Open Access
Journal of the Faculty of Medicine Baghdad
Print ISSN: 0041-9419, Online ISSN: 2410-8057
DOI: https://doi.org/10.32007/jfacmedbaghdad3218
Research Article

Sequencing of Catalytic Serine Protease, Linker, and Activation Peptide Domains-Coding Regions of the *F9* Gene in Iraqi Hemophilia B Patients

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Received: Oct. 2026
Revised: Jan. 2026
Accepted: Jan. 2026
Published Online: March 2026
Published: April 2026

Abstract:

Background: Hemophilia B is an X-linked recessive disorder caused by mutations in the *F9* gene, causing bleeding tendency predominantly in males. The mutational spectrum of the *F9* gene has not been adequately studied in Iraq.

Objectives: To detect the disease-causing variants of exons 6, 7, and 8 and immediate introns of *F9* gene using Sanger sequencing among Iraqi hemophilia B patients and to correlate them with phenotypes.

Methods: Forty Iraqi hemophilia B patients were recruited for this cross-sectional study from The Hereditary Bleeding Disorder Ward in the Children Welfare Teaching Hospital, Medical City, Baghdad, between November 2021 and April 2022 using a consecutive sampling technique. Peripheral blood samples were used for sequencing exons 6, 7, and 8, which encode catalytic serine protease (SP), linker, and activation peptide domains and immediate introns of the *F9* gene using Sanger sequencing.

Results: Nineteen (47.5%) patients had positive conclusive results. Fifteen unique variants were detected; 12 (80%) of them were disease-causing. Nine variants were located in the SP, one in the linker domain, and two in the splice site of intron 6. The most common pathogenic variant was the c.572G>A (p.Arg191His) on the linker domain as seen in six patients, while c.880C>T (p.Arg294Ter) and c.1358G>T (p.Trp453Leu) were the most common pathogenic variants of the SP domain as seen in two patients each. The vast majority were point mutations that are generally similar to the reported phenotype.

Conclusion: Molecular profiling of *F9* gene in the current cohort confirms 12 disease-causing variants, making molecular diagnosis and genetic counseling of hemophilia B possible. It explained the discrepancy between FIX level and clinical course, and variable severity among family members. Integrating genetic data into national registries will expand the molecular database for important health conditions in Iraq, improving healthcare provision through genetic counseling, prevention, and prenatal diagnosis.

Keywords: c.572G>A; *F9* gene; Hemophilia B; Iraq; Sanger sequencing.

Introduction:

Hemophilia B (Christmas disease; OMIM #306900) is an X-linked recessive bleeding disorder, affecting about one in 30,000 male births (1). It results from a deficiency of the coagulation factor 9 (FIX) proteins, a vitamin K-dependent plasma protease that participates in the intrinsic coagulation pathway (2). According to the residual plasma FIX activity, hemophilia B is classified into severe [<1% of normal (<0.01 U/mL)], moderate [1–5% (0.01–0.05 U/mL)], and mild [>6–40% (0.06–0.40 U/mL)] (3). Patients with severe hemophilia B, accounting for approximately 30-45% of patients, usually suffer from recurrent joint, soft-tissue, and muscle bleedings in addition to mucocutaneous bleedings (easy bruising, epistaxis, prolonged bleeding following traumas and surgical procedures (4).

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The classic laboratory findings in hemophilia B include a prolonged activated partial thromboplastin time (aPTT) and a normal prothrombin time (PT) (5). Defects in FIX level are caused by mutations in factor 9 gene (*F9* is the HGNC-approved symbol) (6). *F9* gene (OMIM #300746) is located on the long arm of chromosome X (Xq27.1). It spans about 34 kb of genomic DNA [GRCh38: X:139,530,739-139,563,459] and contains eight exons encoding a 2.8 kb mRNA (7). The transcribed mRNA encodes a precursor protein containing 461 amino acids, which includes an N-terminal signal peptide and a propeptide, added to the mature protein, which includes 6 domains: Gamma carboxyglutamic acid (GLA) domain, two epidermal growth factor-like domains (EGF), linker, activation peptide, and a serine protease (SP) domain. SP domain is encoded by part of exon 6 and most of exons 7 and 8 (8).

In the interactive *F9* database and until March 2023, a total of 1692 unique variants were reported on *F9* gene in all coding and non-coding regions (including promoter, introns, and 3'-untranslated regions (3' UTR) of the *F9* gene (9). The mutations are distributed over the entire *F9* gene, and approximately 56% in the part that encodes the SP domain (8).

Molecular testing in hemophilia B patients helps confirm the diagnosis in query or inconclusive clinical settings, female heterozygote carrier detection, proper genetic counseling, prenatal and pre-implantation genetic diagnosis, correlating the detected mutations with clinical phenotype, and expanding the knowledge about *F9* gene mutations in various ethnic/geographic groups (10,11).

In Iraq, the latest estimate of hemophilia prevalence reported a significant increase in one decade from 7.2/100 000 males in 2007 to 15.9/100 000 males in 2016. Similarly, the incidence of hemophilia had increased from 8.4/100 000 livebirths in 2007 to 16.3/100 000 livebirths in 2016. Severe hemophilia represented 63.4% of all types (12). In 2019, the estimated total number of people with hemophilia (PWH) in 2019 was 2,416 (13).

This study is part of an ongoing project to create and expand a molecular database of common and important health problems in Iraq. It aimed to determine disease-causing variants of the SP, linker, and activation peptide domains-coding regions of *F9* gene and to evaluate clinical and hematological characteristics of unrelated Iraqi Hemophilia B patients in addition to assessing the genotype-phenotype correlation of the detected variants.

