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Molecular Genotyping and Laboratory Analysis of Hemophilia A in West Bank, Palestine

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Molecular Genotyping and Laboratory Analysis of Hemophilia A in West Bank, Palestine

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2021

Dedication

To Allah

To my parents

To my Wife

To my children

To my brothers

To all my friends

For their love and support

Shadi Khalil Mohamad Hasan

Declaration

I certify that thesis submitted for the degree of Master in Clinical Laboratory Sciences, is the result of my own research, except where otherwise acknowledged, and that this study has not been submitted for higher degree to any other university or institution.

Signed

Shadi Khalil Mohamad Hasan

Date:

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Abstract

Background: Hemophilia A (HA) is a sex-linked bleeding disorder resulting from deficiency or dysfunction of coagulation FVIII, a plasma glycoprotein that plays an important role in the blood coagulation cascade. HA occurs in one in every 5000 male births. More than 1200 mutations have been identified in the *F8* gene and associated with HA. These mutations vary from single nucleotide substitution to gross deletions/insertions and inversions. Intron 22 inversion and intron 1 inversion account for approximately 40–50% and 2- 5% of severe HA cases, respectively.

Objectives: Screening for the molecular defects has become a crucial tool in hemophilia care with respect to prediction of the clinical course and safe genetic counseling of relatives. Therefore, the aim of the present study was to genotype the *F8* gene and conduct a laboratory analysis of hemophilia A in the West Bank, in order to provide proper diagnosis and management of patients.

Methods: A total of 79 HA cases were enrolled (73 males and 6 females) from 49 unrelated families. Hematological and coagulation screening tests were conducted for each patient including: Complete blood count (CBC), partial thromboplastin time (PT) and activated partial thromboplastin time (APTT). Coagulation FVIII activity assay (FVIII: C) was performed by using one-stage clotting assay. High molecular weight genomic DNA was prepared using salting out method. Nested Long Distance Polymerase Chain Reaction (NLD-PCR) was performed for detection of *F8* gene intron 22 inversion (Inv22) for all severe HA cases as well as carrier mothers, while multiplex PCR was used for detection of *F8* gene intron 1 inversion (Inv1). DNA sequencing was used to analyze *F8* gene mutations for mild and moderate HA patients as well as for those who were negative for both inv22 and inv1.

Results: Depending on both FVIII activity levels and genotyping of *F8* gene, 54 (74%) cases were grouped as severe, 9 (12.3%) cases as moderate and 10 (13.7%) of the study cases as mild hemophilia A. Analysis of inv22 by NLD-PCR showed that 57.4% of severe HA patients have this inversion, while analysis of inv1 by multiplex PCR revealed that two patients were positive for inv1 with a percentage of 3.7% among all HA severe cases. One sample has been completely screened and the disease-causing mutation has been identified, namely

(c.388G>A) that result in substitution of the amino acid glycine at codon 130 by arginine (p.Gly130Arg) in A1 domain of FVIII protein. In another 4 samples, so far 3 harmless SNPs were identified. Only 3.7% HA patients receive prophylactic FVIII replacement therapy and the rest (86.3%) were on-demand treatment. Most patients (76.7%) have a family history of HA and 23.3% have no family history of HA.

Conclusion: The frequency of *F8* gene inv22 and inv1 were found in 57.4% and 3.7% among severe HA patients, respectively. About 23.3 % of HA patients have no family history and thus the development of HA is attributed to *de novo* mutations. Further investigation of HA cases by DNA sequencing is necessary to detect *F8* gene mutations in cases that were negative for inv22 and inv1.

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List of Abbreviations

HA	Hemophilia A
HB	Hemophilia B
rFVIIa	recombinant FVIIa
PT	Prothrombin Time
APTT	Activated Partial Thromboplastin Time
CBC	Complete Blood Count
mRNA	Messenger Ribo Nucleic Acid
DNA	Deoxyribonucleic acid
HAMSTeRS	The Hemophilia A Mutation Structure Test and Resource Site.
Inv22	Inversion 22
Inv1	Inversion 1
Int1-h1	Intron 1 homolog 1
Int1-h2	Intron 1 homolog 2
Int22-h1	Intron 22 homolog 1
Int22-h2	Intron 22 homolog 2
Int22-h3	Intron 22 homolog 3
BLAST	The Basic Local Alignment Search Tool
EDTA	Ethylene diamine tetra acetic acid
PCR	Polymerase Chain Reaction

IS-PCR	Inverse-Shift polymerase chain Reaction
NLDPCR	Nested Long Distance polymerase chain Reaction
7- deaza-dGTP	7-deaza-deoxyguanosine triphosphate
Bp	Base pair
BU	Bethesda Unit
DEPC	Diethyl pyrocarbonate
I.U	International unit
ORF	Open Reading Frame
UTR	Untranslated Region
SNP	Single nucleotide polymorphism
DDAVP	1-deamino-8-D-arginine vasopressin
WFH	The World Federation of Hemophilia
WHO	World Health Organization

Chapter one

Introduction

1.1. Hemostasis

Hemostasis is a physiological process of blood clot formation that leads to stop bleeding and maintains blood in the fluid state within the vascular compartment. The major role of hemostatic system is to maintain a full balance between the body's tendency toward clotting and bleeding as well as removing the fibrin clots (Versteeg et al 2013). Hemostasis requires a coordinated response from platelets, coagulation factors, blood vessel endothelium and fibrinolytic system (Gale, 2011; Janz & Hamilton, 2006).

The hemostasis process consists of three main components: (i) platelets adhesion, aggregation and platelets plug formation (primary hemostasis) (ii) coagulation cascade that culminates in the deposition of insoluble fibrin at the injury site (secondary hemostasis), and (iii) fibrinolysis that removes blood clots (Gale, 2011; Stassen et al., 2004).

1.1.1. Primary Hemostasis

Primary hemostasis involves adhesion of platelets, activation and plugging at the injury site. After blood vessel damage, a sub-endothelial substance or collagen fibers are exposed, the platelets glycoprotein receptors such as GPVI binds to its ligand collagen which result in platelets adhesion, another receptor called GPIb-IX-V binds to immobilized von Willebrand factor (VWF) by specific interaction between GPIbα and the A1 domain of VWF, that leads to platelets activation (Broos et al., 2011; Ruggeri, 2007).

Platelets activation induces a conformational change of integrins on platelets surface that normally present in their inactive state, especially αIIbβ3, α2β1, α6β1 and αvβ3 integrins. The αIIbβ3 receptor as it present at the highest density on the surface of platelets play an important role in platelet to platelet aggregation and formation of platelets plug through binding to

multiple ligands such as fibrinogen, VWF, fibronectin and collagen (Varga-Szabo et al., 2008).

1.1.2. Secondary Hemostasis

There are two models concerning the secondary hemostasis (coagulation cascade): The classical coagulation cascade model and the cell-based coagulation model (Ferreira et al., 2010).

The coagulation cascade model, proposed in 1964 by Macfarlane, Davie & Ratnoff consists of multiple steps of coagulation factors (Table 1) activation that ends up with cleavage of soluble fibrinogen by thrombin and formation of insoluble fibrin mesh at the site of damaged blood vessel (Furie, 2009). The majority of these factors are precursors of proteolytic enzymes known as zymogens that circulate normally in their inactive form. The coagulation factors are classified into three groups: fibrinogen group (fibrinogen, V, VIII and XIII),Vitamin K –dependent group (II, VII, IX and X) and the contact group (XI, XII, High molecular weight kininogen (HMWK) and prekallikrein) (Palta et al., 2014).

Table 1: Coagulation factors (Palta,2014).

Assigned Roman numbers	Clotting factor name	Function	Plasma half-life (Hours)	Plasma concentration (mg/dl)
I	Fibrinogen	Clot formation	90	3000
II	Prothrombin	Activation of factors I, V, VII, VIII, XI, XIII, protein C, platelets	65	100
III	TF (Tissue-Factor)	Co –factor for FVIIa	----	----
IV	Calcium	Facilitates coagulation factor binding to phospholipids	----	----
V	Proacclerin, labile factor	Co-factor of FX-prothrombinase complex	15	10
VI	Unassigned	----------	-----	----
VII	Stable factor, proconvertin	Activates factors IX and X	5	0.5
VIII	Antihemophilic factor A	Co-factor of FIX-tenase complex	10	0.1
IX	Anti-hemophilic factor B	Activates FX: forms tenase complex with factor VIII	25	5
X	Stuart-Prower factor	Prothrombinase complex with FV: activates FII	40	10
XI	Plasma thromboplastin antecedent	Activates FIX	45	5
XII	Hageman factor	Activates factors XI, VII and prekallikrein	-----	----
XIII	Fibrin-stabilizing factor	Cross-links fibrin	200	30
	Prekallikrein (Fletcher factor)	Serine protease zymogen	35	----
	HMWK- (Fitzgerald factor)	Co-factor	150	----
	vWf	Binds to FVIII, mediates platelets adhesion	12	10µg/ml
	Anti-thrombin III	Inhibits FIIa, FXa, and other proteases	72	0.15-2 mg/ml
	Heparin cofactor II	Inhibits FIIa	60	----
	Protein C	Inactivates FVa and FVIIIa	0.4	----
	Protein S	Co-factor for activated protein C	-----	----

1.1.2.1. Classical coagulation Pathway

During vessel damage the coagulation factors act in specific pathways: The intrinsic pathway (contact) and the extrinsic pathway (tissue factor or TF). These two pathways meet at certain point to form the common pathway that culminates in the formation of stable fibrin clot and stop the bleeding (Palta et al., 2014).

The intrinsic pathway (Figure 1) is so called because all its components are present in the blood. This pathway is considered to be the longer pathway of secondary hemostasis. It begins when blood vessel damage occurs, endothelial collagen exposed which in turn activates factor XII, HMWK and prekallikrein. Factor XII which becomes factor XIIa after activation, acts as a catalyst to activate factor XI to factor XIa. Factor XIa then activates factor IX to factor IXa.

Factor IXa in turn act as a catalyst for turning factor X into factor Xa. When any coagulation factor is activated, it activates many other factors in the next steps. As the cascade move further down, the concentration of that factor increases in the blood. The intrinsic pathway is clinically measured as the activated partial thromboplastin time (APTT) (Gailani & Renné, 2007).

The extrinsic pathway (Figure 1) requires an external factor which is the tissue factor (TF). The extrinsic pathway is the shorter pathway of secondary hemostasis. It begins when a damage to the vessel, triggers the endothelial cells to release tissue factor which in turn activate factor VII to factor VIIa. Factor VIIa activates factor X into factor Xa. At this point both extrinsic and intrinsic pathways become one (common pathway). The extrinsic pathway is clinically measured as the prothrombin time (PT) (Mackman et al., 2007).

The intrinsic and extrinsic pathways converge at the level of factor X activation. This pathway (the common pathway) begins at factor X which is activated to factor Xa. Cleavage of factor X requires a complex called Tenase complex. This complex has two forms: Extrinsic, which consist of factor VII, factor III (tissue factor) and Ca^{+2}, or intrinsic, made up of factor VIII, factor IXa, and Ca^{+2}. Factor Xa activates factor II (prothrombin) into factor IIa (thrombin). Factor V acts as a cofactor for factor Xa to cleave prothrombin into thrombin. Factor IIa (thrombin) activates fibrinogen into fibrin. Thrombin in turn activates other factors in the intrinsic pathway (factor XI) as well as cofactors V and VIII and factor XIII. Fibrin units come together to form fibrin fibers, and factor XIII acts on fibrin fibers to form a fibrin mesh. This mesh helps to stabilize the platelet plug (Hoffman et al., 2005; Lane et al., 2005) .

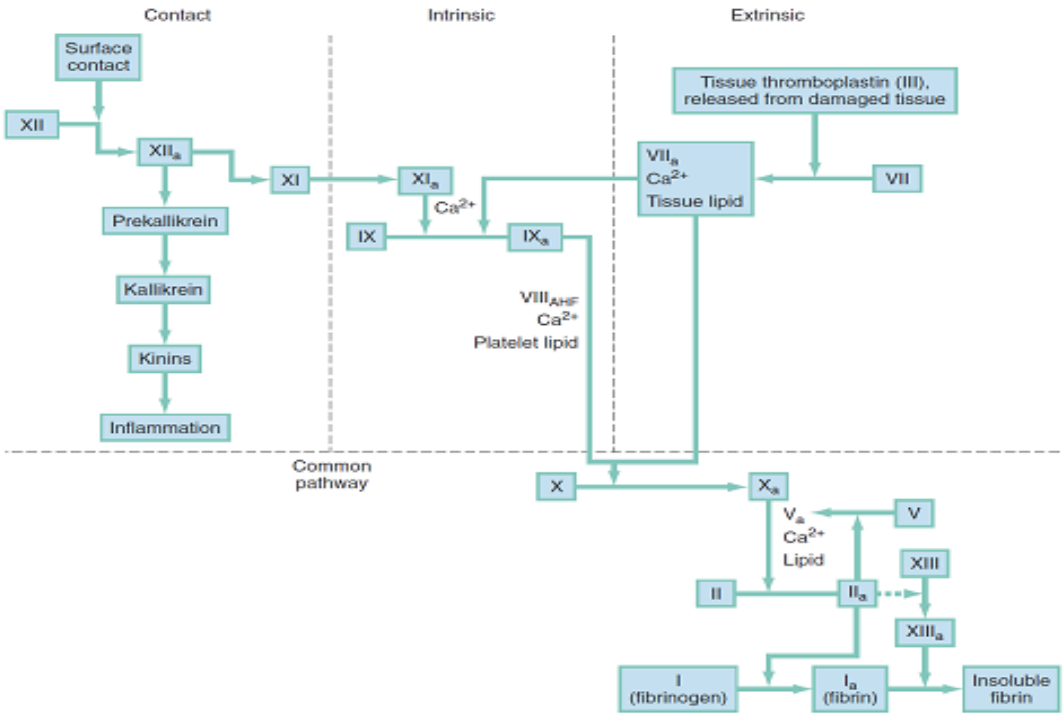


Figure 1: Blood coagulation pathways (Joe. D, 2007).

Although the term coagulation "cascade" was a successful model and a significant advance in the understanding of coagulation, more recent clinical and experimental studies showed that the cascade hypothesis cannot explain many aspects taking place *in vivo*, also clinical

observations do not support a separate intrinsic and extrinsic pathways, outlined in the coagulation cascade (Ferreira et al., 2010). The classical coagulation cascade model suggests that the extrinsic and intrinsic pathways operate as semi-independent pathways, and the interactions between coagulation factors and cells are relatively limited (Ho & Pavey, 2017).

For example, deficiencies of factor XII, prekallikrein or HMWK prolong the activated partial thromboplastin time (APTT) but are not associated with an increased risk of bleeding. On the other hand, deficiency of factor IX causes hemophilia B and severe clinical bleeding. The coagulation cascade model does not explain why the activation of factor X by the extrinsic pathway is not able to compensate for impairment of the intrinsic pathway due to a lack of factor VIII (hemophilia A) or factor IX (hemophilia B). Also, the degree of APTT prolongation in hemophilia patients does not necessarily predict the extent of the bleeding. This is clearly seen in that the activity of the extrinsic pathway of hemophilia patients is normal, as their prothrombin time (PT) remains within the reference range, even though APTT is prolonged and they have bleeding episodes (Ho & Pavey, 2017).

1.1.2.2. Cell-based coagulation model

Recent understanding of the hemostatic system highlights the important role of cells in the coagulation cascade. According to this model, essential coagulation reactions takes place on the cell surface which not only provides a space for the reaction but also provides pro-coagulant substances. Evaluation of this model suggests that the coagulation process actually occurs *in vivo* with interrelationship of physical, cellular and biochemical processes in a series of stages or phases (Smith, 2009).

In the cell-based coagulation model (Figure 2), the TF-bearing cells (such as vascular smooth muscle cells) and platelets as the two main cellular components, in coordination with thrombin and fibrinogen as the main coagulation proteins, play the key role in hemostasis (Ho & Pavey, 2017).

The cell-based model consists of four phases:

Initiation phase: The initiation phase of the coagulation process occurs when the TF-bearing cells are exposed to blood components at the site of injury. FVIIa rapidly binds to the exposed TF, forming the FVIIa/TF complex. The TF-FVIIa complex then activates additional FVII to FVIIa, allowing for even more TF-FVIIa complex activity, which then activates small amounts of FIX and FX. FXa in association with its cofactor, FVa, will form a complex called prothrombinase. The FV can be activated by FXa resulting in FVa which is necessary for the prothrombinase complex. Prothrombinase transforms small amounts of prothrombin (Factor II) to thrombin. Although the amount of thrombin generated is not sufficient to generate sufficient amounts of fibrin, but it is a critical step in the amplification phase of the coagulation system (Ferreira et al., 2010; Ho & Pavey, 2017).

Amplification phase: Once a small amount of thrombin has been generated on the surface of TF-bearing cells, thrombin diffuses away from the TF-bearing cell and activates platelets that have leaked from the blood vessel at the site of injury. Binding of thrombin to platelet surface receptors causes extreme changes in the surface of the platelet, resulting in shape change, shuffling of membrane phospholipids to create a pro-coagulant membrane surface, and the release of chemotactic substances that attract clotting factors to their surface which leads for more activation of platelets. As the permeability of platelet membranes is changed, this allows entry of calcium ions that induce clustering of phospholipids (increasing the pro-coagulant nature of the membrane), promotes binding of more coagulation proteins to the activated membrane surface, and activation of the glycoprotein IIb/IIIa receptors on the platelets surface. The thrombin generated in the initiation phase cleaves FXI to FXIa and activates FV to FVa on the platelet surface. Thrombin also cleaves von Willebrand factor from FVIII, releasing it to mediate platelet adhesion and aggregation. The released FVIII is subsequently activated by thrombin to FVIIIa (Ferreira et al., 2010; Smith, 2009).

