

Mutation Screening of the Factor VIII Gene in Hemophilia A in Saudi Arabia: Two Novel Mutations and Genotype-Phenotype Correlation

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Abstract

Background: Hemophilia A is an X-linked bleeding disorder caused by mutations in the factor VIII gene (F8C). Molecular testing for the factor VIII gene is difficult due to its large size. More than 1000 different mutations have been described in factor VIII gene. In this study we have investigated the factor VIII gene mutations in Saudi Arabian population.

Methods: For genotyping factor VIII cohorts of 110 samples from Saudi Arabian patients undergoing treatment for hemophilia A were collected. All patients were tested for factor VIII coagulant activity on Behring Coagulation System. Genomic DNA was isolated from blood on MagNapure system. Screening for inv-1 was done by multiplex PCR method, and inv-22 was done by ligation (inverse) PCR method. DNA sequencing was performed by Sanger method for all 26 exons of factor VIII gene. PCR products were sequenced on ABI 3500 Genetic analyzer. For molecular simulations we have used softwares such as CHARMM and GROMACS v4.0.527. In order to predict the possible impact of a variation on the function of factor VIII gene the online tools Polyphen 2, and SIFT were used.

Results: Out of 110 cases screened, 2 patients were positive (affected) for inv-1 and 15 patients were positive (12 affected and 3 carriers) for inv-22. Out of 32 cases sequenced for coding exons, 2 novel mutations were found, one novel missense mutation c.355G>C, p. (A119P) in exon 3, and another novel frame shift mutation c.6482delC, p.(P2161Lfs*25) in exon 23. Also known mutations such as, c.409 A>C, p. (T137P) in 2 individual patients in exon 4, another known mutation c.1804C>T, p.(R602*) in 1 patient in exon 12 were found. Genotype-phenotype correlations and computer prediction analysis on these novel mutations and the secondary structure analysis of the factor VIII protein were performed, and compared with the predicted native proteins.

Conclusions: These novel mutations in factor VIII gene and molecular dynamic simulation results to appropriately predict the deleterious effects of these mutations are presented in this study. In addition, for the native and mutant proteins models, the amino acid residues and its secondary structures were determined. Our *In-silico* study suggests that these mutations have significant impact on the structure and function of the factor VIII protein.

Keywords: Hemophilia A; F8 Protein; Saudi Arabia; Blood coagulation; Computer simulation

Introduction

Hemophilia is classified into three types, according to the deficiencies in clotting factors. Hemophilia A (OMIM 306700) occurs when clotting factor VIII is either absent or not present in sufficient amounts [1,2]. Since this is the most common type, it is frequently referred to as classic hemophilia. Hemophilia A is hereditary X-linked recessive, where heterozygous females transmit the affected gene and the disease phenotype presents almost exclusively in males [3,4]. However, several women, those carrying the disease, might have mild symptoms of this disease [5]. Hemophilia B (also called Christmas disease) occurs when clotting factor IX is either absent or not present in sufficient amounts [6-8]. Hemophilia A affects 1 in 5000 males, and hemophilia B affects 1 in 30,000 males [9,10]. Hemophilia C (also known as Rosenthal syndrome) occurs when clotting factor XI is either absent or not present in sufficient amounts, it is extremely rare, it is reported to occur in Ashkenazi Jewish population, and it affects 1 in 100, 000 adults in USA [11,12]. Hemophilia A and hemophilia B are the most common, over 80 percent of patients have hemophilia A (classical hemophilia), and hemophilia B affects about 15 per cent of hemophiliacs. However, in the Arab world the most common type of hemophilia is yet to be determined [13]. Other coagulation factor

deficiencies are less common, with patients suffering either milder bleeding or thrombotic episodes.

The human factor VIII gene was cloned between 1982 and 1984 [14]. Factor VIII is synthesized in the liver, and it circulates in the plasma in a stable complex with von Willebrand factor (vWF), that inhibits the proteolytic degradation of factor VIII. Factor VIII activation occurs on the surface of platelets, and is initiated by thrombin or factor Xa. Activated factor VIII (factor VIIIa) is a cofactor for factor

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IXa activation and enhances the reaction approximately 10,000 fold. Genetic mutations in factor VIII cause deregulation of this protein leading to bleeding disorders [15-19]. Defects in factor VIII gene includes deletions, large DNA inversions, nonsense mutations, ins/del-frame shifts, splice variants and a large number of missense point mutations, all of which can cause defects in the expression, secretion, and/or half-life of factor VIII protein in circulation [20-25]. According to western reports around 1-5% of hemophilia A cases known to have intron-1 inversion in factor VIII gene [19,24], and the intron-22 inversion is the most common mutation in hemophilia A responsible for about 40 - 50% of severe cases [26].

One third of hemophilia A cases are sporadic with no prior family history of the disease [27,28]. Several studies are available in literature describing the mutations in factor VIII gene, from many ethnic groups and different populations [16,17,25,29,30]. Recently, some reports were published from Middle Eastern countries also describing factor VIII gene mutations [31-34]. However, the spectrum and nature of common mutations causing hemophilia A in Arab population specifically in Saudi Arabs is not clear. Therefore, we plan to study the mutations in factor VIII gene from Saudi Arabian hemophilia A patients that could help in screening and treating this genetic disorder. These tests will offer a fast and reliable mutation screening method that can also be applied in the future for prenatal genetic diagnosis to families at risk of having a child with hemophilia.

Materials and Methods

Samples collection and storage

For factor VIII investigation a cohort of 110 samples undergoing treatment at King Faisal Specialist Hospital and Research Centre, Riyadh (KFSH&RC) were collected. Out 110 cases, 75% were severe hemophilia A, 17% cases were mild, and 8% cases were moderate hemophilia A. Severity was determined as described by world federation of hemophilia (<http://www.wfh.org>). Representative hemophilia A patient's clinical characteristics and symptoms are shown in Table-3. All patients were tested for factor VIII coagulant activities that were carried out on Behring Coagulation System (BCS; Siemens, Marburg GmbH, Germany) using chromogenic assay. Ethical approvals were obtained from the participated institutions. Detailed medical history of individuals and families was taken to confirm the pattern of inheritance. British Committee for Standards in Haematology Guidelines (<http://www.bcsgh.org>) were followed for the patient's selection criteria. Genomic DNA was isolated from EDTA whole blood with the MagNA Pure compact nucleic acid isolation Kit-I.

Factor VIII intron 1 and 22 inversion assays

Intron-1 inversion (inv-1) was done by the method of Bagnall et al. PCR amplification was done using Fermentas kit. The primers used in PCR - A were 9FW, 9 CRV, and *int-1h* 2FW; and in PCR -B were, 9FW, *int-1h* 2FW and *int-1h-2* RV shown in Table 1. Factor VIII intron-22 inversion (inv-22) was done by inverse PCR method [35,36], replacing the long PCR assays, as it is more robust than the long-PCR [35] Inverse-PCR (I-PCR) involves 3 steps:

- (1). *BclI* restriction digestion.
- (2). Self-ligation of restriction fragments, two relevant B-rings (self-ligated *BclI* fragments) are formed after *BclI* digestion and ligation, in normal case a 21.5-kb ring that corresponds to the non-rearranged copy of *int-22h-1* (Normal B-ring); and inv-22 containing B-ring, a 20-kb ring that spans a chimeric copy of *int-*

22h composed by a part of *int-22h-1* (intragenic) and part of either *int-22h-2* (proximal) or *int-22h-3* (distal). Each ring is specifically recognized by a specific PCR amplification. Normal B-rings yield product of 487 bp with primers IU and ID; Inv-22 positive B-ring yield products of 559 bp with primers IU and ED.

