A Case study of severe hemophilia B due to pathogenic C.676C>T missense mutation in the *F9* gene

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Abstract. The diversity and frequency of F9 gene mutations in Indonesia remain largely undocumented. In this study, long-read sequencing was performed using the PromethION24 platform developed by Oxford Nanopore Technologies plc (ONT). The research subject was an individual diagnosed with severe hemophilia B. Amplification of the F9 gene was carried out using the forward primer F (5′-CGGAGCCAAATGTTCTTTTC-3′) and the reverse primer R (5′-CACCAACTGCTCATCTCTGG-3′). The three-dimensional conformation of the mutant coagulation factor IX protein was modeled using SWISS-MODEL, and the resulting structure was examined with Discovery Studio and UCSF Chimera. Long-read sequencing analysis revealed a missense mutation (c.676C>T) in the F9 gene, which was subsequently validated through Sanger sequencing. This pathogenic substitution (p.Arg226Trp) results in an amino acid change at residue 226 within exon 6, located in the activation peptide region of the serine protease domain. Structural modeling indicated an alteration in the coil configuration at residue 226, which may interfere with atomic interactions and compromise the activation process of factor IX, thereby contributing to the clinical manifestations of hemophilia B.

1. Introduction

Hemophilia B is an X-linked genetic bleeding disorder caused by mutations in the F9 gene, which lead to decreased or impaired production of coagulation factor IX in circulation [1]. Alterations in the F9 gene are closely associated with the clinical manifestations observed in individuals with hemophilia B, including spontaneous bruising or bleeding that may occur with varying degrees of severity, ranging from severe to mild, or sometimes with minimal bleeding history. The severity of hemophilia B is commonly

categorized into three levels according to the plasma concentration of factor IX. Severe hemophilia B is defined by factor IX activity levels of less than 1%, while moderate hemophilia B is characterized by levels between 1% and 5%. In contrast, mild hemophilia B is identified when factor IX activity ranges from greater than 5% up to less than 40% [2].

Coagulation factor IX is produced by the F9 gene and is composed of several key functional domains, including a gamma-carboxyglutamate (Gla) domain, two epidermal growth factor-like (EGF-like) domains, and a serine protease domain [3]. The Gla domain, characterized by a high content of glutamic acid residues, is essential for binding to phospholipid surfaces and plays a critical role in enabling proper activation of factor IX within the coagulation cascade, particularly through its interaction with factor VIIa [4]. In terms of its genomic structure, the F9 gene consists of eight exons and seven introns [5] and is located on chromosome Xq27.1 [6]. It encodes a protein made up of 461 amino acids [7].

Mutations in the F9 gene may disrupt proper protein folding, which can ultimately impair the function of coagulation factor IX. Kulkarni et al. (2021) reported several missense variants associated with hemophilia B, including c.127C>T, c.195G>A, and c.197A>T [5]. According to the Coagulation Factor Variant Databases (2024), a total of 1244 mutation variants have been identified among 4713 patients. These reported alterations include deletions, insertions, indels, duplications, and point mutations such as missense and nonsense variants. However, information regarding the types and frequency of F9 mutations in Indonesia remains limited.

The prevalence of hemophilia B may increase in the coming decades due to the potential transmission of the defective F9 gene from affected individuals or carriers. Among the various mutation types, missense mutations are the most frequently reported. Many of these variants are considered pathogenic because amino acid substitutions can alter protein structure and conformation, leading to cellular dysfunction and disease development. Therefore, the identification of F9 gene mutations plays an important role in diagnosis, treatment planning, genetic counseling, and further research related to hemophilia B.

2. Materials and Methods

2.1 Research subject

The study participants consisted of individuals diagnosed with hemophilia B who were registered with the Hemophilia Society of the Special Region of Yogyakarta, Indonesia. Ethical clearance for this research was obtained from the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, under approval number KE/FK/1418/EC/2023. One participant included in this study was a 16-year-old male diagnosed with severe hemophilia B, presenting with a coagulation factor IX activity level of 0.9%. Clinically, the patient experienced recurrent knee swelling and spontaneous bleeding episodes occurring approximately three to four times per month. Management involved intravenous administration of coagulation factor IX at a dose of 1500 IU per injection. Repeated bleeding episodes and joint complications may lead to impaired mobility or paralysis, often necessitating the use of assistive devices such as walkers for ambulation.

