



THE AGA KHAN UNIVERSITY

eCommons@AKU

LABRAD

Publications

7-2018

LABRAD : Vol 44, Issue 1 - July 2018

Aga Khan University Hospital, Karachi

Follow this and additional works at: <https://ecommons.aku.edu/labrad>



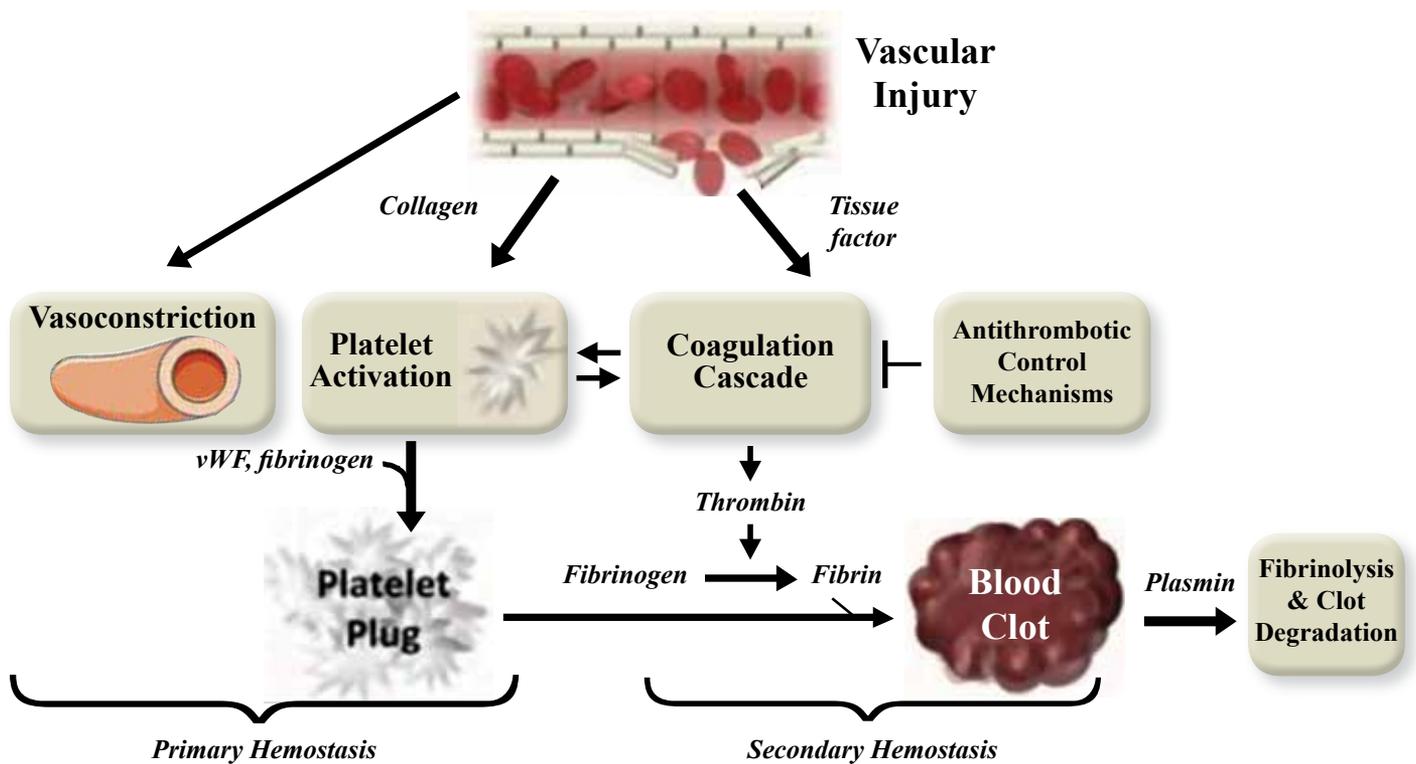
Part of the [Pathology Commons](#), and the [Radiology Commons](#)

LABRAD

JULY 2018

VOL. 44, ISSUE 1

Haemostasis and Thrombosis



آغا خان یونیورسٹی ہسپتال، کراچی

The Aga Khan University Hospital, Karachi



CAP
ACCREDITED
COLLEGE of AMERICAN PATHOLOGISTS

LABRAD

A Publication of the Departments of Pathology & Laboratory Medicine and Radiology

July 2018

Volume 44, Issue 1

Editor

Dr Natasha Ali

Associate Editor

Dr Lena Jafri

Editorial Committee

Department of Pathology and Laboratory Medicine

Dr Nasir Ud Din

Dr Joveria Farooqi

Dr Zahra Hasan

Dr Anila Rashid

Radiology

Dr Naila Nadeem

Dr Dawar Khan

Labrad Administration Office

Farhana Arshad

Department of Pathology and

Laboratory Medicine

Aga Khan University Hospital

Stadium Road, P. O. Box 3500

Karachi 74800, Pakistan

Tel: 92 21 3486 1551

Fax: 92 21 3493 4294, 3493 2095

hospitals.aku.edu/Karachi/clinical-laboratories

How to Investigate a Bleeding Disorder	4
Nucleic Acid Testing at AKUH	6
Lab Diagnosis of von Willebrand Disease	7
Scoring Systems in Diagnosis of Disseminated Intravascular Coagulation	8
Ristocetin Cofactor Activity	10
All You Need to Know About Thrombophilia Screening	11
Utility of ADAMTS-13 Assay	14
Homocysteine as a Risk Factor of Thrombosis	15
Detection of Heparin/Platelet Factor 4 Antibodies	17
Anti-factor Xa Assay	18

From the Editor's Desk

This year's first issue is on Thrombosis and Haemostasis which is a complex physiological process occurring in the human body. Most researchers claim that it is two sides of the same coin. Bleeding and thrombotic disorders are common and challenging medical problems. A good bleeding/haemostatic history will give you the diagnosis in majority of patients. Laboratory investigations then support your differential diagnosis. AKUH's Clinical Laboratory offers a wide panel of bleeding and thrombophilia testing that can be effectively utilized in proper evaluation of the patients with haemostatic abnormalities. This issue is an attempt to

provide physicians with information on haemostatic abnormalities that help in screening and diagnosing of these diseases. The issue includes articles on appropriate work-up of suspected bleeding disorder, transfusion-associated-viral infections such as HIV, hepatitis B and C in patients who require blood product support for treatment, investigations for thrombophilia, acquired bleeding disorders such as paroxysmal nocturnal haemoglobinuria etc. We hope that our readers will gain in depth insight from this thematic issue.

Dr Natasha Ali

Critical International Normalised Ratio

Dr Shabneez Malik
Haematology

Oral anticoagulant therapy is commonly used in the management of venous thrombosis such as deep venous thrombosis of veins and pulmonary embolism.

