Sudan University of Science & Technology

College of Graduate Studies

Detection of Methylene Tetra Hydro Folate Reductase Gene C677T Polymorphism and Coagulation Tests among Sudanese patients with Deep Vein Thrombosis

الكشف عن طفرة جين ميثيلين تتروهيدروفوليت C677T واختبارات التجلط وسط المرضى السودانيين المصابين بالجلطات الوريدية العميقة

A dissertation submitted in partial fulfillment of the requirements for the degree of M.Sc. Degree (Honor) in Medical Laboratory Science (Hematology and Immunohematology)

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B.Sc. In Medical Laboratory Science

Sudan University of Science & Technology 2015

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January, 2019

الآيـة

قَالَ تَعَالَىٰ:

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صدق الله العظيـم

سورة العلق الآيات (1-8)

Dedication

TO MY PARENTS

TO MY HUSBAND

TO MY EXPECTED DAUGHTER

TO MY SISTERS

TO MY FRIENDS

TO MY TEATCHERS

I DIDICAE THIS WORK

Acknowledgment

My grateful thank for Allah who guided me to the straightway in my life

Then I would like to express my appreciation to my supervisor

D. Munsoor Mohammed Munsoor who has cheerfully answered my queries, provided me with materials, checked my examples, assisted me in a myriad ways with writing and helpfully commented on earlier draft of project. Also I am very grateful to my friends and family for their good humour and support throughout the production of this project.

Abstract

This is analytical case control study designed to investigate the relationship between methylene tetra hydro folate reductase MTHFR gene (C677T) polymorphism and deep vein thrombosis in Khartoum state in the period between February, 2018 and January, 2019 .this study included Forty Sudanese patients who previously diagnosed as DVT (14 male 35% and 26 female 65%) compared with Forty healthy people as control (30 male 75% and 10 female 25%).The mean age of case group was 38.08, age range of 21- 40 years, mean age of control group was 29.03, age range 21-40 years. The mean of disease duration in month in study group was 12.13, duration range of 1-12 months. 31 (77.5%) of case group was use heparin as treatment and 9 (22.5%) of case group was use warfarin. The variable frequencies of case group under study included: pregnancy 46.2%, contraceptive pills 84.6% and surgery32.5%. Blood samples were collected from participants in EDTA and tri sodium citrate anticoagulant containers after filling the questionnaire. Then DNA was extracted by using guanidine chloride method, and the frequency of the gene polymorphism in the patients and controls were determined using PCR-restriction fragment length polymorphism, then followed by PT and PTT measurement by coagulometer instrument. The results were analyzed by statistical package for social science (SPSS) computer program. The result showed that all the patients confirmed to have DVT had the wild gene and hence were negative for MTHFR (C677T) gene mutation. There were no positivity for mutation among control group. The result also showed that the mean of prothrombin time (PT) and partial thromboplastin time (PTT) of case group are no significantly different than that of control group (P.value 0.321 and P.value 0.098 reapectively).

مستخلص البحث

هذه دراسة حالة والحالة الضابطة اجريت لكشف العلاقة بين الطفره في جين مختزلة ميثيلين تتروهيدروفوليت MTHFR C677Tو مرض الجلطة الوريدية العميقة في ولاية الخرطوم في الفتره بين شهر فبراير 2018 و يناير 2019 . هذه الدراسة تضم أربعين مريض سوداني تم تشخيصة مسبقا بمرض الجلطة الوريدية العميقة (14 رجل 35% و 26 امراة 65%) و تمت مقارنة النتائج مع اربعين سوداني صحي (30 رجل 75% و 10 امراة 25%) . متوسط العمر في المجموعة الاختبارية كان 38.08 , الفئة العمرية 21-40 سنة و متوسط العمر في المجموعة الضابطة كان 29.0 , الفئة العمرية 21-40 سنة . متوسط مدة المرض بالأشهر في المجموعة الاختبارية كان 12.13 , نطاق المرض 1-12 شهر. 31 (77.5%) من المجموعة الاختبارية كانوا يستخدمون الهيبارين كعلاج و9 (22.5%) من المجموعة الاختبارية كانوا يستخدمون الوارفارين كعلاج . الترددات المتغيرة للمجموعة الاختبارية تحت الدراسة تشمل : الحمل 46.2 % ، حبوب منع الحمل 84.6 % و الجراحة 32.5% .

جمعت عينات الدم الوريدي من كل المتبرعين في حاويتين تحتويان على مضاد التجلط واحده تحتوي على ايثالين ثنائي الامين رباعي حامض الاستيك و الاخرى على ثلاثي سترات الصوديوم لمنع التخثر بعد جمع بياناتهم بالاستبيان الهيكلي . ثم تم استخلاص الحمض النووي منزوع الاوكسجين للتحليل من كل عينه عن طريق كلوريد الغوانيدين ، و تم الكشف عن الطفره الجينية باستخدام سلسلة التفاعل المبلمر (PCR) لكل عينة للمجموعتين المرضية و السليمة و كذلك قياس وقت تخثر البروثرومبين ، و وقت تخثر الثرومبوبلاستين باستخدام جهاز قياس التخثر الالي ،وحللت النتائج باستخدام الحزم الاحصائية للعلوم الاجتماعية المحوسب (SPSS).

اظهرت نتيجة الدراسة ان كل المرضى اللذين تم تشخيصهم بالجلطه الوريدية العميقة ليس لديهم طفره في جين مختزلة ميثيلين تتروهيدروفولات C677T و لا وجود للطفره في المجموعه الضابطة . و اظهرت النتيجة ايضا انة لا يوجد اختلاف ذو دلاله احصائية في متوسط وقت تخثر البروثرومبين و وقت تخثر الثرومبوبلاستين في العينات الاختبارية عند مقارنتها بالعينات الضابطه (القيمة الاحصائية0.321 P و 0.098 P على التوالي).

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Abbreviations

Abbreviation	Full text name
APAs	Antiphospholipid antibodies
APCR	Activated protein C Resistance
aPTT	Activated partial thromboplastin time
AT	Antithrombin
CACL	Calcium chloride
CD	Cluster of differentiation
COCs	Combined oral contraceptives
CSF	Colony stimulating factor
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
ECs	Endothelium cells
EDRF	Endothelium-drive relaxing factor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDPs	Fibrin degradation products
FV	Factor five
FVL	Factor five leiden
GP	Glycoprotein

HCL	Hydrochloride
HIV	Human immunodeficiency virus
HMWK	High molecular weight kininogen
HRT	Hormone replacement therapies
IPG	Impedance plethysmography
KCCT	Kaolin cephalin clotting time
KHCO3	Potassium bicarbonate
MTHFR	Methylenetetrahydrofolate reductase
NACL	Sodium chloride
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor-1
PC	Protein C
PCR	Polymerase chain reaction
PE	Pulmonary embolism
PF3	Platelet factor 3
PK	Prekallikrein
PPP	Platelet poor plasma
PS	Protein S
PT	Prothrombin
PT	Prothrombin time
PTT	Partial thromboplastin time
PTTK	Partial thromboplastin time with kaolin
RCLB	Red cell lysis buffer

SDS	Sodium Deoxycholate
SNPs	Single-nucleotide polymorphisms
TAFI	Thrombin activatable fibrinolysis inhibitor
TE	Tris-EDTA buffer
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TPA	Tissue plasminogen activator
T-PA	Tissue plasminogen activator
TxA2	Thromboxane A2
u-PA	urokinase-plasminogen activator
VDRL	Venereal disease research laboratory
VTE	Venous thromboembolism
VWF	Von willebrand factor
WCLB	White blood cell lysis buffer

Chapter one

Chapter One

1.1 Introduction

Hemostasis is the process of blood clot formation at the site of vessel injury. When a blood vessel wall is disrupted, the hemostatic response must be quick, localized, and carefully regulated. Abnormal bleeding or thrombosis (nonphysiologic blood clotting not required for hemostatic regulation) may occur when specific elements of these processes are missing or dysfunctional (Lane, *et al.*, 2005).

The pathways of thrombin-stimulated fibrin clot formation and plasmin-induced clot lysis are linked and carefully regulated. When they work in coordinated harmony, a clot is laid down initially to stop bleeding, followed by eventual clot lysis and tissue remodeling (Lane, *et al.*, 2005). Abnormal bleeding can result from diminished thrombin generation (eg, due to factor VIII deficiency) or enhanced clot lysis (eg, due to alpha-2-antiplasmin deficiency). Conversely, excessive production of thrombin (eg, due to an inherited thrombophilia) can lead to thrombosis (Lane, *et al.*, 2005).

Although the clotting process is a dynamic, highly interwoven array of multiple processes, it can be viewed as occurring in phases: Endothelial injury and formation of the platelet plug, propagation of the clotting process by the coagulation cascade, Termination of clotting by antithrombotic control mechanisms and Removal of the clot by fibrinolysis. (Furie and Furie , 2008).

Venous thromboembolism (VTE) which is manifested either as deep venous thrombosis (DVT) or pulmonary embolism (PE) is the third most frequent cardiovascular disorder after myocardial infarction and stroke. VTE is a multifactorial disease, usually the result of the interaction between a genetic background predisposing to hypercoagulability and acquired risk factors. Although considered idiopathic in 25-50% of cases, investigations may detect a cause in over 80% of the patients with VTE (Anderson and Spencer, 2003).

Deep vein thrombosis (DVT) is the thrombotic obstruction of deep veins in the lower extremities, and is a significant cause of mortality and morbidity. According to the studies, 1 in 1000 individuals in old age population is complicated by DVT, annually (Esmon, 2009 and Shaheen, *et al.*, 2012).Its a common disorder in the elderly and its incidence rises markedly with age (Kesieme, *et al.*, 2011).

The pathogenesis of DVT is multifactorial and involves environmental, acquired, and genetic factors. In recent years, many authors have confirmed that thrombophilia, either acquired or genetic defined as a predisposition to increased risk of venous and occasionally arterial thromboembolism due to hematological abnormalities, was often responsible for the occurrence of lower extremity DVT (Lane, *et al.*, 1996 and Greaves and Baglin, 2000).

According to some studies, genetic factors account for about 60% of the risk for DVT (Kesieme, *et al.*, 2011). Factor V (FV) Leiden which is the most common cause of inherited thrombophilia, predisposes patients to DVT because of resistance to protein C, Prothrombin 20210, another common cause of hereditary thrombophilia, is also a risk factor for DVT (Seligsohn and Lubetsky, 2001), also Plasminogen activator inhibitor-1 (PAI-1) 4G/5G. The 4G allele is associated with a higher level of PAI-1 in plasma which in turn results in a decreased fibrinolysis activity and therefore a higher tendency to thrombus formation (Jurcut, *et al.*, 2008 and Akhter MS, *et al.*, 2010) and two common polymorphisms in methylene tetrahydrofolate reductase (MTHFR) gene including C677T and A1298C, lead to decreased enzyme activity and therefore elevation of homocysteine level. Several studies have shown that these two polymorphisms might be associated with DVT due to hyperhomocysteinemia (Pop, *et al.*, 2014 and Oztuzcu, *et al.*, 2014).

Among the “traditional” thrombophilia factors, methylenetetrahydrofolate reductase (MTHFR) polymorphisms associated with hyperhomocysteinemia have recently raised interest. The MTHFR gene encodes the enzyme MTHFR, which regulates the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the substrate for remethylation of homocysteine to methionine. The MTHFR gene is located on the short (p) arm of chromosome 1 at position 36.3. There are two common polymorphisms of the MTHFR gene: the MTHFR 677 C>T polymorphism resulting in the substitution of alanine to valine at codon 222 (Ala>Val) and the MTHFR 1298 A>C polymorphism, resulting in a glutamine to alanine substitution (Glu>Ala). The MTHFR C677T polymorphism, especially in the homozygous state, leads to decreased enzyme activity and hyperhomocysteinemia, whereas the MTHFR A1298C polymorphism presents a less well-defined effect, with a lesser decrease of the enzyme function (Anderson and Spencer, 2003).

The prevalence of MTHFR 677C > T is variable depending on the ethnicity and location. The allele frequency was reported to be higher in the Italian and the Hispanics and lower in the American Blacks and sub-saharan Africa (Botto and Yang, 2000). The prevalence of 677 ranges

30.0 to 50.0% (heterozygotes) and 9.0 to 12.0% (homozygotes) in the general population. The exact impact of the 677 mutation on the occurrence of venous thrombosis has been unclear so far, but it is obvious that the presence of hyperhomocysteinemia increases the risk for thrombosis (Den, *et al*., 2005).

The aim of this study is to investigate the relationship between MTHFR gene (C677T) mutation and deep vein thrombosis in Khartoum state in a series of Sudanese patients with lower extremity DVT, This study might help in detection wither this polymorphism is consider as risk factor for DVT or not, at the same time help in clarifying these issues, and to propose recommendations for MTHFR screening in patients with DVT.

1.2 Literature review

1.2.1 Hemostasis:

Hemostasis is one of a number of protective processes that have evolved in order to maintain a stable physiology. It has many features in common with other defense mechanisms in the body, such as the immune system and the inflammatory response (Hoffbrand, *et al.*, 2016).

Hemostasis depends on a system of checks and balances between thrombosis and hemorrhage that includes both procoagulants and anticoagulants. This scale needs to be kept in balance. Thrombosis is an activation of the hemostatic system at an inappropriate time in a vessel. Thrombi formed in this fashion are pathologic and beyond the normal hemostatic mechanism. If physiological anticoagulants are decreased in the circulation there will be a clot. If procoagulants or clotting factors are decreased, the scale will tip toward bleeding. Hemorrhage or excessive bleeding may be due to blood vessel disease, rupture, platelet abnormalities, and acquired or congenital abnormalities. Hemostasis is comprised of the vascular system, platelets, and a series of enzymatic reactions of the coagulation factors (Schetz, 1998).

1.2.1.1 Components of the primary haemostatic system:

1.2.1.1.1 The vascular system:

The vascular system prevents bleeding through vessel contraction, diversion of blood flow from damaged vessels, initiation of contact activation of platelets with aggregation, and contact activation of the coagulation system (Harmening, 1997).

The basic structure of blood vessels can be broken down into three layers: the intima, the media and the adventitia. It is the materials that make up these layers and the size of these layers themselves that differentiate arteries from veins, and indeed one artery or one vein from another artery or vein. The intima is the innermost layer and the surface is covered with a single layer of the endothelium (ECs), which rest on a basement membrane of subendothelial microfibrils that are composed of collagen fibers and some elastin. The media or middle layer contains mainly circularly arranged smooth muscle cells and collagenous fibrils, and is divided from the adventitia by the external elastic lamina. The muscle cells contract and relax, whereas the elastin allows vessels to stretch and recoil. The adventitia or outermost layer is composed of collagen fibers and fibroblasts that protect the blood vessel and anchor it to surrounding structures. (Hoffbrand, *et al.*, 2016)

Endothelial cells are not passive blood vessel wall linings; they are active participants in global hemostasis. Endothelial cells are particularly important in the prevention of coagulation. Endothelial cells express several molecules on their surface membranes that are important in regulation of coagulation. Examples are heparin sulfate and thrombomodulin, which activate anticoagulant systems (antithrombin and the protein C protein S system, respectively).Endothelial cells produce a variety of metabolic products that are critical in the prevention of thrombosis, including tissue plasminogen activator (t-PA), the primary initiator of the fibrinolytic system; tissue factor pathway inhibitor (TFPI), which inhibits coagulation via the TF-VIIa-Xa complex; and prostacyclin, a potent vasodilator and platelet antagonist. Endothelial cells also produce nitric oxide (NO; originally called the endothelial-derived relaxing factor [EDRF]), which is a potent vasodilator and platelet antagonist, and endothelin, which is a potent vasoconstrictor (William, 2002).

