بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate studies

Molecular Detection of Exon 10 Mutations in Factor V Gene In Sudanese Deep Vein Thrombosis by DNA Sequencing

الكشف الجزيئي للطفرات الموجود في اكسون (10) لجين عامل التجلط (5) لدي السودانيين المصابين بتخثر الاوردة الدموية عن طريق تحديد تسلسل الحمض النووي

A Dissertation Submitted for Partial Fulfillment of M.Sc. Degree in Medical Laboratory Science (Hematology and Immunohematology)

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February, 2019

الآية

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قال تعالى:

قَالَ يَا قَوْمِ أَرَأَيْتُمْ إِنْ كُنْتُ عَلَىٰ بَيِّنَةٍ مِنْ رَبِّي وَرَزَقَنِي مِنْهُ رِزْقًا حَسَنًا ۚ وَمَا أُرِيدُ أَنْ أُخَالِفَكُمْ إِلَىٰ مَا أَنْهَاكُمْ عَنْهُ ۚ إِنْ أُرِيدُ إِلَّا الْإِصْلَاحَ مَا اسْتَطَعْتُ ۚ وَمَا تَوْفِيقِي إِلَّا بِاللَّهِ ۚ عَلَيْهِ تَوَكَّلْتُ وَإِلَيْهِ أُنِيبُ

صدق الله العظيم
سورة هود الآية (88)

Dedication

Behind any successful work, there are self-efforts as well as guidance from those who are closed to our hearts

I dedicate my work to my loving parent, and husband for their love, encouragement, prayers of day and night, and supporting me morally and financially

For all those who encouraged me to fly toward my dream

Along with all hard working and respected teachers, and for gentle readers.

Acknowledgment

My grateful thank for Allah who guide me to the Straightway in my life

Then I would like express my appreciation to my supervisor

D. Hisham Nouraldayem who has cheerfully answered my queries, and assisted me in a myriad way with writing and helpfully commented on earlier draft of project.

Special thanks to D. Abdulla Mosa Who provide me with material and continued support.

I would like to thank my friends and family for their good humor and support throughout the production of this project.

Finally, I wish to thank all friend and teacher of research laboratory for their excitement and willingness to provide feedback made the completion of this research an enjoyable experience.

Abstract

This descriptive cross-sectional case study was conduct among Sudanese patients with deep vein thrombosis to screen the presence of the most common mutations in exon 10 of factor V gene in patients of deep vein thrombosis, by DNA Sequencing, in period from August to October 2018. 30 samples were collected from previously diagnosed deep vein thrombosis patients from Omdurman teaching hospital in (3ml) EDTA anticoagulant. Then DNA was extracted by using Guanidine chloride method. Exon 10 was amplified by PCR technique using specific primer. Fifteen samples were sequenced by BGI Company in both strands. Sequences were analyzed by different bioinformatics tools, (Finch TV, BLST, Codon code alignment). The result showed no existence of factor V Leiden mutations among all subject of study, and presence of missense variant Rs6020 (A/G). In conclusion, the variant associated with coagulopathy and osteonecrosis of femoral head in Chinese patients, but exact role in the pathogenicity of DVT in Sudan remain to be identified.

مستخلص البحث

هذه دراسة مقطعية عرضية أجريت على مرضي تخثر الأوردة الدموية في السودان في الفترة ما بين أغسطس الي اكتوبر2018 وذلك لتشخيص وجود الطفرات الوراثية الموجودة في اكسون عشرة من عامل التجلط خمسة بواسطة تحديد تسلسل الحمض النووي. اختير عدد ثلاثون مريضا شخصوا مسبقا بتخثر الاوردة الدموية، جمعت 3 مل من الدم الوريدي من كل مشارك في حاويه مانع تجلط يحتوي على ايثايلين تنائي الأمين رباعي حامض الاستيك. استخرج الحمض النووي منزوع الاكسجين للتحليل الجزئي عن طريق كلوريد الغوانيدين.تم تضاعف الاكسون العاشر باستخدام تقنية الPCR ، ثم تم تحديد تسلسل الحمض النووي ل اكسون عشرة للاتجاهين، ثم تم تحليل النتائج بواسطة عدة برامج حوسبية. وحللت النتائج باستخدام الحزم الإحصائية للعلوم الاجتماعية المحوسب.أوضحت الدراسة ان الطفرة الوراثية factor V Leiden ليست موجودة عند الأشخاص تحت الدراسة المصابين بتخثر الاوردة الدموية، وكشفت أيضا وجود طفرة وراثية اخري (rs6020)A/G، اذ ان هذه الطفرة لها علاقة ب اعتلال خثري وتنخر عظم راس الفخذ لدي الصينيين ولكن الدور الأساسي لهذه الطفرة في تخثر الاوردة الدموية لدي السودانيين يجب ان تدرس.

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Abbreviation

APCR	Activated Protein C Resistant
APTT	Activated Partial Thromboplastin Time
D.W	Distal water
DNA	Deoxyribo Nucleic Acid
DVT	Deep Vein Thrombosis
EDTA	Ethylene Diamine Tetra Acetic acid
FVL	Factor V Leiden
HRT	Hermon Replacement Therapy
OCPs	Oral Contraceptive Pills
ONFH	Osteonecrosis of Femoral Head
PE	pulmonary Embolism
RCLB	Red Cell Lysis Buffer
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variation
TEB	Tris EDTA Borate
VTE	Venous Thromboembolism.

Chapter one

Introduction

1.1 Introduction

Physiological hemostasis is an intricate biological system, where pro-coagulant and anti-coagulate force interplay and preserves blood fluidity when blood vessels are intact or trigger blood clot formation to prevent excessive bleeding when blood vessels are injured (Lippi and Favaloro, 2018). It consists of acomplex regulated system, include vasculare system, coagulation system, fibrinolysis, platelet system¸ kinin system¸ serine protease inhibitor and complement system (Ogedegbe, 2002). Blood coagulation involves the serial activation of plasma serine protease precursors (zymogens) by limited proteolysis, to result in the formation of thrombin. Factor V take part in this process as a component of prothrombinase complex that composed of factor Va, factor Xa, prothrombin, calcium ions and negatively charged phospholipid membrane (Jeimy, 2010). Also, factor V have anticoagulant role, by participation in the inactivation of activated factor VIII (FVIII) (Duga *et al*., 2004). Disorder of hemostasis or unbalanced hemostasis may lead to hypo-coagulation (hemorrhage) or hyper-coagulation (thromboembolic disorders) (York, 2013). Hyper-coagulation is state of increased risk for thrombosis. Hyper-coagulability is also designated thrombophilia (Sun and Kroll, 2018). Thrombophilia is a group of different disorders that cause blood to clot abnormality (Szmelskyj *et al.*, 2015). Thrombophilic abnormalities can be inherited, acquired and "mixed". Inherited thrombophilia includes, deficiency of antithrombin, deficiency of protein

C, deficiency of protein S, factor V Leiden mutation, and prothrombin gene mutation. Acquired thrombophilia include (obesity, pregnancy, cancer and its treatment, anti-phospholipid antibody syndrome, drug-induced thrombosis, and myeloproliferative disorders). Mixed disorders are those with both an inherited and an acquired component, e.g. hyperhomocysteinemia (Anderson *et al.*, 2018).