Previously, laboratories used to report prothrombin time as a part of anticoagulation monitoring. It was then found out that the prothrombin time varied due to the difference in sensitivity of various thromboplastins used in different clinical laboratories. World Health Organization (WHO) devised the term "International Normalised Ratio" (INR) to reduce this inter-laboratory variability. This term is self-explanatory. This is a ratio of patient's prothrombin time divided by reference normal prothrombin time to the power of a coefficient which is known as "international sensitivity index." This relates the sensitivity of a given thromboplastin to the sensitivity of WHO's first international reference preparation of thromboplastin (given an ISI of 1.0).

Monitoring of INR is critical in warfarinised patients. Because of the narrow therapeutic INR, patients on warfarin are stabilised within a range of 2-3. Elevated INR can lead to haemorrhagic

complications on one hand while sub-therapeutic INR can result in thrombosis. Therefore accurate monitoring of INR is crucial in preventing bleeding and thrombosis. The goal of establishing critical INR is to alert the physician to a potentially life threatening bleeding or thrombosis requiring urgent intervention. Critical INR value needs to be established at local level as the line between therapeutic and critical INR is thin.

The Seventh American College of Chest Physicians Conference on Antithrombotic and Thrombolytic Therapy recommended therapeutic intervention in oral anticoagulant patients who have an INR of 5.0 or greater. Therefore, critical INR in many laboratories is 5.0. In 2000 College of American Pathologist survey, 166 laboratories (27 per cent) had chosen a critical INR of 5.0. Similarly an INR below 1.5 should be considered critical for a patient on oral anticoagulation as the patient is at risk for thrombosis.

At AKUH, an INR of 5.0 or above is considered critical. At this INR, the patient is contacted for clinical details including medications. Patients are advised to repeat INR and to consult their primary physicians on emergency basis.

How to Investigate a Bleeding Disorder?

Dr Natasha Ali
Haematology

Bleeding is a very common symptom for which patients approach doctors. At the same time, bleeding disorders are a common reason for a general practitioner to consult a haematologist. Determining the cause of bleeding disorders is challenging due to the variability of symptoms from patient to patient and requires a stepwise approach which traces each case from history to laboratory investigations. Questions usually focus on three main causes of bleeding: impaired vascular integrity, thrombocytopenia or platelet dysfunction and deficiency of clotting factors. The clinical manifestations of any bleeding disorder depend on the nature of defect and its severity.

History

Table 1. Questions for a Patient Suffering from a Bleeding Disorder

Onset of bleeding – to distinguish between hereditary and acquired bleeding disorders
Type of bleeding – whether it is muco-cutaneous or within a cavity
Severity of bleeding – patient is asked whether the symptoms occur spontaneously or during haemostatic challenge e.g. surgical procedures, road traffic accidents, etc.
Frequency of bleeding.
Timing of bleeding episode – whether it occurs immediately or is it delayed.
Are other members in the family affected?
Drug history which particularly includes use of aspirin or NSAIDS
Nutritional history – deficiency of vitamin C
In case of an infant presenting with bleeding, ask the parents regarding prolonged bleeding during child’s circumcision or from his/her umbilical stalk

Physical Examination

The underlying cause can be determined by carefully examining the patients. Usually low platelets or platelet dysfunction produce muco-cutaneous bleedings [Fig. 1] while clotting factor deficiency results in a cavity bleed for example within joints, abdominal cavity, muscle, etc. [Fig. 2].



Fig. 1. Petechiae (small pinpoint cutaneous bleeding due to platelet defect or low platelets)



Fig. 2. Hemarthrosis in a Hemophilia patient – bleeding within the knee joint secondary to defective coagulation

Laboratory Investigations

Certain pre-requisites should be met before collection of blood samples for lab tests. These are listed in Table 2.

Table 2. Pre-analytic Considerations for Coagulation Testing

Patient should not have received recent transfusions (blood, plasma products and platelets)
Patient should not be on medications like Aspirin, NSAIDS or antibiotics (penicillin)
Patient should not be in stress (as it increases factor VIII levels)
Patient should not be pregnant (as it increases Von Willebrand factor antigen levels and factor VIII)

Screening tests: Any patient with a bleeding disorder should be tested for basic coagulation profile. The results of these will determine whether more specific tests are required for diagnosis. These tests are listed in Table 3.

Table 3 Screening Test for Investigating Bleeding Disorders

CBC including platelet count and peripheral film review
Bleeding time
Prothrombin time [PT]
Activated partial thromboplastin time [APTT]
Urea clot lysis test for factor XIII

Specialised tests: Based upon the screening test results, second line investigations are ordered for further work up. Different approaches are used to diagnose a patient depending on clinical details and screening tests. The four possible common scenarios are described below:

Case 1

If only PT is prolonged with normal platelet count and APTT, then factor VII deficiency should be suspected [Fig. 3]. Factor VII assay will help in establishing definitive diagnosis.

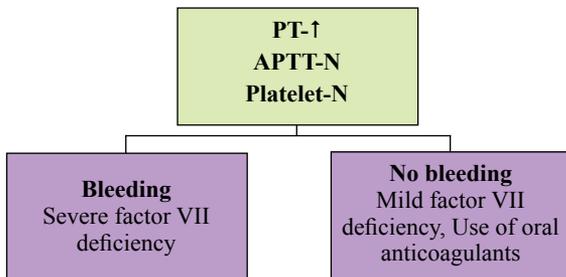


Fig. 3. Algorithm for Isolated Prolonged PT

Case 2

If an isolated APTT elevation is observed and the patient has bleeding tendency then the APTT mixing test can help to differentiate between factor VIII and IX deficiency. Factor XI is also a remote possibility [Fig. 4]. Von Willebrand Disease can lead to raised APTT with elevated bleeding time. Presence of lupus anticoagulants or factor XII deficiency can increase APTT but patient will have no bleeding symptoms in this case.

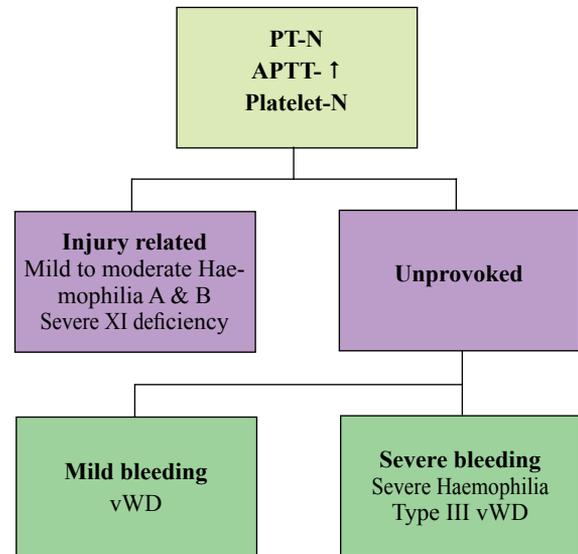


Fig. 4. Algorithm for Isolated Prolonged APTT

Case 3

Combined elevation of PT and APTT raises the possibility of deficiencies of factors in common pathways such as I, II, V and X. Common causes are vitamin K deficiency in neonates or infants and liver disorders in adults. Anticoagulation therapy also leads to raised PT and APTT [Fig. 5].