Cases and Methods

This cross-sectional study recruited 40 unrelated Iraqi Arab hemophilia B patients from The Hereditary Bleeding Disorder Ward in the Children Welfare Teaching Hospital, Medical City, Baghdad, a tertiary referral hospital, during a 5-month period extending between November 2021 and April 2022. All hemophilia B patients were enrolled during their regular outpatient visits to receive prophylactic or therapeutic FIX concentrate regardless of age, gender, residence, or disease severity. Acquired hemophilia B, relatives of already enrolled patients, and those who refuse to voluntarily participate in this study were excluded from this study.

A consecutive (also called consensus) non-probability sampling technique was used in this study by visiting the center twice weekly on the outpatient clinic days, enrolling all hemophilia B patients fulfilling the inclusion criteria and without any of the exclusion criteria, until a target of 40 was reached. This number was calculated by a special equation considering population size, population proportion, confidence level, and margin of error.

Full clinical data were recorded from hospital records and direct interviews of adult patients and guardians of young children. These included basic demographic data, detailed medical history, family history, findings of a thorough clinical examination, relevant laboratory results, including hematological investigations for diagnosis and assessment of disease severity and presence of inhibitors, in addition to biochemical and serological tests when indicated.

From all enrolled patients, a 3-5 mL of peripheral blood was aspirated for molecular diagnosis, which included three steps: DNA extraction, conventional PCR amplification of the target regions (entire exons 6, 7 and 8) and their immediate introns, followed by Sanger sequencing.

DNA extraction was performed using Promega ReliaPrep™ blood gDNA. Three primer sets were designed for PCR amplification, each set for one of the studied exons. Three primer sets were designed for PCR amplification of the target exons (6, 7, and 8), using NCBI Primer Blast. Each set consists of two primers: forward and reverse.

F9_exon 6 Forward:
AGGATGGGCCTCAATCTCAA,
F9_exon 6 Reverse:
GGAGGCCTTCTCACATTGGT,
F9_exon 7 Forward:
CATTCCATTTCTGCCAGCAC,
F9_exon 7 Reverse:
TGACCCTTCTGCCTTTAGCC,
F9_exon 8 Forward:
GGTCAGTGGTCCCAAGTAGTCA,
F9_exon 8 Reverse:
GGCTGGGCCCTTAGAAATG.

Sizes of the PCR products were 442 bp for exon 6, 362 bp for exon 7, and 865 bp for exon 8. PCR protocol was with initial denaturation step at 95ºC for 3 minutes, followed by 30 cycles consisting of denaturation step at 95°C for 30 seconds, annealing at 65°C for 30 seconds, then extension at 72°C for 30 seconds, followed by final extension step at 72ºC for 90 sec. Both steps (DNA extraction and PCR amplification) were performed in the National Center for Teaching Laboratories, Medical City Campus, Baghdad.

Sanger sequencing was carried out using ABI3730XL automated DNA sequencer by Macrogen Company labs., Republic of Korea. Quality control measures for each step, before, during, and after sequencing, were ensured as per international recommendations and guidelines for molecular testing. The digital results were analyzed using Mutation Surveyor software version 5.1.2, performed by a qualified geneticist in Baghdad.

The nomenclature of *F9* variants was based on cDNA reference sequence NM_000133.4 and protein reference sequence NP_000124.1, in accordance with recommendations of the Human Genome Variation Society (HGVS) (14,15). The detected *F9* variants were compared with those published in the Factor IX Variant Database and CDC Hemophilia Mutation Project (CHAMP & CHBMP) (9,16,17). Variants were classified either as class 1 "benign" (B), class 2 "likely benign" (LB), class 3 "variant of uncertain significance" (VUS), class 4 "likely pathogenic" (LP), or class 5 "pathogenic" (P) variants (15).

Ethical considerations: This study was conducted in accordance with the Helsinki Declaration. The study was approved by the research ethics committee in the Department of Pathology and Forensic Medicine, College of Medicine, University of Baghdad (issue no. 200 at 10/11/2021). Another approval was obtained from the Ministry of Health, the Institute of Training and Development as well as from the hospital management. All adult patients, as well as the parents of pediatric patients, have given their informed consent to participate in this study and have their DNA tested.

Statistical Analysis: Microsoft Excel® version 2013 and IBM-SPSS (Statistical Package for Social Sciences) version 22 were used for data entry and analysis. Descriptive statistics were presented as numbers, percentages, range, mean, and standard deviation. Categorical data were analyzed using the Chi-square test. A *P*-value <0.05 was considered statistically significant.

Results

Out of the 40 recruited patients with hemophilia B, 39 (97.5%) were males, and 1 (2.5%) was a female. Their ages ranged between 2 and 50 years, with a mean±SD of 23.0±12.11. Their ages at diagnosis ranged from one week to 38 years, with a mean± SD of 6.6 ±10.65 years. There were 28 (70%) patients with severe, 8 (20%) with moderate, and only 4 (10%) with mild disease (Table 1).

Table 1: Distribution of the hemophilia B cases by age at diagnosis and severity of FIX deficiency

Patients age at diagnosis (years)	Disease severity based on FIX level No. (%)			
	Mild No. (%)	Moderate No. (%)	Severe No. (%)	Total No. (%)
Less than 1	0 (0)	1 (2.5)	13 (32.5)	14 (35.0)
1-5	0 (0)	2 (5.0)	12 (30.0)	14 (35.0)
6-10	2 (5.0)	1 (2.5)	2 (5.0)	5 (12.5)
11-18	0 (0)	1 (2.5)	0 (0)	1 (2.5)
Above 18	2 (5.0)	3 (7.5)	1 (2.5)*	6 (15.0)
Total	4 (10.0)	8 (20.0)	28 (70.0)	40 (100.0)

* = this adult male had severe FIX deficiency but a mild clinical course

At diagnosis, a significant statistical association was found between FIX level and the presenting manifestations (p=0.003). Bruises/ecchymosis and post-circumcision bleeding were the presenting manifestations in severe cases only, while post-traumatic and gum/teeth bleeding were mainly seen in patients with mild / moderate disease. Hematuria was associated with moderate and severe cases, and spontaneous epistaxis was associated with mild and moderate cases (Table 2).