1.2.1.1.2 Platelets:

Platelets, or thrombocytes, are small discoid cells (0.5 to 3.0 μm) that are synthesized in the bone marrow and stimulated by the hormone thrombopoietin. They are developed through a pluripotent stem cell that has been influenced by colony-stimulating factors (CSF) produced by macrophages, fibroblasts, T-lymphocytes, and stimulated endothelial cells. The parent cells of platelets are called megakaryocytes. These large cells (80 to 150 μm) are found in the bone marrow. Megakaryocytes do not undergo complete cellular division but undergo a process called endomitosis or endoreduplication creating a cell with a multilobed nucleus. Each megakaryocyte produces about 2000 platelets. Thrombopoietin is responsible for stimulating maturation and platelet release. This hormone is generated primarily by the kidney and partly by the spleen and liver **(**Southern, *et al.,* 1995).

There is no reservee of platelets in the bone marrow: 80% are in circulation and 20% are in the red pulp of the spleen. Platelets have no nucleus but do have granules: alpha granules, and dense granules (Ciesla, 2007).

Alpha granules contain proteins, such as coagulation factors (fibrinogen, vWF, and factor V), platelet-specific proteins (thromboglobulin, platelet factor 4, platelet-derived growth factor), and others. Dense bodies contain small molecules and ions, such as adenosine diphosphate, adenosine triphosphate, calcium, and serotonin. Platelet granule contents are released when platelets are

activated, providing an immediate source of clotting factors and platelet agonists, which recruit other platelets into the growing platelet plug (William, 2002).

The normal platelet number is approximately 150,000 to 350,000/L (150 to 350 X 109/L). Platelets contain actin filaments, myosin, and other contractile proteins, which help them retain their shape and allow platelet plugs to contract. Platelets have a variety of surface glycoproteins, some of which act as receptors for vWF, fibrinogen, or other adhesive proteins. Many platelet receptors consist of complexes of two or more glycoproteins. The most important platelet receptors are the following:

• GP Ib-IX/V (previously designated GP Ib-IX): The platelet receptor for vWF (Designated CD42 in the Cluster Designation (CD) system).

• GP IIb-IIIa: The platelet receptor for fibrinogen, which also acts as a receptor for vWF, fibronectin, and other adhesive proteins (Designated CD41/CD61 in the CD system). GP IIb-IIIa exists on the resting platelet in a low-affinity or inactive form. After the platelet is activated by initial adhesion, the GP IIb-IIIa undergoes a conformational change to a high-affinity form, and additional IIb-IIIa is transferred from the interior to the exterior of the platelet (William, 2002).

1.2.1.2 Coagulation:

Coagulation is a complex network of interactions involving vessels, platelets, and factors. The ability to form and to remove a clot is truly a system dependent on many synergistic forces. Coagulation is divided into two major systems: the primary and secondary systems of hemostasis. The primary system comprises platelet function and vasoconstriction. The secondary system involves coagulation proteins and a series of enzymatic reactions. Once the coagulation proteins become involved, fibrin is formed, and this reinforces platelet plug formation until healing is complete. The product of the coagulation cascade is the conversion of soluble fibrinogen into an insoluble fibrin clot. This is accomplished by the action of a powerful coagulant, thrombin. Thrombin is formed by a precursor circulating protein, prothrombin. Dissolution of the platelet plug is achieved by the fibrinolytic process (Ciesla, 2007)

1.2.1.2.1 Coagulation factors:

The coagulation factors may be categorized into substrates, cofactors, and enzymes. Fibrinogen is the main substrate. The cofactors accelerate the activities of the enzymes, which are involved in the coagulation cascade. Examples of cofactors include tissue factor, factor V, factor VIII, and

Fitzgerald factor. With the exception of factor XIII, all the enzymes are serine proteases when activated. (Harmening, 1997)

The coagulation factors may also be categorized into 3 groups on the basis of their physical properties. These groups are the contact proteins comprising of factors XII, XI, prekallikrein (PK), and high molecular weight kininogen (HMWK); the prothrombin proteins comprising of factors II, VII, IX, and X; and the fibrinogen or thrombin sensitive proteins comprising of factors I, V, VIII, and XIII (Harmening, 1997) .

1.2.1.2.1.1 Factor I, Fibrinogen:

Substrate for thrombin and precursor of fibrin, it is a large globulin protein (340,000 D) produced by the liver. Its function is to be converted into an insoluble protein and then back to soluble components. When exposed to thrombin, two peptides split from the fibrinogen molecule, leaving a fibrin monomer to form a polymerized clot (Ciesla, 2007).

1.2.1.2.1.2 Factor II, Prothrombin:

It is an alpha2 globulin produced in the liver with a molecular weight of 69,000-D (Rodak, 1995) Precursor to thrombin, in the presence of Ca2, it is converted to thrombin (IIa), which in turn stimulates platelet aggregation and activates cofactors protein C and factor XIII. This is a vitamin K–dependent factor (Ciesla, 2007).

1.2.1.2.1.3 Factor III, Thromboplastin:

Factor III is now referred to as tissue factor, a 45,000 D transmembrane lipoprotein a cofactor found in all tissues but the highest concentrations are found in the brain, liver, lung, and placenta. Normally, no tissue factor is found in plasma, but released from traumatized tissue and carried into the extravascular spaces by escaping blood, where it serves as a cofactor for activation of factor VII in extrinsic coagulation. (Berg, 1993)

1.2.1.2.1.4 Factor IV, Ionized Calcium:

This is active form of calcium of molecular weight of 40-D. It is the mediator of platelets activation and binds vitamin K- dependant factors to phospholipids surfaces (factor III and platelet factor 3 (PF3) (Rodak, 1995).

1.2.1.2.1.5 Factor V, Proaccelerin or Labile Factor:

This is a single-chain glycoprotein of a molecular weight of 286,000-D.It is produced in liver. It attaches to exposed receptors on activated platelets and tissue factor (Berg LH, 1993). As part of common pathway, factor V is necessary for both the tissue factor pathway and the intrinsic pathway conversion of prothrombin to thrombin (Jobe, 1992).

1.2.1.2.1.6 Factor VI:

Factor VI dose not designate any clotting factor. Originally was given to what was thought to be a separate factor. It was subsequently proved that this factor was the same as the modified form of factor V (Vm) (Rodak, 1995).

1.2.1.2.1.7 Factor VII, Proconvertin or Stable Factor:

This is a single-chain glycoprotein of a molecular weight of 53,000-D. It is vitamin K dependant factor, and remains stable for several hours in plasma and serum. It is the initiating serine protease enzyme in the tissue factor pathway (Ruf, *et al.*, 1992).

1.2.1.2.1.8 Factor VIII-von Willebrand factor complex (VIII:vWF):

This factor consists of one factor VIII portion bound to one multimeric von Willebrand factor polymer (vWF). The factor VIII portion is controlled by genes on the X chromosome and has a molecular weight of 260,000-D, produced in several tissues but the major site is hepatic. The vWF portion is a multimeric combination of low-, intermediate- and high molecular weight subunits with varying molecular weight (Berg, 1993).

Because factor VIII is bound to vWF multimers, it is present on the surface of platelets involved in the platelets plug. After modification by factor Xa or thrombin, factor VIII becomes an active

cofactor (VIIIa) in the coagulation scheme. It serves as a cofactor for factor IXa activation of factor X on the platelet factor 3 surface (Bithell, 1987).

1.2.1.2.1.9 Factor IX, Plasma Thromboplastin Component:

A component of the thromboplastin generating system, it influences amount as opposed to rate. It is deficient in hemophilia B, also known as Christmas disease. It is sex linked and vitamin K–dependent produced in the liver (Ciesla, 2007).

1.2.1.2.1.10 Factor X, Stuart-Prower:

Final common pathway merges to form conversion of prothrombin to thrombin, activity also related to factors VII and IX. It is vitamin K–dependent produced by the liver and can be independently activated by Russell's viper venom (Ciesla, 2007).

1.2.1.2.1.11 Factor XI, Plasma Thromboplastin Antecedent:

Essential to intrinsic thromboplastin generating of the cascade, it has increased frequency in the Jewish population. Factor XI is activated by factor XIIa and becomes an essential serine protease enzyme in the intrinsic factor pathway (Ciesla, 2007).

1.2.1.2.1.12 Factor XII, Hageman factor:

It is a single glycoprotein produced by the liver. It is a contact factor that autoactivates upon binding with negatively charged surfaces such as glass, kaolin, and subendothelial tissues. Factor XIIa is the initial enzyme in the contact factor system and acts as the catalyst for several important hemostatic events (Rodak, 1995).

1.2.1.2.1.13 Factor XIII, Fibrin Stabilizing Factor:

In the presence of calcium, this transaminase stabilizes polymerized fibrin monomers in the initial clot. This is the only factor that is not found in circulating plasma (Ciesla, 2007).

1.2.1.2.1.14 High-Molecular-Weight Kininogen:

This surface contact factor is activated by kallikrein (Ciesla, 2007)

1.2.1.2.1.15 Prekallikrein, Fletcher Factor:

This is a surface contact activator, in which 75% is bound to HMWK (Ciesla, 2007).

1.2.1.2.2 Coagulation Inhibitors:

The rampant amplification of the coagulation cascade must be checked and controlled in order to limit clotting to the area where it is needed. Factors that inhibit coagulation include the following.

1.2.1.2.2.1 Blood flow and hepatic degradation of clotting factors:

Normal blood flow dilutes the activated clotting factors below the level required to propagate the cascade. Hepatocytes in the liver digest and destroy the activated clotting factors washed away from the site of clot formation (William, 2002).

1.2.1.2.2.2 Antithrombin:

Antithrombin (AT; previously called antithrombin III [AT III]) is the most important physiologic inhibitor of activated coagulation factors. Antithrombin is synthesized in the liver and endothelial cells. It irreversibly binds to and inhibits thrombin, factor Xa, and other activated clotting factors. Heparin (or heparan sulfate on endothelial cells) binds to and activates AT. By itself, AT has a low affinity for thrombin; however, complexing with heparin increases the activity of AT approximately 1,000-folds (William, 2002).

1.2.1.2.2.3 Protein C and protein S:

Proteins C and protein S are vitamin K–dependent inhibitors of the coagulation cascade that control coagulation by inactivating factors Va and VIIIa. Protein C is activated by the binding of thrombin to thrombomodulin on endothelial cell surfaces; therefore, thrombin, a key mediator of the coagulation cascade, also initiates a key anticoagulant system. When thrombin binds to thrombomodulin, thrombin is no longer able to convert fibrinogen to fibrin; instead, it enzymatically cleaves and activates protein C. Activated protein C, in combination with protein S, inactivates factors Va and VIIIa. Protein S circulates in two forms: free protein S and protein S complexed with a protein involved in the complement system, the C4b binding protein. Free protein S is active, whereas the bound form is not. Increases in the C4b binding protein (as in acute inflammation) decrease the level of free protein S and can be prothrombotic (William, 2002).

1.2.1.3 Primary hemostasis:

Primary hemostasis primarily involves platelets and vWF and results in the formation of a platelet plug. If the endothelial injury is small, this may be adequate to stop bleeding. However, if the injury is greater, participation by the coagulation cascade is required .von Willebrand's factor is synthesized by endothelial cells and mega-karyocytes. It circulates in plasma complexed with the factor VIII clotting factor. It circulates as multimers of various sizes, with molecular weights up to 20 million Daltons. The large multimers are required for normal vWF function; a decrease or absence of the high-molecular-weight multimers results in a bleeding disorder despite the presence of normal levels of total vWF (this is characteristic of one form of von Willebrand's disease [vWD]). When the endothelial layer of a blood vessel is disrupted, subendothelial collagen is exposed to the circulation. Large vWF multimers bind to subendothelial collagen and GP Ib-IX/V (the vWF receptor) on platelet surfaces, resulting in platelet adhesion and activation. GP IIb/IIIa (the fibrinogen receptor) on the platelet surface is converted from a low-affinity to a high affinity form, and additional GP IIb/IIIa is brought to the platelet surface. The platelet granule contents are released, which recruit and activate other platelets. Phospholipase A2 is activated, generating arachidonic acid from platelet membrane phospho-lipids.Arachidonic acid is converted to thromboxane A2 (TxA2) by the enzymes cyclooxygenase and thromboxane synthetase. Thromboxane A2 is a potent vasoconstrictor that stimulates platelet aggregation and causes release of platelet granules. Inhibition of cyclooxygenase (by aspirin and other nonsteroidal anti-inflammatory drugs) blocks the synthesis of thromboxane A2, thus inhibiting platelet aggregation. Inhibition of cyclooxygenase, with subsequent block of TxA2 production, is how aspirin inhibits platelet activation.

• Note that both prostacyclin (a potent vasodilator and platelet antagonist) and TxA2 (a potent vasoconstrictor and platelet agonist) are derived from arachidonic acid via cyclooxygenase. Endothelial cells predominantly produce prostacyclin, whereas platelets predominantly produce TxA2 (William, 2002).

1.2.1.4 Secondary hemostasis:

The term coagulation cascade refers to the sequential activation of coagulation factors, resulting in the conversion of fibrinogen to fibrin and the subsequent polymerization of fibrin into a fibrin

clot. Most of the coagulation factors are serine proteases. They circulate in the plasma as inactive precursors (zymogens), which are converted to the active enzyme by protease cleavage. One coagulation factor cleaves and activates the next factor along the line and so on. Since each active enzyme can activate many molecules of the subsequent factor, there is a geometric increase in the number of molecules activated. Like a small snowball starting at the top of a hill, the end result of the coagulation cascade is an avalanche of activated clotting factors (William, 2002).

1.2.1.4.1 Concepts of the Coagulation Cascade:

The classic concept of the coagulation cascade bases on two separate and independent pathways: the intrinsic pathway measured by the partial thromboplastin time (PTT), and the extrinsic pathway measured by the prothrombin time (PT). The two pathways came together at the activation stage of factor X to Xa, and hence the pathway from factor X down to fibrin was called the common pathway. We now know that there is really only one pathway; the intrinsic pathway is largely a laboratory artifact. However, because we still use the same two main tests to investigate the status of the coagulation cascade, you must understand both pathways in order to interpret the results of laboratory tests of coagulation (William, 2002).