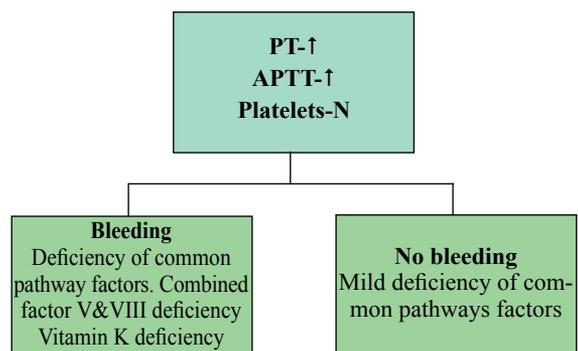


Fig. 5. Algorithm for Combined Elevation of PT and APTT

Case 4

Profound disturbance of primary screening results are indicative of global haemostatic abnormalities such as advanced hepatic disorder or disseminated intravascular coagulation (DIC) [Fig. 6].

In summary, while investigating a patient for suspected bleeding disorder, a thorough clinical history and physical examination is of paramount importance since majority of the disorders can be diagnosed with these two tools. Additionally, appropriate laboratory testing and timely consultation with a haematologist experienced in

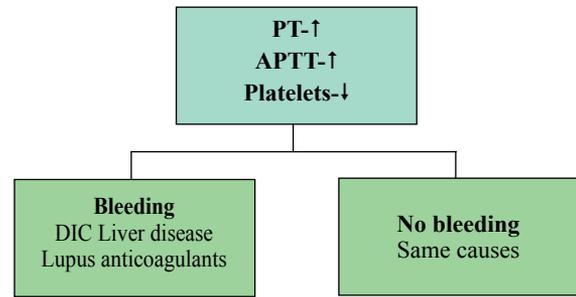


Fig. 6. Algorithm for Elevation of all Primary Screening Investigations.

dealing with bleeding disorders are the key features of successful management of these patients.

Nucleic Acid Testing at AKUH

Sana Chundrigar
Molecular Pathology

In recent years, despite improvements in serological tests for HCV, HBV and HIV infections, instances of viral transmission via transfusion still occur because of donations that take place while a donor is either in the pre-seroconversion window phase, infected with immunovariant viruses, or a non-seroconverting chronic carrier.

What is Nucleic Acid Testing and how does it help?

Nucleic Acid Testing, (NAT) is a highly sensitive molecular method of testing blood donations for viral nucleic acid. It is used to detect low level of viral genetic material present in the blood specimen even before the body begins producing antibodies in response to the virus.

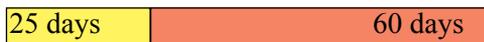
Detection of HIV I and II



Detection of HCV



Detection of HBV



NAT testing
 Ag & Ab Detection

Fig. 1. Window period of HIV, HCV and HBV

NAT significantly reduces the ‘window period,’ which is the time between donor exposure to the virus and the appearance of antibodies [Fig 1]. By reducing the window period it further diminishes occurrence of disease transmission via transfusion. The performance of the NAT assay is essentially dependent on both its analytical and clinical sensitivity and specificity. The analytical sensitivity is generally determined by testing dilutions of standardised materials such as WHO International Standards and subsequent calculations of 95 per cent Limit of Detection (LOD). A NAT can be conveniently performed on a mini-pool which is a pool of six test samples.

Countries that have introduced NAT have encountered a decrease in residual risk of viral transmissions. Although other safety measures such as more stringent selection of blood donors have contributed as well as a marked decrease is evident between pre- and post-NAT implementation. The residual risk for HCV transmission prior to NAT was 0.64 cases per million in France and 3.94 per million in Spain which decreased to 0.1 per million and 2.33 per million respectively after NAT was adopted. HIV NAT yield rates were estimated at 0.3 per million donations in France and Spain as opposed to 0.59 and 2.48 respectively preceding NAT incorporation. There is potential that next generation NAT testing could completely eradicate transfusion risk of HIV, HCV and HBV.

NAT at AKUH

The quality of blood products at the AKUH has further improved by the recent addition of NAT testing in the blood donor screening programme. There is automated pooling of blood donations, sample preparation, amplification (real time polymerase chain reaction or PCR) and detection. [Fig.2].

1. Lysis, stabilisation
2. Capture
3. Wash
4. Elute and deproteination



Fig. 2. Steps in Nucleic Acid Extraction

Before authorising for general use the NAT test was validated on more than 1000 blood donations. So far up to 18,000 blood donations have been screened by NAT assay and unsurprisingly it has captured many window period positive specimens.

Real time data collection allows monitoring of the PCR process in each individual cycle instead of end point measurement after completion of all cycles [Fig.3].

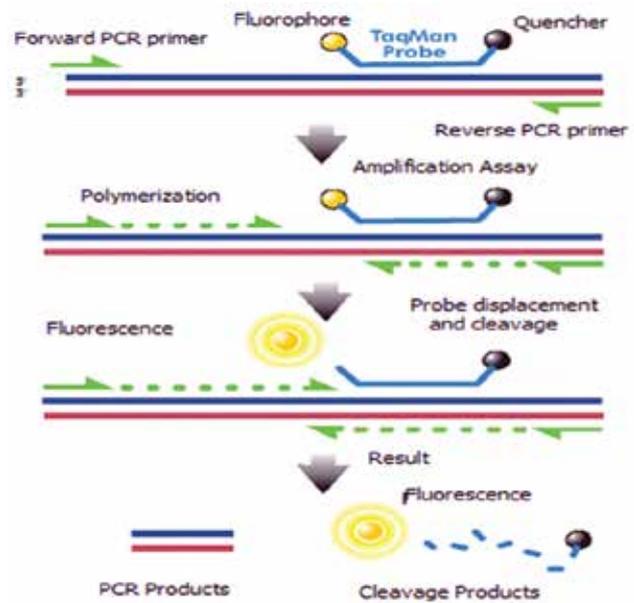


Fig. 3. Principles of the Real Time PCR

Lab Diagnosis of von Willebrand Disease

Bushra Afaq and Dr Bushra Moiz
Haematology

Von Willebrand Disease (VWD) is an autosomally inherited congenital bleeding disorder resulting from deficiency or dysfunction of von Willebrand factor (VWF). The predominant clinical problems are muco-cutaneous bleeds. Therefore, patients usually present with petechiae, oral and gum bleeding, nose bleeding or epistaxis. Excessive menstrual loss or menorrhagia is a common bleeding problem in women of reproductive age. Circumcision acts as a good haemostatic challenge in case of males and therefore boys may be diagnosed earlier than girls. VWD has three clinical types based on von Willebrand Antigen (VWAg) levels.