- (3). Standard multiplex PCR analysis. PCR is achieved by using 3 primers as shown in Table 2. A minimum of 500 ng to 1.0 µg of DNA was used in this assay [36,37].

DNA sequencing by capillary method

Polymerase chain reaction (PCR) products were sequenced for all 26 exons of factor VIII by Sanger method and analyzed on ABI 3500 Genetic analyzer as described by [17,33]. Descriptions of the primers used for amplification and sequencing were reported previously [38,39]. PCR was performed with 100 ng of genomic DNA using a HotStarTaq Plus DNA polymerase (Qiagen) in 20 µls volume. PCR cycling conditions were 40 cycles with denaturation at 95°C for 30 sec; annealing at 60°C for 30 sec; and extension at 68°C for 1 minute. The PCR products were purified by magnetic beads method using Agencourt AMPure XP kit and used in Big Dye Terminator v3.1 cycle sequencing reaction. The Big Dye reaction products were purified with Big Dye X-terminator purification kit, followed by capillary electrophoresis, and the sequences were verified on Applied Biosystem's Sequence Analysis software v5.4 for quality, and the final mutation analysis was performed on CLC genomics workbench. Mutations were designated according to the Human Genome Variation Society guidelines [40]. In order to predict the possible impact of a variation on the function of factor VIII gene the online tools Polyphen 2 (<http://genetics.bwh.harvard.edu/pph2/>); and SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html) were used.

Molecular dynamics (MD) simulation

For molecular simulations and genotype phenotype correlations we have used the softwares such as CHARMM (Chemistry at Harvard Macromolecular Mechanics; <http://www.charmm-gui.org>) and GROMACS v4.0.527. The molecular dynamics (MD) simulations were

PCR-A	Sequence
9fw (20 mer)	5' – GTTGTGGGAATGGTTACGG – 3'
9crv (20 mer)	5' – CTAGCTTGAGCTCCCTGTGG – 3'
<i>int-1h</i> 2fw (21 mer)	5' – GGCAGGGATCTTGTGGTAAA – 3'
PCR-B	
9fw (20 mer)	5' – GTTGTGGGAATGGTTACGG – 3'
<i>int-1h</i> 2fw (21 mer)	5' – GGCAGGGATCTTGTGGTAAA – 3'
<i>int-1h</i> 2rv (24 mer)	5' – TGGGTGATATAAGCTGCTGAGCTA – 3'

Table 1: Primers used for inv-1 testing by PCR method [24].

Primer	Sequence	NC_000023.9	<i>BclI</i> site*
IU (20 mer)	5' – CCT TTC AAC TCC ATC TCC AT – 3'	153779730-50	460
ID (21 mer)	5' – ACA TAC GGT TTA GTC ACA AGT – 3'	153758587-608	27
ED (19 mer)	5' – TCC AGT CAC TTA GGC TCA G – 3'	154257328-47	99
		154349067-86	

*DNA distance (bp) from the 5' end of the primer to the *BclI* restriction site. This DNA distance indicates the contribution of this primer usage to the inverse PCR product.

Table 2: Primers used for inv-22 testing by inverse PCR method [21].

performed with a 5 fs time step using set temperature of 300 K and constant pressure of 1 atm, below periodic solvent boundary conditions. The knowledge based minimization (KoBaMIN) program [41], allowed refining the factor VIII domain A1 structure to evaluate changes as a result of refined protocol. The conjugate gradient technique for 3D structure optimization along with the deviation between the structures was assessed by their RMSD values. This server utilizes GROMACS for force field energy minimization conjugate gradient and Limited reminiscence Broyden Fletcher Goldfarb Shanno (L-BFGS) methods [42]. The free energy simulations were performed with solvent water molecules in close proximity to the solute to get an efficient solvent boundary potential (SSBP). The configuration of ions was first verified by brief Monte Carlo (MC) simulation based on Van Der Waals (VdW) interactions. The Particle Mesh Ewald [43] methodology was utilized for electrostatics observations and a 12 Å cutoff was applied for VdW interactions. The mutations were further studied utilizing (Collaborative Computational Project, number 4) CCP4; QtMG and energy minimization for 3D structures were carried out using NOMAD-Ref server. Molecular Operation Environment (MOE) was used to assess the associated hemophilia-A disease variants in the factor VIII protein. Furthermore, SWISS-PROT and CCP4 (QTMG) analyzed the structure conformational changes of factor VIII protein domains.

The protein stability was determined by Schrodinger (BioLuminate) increased insights. Structure analysis was performed using Have Your Protein Defined (HOPE) [44], developed by the Centre for Molecular and Bio-molecular Informatics (CMBI), department of Bioinformatics, Radboud University. This program was used to observe the impact of mutation variant on the factor VIII protein domain A1. The analysis was based on the parameters of functional contacts like metal, DNA, hydrogen bonds, ionic interactions, and structural areas together with motifs, domains and transmembrane domains. The last three parameters are very important to acquire factual data of actual protein structure resulting in annotated information in UniProtKB DAS servers [45]. Initially, we checked whether the mutation was located in the factor VIII protein based on assigned and corresponding function, non-structural features like post-translational modifications based on a protein sequence in FASTA format. Additionally, we have also compared the predictions of the functional effects that were determined by the SNAP [46,47].

Results

Analysis of samples for Inversion 1 in Factor VIII gene

Representative hemophilia A patient's clinical characteristics and symptoms are shown in Table 3. All of the 110 samples were analyzed for the inv -1 in factor VIII gene, two male patients were positive (affected) for inv-1 and all other patients were wild type (Table 4). Representative PCR gel pictures are shown in Figures 1A and 1B. In Figure 1, PCR bands for wild type alleles (1.9 kb in PCR-A; and 1.2 kb in PCR-B) in panel A; and affected with hemophilia (1.3 kb in PCR-A; and 1.8 kb in PCR-B) in panel B are indicated by arrows. One 25 years old male patient with chronic joint disability in left knee (code no. Inv1-1) had mild hemophilia A with factor VIII base line levels of 5-40%. In Figure 1 panel B the PCR products of a patient affected with hemophilia A were shown, this patient is a 18 years old male patient with chronic joint disability in right knee (code no. Inv-1-2) and he had severe form of hemophilia with factor VIII base line levels of <1% (Table 3). Factor VIII inhibitor was absent in both of these patients.