Table 2: Association of disease severity and patients' presenting manifestations

Disease severity	Easy Bruises & ecchymosis No. (%)	Post-circumcision bleeding No. (%)	Posttraumatic bleeding No. (%)	Spontaneous epistaxis No. (%)	Hematuria No. (%)	Gum/teeth bleeding No. (%)	Total No. (100%)
Mild	0 (0)	0 (0)	2 (50.0)	1 (25.0)	0 (0)	1 (25.0)	4 (10)
Moderate	0 (0)	0 (0)	4 (57.1)	1 (14.3)	1 (14.3)	1 (14.3)	7 (17.5)
Severe	14 (48.3)*	6 (20.7)*	7 (24.1)*	0 (0)	1 (3.4)	1 (3.4)#	29 (72.5)
Total	14 (35.0)	6 (15.0)	13 (32.5)	2 (5.0)	2 (5.0)	3 (7.5)	40 (100.0)

* *p = 0.003; #* = this adult male had severe FIX deficiency but a mild clinical course

A positive family history of hemophilia B (both first and second-degree relatives) was present in 27 (67.5%) patients, and parental consanguinity was present in 25 (62%) patients. Five (12.5%) patients had hepatitis C virus (HCV) positive serology, 4 of them had a severe factor deficiency but all were above 25 years. None of the enrolled patients had FIX inhibitors.

Three cases of clinical interest have been observed in this study:

The first was the only enrolled female in this study. She was 22-yeard-old, and had a strong positive family history (her father, male cousin, 4 sisters, and her aunt), all have documented FIX deficiency. She was diagnosed at the age of 8 years due to bleeding after tooth extraction. Her factor IX level was 6% (mild deficiency), with no inhibitors.

The second is the adult male with severe FIX deficiency (<1% factor IX level) but having a mild clinical course. He did not report a history of a significant bleeding tendency until he is 18 years old, when he presented with hematuria. He had a positive family history and negative test for inhibitors.

The third was a 9-year-old boy with a mild disease, who visited a tertiary hemophilia center for prophylactic FIX administration prior to surgery.

Results of Sanger sequencing:

All 40 patients were tested by Sanger sequencing for the entire exons 6, 7, and 8 in addition to their immediate introns (intron-exon and exon-intron junctions) of *F9* gene. Nineteen (47.5%) patients had conclusive positive results, where a disease-causing variant was detected, 14 (35.0%) had negative results (either no or non-disease-causing variants were

detected), while seven (17.5%) patients had inconclusive sequencing results, where one or more exons showed sequencing failure or poor DNA quality that no decision can be made (Table 3).

Table 3: Summary of *F9* sequencing results of the study group

Patients´ result	No. (%) from total number (n=40)	No. (%) from conclusive results (n=33)
Positive/conclusive molecular results	19 (47.5)	19 (57.5)
• Pathogenic (all exonic)	13 (32.5)	13 (39.3)
• Likely pathogenic	6 (15.0)	6 (18.1)
• Exonic	4 (10.0)	4 (12.1)
• Splice site (intronic)	2 (5.0)	2 (6.1)
Negative molecular results	14 (35.0)	14 (42.4)
• No detected variant	8 (20.0)	8 (24.2)
• Benign (exonic)	1 (2.5)	1 (3.0)
• Likely benign (exonic)	2 (5.0)	2 (6.1)
• Intronic	3 (7.5)	3 (9.1)
Inconclusive molecular results	7 (17.5)	
• Failure in reverse sequencing	1 (2.5)	
• Poor quality DNA in ≥1 exon	6 (15.0)	
Total	40 (100.0)	

Variants' characteristics: A total of 15 unique variants were detected in the recruited hemophilia B patients.

Location:

a. Location on *F9* gene and immediate regions: Three (20%) out of 15 detected variants were located in exon 6 [two (13%) of them were disease-causing variants], one (7%) in exon 7 which was likely pathogenic (LP), and eight (53%) in exon 8 [seven (47%) of them were disease-causing variants] (figures 1&2). In addition, two (13%) splice site likely pathogenic variants in intron 6, and other intronic variants in intron 7 were detected **(**Table 4**)**.