Laboratory assessment for Von Willebrand Disease

Following are the laboratory tests which should be performed for the laboratory diagnosis of VWD.

Bleeding time: It is performed by IVY method. Its reference range is between 1-6 minutes
APTT: A clotting assay having a reference range between 25-35 seconds.
VWAg: A quantitative determination of von Willebrand activity by turbidimetric method. Its reference range is between 50-160 per cent.
F-VIII: A clotting assay having a reference range between 50-150 per cent.
R-COF: A quantitative determination of F-VIII Ristocetin Cofactor activity using aggregometry technique. Its reference value is >40 per cent.
RIPA: Ristocetin platelet induced aggregometry. Normal platelets show response to standard strength of ristocetin but fail to respond to a lower dose.

Results of the above stated laboratory tests are summarized in the Table 1.

Table 1. Expected Laboratory Values in Subtypes of VWD

	Normal	Type 1	Type 2A	Type 2B	Type 2M	Type 2N	Type 3	PLT-VWD
VWF:Ag	N	L or ↓	↓ or L	↓ or L	↓ or L	N or L	absent	↓ or L
VWF:RCo	N	L or ↓	↓	↓	↓	N or L	absent	↓
FVIII	N	N or ↓	N or ↓	N or ↓	N or ↓	↓	1-9 IU/dL	N or L
RIPA	N	often N	↓	often N	↓	N	absent	often N
BT	N	N or ↑	↑	↑	N	N	↑	↑
Platelet Count	N	N	N	N or ↓	N	N	N	↓

↓ Decreased activity in comparison to reference range

↑ More than normal range

N Normal

L Subnormal activity

AKUH's Clinical laboratory offers a variety of tests required for diagnosis and characterisation of VWD.

Scoring Systems in Diagnosis of Disseminated Intravascular Coagulation

Dr Natasha Ali
Haematology

The liver is the most important organ involved in the regulation of haemostasis. Most of the clotting factors, their inhibitors and a number of proteins responsible for fibrinolysis are produced in the liver. Therefore, liver disease impacts both primary and secondary haemostasis pathways through a number of different mechanisms. Abnormalities of the clotting cascade are the predominant features of acute and chronic liver disease. In some patients suffering from hepatic disease, physicians are faced with the diagnostic challenge of an underlying bleeding or thrombotic diathesis due to overlap between various clinical syndromes including Disseminated Intravascular Coagulation (DIC).

According to the International Society of Thrombosis and Haemostasis (ISTH), DIC is a syndrome characterised by a systemic intravascular activation of coagulation, with loss of localisation, arising from different causes. It can originate from and cause damage to the microvasculature, which if sufficiently severe can produce organ dysfunction. ISTH also proposed that the working definition of DIC can be divided into two phases:

- a) Non-overt DIC: represents subtle haemostatic dysfunction
- b) Overt DIC: de-compensated phase of non-overt DIC

Events responsible for DIC in liver disease include liver necrosis, impaired endotoxin clearance, surgery, shock and ascites recirculation. DIC can be recognised as a syndrome suggested by clinical signs and laboratory tests. The characteristic laboratory findings include: prolonged prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), increased levels of fibrin related markers (fibrin degradation products, D-Dimer), decreased platelet count, fibrinogen level, plasma coagulation factors and their inhibitors. More specialised and useful tests include: prothrombin activation fragments and thrombin-antithrombin complex (TAT).

In 2001, ISTH proposed two separate scoring systems for overt and non-overt DIC. The overt DIC score consists of a five steps diagnostic algorithm, in which a specific score, reflecting the severity of the abnormality found, is given to each of the laboratory tests [Table 1]. A score of five or

Table 1. ISTH Score System for Overt DIC

Screening Tests	Findings	Points
PLATELET COUNT	>100,000/ μ L	0
	50,000-100,000/ μ L	1
	<50,000/ μ L	2
PROTHROMBIN TIME	(PT upper limit of reference range) >3 secs	0
	(PT upper limit of reference range) =3-5.9 secs	1
	(PT upper limit of reference range) \geq 6 secs	2
FIBRINOGEN	>100mg/dl	0
	<100mg/dl	1
FIBRIN MONOMER	No increase	0
	Moderate increase	2
	Severe increase	3

more is considered to be compatible with DIC, whereas a score of less than five may be indicative (but not affirmative) for non-overt DIC. Similarly using certain clinical and laboratory findings [Table 2] a score of five or greater is compatible with

Table 2. ISTH Score for non-Overt DIC

Parameter	Findings	Points
Diagnosis	Not associated with DIC	0
	Associated with DIC	2
Platelet count	>100,000/ μ L	0
	<100,000/ μ L	1
Prolongation of PT	< 3 seconds	0
	> 3 seconds	1
Soluble fibrin or FDP	Normal	0
	Increased	1
Antithrombin III	Normal	-1
	Low	1
Protein C	Normal	-1
	Low	1

non-overt DIC and could diagnostically define patients with a poor prognosis due to haemostatic dysfunction, independent of developing overt DIC.

Following steps are suggested for the diagnosis of DIC:

Step 1: Determine if the patient has an underlying condition associated with DIC. If no underlying is present then do not proceed further.

Step 2: Order screening coagulation tests and decide whether patient has overt or non-overt DIC.

Ristocetin Cofactor Activity

Bushra Afaq
Haematology

Ristocetin Cofactor (RCo) measurement is essential for the diagnosis of von Willebrand disease (VWD). VWD is an autosomally-inherited congenital bleeding disorder that is caused by deficiency or dysfunction of von Willebrand factor (VWF), a plasma protein that mediates the initial adhesion of platelets at site of vascular injury and also binds and stabilises blood clotting factor VIII (FVIII) in the circulation. Therefore, defects in VWF can cause bleeding by impairing platelet adhesion or by reducing the concentration of FVIII.

VWD is classified into three major categories:

1. Type 1: a quantitative deficiency of VWF factor (<30 per cent)
2. Type 2: a qualitative defect of VWF having dysfunctional proteins. Type 2 has further subtypes (2A, 2B, 2M, 2N) based on laboratory tests.
3. Type 3: a severe deficiency of VWF (<3 per cent)

The RCo Assay is used in the quantitative determination of RCo activity in citrated plasma. It is particularly useful in detection of type 2A, 2B, and 2M VWD, where the VWF: Ag may be normal or near normal whereas the VWF: RCo is markedly reduced.