2.2 DNA Extraction

Genomic DNA was isolated from saliva samples using the Geneaid DNA Isolation Kit in accordance with the manufacturer's guidelines. In brief, 20 µL of proteinase K was added to 200 µL of the sample and incubated at 60°C to promote cell lysis and protein degradation, followed by the addition of 200 µL of genomic DNA stabilization buffer (GSB). The purification process included two washing steps to eliminate impurities, after which the

DNA was eluted twice with 50 μL of elution buffer each time, yielding a total volume of 100 μL. The quantity and quality of the extracted DNA were then evaluated using a Thermo Scientific NanoDrop spectrophotometer.

2.3 Long-Read Sequencing for mutation detection

Long-read sequencing was performed using the PromethION24 platform (Oxford Nanopore Technologies plc, ONT) to determine the nucleotide sequence of the F9 gene. Prior to sequencing, DNA concentration was measured using a spectrophotometer, with a recommended input of approximately 1–2 μg of DNA at a concentration of ≥50 ng/μL. The priming mixture consisted of a flow cell flush buffer used to cleanse the flow cell and a flow cell tether, which facilitates the anchoring of DNA molecules to the nanopores. For library preparation, 15 μL of sequencing buffer was combined with 5 μL of the DNA library, and Rapid Barcoding Kit V14 was employed for barcoding. Sequencing data were subsequently processed using EPI2ME, with reference alignment performed against the GenBank database (ChrX: 154835792–155022723, GRCh38).

Identification of F9 gene mutations was carried out through long-read nanopore sequencing, which allows the direct analysis of extended DNA fragments. Following sequencing, the resulting data were examined using Integrative Genomics Viewer (IGV) to detect potential mutations within the F9 gene of the study subject. In addition, the Benchling sequencing alignment tool was used to compare nucleotide sequences between the reference (wild-type) and mutant F9 gene. The detected mutation was subsequently validated by Sanger sequencing, which involved designing specific primers targeting the mutation site, PCR amplification of the target region, and determination of the nucleotide sequence for confirmation. Finally, the results obtained from both sequencing approaches were compared to ensure accuracy, consistency, and reliable clinical interpretation of the identified mutation in relation to hemophilia B.

2.4 Primer Design for Gene Amplification of *F9*-Exon 6

Primer design was performed following long-read sequencing to precisely determine the location of the detected mutation. The primers were specifically designed based on mutation site information obtained from the sequencing results. Primer sequences were generated using Primer3Plus, a freely accessible online tool available at https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi, utilizing the reference sequence NC_000023.11. The analysis produced a pair of primers consisting of forward primer F (5′-CGGAGCCAAATGTTCTTTTC-3′) and reverse primer R (5′-CACCAACTGCTCATCTCTGG-3′). These primers were designed to amplify a 594 bp fragment targeting exon 6 of the F9 gene.

2.5 DNA Amplification and Sanger Sequencing of *F9*-Exon 6 Gene

The PCR reaction mixture was prepared in a total volume of 25 μL, consisting of 12.5 μL of Meridian PCR Master Mix, 1 μL each of forward and reverse primers, 7.5 μL of nuclease-free water, and 3 μL of DNA template; amplification was performed using a thermal cycling program that began with an initial denaturation at 95°C for 5 minutes, followed by cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and

extension at 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes to ensure complete DNA synthesis.

Sequencing of the amplified F9 exon 6 fragment was carried out at the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, using the Sanger sequencing method based on the dideoxynucleotide chain-termination principle. For sequencing preparation, a 20 µL mixture containing the primers and PCR product was prepared. The amplified fragment was subsequently visualized using gel electrophoresis and gel documentation to confirm the presence of the target gene before sequencing.