Propagation phase: The propagation phase is characterized by the recruitment of large numbers of platelets to the site of injury, as the result of releasing contents of platelet granules. The activation of the glycoprotein IIb/IIIa receptors on the platelets surface during the amplification phase allowing cross-linkage of platelets by fibrinogen. The propagation phase occurs on the surface of activated platelets. FIXa that was generated by TF-FVIIa in the initiation phase can bind to FVIIIa (generated in the amplification phase) on the platelet surface. Additional FIXa is generated due to cleavage of FIX by FXIa that was generated during amplification on the platelet surface. Once the intrinsic tenase complex forms (FIXa–FVIIIa) on the activated platelet surface, it rapidly begins to generate FXa on the platelet surface. FXa is also generated during the initiation phase on the TF-bearing cell surface, but the majority of FXa is generated directly on the platelet surface through cleavage by the intrinsic tenase complex. The FXa generated on platelets then rapidly binds to FVa and forms the prothrominase complex (FXa–FVa) which cleaves prothrombin to thrombin. This prothrombinase activity results in a burst of thrombin generation leading to cleavage of fibrinopeptide A from fibrinogen, at the time that there is enough thrombin is generated with enough speed to result in a critical mass of fibrin. These soluble fibrin molecules will polymerize into fibrin strands, resulting in an insoluble fibrin matrix (Ferreira et al., 2010; Ho & Pavey, 2017; Smith, 2009).

Termination phase: Once a fibrin clot is formed, the clotting process must be limited to the injury site to prevent thrombosis of the blood vessel. For this purpose, there are four natural anticoagulants involved to control the spread of coagulation process: Tissue factor pathway inhibitor (TFPI), protein C (PC), protein S (PS) and anti-thrombin (AT).

TFPI is a protein secreted by the endothelium, which forms with TF, FVIIa and FXa a quaternary complex, which in turn inactivates the activated coagulation factors and limits the coagulation. Protein C and protein S have the ability to inactivate the procoagulant FVa and FVIIIa cofactors. AT inhibits the activity of thrombin and other serine proteases such as FIXa, FXa, FXIa and FXIIa (Ferreira et al., 2010; Malý et al., 2007).

This new model of hemostasis could explain some clinical aspects of hemostasis that the classical cascade model failed to do so. This new model gives more clear and better

understanding of the *in vivo* coagulation process and is more consistent with the clinical observations of several coagulation disorders (Ferreira et al., 2010).

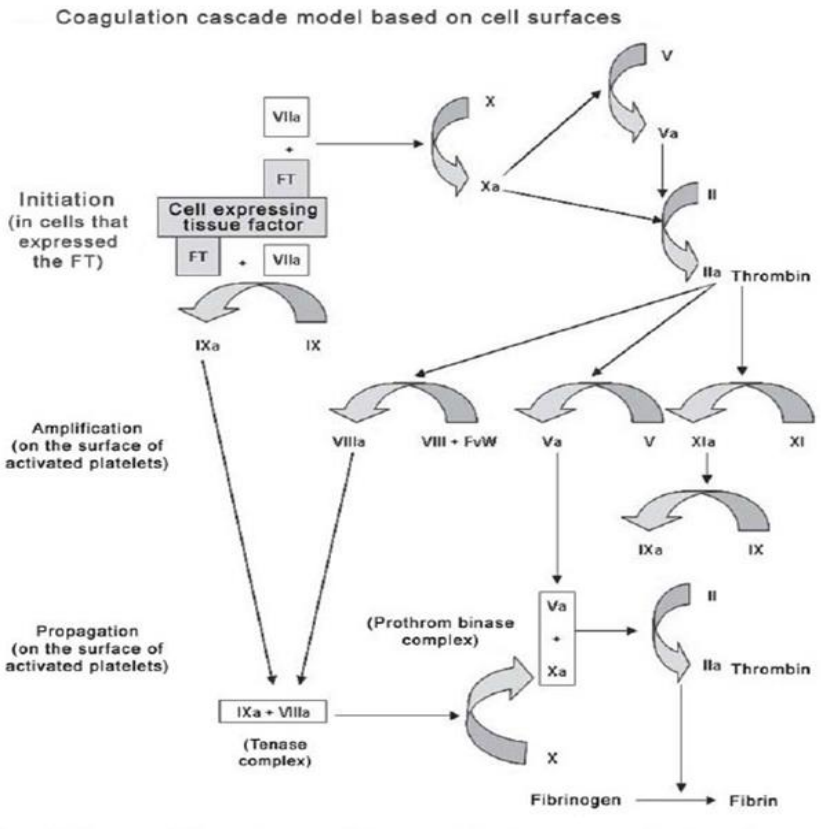


Figure 2: Cell-based coagulation cascade. (Translated and adapted from Vine, AK. Recent advances in hemostasis and thrombosis(Vine, 2009)**.**

1.1.3. Tertiary Hemostasis (Fibrinolysis)

The fibrinolytic system operates to remove blood clots during the process of wound healing and also to prevent blood clots in normally blood vessel. It is mainly composed of plasmin, plasminogen and tissue-type plasminogen activator. Plasmin which is the central serine protease enzyme in the fibrinolytic system and is generated from its zymogen plasminogen by the action of t-PA. At the surface of fibrin clot, both plasminogen and t-PA comes together and bind to the clot. Then, t-PA activates plasminogen and converts it into plasmin which in

turn starts to degrade the fibrin clot. Alpha -2-antiplasmin acts to down-regulated the plasmin in the circulation (Rau et al., 2007; Weisel & Litvinov, 2014).

1.2. Hemophilia

1.2.1. Definition

Hemophilia is a congenital bleeding disorder resulting from deficiency or dysfunction of one of the blood coagulation proteins. There are three types of hemophilia: hemophilia A (HA), hemophilia B (HB), and hemophilia C (HC), which are due to deficiency of coagulation Factor VIII (FVIII), factor IX (FIX), and factor XI (FXI), respectively. Both HA and HB are X-linked recessive disorders, and thus affects males more than females, while HC is an autosomal recessive disorder (Bane et al., 2014; Karim & Jamal, 2013). Acquired hemophilia A is a rare disease resulting from development of autoantibodies against FVIII (Kruse-Jarres et al., 2017).

1.2.2. History of Hemophilia

A bleeding condition that resembles hemophilia was first noticed back in the 2nd century AD, the Babylonian Talmud stated that male boys should not be circumcised if two brothers had already died owing to excessive bleeding from the procedure. In the 12th century, the Islamic physician Albucasis, described a family where males died from bleeding after minor traumas. John Conrad Otto, a physician in the New York Hospital, published in 1803 the first medical description of hemophilia. Nasse in 1820, was the first one who described the inheritance pattern of hemophilia when he stated that hemophilia is transmitted entirely by unaffected females to their sons. It was in 1828 that the term hemophilia was used for description of the hemorrhagic condition, and it was written by Hopff from the University of Zurich, Switzerland (Otto, 1951).

Hemophilia was called the royal disease, since Queen Victoria, who ruled England from 1837 to 1901, was a hemophilia B carrier. She passed the mutation to her eighth son Leopold who suffered from hemophilia B and died of a brain hemorrhage. In addition, Alice and Beatrice, two of Queen Victoria's daughters were carriers of hemophilia B and they transmitted the

disease on to the Spanish, German, and Russian royal families. Furthermore, Alexandra, Alice's daughter, married the Tsar of Russia Nicholas and was the mother of Alexis, the Tsarevich, whose bleeding condition was the main reason for the increasing influence of the monk Rasputin on the Romanov dynasty. Victoria Eugenie, Beatrice's daughter, became Queen of Spain and had two sons, Alfonso and Gonzalo who were affected by hemophilia too (Figure 3) (Franchini & Mannucci, 2014; Stevens, 2005).

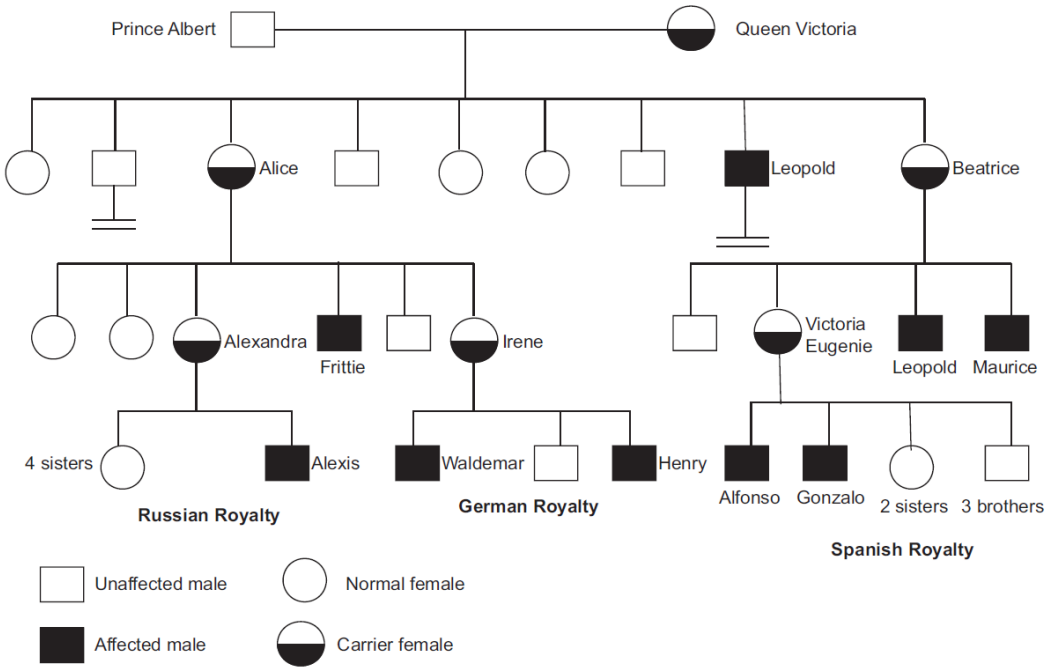


Figure 3: Queen Victoria's abridged family tree. (Franchini & Mannucci, 2014).

In 1936 two doctors from Harvard University, Patek and Taylor, found that by adding an extracted substance from plasma, hemophilia could be corrected so they called the plasma-derived factor as antihemophilic globulin (Boling et al., 1936).

The existence of more than one type of hemophilia was discovered in 1947, when Pavlosky from Argentina found that blood from one hemophilia patient can correct the clotting problem in a second hemophilia patient. After that Biggs et al from Oxford University found that there

was another disease that is different than Hemophilia A, they called it Christmas disease (the name of their patient) or Hemophilia B (Bigges et al., 1952).

1.2.3. Epidemiology of hemophilia

The estimated number of hemophilia patients around the world is approximately 400,000. Hemophilia A represent about 80-85% of hemophilia patients in the world. The incidence of hemophilia does not vary appreciably among populations (Srivastava et al., 2013).

Hemophilia A: Is a sex-linked disorder resulting from deficiency of clotting FVIII, however around 30% of new HA cases are due to *de novo* mutations. HA occurs in one in every 5000 male births (Karim & Jamal, 2013; Somwanshi et al., 2014).

HA is caused by mutations in the *F8* gene that encodes the FVIII protein, a plasma glycoprotein that plays an important role in the blood coagulation cascade (Awidi et al., 2010).

Hemophilia B: Is an X-linked disorder caused by deficiency of clotting FIX. However, up to 30% of cases of HB arise from new mutations. The incidence of HB is approximately one in 25000-30000 male live births with a nearly similar prevalence all over the world (Goodeve, 2015).

HB is caused by mutations in the *F9* gene that encodes the FIX which a glycoprotein of 415 amino acid residues, and plays an important role in the blood coagulation cascade (Karimipour, 2009).

Hemophilia C: It is also known as plasma thromboplastin antecedent (PTA) deficiency or Rosenthal syndrome. It is a very rare congenital disorder due to the deficiency of FXI. It is a mild form of hemophilia with an incidence rate of 1:100,000. The disease is more frequent in the Jewish population with a frequency of 8%. The disease affects both sexes equally and presents at any age (Bane et al., 2014).

HC is caused by mutations in the *F11* gene that encodes the FXI, a160-kD plasma glycoprotein that is the precursor of the trypsin-like protease FXIa (Gaiz & Mosawy, 2018).

1.2.4. Clinical features of hemophilia

Hemophilic patients show easy bruising in early childhood, spontaneous bleeding particularly into the joints and muscles and excessive bleeding following trauma or surgery (Srivastava et al., 2013). The most common sites of bleeding are muscles and joints which cause pain and if not properly treated leads to atrophy of the muscles and malformation of the joints and consequently a permanent disability. When bleeding occurs in brain or CNS it is life-threatening and requires immediate treatment (Graw et al., 2005). However, it is important to correctly diagnose bleeding disorders from the beginning and initiate the appropriate therapy to avoid further complications of the diseases.

Plasma levels of coagulation factors are generally correlated with the severity of hemophilia. Hemophilia A and B are clinically categorized into mild (0.05 to 0.4 IU/ml), moderate (0.01 to 0.05 IU/ml) or severe (<0.01 IU/ml) hemophilia (Srivastava et al., 2013).

Table 2: Relationship of bleeding severity to clotting factor level. (Srivastava et al., 2013).

Disease severity	**FVIII or FIX plasma Levels**	**Clinical manifestations**	**Usual age of Diagnosis**
Severe	<1%	Spontaneous bleeding, predominantly in joints and muscles.	1st year of life
Moderate	2-5%	Occasional spontaneous bleeding. Severe bleeding after trauma or surgery.	Before age 5–6 years
Mild	6-40%	Severe bleeding after major trauma or surgery.	Often later in life

Carrier females usually have a 50% activity of FVIII, but their plasma FVIII level may range from 40 to 60% in extreme cases of lyonization of X-chromosome (Plug et al., 2006). The

frequency of severe, moderate and mild HA is 40%, 10% and 50%, respectively (Graw et al., 2005).

Patients with severe and moderate hemophilia are usually diagnosed shortly after birth while those with mild hemophilia are usually diagnosed by chance or following a major trauma. Thus mild hemophilia patients are usually under diagnosed and their numbers are also underestimated in most reports describing the incidence of hemophilia (Srivastava et al., 2013).

1.2.5. Treatment of hemophilia

Hemophilia treatment involves prophylaxis, control of bleeding episodes, and treatment of factor inhibitors. The main goal of hemophilia treatment is to give the patient an adequate replacement of the deficient coagulation factor protein to prevent bleeding episodes. This is most effectively and efficiently accomplished by the prophylactic administration of clotting factor concentrates, which contain an abundance of the specific deficient coagulation factor (Kessler, 2005).

In 1970s, lyophilized plasma-derived coagulation factor concentrates became available on a large scale. This was an innovation step in hemophilia care because it was possible for patients to take their infusions at home (Franchini & Mannucci, 2014).

Desmopressin (1-deamino-8-D-arginine vasopressin, also known as DDAVP) was first used in 1977 for treatment of patients with mild hemophilia A and von Willebrand disease. It is a synthetic analogue of antidiuretic hormone (ADH). The compound boosts the plasma levels of FVIII and vWF after administration. Desmopressin elevates the plasma factor VIII level two- to four folds above the baseline, most likely by increasing its release from the storage sites. DDAVP is contraindicated in elderly patients and those with vascular disease, because arterial thrombosis is a theoretical risk and has been reported following DDAVP in these circumstances (Mannucci & Bianchi, 2012).

In 1985, virus–inactivated plasma derived coagulation factor concentrates were developed after thousands of hemophilia patients were infected and many of them died of blood-borne

infections. The non-virus-inactivated coagulation factor. concentrates were made from thousands of human blood donations that were contaminated with human immunodeficiency virus (HIV) and hepatitis virus (Franchini & Mannucci, 2012).

The recombinant era for hemophilia began in 1980s with the cloning of the *F8* gene and then expression of its functional protein within mammalian cell lines. Recombinant DNA technology for production of coagulation factors was a promising step for treatment of hemophilia. It was in 1989, when the first recombinant factor VIII was used, whereas recombinant factor IX concentrate was only available in 1997 (Franchini et al., 2013).

At present, treatment of hemophilia is based on prophylactic treatment of patients with clotting FVIII of FIX. This treatment not only improves the quality of the patients' life but also avoids the complications of the disease. Before starting the therapy, the disease severity should be determined and thus the patient can be substituted with the correct amount of the clotting factor (Srivastava et al., 2013). Several studies demonstrated the advantage of continuous FVIII prophylaxis over on-demand treatment in hemophilia A and B patients. While continuous prophylaxis reduces the number of bleeding episodes significantly, but it cannot prevent these episodes completely unless about a 4-fold higher consumption of FVIII is used (Siegmund et al., 2009).

The most significant limitation of treatment with a standard prophylaxis protocol is the short half-life of FVIII, since FVIII protocol require three to four infusions per week (Coppola, 2010).

Gene therapy is a promising alternative for treatment of hemophilia. The ultimate goal of gene therapy is the replacement of a defective gene sequence with a corrected version to eliminate the disease for the lifetime of the patient. Following gene therapy hemophilic patients shall be free from prophylaxis and the need for intravenous delivery of the coagulation factor (Nathwani et al., 2017). There are two approaches for gene therapy: *in vivo* and *ex vivo*, depending on the mechanisms of gene delivery into cells (Matsui et al., 2007).

Several studies have focused on the in vivo delivery of viral vectors into liver cells by using adeno-associated virus (AAV). AAV vectors are derived from a nonpathogenic human

parvovirus. Most of the properties of AAV vectors make them very suitable for the treatment of hemophilia A and B, such as the genomic size of this virus with its moderate packaging capacity (Doshi & Arruda, 2018). However, there are potential side effects for this procedure including adverse immunological reactions, vector-mediated cytotoxicity, germ-line transmission, and oncogenesis (Chuah et al., 2004; Lundstrom, 2019).