The current concept of the coagulation initiated by exposure of TF to the circulation and reaction of TF with factor VIIa. The TF-factor VIIa complex can enzymatically activate factor X to Xa, factor IX to IXa, and factor XI to XIa. The initial activation of factor X to Xa may be important in getting the coagulation cascade started; however, a specific inhibitor produced by endothelium called tissue factor pathway inhibitor (TFPI) rapidly inactivates the TF-VIIa-Xa complex. Therefore, the major action of the TF-VIIa complex in vivo is the activation of factor IX to IXa, which then activates factor X to Xa. Activation of factor XI to XIa by the TF-VIIa complex appears to play a relatively minor role in the coagulation cascade Activation of factor X to Xa and prothrombin (II) to thrombin (IIa) are key steps in the coagulation cascade since both Xa and thrombin have positive feedback activity on earlier steps of the cascade. Factor Xa activates VII to VIIa, increasing the amount of VIIa available to complex with TF. Thrombin converts factor V to Va and factor VIII to VIIIa. It also activates factor XI to XIa and XIII to XIIIa. Thrombin is also a potent platelet agonist. Factor X is activated by a complex of factor IXa, VIIIa, phospholipid, and calcium. Prothrombin is activated by a complex of factor Xa,Va, phospholipid, and calcium. Thrombin cleaves off two small peptides from fibrinogen (fibrinopeptides A and B), converting

fibrinogen to fibrin monomer. Fibrin monomer spontaneously polymerizes to form soluble fibrin polymer, which is then covalently cross-linked by factor XIIIa, converting it to a stable fibrin clot (William, 2002).

1.2.1.4.1.1 Intrinsic System:

It is a parallel pathway for thrombin activation by factor XII. It begins with factor XII, HMW kininogen, prekallekerin and factor XI, which results in activation of factor XI. Activated factor XI further activates factor IX, which then acts with its cofactor (factor VIII) to form tenase complex on a phospholipid surface to activate factor X (Hall, 2010 and Kumar ,*et al.*, 2010).

1.2.1.4.1.2 Extrinsic pathway:

It is considered as the first step in plasma mediated haemostasis. It is activated by TF, which is expressed in the subendothelial tissue (Lasne D et al 2006) Under normal physiological conditions, normal vascular endothelium minimises contact between TF and plasma procoagulants, but vascular insult expose TF which binds with factor VIIa and calcium to promote the conversion of factor X to Xa (Owens and Mackman, 2010)

1.2.1.4.1.3 Common pathway:

Activated factor X along with its cofactor (factor V), tissue phospholipids, platelet phospholipids and calcium forms the prothrombinase complex which converts prothrombin to thrombin. This thrombin further cleaves circulating fibrinogen to insoluble fibrin and activates factor XIII, which covalently crosslinks fibrin polymers incorporated in the platelet plug. This creates a fibrin network which stabilises the clot and forms a definitive secondary haemostatic plug (Hall, 2010 and Kumar, *et al.*, 2010).

1.2.1.5 Fibrinolytic system:

Fibrinolytic system is a parallel system which is activated along with activation of coagulation cascade and serves to limit the size of clot. Fibrinolysis is an enzymatic process that dissolves the fibrin clot into fibrin degradation products (FDPs) by plasmin originating from fibrin bound plasminogen in liver. This reaction is catalysed by tPA or urokinase plasminogen activator (u-PA)

released from vascular endothelium. The release of t-PA is stimulated by tissue occlusion, thrombin, epinephrine, vasopressin and strenuous exercise.

Plasmin activity is tightly regulated by its inhibitor (α-2 antiplasmin) thus preventing widespread fibrinolysis (Cesarman-Maus and Hajjar 2005). In vivo activity of the fibrinolytic system is assessed clinically by measuring the FDP's. D dimers are produced by digestion of cross linked fibrin and are specific indicators of fibrinolysis used in the assessment and diagnosis of pulmonary embolism, DIC or deep vein thrombosis (Colvin, 2004).

Since plasmin has the potential to degrade fibrinogen leading to deleterious consequences, the fibrinolytic activity is limited by following factors: Plasminogen activator inhibitor - It is the main physiological inhibitor of fibriolysis and acts by inhibiting t-PA and u-PA irreversibly, TAFI - It is a plasma proenzyme synthesized by liver and activated by thrombin. It decreases the affinity of plasminogen to fibrin and augments the action of anti-trypsin in inhibiting plasmin and last one is Plasmin inhibitors - α2 antiplasmin and α2Macroglobulin are the glycoproteins that exert action by virtue of plasmin inhibition (Ejiofor, 2013).

1.2.2 Deep vein thrombosis:

A deep vein thrombosis is a blood clot that forms in the major veins of the body – usually the legs. It can affect people of any age but the risk of developing a DVT increases after the age of 40 years.

Life-threatening complications can arise from DVT when blood clots dislodge, travel in the bloodstream, and then lodge in other veins or arteries causing a blockage (this blockage is called an embolism). This can be life threatening, especially when the embolism occurs in the lungs, heart, or brain. Pulmonary embolism (a blood clot in the lung) is the most common of these serious DVT complications (Southerncross, 2019).

Veins are blood vessels that carry blood from the tissues of the body back to the heart. Veins that lie just beneath the skin surface are referred to as “superficial veins” while veins found deep inside the muscles are referred to as “deep veins”. Other veins connect the superficial and deep veins, allowing blood to flow between them (Southerncross, 2019).

When a blood clot occurs in a vein it is referred to as a venous thrombosis. A DVT is a blood clot that occurs in the deep veins. DVTs can occur in any of the deep veins but most commonly occur

in the leg veins. The clot will either partially or completely block the flow of blood through the affected vein. When the blood clot is associated with inflammation of the vein it is referred to as thrombophlebitis.

A DVT is usually more serious than a blood clot in one of the superficial veins, as there is a much greater risk with a DVT that part of the clot may dislodge and circulate through the body (Southerncross, 2019).

1.2.2.1Causes:

General factors that indicate an increased risk of developing a DVT include: Obesity, Smoking, Having previously had a DVT and having a family member who has had a DVT.

A DVT is also more likely to occur when the blood flow through the deep veins is slowed. Immobility is one such factor; blood flow is slowed when a person remains immobile for long periods of time as a result of such things as:

Paralysis, eg: following a stroke or injury , Being bedridden, eg: following surgery or due to illness , Having a leg in a plaster cast or splint and Sitting for long periods of time while travelling, eg: in a bus, train, plane, or car (Southerncross, 2019).

Other factors that can slow blood flow include:

Injury to a vein, eg: as a result of a broken bone or severe muscle injury , Surgery – particularly orthopaedic and cancer surgery , Heart disease – particularly heart failure , Varicose veins and Phlebitis (inflammation of the walls of the vein) (Southerncross, 2019).

A DVT is also more likely to occur where there is some factor that makes the blood more likely to clot, including:

Hormone medications – some research studies have indicated that there may be a small increased risk of DVT associated with some types of oestrogen-containing oral contraceptive pills, as well as some hormone replacement therapies (HRT).

Inherited disorders – such as the deficiency of some blood clotting factors, eg: protein C; or defective blood clotting factors, eg: factor V Leiden.

Inflammatory bowel diseases, eg: Crohn's disease , Certain cancers and Pregnancy (Southerncross, 2019) .

1.2.2.2 Signs and symptoms:

A DVT does not always cause symptoms. If symptoms do occur, the first symptom is usually a cramp-like aching pain in the affected muscles. This pain might worsen when exercising but does not subside with rest. Symptoms of a DVT in the calf muscle may include:

Swelling of the lower leg ,Tenderness of the calf muscle , Localised redness and warmth ,A mild fever and Lower leg veins may become more prominent (darker and raised) and sometimes the skin becomes darker. DVT can also occur in the upper leg, arms or neck and cause similar symptoms in those areas of the body (Southerncross, 2019).

If a pulmonary embolism occurs as a result of a DVT it may produce barely noticeable symptoms such as chest discomfort and mild breathlessness, or more noticeable symptoms such as sharp chest pain, a rapid heart rate, breathlessness, and coughing-up blood (Southerncross, 2019).

1.2.2.3 Diagnosis:

If a DVT is suspected – even if symptoms are mild – it is important to seek medical attention promptly. Accurate diagnosis and appropriate treatment of a DVT are necessary to reduce the risk of potentially life-threatening complications, such as pulmonary embolism (Southerncross, 2019).

Because a DVT can occur without any obvious symptoms, diagnosis can sometimes be difficult. Initially, the doctor will examine the affected area and take a full medical history. The doctor may recommend a blood test called a D-Dimer, which measures a protein essential for blood clotting. If the D-Dimer test is positive, further tests to confirm the diagnosis are likely to be ordered.

The most common diagnostic test for DVT is an ultrasound scan. The specific type of scan used is called a doppler ultrasound, which determines how fast blood is flowing through a blood vessel. The scan is painless and is able to detect up to 95% of DVTs (Southerncross, 2019).

If there is doubt about the diagnosis, venography may be recommended. This diagnostic test involves injecting a specialised dye that can be seen by x-ray as it flows through the veins, allowing them to be easily visualised.

Other tests that may be used to assist with diagnosis include:

Blood tests to check for irregularities in the blood clotting system or for inherited disorders and impedance plethysnography (IPG): This test measures the blood pressure at various places in the leg to identify the location of the blood clot (Southerncross, 2019).

1.2.2.4 Treatment:

The immediate goal of treatment for a DVT is to limit the size and movement of the clot, and to prevent complications. Treatment will depend on the location and severity of the clot. Some small clots may resolve spontaneously without treatment but a DVT is generally treated intensively. Admission to hospital for treatment and observation for signs of complications may be required. Treatment may include:

1.2.2.4.1Medications:

1.2.2.4.1.1 Anticoagulants:

These medications "thin" the blood, reducing its ability to clot. Anticoagulant medications can be administered as a continuous infusion into a vein (intravenously), as an injection under the skin (subcutaneously), or in tablet form (orally). Blood tests to monitor their effectiveness are required regularly and dosage changes may be required.

Anticoagulant treatment, in the form of tablets (e.g. warfarin) or subcutaneous injections (e.g. enoxaparin), is usually maintained for at least three months to be fully effective in treating a DVT. In some cases, it may be required on a long-term basis (Southerncross, 2019).

1.2.2.4.1.2 Thrombolytic agents:

In some cases, these medications, eg: alteplase, are given by intravenous injection to help dissolve the clot. However, they can cause side effects, such as severe bleeding, so are usually used only in life-threatening situations, eg: the presence of a large pulmonary embolus (Southerncross, 2019).

1.2.2.4.2 Compression stockings:

Elasticised compression stockings give support to the lower legs and encourage the return of blood to the heart and helps to reduce swelling. It is generally recommended that compression stockings are worn in situations where immobility is likely (Southerncross, 2019).

1.2.2.4.3 Surgery:

In high-risk cases, where there have been recurrent or severe DVTs, surgery to insert a small filter into the main vein leading to the heart (the vena cava) may be recommended. This traps any blood clots travelling through the blood stream thus preventing the clot travelling to the heart and lungs (Southerncross, 2019).

1.2.2.5 Long-term complications:

After a DVT some people may develop a chronic (long-term) condition called "post-phlebotic syndrome". This is due to damage and scarring to the veins and is characterised by swelling, discomfort, and skin pigmentation in the affected area. It can increase the likelihood of subsequent DVT.

Recurrent pulmonary emboli can lead to a condition called pulmonary hypertension, which is where the blood pressure within the lungs is increased. This can cause serious problems with the functioning of the heart. Certain medications, compression stockings, and, in rare cases, surgery, may be recommended to help treat these long-term complications (Southerncross, 2019).

1.2.3 Thrombophilia:

Thrombophilia can be defined as a predisposition to form clots inappropriately. Thrombotic events are increasingly recognized as a significant source of mortality and morbidity (Hoppe and Matsunaga, 2002). The predisposition to form clots can arise from genetic factors, acquired changes in the clotting mechanism, or, more commonly, an interaction between genetic and acquired factors (Rosendaal, 1999). A hereditary thrombophilia results when an inherited factor, such as antithrombin or protein C deficiency, requires interaction with components that are inherited or acquired before onset of a clinical disorder (Lane, *et al.*, 1996)A homozygous abnormality or combination of two or more heterozygous abnormal factors can lead to clinically

apparent thrombotic disorders at an early age. However, milder heterozygous traits, when existing alone, are more often discovered by laboratory investigation (Lane, *et al.*, 1996). The thrombophilia represent a spectrum of coagulation disorders associated with a predisposition for thrombotic events deep vein thrombosis (DVT) and pulmonary embolism (PE) (Kaandorp, *et al.*, 2009). in recent years, many authors have confirmed that thrombophilia, either acquired or genetic and defined as a predisposition to increased risk of venous and occasionally arterial thromboembolism due to hematological abnormalities, was often responsible for the occurrence of lower extremity DVT(Lane, *et al.*, 1996 , Greaves and Baglin, 2000).They recommended the detection of inherited genetic predisposing factors to thrombophilia in patients with spontaneous, unprovoked vein thrombosis and in patients with venous thrombosis associated with a family history of venous thromboembolism (VTE)(Irani-Hakime, *et al.*, 2000).

1.2.3.1 Inherited thrombophilia:

Heritable thrombophilia describes an inherited tendency for venous thrombosis, i.e. deep vein thrombosis (DVT) with or without associated pulmonary embolus (PE) (Hoffbrand, *et al.*, 2016).

Based on the studies, genetic factors are responsible for approximately 60 % DVT cases (Souto, *et al.,* 2000). Inherited deficiencies of factors associated with anticoagulation including antithrombin, protein C, and protein S were the first to be associated with increased thrombosis, in particular venous thrombosis. Factor V (FV) Leiden which is the most common cause of inherited thrombophilia, predisposes patients to DVT because of resistance to protein C. Prothrombin 20210, another common cause of hereditary thrombophilia, is also a risk factor for DVT. (Seligsohn and Lubetsky, 2001) Two common polymorphisms in methylene tetrahydrofolate reductase (MTHFR) gene including C677T and A1298C, lead to decreased enzyme activity and therefore elevation of homocysteine level. Several studies have shown that these two polymorphisms might be associated with DVT due to hyperhomocysteinemia (Pop, *et al.*, 2014, Oztuzcu, *et al.*, 2014).

Another polymorphism which is known as a risk factor for DVT is Plasminogen activator inhibitor-1 (PAI-1) 4G/5G. The 4G allele is associated with a higher level of PAI-1 in plasma which in turn results in a decreased fibrinolysis activity and therefore a higher tendency to thrombus formation (Jurcut, *et al.*, 2008, Akhter, *et al.,* 2010). Inherited deficiencies of factors

associated with anticoagulation including antithrombin, protein C, and protein S were the first to be associated with increased thrombosis, in particular venous thrombosis.

