will still receive the standard treatment for your illness.

14. Possible current and future uses of the biological material and of the data to be generated from the research and if the material is likely to be used for secondary purposes or would be shared with others, this should be mentioned

All the data and materials obtained from you will be used only for research purposes. It will not be used for secondary purposes nor will it be shared with others.

15. Contact details of Chairman of the IEC for appeal against violation of rights.

Dr. S.L. Hoti,
Director Grade Scientist (Scientist G),
ICMR-National Institute of Traditional Medicine
(Formerly RMRC),
Belgagavi- 590010
Phone No. 0831-2477477
Fax. 0831-2475479

CONSENT FORM-II

PARTICIPANT CONSENT FORM

CASES WITH Hemophilia B

Participant's name:

Address:

Phone No.

Email ID:

Title of the project: Genetic and Molecular Profiling of FIX (FACTOR 9) Gene of Hemophilia B in Karnataka.

The details of the study have been provided to me in writing and explained to me in my own language. I confirm that I have understood the above study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided. Such a use is only for scientific purpose(s).

I have been given an information sheet giving details of the study. I fully consent to participate in the above study.

Signature of the Participant: _____ Date: _____

Signature of the Witness: _____ Date: _____

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Sujayendra S Kulkarni,
J¸ï. J£ï. ªÉÊzÀåQÃAiÀÄ
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JZï.J¸ï.PÉ. D¸ÀàvÉæ ºÁUÀÆ
¸ÀA±ÉÆÃzsÀ£Á PÉÃAzÀæ, ¨ÁUÀ®PÉÆÃl.
zÀÆgÀªÁtÂ¸ÀASÉå: 9886081324

¸ÀA±ÉÆÃzsÀ£Á GzÉÝÃ±À: è ಪುರುಷರಲ್ಲಿ PÀAqÀÄ§gÀÄªÀ ಹಿಮೋಫಿಲಿಯಾ ಬಿ ಜೆನೆಟಿಕ್ □□□□ □□□□□□□□□□□ □□□□□□□□□□□ ಆಫ್ □□□□□□ (ಫ್ಯಾಕ್ಟರ್ ೯) ಜೀನ್ CzsÀåAiÀÄ£À (Genetic and Molecular Profiling of FIX (FACTOR 9) Gene of Hemophilia B in Karnataka).

¸ÀA±ÉÆÃzsÀ£ÉAiÀÄ «zsÁ£À: ರಕ್ತದ್ ಮುಖಾಂತರ ಜಿನನ್ನು ಬೇರ್ಪಡಿಸಿ ಅದರಲ್ಲಿರುವ ಮ್ಯುಟೇಷನ್ ಕಂಡುಹಿಡುಯುವದು.

aQvÁì «zsÁ£ÀPÉÌ ¨ÉÃPÁUÀÄªÀ ¸ÀªÀÄAiÀÄ: 30 ¤«ÄµÀ,

ಭಾಗವಹಿಸುವವರಿಗೆ ಅಧ್ಯಯನದ ನಿರೀಕ್ಷೆಯ ಅಪಾಯಗಳು: ಕಡಿಮೆ ಅಪಾಯಕ್ಕಿಂತ ಹೆಚ್ಚು

aQvÉì¬ÄAzÀ DUÀÄªÀ G¥ÀAiÉÆÃUÀ: ಪುರುಷರಲ್ಲಿ PÀAqÀÄ§gÀÄªÀ ಹಿಮೋಫಿಲಿಯಾ ಬಿ ಜೆನೆಟಿಕ್ □□□□ □□□□□□□□□□□ □□□□□□□□□□□ ಆಫ್ □□□□□□ (ಫ್ಯಾಕ್ಟರ್ ೯) ಜೀನ್ CzsÀåAiÀÄ£À (Genetic and Molecular Profiling of FIX (FACTOR 9) Gene of Hemophilia B in Karnataka).

aQvÉìAiÀÄ «ªÀgÀUÀ¼À£ÀÄß ¤UÀÆqsÀªÁV EqÀ¯ÁUÀÄªÀÅzÀÄ ºÁUÀÆ gÉÆÃVAiÀÄ «ªÀgÀUÀ¼À£ÀÄß ¸ÀA±ÉÆÃzsÀ£ÉAiÀÄ ¸À®ÄªÁV ªÀiÁvÀæ §¼À¸À¯ÁUÀÄªÀÅzÀÄ.

aQvÉìAiÀÄ ¸ÀªÀÄAiÀÄzÀ°è CqÀØ¥ÀjuÁªÀÄUÀ¼ÀÄ GAmÁzÀ°è, gÉÆÃVAiÀÄ ¸ÀA¥ÀÆtð aQvÉìAiÀÄ£ÀÄß GavÀªÁV ªÀiÁqÀ¯ÁUÀÄªÀÅzÀÄ ºÁUÀÆ CzÀgÀ ªÉZÀÑªÀ£ÀÄß ¨sÀj¸À¯ÁUÀÄªÀÅzÀÄ.

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SUJAYENDRA S. KULKARNI
J¸ï. J£ï. ªÉÊzÀåQÃAiÀÄ
ªÀÄºÁ«zÁå®AiÀÄ ªÀÄvÀÄÛ
JZï.J¸ï.PÉ. D¸ÀàvÉæ ºÁUÀÆ
¸ÀA±ÉÆÃzsÀ£Á PÉÃAzÀæ,

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PLAGIARISM VERIFICATION CERTIFICATE

BLDE (DEEMED TO BE UNIVERSITY)

Annexure -I

PLAGIARISM VERIFICATION CERTIFICATE

1. Name of the Student: **Sujayendra. S. Kulkarni Reg No: 17PHD002**

2. Title of the Thesis: **"Genetic and Molecular Profiling of FIX (FACTOR 9) Gene of Haemophilia B in Karnataka"**

3. Department: **Anatomy**

4. Name of the Guide & Designation: **DR. R.S. Bulagouda,**
Professor and Head,
Department of Anatomy,
Shri B. M. Patil Medical College,
Vijayapura.

5. Name of the Co-Guide & Designation:
a) **Dr Sanjeev I. Kolagi**
Professor and Head
Department of Anatomy
S Nijalingappa Medical College and
HSK Hospital, Bagalkote.

b) **Prof. Pramod B Gai**
Director
Karnataka Institute for DNA
Research [KIDNAR]
Dharwad,

6. The above thesis was verified for similarity detection. The report is as follows:

Software used**: Ouriginal Date: 07.01.2023.**

Similarity Index (%): **Six percent (06%) Total word Count:** 30964.

The report is attached for the review by the Student and Guide.

The plagiarism report of the above thesis has been reviewed by the undersigned.

The similarity index is below accepted norms.

The similarity index is above accepted norms, because of following reasons:

Total 24% Similarity found out of which 18% is from his own publications, hence 24-18=6% Similarity. The thesis may be considered for submission to the University. The software report is attached.

Signature of the Guide	Signature of Co-Guide	Signature of Student
Name & Designation	Name & Designation	
Professor & H.O.D. Dept. of Anatomy BLDE (Deemed to be University) Shri B. M. Patil Medical College Vijayapura-586103.	Professor & HOD Dept. of Anatomy S. N. Medical College BAGALKOT	

Verified by (Signature)

Name & Designation

Dr. Prasanna Kumar BM
University Librarian
BLDE (Deemed to be University)
Shri B M Patil Medical College
Vijayapura - 586103

ETHICAL CERTIFICATES

BLDE (DEEMED TO BE UNIVERSITY)

[Declared as Deemed-to-be-University u/s 3 of UGC Act, 1956 vide Government of India Notification No.F.9-37/2007-U.3(A)]

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA

BLDE (DU)/IEC/340/2018-19 21-12-2018

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The ethical Committee of this University met on21st December 2018 at 11 a.m.to scrutinizes the Synopsis/ Research Projects of Post Graduate Student / Under Graduate Student Faculty members of this University / College from ethical clearance point of view. After scrutiny, the following original/ corrected and revised version Synopsys of the thesis/ research projects has been accorded ethical clearance.

Title. Genetic and Molecular Profiling of FIX (FACTOR 9) Gene of Hemophilia B in a Karnataka

Name of the Faculty member /PhD/PG/UG student. Sujayendra S. Kulkarni. PhD

Name of the Guide; Dr. S. P. Gugrigouder, Professor and Principal.

Dr. Sharada Metgud

Chair person
IEC, BLDE (DU),
VIJAYAPURA

Institutional Ethics Committee VIJAYAPURA BLDE (DEEMED TO BE UNIVERSITY)

Dr.G.V.Kulkarni

Member Secretary
IEC, BLDE (DU),
VIJAYAPURA

MEMBER SECRETARY
Institutional Ethics Committee
BLDE (Deemed to be University
Vijayapura-586103, Karnataka

Note:-Kindly send Quarterly progress report to the Member Secretary

Following documents were placed before ethical committee for Scrutinization.