In the *ex vivo* gene therapy, the target cells are first isolated and genetically modified *in vitro.* The *ex vivo* gene therapy avoids the risk of vector-associated adverse effects. However, long-term persistence and efficient *in vivo* engraftment following transplantation of gene-modified cells is a major concern (Chuah et al., 2013; Ohmori et al., 2015).

1.2.6. Complications of treatment

One of the most serious complications of hemophilia treatment is the development of antibodies (inhibitors) to infused FVIII which occurs in a frequency of about 20-40% in severe patients while in mild or moderate hemophilia patients at a lower incidence of 1–13% (Roozafzay et al., 2013). Development of inhibitory mechanisms has been attributed to several factors including the type of *F8* gene mutations and onset and type of treatment. These inhibitors clear the substituted factor from the blood and render the therapy ineffective. So, it is important for these patients to be correctly and accurately diagnosed to establish the level of inhibitors and select the appropriate therapy. Anti-FVIII or inhibitors compromise the treatment regimen and necessitate the use for expensive alternative treatment such rFVIIa (Witmer & Young, 2013).

FVIII inhibitors are alloantibodies, polyclonal IgG. Antibodies can be either inhibitory or non-inhibitory. FVIII inhibitors are usually IgG_4 and IgG_1 subclasses. IgG_1 antibodies are also found in a significant number of patients without functional inhibitors. IgG_4 subclass antibodies are the most predominant functional inhibitory antibodies in hemophilia A patients. FVIII inhibitors do not fix the complement and the IgG_4 antibodies do not precipitate as

complexes in gels. FVIII inhibitory antibodies most often are directed against the A2, A3 and C2 domains of FVIII protein (Lollar, 2004; Miller, 2018).

FVIII inhibitors show different kinetics of interaction with FVIII and inhibition: Type I inhibitors follow second-order kinetics (dose-dependent linear inhibition) and completely inactivate the FVIII protein. Type II inhibitors have complex kinetics and incompletely inactivate the FVIII protein. Type I inhibitors present more commonly in severe hemophilia, while Type II inhibitors are more common in patients with mild hemophilia or in patients with acquired FVIII inhibitor (Miller, 2018; Witmer & Young, 2013).

Development of inhibitory mechanisms has been attributed to several factors including: Genetic–risk factors and treatment-related risk factors. Genetic risk factors which classified into: The type of genetic mutation which is the most significant factor for the formation of inhibitors. The incidence of inhibitor formation in patients with severe hemophilia A is approximately 30%. Null mutations (large deletions, nonsense mutations and intron 22 inversions) result in complete reduction of FVIII protein, and are associated with the overall highest rates of inhibitor formation (21–88%) (Oldenburg et al., 2004). Intron 22 inversion, is the most common severe *F8* gene mutation and is associated with an inhibitor incidence of 40 – 50 % (Awidi et al., 2010; Oldenburg et al., 2004).

Other risk factors for inhibitor development includes the race and ethnicity, where hemophiliacs of African or Hispanic heritage have an increased risk of inhibitor formation. However, the mechanism is not clear yet (Miller et al., 2012).

Immune response traits may also affect the reaction to exogenous FVIII protein. These factors include the major histocompatibility complex (MHC) class II system and polymorphisms of interleukins (ILs), tumor necrosis factor (TNF)-α.The role of MHC phenotype in inhibitor formation is still debated (Pavlova et al., 2009).

Treatment-related risk factors include: the intensity of the first exposure, age at first exposure, prophylaxis, and the type of clotting factor product (recombinant versus plasma-derived).

The intensity of the first FVIII exposure is demonstrated to be a risk factor for inhibitor formation because significant cell injury or inflammation leads to immunologic 'danger signals' which in turn stimulate antigen-presenting cells and amplify an immunologic response which promote the formation of inhibitors. Factors like the source of factor VIII: plasma-derived versus recombinant factor products, early prophylaxis for prevention inhibitor formation were investigated for their role in inhibitor formation but their role is still inconclusive (Witmer & Young, 2013).

The most common methods used to detect and quantify FVIII inhibitors include the Bethesda assay or the Nijmegen-modified Bethesda assay (Verbruggen et al., 2009). The Nijmegen modification of inhibitor assay considered to be an improved specific and sensitive assay over the original Bethesda assay. These assays can detect inhibitors that reduce clotting factor activity (inhibitory). Both assays utilize serial dilutions of a patient's plasma that is incubated with equal volumes of normal plasma for 2 h at 37°C. Then the residual FVIII level of the incubation mixtures is measured. A positive result is when there is a significant decrease in the residual FVIII. The dilutions and residual factor are plotted against each other and the inhibitor titer is obtained by linear regression. By definition one Nijmegen Bethesda unit reduces the FVIII activity level by 50% (WHF, 2007).

1.2.7. Molecular genetics of hemophilia A

1.2.7.1. *F8* gene structure

The *F8* gene is located to the most distal band of the long arm of the chromosome X (Xq28). It is 186 kb in length and comprises 26 exons, encodes about 9010 base-long mRNA (an ORF of 7056 bases and a 3'UTR of 1806 bases) and a precursor protein of 2351 amino acids (mature protein is 2332 residues) (Bowen, 2002). Two exons remarkably large ones which are Exons 14 and 26 are remarkable long with 3106 bp and 1958 bp, respectively (Bowen, 2002; Graw et al., 2005). Introns located within this gene vary in length; the largest are introns 22

and 1 with approximately 32 kb and 23 kb, respectively (Graw et al., 2005). Exons and introns length of FVIII gene are illustrated in (Table 3).

Table 3: Exons and introns of *F8* gene. (Bowen, 2002; Gitschier et al., 1984).

Exon	Length (bp)	Intron	Length (kb)	Exon	Length (bp)	Intron	Length (kb)
1	313	1	22.9	14	3106	14	22.7
2	122	2	2.6	15	154	15	1.3
3	123	3	3.9	16	213	16	0.3
4	213	4	5.4	17	229	17	0.2
5	69	5	2.4	18	183	18	1.8
6	117	6	14.2	19	117	19	0.6
7	222	7	2.6	20	72	20	1.6
8	262	8	0.3	21	86	21	3.4
9	172	9	4.8	22	156	22	32.4
10	94	10	3.8	23	145	23	1.4
11	215	11	2.8	24	149	24	1.0
12	151	12	6.3	25	177	25	22.4
13	210	13	16.0	26	1958		

Until now more than 1200 mutations have been identified in the *F8* gene and were associated with HA. These mutations varies from single nucleotide substitution to gross deletions/insertions and inversions (Bogdanova et al., 2005).

1.2.7.2. Synthesis and structure of coagulation Factor VIII protein

FVIII is a complex heterodimeric glycoprotein that is primarily synthesized by liver cells and secreted to the circulation where it is assembled with vWF for stability and protection against the proteolytic action of activated protein C (APC). FVIII gene is transcribed into mRNA segment with approximately 9 kb in length which comprises a short 5'-UTR, an ORF plus stop codon and a long 3'-UTR with 150, 7056 and 1806 bp, respectively. It is translated into a precursor protein of 2351 amino acids (Bowen, 2002). Following translation, it undergoes extensive glycosylation in the endoplasmic reticulum and sulfation in the Golgi apparatus. The signal peptide is comprised of 19 amino acids that is proteolytically removed from N-terminal sequence to give a mature factor VIII of 2332 amino acids. The mature FVIII protein consists of three homologous A domains, two homologous C domains and a unique B domain, which are arranged in the order A1-A2-B-A3-C1-C2 from the amino-terminus to the carboxyl-terminal. Further processing events of cleavage by thrombin occur to yield a final product of activated FVIII (Bowen, 2002; Graw et al., 2005). Features of *F8* gene and its protein are shown in (Figure 4).

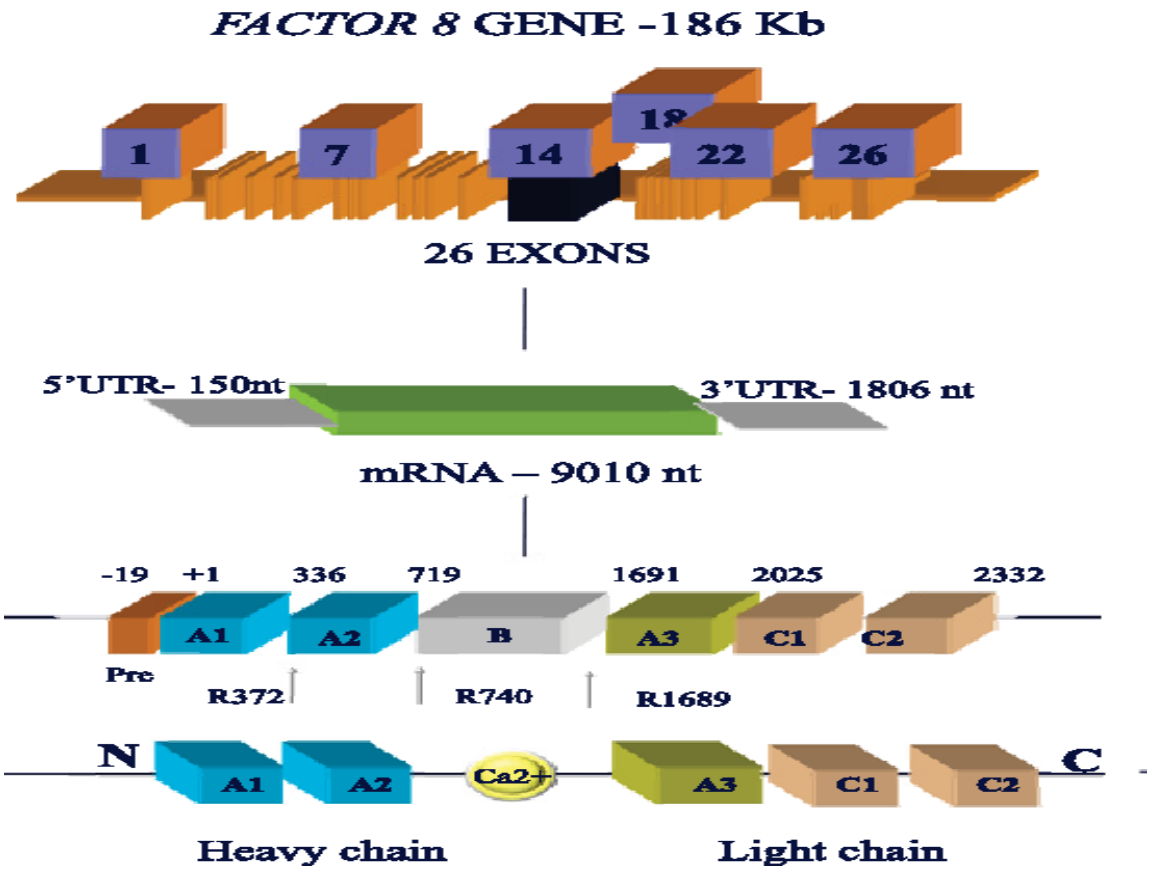


Figure 4: Features of *F8* gene and its protein FVIII. (Jayandharan & Srivastava, 2011).

1.2.7.3. Genetic variations in hemophilia A

Mutations involving intron 22 inversion and intron 1 inversion account for approximately 40–50% and 2- 5% of severe HA cases, respectively. With the exception of these two mutations, nearly every family with HA has its unique mutation that occurs throughout the *F8* gene with single point mutations constituting the majority of mutations (Awidi et al., 2010).

1.2.7.3.1. Intron 22 inversion

Intron 22 inversions originating in male germ cells, is the most frequent inversion that affects FVIII gene. Inv22 is responsible for approximately 40–50% of severe HA cases. (Rosslter et al., 1994). Inv22 results from homologous intra-chromosomal recombination between a 9.5 kb region (int22h-1) within the *F8* locus and with either int22h-2 or int22h-3. Int22h-2 and int22h-3 are telomeric segments located approximately 400 kb and 500 kb upstream of the *F8* gene, respectively (Bagnall et al., 2006; Naylor et al., 1995). Structure of the wild-type and int22-inverted *F8* gene is shown in (Figure 5).

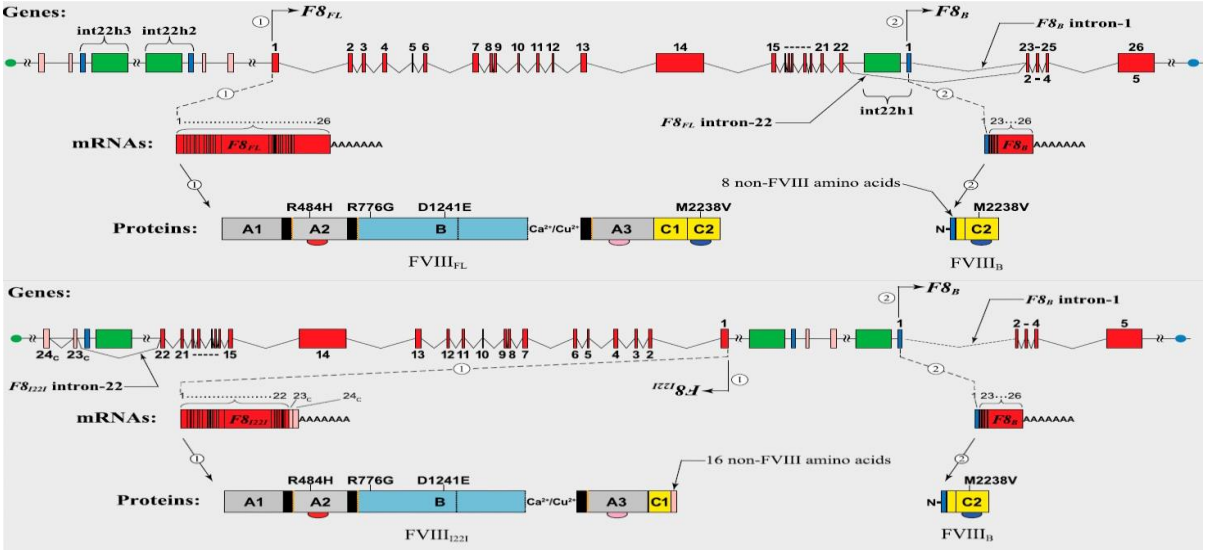


Figure 5: Structure and function of the wild-type and int22-inverted *F8* loci: DNA, mRNA, and protein. (Sauna et al., 2015).

1.2.7.3.2. Intron 1 inversion

Intron 1 inversion is a large molecular defect and is found in 2% to 5% of severe HA cases. Inv1 involves an intra-chromosomal homologous recombination between a region of 1041 bp of intron 1 (Int1h-1) and its extragenic copy (Int1 h-2) that is located approximately 140 kb telomeric the FVIII gene (Figure 6). This inversion prevent the formation of full-length FVIII

messenger RNA that leads to the absence of FVIII protein thus causing severe HA (Antonarakis et al., 1995; Bagnall et al., 2002).

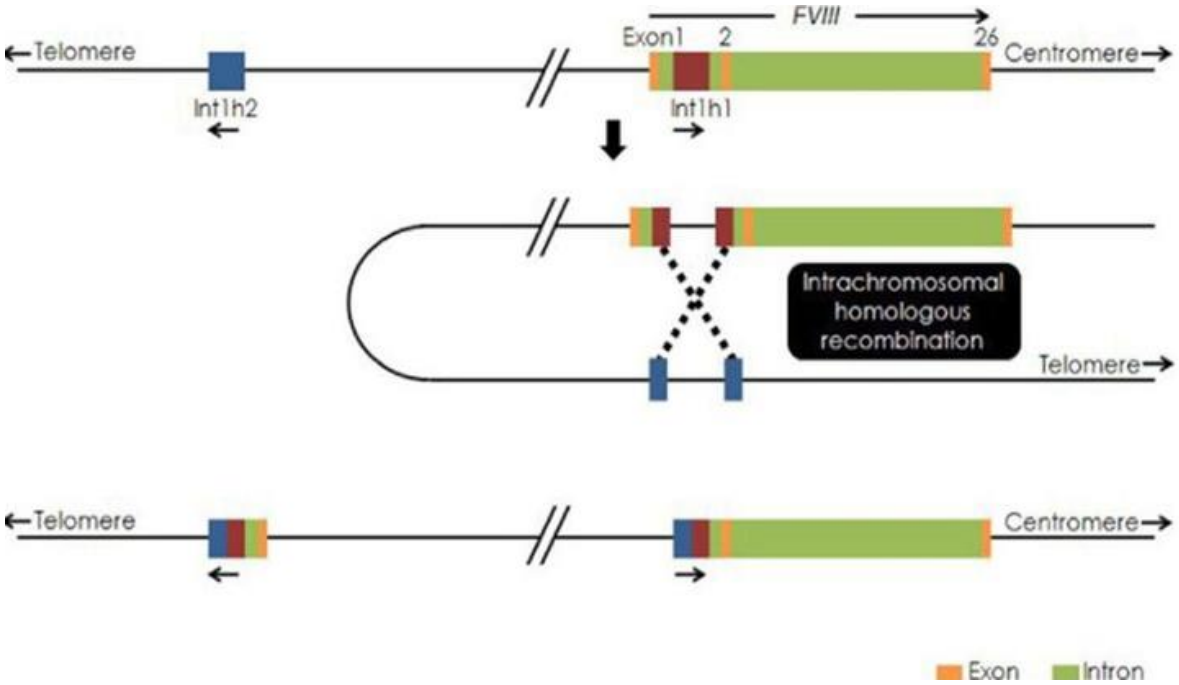


Figure 6: Intron 1 inversion of FVIII gene. Causey, T. (2017, November 1). *Genetic Testing in Common Disorders of Coagulation.* Retrieved from https://slideplayer.com/slide/13620671/.