Venous thrombosis is a common multifactorial disease Dahl back *et al* described an inherited form of Activated protein C resistant (APCR) Shown to occur in 20-60% of patients with venous thrombosis and 2-5%of general population. All inherited cases of APCR so far identified have been caused by single base change, G-A, at nucleotide 1691 of the factor V gene, causing the Arginine at 506 to be replaced by Glutamine. Gln506 form, called factor V Leiden (Rees, 1996). It suspected in individuals with history of venous thromboembolism manifesting as deep vein thrombosis or pulmonary embolism, especially in women with a history of VTE during pregnancy or in association with oral contraceptive use, and in individuals with a personal or family history of recurrent thrombosis (Yan and Rennert, 2013). Deep Vein Thrombosis means thrombosis in the deep veins of extremities and neck, as opposed to superficial vein (Shackford, 2018). FVL is the common cause for APC resistance. Other less common causes include factor V Cambridge, HR2 haplotype, factor V Hong Kong, and factor V liver pool (Segall and liem, 2007).Only factor V Cambridge, liver pool and P.GLU666Asp have been associated with risk of thrombosis(Yan and Rennet, 2013). Other SNP of factor V gene (rs6020), reported have been associated with coagulopathy and osteonecrosis of the femoral head (Kou-ti Peng, 2014).

1.2Rationale:

Deep Vein Thrombosis (DVT), a subset of venous thromboembolism (VTE), is a major cause of morbidity and mortality worldwide. The incidence of venous thromboembolism is estimated to be 1 per 1,000 people and contributes to 60,000-100,000 deaths annually (Stone *et al.*, 2017).

Factor V Leiden (FVL) mutation increased risk for venous thromboembolism (DVT or PE), but thrombosis in unusual location also occur. It is also associated with a 2-to3-fold increase relative risk for pregnancy loss and possibly other obstetric complications (Kujovich, 2011). Factor V Leiden accounting for approximately 90% of the cases with deep vein thrombosis. Factor V Leiden mutation is identified in approximately 20% with first VTE episode and in 50-60% of patients with recurrent VTE (Yan and Rennert, 2013). This study attempts to screen the presence of most common mutations in exon 10 of factor V gene in Sudanese deep vein thrombosis patients by DNA sequencing.

1.3 Objectives:

1.3.1 General objectives

To detect exon 10 mutations in factor V gene in Sudanese deep vein thrombosis patients by DNA sequencing.

1.3.2 Specific objectives

1-To screen the presence of F V Leiden mutations in Sudanese DVT patients by DNA sequencing.

2-To investigate other genetic variants in exon 10 of FV gene in Sudanese patients with DVT by DNA sequencing.

Chapter two

2. Literature review

2.1 Factor V gene

F5gene provide instructions for making a protein called coagulation factor V, with cytogenetic location: 1q24.2, which is the long (q) arm of chromosome 1 at position 24.2 and molecular location: base pairs 169,511,954 to 169,586,630 (Bethesda, 2013). FV gene contains 25 exons which range in size from 27 to 2820 bp. Analysis of the factor V cDNA indicates that the protein contains several types of internal repeats with following domain structure: A1-A2-B-C1-C2. The structure of the gene for factor V is similar to factor VIII gene (Cripe *et al.*, 1992). FV gene spans more than 80 kb with complimentary DNA (cDNA) length of 6,672 bp and encode preprotein of 224 amino acid. FV and FVIII share approximately 40% sequence identity in their A- and C-domains (Emmerich *et al.*, 2016). Any defect in factor V gene result in either an autosomal recessive hemorrhagic diathesis or an autosomal dominant form of thrombophilia, which known as activated protein C resistance (Gray *et al*., 2018).

Factor V is a large single-chain glycoprotein that circulates in human plasma and is also contain in the alpha granules of human platelets, with approximately 18%-25% of total FV found in platelets. The pro-cofactor factor V is proteolyzed by α-thrombin to active cofactor factor Va (Ziedins and Mann, 2018). Thrombin (IIa) cleaves FV at R709, R1018, andR1545 to activate FV (FVa). FVa together with factor Xa forms the prothrombinase complex, which promotes prothrombin (FII)conversation to thrombin on platelet surface. Thrombin generates fibrin clot and

activates FXIII, which by means of its transglutaminase activity crosslinking fibrin fibers. Moreover, thrombin also activates the thrombin activatable fibrinolysis inhibitor (TAFI). These effects contributed to strength and resistance of clot (Corral *et al.*, 2010). Additionally, Thrombin activate protein C when bound to thrombomodulin on the vascular endothelial membrane surface. Activated protein C (APC) with its cofactor protein S and in presence of calcium on membrane surface, inhibit thrombin formation by inactivated surface bound FVa and FVIIIa through proteolysis (Pierce *et al.*, 1999). FVa consist of heavy chain (A1-A2) and light chain (A3-C1-C2) non- covalently linked by calcium ion. APC inactivation FVa via limited proteolysis of heavy chain, which lead to loss of binding affinity for FXa. The rate of APC –catalyzed FVa inactivation is increased ~200-fold in the presence of phospholipids and is modulated by the phospholipid composition (Castoldi and Rosing, 2010).APC normally inactivates coagulation factor V by cutting (cleaving) it at specific sites. This inactivation slow down the clotting process and prevent clots from growing too large. When coagulation factor V is cleaved at particular site (protein position 506), it can work with APC to inactivate factor VIIIa, which is another protein that is essential for normal blood clot (Bethesda, 2013).Activated factor V is initially cleavage at Arg506 and then Arg306 andArg679. Peptide bond cleavage at Arg506 is essential for optimal exposure of cleavage site at Arg306 and Arg679. Cleavage at Arg306 accounts for a70% loss of activated factor V activity and the subsequent Arg679 cleavage is responsible for the loss of remaining. Therefore, any defect on one or more of this three-cleavage site may potentially affect APC inactivation (Liang *et al.*, 1998).

2.2Factor V Leiden

2.2.1Definition

Is a primary hypercoagulable state due to variant (mutated form) of human factor V. This mutated factor is named after the city of Leiden. (Sharma *et al*., 2018).

It is a single point mutation in the gene coding for coagulation factor V results in a form of factor V that resistant to degradation by activated protein C and lead to a relative hypercoagulable state (Price and Ridker., 1997). Factor V Leiden is widely considered the first and most common prothrombotic polymorphism. It may have developed through genetic drift and natural selection in Caucasian, but no similar prothrombotic polymorphism has been described in other populations (Corral *et al.*, 2010). People with factor V leiden thrombophilia have a higher than average risk of developing a type of clot that forms in legs (deep vein thrombosis) or clot that travels through the blood and lodges in the lungs (pulmonary embolism). FV Leiden is the common inherited form of thrombophilia, between 3-8% of Caucasian (white) U.S and European populations carry one copy of the factor V Leiden mutation, and about 1 in 5,000 people have two copies of the mutation. The mutation is less common in other populations (Bethesda, 2013). Evidence suggests that heterozygote for the FV variant has at most a modest effect on risk of recurrent thrombosis after initial treatment of a first VTE. FV thrombophilia (heterozygote or homozygote) is a major factor contributing to pregnancy loss and other adverse pregnancy outcomes {preeclampsia, fetal growth restriction, and placental abruption} (Kujovich, 2018).