RCo Activity in plasma may be determined by the agglutination of a standardised suspension of platelets in the presence of von Willebrand factor using the antibiotic called Ristocetin. The RCo Activity is the in-vitro activity of the von Willebrand factor, which is responsible for the agglutination of platelets in the presence of Ristocetin. Levels of RCo activity are determined by the ability of the test plasma and Ristocetin to induce aggregation in a standardised platelet suspension.

Principle of the test method is reconstituted lyophilised platelets are treated with Ristocetin in the presence of varying dilutions of normal standardised human plasma having known amounts of RCo Activity. A standard curve is thus prepared. Patient

plasma is then tested in the presence of Ristocetin and reconstituted platelets and an aggregation pattern is determined. RCo Activity of test plasma is interpolated from the standard curve [Fig. 1].

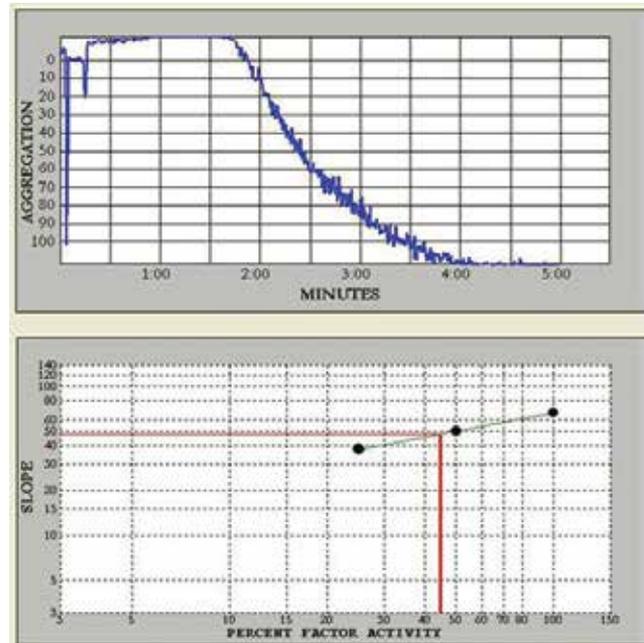


Fig. 1. Standard Curve and Interpolation of Ristocetin Cofactor Activity for the Patient

Results of less than 40 per cent RCo (von Willebrand Factor) are generally considered abnormal and are indicative of von Willebrand disease.

As a quality control of deficient von Willebrand Reference Plasma is included in the test kit to function as an abnormal Control. The RCo value of this Control is less than 30 per cent.

The RCo assay is considered by many investigators to be the single most important assay for VWD. However, a complete diagnosis and determination of the variant forms of this coagulopathy requires an evaluation of family history and lab parameters such as Von Willebrand antigen, Factor VIII activity and bleeding time.

The test is available at Aga Khan University Hospital's clinical laboratory.

All You Need to Know About Thrombophilia Screening

Dr Bushra Moiz
Haematology

What is Thrombophilia?

Heritable thrombophilia is an increased tendency towards venous thrombosis. Initially the terminology was reserved for inherited disorders only but later the term ‘thrombophilia’ was used loosely for both inherited and acquired disorders with a predisposition for either venous or arterial thrombosis.

Assessment of Thrombophilia

1. **Clinical:** Assessment of thrombophilia starts with detailed clinical history and physical examination. The patient should be assessed for acquired risk factors, medication and co-morbidities.
2. **Laboratory:** The tests are listed in Table 1. All the mentioned tests are offered by the clinical laboratory at AKUH.

Selection of Patient for Heritable Thrombophilia

The inherited thrombophilia screening in an unselected patient population is not advisable. Since it is genetic, testing with personal and family considerations therefore patients should be carefully chosen. Not every patient with thrombosis requires this testing. Table 2 and 3 summarise the conditions in which screening should not be performed. The information provided is according to the guidelines published by the British Committee of Standards in Haematology.

What are the Appropriate Timings of Testing?

There is seldom any point in striving to obtain samples for thrombophilia testing when the patient presents with deep venous thrombosis (DVT) or pulmonary embolism (PE). During an acute thrombotic episode, patients usually have low levels of protein C, protein S and antithrombin III

Table 1. Comprehensive Panel of Thrombophilia Testing

	Test	Purpose
Routine	CBC, PT, APTT, Liver function tests and Serum creatinine	To determine safety profile before initiation of heparin and warfarin
Specialised	Protein C, protein S, anti-thrombin III, activated protein C resistance (APCR)	Required in investigation of heritable thrombophilia
	Anticardiolipin antibodies (IgM and IgG), lupus anticoagulants, serum homocysteine levels	Required in acquired thrombophilia
Ancillary	PNH screening (CD55 and 59)	Required for suspected PNH (intravascular haemolysis and pancytopenia)
	HIT	For suspected HIT (heparin induced thrombocytopenia)
	ADMATS-13 assay	Needed for suspected TTP (micro-angiopathic haemolytic anaemia, thrombocytopenia, renal insufficiency and neurological deficit)
	JAK-2 V617F mutation	For suspected myeloproliferative disorder (MPD) with erythrocytosis/leucocytosis/thrombocytosis

Table 2. Conditions Recommended for Thrombophilia Screening

Venous thrombosis at an early age (<40 years)
Venous thrombosis in a patient with thrombosis prone family (>2 symptomatic family members)
Neonates and children with fulminating purpura (purpura fulminans) for protein C and S
Adults with skin necrosis following warfarin therapy for protein C and S
Case finding of asymptomatic relatives for high risk such as protein C and S deficiencies in thrombosis prone families
Women requiring contraceptives or hormone replacement therapy with high risk thrombophilia in symptomatic relative
Pregnancy with previous travel related thrombosis
Asymptomatic pregnant woman with family history of thrombosis which was either unprovoked or provoked by pregnancy, combined oral contraceptives (COC) exposure or traveling

Table 3. Conditions Which Should not be Tested for Heritable Thrombophilia

Unselected patients with first episode of thrombosis
First episode of cerebral venous thrombosis
Arterial thrombosis
Paediatric stroke
Hospital acquired venous thrombosis
Asymptomatic relatives of low risk thrombophilia (factor V Leiden, prothrombin gene mutation).
Retinal vein occlusion
First event of an intra-abdominal vein thrombosis
Pregnant women with previous unprovoked thrombosis
Pregnancy with previous trauma/surgery related thrombotic event
Pregnancy or COC related thrombosis
Unselected patients with upper limb thrombosis
Central Venous Catheters related thrombosis

as the two are consumed in an attempt to natural thrombolysis. This results in false low levels of these proteins. Testing should be delayed for at least one month after discontinuation of oral anticoagulation. Pregnancy and oestrogen reduce protein S levels significantly and increase resistance to APC. If possible, thrombophilia testing should be avoided in inter-current illnesses, pregnancy and with oral contraceptive usage.