Analysis of samples for Inversion 22 in Factor VIII gene

A total of 108 patients (after removing the two inv-1 positives) with

hemophilia A, were analyzed for the inv-22 in factor VIII gene. 15 cases out of 75 severe cases (20%) were positive for inv-22 in the factor VIII gene and all other patients were wild type for inv-22. Out of these 15 positive cases, 3 were carriers (female), and 12 were affected (male) with hemophilia A (Table 4). All these patients have elevated pro-thrombin time (PTT) with severe form of hemophilia with factor VIII base line levels of <1% (Table 3). The inv-22 PCR gels are shown in Figure 2A and 2B for a representative of samples. The clinical characteristics' of representative patients and the summary of positive samples are shown in Tables 3 and 4.

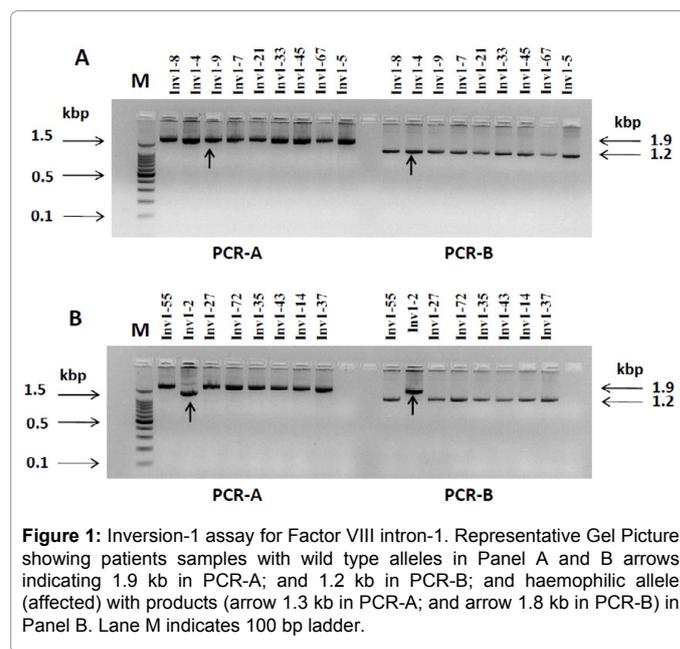


Figure 1: Inversion-1 assay for Factor VIII intron-1. Representative Gel Picture showing patients samples with wild type alleles in Panel A and B arrows indicating 1.9 kb in PCR-A; and 1.2 kb in PCR-B; and haemophilic allele (affected) with products (arrow 1.3 kb in PCR-A; and arrow 1.8 kb in PCR-B) in Panel B. Lane M indicates 100 bp ladder.

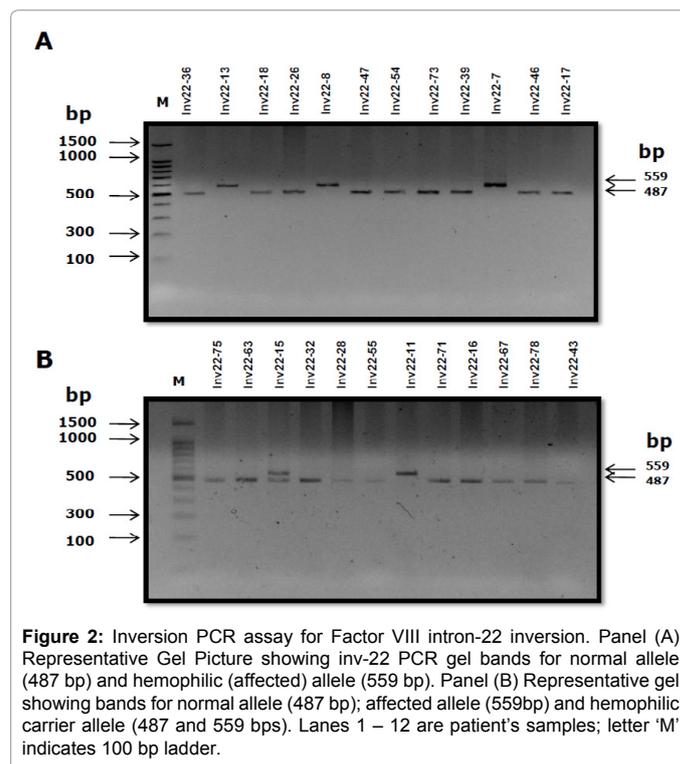


Figure 2: Inversion PCR assay for Factor VIII intron-22 inversion. Panel (A) Representative Gel Picture showing inv-22 PCR gel bands for normal allele (487 bp) and hemophilic (affected) allele (559 bp). Panel (B) Representative gel showing bands for normal allele (487 bp); affected allele (559bp) and hemophilic carrier allele (487 and 559 bps). Lanes 1 – 12 are patient's samples; letter 'M' indicates 100 bp ladder.

Code No.	Gender	Age (Yrs)	Severity	Type of Care	Treatment Products Used	Presence of Joint Disability	PTT
Inv-22-1	Male	18	Severe (<1%)	Episodic	Plasma Derived Clotting Factor	Right Elbow	112
Inv-1-1	Male	25	Mild (5 to 40%)	Prophylaxis	Plasma Derived Clotting Factor	Left Knee	NA
UQU-HA9	Male	6	Moderate (1 to <5%)	Episodic	Plasma Derived Clotting Factor	No	NA
Inv-22-2	Male	25	Severe (<1%)	Episodic	Fresh Frozen Plasma	Right Elbow	85.9
Inv-22-3	Male	32	Severe (<1%)	Prophylaxis	Fresh Frozen Plasma	Right Knee Left Ankle	58.7
Inv-22-4	Male	26	Severe (<1%)	Episodic	Fresh Frozen Plasma	Right Knee	68.3
Inv-1-2	Male	19	Severe (<1%)	Episodic	Plasma Derived Clotting Factor	Right Knee	101
Inv-22-5	Male	17	Severe (<1%)	Prophylaxis	Fresh Frozen Plasma	Right Ankle and Elbow	85.6
UQU-HA6	Male	51	Severe (<1%)	Episodic	Plasma Derived Clotting Factor	Right Knee, Left Elbow, Left Ankle	125.4
Inv-22-6	Male	28	Severe (<1%)	Prophylaxis	Plasma Derived Clotting Factor	Right Elbow	141.6
Inv-22-7	Male	32	Severe (<1%)	Prophylaxis	Plasma Derived Clotting Factor	Unknown	84
Inv-22-8	Male	15	Severe (<1%)	Episodic	Plasma Derived Clotting Factor	Unknown	127.8
Inv-22-9	Male	9	Severe (<1%)	Episodic	Plasma Derived Clotting Factor	Unknown	46.4
UQU-HA7	Male	11	Severe (<1%)	Episodic	Fresh Frozen Plasma	No	150

Table 3: Summary table showing representative Hemophilia A patient's clinical characteristics.