2.2.2 Historical overview

In 1993, a Swedish research team led by B. Dahl back recognized an unusual phenomenon affecting the coagulation system. They study the effect of addition external APC to plasma of patients with VTE. Normally, APC should inactivate clotting factor V and therefore slow down the coagulation process. However, in certain patients studied by Dahl back and his team, this slowing down did not occur. They called this phenomenon "APC resistant", and they originally thought this could be due a deficiency in a yet unknown protein that co- helps APC inactivating FV. One year later, another group of researchers from Holland, led by R.M. Bertina, discovered a missense point mutation in factor V gene, where adenine (A) replaced guanine (G) at nucleotide position 1691 of exon 10 of the factor v gene. They called this mutation as FVL after the Dutch city where they made their discovery in. This nucleotide replacement happened to be in the codon for the amino acid residue arginine 506 (CGA) normally present in the factor V molecule, creating a new codon (CAA) which is translate as glutamine. Due to amino acid change, APC can no longer inactivate FV efficiently, and carriers of FVL develop hypercoagulability, which manifested as VTE (Jadaon, 2011). This mutation result in reduced anticoagulant response to APC, because of FVL is inactivated about 10 times slower than normal FV. This impairment of FVa inactivation increase thrombin generation and explains more than 90% of clinical APCR phenotype (Emmerich *et al.*, 2016). APC resistance is a common inherited risk factor for venous thrombosis. It is present in 20%of patient with deep vein thrombosis, 3%

of health individual and 40%-60%of familial thrombophilia. Heterozygote carrier have seven-fold-increased risk of venous thrombosis, and increased 80-fold in homozygote carrier (Rosendaal *et al.*, 1994).

2.2.3Inheritance

The chance of developing an abnormal blood clot depends on whether a person has one or two copies of the factor V Leiden mutation in each cell. People who inherited two copies of the mutation, one from each parent, have a higher risk of developing a clot than people who inherit one copy of the mutation. Considering that, about 1 in 1,000 people per year in the general population will develop an abnormal blood clot, the presence of one copy of the FVL mutation increase that risk to 3 to 8 in 1,000, and having two copies of the mutation may raise the risk to as high as 80 in 1,000 (Bethesda, 2013). Factor V Leiden thrombophilia inherited as autosomal dominant manner. Homozygote for Leiden variant are inheriting in an autosomal recessive manner (Kujovich, 2018).

2.2.4Symptom

People with factor V Leiden thrombophilia have a higher risk for blood clots. The severity of factor V Leiden thrombophilia varies greatly from person to person. The chance of developing blood clot is affected by a number of factors, such as having family history of clots, a second factor V Leiden gene mutation, a second genetic or acquired blood clotting disorder, and other non-genetic risk factor, include surgery, immobility, cancer and non-O blood group (Bertina, 2017). Symptom of FVL include having a first DVT or PE before 50 years of age, having recurring DVT or PE, venous thrombosis in un usual sites in the body , DVT or PE during or right after pregnancy and history of unexplained pregnancy

loss in the second or third trimester. Use of hormones, such as (OCPs)and (HRT); Taken after menopause, increase risk of developing DVT and PE. Healthy women taking OCPs have a 3-4-fold increased risk of developing DVT or PE compared with women who do not take OCPs and women with FVL who take OCPs have about a 35-fold increased risk of developing a DVT or PE compared with women without FVL and not take HRT. Likewise, postmenopausal women taking HRT have 2-3 fold higher risk of DVT or PE than women who do not take HRT, and women with factor V Leiden who take HRT have a 15 –fold higher risk (Ornstein and Cushman, 2003).The literature suggests that factor V Leiden mutation is associated with 3-to-6 fold increase in risk for primary and recurrent venous thrombosis. In genetically affected persons, this risk is substantially higher among those with co-existent predispositions for thrombosis, including advanced age, OC use, hyperhomocysteinemia, and deficiency of protein C and S (Price and Ridker, 1997).

2.2.5Previous Studies:

Since its discovery, several studies conducted to determine the prevalence of FVL mutation in normal subjects and in patients with VTE, as well as to measure the risk value of this mutation in developing VTE. First reports appear in Europe, on populations of Caucasian origin. They found FVL to present in a quite high percentage of patients VTE (15-65%) and healthy subjects (1-15%). Similar results were obtained when Caucasian where studied in non-European countries like USA, Australia, and Israel. However, when studies where extended to other ethnic groups, FVL found to be very rare in Africans, south-east Asians, Chinese, Japanese, Americans Indians, green land Eskimos and aboriginal of Australia (Stefano *et al*., 1998; Jadon, 2011). Another study done to investigated

the incidence of FVL in non-selected black population of new haven city, and found surprisingly, that the incidence of this mutation in black population is not significantly different from white population. Indicate that the possibility of the introduction of an ancestral Caucasian gene into black population (Pottinger *et al*., 1996). Later on, studies were conducted on Arabs and population live in the Middle East and North Africa (the MENA region), these studies showed high prevalence of FVL in these populations. However, the MENA region is geographically very close to Europe and a lot of human movement from and to Europe, and hence such populations expected to have some Caucasian genes in their DNA (Jadaon, 2011). Heterozygote for FVL occurs in 3-8% of general European populations and US. The Frequency of homozygote FVL in white population is approximately 1 in 5,000. Within Europe, the prevalence varies from 10-20% in southern Sweden and Greece to 2-3% in Italy and Spain. The mutation found in 3.8% of individual in France, but the frequency ranges from 1.3% in southwestern regions to 7.1% northeastern France. In US, it is present in 5.2% of white American, 2.2% of Hispanic American, 1.2% of African American, 0.45% of Asia American, and 1.25% of naïve American (Kujovich, 2011). Factor V Leiden mutation arose the in the eastern Mediterranean basin, the prevalence in Lebanon (14.2%) was similar to prevalence in Syria (13.6%), Greece Cyprus (13.4%), Jordan (12.3%), and turkey (7.4%) (Irani-Hakime *et al*., 2000). Scientists suggested that the mutation occurred in Europe first and then spread to other parts of the world. However, the observed highest prevalence of FVL in eastern Mediterranean countries have raised speculations that FVL might have occurred somewhere there and then spread to Europe (Jadaon, 2011).

Factor V Leiden mutation found to be absence from African native, from Zaire and Cameron. (Franco *et al*., 1999). It is absence from Zulu speaking Africans (South Africa) (Hira, *et al*., 2003) No existence of FVL mutation in the Somali population (Abdi and Osman, 2017).

In Sudan, study done by Ibrahim et al, to analyze the genetic and acquired risk factors for DVT of lower extremities among Sudanese women, and found that FV gene mutations absent from all subjects in the study (Ibrahim *et al*., 2018).