- Copy of Synopsis/Research Projects
- Copy of inform consent form
  Any other relevant documents

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Office of the Institutional Ethics Committee

File No: SNMC/IECHSR/2018-19/A-82/1.1 Date:15 /02/2019

To:

Sujendra Kulkarni

Lecturer

Central Research Laboratory

SNMC, Bagalkot

Topic of Protocol: Genetic and Molecular Profiling of FIX (FACTOR 9) Gene of Hemophilia B in Karnataka

Subject: Approval for conducting the above mentioned study & related documents by IEC.

Dear Dr Harini J

The Ethics Committee (EC) meeting of SNMC was held on 19-01-2019 from 09.30 AM onwards in the Hall of Medical Education Department of S. Nijalingappa Medical College & Hanagal Shri Kumareshwar Hospital & Research Centre, Bagalkot.

Following members of the committee were present:

1. Dr. S. L Hoti, Scientist-G,Director grade scientist ICMR-NITN, Belgaum.	**Chairman**
2. Dr.Yasmeen Maniyar , Professor & HOD of Pharmacology , SNMC, Bagalkot.	**Member**
3. Dr Anita Herur Professor of Physiology,SNMC,Bagalkot	**Member**
4. Dr Ashalata Mallapur Prof &HoD OBG, SNMC,Bagalkot	**Member**
5. Dr Chandrashekar V M Professor of Pharmacology HSK Pharmacy college	**Member**
6. Dr. Chandrashekharayya S. Hiremath, Professor of ENT, SNMC, Bagalkot	**Member**
7. Dr Manjula R Associate professor of Community Medicine	**Member**
8. Mr. Vittal Kamble , Near Vallabhbai chowk, Bagalkot.	**Member**
9 Mr.Jagdeesh, Budihal, advocate Navanagar , Bagalkot.	**Member**
10. Mr. D. G. Bannur, Holebasaveshwar Nilaya, 10th Cross, Vidyagiri, Bagalkot.	**Member**
11. Dr.Vijayamahantesh SN Professor of Forensic Medicine, SNMC, Bagalkot.	**Member Secretary**

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Office of the Institutional Ethics Committee

The Ethical Committee of SNMC reviewed the following documents:

1. Research Protocol entitled Genetic and Molecular Profiling of FIX (FACTOR 9) Gene of Hemophilia B in Karnataka

2. Information sheet for participants of the study (Consent Form –I) and (Consent Form –II) of Genetic and Molecular Profiling of FIX (FACTOR 9) Gene of Hemophilia B in Karnataka

NOTE: It is to be noted that neither PI nor any of the proposed study team members were present during the decision-making procedures of the Ethics Committee, and members who are independent of the Investigator, have voted/ provided opinion on the trial.

Discussion points:
After reviewing the documents submitted by the Principal Investigator, the Committee has decided to grant approval for conducting the above mentioned study.

You are requested to report to the Ethics Committee the Following:
1. Progress of the study at the end of 4 months.
2. Provide a report to the Ethics Committee on completion of the study.

The Ethics Committee of SNMC follows procedures that are in compliance with the requirements of ICH (International Conference on Harmonization) related to GCP (Good Clinical Practice), schedule Y and all other applicable Indian regulations.

If you have any Questions concerning the above, please feel free to contact the undersigned.
Thanks & Regards,

(Dr. Vijayamahantesh SN)
Member Secretary p 2/2

Member Secretary,
IEC
S. N. Medical College
BAGALKOT

PUBLICATIONS

Original Article

Clinical profile of hemophilia B patients from Karnataka

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Abstract

Background: The most prevalent severe inherited hemorrhagic condition is hemophilia, which means "love of blood." Hemophilia A and B are caused by a lack or malfunction of the factor VIII and factor IX proteins. **Objective:** The present study is to determine the prevalence and clinical profile of hereditary coagulation disorder, particularly hemophilia B, in Karnataka. **Methods:** The study comprised 150 HB patients with a mean age of 25, n_{male} = 148 and n_{female} = 2. The samples were collected from hemophilia societies across Karnataka. The detailed history of HB patients was recorded in a predesigned Performa regarding family history, age, time of first bleed, site of the bleed, and bleeding history. **Result:** In our study cohort, the majority of the 58 (38.7%) cases belong to 21–30 years of age. The mean age of onset was 2.0 ± 1.0 years in severe, 7.5 ± 2.8 0 years in moderate, and 10.0 ± 3.5 years in mild HB patients. Out of 150 HB cases, 102 (68%) cases were diagnosed as severe, 30 (20%) as moderate, and 18 (12%) as mild. Mean factor IX levels were 0.6 ± 0.2, 2.5 ± 1.3, and 8.0 ± 2.6 in the severe, moderate, and mild group, respectively. A family history of bleeding was observed in 97 [64.7%] HB patients. Forty-seven (32.3%) HB patients had a history of consanguinity. The most common initial site of bleed was in joints in 86 [57.3%]. **Conclusion:** The present study is one of the fewer studies from Karnataka studying the demographic and clinicopathological features of hemophilia B. Early diagnosis can be only helpful with knowledge of spectral presentation of hemophilia B in a local population.

Keywords: Epidemiology, F9 gene, hemophilia B, Karnataka

Introduction

Bleeding disorders like hemophilia A [classic hemophilia] and hemophilia B [Christmas disease] are the most common inherited disorders worldwide and India. The World Federation of Hemophilia estimates that there are 4,00,000 individuals worldwide with hemophilia; among them, 80% are in India.[1] HA and HB are X-linked diseases and genes which are responsible for HA located in the long arm of the X chromosome [Xq28] and HB located in the long arm of the X chromosome [Xq27].[2,3]

The two most common forms of hemophilia are hemophilia A [HA] and hemophilia B [HB]. HA comprises approximately 80% of total hemophilia cases, and HB comprises 20% of hemophilia cases.[4] Approximately 30% of the patients have no family history and are results of de novo mutations. HA occurs in 1 out of 10,000 male births, while HB occurs in 1 out of

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Received: 04-09-2021 **Revised:** 18-12-2021
Accepted: 31-12-2021 **Published:** 30-06-2022

Access this article online
Quick Response Code:
Website: www.jfmpc.com
DOI: 10.4103/jfmpc.jfmpc_1773_21

How to cite this article: Kulkarni S, Hegde R, Hegde S, Kulkarni SS, Hanagvadi S, Das KK, *et al*. Clinical profile of hemophilia B patients from Karnataka. J Family Med Prim Care 2022;11:2735-8.

25,000 male births.[5,6] One of the most frequent coagulation abnormalities is factor deficiency (factor VIII and factor IX). Both hemophilia A and hemophilia B are inherited in a recessive X-linked manner.[2]

The clinical hallmark of hemophilia is bleeding into soft tissues, muscles, and joints. In developing and crowded nations like India, where patients with hemophilia have limited access to the treatment further, repeated use of blood and blood products as a cheaper alternative to factor concentration increases the risk of transfusion transmitted infections.[1,7]

The present study aims to determine the prevalence and clinical profile of hereditary coagulation disorder, particularly hemophilia B, in Karnataka. To learn more about the prevalence and clinical characteristics of hemophilia, it is necessary to diagnose the disease and manage it. The goal of this research was to learn more about the clinicopathological characteristics of hemophilia patients.

Materials and Methods

A cross-sectional study was conducted during 2018–2020. Ethical approval for the study was obtained from Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura (Ref No.: BLDE (DU)/IEC/340/2018-19) and SNMC institutional ethics committee of human subjects research, Bagalkot (SNMC/IECHSR/2018-19/A-B2/1.1). Totally, 150 HB patients [mean age = 25; n_{male} = 148 and n_{female} = 2] were included in the study. The samples were collected from hemophilia societies across the Karnataka State of India. The detailed history of HB patients was recorded in a predesigned Performa regarding family history, age, time of first bleed, site of the bleed, and bleeding history. Factor assay was carried out by using a "one-stage assay" using a semi-automated clot analyzer. Factor level <1% [<0.01 IU/mL], 1–5% [0.01–0.05 IU/mL], and >5%–<40% [>0.05–<0.4 IU/mL] was defined as severe, moderate, and mild, respectively.[8] The obtained data were tabulated and analyzed via SPSS 15.0 (SPSS Inc., Chicago, USA). Data are presented as mean ± SD.

Student's t test and one-way ANOVA were used to compare the significant difference in mean. For categorical variables, the Chi-square test was used. *P* value < 0.05 is considered statistically significant.