Patient Code	Gender	Clinical Severity	Mutation Type/Effect		Function	Exon/Intronic	Nucleotide/AA Changed	Novel
UQU-HA4	Male	Severe (<1%)	Point	Missense	Delterious	Exon4	c.409A>C, p.(T137P)	No
UQU-HA6	Male	Severe (<1%)	Point	Missense	Delterious	Exon4	c.409A>C, p.(T137P)	No
UQU-HA7	Male	Severe (<1%)	Point	Missense	Delterious	Exon3	c.355G>C, p.(A119P)	Yes
UQU-HA14	Male	Severe (<1%)	Point	Termination	Delterious	Exon12	c.1804C>T, p.(R602*)	No
UQU-HA15	Male	Severe (<1%)	Frame Shift	Termination	Delterious	Exon23	c.6482delC, p.(P2161Lfs*25)	Yes
Inv-1-1	Male	Mild (5 to 40%)		Inv-1	Affected	IVS-1	N/A	No
Inv-1-2	Male	Severe (<1%)		Inv-1	Affected	IVS-1	N/A	No
Inv-22-1	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-2	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-3	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-4	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-5	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-6	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-7	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-8	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-9	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-10	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-11	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-12	Female	Severe (<1%)		Inv-22	Carrier	IVS-22	N/A	No
Inv-22-13	Male	Severe (<1%)		Inv-22	Affected	IVS-22	N/A	No
Inv-22-14	Female	Severe (<1%)		Inv-22	Carrier	IVS-22	N/A	No
Inv-22-15	Female	Severe (<1%)		Inv-22	Carrier	IVS-22	N/A	No

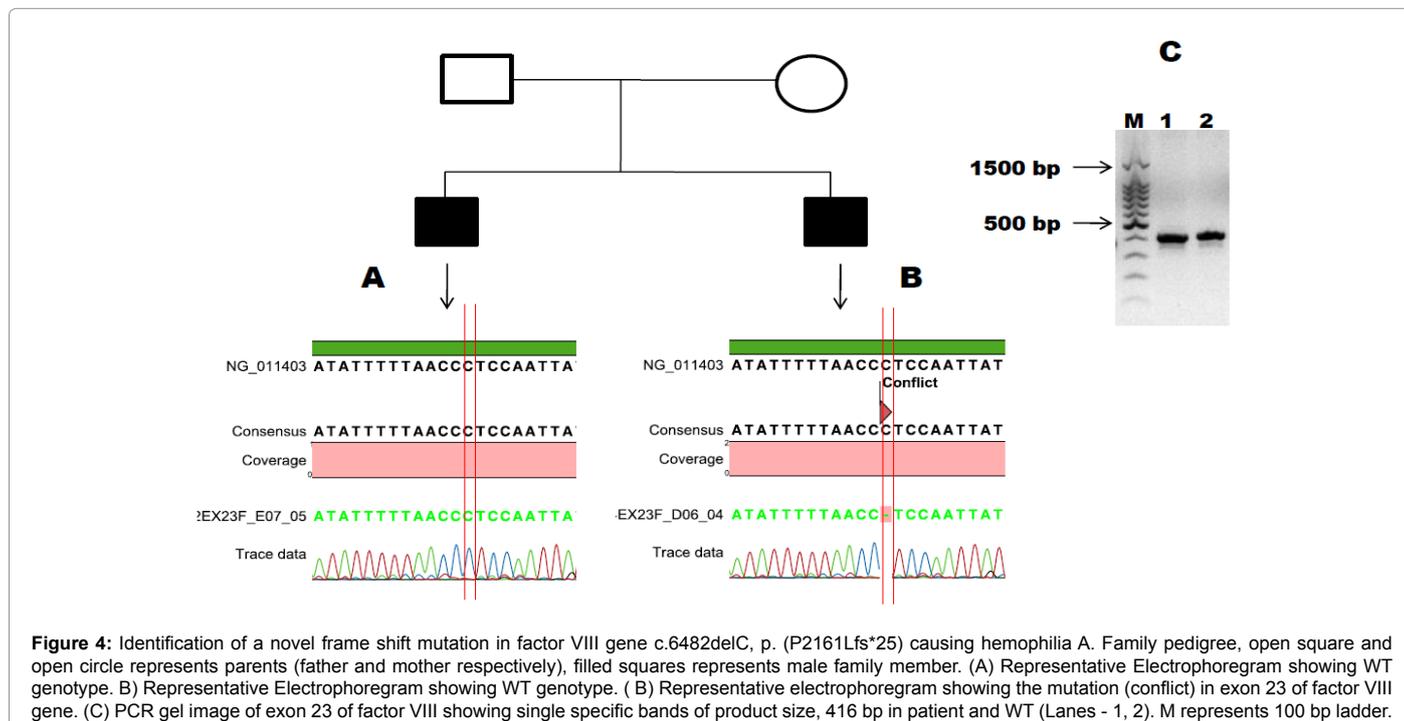
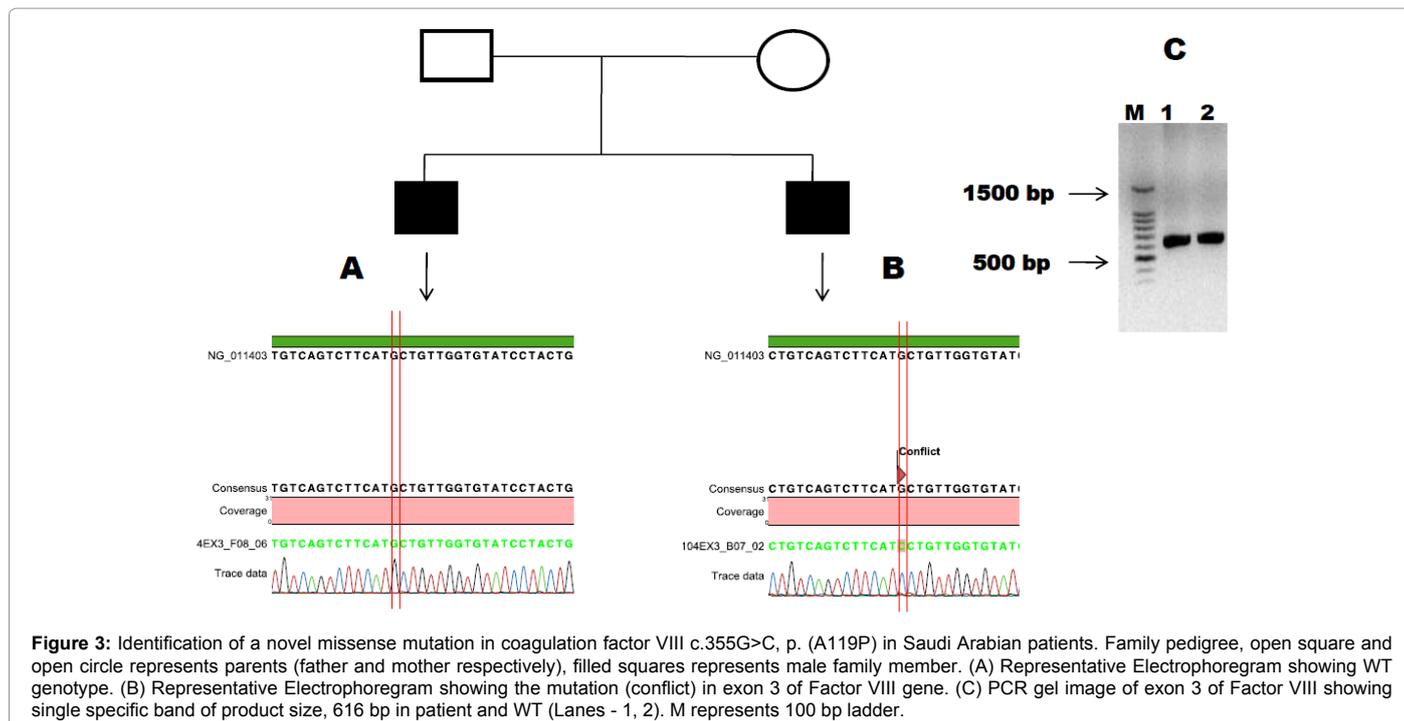
Table 4: Summary of mutations detected in Saudi Arabian Hemophilia A patients.