2.2.6 Diagnosis

1. Medical history of venous thromboembolism in any family members (Campello *et al*., 2016).
2. Activated protein C Resistance test: the main clinical purpose of this test to detect the presence of FV Leiden. Original APC assay involves performing an APTT on individual's plasma in the presence and absence of exogenous APC; the two result are expressed as ratio (APTT+APC/APTT-APC). Activate Protein C mediated cleavage of FVa and FVIIIa prolongs APTT, and this prolongation decrease in the presence of FV Leiden. Ratios >2.0 are typical of normal individuals, while ratio <2.0 are typical of FVL individuals. In this test, colloidal silica is added to the patient's plasma and also addition of excess calcium and phospholipid to activate FXII, followed by XI, IX, VIII, X, and II, culminating in clot formation. The sensitivity of test is only 50-86%, and specificity is 75-98%, due to great number of coagulation factors that participate in clotting reaction. Abnormal level of any of these proteins can interfere with the test result by affecting the APTT. False low ratio (suggest APCR in absence of FVL) are caused in a variety of condition including Acute phase

reaction and pregnancy (elevate FVIII and low PS), hormone replacement therapy or oral contraceptive (low PS), PS interfere when (<20%). Falsely raised ratios can occur with factor deficiency such as (Liver dysfunction, warfarin, vitamin K deficiency, disseminated intravascular coagulation). Or anticoagulant therapy. Lupus anticoagulant (sequestering phospholipids required for clotting). Heparin, rivaroxaban, and direct thrombin inhibitors (dabigatran, argatroban, bivalirudin), interfere with original APC–assay. Platelet contamination of plasma specimen with subsequent freezing and thawing can also altered the result. These interferences result in misclassification, so modified APC-R assay made to limit these interferences. The" modified" second generation APC-R assay, involve the patient plasma is diluted in factor V deficient plasma in addition of heparin neutralizer. These modification increase sensitivity and specificity to almost 100%. These modification methods eliminate the influence of abnormal factor (Kadauke *et al*., 2014).

3. Molecular genetic testing is recommended in individuals receiving direct thrombin inhibitors or direct factor Xa inhibitors, which may interfere with results of APC-R assay, and in individual with following laboratory testing:

- Positive APC-R to confirm the diagnosis, and to distinguish FVL variant heterozygote from homozygotes.
- Borderline APC-R assay to confirm the diagnosis.
- Very low APC-R assay value to differentiate FVL heterozygote, homozygote, and pseudo homozygotes, Strong lupus inhibitor and a markedly prolonged baseline APTT (Kujovich, 2018).

2.3 R455K (Rs6020)

Single nucleotide polymorphisms other than factor V Leiden are associated with coagulopathy and osteonecrosis of the femoral head in Chinese patients. The rs6020 G>A polymorphism in exon 10 of F V gene had a higher risk of having coagulation abnormalities than those without the mutation (Peng *et al*., 2014). In this polymorphism, Adenine convert to guanine at position1628, causing arginine replaced by lysine at position 455 (Vos, 2005). Exposure to risk factors such as alcohol and steroids in patients with the rs6020 polymorphism cause coagulation abnormalities and, subsequently, thromboembolisms in the femoral head. Although the exact molecular mechanism for APC resistance in carrier of rs6020 polymorphism is still unclear, it is associated with coronary artery disease, thrombosis, and preeclampsia had been reported in Asian population. In addition, found five other SNP loci (rs9332595, rs9332647, rs3766110, rs10919186, and rs12040141) in the intron region of the F V gene that were associated with an increased risk of ONFH. These SNPs serve as a screening tool in patients who are at risk for developing osteonecrosis (Peng *et al*, 2014). The minor allele frequency of this mutation is about 30% in Africa, and 0%in Caucasian. The oriental minor allele frequency (represents the average from the Chinese and Japanese data) is about 70% (Vos, 2005).

2.4.H1299R (A4070G)

The A4070G polymorphism is highly conserved F V haplotype with wide geographical distribution. This haplotype marked by R2 polymorphism. An A to G transition at position 4070 in exon 13 of F V gene, which

replaces histidine by arginine at position 1299 of B domain, it was shown to influence circulating F V levels and to contribute to activate protein C (APC) resistance phenotype. This polymorphism is risk factor for venous VTE and influences APC resistance (APCR) in absence of the FV R506Q mutation (Lunghi *et al.,* 1998; Alhenc-Gelas *et al*., 1999). The R2 haplotype was found to be conserved and similarly frequent (0.075) in four different populations (Somali, southern Indians, Italians, and Greek Cypriot) (Lunghi *et al.*, 1998). Other study reported that factor FV R2 polymorphism is not an independent risk factor in thrombosis, but it can be important when associated with FVL in VTE. Compound heterozygosis of FV1691A and FV4070G produce a 3-to-4 increase in VTE risk when compared with FVL alone. Other studies did not find such an association or risk (Ozturk *et al.,* 2013).

2.5. Histidine 1254 Arginine

Genotyping of subjects of Somali origin at the 4070 polymorphic site revealed a new restriction pattern, suggesting the presence of a different polymorphic *Rsa1* restriction site in the same amplicon. Direct sequencing of the polymerase chain reaction (PCR) fragment in these subjects revealed an A to G transition at position 3935 (R3 polymorphism), which predicts the incorporation of Arg in place of His 1254 (CAT /CGT). The 3935G allele could also be detect in Cypriots, whereas non-in Indian and Italian. It is limited geographical distribution. This new polymorphism mimics the R2 polymorphism (His 1299 Arg) but can easily distinguished from it by restriction pattern (Lunghi *et al*., 1998).

2.6. F V Cambridge

A novel DNA sequence mutation in exon 7 of F V Gene, where guanine convert to cytosine at nucleotide position 1091. It affect Arg306, an important APC cleavage site. Lead to amino acid substitution (Arg306 to Thr). This mutation found in a family with strong history of thrombosis. F V Cambridge associated with both APC resistance and increased risk of thrombosis (Liang *et al.*, 1998).

2.7.F V Hong Kong

Also novel DNA sequence mutation in exon 7 of F V gene, where Adenine at position 1090 convert to Guanine, which result in Arg306 - Gly substitution but it is not associated with APC resistance. On other hand, susceptibility to APC cleavage appear to persist for gly306. This mutation has not any clinical significant and not predispose to thrombosis (Liang *et al.*, 1998). Subsequent studies have confirmed a high prevalence (4.7%) of FV Hong Kong among Hong Kong Chinese, and clinical studies suggested that FV Hong Kong have normal APC response so failed to demonstrate it as a risk factor for thrombosis (Norstrum *et al.*, 2002).