Results

One hundred and fifty patients suffering from hemophilia B were included in the present study. Our study cohort includes 148 male and 2 female HB patients. The age of the patients ranges from 5 to 35 years, and the mean age was 25 years. In our study cohort, the majority of the 58 (38.7%) cases belong to 21–30 years of age followed by 11–20 years (41) (27.3%). The mean age of onset of symptoms was 2.0 ± 1.0 years in severe patients, 7.5 ± 2.8 years in moderate patients, and 10.0 ± 3.5 years in mild HB patients [Table 1].

Table 1: Age distribution of patients (n=150)

Age-group	No.	Percentage
1-10	18	12%
11-20	41	27.3%
21-30	58	38.7%
31-40	33	22%

Out of 150 HB cases, 102 (68%) cases were diagnosed as severe, 30 (20%) cases were diagnosed as moderate, and 18 (12%) cases were diagnosed as mild. 2 female HB patients were diagnosed as severe. Mean factor IX level was 0.6 ± 0.2, 2.5 ± 1.3, and 8.0 ± 2.6 in the severe, moderate, and mild group, respectively.

In our study, family history of bleeding was observed in 97 [64.7%] HB patients. Among those, 80 [82.5%] cases belong to the severe group, 10 [10.3%] belong to the moderate group, and 7 [7.2%] belong to the mild group, respectively. Forty-seven (32.3%) HB patients had a history of consanguinity.

The most common initial site of bleed was in joints in 86 [57.3%] HB patients followed by skin in 27 [18%], muscle in 16 [10.7%], epistaxis in 11 [7.3%], and petechiae in 10 [6.7%] hemophilia B patients, respectively. The present study shows 38 (25.3%) HB patients were inhibitor positive. The detailed clinicopathological characteristics are given in Table 2.

Discussion

Bleeding disorders like hemophilia A [classic hemophilia] and hemophilia B [Christmas disease] are the most common inherited disorders worldwide and India. HA is more common than HB. Being an inherited disorder, family history has been observed in 45–47% of cases of hemophilia B patients.[2,9] In our study, 97 [64.7%] HB patients have a family history of bleeding disorder. Remaining may be due to spontaneous mutations or transferred by mildly affected or carrier parents who were asymptomatic and undiagnosed.[9]

The majority of the cases (96%) have bleeding manifestation before 5 years of age[10] with the mean age of onset ranging from 9 to 11 months depending upon severity.[11] In our study, the mean age of onset of symptoms was 2.0 ± 1.0 years in severe patients, 7.5 ± 2.8 0 years in moderate patients, and 10.0 ± 3.5 years in mild HB patients. Karim MA *et al.* 2013 reported that the mean age onset was 15.8 years.[10] All the patients in the present study were male except two females. Hemophilia A and hemophilia B are X-linked disorders; it most commonly affects the male. Females act as a carrier.

In the present study, 102 (68%) HB patients having <1% of factor IX concentration designated as severe, 30 (20%) cases having 1–5% of factor IX concentration designated as moderate, and 18 (12%) cases having >5–<40% of factor IX concentration designated as mild. In the study, Ahmad *et al.*[12] 2008 reported that 69.6% of hemophilia B patients had a severe,

Table 2: Clinicopathological characteristics of hemophilia B patients of our study cohort

Clinicopathological parameters	Severity of disease			P
	Severe (Factor IX <1%) n=102 (68%)	Moderate (Factor IX 1-5%) n=30 (20%)	Mild (Factor IX >5%) n=18 (12%)	
Mean factor IX levels (%)	0.6±0.2	2.5±1.3	8.0±2.6	0.001*
Family history of bleeding (n)	80	10	7	
Mean age of onset (years)	2.0±1.0	7.5±2.8	10.0±3.5	0.001*
Inhibitor positive (n)	23	11	04	
Inhibitor negative (n)	79	19	14	
Bleeding sites				
Joints	55 (54%)	19 (63.3%)	12 (66.7%)	
Skin	17 (16.7%)	6 (20%)	4 (22.2%)	
Muscle	13 (12.7%)	1 (3.3%)	2 (11.1%)	
Epistaxis	7 (6.9%)	4 (13.3%)	0	
Petechiae	10 (9.8%)	0	0	

19.2% had a moderate, and 11.2% had a mild condition. The study by Sahoo *et al.*[9] 2020 and Pawan *et al.*[2] 2021 showed a higher percentage of severe hemophilia cases, i.e., 87% and 84%, respectively.

The initial bleeding site depends upon level factors. In a place where circumcision is a routine practice, post-circulation bleed is the most common initial bleed [51%–62%].[1] Joint bleed [hemarthrosis] is the most common [82%–100%]. Ahmad *et al.*[12] reported the most common presenting features in hemophilia as hemarthrosis in 82%. Hemarthrosis is most common in 72.85% of HB cases followed by skin bleed [77–90%].[10,13-15] In the present study, joint bleeds [hemarthrosis] were the most common clinical manifestation (57.3%), followed by skin bleed in 18%, muscle in 10.7%, epistaxis in 7.3%, and petechiae in 6.7%. The study by Pawan et al. 2021 shows 88% of hemarthrosis and 96% of hematoma in severe HB patients, but our study recorded a low rate of hemarthrosis [54%] and hematoma [16.7%] in severe HB.[2] This may be due to differences in the geographical region and population. In our study, family history of bleeding was observed in 97 [64.7%] HB patients and it is high compared to the study conducted by Pawan *et al.*[2] 2021, and Sahoo *et al.*,[9] 2020 showed positive family history in 45% and 47%, respectively.

Patients with hemophilia B might have a wide range of clinical symptoms and require proper management for the rest of their life. Primary care physicians are the first point of contact for diagnosis and treatment. Our study provides knowledge about hemophilia B prevalence, clinical features, and laboratory diagnosis; this will help the physicians for better diagnosis and early treatment. Only a few clinics offer the testing such as factor concentration and mutation analysis needed to diagnose inherited bleeding problems in our population. Several previous studies from India carried out on hemophilia B were referred to hospitals, and those were not properly determining the severity of the disease in our population. So the present study provides better understanding of concentration of factor levels with respect to severity of the hemophilia B, and it also provides demographic and clinicopathological aspects of hemophilia B in the present study population.

Conclusion

Hemophilia is common worldwide; it shows heterogeneous presentation upon the disease severity over different populations. The present study is one of the fewer studies from the Karnataka region of India studying the demographic and clinicopathological features of hemophilia B. In India, hemophilia inhibitor screening and other genetic tests are not commonly available for the identification and management of the patient. Care of hemophilia is complex, often requiring health management beyond the prevention and treatment of bleeding. Early diagnosis can be only helpful with knowledge of spectra presentation of hemophilia B in a local population.

Key points

Proper detailed early diagnosis of hemophilia B with respect to factor level, family history, severity of the disease, and clinical features is required for the proper treatment and future management.

The present study provides much needed information for physicians and clinicians of care centers for proper early diagnosis and management of the disease.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Payal V, Sharma P, Goyal V, Jora R, Parakh M, Payal D. Clinical profile of hemophilia patients in Jodhpur Region. Asian J Transfus Sci 2016;10:101-4.
2. Pawan PK, Mahima Y, Vijai T, Manjula L. Clinicopathological features of hemophilia in a tertiary care centre of India.

J Family Med Prim Care 2021;10:295-9.