Analysis of mutations in Factor VIII gene by capillary sequencing

32 samples of Saudi Arabian patients were sequenced for factor VIII gene. Out of 32 DNA samples screened, two novel mutations were found, one missense mutation in exon 3, [c.355G>C, p.(A119P)] another was a frame shift mutation in exon 23 [c.6482delC, p.(P2161Lfs*25)]. Also, two known mutations were found, in exon 4, c.409 A>C, p. (T137P) in 2 individual patients, another known mutation in exon 12, c.1804C>T, p.(R602X) in 1 patient were found (Table 4). The patient with the novel mutation in exon 3, was a 11 years, male, and had no joint pains with base line factor VIII levels of <1% (severe form of hemophilia A) with episodic bleedings (Tables 3 and 4). This patient did not have any systemic manifestations. This patient was on more than one types of treatment plan with PTT 150 seconds (Table 3). This patient also tested positive for factor VIII inhibitor. This G>T mutation occurred in codon TGG of tryptophane to change in to TGT

(cysteine) and this is present in A1 domain of factor VIII. This mutation of hydrophobic neutral alanine (GCT) to CCT codes to hydrophobic neutral aminoacid proline. In Figure 3, the electrophoregram is shown in panel A, is for the wild type control, and in panel B, for the patient with mutation in exon 3. In panel C, PCR gel picture is shown for exon 3 product size of 616 bp, the actual exon 3 size is 123 bp.

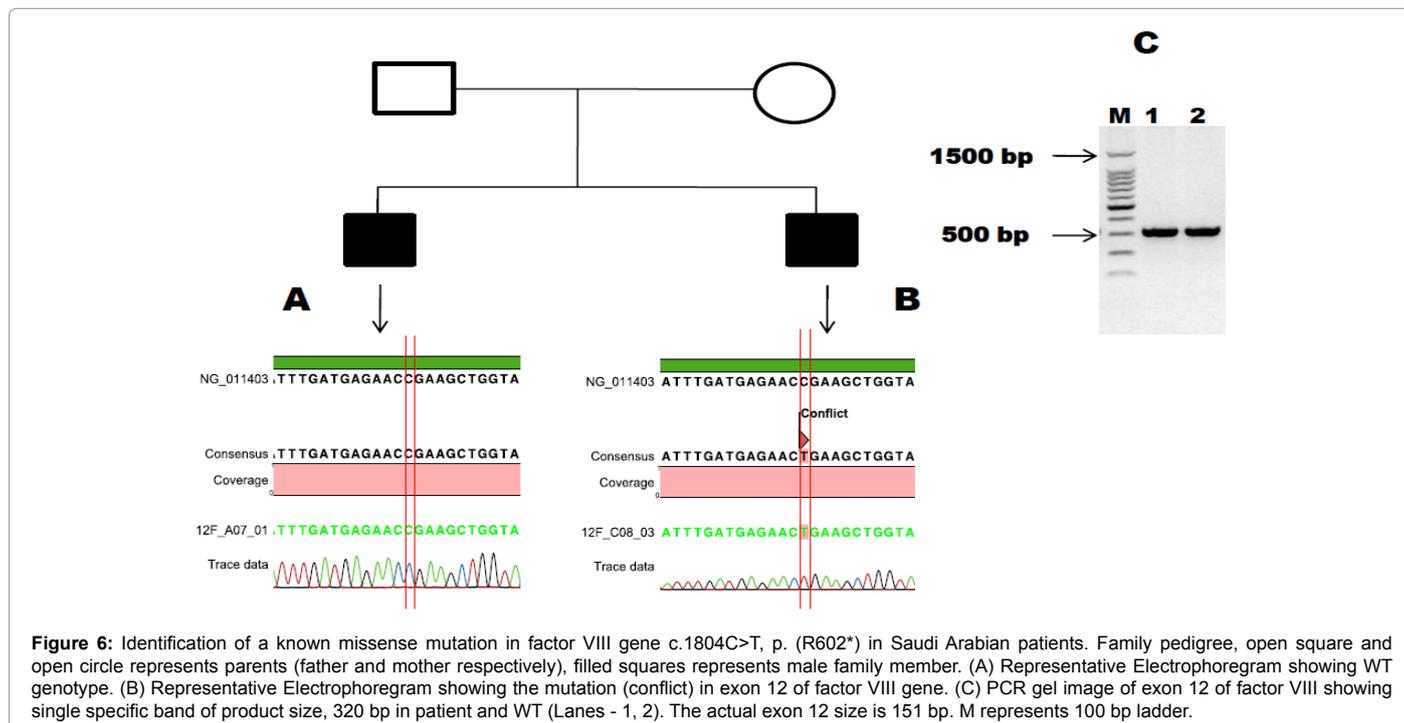
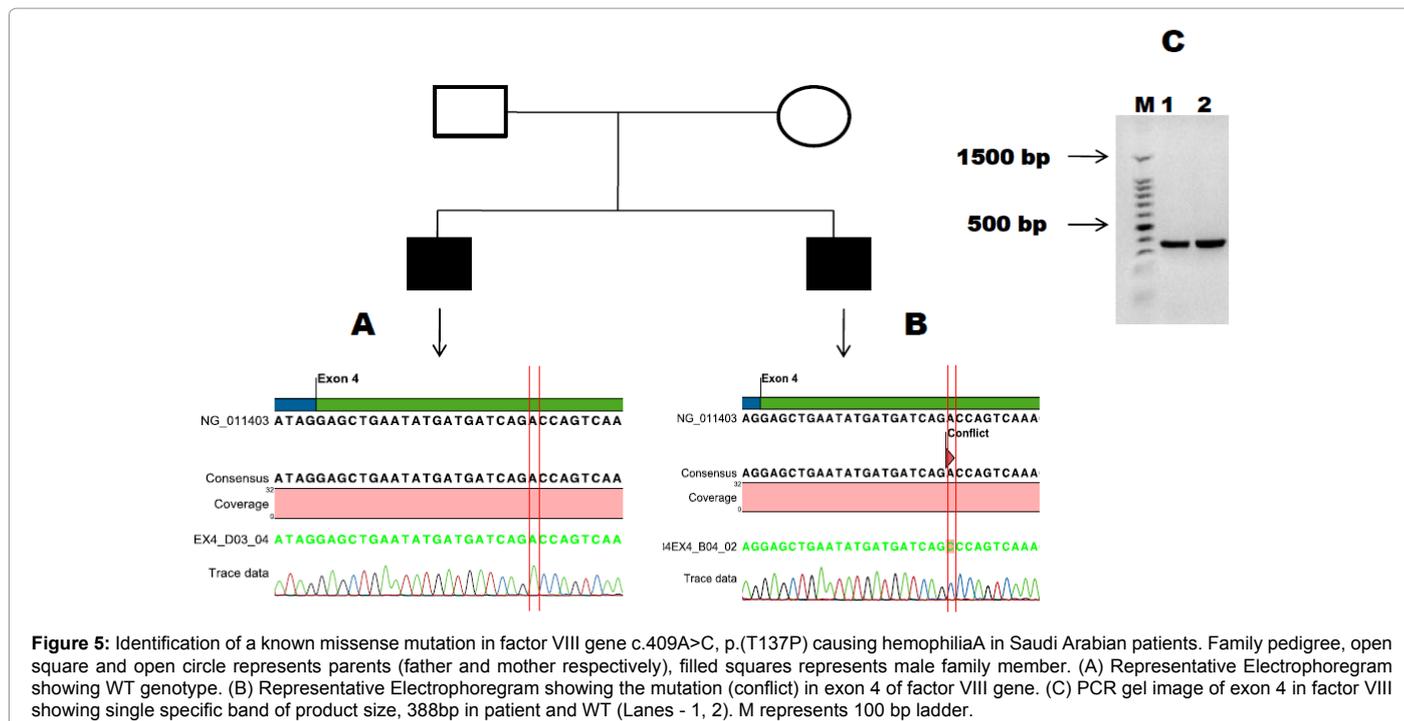
Another novel mutation was identified in one male patient (18 yrs old), this is a frameshift mutation c.6482delC, p.(P2161Lfs*25) in exon 23 causing blood coagulation factor VIII deficiency. This deletion of C in CCT codon of proline makes a frameshift to CTC (Leucine) and coding changes in further 24 (frameshift) makes a stop codon (TGA), at 25th amino acid leading to transcriptional termination and truncated protein. In Figure 4, the electrophoregram is shown in panel A, is for the wild type control, and in panel B, for the patient with mutation in exon 23. In panel C, PCR gel picture is shown for exon 23 product size of 416 bp, the actual exon 23 size is 145 bp. This mutation is associated with severe form of haemophilia. The base line factor VIII levels were