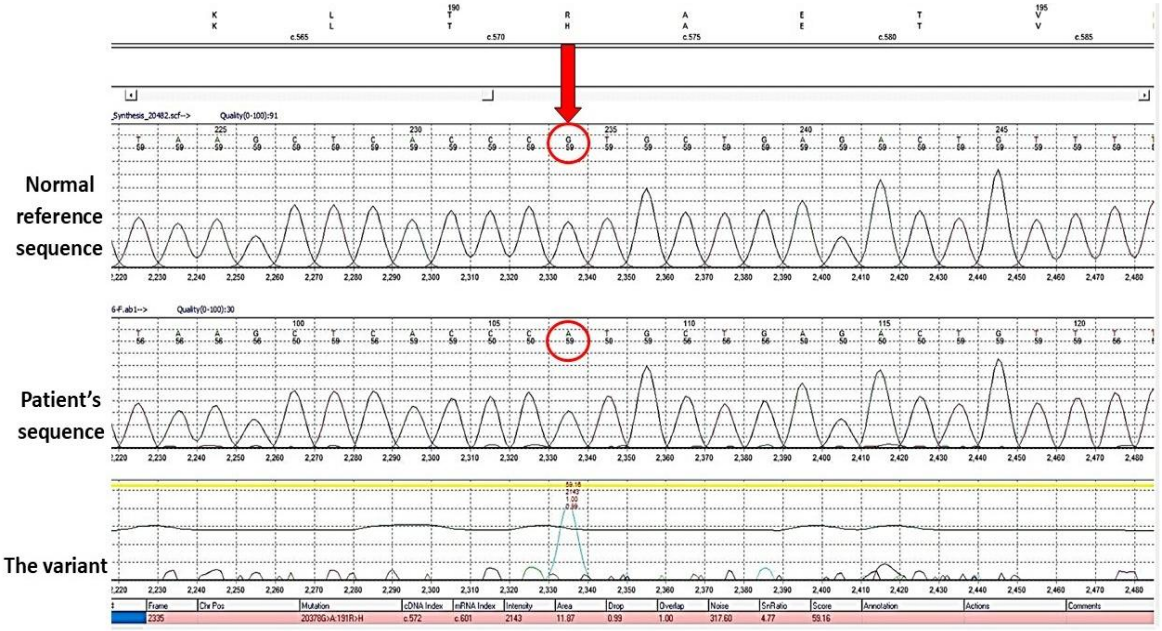

Figure 1: A chromatogram showing the c.572G>A variant in exon 6. A single peak represents a hemizygous allele with the nucleotide change from G to A in the patient's sequence. The red arrow and circles point to the site and type of substitution.

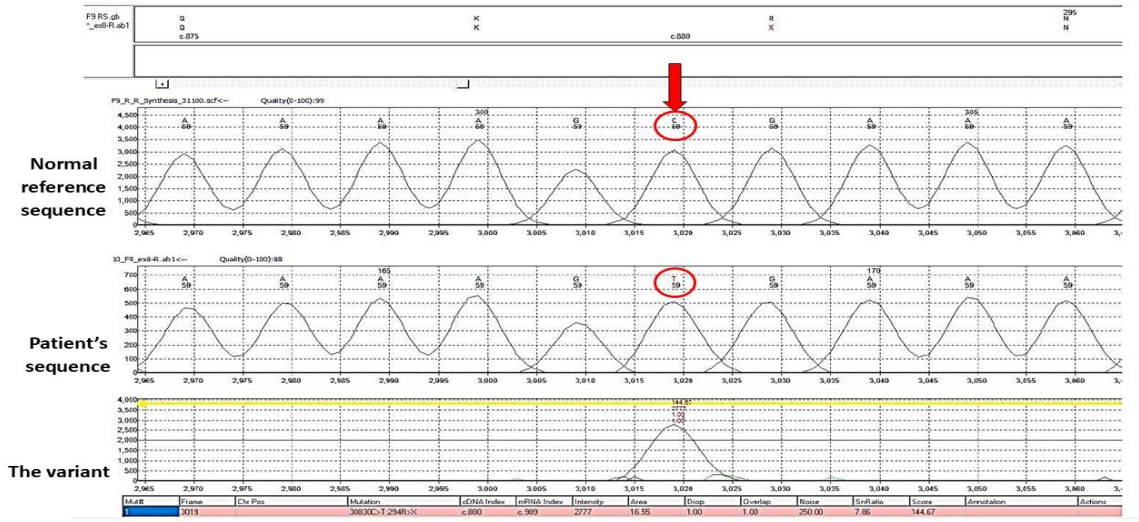

Figure 2: A chromatogram showing c.880C>T variant in exon 8 of the *F9* gene. A hemizygous allele with a single peak and the nucleotide change from C to T is seen in the patient's sequence. The red arrow and circles point to the site and type of substitution.

Table 4: Characteristics of the detected variants of *F9* gene in the study group

Variant (n=15)		No. (%) of variants	Frequency on patients
Location on protein	Serine Protease (SP)	10 (67.0)	15
	Linker	1 (6.7)	6
	Activation peptide	1 (6.7)	7
	Intron and splice site	3 (20.0)	13
Location on *F9* gene	Exonic:	12 (80.0)	28
	• Exon 6	3 (20.0)	14
	• Exon 7	1 (6.7)	1
	• Exon 8	8 (53.3)	13
	• Intronic:	3 (20.0)	13
	• Splice	2 (13.4)	2
	• Non-splice	1 (6.7)	11
Type of variants	Point mutation:	14 (93.3)	27
	• Missense	11 (73.3)	11
	• Non-sense	2 (13.4)	3
	• Silent	1 (6.7)	4
	Deletion:	1 (6.7)	1
Classification	• Pathogenic	6 (40.0)	13
	• Likely Pathogenic	6 (40.0)	6
	• VUS	0 (0)	0
	• Likely Benign	1 (6.7)	4
	• Benign	1 (6.7)	7
	• Benign intronic	1 (6.7)	11

***VUS:** Variant of uncertain significance*

b. Location on factor IX domains: Out of the 15 variants, ten (67%) were located on the SP domain, one (6.7%) on the linker domain, one (6.7%) on the activation peptide, one intronic (intron 7), and two splice site variants (intron 6) (Table 4, Figures 1&2).

Pathogenicity: Among the 15 unique variants detected in the sequenced regions of the recruited patients, six (40%) were pathogenic, six (40%) were likely pathogenic, one (6.7%) was likely benign, one (6.7%) was benign, and one (6.7%) was an intronic variant (Table 4).