3. Kulkarni S, Hegde R, Hegde S, Kulkarni SS, Hanagvadi S, Das KK, *et al.* Mutation analysis and characterisation of *F9* gene in haemophilia- B population of India. Blood Res 2021;56:252-8.
4. Bolton PH, Pasi KJ. Haemophilias A and B. Lancet 2003;361:1801-9.
5. Bhattacharya DK. Haemophilia in the Indian scenario. Int J Hum Genet 2006;633-9.
6. Meena L, Kumar S, Sinha S, Bharti A, Gupta V, Shukla J. A study to determine the prevalence, clinical profile and incidence of formation of inhibitors in patients of hemophilia in North Eastern part of India. J Family Med Prim Care 2019;8:2463-7.
7. Mannucci PM. Hemophilia therapy: The future has begun. Haematologica 2020;105:545-53.
8. Benson G, Auerswald G, Dolan G, Duffy A, Hermans C, Ljung R, *et al.* Diagnosis and care of patients with mild haemophilia: Practical recommendations for clinical management. Blood Transfus 2018;16:535-44.
9. Sahoo T, Naseem S, Ahluwalia J, Marwaha RK, Trehan A, Bansal D. Inherited bleeding disorders in North Indian children: 14 years' experience from a tertiary care center. Indian J Hematol Blood Transfus 2020;36:330-6.
10. Karim MA, Siddique R, Jamal CY, Islam A. Clinical profile of haemophilia in children in a tertiary care hospital. Bangladesh J Child Health 2013;37:90-6.
11. Hazewinkel MH, Hoogerwerf JJ, Hesseling PB, Hartley P, MacLean PE, Peters M, *et al.* Haemophilia patients aged 0-18 years in the Western Cape. S Afr Med J 2003;93:793-6.
12. Ahmad F, Kannan M, Ranjan R, Bajaj J, Choudhary P, Saxena R. Inherited platelet function disorders versus other inherited bleeding disorders: An Indian overview. Thromb Res 2008;121:835-41.
13. Uddin MM, Rahman MJ, Rahman MM, Sultana SA, Shah MS. Clinico-pathological study on haemophilia: An analysis of 50 cases. J Bangladesh Coll Phys Surg 2006;24:50-3.
14. Kim KY, Yang CH, Cho MJ, Lee M. Comprehensive clinical and statistical analysis of hemophilia in Korea. J Korean Med Sci 1988;3:107-15.
15. Qasim Z, Naseem L, Asif N, Hassan K. Haemophilia; pattern of clinical presentation and disease severity. Int J Pathol 2013;11:58-63.

BLOOD RESEARCH
VOLUME 56 · NUMBER 4
December 2021
ORIGINAL ARTICLE

Mutation analysis and characterisation of *F9* gene in haemophilia- B population of India

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p-ISSN 2287-979X / e-ISSN 2288-0011
https://doi.org/10.5045/br.2021.2021016
Blood Res 2021;56:252-258.

Received on January 22, 2021
Revised on August 18, 2021
Accepted on August 25, 2021

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Background
Hemophilia B (HB) is an X-linked bleeding disorder resulting from coagulation factor IX defects. Over 3,000 pathogenic, HB-associated mutations in the F9 gene have been identified. We aimed to investigate the role of F9 variants in 150 HB patients using sequencing technology.

Methods
F9 gene sequences were amplified from peripheral blood-derived DNA and sequenced on an Applied Biosystems (ABI) 3500 Sanger sequencing platform. Functional and structural predictions of mutant FIX were analyzed.

Results
Among 150 HB patients, 102 (68%), 30 (20%), and 18 (12%) suffered from severe, moderate, and mild HB, respectively. Genetic analysis identified 16 mutations, including 3 novel mutations. Nine mutations (7 missense and 2 stop-gain) were found to be pathogenic. Only 3 mutations (c.127C>T, c.470G>A, and c.1070G>A) were associated with different severities. While 2 mutations were associated with mild HB cases (c.304C>T and c.580A>G), 2 (c.195G>A and c.1385A>G) and 3 mutations (c.223C>T, c.1187G>A, and c.1232G>A) resulted in moderate and severe disease, respectively. Additionally, 1 mutation each was associated with mild-moderate (c.*1110A>G) and mild-severe HB disease (c.197A>T), 4 mutations were associated with moderate-severe HB cases (c.314A>G, c.198A>T, c.676C>T, and c.1094C>A). FIX concentrations were lower in the mutated group (5.5±2.5% vs. 8.0±2.5%). Novel p.E66D and p.S365 mutations were predicted to be pathogenic based on changes in FIX structure and function.

Conclusion
Novel single nucleotide polymorphisms (SNPs) largely contributed to the pathogenesis of HB. Our study strongly suggests that population-based genetic screening will be particularly helpful to identify risk prediction and carrier detection tools for Indian HB patients.

Key Words Hemophilia B, F9 gene, Stop-gain mutation, Missense mutation, India

INTRODUCTION

Hemophilia is an X-linked blood disorder that causes sustained bleeding after injuries or trauma. Symptoms mainly include bleeding in the joints and muscles. The term hemophilia was first used by Friedrich Hopff, University of Zurich, in 1828 [1, 2]. Hemophilia B (HB) is caused by defects in coagulation factor IX. It is also called "Christmas disorder", after it was identified by Stephen Christmas in 1952 [3]. FIX activity can be classified as severely (<1%), moderately (1–5%), and mildly (5–30%) impaired [1, 4, 5]. HB is less common

than hemophilia A (HA) (1 in 25,000 males vs. 1 in 5,000 males worldwide) [6, 7]. Approximately 14,000 people with hemophilia have been registered at the Haemophilia Federation of India; however, hemophilia remains under-diagnosed and many cases are thus not registered. HA occurs in 1 out of 10,000 male births, while HB occurs in 1 out of 30,000 male births [8].

The F9 gene (Xq27.1-q2.2) is approximately 33.5 kb in length and contains eight exons [6, 9]. FIX is homologous to clotting factors VII (FVII) and XI (FXI). It plays a crucial role in blood coagulation and is synthesized in the liver as a vitamin K-dependent serine protease (SP) precursor [1]. FIX circulates in plasma as a glycoprotein. During blood coagulation, inactive FIX is converted into an active SP called Factor IXa. This activation process occurs in two ways: through intrinsic and extrinsic pathways. In the course of the intrinsic pathway, Factor IXa activates FIX in the presence of calcium ions. During the extrinsic pathway, FIX is activated by factor VIIa in the presence of calcium and lipoprotein [10]. FIX protein is composed of a γ-carboxyglutamic acid-rich (Gla) domain, two epidermal growth factor-like domains (EGF1 and EGF2), and an SP domain [6].

Recently, more than 3,000 pathogenic mutations and neutral polymorphisms have been identified in the F9 gene, and these mutations have been documented in various online hemophilia databases [6, 11, 12]. In this study, we analyzed the F9 genes of 150 HB patients from the hemophilia societies of Karnataka, India, for molecular changes using sequencing technology.

MATERIALS AND METHODS

Sample collection

This study was approved by the Institutional Ethical Committee of Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura (Ref. No.: BLDE (DU)/IEC/340/2018-19), and the SNMC Institutional Ethics Committee of Human Subject Research, Bagalkot (Ref. No.: SNMC/IECHSR/2018-19/A-B2/1.1). Written informed consent was obtained from all HB patients before blood sample collection. A total of 150 HB patients were included in this study, which were followed up at 12 different hemophilia societies across the Karnataka state of India. A detailed clinical history was obtained from all HB patients.

F9 gene analysis

Peripheral blood from HB patients was collected in EDTA vacutainers (BD, Franklin Lakes, NJ, USA). Prior to DNA isolation, the FIX concentration was measured and inhibitor assay were performed. DNA was extracted from peripheral blood using a blood and tissue DNA extraction kit (QIAGEN, Hilden, Germany). All exonic regions were amplified and the products were sequenced on an Applied Biosystems (ABI) 3500 Sanger sequencing platform using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). Results were analyzed using DNA sequence analysis software v5.4.

Bioinformatics analysis

The pathogenicity of the novel non-synonymous variants was analyzed using bioinformatics tools, such as PROVEAN

Table 1. Clinicopathological characteristics of the study population.

Clinicopathological parameters	Disease severity		
	Severe (Factor IX <1%), N=102 (68%)	Moderate (Factor IX 1–5%), N=30 (20%)	Mild (Factor IX >5%), N=18 (12%)
Mean factor IX levels (%)	0.6±0.2	2.5±1.3	8.0±2.6
Family history of bleeding (N)	80	10	7
Mean age of onset (yr)	2.0±1.0	7.5±2.8	10.0±3.5
Mutation frequency (within group)	48% (49/102)	86.7% (26/30)	83.3% (15/18)
Inhibitor-positive (N)	23	11	4
Inhibitor-negative (N)	79	19	14
Hemoglobin concentration	Normal	Normal	Normal
Platelet count	Normal	Normal	Normal
Prothrombin time	Normal	Normal	Normal
Activated partial thromboplastin time	High	High	High
Bleeding sites			
Joints	55 (54%)	19 (63.3%)	12 (66.7%)
Gum	17 (16.7%)	6 (20%)	4 (22.2%)
Muscle	13 (12.7%)	1 (3.3%)	2 (11.1%)
Epistaxis	7 (6.9%)	4 (13.3%)	0
Petechiae	10 (9.8%)	0	0

(http://provean.jcvi.org/seq_submit.php), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml), PHD SNP (https://snps.biofold.org/phd-snp/phd-snp.html), SNPs & GO (https://snps.biofold.org/snps-and-go/snps-and-go.html), PANTHER (http://www.pantherdb.org/), and SNAP2 (https://www.rostlab.org/services/snap/). The conservation property of missense variants was investigated using the Clustal Omega multiple sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). A 3D model of wild type and mutant FIX proteins was predicted using the Swiss model (https://swissmodel.expasy.org/), and the results were visualized and analyzed using the UCSF Chimera program.