2.8 FV-Glu119Stop and FV liver pool

Are two novel point mutation, 529G>T and 1250T>C, resulting in FV-Glu119Stop and FV-Ile359Thrsubstitutions inherited on different alleles. FV-Ile359Thr (liver pool) substitution confers prothrombotic risk and APC resistance when co- inherited with FV-Glu119Stop. The Ile359Thr substitution creates a new consensus sequence for N-linked glycosylation within factor V heavy chain at position Arg357, and that is abnormal glycosylation might disrupt APC-mediated proteolysis of the variant FV and FVa. The anticoagulant APC cofactor function of FV-Ile359Thr

molecule was impaired. The APC mediated cleavage of Arg506 has been reported to enhance the cofactor activity of FV. It hypothesized that proteolysis at Arg506 expose binding sites for APC, PS, or even FVIIIa. The rate of cleavage of Arg506 in FVa-Ile359Thr is normal, and the mechanism by which the APC cofactor activity of FVa-Ile359Thr variant is decreased is at present unknown. Ile359Thr mutation affect anticoagulation by two mechanism, impeding the APC-mediated down regulation of FVa molecule and being a poor APC cofactor for down regulatory of FVIIIa. These findings explain the association of the FV-Ile359Thr mutation with thrombosis (Steen *et al*., 2004).

2.9. Factor V HR2 Haplotype

Is a complex haplotype of FV, which include 13 different polymorphisms throughout the gene, seven of the 13 base changes predict an amino acid change in the FV, and lead to functional modifications of the protein. FVHR2 has decreased co-factor activity for APC –mediated degradation of FVIIIa, and increase ratio of factor V1 to factor V2, the former being more procoagulant isoform. FVHR2 increased resistance to APC and reduce FV antigen and/or coagulation activity. It frequent throughout Asia, Europe and in native African populations ranging from 5-to-17% with high prevalence in Indian from Costa Rica. It suggests that FVHR2 is a very mild prothrombotic factor and the association of FV Leiden and HR2 haplotype seems not to be increase significantly the risk of venous thrombosis carried by isolated heterozygote FVL (Castaman *et al.*, 2003).

2.10. FVGlu666Asp

This mutation has recently been reported to co-segregate with APC resistance in Chinese pedigree with thrombosis. The underlying mechanism is still unknown (Castoldi and Rosen, 2010). This mutation

found to be very rare in Indian population. However, Xing-Guang et al described low prevalence of FV Glu666Asp mutations in Chinese patients with DVT as well as they did not find significant association of Glu666Asp mutation with APC-R (Sharma *et al*., 2017).

2.11. Deep vein thrombosis

The term thrombosis refers to the formation, from constituents of blood, of abnormal mass within the vascular system of a living animal. When this process occurs within the deep veins, it is referred to as deep vein thrombosis (DVT) (Kesieme*etal.*,2011).

It occurs most often in the legs, although they can also occur in other parts of the body, including the brain, eye, liver, and kidneys. These clots will break away from their original site and travel through the blood stream and lodge in the lungs, where they are known as pulmonary emboli (Bethesda, 2013).

Deep vein thrombosis is a major and a common preventable cause of death worldwide it affects approximately 0.1% of persons per year. It is predominantly a disease of the elderly and slightly male preponderance. Lower extremity DVT can be symptomatic or asymptomatic. Symptomatic patients always present with lower extremity pain, calf tenderness, and lower extremity swelling. Stasis and endothelial injury are important in deep vein thrombosis (DVT) following trauma or surgery while hypercoagulability is responsible spontaneous deep vein thrombosis. At least 96% patients treated for venous thromboembolism (VTE) have at least one risk factor. The approach to making a diagnosis currently involves, D-dimer testing, and compression ultrasonography. Prophylaxis is very important and can be mechanical and pharmacological. The mainstay of treatment is anticoagulant therapy.

Low molecular-weight heparin, unfractionated heparin, and vitamin K antagonists have been the treatment of choice. Currently anticoagulants specifically targeting components of the common pathway have been recommended for prophylaxis. These include fondaparinux, a selective indirect factor Xa inhibitor and oral selective direct thrombin inhibitors (dabigatran) and selective factor Xa inhibitors (rivaroxaban and apixaban) (Kesieme *et al.*,2011*)*.

Chapter Three

3. Materials and methods

3.1 Study design

Descriptive cross-sectional study

3.2 Study area

The study was carried out in Khartoum state, sample were collected from Omdurman teaching hospital.

3.3 Study period

The study was conduct during the period from 16 August to 25 of October 2018.

3. 4 Sample size

Thirty- (30) Sudanese patients with deep vein thrombosis.

3.5 Sampling

A total 3ml of venous blood samples were collected in plastic container containing EDTA anticoagulant from each participant using sterile disposable syringe and applying standard septic no traumatic vein puncture technique. Demographic and clinical data were collected using a specific questionnaire. The samples were preserved at -20 ^{o}C for DNA extraction.

3.6 Inclusion criteria

Sudanese patients with confirmed diagnosis deep vein thrombosis using duplex ultra sound.

3.7 Exclusion criteria

Other thrombosis rather than deep vein thrombosis in lower leg, deep vein thrombosis patients with hyper tension or heart disease, and alcohol consumption deep vein thrombosis patients.

3.8 Ethical consideration

Ethical approval to conduct this study was obtained from College of Graduate Studies, Sudan University of science and technology.

3.9 Methodology

3.9.1DNA extraction for polymerase chain reaction

DNA was extracted by guanidine chloride method, the entire volume of EDTA blood sample (3ml) placed in a tube.10 ml of the red cell lysis buffer (RCLB) (8.3 gm of NH4CL, 1gm KHCO3 1.8ml 5%EDTA and liter of distilled water (D.W) was added, mixed well and centrifuged at 6000 rpm for 5 minutes. This step repeated until a clear pellet of white blood cells appeared at bottom of the tube. Samples were centrifuge at high speed 3000 rpm for 10 minutes for collection of pellets. 2ml of the white blood cell lysis buffer (WCLB) (1.576 gm Tris- HCL, 1.088gm EDTA, 0,0292 gm NaCl, 0.2% SDS, and 100 ml distill water). 25µl of proteinase k, 1ml guanidine chloride and 300µl NH4 acetate was added to the clear white pellets. The mixture incubated at 37ºC overnight. Cooled to room temperature and then transferred to 2 ml pre+ chilled chloroform in 30 ml falcon tube. The mixture mixed well by the vortexing and centrifuged at 2500 rpm for 5 minutes. The upper layer was collected in new tube and 10 ml of cold Absolute Ethanol was added, the mixture was shake well and kept at- 20 ºC for 2 hour. Centrifuged at 3000 rpm for 20 minutes, the supernatant was carefully drained and the tube was inverted on tissue paper for 5 minutes, the pellet was washed with 4ml of 70% ethanol and centrifuged at 3000 rpm for 15 minute. The supernatant was discarded and the pellet was allowed to dry for 1 hour then re-suspended in100µL Tris EDTA (0.0IM) and 100ml distilled water), mixed well by

vortexing, and the obtained DNA was aliquot as stock solution,(stored at -20ºC).

3.9.2 DNA Storage

DNA was preserved at -20 ºC until PCR was performed.

3.9.3 Preparation of 10X Tris borate EDETA (TBE) buffer

Amount of 108gm Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M EDTA then dissolve on one liter deionized water PH 8.0.

3.9.4 Preparation of 1X TBE buffer

Ten ml of 10X TBE buffer was added to 90 ml deionized water,and heated until completely dissolved.