Type: Of the 15 detected variants, 11 were missense, one was a silent mutation, two were nonsense (stop-gain), and one was an in-frame deletion.
The five pathogenic SP mutations were all missense, while out of the four likely pathogenic SP mutations, three were missense and one was of deletion type, and the only likely benign variant was a silent substitution type (Figures 1&2).
Three types of intronic variants were detected: two splice sites (intron 6), both of which were missense. Additional intronic variants were detected in intron 7 of 11 patients; these variants are benign and have no effect on the encoded protein.

Detailed data of all variants in exons 6, 7 and 8 of the *F9* gene detected in the current study are shown in Table 5.

Locations of variants detected in this study are depicted in Figure 3.

Table 5: Data of all detected variants of exons 6, 7 and 8 of *F9* gene in the current study

#	DNA change	Amino acid change	Exon/ intron	Type of the change	Reference SNP	Effect of the change	Domain in the *F9* gene	Frequency in the study	Frequency in the database	Reference
1	c.572G>A	p.Arg191His	6	Substitution/missense	rs137852238	Pathogenic	Linker	6	85	FDB [9]
2	c.880C>T	p.Arg294Ter	8	Substitution/nonsense	rs137852248	Pathogenic	SP	2	70	FDB [9]
3	c.1358G>T	p.Trp453Leu	8	Substitution/missense	None	Pathogenic	SP	2	1	FDB [9]
4	c.1183T>G	p.Phe395Val	8	Substitution/missense	None	Pathogenic	SP	1	2	FDB [9]
5	c.1187G>A	p.Cys396Tyr	8	Substitution/missense	rs137852273	Pathogenic	SP	1	8	FDB [9]
6	c.1135C>T	p.Arg379Ter	8	Substitution/nonsense	None	Pathogenic	SP	1	65	FDB [9]
7	c.689_691delGAG	NA	6	Inframe deletion	None	LP	SP	1	7	FDB [9]
8	Splice Intronic c.724-1G>T	NA	6	Substitution/missense	None	LP	Intron 6	1	NR	FDB [9]
9	Splice intronic c.723+2T>C	NA	6	Substitution/missense	None	LP	Intron 6	1	2	FDB [9]
10	c.760G>A	p.Gly254Ser	7	Substitution/missense	rs866538308	LP	SP	1	4	FDB [9]
11	c.1245T>A	p.His415Gln	8	Substitution/missense	None	LP	SP	1	4	FDB [9]
12	c.1331A>G	p.Tyr444Cys	8	Substitution/missense	None	LP	SP	1	223	CDC [14]
13	c.1050T>C	p.Ser350=	8	Substitution/silent	None	LB	SP	4	4	Varsome [32]
14	c.580A>G	p.Thr194Ala	6	Substitution/missense	rs6048	Benign	Activation peptide	7	62	Varsome [32]
15	Intronic variants	NA	7	Substitution/missense	None	NA	Intron 7	11	NR	NA

***SP** = serine protease; **LP** = likely pathogenic; **LB** = likely benign; **FDB** = FactorIX Database; **CDC** = Centers for Disease Control and Prevention; **NR** = Not reported; **NA**= Not applicable; **SNP**: Single nucleotide polymorphism*

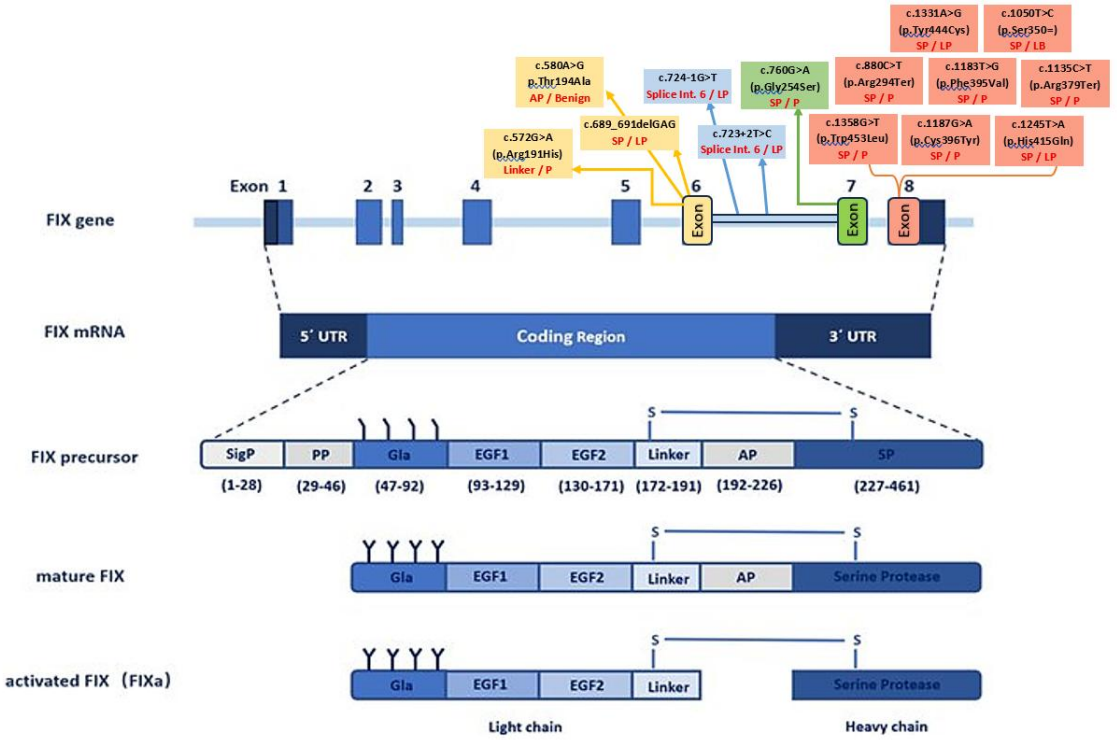

Figure 3: The *F9* gene, FXI domains, and relative sites of the variants detected in the current study

Discussion

Detection and characterization of disease-causing mutations are important for understanding the genetic etiology of human diseases, especially those causing significant health problems. They also aid in patients' management, informed decision making, and family planning. Identification of genetic variants, estimating allele frequency in different population groups, and facilitating data sharing between different researchers are additional benefits (14).