<1% in this patient, with joint disability in right knee, this patient did not have any systemic manifestations. This patient was on prophylaxis treatment with plasma derived clotting factor concentrate.

In exon 4 a known mutation in 2 patients with ages of 51 yrs and 29 years respectively was found. This mutation c.409 A>C, p.(T137P); (ACC>CCC) is associated with severe form of haemophilia (33,34). Both cases have factor VIII base line levels of <1%, and have chronic joint disabilities patient also had HIV and was a diabetic. A

representative electrophoregram for one family is shown in Figure 5. In Figure 5 panel A, is for the wild type control, and in panel B, for the patient with mutation in exon 4. In panel C, PCR gel picture is shown for exon 4, PCR product size is 388 bp, the actual size of the exon 4 is 213 bp. In Figure 6, another known mutation is shown, this is a missense mutation in exon 12, c.1804 C>T, p.(R602*). This patient was a 8 years old male Saudi with episodic bleeding, he has factor VIII levels of less than 1% and being treated with more than one product for hemophilia (Tables 3 and 4). This patient did not have joint pains, but



had systemic manifestations such as recurrent urinary tract infections. This mutation in arginine codon (CGA>TGA) makes a stop codon causing a truncated protein production. This mutation is reported to be associated with severe to moderate form of haemophilia. The electrophoregrams and the gel picture is shown below in Figure 6.

Molecular dynamic simulation studies of missense mutation in exon 3, [c.355G>C, p.(A119P)]

The annotation for the missense mutation within the gene on X

chromosome was performed. This mutation provides information for the translated regions of the factor VIII gene in exon 3. Factor VIII gene coding regions of the protein structure retrieved from the Protein Data Bank (PDB) database, we resolved the structure from UniProtKB (P00451; FA8_HUMAN; PDB ID: 2R7E). This structure was typically from five domains (A1- A2-A3; C1-C2), and two chains A-B, but our targeted sequence aligned with only with the "Chain A" within a domain A1 with regard to the structure assembly simulations (Figures 7A-7D). The factor VIII associated protein domain "Chain A" mutated

structure on was within Loop at Ala119Pro. The predicted factor VIII evaluation was determined using a Ramachandran plot with a favored region (90.0%), an allowed area (2.0%), and an outlier region (11.4%). We found greater than 95% of the residues in the most favored regions, which showed that the quality of the structure was comparable to the template. The residues were scanned, and loop mutation analysis was performed for factor VIII gene. The consensus method was used on an additional critical residue with a hot residue to increase the thermodynamic stability of factor VIII, and this technique was simple in comparison to previously described strategies (Figures 7A-7D).

The energy minimization of factor VIII before the mutation was performed at -321 951.904 kJ/mol, which decreased to -297 519.203 kJ/mol after mutation of Ala119 in factor VIII gene, these energy changes were calculated by using BioLuminat. The energy minimization was based on the set of selected polar and non-neutral residues for these screenings. We fixed the refinement cut of 0.00 Å with an implicit solvent minimization. The result of decreasing the minimized energy value of the Loop: Ala119 Pro showed that the substitution of Ala to Pro119 may contribute to improved compactness and may increase protein folding. Further loop mutation was based on the implicit solvent according to MD simulations. Compared to the results at a temperature of 300K, the deviation of the factor VIII protein increased from 2.0 Å to 10 ns. In contrast, the deviation of mutation Ala119Pro was maintained at 1.2 Å until the end of the simulation (t=10 ns), which

showed that Ala119 Pro had reached its folded state. The small peak at 1.3ns indicated that Ala119 Pro stabilized the structure. Our results showed that the Ala119 Pro structure was stable and could maintain its conformation at 300 K, at a pressure bar of 1.01225 with a surface tension 4,000.0 Å, in a total simulation time (ns) of 1.2/elapsed 0.0, and recording interval (ps) energy of 1.2. However, there were subtle changes that were observed between wild type and mutated factor VIII (Loop: Ala119 Pro), as revealed by the superimposed structures with a RMSD value of 1.516 Å, which were determined using MOE. Ala119 of the factor VIII protein showed a higher solvent accessibility than Pro119 in the protein structure. In a further analysis of annotated predicted solvent accessibility and pre-calculated packing density, Ala119 Pro was found to increase the density and lessen the internal cavities compared to the wild type form. Thus, substitution of a residue (Ala119) with Pro119 enhanced the packing and compactness of the mutant structure.