3.9.5 Preparation of ethidium bromide solution

Ten milligrams of ethidium bromide powder were dissolved into 500ml deionized water, and kept into brown bottle.

3.9.6Preparation of agarose gel

Amount of 2 gm of agarose powder (Intron Biotechnology, Korea) dissolved by boiling in 100 ml 1 X TBE buffer, then was cooled to 55C in water bath. Then 2 ml of (10mg\ml) ethidium bromide were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed.

Polymerase chain reaction amplification

The primers sequence were designed by NCBI primer blast (http://www.ncbi.nlm.nih.gov/tools/primer.blast/). Amplification was done using TECHNE Ltd peltire thermal cycler (Germany), DNA

amplifies was done using Maxime PCR premix kit (iNtRON, Korea). The PCR assay was carried out in a total volume of 20ml, using premix master mix tube (Maxime PCR premix kit (i-Taq) for each sample, in Ependorf tube as follow: 1ml of each reverse and forward PCR primers, 4ml of template DNA, and 14ml of water for injection. The amplification conditions include three steps: heating at 94^{o}C for 7min; 35 cycles of denaturation at 94^{o}C for30 sec, annealing at 60^{o}Cfor 30sec, and extension at 72^{o}C for 30 sec; and the final extension at 72^{o}C for 10 min (Ibrahim *et al.*, 2018)

Table (1): primer used for amplification of factor V Leiden gene (NCBI Blast primer, 2018).

Primer name	Primer sequence	Product size bp
F5 forward	5-TGGTTCCAGCGAAAGGTTATT-3	388
F5 reverse	5-TGCCCCATTATTTAGCCAGGA-3	

3.9.7 Visualization of the DNA product

The gel-casting tray was put into the electrophoresis, tank flooded with 1 XTBE buffer just to cover the gel surface, 3ml of PCR product was applied into the gel, 2 ml of 50-bpDNA ladder (iNtRON, Korea), was used as molecular weight marker added to well in each run. The electrophoresis was carried out at 150 volts for 10 minutes, the gel tray was removed from the electrophoresis apparatus, and the buffer was discarded. Then the gel was visualized for DNA bands by U.V transilluminater and photographed (Uvitec-UK).

DNA Sequencing

The PCR products of 15 best bands were send for Sanger dideoxy sequencing by BGI Company.

3.10 Statistical analysis:

All outcome data were analyzed by using statistical package for social sciences (SPSS; version 20). The outcome data arranged in tables and then entered into SPSS according to program guidelines analyzed by correlation. *P*value less than 0.05 was consider significant for the association between the variable.

Chapter Four

4.The results

4.1 Demographic data:

A total of 45 sample, 30 samples of deep vein thrombosis patients and 15 sample of health control were collected during the period 16 august To 25 October. Convential PCR technique was done for this sample to amplify F5 gene using specific primer.

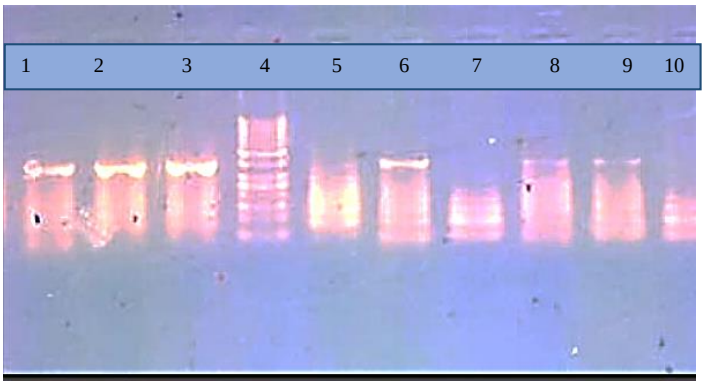

Figure1: Amplification of factor V gene. Length of band :(388).
Lane 1-3 and 6-19 are bands of exon 10, lane 4 is DNA ladder (50) bp.
5, 7 and 10 are negative bands.

DNA Sequencing

DNA Sequencing explore the genetic variation that might be associated with thrombosis in a sudanese patients with deep vein thrombosis . samples were sent for sequence by BGI Company. The result show that no evidance for existence of factor V Leiden in all subject of study. Also identified a heterozygote single nucleotide variation (SNV) in exon 10 (Rs6020) of coagulation factor V in this patients. The mutation causes

substitution of a residue Arginine at position 455 with Glutamine. This mutation had no significant association with disease.

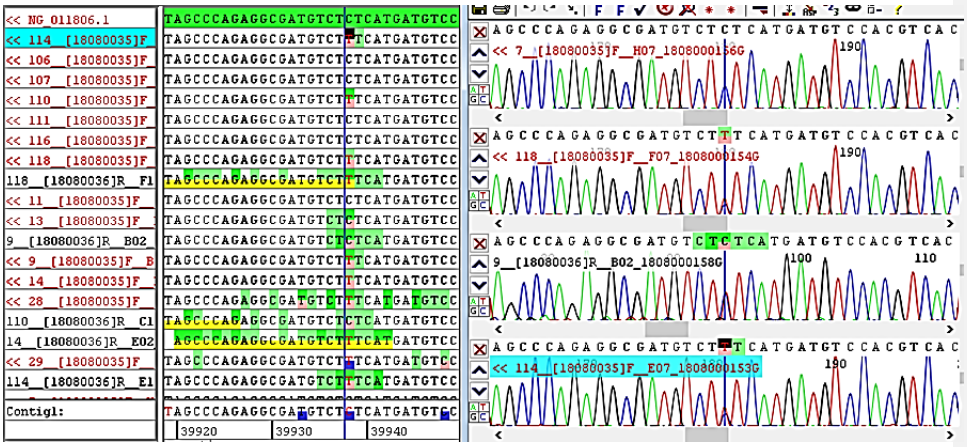

Figure2: Alignment and chromatogramof exon 10 of FV Leiden, DNA Sequencing, the line show the (rs6020C>T) SNP.

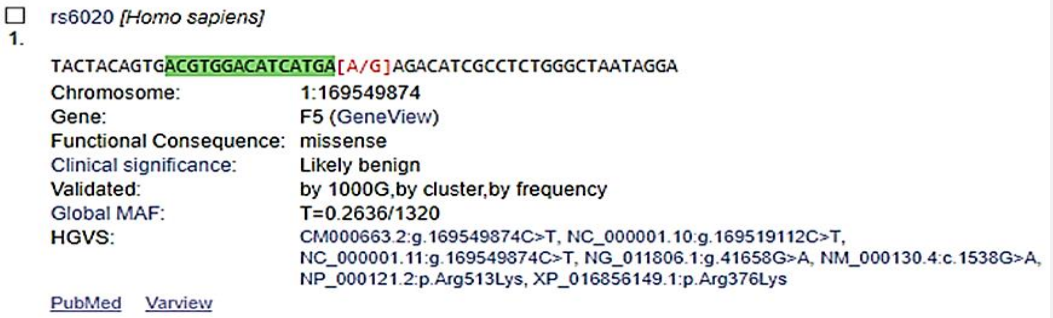

Figure 3: SNP of FVL 1691G>A (rs6020).