Due to the higher cost of sequencing the entire *F9* gene, regions with a higher reported mutation rate were chosen for this study. The catalytic serine protease (SP) domain (exons 8, 7, and parts of exon 6) of *F9* gene was targeted in this study as this domain-coding region accounts for 56% of lesions in *F9* gene. The linker and activation peptides domains coding-regions lie within the remaining parts of exon 6. That's why the entire exons 6, 7, and 8 in additions to their immediate regions, were subjected to sequencing (9).

Basic demographic characteristics of the patients: The association of disease severity with younger age at diagnosis in the current study is consistent with the results of other studies. An epidemiological study reported that hemophilia B median age at diagnosis was 12 months, which is close to the current study. An Egyptian study found that the ages at diagnosis ranged from 22 days to 3 years (17,18). Younger ages at diagnosis are likely related to prevalent causative variants causing severe factor 9 deficiency and therefore severe phenotype with earlier age of onset, or issues related to diagnostic facilities. A positive family history in many patients make families more concerned with even minimal bleeding tendencies.

Clinical characteristics: The higher frequency of severe cases compared to moderate and mild in the current study, can be a reflection of the true distribution of the cases and/or may be due to the fact that severe cases regularly visit the hospital to receive prophylactic treatment, and therefore, have a higher chance of being recruited to the study. An earlier Iraqi epidemiological study showed that 42% of the registered patients with hemophilia B were severe, 39% were moderate, and 19% were mild cases (19).

The global *F9* database shows that 43% of registered cases had severe hemophilia B, 23% had moderate disease, 16% had mild disease, and 18% had uncertain disease (9).

Several cohorts from US, India, and Saudi Arabia on hemophilia B patients found that severe cases represented 40%, 68%, and 61.5% respectively; moderate disease represented 39%, 20%, and 5%; and mild disease represented 21 %, 12%, and 33% (20,21). The differences between those studies in terms of the severity distribution within each sample may be due to larger sample sizes, presence of a large percentage with uncertain severity, geographic and ethnic variations, and varying sampling techniques.

The statistically significant association between FIX level and the presenting manifestations in the current study may be sue to overlooking easy bruising and small bleedings while a more severe bleeding, especially following trauma is reported. The presenting manifestations in this study differ from an Indian study where hemarthrosis was the most common presenting feature (22). Hemarthrosis was

not reported in the current study as a presenting but as a late clinical feature in most patients.

A positive family history of hemophilia B was found in 27 patients (67.5%), and parental consanguinity was found in 25 of them (62%); in two Iraqi studies from Al-Hilla and Baghdad, the presence of family history was reported in 53% and 71%, and parental consanguinity was 67% and 40.7% (17,23). Knowing that the consanguinity rate of the general population in Baghdad ranged between 40-45% (24), it can be seen that the average consanguinity of hemophilia B families is higher than the general population figures contrary to other studies in Iraq and Turkey (25,26). Consanguinity, however, has little effect on X-linked recessive disease in males, but has a significant effect on manifesting females. Studies in the US and India showed a positive family history of the disease in 70%, and 65% (27,28).

In this study, almost all patients who had hepatitis C virus (HCV) positive serology had severe disease and all were above 25 years. In earlier Iraqi and Pakistani studies, HCV-positive patients were 14% and 15% respectively, which is consistent with this study (29,30). There was no independent risk factor for HCV infection in the current study, and the most likely cause is the recurrent transfusion of blood products, which was practiced when FIX concentrate was not available (times of war and embargo). Older patients are more likely to receive blood components than pediatric age group at that time.

Molecular and sequencing results: According to Table 5, the most common disease-causing variant detected in this study was the pathogenic variant on exon 6 [c.572G>A (p.Arg191His)], as seen in six cases. This variant was reported in 85 cases in the global *F9* database (9). Two pathogenic variants on exon 8, c.880C>T (p. Arg294Ter) and c.1358G>T (p.Trp453Leu), were reported in the current study in two cases each, but in 70 and once in the global *F9* database, respectively (9).

Other pathogenic variants on exon 8, c.1183T>G (p. Phe395Val), c.1187G>A (p. Cys396Tyr), and c.1135C>T (p. Arg379Ter), were detected in the current study in one case each but were reported in two, 65, and eight times in the global *F9* database, respectively (9).

The intronic variants detected in this study are of 2 types: the splice-site (n=2) and others (n=7). The former is deleterious (disease-causing); their presence is diagnostic of hemophilia B. The other 7 intronic variants, reported in 11 patients are, however, non-deleterious in all databases. Their presence is of no clinical value and is part of normal genetic variations.

Six likely pathogenic variants, each were detected once in the current study, c.689_691delGAG, c.724-1G>T, c723+2T>C, c.760G>A (p.Gly254Ser), c.1245T>A (p.His415Gln), and c.1331A>G (p.Tyr444Cys), but reported in seven, none, two, four, four, and 223 in the database (9,14).

One likely benign, the silent variant, c.1050T>C (p.Ser350=), was reported in four patients in this study and four times in Varsome database. (31) One benign variant c.580A>G (p. Thr194Ala) was detected in seven patients in this study and reported 62 times in Varsome (31).