1.2.3.1.1 Causes of inherited thrombophilia:

1.2.3.1.1.1 Prothrombin gene mutation (G20210 mutation):

Prothrombin is a protein in the blood that is required for the blood to clot. It is also called factor II. It is a vitamin K-dependent protein which is synthesized in the liver and circulates with a half-life of approximately three to five days. Vitamin K acts as a cofactor for posttranslational gamma-carboxylation of prothrombin which is required for functional activity. Blood clots are composed of a combination of blood platelets and a meshwork of the blood clotting protein fibrin. Prothrombin is a blood clotting protein that is needed to form fibrin. If somebody has too little prothrombin, he or she has a bleeding tendency (Elizabeth, *et al.*, 2013).

Prothrombin gene (G20210A) mutation is associated with an increased risk of thrombosis and it is the most identifiable risk factor for venous thrombosis and is in fact the second most common genetic defect for inherited thrombosis, with Factor V Leiden being the most common. It is an autosomal dominant disorder, with Heterozygotes being at a 3- to 11-fold greater risk for thrombosis in both men and women and for all age groups. Although homozygosity is rare, inheritance of two 20210A alleles would increase the risk for developing thrombosis (Salwa and Joseph, 2006).

The mutation leads to an increased amount of thrombin circulating in the person's blood stream. The exact mechanism by which the prothrombin gene mutation results in a thrombophilic state is unclear. It is thought that the increased amount of circulating prothrombin provides a springboard upon which the clotting cascade can get started and that, in some circumstances, it may run out of control because of that springboard potential. The prothrombin gene mutation (PM) is signaled by a defect in clotting factor II at position G20210A and the human prothrombin gene spans 21 kb on chromosome 11p11-q12 and consists of 14 exons and 13 introns, which account for 90 percent of the sequence. This mutation occurs as a result of the G to A transition at nucleotide 20210 in the prothrombin gene. The reported prevalence in Europe is around 2 %to 6% and the risk of venous thrombosis to heterozygous carriers is three times the normal population (Poort, *et al.*, 1996).

1.2.3.1.1.2 Factor V Leiden mutation:

Factor V is one of the essential clotting factors in the coagulation cascade. Its active form, factor Va, acts as a cofactor allowing factor X to stimulate the conversion of prothrombin to thrombin. Thrombin is then able to cleave fibrinogen to fibrin and a fibrin clot is formed. Activated protein C is a natural anticoagulant it limits the extent of clotting by destroying factor V and reducing further thrombin formation. Factor V Leiden (FVL) mutation (named after the Dutch university where it was discovered) is a point mutation in the gene for clotting factor V (Van and Levi, 2013). It has autosomal dominant inheritance and is the most common cause of inherited thrombophilia the mutation of Factor V Leiden causes acquired protein C resistance, resulting in thrombophilia both in veins and spiral arteries of the placenta. This may lead to placenta abruption and consequently results in miscarriage heterozygotes have a three to five times increased risk of thrombosis. Most women with factor V Leiden thrombophilia have normal pregnancies and Homozygotes are much less common but have a much higher thrombotic risk, around eight times increased risk (van Vlijmen, *et al.*, 2011). Factor V Leiden mutation is the most common hypercoagulable disorder occurring in 5% of the white population. This mutation leads to a form of factor V that when activated to factor Va is resistant to degradation by activated protein C. There is increased procoagulants activity and therefore increased risk of Thromboembolism (Kujovich, 2011). FVL according to epidemiology is present in around 5% of Caucasians and It is rare or absent in people of black African, Far East Asian, native Australian and native American origin and 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 inherit 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 (Van and Levi, 2013).

1.2.3.1.1.3 Methylene tetra hydrofolate reductase deficiencies:

Methylene tetrahydrofolate reductase (MTHFR) is one of the main regulatory enzymes in the metabolism of homocysteine that catalyses the reduction of 5,10- methylenetetrahydrofolate to 5-methyltetrahydrofolate (Forges,*etal*,2007). The 5,10-Methylenetetrahydrofolate reductase (MTHFR) locus is mapped to chromosome 1 (1p36.3) (Goyette, *et al.*, 1994). The MTHFR converts 5,10methylenetetrahydrofolate to 5-methyltetrahydrofolate which produces methyl donor for the conversion of homocysteine to methionine (Goyette, *et al.*, 1998). This conversion is

catalysed by methionine synthase that is found in all mammalian tissues where Vitamin B_{12} is used as a cofactor. In dietary protein, methionine is incorporated and serves as a precursor of Sadenosylmethionine which is a universal methyl donor for the conversion of methionine back to homocysteine. Likewise, the homocysteine could also be converted to cysteine in the transsulfuration process via vitamin B_6 dependent pathway (Selhub, 1999).

MTHFR gene produces an enzyme called methylene tetrahydrofolate reductase and mutation in the gene inhibits the production of this enzyme, result in hyperhomocystinemia, which is an elevated level of an enzyme homocysteine found in blood plasma. When the body is deficient in methylene trahydrofolate reductase, its ability to absorb folate, such as folic acid, is inhibited. Folic acid and B9 are both essential to the development and health of the fetus (Foka, *et al.*, 2000). Deficiency in the homocysteine metabolism pathway resulting in an elevation of homocysteine level in plasma (hyperhomocystinemia) has been regarded as a cause of Thrombophilia (Michael, 2003). Mutations in MTHFR gene lead to decreased activity of enzyme and hyperhomocystinemia, which induces platelet aggregation through promotion of endothelial oxidative damage. Although several mutations within the MTHFR gene but C677T and A1298C mutations are the two most common mutations (Mtiraoui, *et al.*, 2006). the MTHFR C677T polymorphism resulting in the substitution of alanine to valine at codon 222 (Ala>Val) and the MTHFR A1298C polymorphism, resulting in a glutamine to alanine substitution (Glu >Ala), The MTHFR C677T polymorphism, especially in the homozygous state, leads to decreased enzyme activity and hyperhomocysteinemia, whereas the MTHFR A1298C polymorphism presents a less well-defined effect, with a lesser decrease of the enzyme function (Lovricevic, *et al.*, 2004). Single-nucleotide polymorphisms (SNPs) in metabolic pathways, which regulate enzymes such as MTHFR, are considered to be risk factors for thrombophilia. MTHFR is the key enzyme in folate, methionine, and homocysteine metabolism. The disturbances in MTHFR activity could be the cause of increased serum level of homocysteine. Hyperhomocystinemia is a risk factor for changes in coagulation cascade through direct Cytotoxic influence on endothelium, atherogenesis, activation of coagulation factor V and VII, increased level of thrombin, platelet aggregation, and a tendency toward venous thrombosis. (Spiroski, *et al.*, 2008).

The C677T polymorphism is a point mutation at the position 677 on MTHFR gene with the substitution of cysteine to thymine nucleotide at that position. This point mutation causes the substitution of alanine to valine in the MTHFR enzyme (Rosenberg, *et al.*, 2002).The common C677T missense mutation in the MTHFR gene, which converts an alanine to a valine residue, decreases the enzymatic activity and leads to high homocysteine and low folate levels in plasma (Botto and yang, 2000). Homocysteine is metabolised by either the transsulfaration pathway (excess homocysteine is converted to methionine) or the remethylation pathway (recycling of homocysteine to form methionine). Increased homocysteine is an independent risk factor for venous thrombo-embolism (Perry, 1999). The 677 C to T MTHFR mutation results in a thermo labile enzyme with reduced activity for the remethylation of homocysteine. The homozygous form of the mutation induces a state of hyperhomocystinemia (Kujovic, 2004).

The prevalence of the T allele varies among races and ethnic groups: it is more common in Caucasians and Asians (30%) compared to African Americans (11%) (Lovricevic, *et al.*, 2004). In Europe, the lowest frequency is found in Finland (25.1%) and the Netherlands (27.4%) and the highest in Italy (45%; 47.3% in Sicily), France (34%-36%), Hungary (33.7%) and Spain (33%) (Wilcken, *et al.*, 2003).

The incidence of the MTHFR C677T polymorphism in the general population is about 45% and 15% for heterozygous and homozygous genotypes, respectively. The prevalence of homozygotes for the MTHFR 677TT polymorphism in Europe varies between 5 and 15%; the heterozygous MTHFR C677T genotype presents the highest prevalence in Italy (44%) and the lowest in Norway (28%) (Lovricevic, *et al.*, 2004). The role of MTHFR polymorphisms in VTE is controversial: some authors have shown an association between the MTHFR C677T polymorphism and VTE (Jang, *et al.*, 2012), whereas others have proven the contrary (Bezemer, *et al.*, 2007).

1.2.3.1.1.4 Factor XII:

Factor XII or Hageman factor (named after the first patient found to have this deficiency) is the zymogen of a serine protease that initiates the contact activation reactions and intrinsic blood coagulation in vitro. Severe factor XII deficiency (factor XII activity less than 1 percent of normal) is inherited as an autosomal recessive trait. Affected patients have marked prolongation in the activated partial thromboplastin time (aPTT) and increased thrombotic tendency likely due to

reduced plasma fibrinolytic activity. Evaluating 14 Swiss families in 1991,Lammle, *et al* demonstrated that homozygous factor XII deficiency may be associated with an increased risk for venous thromboembolic disease. However, they suggested that partial factor XII deficiency was not, by itself, a strong risk factor for thrombosis (Lammle, *et al.*, 1991). In 2004, Girolami et al looked at reported cases of homozygous factor XII deficiency among Italian patients and noted 11 cases of venous thrombosis had been described. All but four of the cases were noted to be associated with various other risk factors such as pregnancy, the postpartum period, surgery, trauma, AT deficiency, or, heterozygous factor V Leiden. They concluded that the role played by FXII deficiency in the pathogenesis of venous thrombosis is minor, if any (Girolami, *et al.*, 2004).

1.2.3.1.1.5 Protein C and Protein S deficiencies:

Protein C inactivates factor Va and VIIIa involved in the anticoagulant process and this function is enhanced in the presence of protein S. Protein C deficiency results from a decrease in protein C antigen or the activity of protein C also Protein C is a 62-kD, vitamin K-dependent glycoprotein synthesized in the liver. It circulates in the blood as an inactive zymogene at a concentration of 4 μg/ml. Its activation into the serineprotease like enzyme, activated protein C (aPC), is catalyzed by thrombin when it is bound to the endothelial proteoglycan thrombomodulin (Dahlback, 2008) Protein S is a vitamin K-dependent, single-chain glycoprotein, which is synthesized in the liver and vascular endothelium, and acts mainly as a cofactor to aPC in the inactivation of FVIIa and FVa. Protein S is the principle cofactor of activated protein C, and deficiency states mimic protein C deficiency with increased fibrin formation Protein. Bind directly to inhibit factors Va, VIIIa, and Xa. Proteins exists in two distinct forms in plasma the free form accounts for 35 to 40% of total protein S , whereas the remainder is found in a form bound to C4b binding protein. Only the free protein S can serve as a cofactor for protein. The plasma level of protein S depends upon age, sex, lipid levels, estrogen, oral anticoagulant usage and the presence of acute thrombosis. In the plasma, around 60% of circulating protein S is bound to C4b binding protein, and only free protein S can function as a cofactor to aPC. Heritable protein S deficiency is transmitted as an autosomal trait. Those with heterozygous deficiency are at increased risk of venous thrombotic events (VTE), as well as warfarin-induced skin necrosis (Clark and Greer, 2006). Proteins S deficiency results from a decrease in the concentration or the function of protein S (Ten and Van, 2008). Protein S plays a role in inhibition of the clotting cascade. Protein S and C inactivate factors VIIIa and Va, required

cofactors for factors IXa and Xa. This is important because the most important natural inhibitor of clotting, the tissue factor pathway inhibitor, can be short circuited by factor IXa; so inhibition of the clotting cascade requires inhibition of factors IXa and Xa. This is achieved with the complex of activated protein C and protein S (Mary and Peter, 2005).

Sixty percent of protein S circulates in a protein bound form, and only the remaining 40% free form is biologically active. Certain conditions, such as pregnancy, inflammation, and surgical stress, lead to increased levels of the complement 4b-binding protein, which binds to protein S, and thereby decrease protein S activity. In addition, pregnancy is a thrombogenic state because of other alterations in the coagulation pathway (Namee, *et al.,* 2012).

In a study of 11 infants in Denver, Colorado in 1988, Manco-Johnson et al suggested that homozygotes for protein C deficiency can develop a severe thrombotic tendency in infancy characterized as purpura fulminans (Manco-Johnson, *et al,* 1988). Heterozygotes for protein C deficiency have an increased risk of developing warfarin-induced skin necrosis (Chan, *et al.,* 2000). Protein C deficiency has been implicated in adverse pregnancy outcomes such as DVT, preeclampsia, intrauterine growth restriction and recurrent pregnancy loss (Greer IA 2003). Family studies from the Netherlands and the US have shown that family members who are PC deficient are at an 8–10 fold increased risk of venous *thrombosis,* and, by age 40, 50% or more will have experienced a thrombotic event (Broekmans, *et al.,*1985 , Bovill, *et al.,* 1989).

Protein S deficiency is at least as common as antithrombin and protein C deficiency (Heijboer, *et al.,* 1990). The clinical manifestations are similar to those seen with antithrombin and protein C deficiency. Thrombosis occurs in heterozygotes whose levels of functional protein S are in the range of 15–50% of normal. In 1987, Engesser and colleagues conducted a study on 12 Swedish families with 136 members and found 71 of them to be heterozygous for Type I protein S deficiency. 55% of those who carried the defect were found to have had a thrombotic event and 77% of those were recurrent (Engesser, *et al.*, 1987).

1.2.3.1.1.6 Antithrombin III deficiencies:

Antithrombin is a potent inhibitor of the reactions of the coagulation cascade. Although the name, antithrombin, implies that it works only on thrombin, it actually serves to inhibit virtually all of the coagulation enzymes to at least some extent. The primary enzymes it inhibits are factor Xa, factor IXa and thrombin (factor IIa). It also has inhibitory actions on factor XIIa, factor XIa and the complex of factor VIIa and tissue factor. Its ability to limit coagulation through multiple interactions makes it one of the primary natural anticoagulant proteins (Hyers, 2001). Antithrombin acts as a relatively inefficient inhibitor on its own. However, when it is able to bind with heparin, the speed with which the reaction that causes inhibition occurs is greatly accelerated; this makes the antithrombin-heparin complex a vital component of coagulation. This interaction is also the basis for the use of heparin and low-molecular-weight heparins as medications to produce anticoagulation. There are two primary types of antithrombin deficiency: type I and type II. Type I antithrombin deficiency is characterized by an inadequate amount of normal antithrombin present. In this case, there is simply not enough antithrombin present to inactivate the coagulation factors. In type II antithrombin deficiency, the amount of antithrombin present is normal, but it does not function properly and is thus unable to carry out its normal functions. In many cases, the antithrombin in type I deficiencies has a problem binding to heparin, although there have been multiple other changes to the antithrombin molecule described. (Brenner, *et al.*, 2000)

The clinical relevance of a distinction between antithrombin I and antithrombin II deficiency lies in the higher risk of thrombosis associated with the type I variety. Antithrombin III is the most important inhibitor of thrombin, factor Xa, IXa and XII a. Antithrombin III deficiency results from the decrease in the concentration or the function of antithrombin III (Patnaik and Moll, 2008).