polymorphism	Aligner	Contig1	Contig1	39933	39933	1 diffs (0 homo / 1 hetero); 23 not mutated
heterozygoteCT	Aligner	114_[18080035]F_E07_180800153G	Contig1	39938	39938	Heterozygous 39921C>T Leu13307Leu
heterozygoteCT	Aligner	110_[18080035]F_C07_180800151G	Contig1	39938	39938	Heterozygous 39921C>T Leu13307Leu
heterozygoteCT	Aligner	118_[18080035]F_F07_180800154G	Contig1	39938	39938	Heterozygous 39921C>T Leu13307Leu
heterozygoteCT	Aligner	13_[18080035]F_D08_180800160G	Contig1	39938	39938	Heterozygous 39921C>T Leu13307Leu
heterozygoteCT	Aligner	14_[18080035]F_E08_180800161G	Contig1	39938	39938	Heterozygous 39921C>T Leu13307Leu
heterozygoteCT	Aligner	28_[18080035]F_F08_180800162G	Contig1	39938	39938	Heterozygous 39921C>T Leu13307Leu
heterozygoteCT	Aligner	9_[18080035]F_B08_180800158G	Contig1	39938	39938	Heterozygous 39921C>T Leu13307Leu
heterozygoteCT	Aligner	9_[18080036]R_B02_180800158G	Contig1	39938	39938	Heterozygous 39921C>T Leu13307Leu
heterozygoteCT	Aligner	110_[18080036]R_C11_180800151G	Contig1	39938	39938	Heterozygous 39921C>T Leu13307Leu
heterozygoteCT	Aligner	106_[18080036]R_A11_180800148G	Contig1	39938	39938	Heterozygous 39921C>T Leu13307Leu
heterozygoteCT	Aligner	114_[18080036]R_E11_180800153G	Contig1	39938	39938	Heterozygous 39921C>T Leu13307Leu
homozygoteTT	Aligner	29_[18080035]F_G08_180800163G	Contig1	39938	39938	Homozygous 39921C>T Leu13307Leu

Table(2): Show the number and heterozygosity found in case and control.

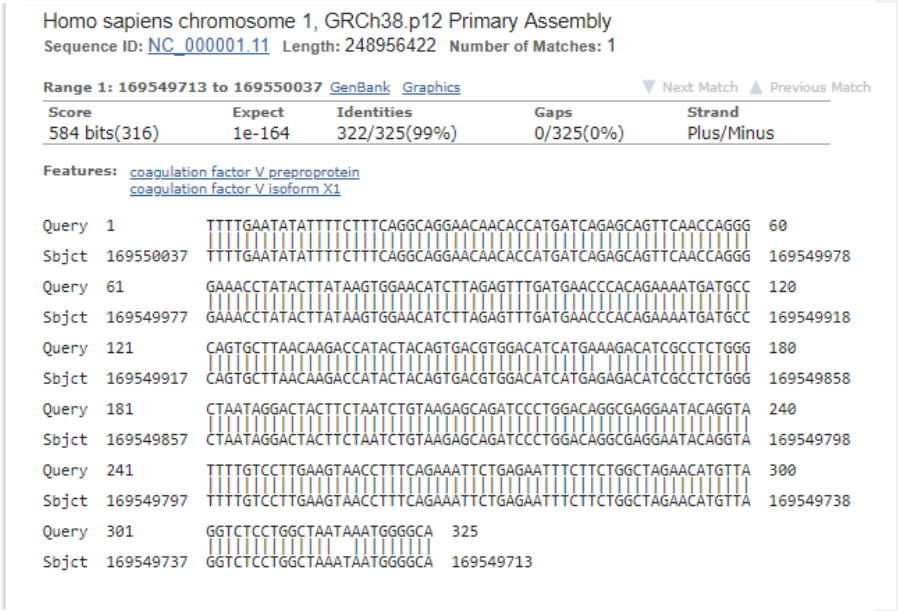

Figure4: Pairwise alignment of exon 10 of FV with homo spaniens chromosome 1,the gabs show A/G position of SNP rs(6020).

Table(3): correlation between the rs6020 mutation and DVT

	Rs6020	Samples	Pv
Pearson correlation	1	.316	.407
N	9	9	

p.value= .407

there is no correlation between the mutation and development of deep vein thrombosis.

Chapter five

5. Discussion , Conclution and Recommendations

5.1 Discussion

Factor V Leiden is the most prevelant inhereted cause of venous thrombosis. The prevelance rate in general population varies from 0 -15% according to Ethnicity and Geographic distribution (Roberts *et al*., 2009; Montagnana *et al*., 2010). The mutation is extremely rare in Asian, African, indigenous australian population, relatively high in white americans (5.2%) (kujovich, 2011), And high in mediterranean populations (13.6% in Syria, 12.3% in Jordon, and 13.4% in Greece) (Irani-Hakime *et al*., 2000). Lebnanon exhibits one of the highest frequencies of FVL mutation in the Eastern Mediterranean and in the world with prevalence of 14.4% in the general population (Irani-Hakime *et al*., 2000; kreidy, 2012). Factor V Leiden is the most common genetic risk factor for VTE, found in 20-25%of patients with VTE and in 50% of patients with famalial thrombophilia (Kujovich, 2011). Heterozygote carrier have 7 fold increase risk of venous thrombosis, and increase 80 fold in homozygote carrier (Rosendaal *et al*., 1994). Testing for factor V leiden (FVL) is now one of the most frequently ordered molecular genetic tests. In this study demonstrate the existance of common genetic frequency for thrombosis in sudanese population of DVT patients DNA sequencing , using limited number of samples.

First, the result show that the most common venous thromboembolism (VTE) genetic risk factors in caucasian, factor V leiden (FVL) is non-existant in samples of study. This study agreed with study of Ibrahim *et*

al.(2018) who reported that total absence of FVL mutation among Sudanese women and this mutation did not account for the incidence of DVT in sudanese. This study agreed with Franco *et al.*(1998) who reported the absence of FVL in Africans. Also this study agreed with stefano *et al.*(1998*)* that reported present of factor V leiden in 5% of caucasian and is virtually absence in Africans. Also these finding agree with Hira *et al.*(2003*)* conclude that the variats of the factor V Leiden (R506Q) seen in other population groups are either non-existent or extremely rare within african populations.

Second, this study demonstrate missense mutation (rs6020) A/G in exon 10 of coagulation factor V, present in nine patients. Which led to change in coding amino acid (adenine-A) to (guanine-G), as (GGAC/GGGC) at position 1628, causing arginine replaced by lysine at position 455. This mutation cause coagulation abnormality and osteonecrosis of femoral head in Chinese pateints. The frequency of this mutation is about 0% in caucasian, 30% in Africa and 70% in average from Chinese and Japanese,so serve as screening tool in patients who are at risk for developing osteonecrosis in China. In the present study, the mutation not represent risk factor for developing DVT among sudanese patients. That may due to varient in ethinic group or genetic factors.

The limitation of this study was the small sample size and financial constriants. Therefore, we recommended further studies in a larger number of Sudanese patients and control to further explore these finding.