1.2.8. Diagnosis of hemophilia

Establishing the correct diagnosis of a hemophilia patient is essential for identification of the type of hemophilia as well as the severity of the disease. For this purpose, a panel of tests are usually done and includes coagulation screening and genetic testing.

Primary coagulation screening tests includes platelets count, prothrombin time (PT) which is normal and activated partial thromboplastin time (APTT) which is typically prolonged in these patients. Secondary and more specific tests include clotting FVIII activity assay and determination of the presence and titer of inhibitors (Tantawy, 2010). Further testing includes DNA analysis to establish the genetic mutations of *F8* gene that can provide a definitive diagnosis and allows genotype-phenotype correlation analysis (Lillicrap, 2013; Nichols et al., 1996).

Diagnosis of carrier females using primary and secondary tests listed above may not provide a definitive diagnosis and genetic testing in these cases will be indispensable (Lillicrap, 2013).

1.3. Literature Review

HA is the most severe and most common inherited bleeding disorders. Studies of the molecular basis of HA from many countries around the world have yielded a remarkable heterogeneity of mutations affecting *F8* gene. In addition, the inhibitor development of some HA patients has been tackled by several studies that have deciphered some immunological and genetic factors that influence inhibitor development.

A study in Palestine, (Abu Arra et al., 2020) included 77 HA patients from 52 unrelated families in West Bank area, revealed that the percentage of severe, moderate and mild HA were 41.7%, 22.2% and 36.1%, respectively. Severe HA patients were screened for Inv22 using Sub Cycling-PCR technique, revealed that 37% of these patients have this mutation. Among the severe HA patients with positive Inv22, 45.5% (5/11) had developed FVIII inhibitors.

Awidi and his coworkers (Awidi et al 2010) from Jordan studied 142 HA patients from 42 unrelated families including 117 cases of severe HA. The authors found that 52 % of severe HA have int22 inv and 2% of severe HA patients have int1 inv. In addition, they reported 19 different mutations comprising single point mutations and frame-shift mutations, of which 15 were novel mutations. Of all severe HA patients, 17 patients (14.5%) were positive for FVIII inhibitor which represent a moderate percentage compared to other countries (Awidi et al., 2010).

In Lebanon, Khayat et al (2008) studied 79 HA patients from 55 unrelated families. The authors found that 29% of HA patients have int22 inv and 2.5% have int1 inv. In addition, the authors reported 32 different mutations comprising single point mutations, small and large deletions as well as splice mutations. From the 32 mutations, 21 mutations were novel mutations. FVIII inhibitors were found in three patients (3.7%), one of them with intron 22 inversion and the other two with nonsense mutations, revealing a low incidence of FVIII inhibitor among Lebanese HA patients (Khayat et al., 2008).

A study from Saudi Arabia, analyzed 110 HA patients. Of all patients, 15 patients (13.6%) showed int22 inv and 2 patients (1.8 %) showed int1 inv. Out of 32 cases sequenced for coding exons, 2 novel mutations were found (Al -Allaf et al., 2016). In another study from Saudi Arabia, the authors found that 43 patients out of 147 (29.3 %), mostly severe HA, have FVIII inhibitor (Owaidah et al., 2017).

A study in Iraq, showed that 25 HA patients were identified with different mutations representing different exons including exons: 18, 22, 23, 24 and intron 22. Most mutations detected were point mutations then inversion mutations followed by frame shift mutations. Most mutations located in exon 24 (45.2 %) and intron 22 (22.6%) (Hassan & Jabber, 2019).

A study of Tunisian HA patients has reported 23 different mutations in *F8* gene from 28 HA patients belonging to 22 unrelated families. The identified mutations included 5 intron 22 inversions, 7 insertions, 4 deletions and 7 substitutions. The distribution of mutations (n=18) other than inv22, showed that 9 are located in exon 14, the most mutated exonic sequence in the *F8* gene and 8 were novel mutations (Elmahmoudi et al., 2012).

In a study from Pakistan, 92 HA patients were screened for mutations in *F8* gene. The *F8* gene mutations were heterogenous and included point mutations (including missense, nonsense, and splice site), inversions (Inv22 and Inv1) and frame shift (deletions and duplications). Forty-two percentage of HA patients have point mutations and 20% of patients have inv22 and 29% of HA patients have severe disease (Campus, 2014).

A study in Colombia revealed that, intron 22 inversion was detected in 14 out of 33 HA male patients (42.4%) unrelated cases. Three out of 33 samples (9.1%) were positive for inv1. Single nucleotide/small frame-shift variants were present in 11 patients (33.3%) and 3 HA patents had large structural variants (Yunis et al., 2018).

A study of Korean HA patients reported 33 mutations in *F8* gene in 38 HA patients, which constitute a high heterogeneity of HA in Korean population. Of these mutations, Inv22 constituted 39.5% of these mutations while inv1 accounted for only 2.6 % of all mutations. Most of the mutations (44.8%) reported in Korean HA patients were novel mutations (Hwang et al., 2009).

A study in France analyzed 120 HA patients from 94 unrelated families and identified a total of 47 mutations in the *F8* gene of which 26 were novel mutations. Inv22 was detected in 47% of patients. None of the French HA patients in this study showed inv1. In this study 18 patients (15%) developed FVIII inhibitors, 6 out of these 18 patients have inv22 and 5 patients have a non-missense mutation and 2 patients have novel mutations (Repessé et al., 2007).

F8 gene mutations were also determined in 42 unrelated Moldavian HA families in 2009, about 30.9 % of these mutations were inv22, 2.4 % were inv1 and the rest of mutations were 2 deletions, 6 frame shift, 16 missense and 2 non-sense and 2 splicing mutations. Out of 26 different mutations in this study, 12 were novel mutations. Inhibitor development was observed in 2 patients with inv22 and one with another deletion mutation (Sirocova et al., 2009).

Genetic analysis for HA was carried in 37 Albanian patients in 2007. The FVIII inv22 was detected only in 2/19 cases (10.5%) apparently unrelated patients with severe HA, while inv1 was not detected in this cohort. A total of 19 different gene mutations were identified. Ten mutations were novel: 4 null mutations in severe HA patients, and 6 missense mutations (Castaman et al., 2007).

The results of these and other studies further emphasize the genetic heterogeneity of HA. More than 1200 mutations in the *F8* gene are listed in the HAMSTeRS database. Among these, the most common defect is an inversion in intron 22 and intron 1. The intron 22 inversion is detected in 40–50% of severe HA patients, however the percentage varies among different populations and ethnic groups. Differences in reported prevalence rates from different countries were attributed in some studies to the limited number of studied cases, the ethnic variations, and the inclusion of patients with moderate and mild FVIII activity results. In the absence of Inv22 mutation, other genetic testing should be considered, in order to elucidate the causes of the disease among severe hemophilia A patients in our area, including Inv1 mutation on the same chromosome and full gene sequencing. In contrast inversions of intron 1 are found in much lower percentages compared to inv22 (Bowen, 2002). Thus, in the molecular diagnosis of HA patients, testing for inv22 is the primary test performed for severe

HA patients followed by testing for inv1. Aside from these two inversions, a large number of mutations are possible and thus DNA sequencing is usually the most appropriate technique for identification of these mutations.

1.4. Objectives of the study

The aim of this research is to genotype the *F8* gene and conduct a laboratory analysis of hemophilia A in the West Bank of Palestine, in order to provide proper diagnosis and management of HA patients. The outcome of this study will allow us to determine the spectrum of *F8* gene mutations causing HA in Palestine. The results can also be used to establish a national registry for hemophilia patients. Such a national registry will enable the local health authorities to use the registry in the planning for the needs of the patients and prevent misuse of therapy.

The specific objectives of this study are:

- To determine the spectrum of *F8* gene mutations among hemophilia patients in the West Bank region, Palestine.
- To provide necessary genetic information necessary for developing a laboratory test for screening of *F8* gene mutations.
- To provide necessary genetic information about *F8* gene mutations that can be used for genetic counseling of hemophilia patients.
- To provide data for establishing a national registry for hemophilia A in West Bank region, Palestine.

Chapter two

Materials and Methods

2.1. Materials

All reagents, chemicals and instruments used in this study are listed in Table 4.

Table 4: List of instruments and materials used in this study.

Item	Manufacturer/country
Lyophilized PCR master mix	AccuPower® HotStart PCR PreMix, BIONEER, Korea
Gel purification kit	AccuPower® Gel Purification kit, BIONEER, Korea
PCR primers	Metabion, Germany
1kb DNA ladder	Gene Direx ®
50 bp DNA ladder	Gene Direx®
Agarose	Lifegene company
Ethidium bromide	Sigma
Thermal cycler	T100 Bio-Rad company
Gel documentation system	Bio-Rad GEL DOC 2000. USA
Tris base	Sigma
DEPC-water	Hy-labs
Dimethyl Sulfoxide (DMSO)	Sigma
Expand polymerase mix	Roche Diagnostic 26614221
7-deaza –dGTP	Roche 13797031
6 X Loading Dye	Takara company
Chloroform	SDFCL company
SDS (Sodium dodecyl sulfate)	Sigma
Triton X-100	Sigma
$MgCl_2$	Sigma
Sodium citrate	Merck
Sucrose	Sigma
NaCl (sodium chloride)	SDFCL company
EDTA	Sigma

2.2 Methods

2.2.1. Study population and design

This was a case series retrospective multi-center study. Hemophilia patients registered in the Palestinian Society for Bleeding Disorders records (Al-Bireh, National Diabetes Society Building, 4th floor) were used as a basis for contacting patients and recruiting them at main hospitals in each city. Hospitals that participated in this study are: Al-Watani Hospital (Nablus), Anabta Zakat Committee (Tulkarim), Qalqilia Hospital (Qalqilia), Palestine Medical Complex (Ramallah) and Alia Hospital (Hebron).

2.2.2. Questionnaire

The questionnaire aimed to collect demographic information and medical history of the patients. Patients who accepted to participate in the study, were asked to attend to the nearest clinical care center. Patients were asked to provide a written consent. For patients younger than 18 years old, the guardian (either father or mother) was asked to provide the information needed to complete the questionnaire and to provide the written consent form (see Appendix 1).

2.2.3. Specimen collection, transport and preservation

Three tubes were collected from each patient, one EDTA tube (for CBC) and genomic DNA extraction, the two citrated tubes were used for (PT, APTT and FVIII activity assay) measurements. EDTA tubes were kept in the refrigerator at 4°C until tested. The two citrated tubes were immediately centrifuged at 2000xg RCF for 10 minutes at RT, then the plasma from each tube was put in 12X75 mm plastic tubes with caps and stored at - 40°C or less.

Hematological and coagulation screening tests were done for each patient including: Complete blood count (CBC), partial thromboplastin time (PT) and activated partial thromboplastin time (APTT). Coagulation FVIII activity assay (FVIII:C) was performed by using one-stage clotting assay using commercial kits and standard procedures (Dacie & Lewis, 2017). FVIII: C was performed using STA Compact Max® analyzer (Stago Company, France). Based on FVIII: C levels, samples were categorized as severe (FVIII: C $\leq$1%), moderate (FVIII: C 2-5%) and mild (FVIII: C 6-40%) HA.

2.2.4. Preparation of genomic DNA

Genomic DNA was prepared as described by (Bowen & Keeney, 2003). Briefly, 500 µL of whole blood were transferred into 1.5 mL microcentrifuge tube, followed by addition of equal volume of cell lysis buffer (10 mM Tris-HCl, 11% w/v sucrose pH 8.0, 5mM $MgCl_2$, and 1 % w/v Triton X-100). The mixture was vortexed briefly and incubated at room temperature for 2 minutes, followed by centrifugation at 2000xg for 2 minutes. The supernatant was discarded, the pellet (nuclei) was re-suspended in 500 µl of cell lysis buffer and centrifuged at 2000xg for 2 minutes. The supernatant was discarded, the pellet was re-suspended in 300ul of nuclei lysis buffer (10mM Tris-HCL, pH 8.0, 10mM EDTA, 10mM Sodium citrate, 1% w/v SDS) and gently mixed by repeated pipetting. Then, 100 µl of 6M NaCl and 500 µl of chloroform were added to the mixture, mixed by inversion until a uniform emulsion is formed. The mixture was centrifuged at 2000xg for 5 minutes at room temperature, to separate the aqueous and organic layers. The upper aqueous layer (~300 µl) was transferred to a clean 1.5-ml tube. About 600 µl of absolute ethanol was added, mixed by gentle inversion until the DNA is precipitated as a small fibrous ball.

The DNA fibrous ball was transferred by using a micro-hook made from glass rod or using a stainless-steel pin to a clean tube containing 30µl of sterile distilled water. Finally, the DNA sample was dissolved for 1 hour at room temperature.

2.2.5 Assessment of DNA quality and quantity

The quality and quantity of DNA samples were determined spectrophotometrically using NanoDrop Lite spectrophotometer (ThermoFisher Scientific, Wilmington, USA). Additionally, ~1 µl of DNA sample was mixed with 1 µl of 10X loading dye and loaded on 1% agarose gel parallel to 1 kb DNA ladder. Electrophoresis was performed at 85 volts and gels were visualized using Gel Documentation XR System (Bio-Rad, CA, USA). DNA samples were stained using ethidium bromide.

2.2.6 Genotyping of *F8* gene

All HA samples with FVIII: C ≤ 5% were tested for inv22, if negative then they were tested for inv1. Any sample that was negative for both inv22 and inv1 were then investigated by DNA sequencing along with HA samples with FVIII: C > 5%.

2.2.6.1. Inverse-shifting polymerase chain reaction (IS-PCR) for detection of *F8* gene introns 22 and 1 inversions

Inverse-Shifting PCR is a type of inverse-PCR that present a rapid *in vitro* amplification of DNA sequences that flank a region of known sequence. The method uses the polymerase chain reaction (PCR), but the primers are oriented in the reverse direction of the usual orientation. The template for the reverse primers is a restriction fragment that has been ligated upon itself to form a circle which is considered as the key step for all inverse-PCR protocols (Ochman, 1988; Rossetti et al., 2008). The principle of IS-PCR as well as the primer position used for detection of introns 22 and 1 inversions are illustrated in (Figure 7).

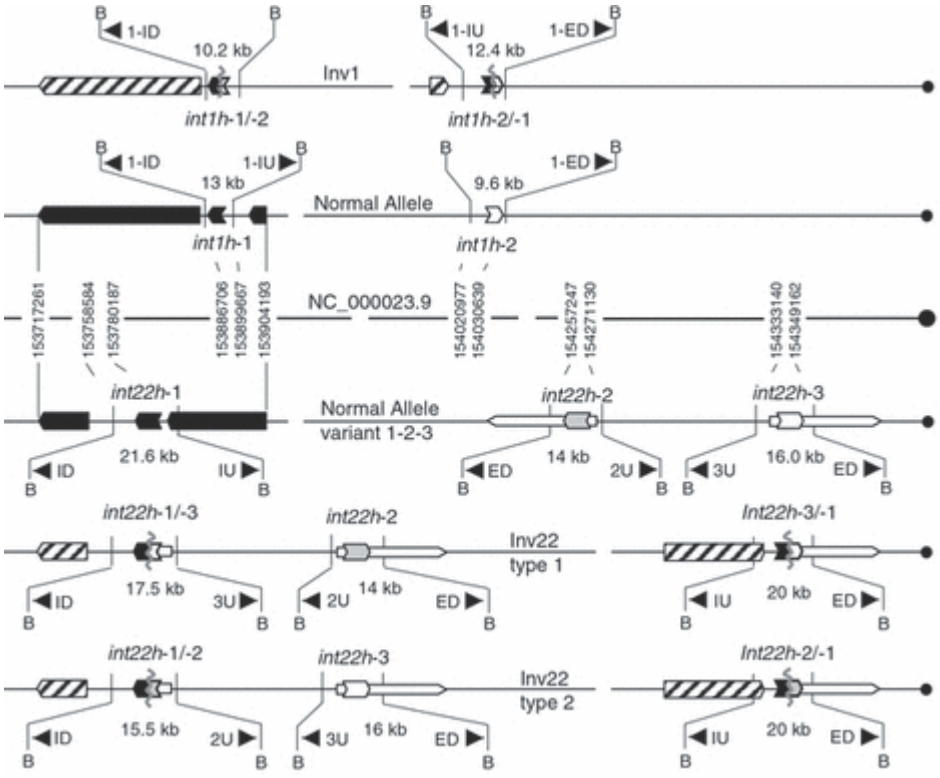


Figure 7: IS-PCR based system for genotyping int22h-and int1h-related rearrangements. Adapted from (Rossetti et al., 2008).

In IS-PCR method about 2 µg of genomic DNA was digested with 20 units of *BclI* restriction enzyme (New England, Bio labs Company, U.K) in 50 µl solution for at least 4 hours at 50°C. Digested DNA was purified using PCR/Gel Purification kit (Bioneer Company, Korea). Purified DNA fragments (including fragments with cohesive ends of *BclI*) were ligated by using T4 DNA ligase (New England, Bio labs Company, U.K) in a 400 µl solution at 16°C for 12-14 hours or overnight. The ligated DNA fragments were purified using the PCR/Gel purification kit (Bioneer Company, Korea).

This purified and digested/ligated DNA product was used as a template for both Intron 22 and intron 1 inversions detection by using different PCR protocols as described in the next sections.

2.2.6.1.1. IS-PCR for detection of *F8* gene intron 22 inversion

For the analysis of inv22, four primers were used as described earlier (Table 5) (Rossetti et al., 2008).

Table 5: Primers used for detection of inv22 by IS-PCR.