Molecular dynamic simulation studies of frame shift mutation in exon 23 [c.6482delc, p.(p2161fs*25)]

The annotation for the frame shift mutation c.6482delC, p.(P2161Lfs*25) in exon 23 within the gene on X chromosome was performed. This structure, belonged to factor VIII gene coding regions of the protein structure retrieved from the Protein Data Bank (PDB) database, we resolved the structure from UniProtKB (P00451 (FA8_HUMAN), (PDB ID: 2R7E). This structure was typically from five

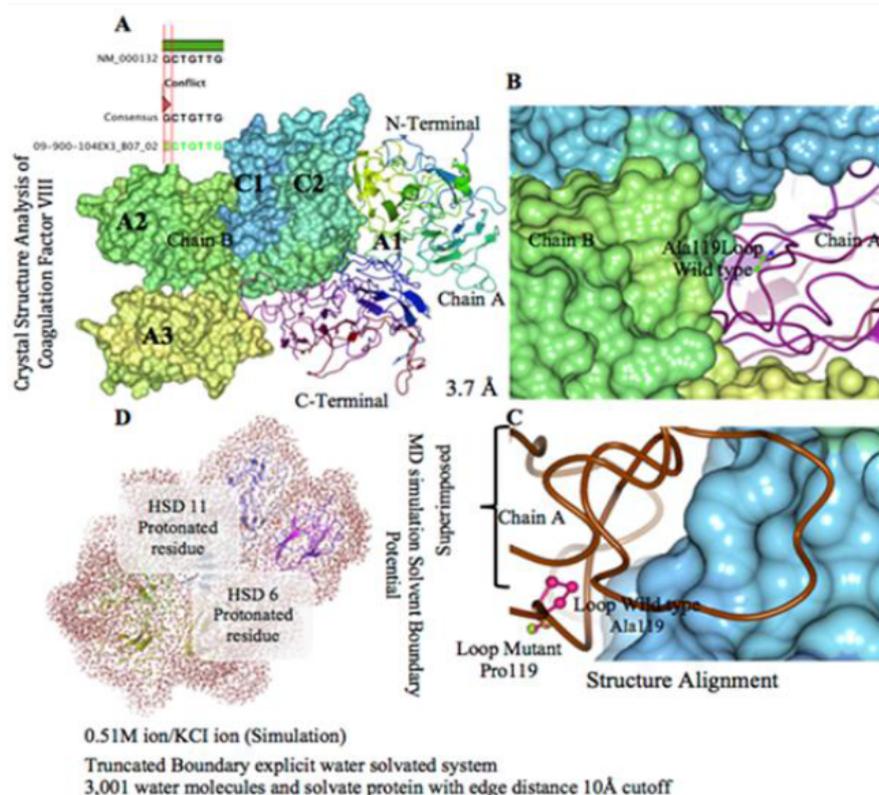


Figure 7: Crystal structure of Factor VIII domains and molecular dynamics (MD) simulation showing Ala119Pro mutation located in the interface the A1 domain. (A) Crystal structure of coagulation domains F8 (PDB ID 2R7E), the domains are individually labeled and in different colors. Indicate the positions of the mutations studied and sequence alignment with sample reads shown in the mutation G>C. B) Close-up views of the positions of the mutation with the mutated and nearby residues shown as ball and stick models as dashed circle. C) The Ala119Pro mutation located in the interface the A1 domain. A and Tertiary structure of the wild type chain A of factor VIII protein showing hemophilia disease causing mutations i.e. grey Ala119. Green Pro119, on loop region near with alpha helix10 modeling. D) The visual inspection also allow to identify the side chain of a histidine residue involved in the hydrogen bonding with surrounding molecules and in that case the δ nitrogen of the histidine (HSB) is protonated residue. The MD simulations system used in calculations are; water box surrounding the entire protein (middle).

domains (A1- A2-A3; C1-C2), but our targeted position not cover on these domains. We predict the truncated protein by homology method (Figures 8A-8C). The mutation for corresponding positions were viewed individually to examine the altered model structures achieved utilizing CCP4 (QtMG). The predicted factor VIII structure was evaluated using a Ramachandran plot with a favored region (97.0%), an allowed area (2.0%), and an outlier region (10.4%). We found greater than 96% of the residues in the most favored regions, which showed that the quality of the structure was comparable to the template [48]. The residues c.6482delC, p. (P2161Lfs*25) were scanned, and Helix mutation analysis was performed for factor VIII gene [49]. Mutation of p.(P2161Lfs*25) in factor VIII and another thermo stable site were targeted as critical residues and were substituted using Schrodinger (BioLuminate). The energy minimization of factor VIII before the mutation was performed at -521 891.904 kJ/mol, which decreased to -317 569.201 kJ/mol after mutation, as calculated by using BioLuminate. The energy minimization was based on the set of selected polar and non-neutral residues for these screenings. We fixed the refinement cut of 0.00Å with an implicit solvent minimization. The result of decreasing the minimized energy value of the Loop: p. (P2161Lfs*25) showed that the frame shift may contribute to improved compactness and may increase protein folding. Compared to the results at a temperature of 300 K, the deviation of the factor VIII protein increased from 2.0 Å to 10 ns. In contrast, the deviation of frame shift mutation c.6482delC, p. (P2161Lfs*25) was maintained at 1.2 Å until the end of the simulation (t=10 ns), which is reached its folded state.

Discussion

Hemophilia A is an inherited disorder of blood coagulation factor VIII, and haemophilic patients suffer from prolong bleeding time after trauma or injuries. The aim of this study was to investigate mutations in the factor-VIII gene in Saudi Arabian population. There are few previous reports about hemophilia A screening in the Middle Eastern population, specifically in Saudi Arabian Population by Owaidah, et al. in Lebanese population by Khayat, et al. in Tunisian population by Elmahmoudi, et al. [50] and in Jordanian population by Awidi, et al. [31]. In the present study mutational screenings of factor VIII gene sequencing analysis, and intronic rearrangements such as, inv-1 and inv-22 analysis were performed in the hemophilia A patients of Saudi Arabian population. The factor VIII mutation database includes more than 1000 unique mutations in hemophiliac patients [51]. We have sequenced 32 DNA samples of Saudi Arabian origin, and this study has identified two novel mutations that were not reported in either of these databases. Also, the mutation c.355G>C, p. (A119P) in exon 3 and another mutation c.6482delC, p.P2161Lfs*25 in exon 23 were not reported in any current literature search using the PubMed/Medline database to the best of our knowledge. These potentially deleterious mutations correlated well with the severity of phenotype. The molecular pathology of these novel mutations was studied by molecular modeling. Two previously reported mutations in exon 4 and exon 12 respectively, were also found in Saudi Arabian cases.