2.7 Visualization of the 3D Structure of Mutant Coagulation Factor IX Protein

The three-dimensional (3D) structure of the mutant coagulation factor IX protein was predicted using the SWISS-MODEL web server, which is freely available at https://swissmodel.expasy.org. The modeling process generated several candidate protein structures, each corresponding to a specific template. Among these models, the structure showing the highest sequence similarity to the template was selected for further analysis and visualization using Discovery Studio software. Visualization of the predicted 3D structure was performed to provide insights into the biochemical properties and functional characteristics of the protein. This analysis aimed to evaluate the potential structural consequences of the amino acid substitution caused by the mutation. Additional structural examination and refinement were subsequently conducted using UCSF Chimera to further assess the possible impact of the mutation on protein conformation and interactions.

2.8 Data Analysis

Data analysis in this study was performed computationally using several bioinformatics tools. The long-read sequencing data were analyzed using IGV (Integrative Genomics Viewer) and EPI2ME, while the Sanger sequencing results were examined using GeneStudio. Additional analyses were carried out through online servers and services integrated with the GenBank database, including the Basic Local Alignment Search Tool (BLAST) and ExPASy. Furthermore, the three-dimensional structure of the mutant coagulation factor IX protein was evaluated using UCSF Chimera to assess potential structural changes associated with the identified mutation.

3. Results and Discussion

3.1 The *F9* Gene Mutation Variants by Long-Read Sequencing

The F9 gene covers about 32,721 base pairs (NC_000023.11) and is organized into eight exons separated by seven introns. Each of these exons plays a specific role in building different functional parts of the coagulation factor IX protein. Exon 1 encodes a signal peptide that guides the protein out of hepatocytes, ensuring it is properly secreted. Exon 2 includes sequences for the propeptide and the gamma-carboxyglutamate (Gla) domain, which are essential for calcium binding and proper function. Exon 3 continues this by encoding the C-terminal portion of the Gla domain along with a short hydrophobic helical segment. Moving further, exons 4 and 5 produce two epidermal growth factor-like (EGF-like) domains that contribute to protein stability and interactions. Exon 6 encodes the activation peptide region, which is important for converting factor IX into its active form. Finally, exons 7 and

8 encode the catalytic serine protease domain the core functional region responsible for the protein's role in blood coagulation.

Due to its relatively large size, the F9 gene presents challenges for analysis using the Sanger sequencing method, as it requires multiple primer sets to amplify different regions of the gene. In contrast, long-read sequencing provides a more efficient approach for determining the nucleotide sequence of this gene. In this study, sequencing results were integrated with the GenBank reference database (ChrX: 154835792–155022723) using the GRCh38 genome assembly. Comprehensive sequencing was also performed on several gene panels located on the X chromosome, including the F9 gene. The mutations identified in the F9 gene through long-read sequencing are summarized in Table 1.

Table 1. Detailed variants obtained from long-read sequencing

Gene Target	Location	Type	cDNA	Variant
F9	Exon 6	Missense	c.676C>T	Pathogenic

The c.676C>T variant has been reported to be associated with hemophilia B, an X-linked recessive disorder that disrupts the normal blood coagulation process [9]. This genetic alteration results in a bleeding disorder characterized by increased susceptibility to bleeding and prolonged bleeding episodes. The clinical severity of the condition depends largely on the extent to which the mutation compromises the function of coagulation factor IX. When factor IX is deficient or functionally impaired, it cannot effectively interact with factor VIIIa to convert factor X into factor Xa within the coagulation cascade [10]. As a consequence, the subsequent conversion of prothrombin to thrombin is disrupted, which ultimately delays fibrin formation, an essential step required for effective blood clot formation.

3.2 The *F9*-exon 6 Gene Mutation (c.676C>T)

The mutations detected through long-read sequencing were subsequently validated using the Sanger sequencing method. The analysis confirmed the presence of a missense mutation located in exon 6 of the F9 gene, specifically the c.676C>T variant (Figure 1). According to the ClinVar pathogenic variant database, this substitution (c.676C>T; p.Arg226Trp) is classified as a pathogenic variant associated with hemophilia B [11].