Primer	Sequence 5'-----3'	NC-000023.9	Length, bp
F822-ID	ACA TAC GGT TTA GTC ACA AGT	153758587-608	21
F822-IU	CCT TTC AAC TCC ATC TCC AT	153779730-50	20
F822-2U	ACG TGT CTT TTG GAG AAG TC	154270775-95	20
F822-3U	CTC ACA TTG TGT TCT TGT AGT C	154333426-48	22

The amplification of the inv22 was performed as described earlier (Rossetti et al., 2008) with slight modifications. Briefly, the PCR was performed using Hot-start PCR master mix (AccuPower® HotStart PCR PreMix, BIONEER, Korea) in a total volume of 20 µl containing 10µM of each primer (1.2 µl of primer mix), 6 µl of digested /ligated DNA and 12.8 µl DEPC-Water. Thermocycling involved an initial denaturation step at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 1 min, followed by 90 sec at 72°C, and a final extension step at 72°C for 5 min. IS-PCR products were analyzed by loading 5 µl of the samples on 3% agarose gel electrophoresis. Expected results are 487 bp for normal *F8* gene intron 22; 333bp for inv22 type1 and 385 bp for inv22 type 2.

2.2.6.1.2. IS-PCR for detection of *F8* gene intron 1 inversion

For the analysis of inv1 three primers were used (Table 6) as described earlier (Rossetti et al., 2008).

Table 6: Primers used for detection of inv1 by IS-PCR.

Primer	Sequence 5'-----3'	NC-000023.9	Length, bp
F81-ID	TCT GCA ACT GGT ACT CAT C	153886959-77	19
F81-IU	GCC GAT TGC TTA TTT ATA TC	153899635-54	20
F81-ED	GCC TTT ACA ATC CAA CAC T	154030453-71	19

PCR was performed using Hot-start PCR master mix (AccuPower® HotStart PCR PreMix, Bioneer, Korea) as described earlier (Rossetti et al., 2008) with slight modifications. Briefly, the PCR reaction with a total volume of 20 µl reaction, contained 10µM of each primer (1.2 µl of primer mix) with 5 µl of digested /ligated DNA and 13.8 µl DEPC-Water. Thermocycling involved an initial denaturation step at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 1 min, and extension at 72°C for 90 sec; followed by a final extension step at 72°C for 5 min. IS-PCR products were analyzed by loading 5 µl of the samples on 3% agarose gel. Expected results are 304 bp for normal *F8* gene intron 1 and 224 bp for inv1.

2.2.6.2. Nested Long Distance Polymerase Chain Reaction (NLD-PCR) for detection of *F8* gene intron 22 inversion

Although the IS-PCR method was successfully used to genotype most of the study samples for inv22 and inv1, it requires a high quality and quantity of high molecular weight genomic DNA. However, few samples did not yield a result using IS-PCR and were repeated more than twice which consumed most of the DNA samples of the respective samples. Therefore, to avoid the need for collecting new blood samples, we sought to genotype these samples using another method that does not high amounts of DNA. Therefore, the NLD-PCR was adopted which was a good alternative for IS-PCR. Furthermore, the NLD-PCR was used to confirm the results obtained by the IS-PCR.

It is noteworthy to mention that the original multiplex LD-PCR described by Liu & Sommer in 1998 (Liu & Sommer, 1998), gave a very low yield and a clear result only in few samples despite extensive optimization of the original protocol. Therefore, this method was abandoned and neither its optimization procedure nor results obtained with this method were described in this study.

For the NLD-PCR (Wang et al., 2020), genotyping of inv22 was performed first by a modified multiplex LD-PCR then the amplicons of this reaction were used as templates for the nested and multiplex PCR. In the first reaction LD-PCR, three primers were used, these 3 primers (HemN-P, -Q and B) (Table 7). The NLD-PCR uses also 3 additional inner primers (HemN-P1, -Q1 and -B1) (Table 7) designed to hybridize to sequences flanked by the outer primers (HemN-P, -Q and B). Schematic representation of *F8* intron 22 inversion and primer design are shown in Figure (8).

Table 7: Primers used for multiplex NLD-PCR.

Primer	**Sequence 5'-----3'**	**GenBank Accession No**	**Length**
HemN-P	GCC CTG CCT GTC CAT TAC ACT GAT GAC ATT ATG CTG AC	AF062514	38
HemN-Q	GGC CCT ACA ACC ATT CTG CCT TTC ACT TTC AGT GCA ATA	X86012	39
HemN-B	CCC CAA ACT ATA ACC AGC ACC TTG AAC TTC CCC TCT CAT A	AF062516	40
HemN-P1	GGA AAG AGG TAG GCA GGA GCC AAG AC		26
HemN-Q1	TGG CTC TGT ATC CCC ACC CAA ATC T		25
HemN-B1	TGT TGT CAT TGT CTG GCT CCT TGT CTG		27

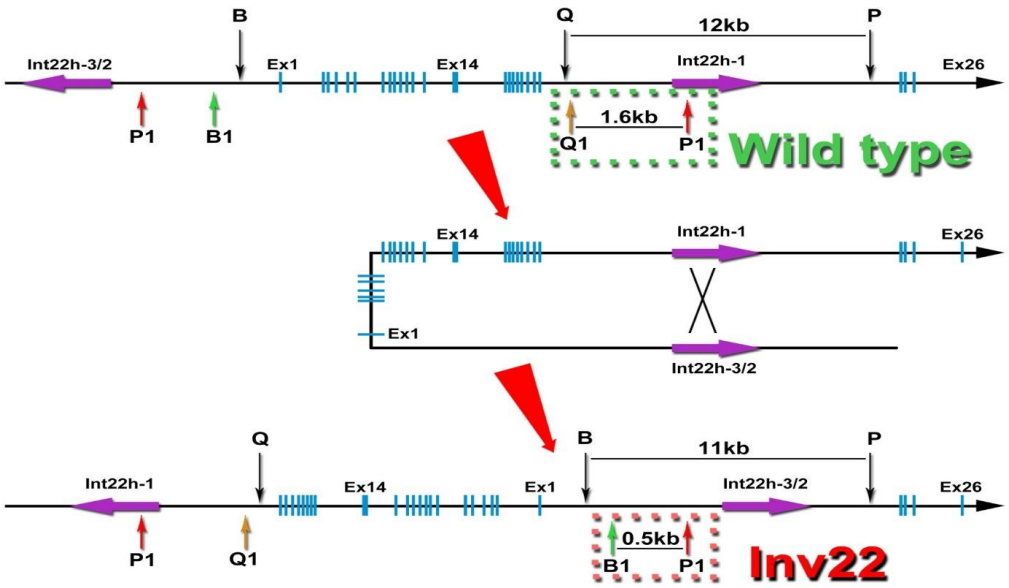


Figure 8: Schematic representation of *F8* intron 22 inversion and primer design. (Wang et al., 2020).

Multiplex LD-PCR was performed in 20 µl reaction volume containing 2 µl of 10X buffer 2, 1.6 µl of 5 mM solution of dGTP/7-deaza-dGTP, 0.8 µl of 10 mM solution of (dTTP, dATP and dCTP), 0.4 µl of 10 µM HemN-P primer, 0.4 µl of 10µM HemN-B primer, 0.4 µl of 5 µM HemN-Q primer, 0.35 µl of 5U/ µl of expand-long DNA polymerase (Roche company, Germany), 0.4 µl of DMSO (100%), 1 µl of genomic DNA and 12.65 µl DEPC-Water.

Thermocycling involved an initial denaturation step at 94°C for 1 min followed by 10 cycles of denaturation at 98°C for 10 sec, annealing at 68°C for 12 mins; followed by 20 cycles of denaturation at 98°C for 10 sec, annealing at 68°C for 12 mins with an increment of 20 secs per cycle, then a final extension step at 72°C for 10 mins.

LD-PCR products were then used as templates in the nested PCR. The nested PCR was performed using Hot-start PCR master mix (AccuPower® HotStart PCR PreMix, Bioneer, Korea) in a total of 20 µl reaction volume, that contained 0.8µl of 10 µM HemN-P1, -B1 and -Q1 primers, 1µl of LD-PCR product and 16.6µl DEPC-Water. Thermocycling involved an initial denaturation step at 94°C for 5 min followed by 30 cycles: denaturation at 94°C for 30 sec, annealing at 68°C for 30 sec, and extension at 72°C for 90 sec; then a final extension step at 72°C for 3 mins. Nested PCR products were analyzed by mixing 0.5µl of nested-PCR

product + 4.5 µl of D.H_2O and 1 µl of 6X loading dye, and loaded on 2 % agarose. Expected results are 1621 bp for normal *F8* gene intron 22 and 540 bp for inverted intron 22.

2.2.6.3. Multi-plex PCR for *F8* gene intron 1 inversion

For inv1 analysis, two PCR reactions were performed on each of genomic DNA samples, one for int1h-1 region and the other for int1h-2 region (Faridi et al., 2012). For analysis of int1h-1 and int1h-2 regions four primes were used F81-9F: *GTT GTT GGG AAT GGT TAC GG*, F81-9CR: *CTA GCT TGA GCT CCC TGT GG*, int1h-2F: *GGC AGG GAT CTT GTT GGT AAA* and int1h-2R: *TGG GTG ATA TAA GCT GCT GAG CTA.* The position of these 4 primers are shown in (Figure 8). For analysis of int1h-1 these primers were used: F81-9F, F81-9CR and int1h-2F, while for analysis of int1h-2 these primers were used: F81-9F, int1h-2R and int1h-2F.

Amplification for both int1h-1 and int1h-2 was performed using Hot-start PCR master mix (Bioneer, Korea) in a total of 20µl reaction volume that contained 0.4 µl of 10 µM of each primer, 0.4 µl of DMSO, 3 µl genomic DNA and 15.4 µl DEPC-Water. Thermocycling involved an initial denaturation step at 94°C for 5 mins followed by 30 cycles: denaturation at 94°C for 30 sec, annealing at (56°C for int1h-1 and 59°C for int1h-2) for 30 sec, extension at 72°C for 2 min, and a final extension step at 72°C for 5 mins. PCR products were analyzed by loading 5 µl of the samples on 2% agarose gel.

The nested PCR yields 1908bp and 1191bp in wild type int1h-1 and inth-2, respectively (Figure 8). While in Inv1, a 1323 bp and 1776 bp are obtained in case of int1h-1 and int1h-2, respectively (Figure 8).

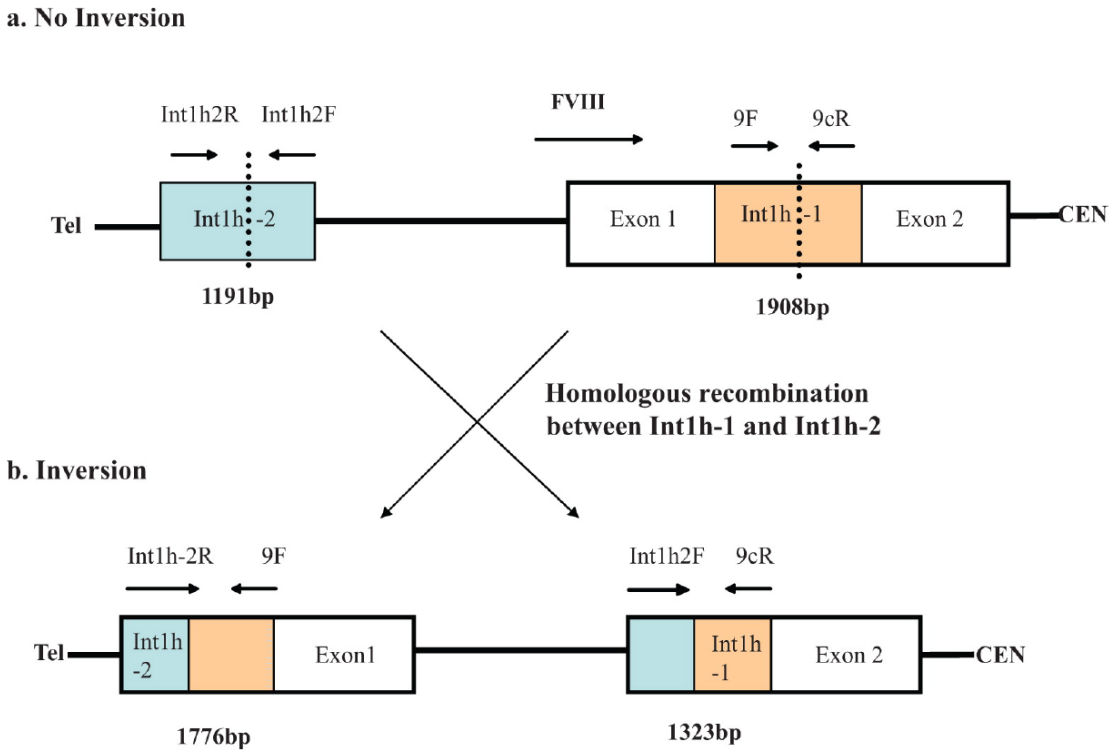


Figure 9: Schematic of int1h-1 and int1h-2 relative to intron 1 of *F8* gene (A: wild type intron 1, B: Inverted intron 1). (Faridi et al., 2012).

2.2.6.4. Amplification of *F8* gene exonic sequences

Since there are 26 exons of *F8* gene, individual exons were amplified separately using PCR primers described earlier (Awidi et al., 2010) and shown in (Table 8). Individual exons were amplified using Hot-start PCR master mix (Bioneer, Korea) in a total of 20 µl reaction volume that contained 1 µl of 5µM of primer mix (forward and reverse primer) of the respective exon, 3µl genomic DNA and 16µl DEPC-Water. Thermocycling of most of these exons involved an initial denaturation step at 95°C for 5 mins followed by 35 cycles: denaturation at 95°C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 1 min, then a final extension step at 72°C for 5 mins.

The amplification of exons 14a, 14c and 14f was performed using a touch- down PCR which involved:

- Initial denaturation step at 94°C for 5 mins;
- 2 cycles: 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min;
- 2 cycles: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min;
- 2 cycles: 94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min;
- 35 cycles: 94°C for 30 sec, 53°C for 30 sec, and 72°C for 1 min;
- Final extension step at 72°C for 5 mins.

PCR products were loaded on 1% agarose gel, target fragments were excised and purified as described in the next section.

Table 8: Sequence and location of PCR primers used for analysis of *F8* gene exonic sequences.
The sequences of PCR primers were based on GenBank accession number NG011403.1.

Primer name	**Primer sequence 5'→3'**	**Length (bp)**	**Coordinate on *F8* gene**
HA 1F	TAG CAG CCT CCC TTT TGC TA	20	4921-4940
HA 1R	CTA ACC CGA TGT CTG CAC CT	20	5400-5381
HA 2F	CAT TAC TTC CAG CTG CTT TTT G	22	28059-28080
HA 2R	TTT GGC AGC TGC ACT TTT TA	20	28348-28329
HA 3F	GCA TGC TTC TCC ACT GTG AC	20	30529-30548
HA 3R	GCC ACC ATT ACA AAG CAC AC	20	30827-30808
HA 4F	CAT GTT TCT TTG AGT GTA CAG TGG	24	34516-34539
HA 4R	TTC AGG TGA AGG AAC ACA AAT G	22	34887-34866
HA 5F	TCT CCT CCT AGT GAC AAT TTC C	22	40347-40368
HA 5R	CCC ATC TCC TTC ATT CCT GA	20	40605-40586
HA 6F	GCG GTC ATT CAT GAG ACA CA	20	42863-42882
HA 6R	CCG AGC TGT TTG TGA ACT GA	20	43120-43101
HA 7F	TGT CCT AGC AAG TGT TTT CCA TT	23	58101-58123
HA 7R	AAT GTC CCC TTC AGC AAC AC	20	58500-58481
HA 8F	CAC CAT GCT TCC CAT ATA GC	20	60903-60922
HA 8R	ATG GCT TCA GGA TTT GTT GG	20	61386-61367
HA 9F	TTT GAG CCT ACC TAG AAT TTT TCT TC	26	61526-61551
HA 9R	GGT ATT TTA GAA ACT CAA AAC TCT CC	26	61825-61800
HA 10F	TTC TTG TTG ATC CTA GTC GTT TT	23	66499-66521
HA 10R	GCT GGA GAA AGG ACC AAC ATA	21	66748-66728
HA 11F	CCC TTG CAA CAA CAA CAT GA	20	70473-70492
HA 11R	TTT CTT CAG GTT ATA AGG GGA CA	23	70834-70812
HA 12F	TGC TAG CTC CTA CCT GAC AAC A	22	73617-73638
HA 12R	CAT TCA TTA TCT GGA CAT CAC TTT G	25	73914-73890
HA 13F	CAT GAC AAT CAC AAT CCA AAA TA	23	79747-79769
HA 13R	CAT GTG AGC TAG TGG GCA AA	20	80110-80091