5.2 Conclution:

This study show that the most common genetic predictor used to assess risk for venous thromboembolism (VTE) in caucasian is abscent in all samples of study.

This study also reveals heterozygote mutation (rs6020) that found in patients with DVT.

To conclude, this study need for further research of these mutation amongst a larger sudanese population , so as to investigate their distribution variants throught the population and their clinical significants.

5.3 Recommendation:

- Further studies on this mutation will be necessary before its role in deep vein thrombosis can be confirmed.
- Another study must be done including large sample size.
- Another study must be done in other exons of F5 gene to discover presence of others genetic risks factors associated with disease.

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Appendices

Appendix (1): Questionnaire

Sudan University of Science and Technology

College of Graduate Studies

Molecular Detection of Exon 10 Mutation in Factor V Gene

In Sudanese Deep Vein Thrombosis by DNA Sequencing

Duration of research: ……

Name: ……. …… No: …….

Gender: male □ female □

Age (in years) …….

Tribe ……

Mobile number ……

Affected leg: right □ left □

Duration of the disease (DVT) ……

Clinical and lab finding at the time of diagnosis: …….

……..

Treatment: ……

Recurrence: ……..

Exclusion factors:

Cardiac disease: Yes □ No □

Alcohol consumption: Yes □ No □

Diabetes: Yes □ No □

Hypertension: Yes □ No □

Pregnancy: Yes □ No □

Family history of thromboembolism Yes □ No □

Physiological factor:

Smoking: Yes □ No □

Obesity: Yes □ No □

Surgery: Yes □ No □

Others (if) any ……

Laboratory results:

A) Coagulation profile:

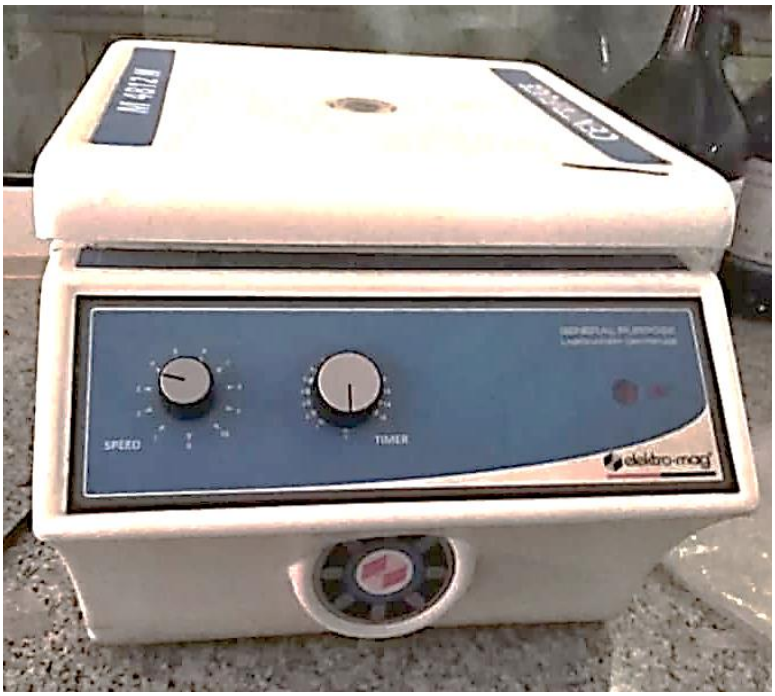
1. PT……..
2. APTT……
3. D. Dimer……

B) PCR:

Exon 10 amplification

Appendix (2): Sensitive Balance

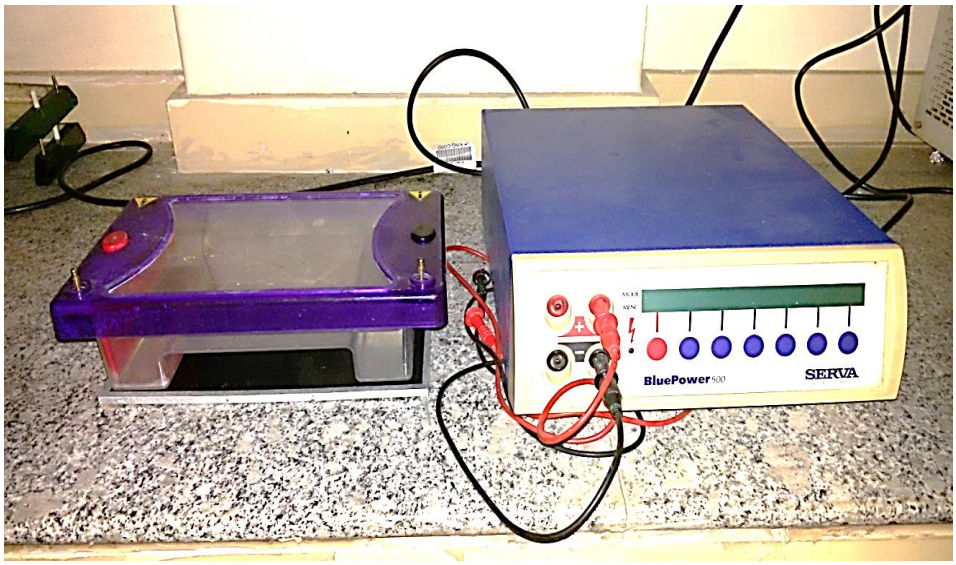
Appendix (3): Centrifuge



Appendix (4): PCR machine

Appendix (5): UV light machine

Appendix (6): Gel electrophoresis



Appendix (7):

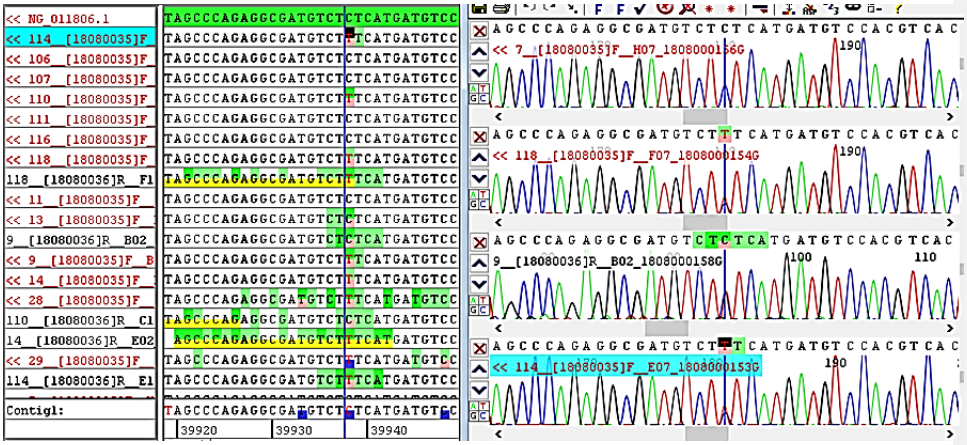


Figure 2: Alignment and chromatogramof exon 10 of FV Leiden, DNA Sequencing, the line show the (rs6020C>T) SNP.