RESULTS

Of the 150 HB patients included in this study, 102 (68%; FIX concentration, 0.6±0.2; age of onset, 2.0±1.0 y), 30 (20%; FIX concentration, 2.5±1.3; age of onset, 7.5±2.8 y), and 18 (12%; FIX concentration, 8.0±2.6; age of onset, 10.0±3.5y) suffered from severe, moderate, and mild HB, respectively. The detailed clinicopathological parameters are summarized in Table 1. In our study, we recorded 16 mutations. Of those, 1 was a synonymous mutation, 12 were missense mutations, 2 were stop-gain mutations, and 1 was a 3' UTR variant. Notably, 13 (81.25%) mutations were previously reported, but 3 (18.75%) were novel mutations, which had not been entered into any of the human SNP databases. The majority of the mutations were found in exon 8 of the gene and largely comprised missense mutations. Exon 8 showed a high number of mutations compared to other exons of the F9 gene (Fig. 1, Table 2).

The novel missense mutation c.198A>T was found in the F9 genes of 4 patients with moderate HB, with a mean FIX concentration of 2.0±0.5 and a mean age of onset of 7±1.0, as well as in 7 patients with severe HB, with a mean FIX concentration of 0.3±0.5 and a mean age of onset of 1.5±0.5. This mutation was observed at a high rate among

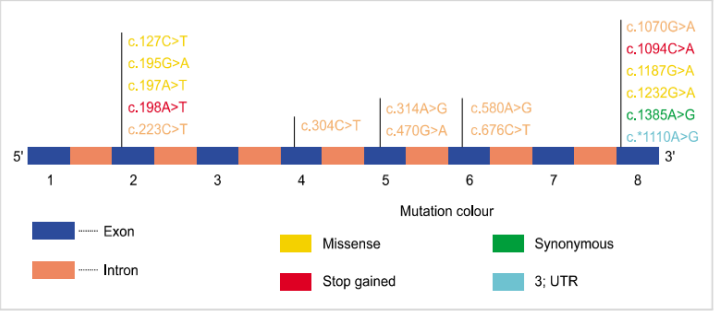

Fig. 1. Graphical representation of the distribution of the mutations within the Factor 9 (F9) gene.

Table 2. List of mutations recorded in the F9 genes of our study cohort.

Mutation type	Nucleotide change	cDNA position	Amino acid change	Exon	Status	Frequency, N (%)
Missense	g.11313C>T	c.127C>T	p.R43W	2	Reported (rs1603264205)	9 (6.0%)
Missense	g.11381G>A	c.195G>A	p.M65I	2	Reported (rs763568424)	1 (0.66%)
Missense	g.11383A>T	c.197A>T	p.E66V	2	Reported (CM940423)	3 (2.0%)
Missense	g.11384A>T	c.198A>T	p.E66D	2	Not reported	12 (8.0%)
Stop-gain	g.11409 C>T	c.223C>T	p.R75[a]	2	Reported (rs137852227)	2 (1.33%)
Missense	g.15369 T>C	c.304C>T	p.C102R	4	Reported (CM960574)	2 (1.33%)
Missense	g.22664 T>C	c.314A>G	p.G143R	5	Reported (CM940499)	4 (2.66%)
Missense	g.22706 G>A	c.470G>A	p.C157Y	5	Reported (rs1367198680)	17 (11.33%)
Missense	g.25386 A>G	c.580A>G	p.T194A	6	Reported (rs6048)	3 (2.0%)
Missense	g.25482C>T	c.676C>T	p.R226W	6	Reported (rs137852240)	6 (4.0%)
Missense	g.36020 G>A	c.1070G>A	p.G357E	8	Reported (rs137852275)	8 (5.33%)
Stop-gain	g.36044C>A	c.1094C>A	p.S365[a]	8	Not reported	15 (10.0%)
Missense	g.36137G>A	c.1187G>A	p.C396Y	8	Reported (rs137852273)	1 (0.66%)
Missense	g.36182 G>A	c.1232G>A	p.S411N	8	Reported (rs137852276)	3 (2.0%)
Synonymous	g.36335 A>G	c.1385A>G	p.Ter462=	8	Reported (rs561793582)	1 (0.66%)
3′ UTR	g.37446A>G	c.*1110A>G	·····	8	Not reported	3 (2.0%)

[a]NG_007994.1, NM_000133.4, and NP_000124.1 reference sequences wereused for genomic DNA variant nomenclature, coding region variant nomenclature, and protein variant nomenclature, respectively. All nomenclatures were made according to Human Genome Variation Society (HGVS) guidelines.

patients with moderate HB. The novel stop-gain mutation c.1094C>A was found in 3 patients with moderate HB, with a mean FIX concentration of 2.6±0.8 and a mean age of onset of 6.5±0.5, as well as in 12 patients with severe HB, with a mean FIX concentration of 0.05±0.15 and a mean age of onset of 2±1.0. This mutation was observed at a high rate among patients with severe HB. The following, previously described pathogenic mutations have been observed: c.127C>T in 3 mild (FIX concentration, 8.1±1.9), 4 moderate (FIX concentration, 2.1±0.2), and 2 severe (FIX concentration, 0.65±0.15) cases; c.470G>A in 3 mild (FIX concentration, 7.6±2.7), 5 moderate (FIX concentration, 2.9±0.9),

Table 3. Genotypic and phenotypic associations of the mutations recorded in the study population.

Mutation type	cDNA position	Patients			Factor IX (IX) concentration level (%)	Age of onset	Inhibitor-positive (N)	Inhibitor-negative (N)	Clinical significance
		m	M	S					
Missense	c.127C>T	03	04	02	6.5, 8.0, 10 (m) 2.0, 2.3. 1.8, 2.0 (M) 0.8, 0.5 (S)	10, 12, 8 (m) 7, 6, 9, 9 (M) 1, 1.5 (S)	2 (m) 2 (M) -	1 (m) 2 (M) 2 (S)	Pathogenic
Missense	c.195G>A	00	01	00	3.0 (M)	7.5 (M)	1 (M)	-	Likely pathogenic
Missense	c.197A>T	02	00	01	6.0, 9.0 (m) 0.3 (S)	13, 11 (m) 1 (S)	-	2 (m) 1 (S)	Likely benign
Missense	c.198A>T	00	04	07	1.5, 2.0. 2.5, 2.0 (M) 0.8, 0.5, 0.1, 0.6, 0.6, <0.01, 0.01 (S)	6, 7, 7, 8 (M) 1, 2, 1, 1.5, 2, 2, 1.6 (S)	4 (M) 4 (S)	- 3 (S)	Pathogenic
Stop-gain	c.223C>T	00	00	02	<0.01, <0.01 (S)	1, 1 (S)	1 (S)	1 (S)	Pathogenic
Missense	c.304C>T	02	00	00	12.0, 10.0 (m)	13, 10 (m)	2 (m)	-	Likely pathogenic
Missense	c.314A>G	00	01	03	2.0 (M) 0.2, 0.1, 0.01 (S)	8 (M) 1, 2, 1 (S)	-	1 (M) 3 (S)	Pathogenic
Missense	c.470G>A	03	05	09	5.0, 7.5, 10.3 (m) 2.0, 3.5, 2.5, 1.8, 4.0 (M) 0.8, 0.5, 0.01, 0.2, <0.01, <0.01, 0.4, 0.2, <0.01 (S)	6.5, 10, 11 (m) 5, 7, 7, 9, 7 (M) 1, 2, 2, 3, 2, 2, 2, 1, 2 (S)	3 (m) 1 (M) 2 (S)	- 4 (M) 7 (S)	Pathogenic
Missense	c.580A>G	03	00	00	10.7, 9.0, 9.0 (m)	10, 8, 8 (m)	-	3 (m)	Likely benign
Missense	c.676C>T	00	01	05	4.0 (M) 0.5, 0.1, <0.01, 0.01, <0.01 (S)	5.5 (M) 3, 1, 2, 2, 1 (S)	1 (M) 1 (S)	- 3 (S)	Pathogenic
Missense	c.1070G>A	01	03	04	6.5 (m) 2.0, 3.8, 2.5 (M) 0.01, 0.1, <0.01, <0.01 (S)	12 (m) 6, 7, 7 (M) 2, 2, 2, 1 (S)	- 2 (M) 2 (S)	1 (m) 1 (M) 1 (S)	Pathogenic
Stop-gain	c.1094C>A	00	03	12	2.6, 3.5, 1.8 (M) 0.01, 0.01, 0.1, 0.2, <0.01, <0.01, 0.1, 0.01, <0.01, 0.01, <0.01, <0.01 (S)	7, 7, 6 (M) 1, 2, 2, 2, 2, 3, 1.5, 3, 2, 2, 2.5, 1 (S)	- 10 (S)	3 (M) 2 (S)	Pathogenic
Missense	c.1187G>A	00	00	01	0.6 (S)	2 (S)	-	1 (S)	Benign
Missense	c.1232G>A	00	00	03	0.6, 0.3, 0.1 (S)	2, 1, 1.5 (S)	1 (S)	2 (S)	Pathogenic
Synonymous	c.1385A>G	00	01	00	4.0 (M)	10 (M)	1 (M)	-	Benign
3′ UTR	c.*1110A>G	01	03	00	12.0 (m) 5.0, 4.0, 3.8 (M)	13 (m) 7, 7.5, 9 (M)	1 (m) 1 (M)	- 2 (M)	-

Abbreviations: m, mild; M, moderate; S, severe.