How should I collect Blood Samples?

The blood sample should be collected in standard sodium citrate bottle/blue top vacutainer in a ratio of 9:1 for lupus anticoagulant, protein C, S, APC, and AT-III. EDTA blood sample is required for HIT, PNH and JAK-2. Clotted blood sample is needed for anticardiolipin antibodies and homocysteine.

Methodology assays used at AKUH Clinical Laboratory

At AKUH, functional assays are used for heritable thrombophilia which have acceptable accuracy and precision. Table 4 shows the various tests and their methodologies used at AKUH.

How should I Interpret the Tests?

The thrombophilia screening should be supervised by experienced laboratory staff and the clinical significance should be made in consultation with an experience haematologist who is aware of relevant factors that may affect test results in each case. The factors affecting thrombophilia testing resulting in spurious assessment is listed in Table 5.

Do I need Repeating the Tests?

Repeat testing for protein C, S and anti-thrombin III is indicated and a low level should be confirmed on one or more separate samples. Diagnosis of deficiency should not be made on a single abnormal test.

Would the Testing affect Patient Management?

Initiation and intensity of anticoagulation following diagnosis of acute venous thrombosis in a patient should be the same with or without thrombophilia. Testing of thrombophilia in selected patients with strong family history of unprovoked thrombosis may influence decision regarding duration of anticoagulation.

Table 4. Tests and their Methodologies Used at AKUH Clinical Laboratories

Clinical utility	Tests	Method
Heritable thrombophilia	Protein C	Chromogenic assay
	Protein S	Clotting based assay
	Antithrombin III	Chromogenic assay
	APCR	Clotting based assay
Acquired thrombophilia	Lupus anticoagulant	Clotting based assay
	Anticardiolipin antibodies	ELISA
	Serum homocysteine	Immunogenic microparticulate assay
PNH	PNH screening	Gel immunoassay
HIT	HIT screening	Gel immunoassay
TTP	ADAMTS-13	ELISA
MPD	JAK-2 V 617 F	PCR

Table 5. Conditions Giving Rise to Misleading Results in Thrombophilia Screening

Acute thrombosis
Anticoagulants- heparin and warfarin
Direct thrombin inhibitors e.g. argatroban, lepirudin, bivalirudin
Liver disease
Pregnancy and COC

Utility of ADAMTS-13 Assay

Dr Farheen Karim Mahar
Haematology

A Disintegrin and Metalloprotease with Thrombospondin Type 1 Motif, 13 (ADAMTS-13) is an enzyme which is released primarily from the stellate cells of the liver but is also found in platelets and endothelial cells. It is involved in the regulation of the size of von Willebrand factor (VWF) in plasma.

ADAMTS-13 maintains primary haemostasis by proteolyzing ultra-large von Willebrand Factor (ULvWF). The latter is the hyperactive form of vWF which is released from storage granules (Weibel-Palade bodies) of endothelial cells in response to inflammatory stimulation. The ULvWF has more affinity for platelets favouring platelet aggregation at sites of high shear stress with subsequent formation of micro vascular thrombi. The ULvWF relies upon ADAMTS-13 for its cleavage and its conversion into a less active form.

Deficiency of ADAMTS-13 may be congenital (Upshaw-Schulman syndrome) or more often than not acquired. An acquired deficiency is attributed to the presence of auto-antibodies against ADAMTS-13 which may either inhibit ADAMTS-13 function or may cause rapid clearance of circulating ADAMTS-13. Inherited or acquired deficiency of ADAMTS-13 impairs ULvWF cleavage. Deficient proteolysis of ULvWF results in disseminated platelet-rich thrombi in the microcirculation which in turn cause typical thrombotic micro-angiopathies resulting in end organ ischemia.

The laboratory assessment of ADAMTS-13 levels is useful since severe deficiency of ADAMTS-13 has been proposed as a specific laboratory marker of thrombotic thrombocytopenic purpura (TTP) or Upshaw-Schulman syndrome. It is also seen in conditions like sepsis, disseminated intravascular coagulation (DIC) and metastatic malignancy. Mild to moderate deficiency is seen in chronic inflammation, hepatic dysfunction and pregnancy.

Given that severe secondary deficiency of ADAMTS-13 might correlate with development of widespread microvascular thrombi and end organ injury, determination of ADAMTS-13 activity at the time of hospital admission in patients with severe conditions like sepsis and DIC will help in understanding the extent of the disease. It also raises the possibility of novel supportive therapies for patients with sepsis and DIC such as ADAMTS-13 supplementation and plasma therapy because sepsis may have the same pathophysiology of severe ADAMTS-13 deficiency for thrombotic microangiopathies as idiopathic TTP.

The test is currently offered by clinical laboratories of AKUH. It is based on the principle of enzyme linked immuno sorvent assay (ELISA) technique. Two to three millilitres of blood collected in citrate anticoagulant is required for the test. The reference range developed at AKUH for ADAMTS-13 is 175-365 ng/dl. There is no difference in reference range between males and females or adults and children.

Homocysteine as a Risk Factor of Thrombosis

Rakshshanda Maher
 Chemical Pathology

Elevated plasma homocysteine is associated with increased incidences of cardiovascular mortality, stroke, dementia and Alzheimer’s disease, bone fracture and higher prevalence of chronic heart failure. It was also shown that elevated plasma homocysteine is a risk factor for preclampsia and neural tube defects (NTD). Homocysteine has been implicated in atherosclerotic and thrombotic vascular disease in the general and in end-stage renal disease (ESRD) population as well.

Homocysteine is a sulphur containing amino acid, formed normally during metabolism of another sulphur containing amino acid methionine. It is metabolised by pathways, remethylation and trans-sulfuration of methionine (Fig 1). Approximately 50

An elevated plasma homocysteine level has been found in patients with thrombotic disorders. In actual thrombosis is a formation of blood clot inside the blood vessel, obstructing the blood flow through the circulating system. It is mainly classified into venous and arterial thrombosis. The mechanism by which homocysteine contributes to atherothrombosis is complex and in vivo relevance is uncertain. However in the *Journal of Clinical Investigation-2009*, Jacoviner and his colleagues have reported a unique mechanism in vivo by which homocysteine may contribute to vascular disease. Accordingly, there is impairment in the synthesis of endothelial surface plasminogen which is the major fibrinolytic agent. This leads to micro vascular dysfunction and subsequent macro vascular occlusion in individuals with hyperhomocystenemia.