Primer name	Primer sequence 5'→3'	Length (bp)	Coordinate on *F8* gene
HA 14aF	CTG GGA ATG GGA GAG AAC CT	20	95965-95984
HA 14aR	ATG TCC CCA CTG TGA TGG AG	20	96531-96512
HA 14bF	GAT CCA TCA CCT GGA GCA AT	20	96446-96765
HA 14bR	GGG CCA TCA ATG TGA GTC TT	20	97044-97025
HA 14cF	AGC TCA TGG ACC TGC TTT GT	20	96922-96941
HA 14cR	CAT TCT CTT GGA TTA ATG TTT CCT T	25	97616-97592
HA 14dF	TCC AAG CAG CAG AAA CCT ATT	21	97495-97515
HA 14dR	AGT AAT GGC CCC TTT CTC CT	20	98089-98070
HA 14eF	GGA TGA CAC CTC AAC CCA GT	20	97990-98009
HA 14eR	CCT TCC ACG AGA TCC AGA TG	20	98559-98540
HA 14fF	TCC CTA CGG AAA CTA GCA ATG	21	98508-98525
HA 14fR	TCA CAA GAG CAG AGC AAA GG	20	99193-99174
HA 15F	TGA GGC ATT TCT ACC CAC TTG	21	121089-121109
HA 15R	CCA AAA GTG GGA ATA CAT TAT AGT CA	26	121387-121362
HA 16F	CAG CAT CCA TCT TCT GTA CCA	21	122547-122567
HA 16R	AAA GCT TCT TAT TGC ACG TAG G	22	123014-122993
HA 17F	AGG TTG GAC TGG CAT AAA AA	20	123108-123127
HA 17R	CCC TGG ATC AAG TCT CAT TTG	21	123503-123483
HA 18F	TGG TGG AGT GGA GAG AAA GAA	21	123570-123590
HA 18R	AGC ATG GAG CTT GTC TGC TT	20	123931-123912
HA 19F	AAC CAA TGT ATC TCA TGC TCA TTT T	25	125489-125513
HA 19R	GGA AGA AAG CTG TAA AGA AGT AGG C	25	125736-125712
HA 20F	TTT GAG AAG CTG AAT TTT GTG C	22	126224-126245
HA 20R	GAA GCA TGG AGA TGG ATT CAT TA	22	126452-126430
HA 21F	CCA CAG CTT AGA TTA ACC TTT CTC A	25	127673-127697
HA 21R	TGA GCT TGC AAG AGG AAT AAG TAA	24	127933-127910
HA 22F	TCA GGA GGT AGC ACA TAC AT	20	131432-131451
HA 22R	GTC CAA TAT CTG AAA TCT GC	20	131718-131699
HA 23F	TTG ACA GAA ATT GCT TTT TAC TCT G	25	164432-164456
HA 23R	TCC CCC AGT CTC AGG ATA ACT	21	164725-164705
HA 24F	ACT GAG GCT GAA GCA TGT CC	20	165806-165825
HA 24R	CCC AAC CAC TGC TCT GAG TC	20	166055-166036
HA 25F	TGG GAA TTT CTG GGA GTA AAT G	22	167055-167076
HA 25R	AAG CTC TAG GAG AGG TGG TAT TTT T	25	167354-167330
HA 26F	CTG TGC TTT GCA GTG ACC AT	20	189909-189928
HA 26R	TTC TAC AAC AGA GGA AGT GGT GA	23	190465-190443

2.2.6.5. Purification of PCR amplicons from agarose gels

The DNA fragments were amplified from agarose gels, using the Gel Purification kit (Bioneer, S. Korea) according to the manufacturer's instructions. Briefly, three volumes of the gel binding buffer were added to gel slice, incubated at 60°C for 10 minutes and the mixture

was mixed every 3 minutes. One volume of absolute isopropanol was added to the mixture, mixed gently, the mixture was transferred to the DNA binding column tube and centrifuged for 1 minute at 13,000 rpm. The flow through was poured off and the DNA binding column was washed with 500 µL of buffer 2, centrifuged for 1 minute at 13,000 rpm, the filtrate was poured off and this step was repeated twice. The DNA binding column was dried by centrifugation at 13,000 rpm for 3 minutes to remove the residual propanol. The DNA binding filter was transferred to a new 1.5 mL micro-centrifuge tube, 45 µL of elution buffer were added to the center of the binding column, incubated at least 2 minutes at room temperature, and then the DNA fragment was eluted by centrifugation at 13,000 rpm for 2 minutes. Purified DNA fragments were used for DNA sequencing.

The quality and quantity of the purified amplicons were assessed by running of 1.5% agarose gel as well as determination of concentration and A260/280 ratio spectrophotometrically.

2.2.6.6. DNA sequencing

The purified amplicons were sequenced using the Forward or/and reverse primers. For this purpose, the PCR products along with either forward or reverse primers were sent for DNA sequencing to Hy-labs laboratories in Jerusalem. The DNA sequencing was performed using Sanger sequencing on Genetic Analyzer AB3700 (Applied Biosystems, Foster City, CA, USA). The DNA sequence results were analyzed visually and then using the BLAST bioinformatics tool.

2.2.7. Ethical consideration

The principles of Helsinki declaration were applied throughout this study. An informed consent was obtained from individual participants or their guardians in case of minors. The study was approved by the Palestinian Ministry of Health.

Chapter three

Results

3.1. Study samples

This study aimed to determine the spectrum of *F8* gene mutations among hemophilia patients as well as to provide necessary genetic information for developing a laboratory test for screening for *F8* gene mutations in the West Bank region, Palestine. In order to achieve this, the medical files or patients' records at the Ministry of Health and Palestinian Society for Bleeding Disorders in the West Bank were reviewed to collect data about Hemophilia A patients. Two hundred potential cases were found, all patients were contacted and asked to participate in this study and 79 patients representing 49 unrelated families accepted to participate in this study. The rest of the patients either declined to participate or their contact information were not valid.

Table 9 shows the general characteristics of the study cases. The patients' age ranged from one year to 62 years with a mean 19.1 years. The study cases represent a young population, where 45 patients (56.9 %) were below 20 years old and 34 patients (43.1%) were above 20 years old.

Table 9: General characteristic of the 79 hemophilia A patients.

Variable		No.	Percentage (%)
Gender	Males	73	92.4
	Females	6	7.6
Age group (in years)	0.1 – 10	20	25.3
	11 – 20	25	31.6
	21 – 30	18	22.8
	31 – 40	9	11.4
	41 – 50	4	5.1
	51 – 60	2	2.5
	61 – 70	1	1.3

The geographic distribution of hemophilia A cases in the West Bank is shown in Figure 10. HA cases were collected from 7 governorates. The highest number of cases has been collected from Hebron and Ramallah districts, respectively.

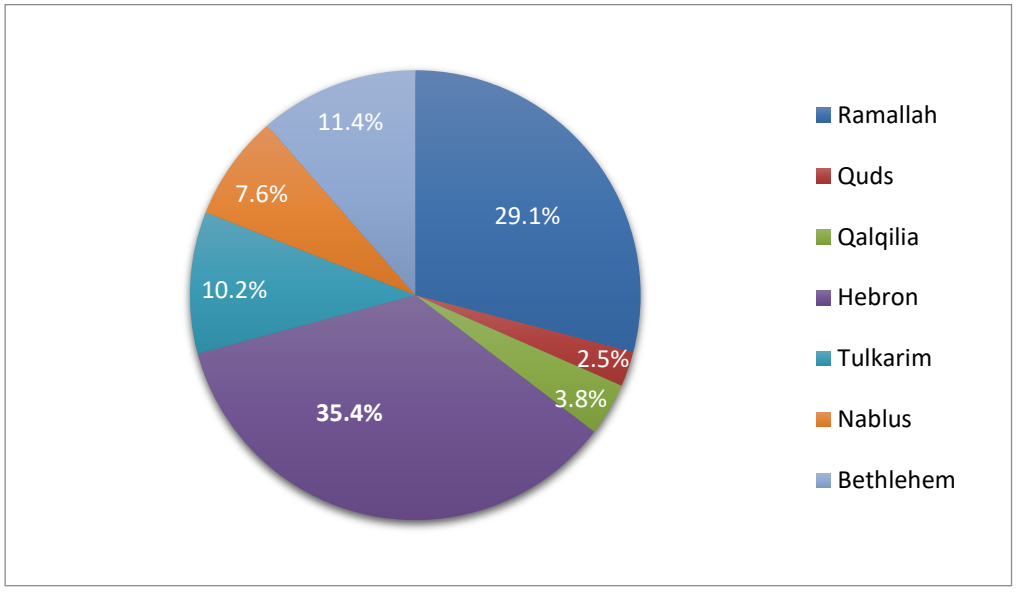


Figure 10: Distribution of hemophilia A cases based on residence place.

3.2. Clinical findings

Analysis of the study populations showed that most patients received on demand-treatment (86.3%) and a small proportion received (13.7%) prophylactic treatment with coagulation factor FVIII concentrates. Among the severe HA patients (n=54), 45 patients (83%) received on-demand treatment and 9 patients (17%) were on prophylactic treatment. On the other hand, from the 19 patients with mild or moderate HA, 18 patients (95%) were receiving on-demand treatment.

As for the inheritance of HA, 56 (76.7%) HA patients have a family history of HA. In contrast, 17 (23.3%) patients have family history of HA which indicates that they have had a *de novo* mutation.

Assessment of the general status of HA patients, revealed that 31 HA patients (42.5%) have Hb levels below the reference range of their age group and thus are considered anemic (WHO, 2011). Additionally, 34 patients (46.6%) of HA patients have joint problems and 16 patients (21.9%) have bone surgery.

Analysis of the age at diagnosis of HA patients, revealed that 38 severe HA patients (38/54=70.4%) were diagnosed during their first year of life and 16 severe HA patients (29.6%) were diagnosed after the first year of age. In contrast, 10 from 19 patients with mild and moderate HA (52.6%) were diagnosed during their first year of life and the rest (9 patients; 47.4%) were diagnosed after the first year of age.

3.3. Hematological findings

The primary coagulation tests for HA patients (73/79) showed that their platelets count and PT values fall within the reference ranges, while APTT values for these patients were prolonged

with a mean of 87 seconds (median= 85; range = 46-144). However, carrier females (6/79) showed normal platelets count (mean= 236; median = 224 and range was 185-305), PT (mean = 12.4; median = 11.9 and range = 10.6-15.2) and APTT (mean= 41, median= 40, range = 37-45). Depending on both FVIII activity level and *F8* gene genotype, HA patients (73/79) were clinically grouped into severe, moderate and mild hemophilia A (Table 10).

Table 10: Grouping of hemophilia A patients based on disease severity.

Disease severity	No. of cases, n (%)	FVIII:C %		
		Mean	Median	Range
Severe	54 (74.0%)	1%	1%	2
Moderate	9 (12.3%)	3%	3%	2
Mild	10 (13.7%)	12%	8%	37
Total	73* (100%)			

*This number represent the HA patients excluding the 6 carrier females.

3.4. Genotyping of Hemophilia A patients

Null mutations or gross rearrangements of *F8* gene such as inv22 and inv1 are associated severe hemophilia A. Thus, all HA patients with FVIII: C <5% were screened first for inv22 and if negative for inv22 were further tested for inv1. Samples negative for both inv22 and inv1, were then screened for other mutations by DNA sequencing.

3.4.1. Intron22 and intron1 inversions

Severe cases of HA were first tested for inv22 using IS-PCR. However, this method did not give a successful result in few cases and thus another method (nested LD-PCR) was used as described in the next paragraph.

Out of 22 HA severe cases that were tested for inv22 using IS-PCR, eight HA cases were positive for inv22, while 14 cases were negative for inv22. A representative agarose gel photograph for IS-PCR results is shown in Figure 11.

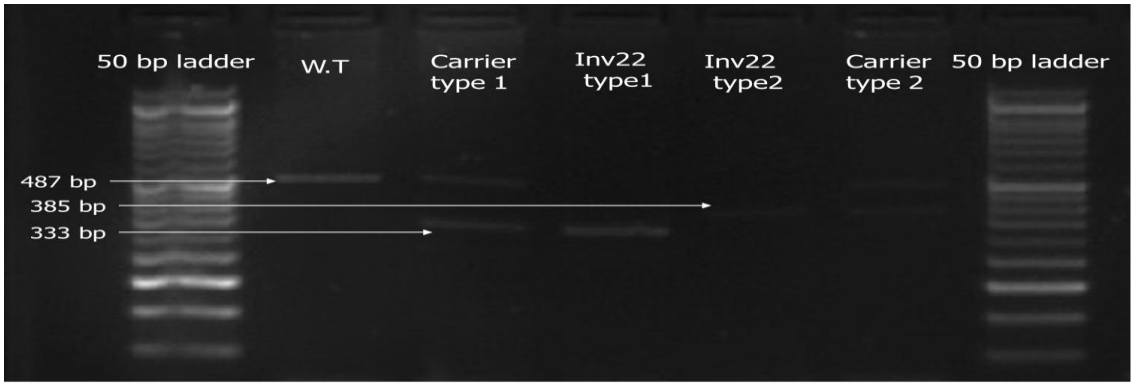


Figure 11: Representative agarose gel for inv22 by IS-PCR method.

The NLD-PCR was used to test all severe HA patients (54 patients) for inv22. Samples analyzed with IS-PCR were mostly repeated using the NLD-PCR for confirmation and as a quality control measure for the NLD-PCR method. The two methods showed concordant results.

In addition, four carrier females who were mothers for severe HA patients were tested for inv22. As a negative control, three healthy individuals were included in the testing for inv22.

Taken altogether, thirty-one (31/54; 57.4%) patients with severe HA were positive for inv22, while 23 patients with severe HA were negative for inv22. Among the carrier females, three were positive and three were negative for inv22. Representative agarose gel for inv22 results using NLDPCR is shown in Figure 12.

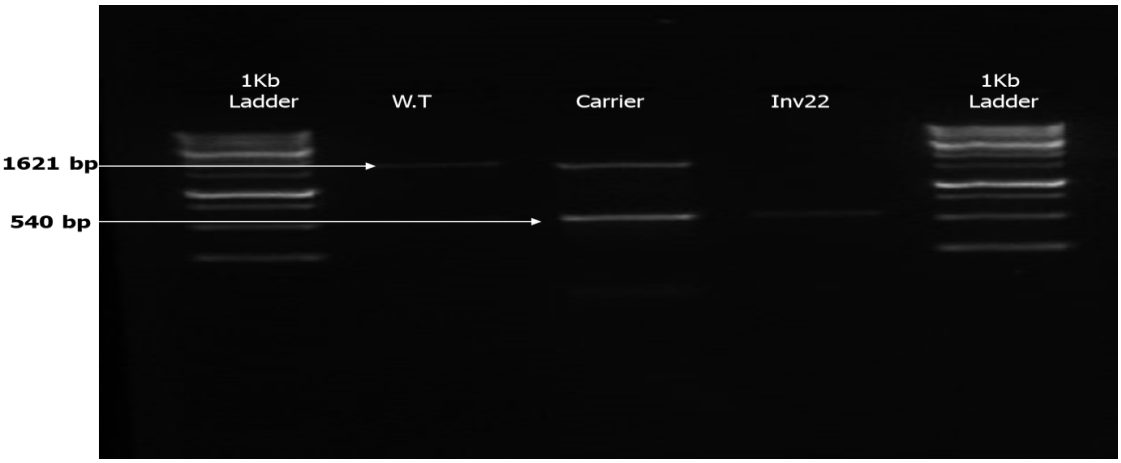


Figure 12: Representative agarose gel for inv22 results obtained using NLDPCR method.

Severe HA patients who were negative for inv22 were then tested for inv1 of *F8* gene using two multiplex PCRs, one for the int1h-1 and the second for int1h-2. Two patients were positive for inv1 with a percentage of 3.7% (2/54) among all HA severe cases. Additionally, one carrier female (the mother of two patient with inv1) was also found to be positive for inv1 mutation. Two female carriers were negatives for both inv22 and inv1 mutations. Figure 13 represents an agarose gel electrophoresis for inv1 results by multiplex PCR method.

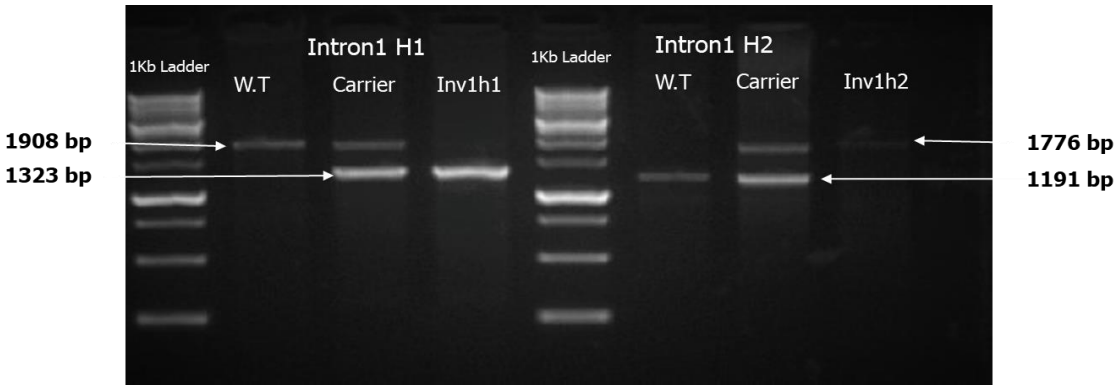


Figure 13: Representative agarose gel for inv1 results obtained by the multiplex PCR method.

Table 11 summarizes the characteristics of all severe HA patients as well we their inv22 and inv1 genotyping results. Additionally, Table 12 summarizes the characteristics of moderate and mild HA patients.

Table 11: The characteristics of all severe HA patients.

Sample code	FVIII: C (%)	Treatment type	Family history	Age at diagnosis (years)	Inv22	Inv1
R01	1%	On-demand	No	4.0	Neg	Neg
R03	2% *	On-demand	Yes	NA	**Pos**	------
R04	1%	On-demand	Yes	0.16	**Pos**	------
R05	2%*	On-demand	Yes	0.1	**Pos**	Neg
R06	1%	On-demand	Yes	0.1	Neg	Neg
R07	1%	Prophylaxis	Yes	0.3	Neg	Neg
R08	1%	On-demand	No	3.0	**Pos**	------
R09	1%	On-demand	Yes	0.75	Neg	Neg
R10	1%	On-demand	No	11.0	Neg	------
R12	1%	Prophylaxis	No	2.0	Neg	Neg
R13	1%	On-demand	No	1.0	Neg	Neg
R14	1%	On-demand	No	0.1	Neg	Neg
R20	1%	On-demand	Yes	NA	Neg	Neg
R21	1%	On-demand	Yes	NA	Neg	Neg
R22	1%	On-demand	Yes	NA	Neg	Neg
R23	1%	On-demand	Yes	2.0	**Pos**	------
T01	1%	On-demand	No	0.1	**Pos**	------
T02	2%*	On-demand	Yes	0.1	**Pos**	------
T03	1%	On-demand	Yes	0.1	**Pos**	------
T04	2%*	On-demand	Yes	17	**Pos**	Neg
T06	1%	On-demand	No	0.1	Neg	Neg
T07	1%	On-demand	No	1.0	Neg	Neg
T08	2%*	On-demand	Yes	0.1	**Pos**	------
BL01	1%	Prophylaxis	Yes	1.5	**Pos**	------
BL02	3%*	Prophylaxis	Yes	0.1	**Pos**	------
BL03	1%	Prophylaxis	Yes	0.1	**Pos**	------
BL04	1%	Prophylaxis	Yes	0.1	**Pos**	------
BL05	1%	Prophylaxis	Yes	0.75	Neg	Neg
BL07	2%*	Prophylaxis	Yes	7.0	**Pos**	Neg
N04	2%*	On-demand	Yes	34	**Pos**	------
Q03	1%	On-demand	Yes	0.5	**Pos**	------
QL04	1%	On-demand	Yes	NA	**Pos**	------
QL05	1%	On-demand	Yes	0.1	**Pos**	------
QL06	1%	On-demand	Yes	0.1	**Pos**	------
H01	1%	On-demand	Yes	NA	**Pos**	------
H02	1%	On-demand	Yes	1.0	Neg	Neg

*****Depending on *F8* genotyping. NA: not available.

Table 11: continued.