Table 4. Pathogenicity predictions of the p.E66D mutation.

Mutation	PROVEAN[a]	SNAP2[b]	PolyPhen2[c]	PHD-SNP[d]	SNP&GO[e]	PANTHER
E66D	Deleterious Score: -2.540	Effect Score: 37	Probably damaging Score: 0.999	Disease Score: 4	Disease Probability: 0.705	Probably damaging

[a]PROVEAN: "Deleterious" if the prediction score was ≤2.5, "Neutral" if the prediction score was ≥2.5. [b]NAP2: "Neutral" if the score ranged from 0 to -100. "Effect" if the score was between 0 and 100. [c]PolyPhen2: "Probably damaging" is the most disease-causing ability, with a score near 1. "Possibly damaging" signifies less disease-causing ability with a score of 0.5–0.8. "Benign", which does not alter protein function, with a score closer to 0. [d]PHD-SNP: if the probability is >0.5, mutation is predicted as "Disease" and if less than <0.5, mutation is predicted to be "Neutral". [e]SNP & GO: Probability of >0.5 is predicted to be a disease-causing nsSNP.

and 9 severe (FIX concentration, 0.1±0.7) cases; c.676C>T in 1 moderate and 5 severe (FIX concentration, 0.1±0.7) cases; c.1070G>A in 1 mild, 3 moderate (FIX concentration, 2.8±0.8), and 4 severe (FIX concentration, 0.05±0.1) cases; c.223C>T in 2 severe cases; and c.314A>G in 1 moderate and 3 severe (FIX concentration, 0.1±0.09) cases. The remaining mutations were recorded at a low frequency. Detailed genotype-phenotype associations are explained in Table 3.

The novel missense mutation p.E66D was shown to be harmful to FIX protein function by PROVEAN, SNAP2, PholyPhen2, SNP&GO, PHD-SNP, and PANTHER (Table 4). Multiple sequence alignment of this novel missense mutation indicated that it was present in the highly conserved residue of the FIX protein (Fig. 2). Homology modeling of the protein structure was conducted using the Swiss model server, which was then visualized and analyzed using chimera program. In p.E66D, the mutant residue is smaller than wild type residue, which might lead to the loss of interactions with the metal ion: "calcium 4 or magnesium 1; via 4-carboxyglutamate" (Fig. 3). The novel stop-gain mutation, p.S365*, results in a premature stop codon that leads to a truncated FIX variant of 365 amino acids, corresponding to a loss of approximately 21% of the wild type FIX protein (Fig. 3).

DISCUSSION

HB is a bleeding disorder that causes abnormal or poor blood clotting. Pathogenic variants of the F9 gene are known to cause this disorder [13]. FIX is an essential element in the intermediate stage of the blood coagulation cascade. Nearly one third of hemophilia cases occur without any previous family history, but mainly due to new genetic variations [7]. In 1.3% to 7.8% of hemophilia cases, more than one mutation in the F9 gene have been reported [14], and, in accordance with other reports, most of the mutations were single nucleotide variants [15]. Missense mutations were the most common single nucleotide variants in HB, accounting for more than 58.4%, whereas 15.4% were frameshift mutations resulting from deletions, insertions, or duplications. Nonsense and splice mutations accounted for 8.3% and 9.4% of the cases, respectively [6].

In the present study, we recorded a total of 16 mutations, of which 15 (93.75%) were coding sequence variants. The majority of them (81.25%) were missense variants.

We recorded the stop-gain mutation p.R75W in 2 patients with severe HB, which have been described previously by Parrado Jara et al. (2020) [5], Radic et al. (2013) [16], Kwon et al. (2008) [17], Saini et al. (2015) [18], and Koeberl et al. (1990) [19]. This mutation is known to result in the loss of >80% of total FIX protein and to increase the probability of an immune response. The p.R226W mutation affects zymogen activation processes by altering the cleavage of Factor XIa [20, 21], which was recorded in 1 moderate and 5 severe (FIX concentration, 0.1±0.7) cases within our study group. The p.C396Y mutation was shown to disturb the environment of the active site that is involved in the conversion of the zymogen into an active enzyme, which was observed only in one severe case of HB [22]. We also recorded the p.T194A mutation in 3 mild HB cases (FIX concentration, 9.8±0.8) and the p.C357Y missense mutation, which were

Fig. 2. Multiple sequence analysis of the Factor IX (FIX) protein. The arrow indicates the position of the p.E66D mutation.

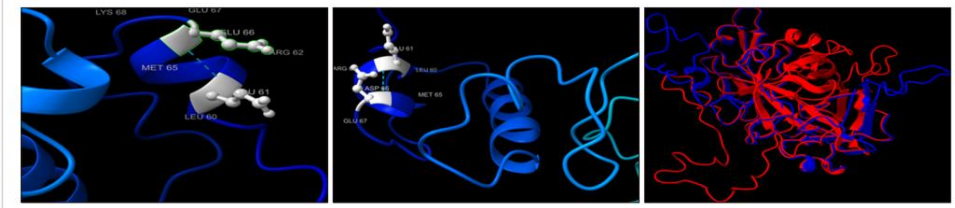

Fig. 3. Segments of a 3D model of wild type (upper left panel) and mutant (p.E66D; upper right panel) Factor IX. A superimposed 3D model of wild type (red) and mutant (p.S365*; blue) Factor IX are shown in the lower panel.

predicted to be detrimental to FIX protein function in the blood coagulation process in previous studies. We recorded the latter mutation in 1 mild, 3 moderate (FIX concentration, 2.8±0.8), and 4 severe (FIX concentration, 0.05±0.1) cases [23-26]. R43 is a mutation hotspot that accounted for 73.5% of HB cases in the present study [27], and we recorded the R43W mutation in c.127C>T in 3 mild (FIX concentration, 8.1±1.9), 4 moderate (FIX concentration, 2.1±0.2), and 2 severe (FIX concentration, 0.65±0.15) cases.

The novel missense mutation p.E66D was recorded in 4 patients with moderate HB, with a mean FIX concentration of 2.0±0.5, and in 7 patients with severe HB, with a mean FIX concentration of 0.3±0.5. It was found to be harmful to the function of the FIX protein by in silico pathogenicity prediction tools. Moreover, this novel mutation was found to be present in highly conserved regions of the FIX protein. The mutant p.E66D residue was found to be smaller than the wild type residue. This might have led to the loss of interactions with the metal ion: "calcium 4 or magnesium 1; via 4-carboxyglutamate". Hence, this missense variant has detrimental effects on normal FIX activation and blood coagulation processes. According to the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines, p.E66D is classified as "pathogenic" [28]. Along with missense variants, we also recorded one novel stop-gain mutation, p.S365*, in 3 moderate HB cases, with a mean FIX concentration of 2.6±0.8, as well as in 12 severe HB cases, with a mean FIX concentration of 0.05±0.15. This led to the loss of 21% of FIX protein, which resulted in the loss of most of the peptidase S1 activity of the FIX protein. According to the ACMG/AMP guidelines, p.S365* is classified as "pathogenic." Only one 3' UTR variant was recorded in our study cohort. Functional analysis of the mutations was the major limitation of our study.