1.2.3.1.1.7 Plasminogen Activator Inhibitor 1 (PAI1):

Plasminogen activator inhibitor-1 is the principal inhibitor of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), the activators of plasminogen and hence fibrinolysis. Plasminogen activator inhibitor 1 (PAI-1) inhibits plasminogen activators (u-PA and t-PA) by forming stable complexes endocytosed via a low-density lipoprotein receptor super family member-dependent mechanism. PAI-1 circulates actively in plasma and latently in platelets but is also secreted and deposited into the matrix by several cells, where it participates in tissue repair

processes. Endothelial PAI-1 expression is modulated by a 4G/5G polymorphism in the PAI-1 promoter, which is 675 bp upstream from the start site of transcription. Angiotensin II plasma levels also influence PAI-1 expression (Buchholz, *et al.*, 2003).

High levels of PAI-1 may be associated with an increased risk of arterial thrombosis due to inhibition of fibrinolysis (Francis, 2002). Overall, the data available on the relationship between PAI-1 and the risk of thrombosis is conflicting. In 1996, Schulman and Wiman studied over 900 Swedish patients over 6 months after their initial presentation with a thrombotic event and found that elevated PAI-1 levels correlated with the risk of recurrence (Schulman and Wiman, 1996).

1.2.3.1.2 Screening for Inherited Thrombophilias:

Screening for an inherited thrombophilia should be considered in patients with thrombosis and a family history of thrombosis, thrombosis in unusual sites, thrombosis at an early age (less than ~40 to 45 years), or thrombosis without an obvious predisposing cause .The testing protocol would depend on the ethnic background of the patient, the particular thrombotic manifestation, and whether a specific inherited thrombophilia has been identified in relatives. As a general rule, tests should include an assay for APC resistance, AT and protein C and S levels, and possibly an assay for the prothrombin G20210A mutation .Acquired thrombophilic conditions, such as a lupus anticoagulant, should also be considered (William, 2002).

1.2.3.2 Acquired thrombophilia:

Acquired thrombophilias are hypercoagulable states secondary to various aetiologies.

1.2.3.2.1 Acquired hyperhomocystinemia:

Hyperhomocystinemia has been underlined as an emerging risk factor for several diseases such as arterial and/or venous thrombosis Hyperhomocystinemia may be acquired secondary to dietary and lifestyle factors such as a reduced intake of folate, vitamin B6 or vitamin B12, excessive caffeine consumption and excessive coffee intake.

The acquired form of hyperhomocystinemia may also result from certain medical conditions such as hypothyroidism or renal impairment. Inherited and acquired conditions have been involved to explain pathophysilogy as gene polymorphism .The Homocysteine Lowering Trial Collaboration has suggested that endothelial dysfunction, alteration of platelet reactivity and disruption of

prostacyclin pathways, may be some of the mechanisms responsible for the reported venous thrombosis risk as well as the theoretical risk of pregnancy loss (Clark, *et al.*, 2007).

1.2.3.2.2 Acquired activated protein C resistance:

APCR is the most prevalent risk factor for thrombosis. The presence of the factor V Leiden mutation produces a protein that is intrinsically resistant to activated protein C, causing the pathological phenotype .The pathophysilogy underlying APCR not caused by the FVL mutation is still not completely understood. In different studies, it has been suggested that acquired factors might be the cause of APCR in the absence of FV Leiden .A number of coagulation factors can affect the activated partial thromboplastin time (aPTT). Previous literature suggested a possible positive correlation between levels of factors V, VIII and IX and acquired APCR. Protein S and protein C, levels can (or may) affect acquired APCR. (Sara, *et al.*, 2011).

1.2.3.2.3 Lupus Anticoagulant/Antiphospholipid Antibody Syndrome:

Lupus anticoagulants are antibodies directed against proteins bound to phospholipids, which interfere with clotting assays .A closely related but not synonymous term is antiphospholipid antibodies (also called anticardiolipin antibodies). Lupus anticoagulants interfere with phospholipid-dependent clotting assays, usually the PTT .Occasionally, both the PTT and PT are prolonged and, rarely, just the PT. The term lupus anticoagulant derived from the fact that the antibodies were first described in two women who had systemic lupus erythematosus and a coagulopathy. It is now known that lupus anticoagulants are found in many patients who do not have lupus and in the majority of cases are associated with little risk of bleeding. In fact, lupus anticoagulants are associated with a significant risk of thrombosis, including thrombi in deep veins of the lower extremities, arteries, cerebral vessels, and unusual sites such as the mesenteric vessels and the portal or hepatic veins. Lupus anticoagulants appear to be a subset of antiphospholipid antibodies (APAs). Not all APAs interfere with phospholipid-dependent clotting tests; those that do are lupus anticoagulants. Antiphospholipid antibodies are usually detected by immunologic methods, such as an ELISA. Lupus anticoagulants are detected by clot-based tests such as the PTT, dilute Russell viper venom time, or a variety of others. Nearly all patients with lupus anticoagulants have a positive assay for APA (≥90%); however, many patients with a positive APA do not have lupus anticoagulants. There is not a good correlation between the titer of APA and the strength of the lupus anticoagulant in the individual case (ie, the patient may have a low titer APA by ELISA

but striking prolongation of the PTT, or vice versa). The APA is actually directed against a protein stuck on the phospholipid rather than the phospholipid itself; probably the most common protein is the 2-glycoprotein I (also called apolipoprotein H). Antibodies may also be directed against prothrombin, protein C or S,and others. Lupus anticoagulants/APAs are seen in some patients with lupus (often patients with a "biologic false-positive" VDRL test), in patients with other autoimmune diseases, with a variety of infections, with certain medications (antiarrhythmic drugs such as quinidine and procainamide, psychiatric drugs such as phenothiazines, and others),with HIV infection,and also for no apparent reason (idiopathic). Pathologic bleeding in people with lupus anticoagulants is uncommon; however, some patients also have a decrease in the prothrombin level, and these patients may have bleeding problems. Thrombosis is a much greater risk. In many cases, the lupus anticoagulant is detected by a PTT ordered as a preoperative screen, in which case it is more a nuisance than anything else (the surgeon has to hold the procedure until you can work up the coagulopathy). The APA syndrome includes a lupus anticoagulant or moderate- to high-titer APA together with some combination of venous and/or arterial thrombosis, central nervous system events (transient ischemic attacks, strokes, amaurosis fugax, others), thrombocytopenia, recurrent fetal loss, cardiac valvular abnormalities (nonbacterial vegetations on valves, most often the mitral valve), and livedo reticularis. It used to be believed that patients with lupus anticoagulants due to a medication or infection did not have a significantly increased risk of thrombosis; unfortunately, this may not be absolutely true. However, it is important to remember that many (possibly most) patients with lupus anticoagulant do not have thrombotic episodes (William, 2002).

Screening tests for detection of lupus anticoagulants include the PTT, dilute PTT, dilute Russell viper venom time, kaolin clotting time, and others. Several tests are available to confirm the phospholipid dependence of the inhibitor; one is the platelet neutralization procedure (PNP). In this test, exogenous phospholipid (such as reagent platelet membrane phospholipid) is added to the clotting test to overwhelm the antibody. The result of the plasma plus phospholipid mixture is compared to a plasma plus saline mixture (the saline is added to control for the dilutional effect of the extra reagent phospholipid). If the plasma plus phospholipid mixture shortens the clotting test significantly, the test is positive for a lupus anticoagulant. If the test used is the PTT, shortening by more than 6 seconds compared to the plasma plus saline control is usually considered positive (William, 2002).

1.2.3.2.4 Malignancies:

Many malignancies are associated with thrombophilia. Patients with malignancies may have several predisposing factors for thrombosis, including chronic DIC, debilitation and inactivity, medications, frequent surgical procedures, and abnormal blood vessel walls. Evidence of chronic DIC has been found in up to 50% of patients with metastatic carcinomas (William, 2002).

1.2.3.2.5 Oral Contraceptives:

The relationship of oral contraceptives to thrombosis has become a big issue of late. Women taking oral contraceptives appear to have ~3- to 4-fold increased risk of thrombosis (primarily deep venous thrombosis in the lower extremities) compared to women not taking oral contraceptives. Oral contraceptives can interact with inherited thrombophilia, notably factor V Leiden (William, 2002).

1.2.3.2.6 Miscellaneous:

A variety of other conditions confer an increased risk of thrombosis, including pregnancy, surgery, sepsis, the nephrotic syndrome, and many others. In pregnancy, the blood levels of many clotting factors are increased. Women may be less active or confined to bed rest, and the gravid uterus presses on the veins from the lower extremities, predisposing to stasis. Surgery increases the risk of thrombosis by several means. The risk is particularly high with hip replacements and other orthopedic surgeries. Sepsis can be thrombogenic due to decreased levels of protein C and free protein S. The nephrotic syndrome may be thrombogenic because of loss of antithrombin in the urine (William, 2002).

1.2.4 Previous studies:

Study of (Spiroski, *et al.,* 2008) which analyze the association of methylene tetra hydro folate reductase polymorphisms (MTHFR-677) with deep venous thrombosis in Macedonians and found no statistical significance (P value 0.412) between them.

Study of (Ghaznavi, *et al.,* 2015) which investigate the correlation between methylene tetra hydro folate reductase (MTHFR) C677T polymorphism with DVT risk in an Iranian population and found Neither MTHFR 677CT heterozygotes (P=0.37) nor MTHFR 677TT homozygotes (P=0.17) was significantly associated with DVT.

Study of (Jusić-Karić, *et al.,* 2016) which investigate frequency and association of 677 (C>T) MTHFR with deep vein thrombosis in the population of Bosnia and Herzegovina, they found no statistical significance (P value 0.368).

Study of (Hosseini, *et al.,* 2015) which assess the impact of, methylene tetra hydro folate reductase (MTHFR) C677T on occurrence of DVT in a population of Iran and found significantly associated increase DVT risk (P value <0.001).

Study of (Hotoleanu, *et al.,* 2013) which assess the frequency of the methylene tetra hydro folate reductase (MTHFR) C677T polymorphism in idiopathic venous thromboembolism (VTE) in a Romanian population and the associated risk of VTE, they found statistical significance in homozygous MTHFR 677TT genotype (P value 0.021) but there is no statistical significance in heterozygous MTHFR C677T genotype (P value 0.492).

Study of (Rouhi-Broujeni, *et al.,* 2016) which investigate the association between homozygous MTHFR C677T and VTE in Shahrekord, southwest Iran and found there is statistical significance association (P value 0.03).

1.2.5 Rationale:

Lower extremity deep venous thrombosis (DVT) remains a common and serious medical condition manifesting in patients with recognized or unrecognized risk factors and complicates the outcome of critically ill patients and the postoperative recovery of surgical patients. In recent years, many authors have confirmed that thrombophilia, either acquired or genetic defined as a predisposition to increased risk of venous and occasionally arterial thromboembolism due to hematological abnormalities, was often responsible for the occurrence of lower extremity DVT (Lane, *et al.*, 1996 and Greaves and Baglin, 2000).

Among the "traditional" thrombophilia factors (such as protein C, protein S or antithrombin III deficiency, Factor V Leiden or Prothrombin G20210A mutations), methylenetetrahydrofolate reductase (MTHFR) polymorphisms associated with hyperhomocysteinemia have recently raised interest. The C677T mutation is the most common polymorphism of methylene tetrahydrofolate reductase (MTHFR) and its lead to decreased enzyme activity and therefore elevation of homocysteine level. Several studies have shown that this polymorphism might be associated with DVT due to hyperhomocysteinemia. MTHFR C677T mutation has been reported in different population worldwide. However, in Sudan there is no available literature regarding this issue.

So this study take place as starter for coming studies to verify the relation between the MTHFR C677T mutation and deep vein thrombosis in Khartoum state in a series of Sudanese patients with lower extremity DVT.

1.2.6Objective:

1.2.6.1 General objective:

To study the association of MTHFR (C677T) polymorphism with deep vein thrombosis and detection of coagulation profile in Sudanese patients with DVT.

1.2.6.2 Specific objective:

1. To detection of MTHFR gene (C677T) polymorphism in cases and controls.
2. To compare the result of MTHFR gene (C677T) polymorphism between case and control group.
3. To measure PT and PTT in cases and controls.
4. To compare the result of PT and PTT between case and control group.

Chapter Two

Chapter Two

Materials and Methods

2.1 Materials:

2.1.1 Study design:

Analytical case control study conducted in Khartoum state during the period of February 2018 to January 2019 to study the association of MTHFR gene (C677T) polymorphism and deep vein thrombosis.

2.1.2 Study population:

Forty Sudanese patients diagnosed with DVT by Doppler ultrasound were included in this study.

2.1.3 Inclusion Criteria:

Sudanese patients with DVT, both male and female.

2.1.4 Exclusion criteria:

Known patient with other thrombophilic condition.

2.1.5 Ethical consideration:

The consent of the selected individuals to the study was taken after being informed with all detailed objectives of the study and its health benefit in future.

2.1.6 Data collection:

Data were collected using self-administered pre-coded questionnaire which was specifically designed to obtain information.

2.1.7 Data presentation:

The data were presented in tables and figures.

2.1.8 Sampling:

Non-probability sampling method was used (only who accepted study tests) (volunteers) were involved in sample, samples collected from Omdurman hospital.

2.1.9 Data analysis:

Data were entered and analyzed by SPSS program (version: 21.0).

2.2 Methodology:

2.2.1 Sample collection:

5 ml of venous blood was collected from each participant into EDTA and tri-sodium citrate container, blood specimens were labeled with patient name, number, date and time of collection.

2.2.2 Hematological analysis:

2.2.2.1Preparation of platelet poor plasma (PPP):

2.5 ml of venous blood was collected into container containing 0.5 ml tri sodium citrate as anticoagulant, and then the blood is centrifuged, after thoroughly mixing, for 15 minutes at 3000 rpm to obtain platelets poor plasma (PPP).

2.2.2.2 Prothrombin Time:

2.2.2.2.1 Principle:

The PT test measures the clotting time of plasma in the presence of an optimal concentration of tissue extract (thromboplastin) and indicates the overall efficiency of the extrinsic clotting system. Although originally thought to measure prothrombin, the test is now known to depend also on reactions with factors V, VII, and X and on the fibrinogen concentration of the plasma.

An aliquot of test platelet-poor plasma was incubated at 37C° with a reagent containing a tissue factor, phospholipid (thromboplastin), and CaCl2. The time required for clot formation was measured by coagulometer the results was reported in seconds (prothrombin time).

2.2.2.2.2 Reagents:

1) Thromboplastin:

Thromboplastins were originally tissue extracts obtained from different species and different organs containing tissue factor and phospholipid. Because of the potential hazard of viral and other

infections from handling human brain, it should no longer be used as a source of thromboplastin. The majority of animal thromboplastins now in use are extracts of rabbit brain or lung.