Most studies so far have indicated normal plasma homocysteine level to be in the range of 5 to 15 $\mu\text{mol/L}$. However this does not imply that there is no risk associated with homocysteine levels between 10 to 15 $\mu\text{mol/L}$.

In 1990, the cut-off for homocysteine was greater than 20 $\mu\text{mol/L}$. This number continued to go lower, and today, a level somewhere around 12 $\mu\text{mol/L}$ is considered as the recent cut-off. Homocysteine analyses in The National Health and Nutrition Examination Survey III (NHANES) results shed some light into what constitutes ‘normal’ homocysteine levels in the US population. A study conducted in 1999 by NHANES investigators (published in *Ann Intern Med* 1999; 131:331) showed that homocysteine

levels continue to increase as a person ages, and men have a higher mean concentration than women.

The AKUH Clinical Laboratory in Karachi, measures the quantitative anticardiolipin IgG & IgM are measured using the DIASTAT anti-cardiolipin kit by indirect enzyme immunoassay method. The ACA panel includes only IgG and IgM antibodies. The incidence of IgAACA is extremely low and their determination is not helpful in the investigation of thrombosis or other manifestations of the APLA syndrome.

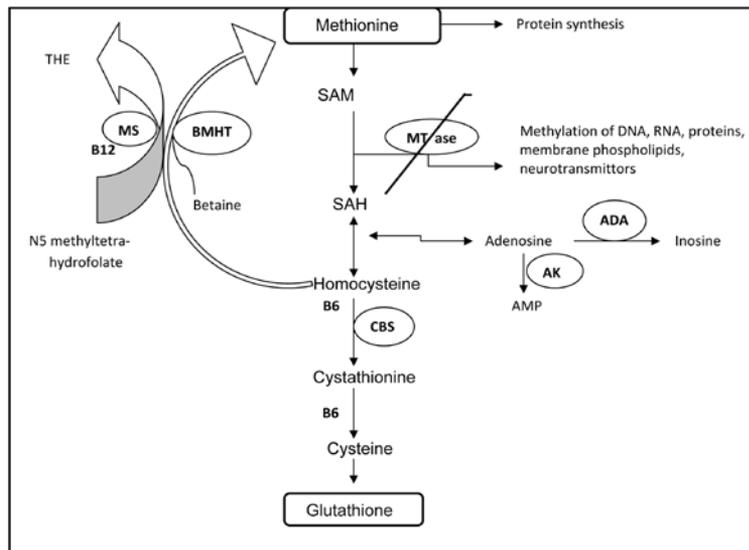


Fig. 1. Metabolism of Homocysteine

per cent of methionine is metabolised to cystathionine by cystathionine beta synthetase (CBS). This is a one way reaction that permanently removes homocysteine from the methionine cycle and initiates the transsulfuration pathway for methyl group either from N5 methyltetrahydrofolate (in all tissues) or from betaine (in liver and kidneys) to form methionine. The former methylation step requires folate and vitamin B12 as cofactors in trans-sulfuration pathways and is effective when there is excess of methionine or cysteine synthesis is required.

CD55 and CD59 for the Diagnosis of PNH

Fatima Azra Ausat
Haematology

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell disorder induced by exposure to chemicals or certain antibiotics and characterised by chronic intravascular haemolysis, venous thrombosis, defective haematopoiesis, frequent episodes of infection and rarely, leukemic transformation.

Pathophysiology of PNH

Patients with PNH have a defective gene called PIG-A, involved in the biosynthesis of glycosylphosphatidylinositol (GPI). Without GPI, important regulatory proteins (e.g. CD55 or “DAF” and CD59 “MIRL”) cannot bind to the surface and protect blood cells from attacks of various proteins called complements. This may result in a breakdown of erythrocytes and release of haemoglobin which causes the urine to turn dark during an episode (or “paroxysm”) of haemolysis, though this is not found in all cases.

There are certain diagnostic tests which are used in the diagnosis of PNH including Ham’s test, sucrose lysis test, and CD55 and CD59 analysis by flow cytometry and by using gel card technique.

CD55 and CD59 determination by gel card technique

Gel agglutination assay for PNH is a simple method for screening red cells for deficiency of GPI linked protein. For optimal results, the determination should be performed using a freshly drawn blood sample preferably into EDTA, citrate or heparin, CPD-A anticoagulant.

Gel card tubes contain monoclonal antibodies against CD55 and CD59; if cells express both antigens the cells agglutinate by forming antigen antibody complexes and bind the mouse antibodies. After centrifugation through the gel, cells carrying antibodies, confirming the presence or MIRL or DAF, will show a positive reaction, grade three-four in the micro-tubes indicates the presence of a normal

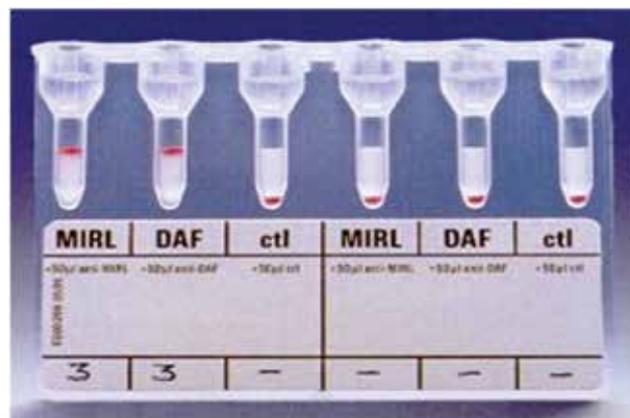


Fig. 1. Gel card showing results for a normal person (three gel tubes on the left) and from a patient suffering from PNH (three gel tubes on the right)

cell population. Cells deficient in MIRL or DAF show a negative reaction as a compact button at the bottom of the micro tube [Fig.1]. Double population of cells indicates a weak positive PNH test. Whenever it is necessary to know the amount of cells affected, a quantitative test procedure such as flow cytometry should be performed following a positive ID-PNH test.

PNH is only diagnosed where both MIRL and DAF are shown to be deficient.

Detection of Heparin/Platelet Factor 4 Antibodies

Safia Naz
Haematology

Heparin-induced thrombocytopenia (HIT) is the development of thrombocytopenia due to the administration of Heparin. HIT predisposes to thrombosis and when thrombosis is identified the condition is called “Heparin-induced thrombocytopenia and thrombosis (HITT).” Clinical features associated with HIT syndrome primarily include a decreased platelet count, risk of venous or arterial thromboembolic complication and the development of antibodies against a complex of heparin and PF4. HIT is a life and limb threatening condition. The diagnosis is made on clinical suspicion requiring laboratory confirmation. The testing is recommended in all patients whose platelet count drop by 50 per cent of baseline within three to five days of heparin administration. HIT is observed both with unfractionated heparin and with low molecular weight heparin though the risk is less with latter.