Of the 150 HB patients, 90 (60%) had mutations in the F9 gene, including mild, moderate, and severe conditions. In the mild, moderate, and severe groups, 15 (10%), 26 (17%), and 49 (32%) of the patients showed mutations in the F9 gene. Among the 3 groups, the severe group showed a high rate of mutations (32%) compared to the other 2 groups; in our study cohort, the number of patients with severe HB was high, followed by moderate and mild groups (Table 1). Of the 16 mutations detected, 9 were identified as pathogenic. All of the 49 hemophilic patients with high mutation rates featured these 9 pathogenic variants: c.127C>T, c.198A>T, c.223C>T, c.314A>G, c.470G>A, c.676C>T, c.1070G>A, c.1094C>A, and c.1232G>A. These corresponded to the mutation locations causing the pathogenic effects that may have led to severe symptoms observed in hemophilic patients of our study population. Both c.1187G>A and c.1385A>G mutations were recorded as benign, and one moderately affected patient was diagnosed with the c.1385A>G variant, whereas the c.1187G>A mutation was found in a patient with severe HB. Two mutations, i.e., c.195G>A and c.304C>T, were likely pathogenic, and the c.195G>A variant was found in one patient with moderate hemophilia. In two patients with mild disease, the c.304C>T mutation was found. The likely benign mutations c.197A>T and c.580A>G were recorded in 2 mild, 1 severe, and 3 mild cases of hemophilia, respectively.

Additionally, 2 stop-gain mutations and 7 missense mutations were major pathogenic variations or disease-causing mutations recorded in our study; patients who were carriers of these mutations showed more severe conditions compared to the other 2 groups (mild and moderate). Likely pathogenic and benign mutations were also missense mutations, and all the patients carrying these mutations were mildly to moderately affected by HB, except for 2 patients. Only 3 mutations (c.127C>T, c.470G>A, and c.1070G>A) were associated with different severities. Moreover, 2 mutations were only associated with mild HB (c.304C>T and c.580A>G), 2 mutations were associated with moderate HB (c.195G>A and c.1385A>G), and 3 mutations were only associated with severe HB (c.223C>T, c.1187G>A, and c.1232G>A). One mutation each was associated with mild-moderate (c.*1110A>G) and mild-severe HB (c.197A>T). In addition, 4 mutations were associated with moderate-severe HB (c.314A>G, c.198A>T, c.676C>T, and c.1094C>A). Among the patients with severe HB, 48% (49/102) featured mutations in the F9 gene, followed by 86.6% (26/30) and 83.3% (15/18) of moderate and mild HB cases, respectively.

In our study, we also observed clinical differences between the mutated and wild type F9 groups. The main clinical symptoms were lower FIX concentration levels (5.5±2.5%) in the mutated groups compared to the control group (8.0±2.5%). The inhibitor-positive rate was also high in patients carrying F9 mutations compared to patients with wild type F9 (40/38), but inhibitor-negative numbers were decreased in the mutated hemophilic patients (46/112). Different studies conducted on HB patients recorded remarkable allelic heterogeneity, consisting of similar mutations, which were associated with different phenotypes. Similar to the results obtained by previous studies, we also found that the same mutations resulted in multiple phenotypes/severities [29]. Studies conducted between 1993 and 2014 with 20 or more unrelated patients with HB showed that the F9 detection rate varied from 83% to 100% using PCR and Sanger sequencing [4], whereas in our study population, the mutation rate was 60%. Phenotypic heterogeneity and mutation rates among HB patients are largely unknown. External influences, such as the modification of genes, environmental factors, or epigenetic effects, are major factors in both cases.

Our study strongly suggests that the majority of HB cases feature pathogenic single nucleotide variations, which may be novel or previously recorded. In many cases, novel single nucleotide variants are involved. Population-based screening of mutations will help establish inhibitor risk prediction and carrier detection strategies in India.

ACKNOWLEDGMENTS

We thank all hemophilia societies and hemophilia B patients and their families for participating in this study. We thank the Karnataka Institute for DNA Research, Dharwad, India, for providing instrumentation support for the research work. We also thank Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura and S. Nijaliangappa Medical College, HSK Hospital and Research Center, Bagalkot, for their continuous support throughout this research project.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

REFERENCES

1. Yu T, Dai J, Liu H, et al. Spectrum of F9 mutations in Chinese haemophilia B patients: identification of 20 novel mutations. Pathology 2012;44:342-7.
2. Rosendaal FR, Smit C, Briët E. Hemophilia treatment in historical perspective: a review of medical and social developments. Ann Hematol 1991;62:5-15.
3. Mannucci PM. Treatment of hemophilia: recombinant factors only? No. J Thromb Haemost 2003;1:216-7.
4. Goodeve AC. Hemophilia B: molecular pathogenesis and mutation analysis. J Thromb Haemost 2015;13:1184-95.
5. Parrado Jara YA, Yunis Hazbun LK, Linares A, Yunis Londoño JJ. Molecular characterization of hemophilia B patients in Colombia. Mol Genet Genomic Med 2020;8:e1210.
6. Yuen LK, Zakaria Z, Yusoff YM, Esa E, Afandi FM, Karim FDA. A novel missense mutation of F9 gene in hemophilia B Patients. J Blood Disord Transfus 2017;8:383.
7. Bhattacharya DK. Haemophilia in the Indian Scenario. Int J Hum Genet 2006;6:33-9.
8. Kumar S, Sinha S, Bharti A, Meena LP, Gupta V, Shukla J. A study to determine the prevalence, clinical profile and incidence of formation of inhibitors in patients of hemophilia in North Eastern part of India. J Family Med Prim Care 2019;8:2463-7.
9. Yi S, Yang Q, Zuo Y, et al. A novel missense mutation in F9 gene causes hemophilia B in a family with clinical variability. Blood Coagul Fibrinolysis 2020;31:121-6.
10. Geddes VA, MacGillivray RT. The molecular genetics of hemophilia B. Transfus Med Rev 1987;1:161-70.
11. Li T, Miller CH, Payne AB, Craig Hooper W. The CDC hemophilia B mutation project mutation list: a new online resource. Mol Genet Genomic Med 2013;1:238-45.
12. Simhadri VL, Hamasaki-Katagiri N, Lin BC, et al. Single synonymous mutation in factor IX alters protein properties and underlies haemophilia B. J Med Genet 2017;54:338-45.
13. Zahari M, Sulaiman SA, Othman Z, Ayob Y, Karim FA, Jamal R. Mutational profiles of F8 and F9 in a cohort of haemophilia A and haemophilia B patients in the multi-ethnic Malaysian population. Mediterr J Hematol Infect Dis 2018;10:e2018056.
14. Huang L, Li L, Lin S, et al. Molecular analysis of 76 Chinese hemophilia B pedigrees and the identification of 10 novel mutations. Mol Genet Genomic Med 2020;8:e1482.
15. Abla Z, Mouloud Y, Hejer EM, et al. Mutations causing hemophilia B in Algeria: identification of two novel mutations of the factor 9 gene. Biodiversitas 2018;19:52-8.
16. Radic CP, Rossetti LC, Abelleyro MM, et al. Assessment of the F9 genotype-specific FIX inhibitor risks and characterisation of 10 novel severe F9 defects in the first molecular series of Argentinian patients with haemophilia B. Thromb Haemost 2013;109:24-33.
17. Kwon MJ, Yoo KY, Kim HJ, Kim SH. Identification of mutations in the F9 gene including exon deletion by multiplex ligation-dependent probe amplification in 33 unrelated Korean patients with haemophilia B. Haemophilia 2008;14:1069-75.
18. Saini S, Hamasaki-Katagiri N, Pandey GS, et al. Genetic determinants of immunogenicity to factor IX during the treatment of haemophilia B. Haemophilia 2015;21:210-8.
19. Koeberl DD, Bottema CD, Sarkar G, Ketterling RP, Chen SH, Sommer SS. Recurrent nonsense mutations at arginine residues cause severe hemophilia B in unrelated hemophiliacs. Hum Genet 1990;84:387-90.
20. Suehiro K, Kawabata S, Miyata T, et al. Blood clotting factor IX BM Nagoya. Substitution of arginine 180 by tryptophan and its activation by alpha-chymotrypsin and rat mast cell chymase. J Biol Chem 1989;264:21257-65.
21. Bertina RM, van der Linden IK, Mannucci PM, et al. Mutations in hemophilia Bm occur at the Arg180-Val activation site or in the catalytic domain of factor IX. J Biol Chem 1990;265:10876-83.
22. Taylor SA, Deugau KV, Lillicrap DP. Somatic mosaicism and female-to-female transmission in a kindred with hemophilia B (factor IX deficiency). Proc Natl Acad Sci U S A 1991;88:39-42.
23. Li Y, Bezemer ID, Rowland CM, et al. Genetic variants associated with deep vein thrombosis: the F11 locus. J Thromb Haemost 2009;7:1802-8.
24. Graham JB, Lubahn DB, Lord ST, et al. The Malmö polymorphism of coagulation factor IX, an immunologic polymorphism due to dimorphism of residue 148 that is in linkage disequilibrium with two other F.IX polymorphisms. Am J Hum Genet 1988;42:573-80.
25. McGraw RA, Davis LM, Noyes CM, et al. Evidence for a prevalent dimorphism in the activation peptide of human coagulation factor IX. Proc Natl Acad Sci U S A 1985;82:2847-51.
26. Miyata T, Sakai T, Sugimoto M, et al. Factor IX Amagasaki: a new mutation in the catalytic domain resulting in the loss of both coagulant and esterase activities. Biochemistry 1991;30:11286-91.
27. Gao W, Xu Y, Liu H, et al. Characterization of missense mutations in the signal peptide and propeptide of FIX in hemophilia B by a cell-based assay. Blood Adv 2020;4:3659-67.
28. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-24.
29. Chavali S, Sharma A, Tabassum R, Bharadwaj D. Sequence and structural properties of identical mutations with varying phenotypes in human coagulation factor IX. Proteins 2008;73:63-71.