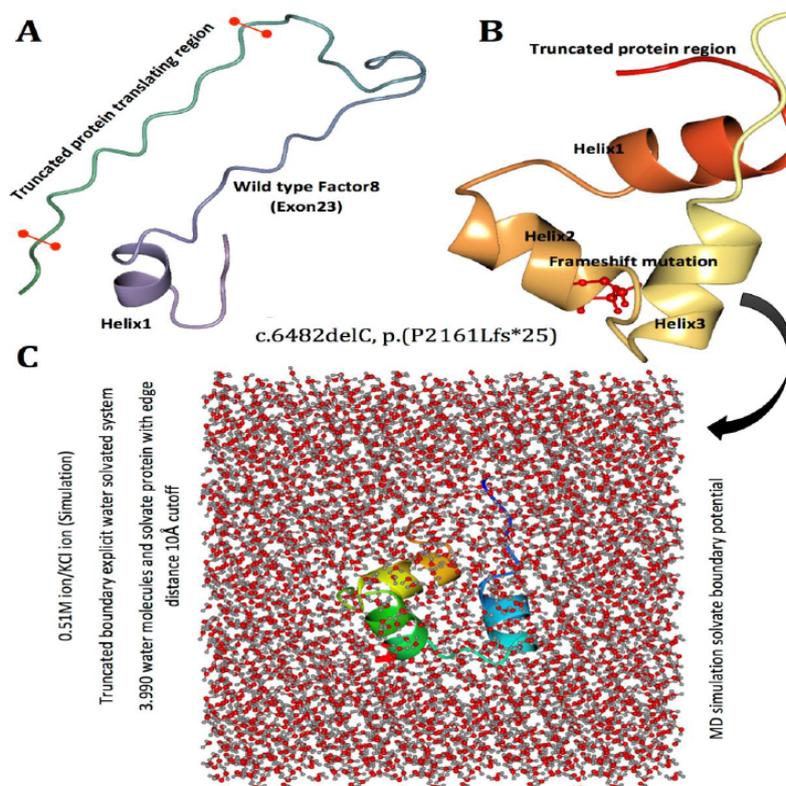


Figure 8: Molecular dynamics (MD) simulation showing the tertiary structure of truncated factor VIII protein for the factor VIII frame shift mutation p.(P2161Lfs*25) on the helix1, truncated boundary explicit water solvated and hydrogen atoms are in color. (A) Structural Model of coagulation domains of factor VIII gene. The domains are individually labeled and in different colors. (B) Damaged truncated region structure views and the mutation shown as ball and stick models as dashed circle. Mutation c.6482delC, p. (P2161Lfs*25) located in the Helix2. The Tertiary structure of truncated factor VIII protein model showing hemophilia A frame shift mutation p.(P2161Lfs*25) on the helix1. (C) The visual inspection allow to identify the side chain of a histidine residue involved in the hydrogen bonding with surrounding molecules and the δ nitrogen of the histidine (HSB) is protonated. The MD simulations system used in calculations are; water box surrounding the entire protein (middle).

The hemophilia disease severity is predicted by type of mutation found in factor VIII gene. Mutations that cause severe disruption of factor VIII protein structure or alter an important functional site will result in disease severity. The prevalence of factor VIII *inv-1* in Saudi Arabian population is not yet clearly understood. There were two previously reported studies by Abu-Amero, et al. and Owaidah, et al. on factor VIII mutation analysis; however they have not performed *inv-1* analysis in that. Our report appears to be the first report of *inv-1* detection in Saudi Arabian population. We have screened 110 patients for *inv-1* by multiplex PCR method, and found a frequency of 1.8% of *inv-1* positive in Saudi Arabian patients. Intron-1 inversion mutation is the second most common mutation in severe hemophilia A in the factor VIII gene. It was initially reported to be present in approximately 5% of patients [52] other studies reported around 2-3% of hemophilia cases known to have intron-1 inversion. However, in the UK severe hemophilia A population it was subsequently found to have a frequency of 1.8% [53]. The data obtained in this study is agreement with these published reports.

In severe hemophilia A *inv-22* is the most prevalent mutation in factor VIII gene. It is well known that the protocol such as, long-PCR for detection of *inv-22* is not a straight forward procedure [52,54]. This kind of inversions is detected by Southern blotting, which is slow and labor-intensive. The prevalence of *inv-22* in hemophilia A in different countries is quite variable with a range of 17.6% to 46.8% [55]. Keeney, et al. [56] have reported 20% positive with *inv-22* in British population. In another study Oldenburg, et al. [57] reported that factor VIII intron-22 rearrangements which accounts for 23%. Diagnostic test of IS-PCR detected *int22h*-related rearrangements in 33.3% (23.3% rearrangements and 10% of deletion 22) of hemophilia A in Egyptian patients (36). In previous studies where a 40-45% of *inv-22* positive cases were reported used the long-PCR method. In the Turkish population, this inversion mutation accounts for 42% of severe cases [58]. There is no extensive study to describe the prevalence of *inv-22* in Saudi patients. One report by Abu-Amero, et al. (34) used only six cases from Saudi Arabia in a total of 20 Arab patients for analysis on *inv-22*, and reported 45% are positive.

In the present study, we have used the inverse PCR method published by Rossetti, et al. (35). A new version of this method known as inverse-shifting PCR (IS-PCR) published by the same group [21] detects both proximal and distal inversions, and also insertions and deletions resulting from additional *int-22h* recombination events. This test involves four primers set (ID, IU, 2U, and 3U) as described in Rossetti, et al [21]. However, we have used three primers set (ID, ED, and IU) as reported in *inv-22* method of 2005. This could be the reason for this discrepancy in frequencies. It is also reported that the results of IS-PCR match perfectly with the long-PCR results [21,36]. However, it remains to be investigated thoroughly all types of *int-22h* related rearrangements which includes inversions, deletions, and duplications in Saudi Arabian patients.

Our *In-silico* study suggests that the novel mutations we found have significant impact on the structure and function of the factor VIII protein. We have also studied the molecular structure by MD simulations by immersing them in a solvent using superior simulation parameters as well as energy minimization to analyze the simulation in terms of potential energies, structural fluctuations, coordinate stability and geometrical features. We have observed that the mutation in exon 3 was located within a domain, annotated in UniProt as: "F5/8 type A 1; Plastocyanin-like 1". The mutation introduces an amino acid with

different properties, which can disturb this domain and abolish its function. The proline that is introduced by this mutation might abolish the required flexibility of the protein at this position. The mutation was located in a region with known splice variants, described as: "In isoform 2". The mutant residue was located near a highly conserved position, this residue was part of an interpro domain named "Cupredoxin" (IPR008972), and this was also buried in the core of a domain. The differences between the wild type and mutant residue might disturb the core structure of this domain.

In summary, our study has identified two novel mutation *c.355G>C*, p. (A119P) and *c.6482delC*, p.(P2161Lfs*25) in Saudi Arabian patients that were not reported previously in any factor VIII databases. Our results showed that this is a deleterious mutation in factor VIII gene and it is associated with pathological conditions and clinically significant as determined by Molecular Dynamics Simulation studies. These potentially deleterious mutations correlated well with the severity of phenotype of the patient. Pathogenic characteristics of this novel mutation was determined based on the standard criteria using PolyPhen database, and found to be deleterious in function, as this correlates with the patient's severity of hemophilia. The molecular pathology of these novel mutations were studied by Molecular Dynamics Simulation. We found a frequency of 1.8% of *inv-1* positive in Saudi Arabian patients, and this is the first investigation of *inv-1* in Saudi Arabian patients. Intron-1 and intron-22 inversions are the most common mutations in severe hemophilia A in factor VIII gene. However, there is no extensive study to describe the prevalence of these inversions in Saudi Arabian population. We have found 20% were positive in severe cases (15 out of 75) for *inv-22* in the factor VIII gene; this observation is in contrary to the published reports. We suggest that further studies are needed with large number of patients.

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Authors Contribution

FAA. Conceived the idea, designed the research and analyzed data. MMT, MA, AB, MA and ZA. Performed experiments and analyzed data. FAA, TO, MMT, and ZA. Wrote the paper. HA, FAB. and TO. Contributed to vital reagents.

Details of Nature of Conflict of Interest

All authors agreed with the contents of this manuscript and all authors declare no conflict of interest in publishing this manuscript.

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