Several tests are available for detection of Heparin/PF4 antibodies in the laboratory [Table 1]. Aga Khan University Hospital, Clinical Laboratory uses ID-PaGIA Heparin/PF4 Antibody Gel Test for detection of these antibodies against this complex.

The ID-PaGIA Heparin/PF4 antibody test is a particle gel immuno assay (PaGIA). High density synthetic polymer particles are coated with heparin/platelet factor 4 complex (HPF4). When these particles are mixed with patient serum-specific antibodies of any immunoglobulin class, they react with the HPF4A on the bead surface, causing agglutination. When the reaction mixture is centrifuged through a gel filtration matrix, agglutinated particles are trapped on top of the gel or distributed within the gel (positive reaction) while free or non- agglutinated particles form a pellet at the bottom of the micro tube (negative reaction). Due to the strong red colour of these particles, the result can be read visually with ease [Fig. 1].

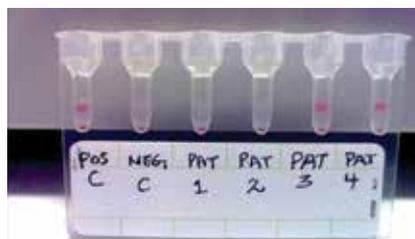


Fig. 1. Heparin/HP4 Gel Card showing Negative results for Patient One and Two and Positive results for Patient Three and Four.

Table 1. Recommendation for laboratory Testing for Heparin Induced Thrombocytopenia Antibodies

Test	Methodology
Particle gel immunoassay ID-Heparin/PF4 antibody test (ID-H/PF4)	Patient serum and PF4 heparin-coated micro-beads are added to the incubation chamber of the micro-column card and after five minutes, the card is centrifuged. A strong positive result indicated by the agglutinated micro-beads remaining top of the column.
Enzyme linked immunosorbent assay test (ELISA)	It detects all circulating antibodies that bind Heparin PF4 complexes and may also falsely identify antibodies that do not cause HIT. Therefore, ELISA is more sensitive but less specific requiring further testing.
Platelet aggregation test	Citrated platelet rich-plasma is used as an initial test for HIT, a positive result generally supports the diagnosis of HIT and further testing is not required. However negative test does not exclude HIT in a patient with a moderate or high pre-test probability for HIT. In this situation washed platelets activation assay should be performed.
Serotonin release assay/washed platelets activation assays	SRA is considered to be gold standard and is specific for the HIT antibodies. The platelets are washed and mixed with serum and heparin. The sample is then tested for the release of serotonin, a marker of platelet activation. If serotonin release assay shows high serotonin release, the diagnosis of HIT is confirmed.

Anti-factor Xa Assay

Dr Natasha Ali
Haematology

Heparin is used widely for the prevention and treatment of thromboembolic diseases, during procedures of haemodialysis and cardiopulmonary bypass surgeries. Two types of heparin preparations widely in use today are unfractionated heparin (UFH) and low molecular weight heparin (LMWH). The inconvenience and limited precision of monitoring of (UFH) therapy has contributed to the increasing use of LMWH preparations. The pharmacokinetic properties of LMWH are different from (UFH) and allows for easily monitored anticoagulant effects of a given dose. Monitoring is, however, recommended in clinical settings like renal insufficiency, obesity, paediatric patients and those on prophylactic anticoagulant therapy in conditions like malignancy or anti-phospholipid syndrome. Anti-factor Xa assay is used to monitor LMWH therapy.

Principle of anti-factor Xa is based on the fact that both factor Xa and antithrombin III are added in excess amounts to the test plasma and the residual factor Xa activity is inversely proportional to the heparin concentration. The test is based on the assumption that the patient has a normal concentration of antithrombin III.

The test is performed by adding a known amount of factor Xa and antithrombin to the plasma of the patient. If heparin or LMWH is present in the patient plasma, it will bind to antithrombin and inhibit factor Xa. The amount of residual factor Xa is inversely proportional to the amount of heparin in the plasma. The amount of residual factor Xa is detected by adding a chromogenic substrate that resembles the natural substrate of factor Xa. Factor Xa cleaves the chromogenic substrate, releasing a coloured compound that can be detected by a spectrophotometer. Results are reported as anticoagulant concentration in anti-factor Xa units/ml, such that high anti-factor Xa values indicate high levels of anticoagulation. Deficiencies of antithrombin in the patient do not affect the assay, because excess antithrombin is provided in the reaction.

Specimen is collected in sodium citrate bottle (blue top) about four hours after subcutaneous injection

of LMWH. Blood sample should be delivered to the laboratory immediately or else falsely low values may occur (because platelets release platelet factor 4 (PF4) which can neutralise heparin or LMWH). For the same reason, plasma must be separated from cells as soon as possible, ideally within one hour of specimen collection. Plasma can be stored for two hours at room temperature or can be frozen for later testing.

Laboratory is specifically notified as to which drug should be measured (heparin, LMWH). Limitations of this test include cost issues and less ready availability than the activated partial thromboplastin time (APTT) for heparin monitoring.

Table 1. Reference Ranges for Anti-Factor Xa

Heparin: 0.3-0.7 units/ml
LMWH: 0.4-1.1 units/ml for twice daily subcutaneous dosing. For once daily subcutaneous LMWH dosing, the therapeutic range is less certain but is approximately 1-2 units/ml.
Target range for DVT prophylaxis (prevention): There is no defined target range for prophylaxis of deep vein thrombosis DVT because such anticoagulation is not usually monitored. When anti-Xa levels have been measured, mean values are usually <0.45 units/ml.
Therapeutic range UFH: 0.3-0.7 U/L
Therapeutic range LMWH: 0.5-1.0 U/L
Prophylactic range UFH: 0.1-0.29 U/L
Prophylactic range LMWH: 0.20-0.49 U/L

Patients not on anticoagulants: 0 unit/ml.
Therapeutic range for anti-factor Xa for a deep venous thrombosis (DVT) patient differs according to the type of preparation used.:

Causes of Sub-Therapeutic Anti-factor Xa Level

1. Specimen drawn at incorrect time (collection times are four hours after injection of LMWH, six hours after injection of danaparoid).
2. Specimen transportation time longer than two hours.
3. Patient receiving prophylactic dose, therefore,

therapeutic range is not applicable and anti-Xa level is actually appropriate for dose.

Causes of Supra Therapeutic Anti-factor Xa Level

1. Renal failure (with LMWH or danaparoid) because of decreased renal clearance.
2. Heparin contamination, if specimen was drawn from an IV heparinised line.



hospitals.aku.edu/Karachi/clinical-laboratories