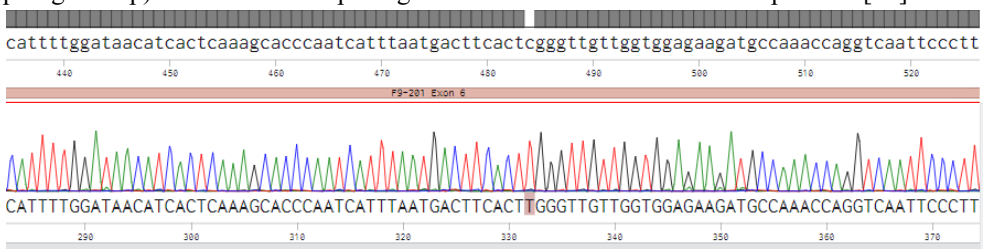

Figure 1. Location of F9 gene mutation

The F9 gene encodes coagulation factor IX, which circulates in the bloodstream as an inactive zymogen until its activation peptide is released through proteolytic cleavage, allowing the molecule to adopt an active serine protease conformation [12]. Exon 6 specifically encodes the activation peptide, indicating that mutations occurring within this region may compromise the function of the serine protease domain, one of the catalytic

components of factor IX [8]. Disruption of this domain can significantly impair the blood coagulation process.

The missense variant c.676C>T results in a nucleotide substitution at position 676, causing the replacement of arginine with tryptophan at amino acid position 226 (p.Arg226Trp) [13]. Residue Arg226 is positioned close to the cleavage site of the activation peptide and is known to be highly conserved, indicating its functional importance. Alterations within this region may destabilize the structure and function of coagulation factor IX, which can ultimately manifest clinically as hemophilia B.

3.3 Structure 3D Protein Target

The coagulation factor IX protein is composed of several distinct structural regions. It starts with a signal peptide at amino acids 1–28, followed by a propeptide region spanning residues 29–46. The mature protein, which contains 415 amino acids, is further organized into multiple functional domains, including the gamma-carboxyglutamate (Gla) domain (47–92), two epidermal growth factor-like (EGF-like) domains (93–171), a connecting segment (172–191), an activation peptide (192–226), and the serine protease domain (227–461) [7]. Analysis of the protein sequence identified a mutation at position 226, where arginine is substituted by tryptophan (Figure 2).

1	mqrvnmimae	spgliticll	gyllsaectv	fldhenanki	lnrpkrynsg	kleefvqgnl
61	erecmeekcs	feearevfen	terttefwkq	yvdgdqcesn	pclnggsckd	dinsyecwcp
121	fgfegkncel	dvtcniknqr	ceqfcknsad	nkvvcscteg	yrlaenqksc	epavpfpcgr
181	vsvsqtsklt	raetvfpdvd	yvnsteaeti	ldnitqstqs	fndftrvvgg	edakpgqfpw
241	qvvlngkvda	fcggsivnek	wivtaahcve	tgvkitvvag	ehnieeteht	eqkrnvirii
301	phhnynaain	kynhdialle	ldeplvlnsy	vtpiciadke	ytniflkfgs	gyvsgwgrvf
361	hkgrsalvlq	ylrvplvdra	tclrstkfti	ynnmfcagfh	eggrdscqgd	sggphvteve
421	gtsfltgiis	wgeecamkgk	ygiytkvsry	vnwikektkl	t	

Figure 2. The protein sequence of Coagulation Factor IX with reference number NP_000124.1

The coagulation factor IX protein is composed of 461 amino acids and has 3628 atoms. The template used to construct the 3D structure of coagulation factor IX protein is Q95ND7.1.A. The results of the 3D protein target structure modelling can be observed in Figure 3.