2) CaCl2:0.025 mol/l.

2.2.2.3 Activated Partial Thromboplastin Time:

Other forms of the APTT test are known as the partial thromboplastin time with kaolin (PTTK) and the kaolin cephalin clotting time (KCCT), reflecting the methods used to perform the test.

2.2.2.3.1 Principle:

The test measures the clotting time of plasma after the activation of contact factors but without added tissue thromboplastin and so indicates the overall efficiency of the intrinsic pathway. The test depends not only on the contact factors and on factors VIII and IX, but also on the reactions with factors X, V, prothrombin, and fibrinogen. It is also sensitive to the presence of circulating anticoagulants (inhibitors) and heparin.

An aliquot of undiluted, Platelet poor plasma [PPP] was incubated at 37°C then phospholipid (cephalin) and a contact activator (e.g. Kaolin, micronized silica or ellagic acid) were added followed by calcium (all pre-warmed to 37°C). Addition of calcium initiates clotting and timing begins. The aPTT result was reported as the time required for clot formation after the addition of CaCl2 and then measured by coagulometer.

2.2.2.3.2 Reagents:

1) Kaolin:

5 g/l (laboratory grade) in barbitone buffered saline, pH 7.4.A few glass beads were added to aid resuspension. The suspension is stable at room temperature. Other insoluble surface active substances such as silica, celite, or ellagic acid can also be used.

2) Phospholipids:

Cephalin as phospholipids substitution.

3) CaCl2:

0.025 mol/l.

2.2.3 Molecular analysis:

The detection of MTHFR gene was analyzed by Polymerase chain reaction (PCR) method.

2.2.3.1 DNA Extraction by Guanidine chloride method:

It was extracted by Guanidine chloride method , the 2.5 ml of EDTA blood samples was placed in a tube , 10 ml of the Red cell lysis buffer (RCLB) (8.3 gm of NH4CL, 1 gm KHCO3, 1.8ml 5% EDTA and liter of distilled water) was added , mixed well and centrifuged at 6000 rpm for 5 minutes, this step was repeated until a clear pellet of white blood cells appeared at the bottom of the tube , samples were centrifuged at high speed 3000 rpm for 10 minutes for collection of pellets , 2 ml of the white blood cell lysis buffer (WCLB) (1.576 gm Tris- HCl, 1.088 gm EDTA, 0.0292 gm NaCl, 0.2% SDS, and 100 ml distilled water) , 10 µl of protienase K , 1 ml guanidine Chloride and 300 µl NH4 acetate was added to the clear white pellets , the mixture was incubated overnight at 37°C , cooled to room temperature , then transferred to 2 ml pre chilled chloroform in 15 ml falcon tubes , the mixture was mixed well by vortexing and centrifuged at 2500rpm for 5 minutes, the upper layer was collected in new tube and 10 ml of cold Absolute Ethanol was added , the mixture was shaked well and kept overnight at -20°C, centrifuged at 3000 rpm for 20 minutes , the supernatent was carefully drained and the tube was inverted on a tissue paper for 5 minutes , the pellet was washed with 4 ml of 70% Ethanol and centerfuged at 3000 rpm for 15 minutes , the supernatent was discarded and the pellet was allowed to dry for 1 hour then re-suspended in 100µl Tris EDTA (2.42 Tris buffer2.42 Tris base, 0.57 ml acetic acid, 50µl EDTA (0.01M) and 100 ml distilled water), mixed well by vortexing , and the obtained DNA was aliqout as stock solution , (stored at -20°C).

2.2.3.2 Determination of the DNA quality and purity:

Part of the DNA solution was mixed with loading dye by ratio 1 in 5 and DNA quality and purity was determined using 1% Agarose gel electrophoresis

2.2.3.3 DNA storage:

DNA was preserved at -20°C until PCR was performed.

Table 2.1The primers sequence

Primer Name	Primer sequence	Product size bp
Forward primer	(5' TGA AGG AGA AGG TGT CTG CGG GA-3')	198bp
Reverse primer	(5'AGG ACG GTG CGG TGA GAG AGT G -3')	

PCR mixture of 20µ was prepared using premix master mix tube (Maxime PCR PreMix Kit (i-Taq)) for each sample, in Ependorf tube as follow:

Table 2.2 PCR mixture:

Reagent	Volume µ
Distilled water	12
Forward primer	1
Reverse primer	1
DNA sample	6
Total volume	20

The PCR protocol as follow:-

Table 2.3 The PCR protocol:

Profile	Temperature	Time duration	Number of cycles
Initial Denaturation	94°C	3 minutes	1
Denaturation	94°C	30 seconds	40
anneling	60°C	30 seconds	
Extension	72°C	30 seconds	
Final Extension	72°C	4 minutes	1

2.2.3.4 Detection of PCR product:

Detection of the product done by gel electrophoresis by using 1.5% agarose gel which stained by ethidium bromide, and 1X Tris EDTA buffer (TE) used as running buffer, 5µl of the product was applied into the gel, the voltage was 100 volt for 30 minutes and DNA ladder (50bp) was used as molecular weight marker

2.2.3.5 MTHFR digestion:

0.2ul of restriction enzyme (Hindfl) was added to 4.8ul of distil water , 1ul of enzyme buffer and 5ul op PCR product a quick spinning is needed, Incubated at 37 ºC 18 hours, and the reaction was Stopped with 4 µl prom phenol blue dye, then 18 µl digested products was loaded into 2% agarose

2.2.3.6 Results interpretation:

The wild-type DNA yields a solitary gave (198 bp) bands and heterozygous yields three bands (198, 175, 23 bp) respectively, and homozygous mutation yield tow band of (175, 23 bp).

Chapter Three

Chapter Three

Results

This data were collected from 40 cases (14 male 35% and 26 female 65%) confirmed to have DVT and 40 control (30 male 75% and 10 female 25%) as showed in figure (3.1). The mean age of case group was 38.08, age range of 21- 40 years, mean age of control group was 29.03, age range 21-40 years as showed in figure (3.2). The mean of disease duration in month in study group was 12.13, duration range of 1-12 months. 31 (77.5%) of case group was use heparin as treatment and 9 (22.5%) of case group was use warfarin. The variable frequencies of case group under study included: pregnancy 46.2%, contraceptive pills 84.6%, surgery32.5% as showed in table (3.1).

All the patient confirmed to have DVT had the wild gene and hence were negative for MTHFR (C677T) gene mutation, and there were no positivity for mutation among control group .this result showed no statistical differences in present of MTHFR mutation between case and control group.

The screening test fore case and control showed no significant different in the mean of PT (P.value 0.321) and PTT (P.value 0.098) between case and control group table (3.2) .

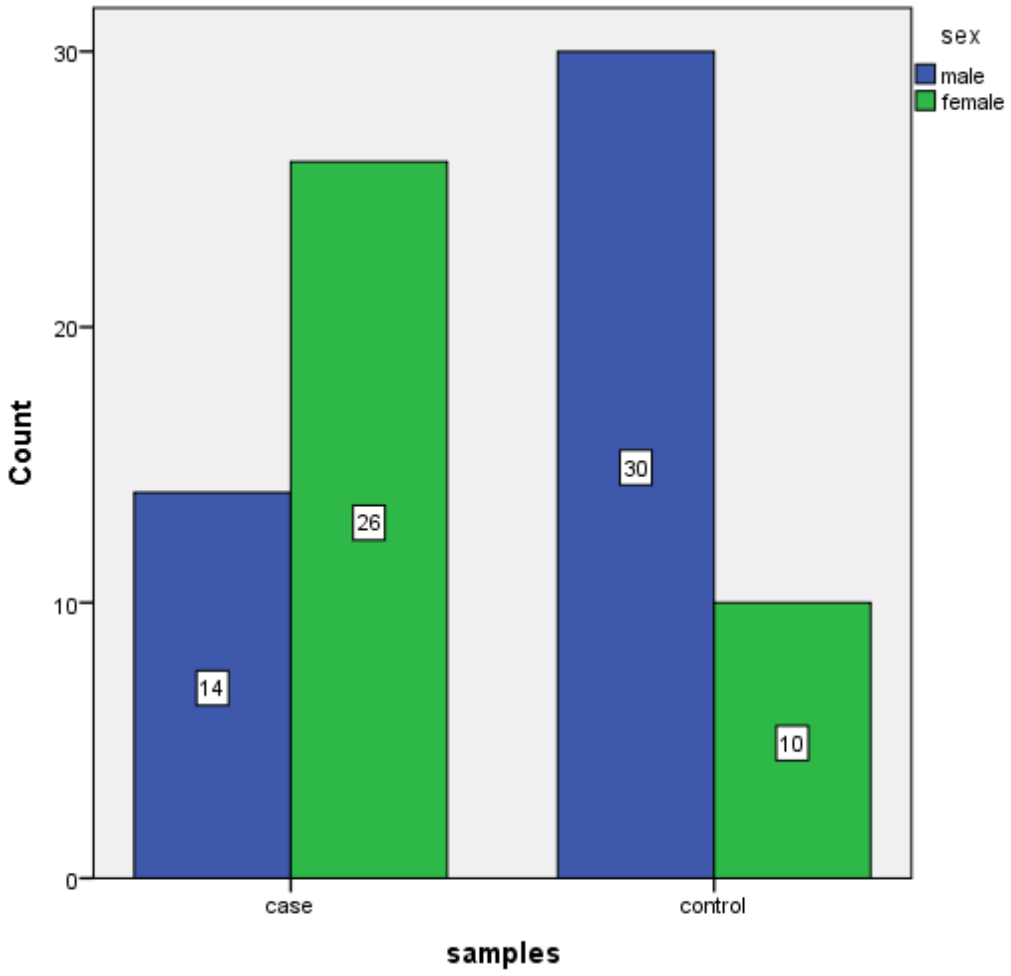

Figure (3.1): Distribution of sex among case and control group.

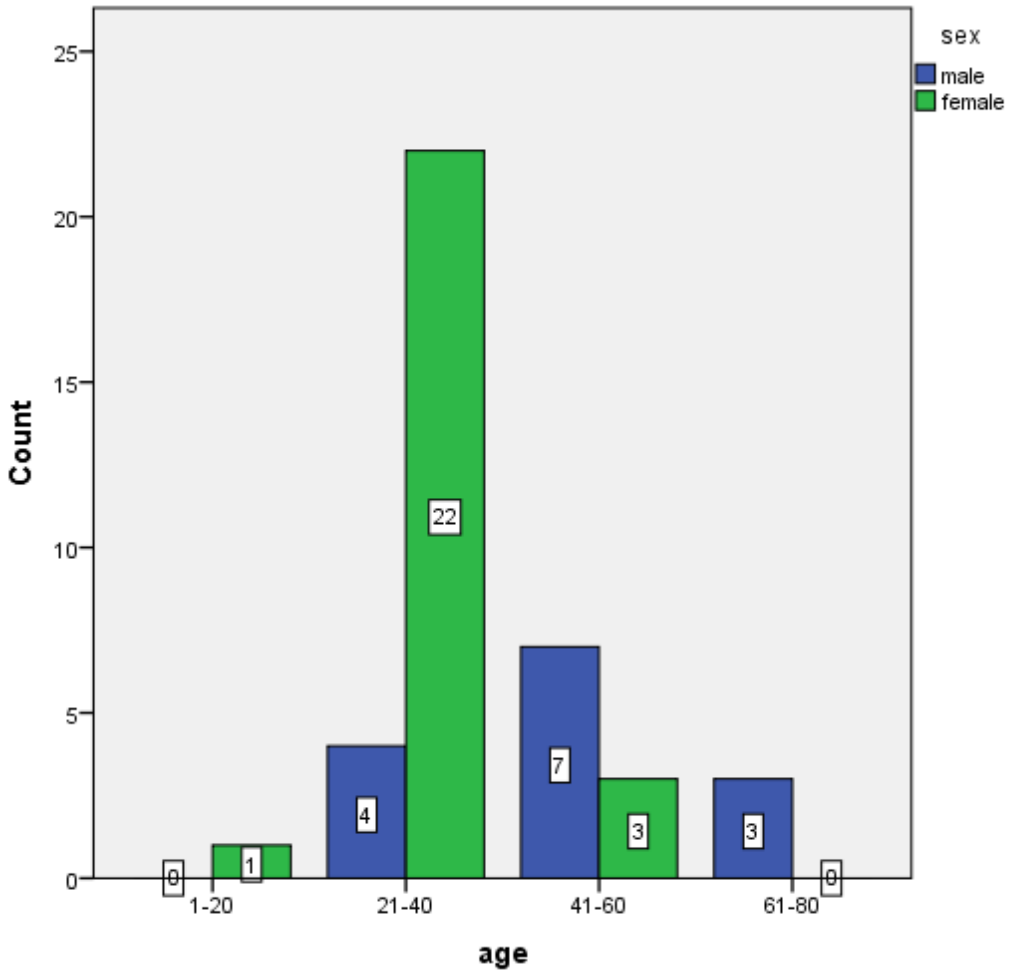

Figure (3.2): Distribution of age group among case.

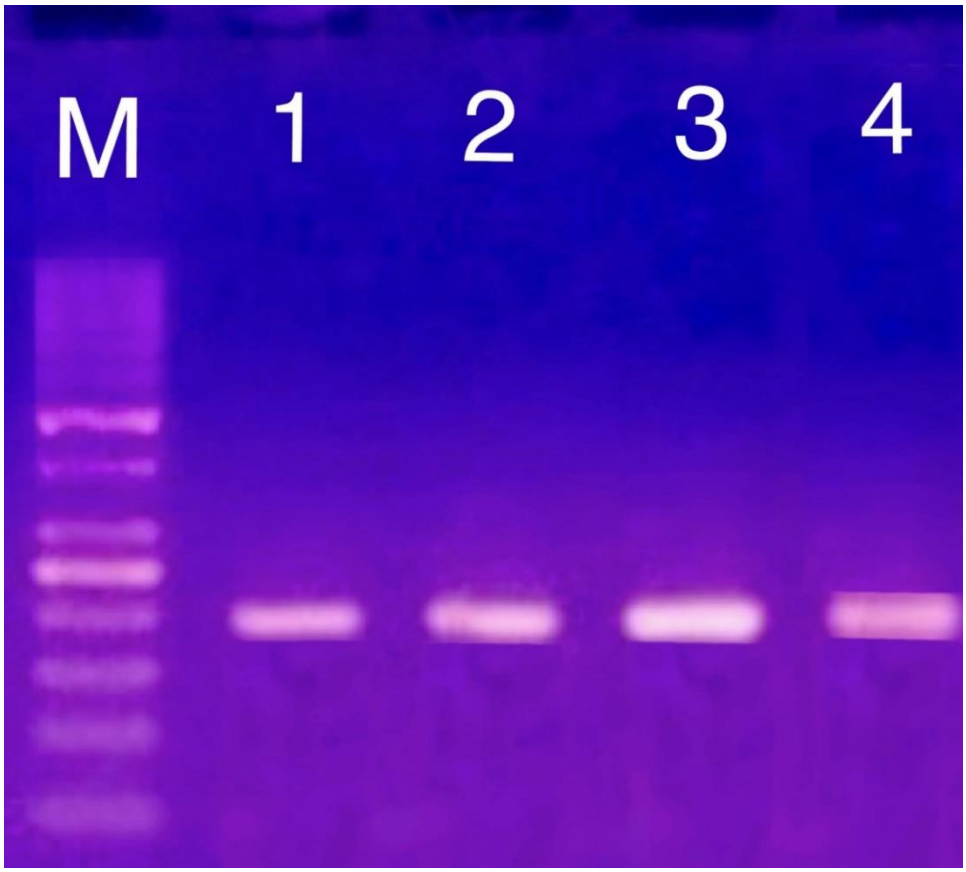

Figure (3.3): PCR amplification of MTHFR gene mutation

Digestion of MTHFR gene with Hinf1 on 2% agarose gel dissolved in 1X TBE buffer, stained with ethidium bromide, Lane M molecular weight marker 50bp , Lane 1 undigested (198bp), Lane 2,3and 4 were wild type (CC).