Sample code	FVIII: C (%)	Treatment type	Family history	Age at diagnosis (years)	Inv22	Inv1
H03	1%	On-demand	Yes	1.0	**Pos**	------
H04	1%	On-demand	Yes	0.1	**Pos**	------
H05	2%*	On-demand	Yes	0.25	**Pos**	------
H06	1%	On-demand	Yes	0.1	**Pos**	------
H07	2%*	On-demand	Yes	0.1	**Pos**	------
H08	1%	On-demand	Yes	0.1	**Pos**	------
H09	1%	On-demand	Yes	0.1	Neg	Neg
H11	1%	On-demand	Yes	0.1	**Pos**	------
H13	2% *	On-demand	Yes	0.1	**Pos**	------
H14	1%	On-demand	Yes	0.6	**Pos**	------
H16	1%	On-demand	Yes	1.0	Neg	**Pos**
H18	2%*	Prophylaxis	Yes	0.1	**Pos**	------
H20	1%	On-demand	Yes	NA	Neg	Neg
H21	1%	On-demand	Yes	0.1	Neg	Neg
H25	1%	On-demand	Yes	0.1	Neg	Neg
H28	1%	On-demand	Yes	0.1	Neg	Neg
H29	1%	On-demand	Yes	0.1	Neg	Neg
H30	1%	On-demand	Yes	0.1	Neg	**Pos**

*Depending on *F8* genotyping. NA: not available.

Table 12: The characteristics of both moderate and mild HA patients.

Sample code	FVIII: C (%)	Treatment Type	Family history	Age at diagnosis (years)	Disease severity
R02	3%	On-demand	No	NA	Moderate
R18	NA	On-demand	No	2.0	Moderate
N01	3%	On-demand	No	NA	Moderate
N02	2%	On-demand	Yes	0.3	Moderate
N03	3%	On-demand	Yes	2.0	Moderate
N05	4%	On-demand	Yes	25.0	Moderate
N06	3%	On-demand	Yes	2.0	Moderate
H17	3%	On-demand	Yes	1.0	Moderate
H22	2%	On-demand	Yes	0.1	Moderate
R15	8%	On-demand	No	0.16	Mild
R16	9%	On-demand	No	14.0	Mild
R17	6%	On-demand	No	0.1	Mild
R19	6%	On-demand	No	0.1	Mild
T05	11%	On-demand	No	0.1	Mild
BL06	43%	Prophylaxis	Yes	0.1	Mild
Q02	13%	On-demand	Yes	4.0	Mild
H15	8%	On-demand	Yes	0.1	Mild
H23	6%	On-demand	Yes	0.1	Mild
H24	8%	On-demand	Yes	NA	Mild

Severe HA sample patients (21/54) who were negative for both inv22 and inv1, are further investigated for the presence of other *F8* gene mutations using Sanger DNA sequencing alongside moderate and mild hemophilia patients (see next section).

3.4.2. Detection of *F8* gene mutations by DNA sequencing

HA samples that where negative for inv22 and inv1 rearrangements could have one of over 1200 mutations reported in the *F8* gene as well as novel mutations. Thus, the Sanger DNA sequencing was used to screen these samples. . For this purpose, individual exons including exon/intron junctions were amplified by PCR and subjected for DNA sequencing.

The DNA sequencing strategy was to sequence one exon in a group of 10 samples concurrently. The results of DNA sequencing are not yet finished, but the preliminary results are summarized in (Table 13). In the first group of samples (n=10) that were screened for *F8* gene mutations, exons 3, 8, 11, 14 and 15 were almost completely screened. These exons were chosen based on the incidence of *F8* gene mutations in neighboring countries (Awidi et al., 2010; Khayat et al., 2008). So far, only one sample has been completely screened and the disease-causing mutation has been identified (sample R02; Table 13). In another 4 samples, so far 3 harmless SNPs were identified as detailed in Table 14, but the disease-causing mutation/s has/have not been determined yet.

The p.Gly130Arg mutation causes an amino acid substitution in A1 domain of FVIII protein and is associated with a severe phenotype of HA. It affects the splice site at the exon3 /intron 3 junction and disturbs normal splicing. This mutation has been described earlier in the CHAMP database and also reported in other countries including China (He et al., 2013; Tariq Masood Khan & Sohail Taj, 2019).

Table 13: Description of *F8* gene mutations detected in HA patients in this study excluding inv22 and inv1.

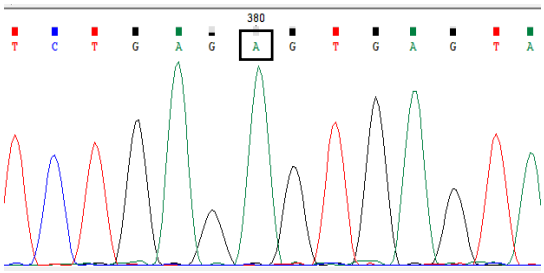
Sample code	Nucleotide change[1]	Amino acid change[2]	Exon #/ protein domain[2]	Novel mutation/ SNP	No. of patients (No. of families)[4]	Phenotype / effect on protein structure[3]	Phenotype in CHAMP db	Known risk of inhibitor development	Family history
R02	c.388G>A	p.Gly130Arg	3 / A1	No	1 (1)	Moderate / DC[3]	Severe	No	No

[1]Nucleotide location refers to HGVS cDNA reference sequence NM_000132.3 and conforms to the convention that "A" in the initiation codon ATG=+1. [2]Amino acid numbering is based on HGVS protein (preproprotein, 2351 amino acids) reference sequence NP_000123.1. [3]Refers to effect of the described change on protein structure as predicted by PolyPhen-2 and Mutation Tasters bioinformatics tool and could be disease causing (DC) or benign (harmless). [4]Preliminary data obtained from a small group of the study samples and thus are not finalized.

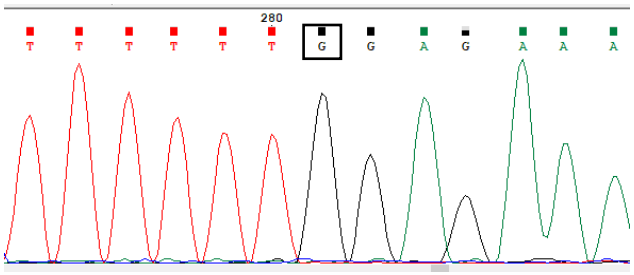
Table 14: Description of neutral *F8* gene SNPs detected in HA patients in this study.

Sample code	Nucleotide change[1]	Amino acid change[2]	Exon #/ protein domain[2]	Novel SNP	Effect on protein structure[3]	No. of patients (No. of families)[4]	CHAMP db info CHAMP	Disease causing mutations in same patient
H18	c.3864A>C	p.Ser1288Ser	14/ B	Yes	Benign	1 (1)	Not listed	Inv22
N01, N03	c.3780C>G	p.Asp1260Glu	14/ B	Yes	Benign	2 (2)	Not listed	ND
R15, R16	c.2511T>C	p.Asp837Asp	14/ B	Yes	Benign	2 (2)	Not listed	ND

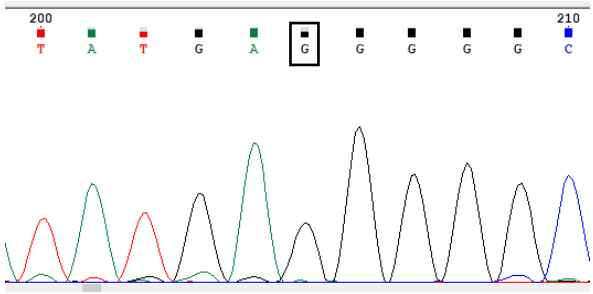
[1]Nucleotide location refers to HGVS cDNA reference sequence NM_000132.3 and conforms to the convention that "A" in the initiation codon ATG=+1. [2]Amino acid numbering is based on HGVS protein (preproprotein, 2351 amino acids) reference sequence NP_000123.1. [3]Refers to effect of the described change on protein structure as predicted by PolyPhen-2 and Mutation Tasters bioinformatics tools and could be disease causing (DC) or benign (harmless). [4]Preliminary data obtained from a small group of the study samples and thus are not finalized. ND: not determined yet.



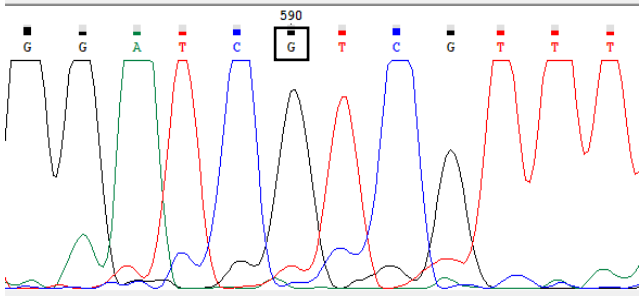
A.



B.



C.



D.

Figure14: Representative chromatograms of identified mutations and SNPs. A: Sample R02, p.Gly130Arg G>A; Disease causing mutation. **B**: Sample H18; c.3864A>C, p.Ser1288Ser.Antisense strand. **C**: Sample N01, p.Asp1260Glu, c.3780C>G. Sense strand. **D:** Sample R15, c.2511T>C, p.Asp837Asp. Antisense strand.

Chapter four

Discussion

Hemophilia A is the most severe and most common inherited bleeding disorders. Studies of the molecular basis of HA from many countries around the world have yielded a remarkable heterogeneity of mutations affecting *F8* gene. More than 1200 types of FVIII mutation have been reported. Among these, the most common defect is an inversion in intron 22 and intron 1. Intron 22 inversion is detected in 40–50% of the severe HA patients, while inversion of intron 1 is found in 2-5% (Bagnall et al., 2002; Bowen, 2002). Aside from these two inversions, a large number of mutations are possible such as point mutations (missense, nonsense and splice site) and frame shifts (deletions and duplications) (Tariq Masood Khan & Sohail Taj, 2019). The aim of this research is to study the molecular genotyping and laboratory analysis of hemophilia A in the West Bank region, in order to provide a proper diagnosis and management of patients.

The medical records of the Ministry of Health and the Palestinian society for Bleeding Disorders, showed that there are 200 cases of HA. All potential cases were contacted but only 79 cases responded and were included in this study. The rest of the patients either declined to participate in the study or their contact address were not valid, emphasizing the need for good file`s reassessment and patient's information to be updated.

A total of (79) HA patients were investigated in this study. HA patients were recruited from 7 governorates, with the highest numbers being recruited from Hebron (35.4%), followed by Ramallah (29.1%) (Figure 10). The average age of HA patients was 19.1 years old and more than half of them (56.9%) were less than 20 years old indicating these patients are mostly youth population. Thus, further attention must be focused in this group of patients in an attempt to improve their life-expectancy and quality of life.

Coagulation studies on HA patients from Palestine showed that all have normal platelets count and PT, while APTT was typically prolonged with a mean of 87 seconds. These findings are consistent with the typical picture for HA patients (Sadaria, 2016).

Depending on both FVIII: C activity levels and genotyping of *F8* gene, 54 (74%) cases were grouped as severe, 9 (12.3%) cases as moderate and 10 (13.7%) as mild HA cases. HA patients with severe phenotype needs regular medical treatment and in need for regular FVIII substitution therapy, thus they are mostly registered and reported in the medical files of the local health care authorities. However, HA patients with moderate phenotype are less frequently reported in the medical files, while mild cases of HA are the least group of HA patients reported. Indeed, mild HA cases are usually underestimated in most studies (Benson et al., 2018; Srivastava et al., 2013) due to the fact that they can lead to normal life and they are recognized only when they experience a major surgery or trauma. Thus, more efforts are needed to properly diagnose patients with mild bleeding disorders which will avoid any disease complications when they experience major trauma or surgery.

Mutations affecting the *F8* gene are heterogeneous and so far, more than 1200 mutations have been reported in this gene. Among these mutations, inv22 is the most common mutation affecting HA patients. Indeed, 40-50% of severe cases of HA have inv22 and approximately 2-5% have inv1 (Bagnall et al., 2002; Bowen, 2002).

In this study, inv22 was detected in 31 of HA severe patients (57.4%). This frequency is similar to that reported in Saudi Arabia (50%) (Owaidah et al., 2009), as well as among HA sever patients in Jordan (52%) (Awidi et al., 2010), and among Arab patients with severe hemophilia A (55%) (Abu-Amero et al., 2008). The frequency of inv22 in this study is higher than frequencies reported in Lebanon (29%) (Khayat et al., 2008), Tunisia (22.7%) (Elmahmoudi et al., 2012) and in Iraq (28%) (Hassan & Jabber, 2019). Additionally, the frequency of inv22 in this research is markedly higher than that reported in a recent study in West Bank region (37%) (Abu Arra et al., 2020). This difference may be explained partially by the criteria set for testing samples for inv22, where in this study all HA cases with FVIII:C <5 % (Sherief et al., 2020) were tested for inv22. From 18 HA patients previously categorized

as moderate HA patients (based on the medical files of these patients), 12 of them were positive for inv22 positive. The grouping of these 12 samples as moderate HA can be due to erroneous judgment either due to inaccuracy of FVIII: C testing or patients were tested after a short time of FVIII substitution therapy. The true classification of HA cases is better established directly after first diagnosis and before starting the FVIII substitution therapy. Once FVIII substitution therapy is established it might be difficult to correctly determine disease severity (Srivastava et al., 2013; WHF, 2007) . Table 15 compares the frequency of *F8* gene inv22 in this study to neighboring countries as well as worldwide. The frequency of inv22 shows a wide variation ranging from 22.7% in Tunisia to 57% in this study. This variation can be attributed to different inclusion criteria for severe HA cases as well as to the different representation of HA cases in the study cohort. Since most of studies that tackled this issue were case-series studies and thus the recruitment of cases was neither inclusive nor representative.

Table 15: Frequencies of inv22 in HA patients worldwide

Country	**Frequency of *F8* gene inv22**	**Reference**
Palestine (West bank)	57.4 %	Current study
Palestine (West bank)	37%	(Abu Arra et al., 2020)
Jordan	52%	(Awidi et al., 2010)
Lebanon	29%	(Khayat et al., 2008)
Saudi Arabia	50%	(Owaidah et al., 2009)
Iraq	28%	(Hassan & Jabber, 2019)
Iraqi Kurdish	46.7%	(Abdulqader et al., 2020)
Egypt	46.1	(Abou-Elew et al., 2011)
Tunisia	22.7%	(Elmahmoudi et al., 2012)
Arabs	55%	(Abu-Amero et al., 2008)
Turkey	42%	(El-Maarri et al., 1999)
Iran	47%	(Roozafzay et al., 2013)
Pakistan	44%	(Muhammad Khan et al., 2014)
South Korea	39.5 %	(Hwang et al., 2009)
France	47%	(Repessé et al., 2007)
Moldavia	30.9%	(Sirocova et al., 2009)
Albania	10.5%	(Castaman et al., 2007)
Mexico	47.2 %	(Luna-Záizar et al., 2018)
Hungary	52%	(Andrikovics et al., 2003)

Of the 4 HA carrier mothers included in this study, three were positive and one was negative for inv22. Since hemophilia is an X linked disease, these mothers have two X chromosomes, one of them is abnormal because it carries mutant *F8* gene and passed it to their sons. The other X chromosome is normal and responsible for production of functional FVIII protein. Carrier females usually have a 50% activity of FVIII, but may range also from 40% to 60% (Plug et al., 2006). The FVIII: C for one of these mothers was 100% (she is a mother of two HA severe patients), this remarkable represents an extreme case of X-chromosome lyonization. In carrier females, the FVIII level represent a balance between the normal X chromosome and the abnormal X chromosome carrying the mutated *F8* gene (Heard, 2004). Two female carriers were negatives for both inv22 and inv1mutations, this indicate that they have other mutation in their *F8* gene which need to be investigated by DNA sequencing.

In this study, two patients were positive for inv1 with a percentage of 3.7% among all HA severe cases. This finding is consistent with previous reports that reported inv1 among 2-5% of severe HA cases (Bagnall et al., 2002; Rossetti et al., 2004). The frequency of inv1 was 1.8% in Saudi Arabia (Al -Allaf et al., 2016), 2% in Jordan (Awidi et al., 2010), and 3.3 % in Iraqi Kurdish severe HA patients (Abdulqader et al., 2020). In our study, one carrier mother was positive for inv1 mutation (she is a mother of the two HA severe patients who were inv1 positives).