CHBMP *F9* database showed that missense mutations account for 58% of the total mutation list, nonsense in 8%, inframe deletion/change in 2%, and splice site mutations in 10% (14). An Indian study reported that 81% of the detected mutations were missense variants (18). Another study in China reported missense mutations in 64%, nonsense mutations in 15%, small deletions in 11.5%, splice site mutations in 4%, and small insertions in 2.5% (32). There is a slight discrepancy among these studies with the current study, which may be related to sample size, sequencing the entire *F9* gene, and different population characteristics.

Inconclusive sequencing results may be attributed to degradation during amplicon transport or storage. Sequencing requires higher level of integrity and purity than conventional PCR.

Serine protease (SP) domain of the *F9* gene: Ten out of 15 (67%) variants were detected in this domain. In the *F9* database, 56% of mutations were related to this domain, and also in a Chinese study, which was 55% (9,32). Nine (60%) of the SP variants were pathogenic seen in 11 patients and one was a silent variant c.1050T>C (p. Ser350=), giving a disease detection rate of 33% for the SP-coding region. The disease-causing mutations detected in the SP domain of the *F9* gene showed moderate to severe levels of FIX deficiency in spite of the fact that the database reported all the severity levels in this domain (9). This observation can be due to the fact that 96% of patients in this study had moderate-severe phenotype. Larger-scale, more inclusive studies can highlight the actual state.

Linker and activation peptide domains of *F9* gene: Pathogenic variant (c.572G>A, p. Arg191His) was detected in six patients on the linker domain, giving a disease detection rate of 18% for this domain. One benign variant (c.580A>G, p.Thr194Ala) was detected in seven patients in the activation peptide domain. The activation peptide domain had a small rate of genetic variation and no disease-causing variation in this study, compared to 3.4% reported in the database (9).

Genotype-phenotype correlation: Most (73%) detected pathogenic mutations were missense mutations. The severity of the disease in patients with missense mutations ranged from mild to severe, as reported in a US cohort and in the available database (FDB, CDC) (9,14,19). Moreover, all nonsense mutations (13%) were detected in severe cases (33). This outcome is expected, as non-sense variants cause a short-truncated protein, with a significant impairment of FIX function.

The in-frame deletion (c.689_691delGAG), was found in a severe hemophilia B patient in this study

and reported in the CDC database as a severe disease too (14).

The two splice site mutations (c.724-1G>T, c723+2T>C) were detected in severe cases similar to those reported in the database. The missense variant c.572G>A was detected in exon 6 (linker domain) of the gene and seen in six patients (3 had mild disease, and 3 had moderate disease), and was reported in the CDC as mild/moderate, similar to the current findings (14).

Generally, there is a similarity in the type of mutation (genotype), factor IX level, and the disease severity (phenotype), especially with the nonsense and in-frame deletion types, between this study and what is reported in the database and published literature.

Additional observations of clinical relevance:

1. The adult male with severe hemophilia B and a mild clinical course:

Molecular diagnosis of this patient revealed the presence of a missense pathogenic variant (c.1358G>T; p. Trp453Leu) in exon 8, which settled the diagnosis of hemophilia B. This same variant was also identified in another patient in this study who presented at 6 months of age with gum and tooth bleeding. This individual variation can be attributed to the fact that clotting factor levels are typically quantified via antigen assays (e.g., ELISA for protein amount), whereas biological activity is assessed through functional tests like one-stage clotting (OC) or chromogenic substrate (CS) assays that evaluate procoagulant efficacy. In hemophilia, mutations can produce normal or near-normal antigen levels of factor IX but severely reduced activity i.e. cross-reacting material (CRM)-negative or low antigen with some residual function (CRM-reduced), explaining phenotypic variability. The latter is exemplified by missense variants commonly seen in *F9* gene. In addition, assay discrepancies, such as between OC and CS methods, highlight that activity does not always correlate directly with antigen levels, impacting diagnosis and therapy monitoring in such conditions. This distinction is critical for patients where protein quantity mispredicts bleeding risk (34). Moreover, coexisting prothrombotic conditions, such as factor V Leiden mutation, protein C or S deficiency, or prothrombin G20210A, can counterbalance the bleeding diathesis, resulting in milder or delayed symptoms even with severe factor deficiency. Clinical bleeding criteria sometimes classify these patients as having moderate or mild phenotypes despite factor levels below 1% (35). These prothrombotic conditions were obviously not tested in this study.

2. The female patient with hemophilia phenotype:

Molecular diagnosis of this female revealed a heterozygous state of a likely pathogenic missense variant on the splice site of intron-exon junction 7 (c.724-1G>T). To explore the genetic constitution of this family, other family members were requested to be sequenced; yet, they declined.

Based on the available data, several explanations can be presumed for this female. A compound heterozygosity, where only one variant was detected, and the other is located on an untested region of *f9* gene. This is the most likely explanation, as homozygosity was excluded. A skewed X-inactivation can also be another possibility, but her 4 manifesting sisters make this suggestion less likely. Even less likely are other possibilities where preferential X- inactivation due to an abnormal X-chromosome exists (36,37). However, several retrospective and clinical studies and case histories reported serious bleeding problems in heterozygote carrier females, even with mild hemophilia B (38).

3. The male patient with a mild disease visiting a tertiary hemophilia center:

This patient had a mild FIX deficiency (factor level 12.5%) who visited the tertiary hemophilia center as he had a strong positive family history of three affected brothers, one affected uncle, and a dead affected uncle. He had history of bleeding following trauma. At time of the interview, he was about to have a dental procedure, for which he was investigated and prophylactic doses of FIX concentrate was recommended. He had c.572G>A (p.Arg191His) pathogenic missense variant on exon 6.