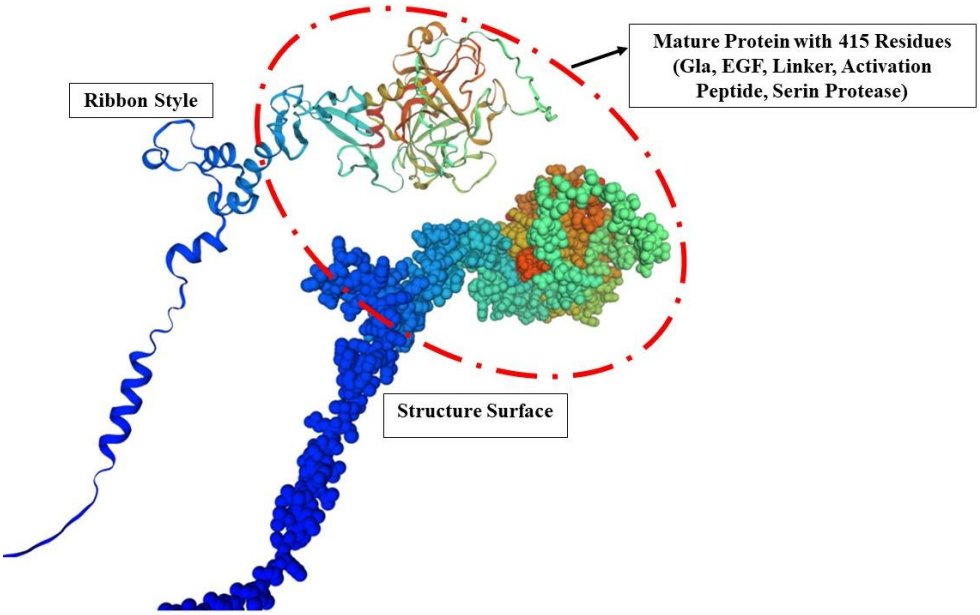

Figure 3. 3D Structure of Mutant Coagulation Factor IX Protein

The 3D structural modeling of the target protein revealed a sequence identity of 99.57%, which is slightly lower than that of the predicted wild-type structure (99.78%). Sequence identity between the model and its template is an important parameter for evaluating how closely the predicted structure represents the actual protein conformation. Generally, a model is considered reliable when the sequence identity exceeds 30%, whereas identities below this threshold may result in models with substantially reduced structural accuracy [14].

The Global Model Quality Estimate (GMQE), which ranges from 0 to 1, reflects the expected reliability of the predicted structure by assessing the compatibility between the target sequence and the template [15]. In this study, the GMQE value for the modeled protein was 0.80, suggesting a high level of structural reliability. The Ramachandran plot analysis showed a value of 87.58%, which is slightly lower than that observed for the wild-type protein structure. Model quality was further assessed using the MolProbity Score, a widely used metric for validating structural accuracy. Typically, a MolProbity score below 2.0 indicates a high-quality structural model, with lower scores reflecting better structural quality. The predicted protein model yielded a MolProbity score of 1.52, indicating good structural reliability. Overall, the comprehensive evaluation of the target protein model is summarized in Table 2.

Table 2. Evaluation of protein target model

Comparisons	**Wild-type**	**Mutant**
GMQE	0.80	0.80
Sequence Identity	99.78 %	99.57%
Ramachandran Favoured	87.80 %	87.58%
MolProbity Score	1.49	1.52

3.4 Pathogenic Variant (p.Arg226Trp)

The c.676C>T variant (p.Arg226Trp) results in a non-conservative amino acid substitution. This mutation has previously been reported in several individuals with factor IX coagulation deficiency [11,13]. The p.Arg226Trp variant is associated with the clinical manifestations of hemophilia B and has been shown to reduce factor IX activity to less than 10% of normal levels [13]. Other substitutions occurring at the same residue, such as p.Arg226Glu and p.Arg226Gly, have also been identified in patients with factor IX deficiency [11,13]. Overall, the F9 gene variant c.676C>T is classified as pathogenic and is strongly linked to the clinical phenotype observed in hemophilia B patients [9,16–18].

Visualization of the predicted three-dimensional protein structure indicates that the mutation influences the coil conformation at residue 226 (Figure 4). The p.Arg226Trp substitution may alter atomic interactions within the protein, potentially leading to functional loss, protein aggregation, reduced structural stability, and impaired enzymatic activity of coagulation factor IX. Notably, this variant is located within a critical region of the activation peptide, where mutations may interfere with the proper activation of the protein. Consequently, such alterations can reduce or abolish the functional activity of coagulation factor IX, contributing to the development of hemophilia B.