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ORGANIZED BY
DEPARTMENT OF PATHOLOGY
B.V.V.SANGHA'S, S. NIJALINGAPPA MEDICAL COLLEGE, BAGALKOT

THIS IS TO CERTIFY THAT

MR. SUJAYENDRA KULKARNI

Bearing Registration Number - Registered with Medical Council,

has presented a **ORAL PAPER** titled

Molecular Characterization of the Factor IX Gene in 28 Karnataka Hemophilia B Patients

during **46th annual conference of Karnataka chapter – Indian Association of Pathologists and Microbiologists (KCIAPM)** held on **20th, 21st & 22nd September 2019**
at S. Nijalingappa Medical College, Bagalkot

Dr. Ravi Kumar	Dr. Vijay Shankar	Dr. Ashok S Mallapur	Dr. S S Inamdar	Dr. Prabhu M H	Dr. Gududur Ajay Kumar
President, KCIAPM	Secretary, KCIAPM	Principal, SNMC Bagalkot	Organizing Chairman	Organizing Secretary	KMC Zonal Chairman

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BLDE (Deemed to be University)

SHRI B. M. PATIL MEDICAL COLLEGE,HOSPITAL AND RESEARCH CENTRE, VIJAYAPUR

International Hypoxia Symposia 2019

(Celebration of Nobel Prize for Physiology or Medicine 2019 to Hypoxia Biologists)

Theme: Oxygen Cell Signalling: Mechanisms to therapeutics

Organized by: Laboratory of Vascular Physiology and Medicine, Department of Physiology

Certificate

This is to certify that Dr. Sujayendra Kulkarni

has participated as **Delegate/Organizer** in Symposia conducted on November 23rd 2019, at BLDE(DU) Shri. B. M. Patil Medical College, Hospital & Research Centre, Vijayapura, Karnataka, India.

Dr. Aravind Patil, Dean Faculty of Medicine & Principal

Dr. Sumangala Patil, Organizing Chairperson

Dr. Lata M Mullur, Convener

IABSCON 2020

9th ANNUAL INTERNATIONAL CONFERENCE of INDIAN ACADEMY OF BIOMEDICAL SCIENCES

27th, 28th, 29th February 2020

Organized By
DEPARTMENT OF BIOCHEMISTRY
D. Y. PATIL MEDICAL COLLEGE, KOLHAPUR (MAHARASHTRA, INDIA)

A Constituent College of
D.Y. PATIL EDUCATION SOCIETY
Deemed To Be University, Kolhapur-Reaccredited by NAAC with 'A' Grade

CERTIFICATE

Type of Conference-Multi speciality (CPD Code-MMC/MAC/2020/F-014720)

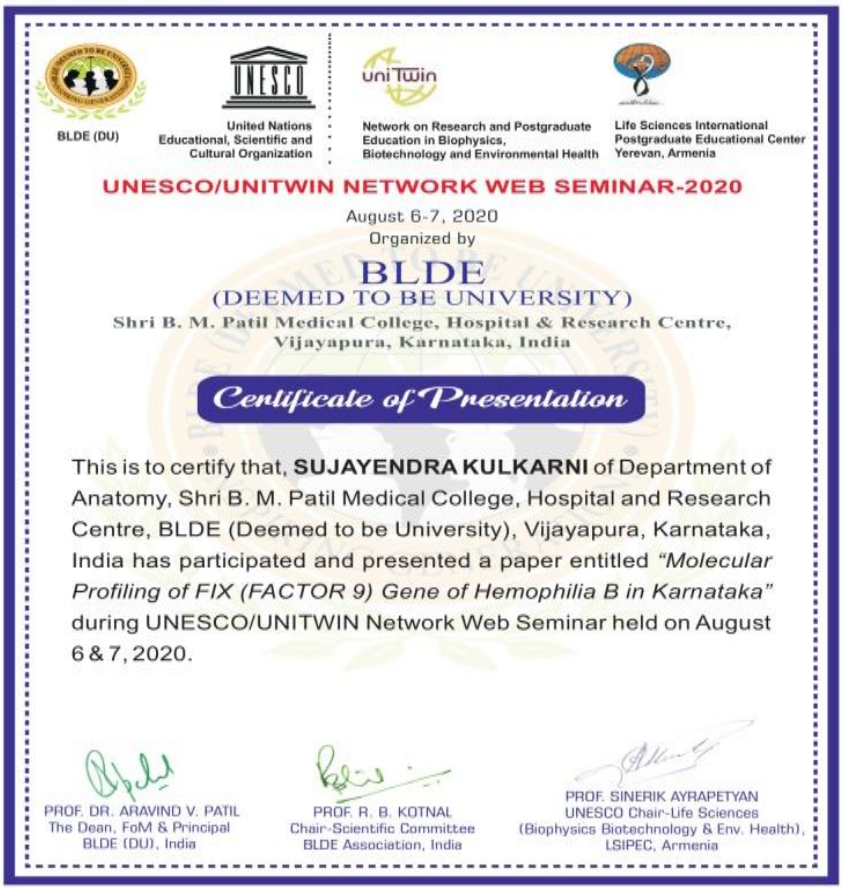
This is to certify that

Dr/Mr/Ms Sujayendra S. Kulkarni Third Prize

has Participated as Delegate / Delivered an Oral / ~~Poster~~ presentation in the 9th Annual International Conference of Indian Academy of Biomedical Sciences.

He / She is granted 4 CPD credit points by Maharashtra Medical Council.

Prof. (MS) Syed Mudassar, President, IABS
Prof. Abbas Ali Mehadi, Secretary, IABS
Prof. Bipin M. Tiwale, Organizing Secretary
Dr.R.K.Sharma, Organizing Chairman
Dr.Sandeep S. Kadam, MMC Observer (MMC/MAO-01898/2018)
Dr.Shailesh Kore, MMC Observer (MMC/MAO-00603/2015)



BLDE (DU)

United Nations Educational, Scientific and Cultural Organization

Network on Research and Postgraduate Education in Biophysics, Biotechnology and Environmental Health

Life Sciences International Postgraduate Educational Center Yerevan, Armenia

UNESCO/UNITWIN NETWORK WEB SEMINAR-2020

August 6-7, 2020

Organized by

BLDE

(DEEMED TO BE UNIVERSITY)

Shri B. M. Patil Medical College, Hospital & Research Centre, Vijayapura, Karnataka, India

Certificate of Presentation

This is to certify that, **SUJAYENDRA KULKARNI** of Department of Anatomy, Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura, Karnataka, India has participated and presented a paper entitled *"Molecular Profiling of FIX (FACTOR 9) Gene of Hemophilia B in Karnataka"* during UNESCO/UNITWIN Network Web Seminar held on August 6 & 7, 2020.

PROF. DR. ARAVIND V. PATIL
The Dean, FoM & Principal
BLDE (DU), India

PROF. R. B. KOTNAL
Chair-Scientific Committee
BLDE Association, India

PROF. SINERIK AYRAPETYAN
UNESCO Chair-Life Sciences
(Biophysics Biotechnology & Env. Health),
LSIPEC, Armenia

41 Annual Conference of Indian Association of Biomedical Scientists

International Virtual Conference on

RECENT TRENDS IN BIOMEDICAL RESEARCH

12- 14th March, 2021

CERTIFICATE

This is to certify that Sujayendra Kulkarni Department of Anatomy, Shri B M Patil medical college, Hospital and Research centre, BLDE(Deemed to be University) Vijayapura

has participated as and presented Oral Presentation entitled

Insilco analysis of Novel missense mutations of FIX gene recorded in Hemophilia B population of Karnataka

in the "International Virtual Conference on "RECENT TRENDS IN BIOMEDICAL RESEARCH" Organized by the Indian Association of Biomedical Scientists held during 12-14 March 2021.

Dr. G. K. Pal
President
IABMS

Dr. E. Padmini
General Secretary, IABMS
Organizing Secretary