Taken altogether, inv22 and inv1 were found in 33 severe HA cases and thus the disease-causing mutation in *F8* gene in the remaining 40 patients remains to be determined using DNA sequencing. For this purpose, we have started to screen samples for *F8* gene mutations using Sanger DNA sequencing. So far, we have completely sequenced one sample and found the disease causing mutation (p.Gly130Arg). The mutation p.Gly130Arg is an amino acid substitution affecting the A1 domain of FVIII protein and disturbs normal splicing as it occurs close to the exon 3/ intron 3 junction. This mutation has been reported previously and is listed in CHAMP database for *F8* gene mutations. The process for detecting *F8* gene mutations in the rest of HA cases (n=39) is still progressing and we hope to complete it soon.

The DNA sequencing results have been also identified three neutral *F8* gene SNPs. Also, these SNPs are neutral as determined using bioinformatics tools (Mutation Tasters 2), they

may useful for future studies that tend to track the origin of certain *F8* gene mutations or population genetic analysis.

The results of this study showed that both inv22 and inv1 are the main causative mutations in severe HA. The genotype–phenotype correlation of the inv22 and inv1 mutations in the investigated patients with HA showed that, among the severe cases, 57.4% (31/54) had inv22 and 3.7% (2/54) had inv1. Inv22 and inv1 mutations are recognized as large structural rearrangements in the FVIII gene that interfere with the production of full-length FVIII mRNA thus leading to severe disease (Andrikovics et al., 2003).

In conclusion, 73 HA cases and 6 carrier females were included in this study. Only 13.7% HA patients receive prophylactic FVIII replacement therapy and the rest (86.3%) receive on-demand treatment. Most patients (76.7%) have a family history of HA and 23.3 % have no family history of HA. Based on disease severity, samples were grouped as severe (n=54), moderate (n=9) and mild (n=10) HA. *F8* gene inv22 was found in 31 severe HA cases (57.4%) and inv1 was found in 2 severe HA cases (3.7%). The disease-causing mutation in *F8* gene in the rest of HA cases remains to be determined by DNA sequencing.

Recommendations

- Determine FVIII inhibitor antibody titer and examine its correlation to disease severity.
- Determine the disease-causing mutation in *F8* gene in samples that were negative for inv22 and inv1.
- Establish the disease severity in HA patients directly after first diagnosis.
- Establish a national registry for HA patients and well-organized medical files with regular updates. This should facilitate health management of HA patients.
- Determine the FVIII inhibitor development and its titer in HA patients after the first couple of FVIII replacement doses. Since these patients needs special treatment to induce immune tolerance and avoid disease complications.

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التنميط الجيني الجزيئي والتحليل المخبري للهيموفيليا(أ) في الضفة الغربية–فلسطين

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الملخص

خلفية البحث: الهيموفيليا(أ) هو مرض نزف الدم المرتبط بالجنس الناتج عن نقص أو خلل في عامل التخثر الثامن وهذا العامل هو بروتين سكري في البلازما ويلعب دورًا مهمًا في عملية تجلط الدم .يصيب مرض الهيموفيليا واحد من كل 5000من المواليد الذكور .تم التعرف على أكثر من 1200 طفرة في جين العامل الثامن والتي ارتبطت في مرض الهيموفيليا (أ) .تتنوع هذه الطفرات من استبدال النوكليوتيدات المفردة إلى عمليات الحذف / الإدراج والانعكاس الإجمالي . يمثل انعكاس انترون 22 وانعكاس انترون 1 حوالي50-40 % و5-2% من حالات الهيموفيليا (أ) الحادة على التوالي .

الأهداف :هدفت هذه الدراسة إلى تحديد النمط الجيني لجين العامل الثامن وإجراء الفوصات المخبرية للهيموفيليا(أ) في الضفة الغربية، من أجل توفير التشخيص والعلاج المناسبين للمرضى .

طرق البحث: تمت دراسة ما مجموعه 79 حالة من حالات الهيموفيليا (أ) كان منهم 73ذكور و 6إناث من 49 عائلة مختلفة لا تجمعها قرابة. تم إجراء فحوصات الدم وفحص التخثر لكل مريض وشملت :تعداد الدم الكامل(CBC) ، وقت الثرومبوبلاستين الجزئي (PT) ووقت الثرومبوبلاستين الجزئي المنشط (APTT) .تم إجراء قياس مستوى الدم من العامل الثامن (FVIII: C) باستخدام مقايسة التخثر على مرحلة واحدة .تم تحضير الحمض النووي الجيني DNA عالي الوزن الجزيئي باستخدام طريقة التمليح .تم إجراء تفاعل البلمرة المتسلسل المتداخل (NLD-PCR) للكشف عن انعكاس الانترون 22 (Inv22) لجميع حالات مرضى الهيموفيليا (أ) الحادةوكذلك الأمهات الحاملات للصفة الوراثية لمرض الهيموفيليا، بينما تم استخدام تفاعل البلمرة المتعدد (Multi-plex PCR)للكشف عن انعكاس انترون 1 (Inv1). تم استخدام تسلسل الحمض النووي DNA Sequencing لتحليل الطفرات الجينية لجين العامل الثامن لمرضى الهيموفيليا (أ) ذوي الحالات المتوسطة والخفيفة وكذلك لأولئك الذين كانوا سلبيين لكل من inv22 وinv1 .

النتائج :اعتمادًا على كل من مستويات الدم للعامل الثامن (FVIII:C) والتنميط الجيني لجين العامل الثامن، فقد تم تصنيف 54 (74%) حالة على انها من النوع الحاد بينما 9 حالات (12.3%) هي من النوع المتوسط وعشر حالات (13.7%) من النوع الخفيف. تحليل inv22 بواسطة NLD-PCRأظهر أن57.4 % من مرضى ال(أ)هيموفيليا الحاد لديهم هذه الطفرة،

بينما أظهر تحليل inv1 بواسطة تفاعل البلمرة المتعدد (Multi-plex PCR) أن مريضين كانا موجبين لـ inv1 بنسبة 3.7% بين جميع حالات الهيموفيليا من النوع الحاد.

تم فحص عينة واحدة بالكامل وتم تحديد الطفرة المسببة للمرض، وهي (c.388G> A) التي تؤدي إلى استبدال الحمض الأميني الجلايسين في الكودون 130 بالأرجينين (Gly130Arg) في المنطقة A1من بروتين العامل الثامن. في 4 عينات أخرى، تم تحديد ثلاثة من الطفرات الغيرمؤذية .يتلقى حوالي 3.7 % فقط من مرضى الهيموفيليا العلاج الوقائي البديل لعامل التخثر الثامن ويتلقى الباقي 86.3% العلاج عند الطلب .معظم مرضى الهيموفيليا76.7.% ليس لديهم تاريخ عائلي للمرض بينما 23.3% لديهم تاريخ عائلي للمرض.

خاتمة: نسبةوجود انعكاس الانترون22 وانعكاس الانترون1 للعامل الثامن في هذه الدراسة كان 57.4% و3.7% على التوالي في حالات الهيموفيليا من النوع الحاد. 23.3% من مرضى الهيموفيليا ليس لديهم تاريخ عائلي للمرض وبالتالي فإن ظهور المرض لديهم يُعزى إلى طفرات جديدة. هناك حاجة لاجراء المزيد من الفحوصات للحالات التي كانت سلبية ل inv22و inv1عن طريق تسلسل الحمض النووي DNA sequencingللكشف عن الطفرات المسببة للمرض.

Appendix 1

List of Primers

Primer name	Primer sequence 5'→3'	Length (bp)
HA 1F	TAG CAG CCT CCC TTT TGC TA	20
HA 1R	CTA ACC CGA TGT CTG CAC CT	20
HA 2F	CAT TAC TTC CAG CTG CTT TTT G	22
HA 2R	TTT GGC AGC TGC ACT TTT TA	20
HA 3F	GCA TGC TTC TCC ACT GTG AC	20
HA 3R	GCC ACC ATT ACA AAG CAC AC	20
HA 4F	CAT GTT TCT TTG AGT GTA CAG TGG	24
HA 4R	TTC AGG TGA AGG AAC ACA AAT G	22
HA 5F	TCT CCT CCT AGT GAC AAT TTC C	22
HA 5R	CCC ATC TCC TTC ATT CCT GA	20
HA 6F	GCG GTC ATT CAT GAG ACA CA	20
HA 6R	CCG AGC TGT TTG TGA ACT GA	20
HA 7F	TGT CCT AGC AAG TGT TTT CCA TT	23
HA 7R	AAT GTC CCC TTC AGC AAC AC	20
HA 8F	CAC CAT GCT TCC CAT ATA GC	20
HA 8R	ATG GCT TCA GGA TTT GTT GG	20
HA 9F	TTT GAG CCT ACC TAG AAT TTT TCT TC	26
HA 9R	GGT ATT TTA GAA ACT CAA AAC TCT CC	26
HA 10F	TTC TTG TTG ATC CTA GTC GTT TT	23
HA 10R	GCT GGA GAA AGG ACC AAC ATA	21
HA 11F	CCC TTG CAA CAA CAA CAT GA	20
HA 11R	TTT CTT CAG GTT ATA AGG GGA CA	23
HA 12F	TGC TAG CTC CTA CCT GAC AAC A	22
HA 12R	CAT TCA TTA TCT GGA CAT CAC TTT G	25
HA 13F	CAT GAC AAT CAC AAT CCA AAA TA	23
HA 13R	CAT GTG AGC TAG TGG GCA AA	20
HA 14aF	CTG GGA ATG GGA GAG AAC CT	20
HA 14aR	ATG TCC CCA CTG TGA TGG AG	20
HA 14bF	GAT CCA TCA CCT GGA GCA AT	20
HA 14bR	GGG CCA TCA ATG TGA GTC TT	20
HA 14cF	AGC TCA TGG ACC TGC TTT GT	20
HA 14cR	CAT TCT CTT GGA TTA ATG TTT CCT T	25
HA 14dF	TCC AAG CAG CAG AAA CCT ATT	21
HA 14dR	AGT AAT GGC CCC TTT CTC CT	20
HA 14eF	GGA TGA CAC CTC AAC CCA GT	20
HA 14eR	CCT TCC ACG AGA TCC AGA TG	20
HA 14fF	TCC CTA CGG AAA CTA GCA ATG	21
HA 14fR	TCA CAA GAG CAG AGC AAA GG	20

HA 15F	TGA GGC ATT TCT ACC CAC TTG	21
HA 15R	CCA AAA GTG GGA ATA CAT TAT AGT CA	26
HA 16F	CAG CAT CCA TCT TCT GTA CCA	21
HA 16R	AAA GCT TCT TAT TGC ACG TAG G	22
HA 17F	AGG TTG GAC TGG CAT AAA AA	20
HA 17R	CCC TGG ATC AAG TCT CAT TTG	21
HA 18F	TGG TGG AGT GGA GAG AAA GAA	21
HA 18R	AGC ATG GAG CTT GTC TGC TT	20
HA 19F	AAC CAA TGT ATC TCA TGC TCA TTT T	25
HA 19R	GGA AGA AAG CTG TAA AGA AGT AGG C	25
HA 20F	TTT GAG AAG CTG AAT TTT GTG C	22
HA 20R	GAA GCA TGG AGA TGG ATT CAT TA	22
HA 21F	CCA CAG CTT AGA TTA ACC TTT CTC A	25
HA 21R	TGA GCT TGC AAG AGG AAT AAG TAA	24
HA 22F	TCA GGA GGT AGC ACA TAC AT	20
HA 22R	GTC CAA TAT CTG AAA TCT GC	20
HA 23F	TTG ACA GAA ATT GCT TTT TAC TCT G	25
HA 23R	TCC CCC AGT CTC AGG ATA ACT	21
HA 24F	ACT GAG GCT GAA GCA TGT CC	20
HA 24R	CCC AAC CAC TGC TCT GAG TC	20
HA 25F	TGG GAA TTT CTG GGA GTA AAT G	22
HA 25R	AAG CTC TAG GAG AGG TGG TAT TTT T	25
HA 26F	CTG TGC TTT GCA GTG ACC AT	20
HA 26R	TTC TAC AAC AGA GGA AGT GGT GA	23
F822-ID	ACA TAC GGT TTA GTC ACA AGT	21
F822-IU	CCT TTC AAC TCC ATC TCC AT	20
F822-2U	ACG TGT CTT TTG GAG AAG TC	20
F822-3U	CTC ACA TTG TGT TCT TGT AGT C	22
F81-ID	TCT GCA ACT GGT ACT CAT C	19
F81-IU	GCC GAT TGC TTA TTT ATA TC	20
F81-ED	GCC TTT ACA ATC CAA CAC T	19
HemN-P	GCC CTG CCT GTC CAT TAC ACT GAT GAC ATT ATG CTG AC	38
HemN-Q	GGC CCT ACA ACC ATT CTG CCT TTC ACT TTC AGT GCA ATA	39
HemN-B	CCC CAA ACT ATA ACC AGC ACC TTG AAC TTC CCC TCT CAT A	40
HemN-P1	GGA AAG AGG TAG GCA GGA GCC AAG AC	26
HemN-Q1	TGG CTC TGT ATC CCC ACC CAA ATC T	25
HemN-B1	TGT TGT CAT TGT CTG GCT CCT TGT CTG	27
F81-9F	GTT GTT GGG AAT GGT TAC GG	20
F81-9CR	CTA GCT TGA GCT CCC TGT GG	20
Int1h-2F	GGC AGG GAT CTT GTT GGT AAA	21
Int1h-2R	TGG GTG ATA TAA GCT GCT GAG CTA	24

Appendix 2

رقم العينة: ..... اسم المستشفى: ..... التاريخ: .....

استبانة بحثية

(مقابلة مع المرضى)

عنوان الدراسة: تحديد الطرز الجينية والتحليل المخبري لمرضى الهيموفيليا في الضفة الغربية، فلسطين

الهيموفيليا أو مرض الناعور هي مرض وراثي ناتج عن نقص او اختلال في عامل التخثر الثامن (هيموفيليا نوع أ) او عامل التخثر التاسع (هيموفيليا نوع ب).

وتهدف الدراسة الى تحديد مرضى الهيموفيليا ومن ثم إعادة تشخيص المرضى باجراء الفحوصات المخبرية الضرورية مثل تعداد الدم الكامل (CBC) ومستوى عامل التخثر في الدم، وقياس مستوى الاجسام المضادة لعامل التخثر (ان وجدت) ومن ثم تحليل المادة الوراثية (DNA analysis) للوصول الى تشخيص نهائي ودقيق مما يتيح لهم الحصول على العلاج المناسب، اضافة الى التعرف على نوع الطفرات التي تسبب الهيموفيليا في فلسطين مما يسهل تطوير بروتوكول علاجي وتشخيصي لهؤلاء المرض في المستقبل. اضافة الى انشاء سجل وطني لمرضى الهيموفيليا في فلسطين مما يساهم في رصد احتياجات المرضى وتعزيز الخدمات المقدمة لهم.

ولذلك نرجو من حضرتك التعاون معنا من خلال المشاركة في هذه الدراسة (وفي حالة كون المريض طفلا نرجو السماح لها أو له بالمشاركة). وتشمل المشاركة الاجابة عن الاسئلة المرفقة ادناه والتبرع بعينة دم، اضافة الى التوقيع على طلب المشاركة في الدراسة.

ونؤكد ان المعلومات التي سوف تقدمها للدراسة وعينة الدم التي سوف تتبرع بها سوف تستعمل لأغراض البحث العلمي فقط وسوف يتم التعامل معها بسرية تامة.

كما يمكن للمريض المشارك في الدراسة طلب الحصول على نتيجة فحصه وتشخيصه من خلال ترك عنوانه مع الباحث ان رغب في ذلك.

الباحث: شادي حسن، برنامج ماجستير العلوم الطبية المخبرية، جامعة بيرزيت

المشرف على الدراسة: الدكتور محمود سرور، برنامج ماجستير العلوم الطبية المخبرية، جامعة بيرزيت

أسئلة الاستبانة

عنوان الدراسة: تحديد الطرز الجينية والتحليل المخبري لمرضى الهيموفيليا في الضفة الغربية، فلسطين

اسم المريض	
الجنس	□**انثى** □ **ذكر**
مكان الاقامة (القرية، المدينة) رقم التلفون	
عمر المريض	
تشخيص أو نوع الهيموفيليا	
شدة المرض	
عمر المريض عند أول تشخيص	
تاريخ أول عملية علاج بعامل التخثر	
تاريخ آخر عملية علاج بعامل التخثر	
عدد مرات العلاج في الشهر	
نوع او شكل العلاج	**عند الحاجة أو وقائي**
هل تعاني من مشاكل في المفاصل، حدد المفصل ان وجد؟	
هل سبق وأجريت عمليات جراحية؟ يرجى تحديد نوع واسم العملية وفي اي عمر؟	
هل تعاني من أمراض مزمنة (يرجى التحديد ان وجدت)؟	

هل الاب والام اقارب؟	
هل يحمل أحد والديك صفة مرض الهيموفيليا	
طول المريض (سم)؟	
وزن المريض (كغم)؟	
هل ترغب بالحصول على نتيجة التحاليل لعينة الدم التي تتبرع بها اليوم (يرجى تحديد طريقة التواصل مثل بريد الكتروني أو فاكس)؟	
هل ترغب باضافة معلومات أخرى؟	

------ انتهت الاسئلة ------

اقرار بالمشاركة في الدراسة

انا الموقع ادناه أقر بالمشاركة (وفي حال كان المريض طفلا اسمح لها او له بالمشاركة) في الدراسة بعنوان: **تحديد الطرز الجينية والتحليل المخبري لمرضى الهيموفيليا في الضفة الغربية، فلسطين.** علما انه تم اطلاعي على الهدف من الدراسة وان المعلومات التي قدمتها للدراسة وعينة الدم التي تبرعت بها سوف يتم استخدامها لأغراض البحث العلمي فقط.

اسم المريض:

التوقيع:

التاريخ:

--- انتهى الاقرار ------