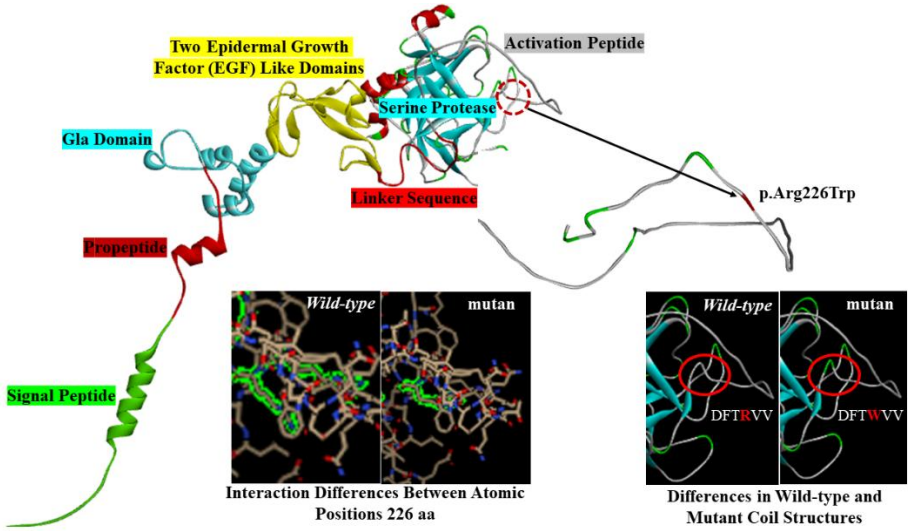

Figure 4. 3D Visualization of the Pathogenic Variant p.Arg226Trp in Coagulation Factor IX

Arginine is a basic and polar amino acid, whereas tryptophan is generally neutral and relatively less polar. Substitution of arginine with tryptophan can therefore introduce substantial alterations in atomic interactions within the protein structure. This amino acid change may disrupt electrostatic interactions that are important for maintaining protein stability. In addition, tryptophan contains a larger and more hydrophobic indole side chain compared with the guanidinium group of arginine, which increases its tendency to participate in hydrophobic interactions rather than polar interactions.

Residue Arg226 is highly conserved and functions as part of the cleavage site within the activation peptide [11,18]. Consequently, substitution of arginine with tryptophan at position 226 may interfere with the proper activation of the serine protease domain, which is essential for the catalytic activity of coagulation factor IX. Taken together, these structural and biochemical changes suggest that the p.Arg226Trp variant is highly likely to be associated with factor IX deficiency and may contribute to the clinical phenotype of severe hemophilia B.

From a clinical perspective, the identification of the F9 c.676C>T (p.Arg226Trp) variant has important implications for genotype–phenotype correlation and diagnostic evaluation. Detection of this variant may improve prognostic assessment, support the development of personalized treatment strategies, such as earlier initiation of prophylactic therapy and optimized peri-procedural management, and assist in counseling regarding the potential risk of inhibitor development compared with other mutation types.

In the diagnostic context, recognizing Arg226 as a mutational hotspot may facilitate prioritized targeted analysis of the F9 gene in patients presenting with reduced factor IX activity. This approach can also enable cascade carrier screening and prenatal testing within affected families, while improving variant classification when integrated with segregation analysis and functional evidence. Furthermore, comprehensive investigation of the F9 c.676C>T (p.Arg226Trp) variant, incorporating standardized factor IX activity assays, structural modeling, and family segregation studies, may refine genotype–phenotype relationships in hemophilia B and provide stronger support for its pathogenic classification.

4. Conclusions

A pathogenic missense mutation (c.676C>T) was identified in exon 6 of the F9 gene in a patient diagnosed with severe hemophilia B. This nucleotide substitution results in an arginine-to-tryptophan amino acid change at position 226 (p.Arg226Trp), which may influence the structural conformation and functional properties of coagulation factor IX. Comparative analysis between the mutant and wild-type 3D structures of factor IX indicates a conformational alteration in the coil region at residue 226, potentially leading to modifications in atomic interactions within the protein structure.

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