Table (3.1): Frequency of risk factors among case group

Risk factors	Yes N (%)	No N (%)	Total
Surgery	13 (32.5%)	27 (67.5%)	40
Pregnancy	12 (46.2%)	14(53.8%)	26
Contraceptive	22 (84.6%)	4 (15.4%)	26

Table (3.2): Relative distribution of PT and PTT in case and control group.

parameter	Sample	N	Mean	Std.	P.value
PT	Case	40	43.0	189.75	0.321
	Control	40	12.8	1.30	
PTT	Case	40	33.7	9.20	0.098
	Control	40	31.0	3.46	

Chapter Four

Chapter four

Discussion, conclusion and recommendation

4.1 Discussion:

Analytical case control study was conducted In Khartoum state during the period of February 2018 to January 2019 to study the prevalence of MTHFR gene (C677T) mutation in deep vein thrombosis patient. A 40 sample were collected from Sudanese patient according to inclusion criteria and considered as case, and 40 samples were collected from healthy people, and considered as control.

In this study the mean age of case group was (38.08), this result confirm the consideration of old age as risk factor for DVT and this agree with (Esmon, 2009 and Shaheen, *et al.*, 2012) they found according to the studies, 1 in 1000 individuals in old age population is complicated by DVT, annually and also with (Kesieme, *et al.*, 2011) who found DVT is a common disorder in the elderly and its incidence rises markedly with age.

About the risk factor of population under study: 46.2% of female in case group was pregnant, women are up to 5 times more likely to develop DVT during pregnancy than when not pregnant (Marik and Plante, 2008), this also agree with (Evans and Ratchford, 2014) they found that pregnancy increase the chance of developing VTE by about four to five fold .The frequency of VTE is similar in all three trimester but higher during postpartum period (Martinelli, *et al.*, 2002).Women who use contraceptive pills were 84.6% of female in case group, women taking combined oral contraceptive pills have as with any other hormonal contraception, an increased risk of venous thromboembolism, it estimated that the risk of VTE increased 3to 5 times in individuals using second-generation COCs and up to 6 to 8 times in those using third-generation COCs (Piparva and Buch, 2011). Also there were 32.5% of case group underwent surgery, surgery lead to 6-fold increased risk to venous thromboembolism (Rosendaal,1999), the risk of DVT is different depending on the type of surgery performed ,general surgery has 15-30% risk for developing DVT in the absent of anticoagulant or mechanical prophylaxis. Vascular surgery may be complicated with VTE in up to 30% of cases, this percentage being reduced to 2.8% in case of drug prophylaxis (Geerts, *et al.*, 2008). Orthopedic surgery and surgery performed in patients with multiple injuries have the greatest risk of developing DVT (Gould, *et al.*, 2012).

About the numeric data the mean of PT in study group was (43.0 second) which increase than that of control group (12.8 second) but without statistical significance (P value 0.321),and the mean of PTT in study group was (33.7 second) which slightly increase than that of control group (31.0 second) but without statistical significance (P value 0.098).this increase in the mean of PT and PTT in case group occur because the patients in this study are on heparin and warfarin treatment which cause PT and PTT prolongation.

In this study all the patient conformed to have DVT had the wild gene and hence were negative for MTHFR(C677T) gene mutation, and there were no positivity for mutation among control group .This result showed no statistical differences in present of MTHFR mutation between case and control group, that is mean there is no association between MTHFR (C677T) gene mutation and DVT in this study group. This result is consistent with the study of (Spiroski,*etal*,2008) which analyze the association of methylene tetra hydro folate reductase polymorphisms (MTHFR-677) with deep venous thrombosis in Macedonians with no statistical significance (P value 0.412) , and consistent with the study of (Ghaznavi, *et al.*, 2015) which investigate the correlation between methylene tetra hydro folate reductase (MTHFR) C677T polymorphism with DVT risk in an Iranian population and found Neither MTHFR 677CT heterozygotes (P=0.37) nor MTHFR 677TT homozygotes (P=0.17) was significantly associated with DVT, also consistent with the study of (Jusić-Karić, *et al.*, 2016) which investigate frequency and association of 677 (C>T) MTHFR with deep vein thrombosis in the population of Bosnia and Herzegovina with no statistical significance (P value 0.368).and not consistent with the study of (Hosseini, *et al.*, 2015) which assess the impact of, methylenetetrahydrofolate reductase (MTHFR) C677T on occurrence of DVT in a population of Iranian patients with significantly associated increase DVT risk (P value <0.001) , and not consistent with the study of (Hotoleanu, *et al.*, 2013) which assess the frequency of the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism in idiopathic venous thromboembolism (VTE) in a Romanian population and the associated risk of VTE with statistical significance in homozygous MTHFR 677TT genotype (P value 0.021) but there is no statistical significance in heterozygous MTHFR C677T genotype (P value 0.492) .Also not consistent with the study of (Rouhi-Broujeni, *et al.*, 2016) which investigate the association between homozygous MTHFR C677T and VTE in Shahrekord, southwest Iran with statistical significance (P value 0.03) .this discrepancies are most probably due to small sample size in this study, non-equal distribution of patients age, ethnic affiliation and different geographic prevalence of this mutation.

4.2 Conclusion:

The (MTHFR) C677T polymorphism is not associated with DVT in this study group, and the mean of PT and PTT of case group are insignificance different than that of control group.

4.3 Recommendation:

Another study should conducted to evaluate the role of MTHFR C677T polymorphism in thrombotic complication among patients with assessment of homosestine level.

Equal distribution of age group should be made in case and control group.

Sample size should be increase.

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Appendices

Appendices

Appendix (1)

Questionnaire for DVT Patients

Sudan University of Science and Technology

College of Graduate studies

Detection of Methylene Tetra Hydro Folate Reductase Gene C677T Polymorphism and Coagulation Tests among Sudanese patients with Deep Vein Thrombosis

Duration of research:

Name: …….. **No: ……**

Gender: ……..…

Age: ……....

Residence: ……..

Mobile number: ……....

Duration of the disease (DVT): ……

Treatment: ……

Pregnancy: ……

Contraceptive pills: ...……..

Surgery: …….....

Others (If any): ……

Laboratory results:

A) PCR

(MTHFR) C677T……

B) Coagulation profile:

1. PT…….

2. APTT……

Remarks:……...
……
……
……

Date: …….. Signature: ……

Thank you

Appendix 2

CONSENT TO PARTICEPATE IN RESEARCH STUDY

Study title: Detection of Methylene Tetra Hydro Folate Reductase Gene C677T Polymorphism and Coagulation Tests among Sudanese patients with Deep Vein Thrombosis.

Investigator: Hanaa Mohamed Ebrahim Altayeb .

Short-Form Written Consent

I confirm that the researcher has explained the element of informed consent to the participant.

The subject know that there participation is voluntary. The purpose of research as well as the risks and benefits have been explained. The procedure as well as the time commitment have been out lined. The participant understands issues of confidentiality.

Witness name: …….

Witness signature: …….

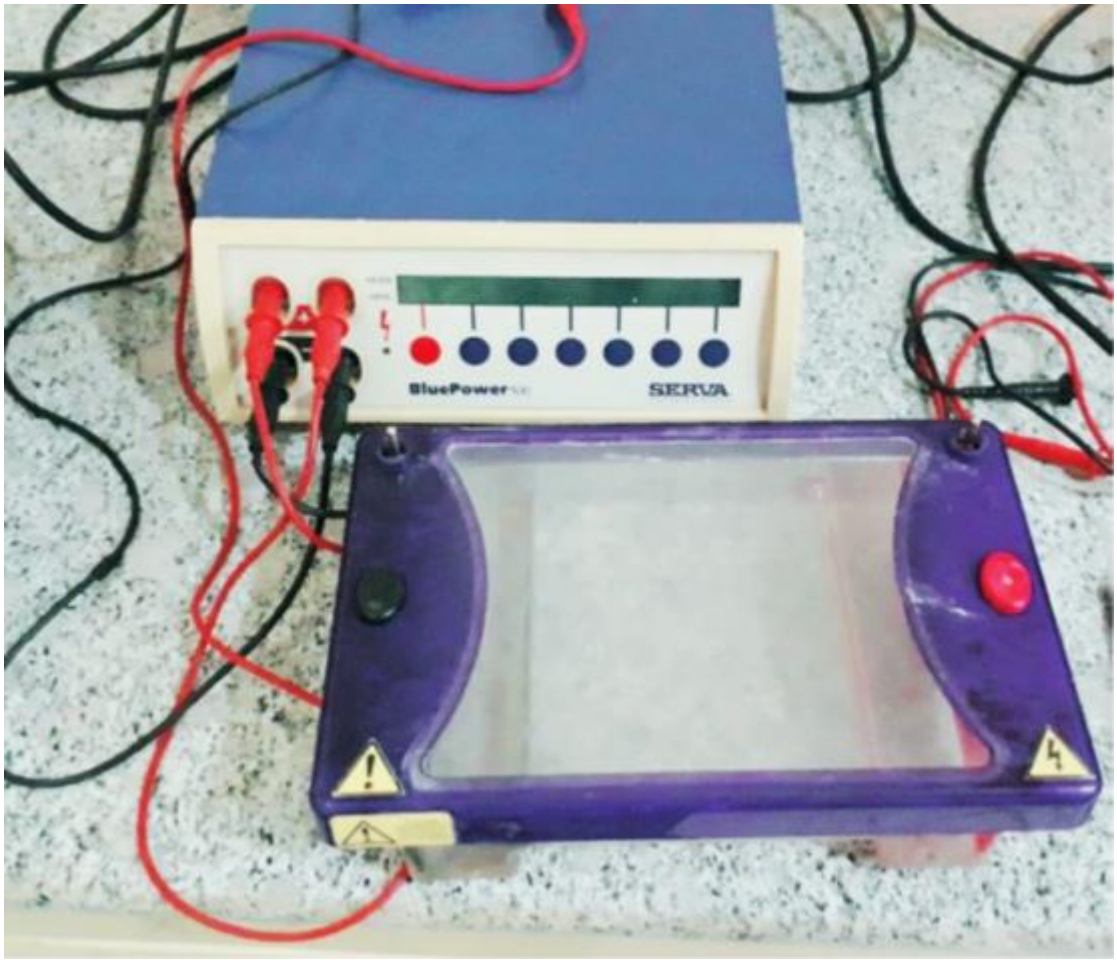
Participant name: ……..

Participant signature: …….

Appendix 3:

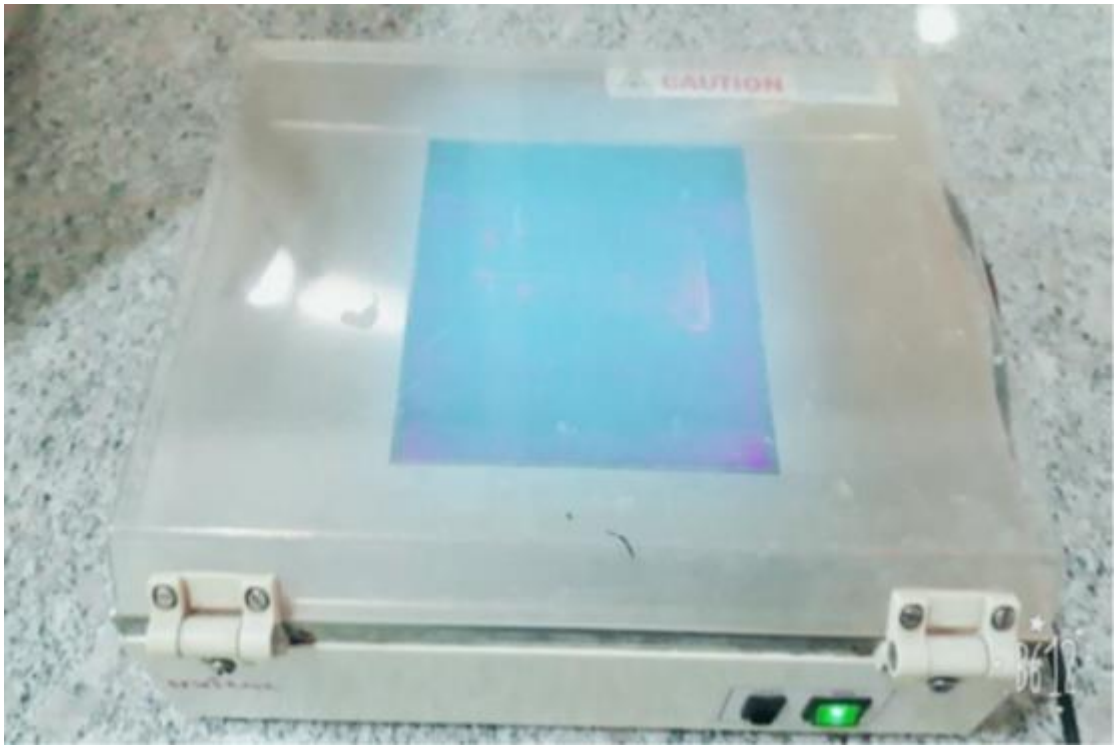
PCR Machine

Appendix 4:



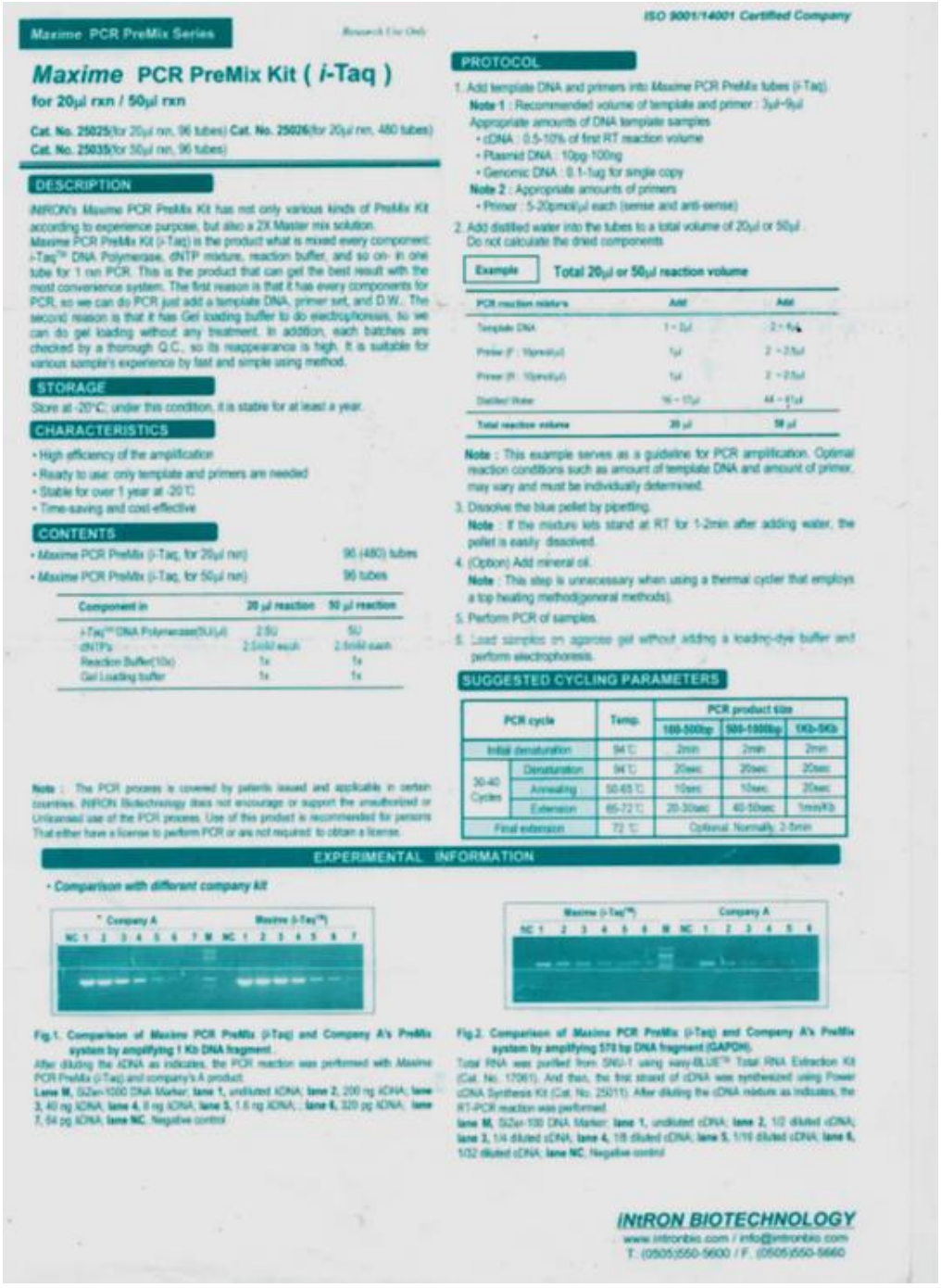
Gel Electrophoresis

Appendix 5:



UV Light Machine

Appendix 6:



ISO 9001:14001 Certified Company

Maxime PCR PreMix Series

Research Use Only

Maxime PCR PreMix Kit (*i*-Taq)

for 20μl rxn / 50μl rxn

Cat. No. 25025(for 20μl rxn, 96 tubes) **Cat. No. 25026**(for 20μl rxn, 480 tubes)
Cat. No. 25035(for 50μl rxn, 96 tubes)

DESCRIPTION

INTRON's Maxime PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution.
Maxime PCR PreMix Kit (i-Taq) is the product what is mixed every component: i-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W.. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

STORAGE

Store at -20°C; under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20℃
- Time-saving and cost-effective

CONTENTS

- *Maxime* PCR PreMix (i-Taq, for 20μl rxn) 96 (480) tubes
- *Maxime* PCR PreMix (i-Taq, for 50μl rxn) 96 tubes

Component in	20 μl reaction	50 μl reaction
i-Taq™ DNA Polymerase(5U/μl)	2.5U	5U
dNTPs	2.5mM each	2.5mM each
Reaction Buffer(10x)	1x	1x
Gel Loading buffer	1x	1x

Note : The PCR process is covered by patents issued and applicable in certain countries. INTRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license.

PROTOCOL

1. Add template DNA and primers into *Maxime* PCR PreMix tubes (i-Taq).
   Note 1 : Recommended volume of template and primer : 3μl~9μl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   Note 2 : Appropriate amounts of primers
   - Primer : 5-20pmol/μl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20μl or 50μl.
   Do not calculate the dried components

Example Total 20μl or 50μl reaction volume

PCR reaction mixture	Add	Add
Template DNA	1 ~ 3μl	2 ~ 4μl
Primer (F : 10pmol/μl)	1μl	2 ~ 2.5μl
Primer (R : 10pmol/μl)	1μl	2 ~ 2.5μl
Distilled Water	16 ~ 17μl	44 ~ 41μl
Total reaction volume	20 μl	50 μl

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

PCR cycle		Temp.	PCR product size		
			100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation		94℃	2min	2min	2min
30-40 Cycles	Denaturation	94℃	20sec	20sec	20sec
	Annealing	50-65℃	10sec	10sec	20sec
	Extension	65-72℃	20-30sec	40-50sec	1min/Kb
Final extension		72℃	Optional, Normally, 2-5min		

EXPERIMENTAL INFORMATION

- *Comparison with different company kit*

Fig.1. Comparison of Maxime PCR PreMix (i-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment.
After diluting the cDNA as indicates, the PCR reaction was performed with Maxime PCR PreMix (i-Taq) and company's A product.
Lane M, SiZer-1000 DNA Marker; **lane 1**, undiluted cDNA; **lane 2**, 200 ng cDNA; **lane 3**, 40 ng cDNA; **lane 4**, 8 ng cDNA; **lane 5**, 1.6 ng cDNA; , **lane 6**, 320 pg cDNA; **lane 7**, 64 pg cDNA; **lane NC**, Negative control

Fig.2. Comparison of Maxime PCR PreMix (i-Taq) and Company A's PreMix system by amplifying 578 bp DNA fragment (GAPDH).
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
lane M, SiZer-100 DNA Marker; **lane 1**, undiluted cDNA; **lane 2**, 1/2 diluted cDNA; **lane 3**, 1/4 diluted cDNA; **lane 4**, 1/8 diluted cDNA; **lane 5**, 1/16 diluted cDNA; **lane 6**, 1/32 diluted cDNA; **lane NC**, Negative control

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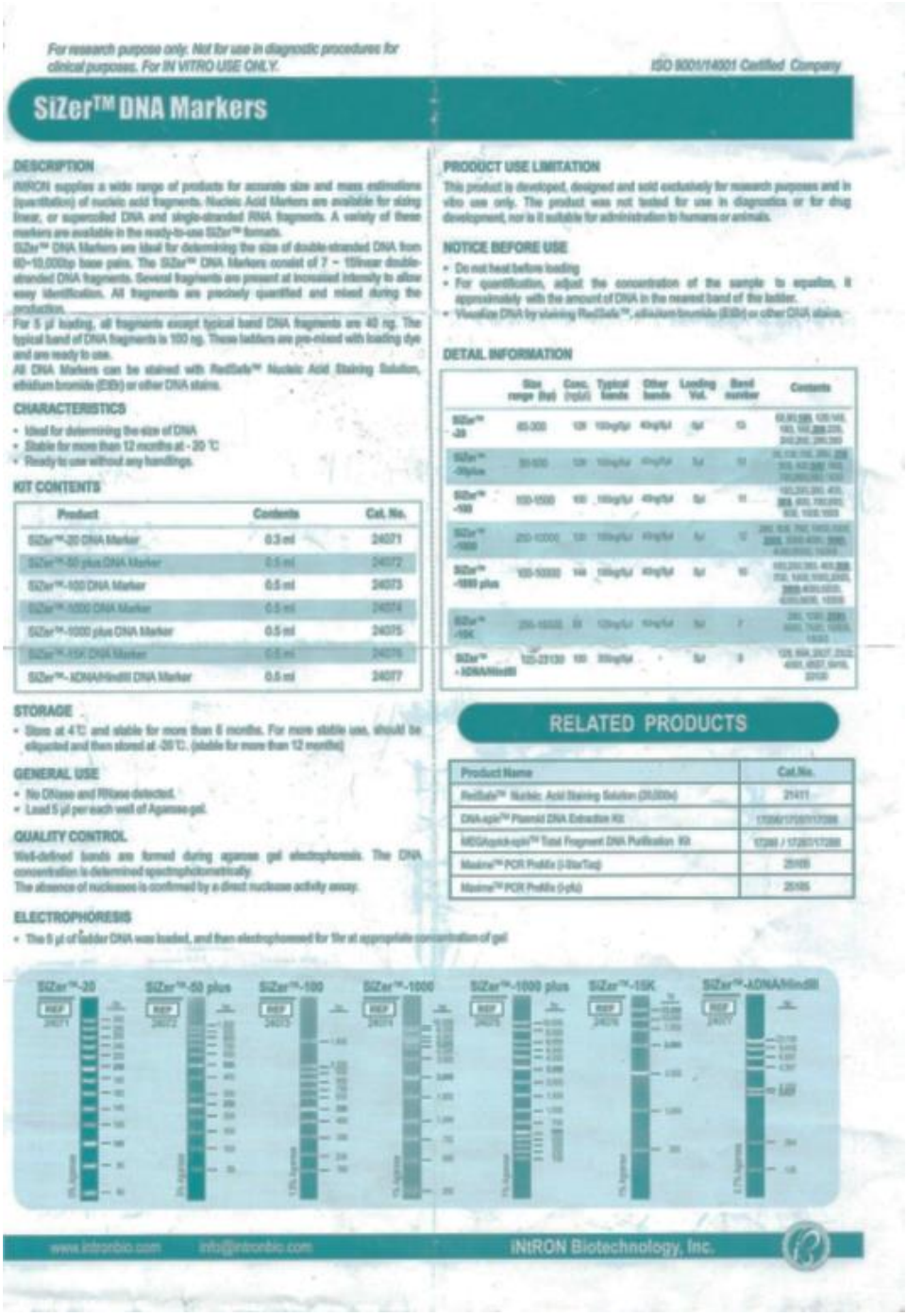
Maxime PCR PreMix Kit (i-Taq))



For research purpose only. Not for use in diagnostic procedures for clinical purposes. For IN VITRO USE ONLY.

ISO 9001/14001 Certified Company

SiZer™ DNA Markers

DESCRIPTION

iNtRON supplies a wide range of products for accurate size and mass estimations (quantitation) of nucleic acid fragments. Nucleic Acid Markers are available for sizing linear, or supercoiled DNA and single-stranded RNA fragments. A variety of these markers are available in the ready-to-use SiZer™ formats.

SiZer™ DNA Markers are ideal for determining the size of double-stranded DNA from 60~10,000bp base pairs. The SiZer™ DNA Markers consist of 7 ~ 15linear double-stranded DNA fragments. Several fragments are present at increased intensity to allow easy identification. All fragments are precisely quantified and mixed during the production.

For 5 µl loading, all fragments except typical band DNA fragments are 40 ng. The typical band of DNA fragments is 100 ng. These ladders are pre-mixed with loading dye and are ready to use.

All DNA Markers can be stained with RedSafe™ Nucleic Acid Staining Solution, ethidium bromide (EtBr) or other DNA stains.

CHARACTERISTICS

- Ideal for determining the size of DNA
- Stable for more than 12 months at - 20 ℃
- Ready to use without any handlings.

KIT CONTENTS

Product	Contents	Cat. No.
SiZer™-20 DNA Marker	0.3 ml	24071
SiZer™-50 plus DNA Marker	0.5 ml	24072
SiZer™-100 DNA Marker	0.5 ml	24073
SiZer™-1000 DNA Marker	0.5 ml	24074
SiZer™-1000 plus DNA Marker	0.5 ml	24075
SiZer™-15K DNA Marker	0.5 ml	24076
SiZer™- λDNA/HindIII DNA Marker	0.5 ml	24077

STORAGE

- Store at 4℃ and stable for more than 6 months. For more stable use, should be aliquoted and then stored at -20℃. (stable for more than 12 months)

GENERAL USE

- No DNase and RNase detected.
- Load 5 µl per each well of Agarose gel.

QUALITY CONTROL

Well-defined bands are formed during agarose gel electrophoresis. The DNA concentration is determined spectrophotometrically.

The absence of nucleases is confirmed by a direct nuclease activity assay.

ELECTROPHORESIS

- The 5 µl of Ladder DNA was loaded, and then electrophoresed for 1hr at appropriate concentration of gel

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and in vitro use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

NOTICE BEFORE USE

- Do not heat before loading
- For quantification, adjust the concentration of the sample to equation, it approximately with the amount of DNA in the nearest band of the ladder.
- Visualize DNA by staining RedSafe™, ethidium bromide (EtBr) or other DNA stains.

DETAIL INFORMATION

	Size range (bp)	Conc. (ng/µl)	Typical bands	Other bands	Loading Vol.	Band number	Contents
SiZer™ -20	60-200	120	100ng/5µl	40ng/5µl	5µl	15	60,80,100, 120,140, 160, 180,200,220, 240,260, 280,300
SiZer™ -50plus	50-500	120	100ng/5µl	40ng/5µl	5µl	15	50, 100, 150, 200, 250, 300, 350, 400,450, 500, 550, 600, 650, 700
SiZer™ -100	100-1500	100	100ng/5µl	40ng/5µl	5µl	11	100,200,300, 400, 500, 600, 700,800, 900, 1000,1500
SiZer™ -1000	250-10000	120	100ng/5µl	40ng/5µl	5µl	12	250, 500, 750, 1000,1500, 2000, 3000,4000, 5000, 6000,8000, 10000
SiZer™ -1000 plus	100-50000	144	120ng/5µl	40ng/5µl	5µl	10	100,200,300, 400,500, 700, 1000,2000, 3000,4000,5000, 6000,8000, 10000
SiZer™ -15K	250-15000	80	120ng/5µl	40ng/5µl	5µl	7	250, 1000, 2000, 3000, 7000, 10000, 15000
SiZer™ -λDNA/HindIII	120-23130	100	80ng/5µl	-	5µl	8	125, 564, 2027, 2322, 4361, 6557, 9416, 23130

RELATED PRODUCTS

Product Name	Cat.No.
RedSafe™ Nucleic Acid Staining Solution (20,000x)	21411
DNA-spin™ Plasmid DNA Extraction Kit	17260/17261/17262
MEGAspin-spin™ Total Fragment DNA Purification Kit	17260 / 17261/17262
Maxime™ PCR PreMix (i-StarTaq)	25105
Maxime™ PCR PreMix (i-pfu)	25155

Maxime PCR PreMix Kit (i-Taq))