Limitations

This study did not sequence the entire *F9* gene; negative results do not exclude hemophilia B in symptomatic patients. Severe phenotypes may have been overrepresented due to the sampling technique used in this study. National database requires a larger sample size, multicenter studies, recruiting diverse geographic and ethnic populations, with complete *F9* gene sequencing. This has not been achieved in this local cross-sectional study.

Conclusions

The molecular profiling of *F9* variants in the current cohort confirms 12 disease-causing variants, making molecular diagnosis and genetic counseling of hemophilia B easier. It can also explain the discrepancy between FIX level and clinical course, as well as variable severity among family members. Integrating such genetic data into national registries will expand the molecular database for important health conditions in Iraq, improving healthcare provision, through genetic counseling and prevention and enables prenatal diagnosis.

Authors' declaration

We confirm that all the Figures and Tables in the manuscript belong to the current study.

The project was approved by the local Ethical Committee in the Dept. of Pathology & Forensic Medicine, College of Medicine, University of Baghdad (issue No. 200 at 10/11/2021).

Conflict of Interest: Bassam M. Sadik Al-Musawi is an Editorial Board Member in the journal, but did not participate in the peer review or any other process other than being a corresponding author.

Funding: No specific grant from a public, private, or nonprofit funding organization was obtained for this study.

Data availability: Upon reasonable request, the corresponding author will make the data sets generated and/or analyzed during the current work available.

Authors' contributions:
Bassam M. Sadik Al-Musawi (study conception and design). Huda H. Mohammed Obaida (Data collection). Huda H. Mohammed Obaida (The first draft of the manuscript). Data analysis & interpretation (Bassam M. Sadik Al-Musawi & Huda H. Mohammed Obaida) and all authors commented on previous versions of the manuscript. Bassam M. Sadik Al-Musawi & Huda H. Mohammed Obaida (approved the final manuscript).

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How to Cite this Article?

Obaida HH, Al-Musawi BM. Sequencing of catalytic serine protease, linker, and activation peptide domains-coding regions of F9 gene in Iraqi hemophilia B patients. J Fac Med Baghdad [Internet]. Available from: https://iqjmc.uobaghdad.edu.iq/index.php/19JFacMedBaghdad36/article/view/3218

التفاعل المتوالي لمناطق الترميز لمجالات الانزيم البروتيني سيرين المحفّز، والرابط، وببتيد التنشيط لمورث العامل التاسع لدى مرضى الهيموفيليا نوع ب العراقيين

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الخلاصة:

الخلفية: الهيموفيليا ب هو مرض وراثي متنحي مرتبط بالكروموسوم X ناتج عن طفرات في المورث *F9* يسبب ميلاً لنزف الدم بشكل رئيسي عند الذكور. لم يُدرس طيف طفرات المورث *F9* بشكل كافٍ في العراق.

الأهداف: الكشف عن التغايرات المسببة للمرض في مناطق الترميز في الأكسونات 6 و 7 و 8 والانترونات المتاخمة لها في مورث العامل التاسع باستخدام تسلسل سانكر لدى مرضى الهيموفيليا ب العراقيين ودراسة ارتباطها بالمظاهر السريرية.

المنهجية: اختير أربعون مريضًا عراقياً مصاباً بمرض الهيموفيليا ب لهذه الدراسة المستعرضة من قسم اضطرابات النزف الوراثية في مستشفى حماية الأطفال التعليمي في مدينة الطب في بغداد بين نوفمبر 2021 وأبريل 2022 باستخدام تقنية العينة المتتالية. استُخدمت عينات دم محيطي لفحص تسلسل الإكسونات 6 و 7 و8 والتي تُرمّز لنطاقات الانزيم البروتيني سيرين المحفّز، والرابط، والببتيد التنشيطي فضلاً عن الإنترونات المتاخمة لها من مورث العامل التاسع باستخدام تسلسل سانكر.

النتائج: أظهرت تسعة عشر مريضاً (47.5%) نتائج إيجابية حاسمة. اكتشف 15 تغايرًا فريدًا، 12 (80%) منها كان مسبباً للمرض. وُجدت تسعة تغايرات في نطاق الانزيم البروتيني سيرين المحفز، وواحد في نطاق الرابط، واثنان في موقع ربط الإنترون السادس. كان المتغير الممرض الأكثر شيوعًا هو c.572G>A (p.Arg191His) والموجود في نطاق الرابط، والذي لوحظ لدى ستة مرضى، في حين كانت التغايرات c.880C>T (p.Arg294Ter) و c.1358G>T (p.Trp453Leu) هما التغايران الممرضان الأكثر شيوعاً في نطاق الانزيم البروتيني سيرين المحفز واللذان لوحظا في مريضين لكل منهما. كانت الغالبية العظمى تغايرات نقطية متوافقة عموماً للنمط الظاهري المسجل.

الاستنتاجات: أكد التحليل الجزيئي لتغايرات مورث العامل التاسع في هذه الدراسة وجود اثنا عشر تغايراً مسبباً للمرض، مما يجعل التشخيص التشخيص الجزيئي والاستشارة الوراثية لمرضى الهيموفيليا من نوع ب ممكناً، كما يفسر التباين بين مستوى عامل IX والمسار السريري، وشدة المرض المتفاوتة بين أفراد العائلة الواحدة. إضافة هذه البيانات الوراثية ودمجها في السجلات الوطنية سوف توسع قاعدة البيانات الجزيئية للأمراض المهمة في العراق، مما يحسن تقديم الرعاية الصحية من خلال الإرشاد الوراثي والوقاية والتشخيص ما قبل الولادة.

الكلمات المفتاحية: c.572G>A، مورث العامل التاسع، الهيموفيليا ب، العراق، تسلسل سانكر