ANTIPHOSPHOLIPID ANTIBODIES IN PATIENTS WITH VENOUS THROMBOSIS AT KENYATTA NATIONAL HOSPITAL

A dissertation submitted to the University of Nairobi in part fulfillment of the degree of Master of Medicine in Human Pathology

Dr. Anne Kasyoka Barasa
MBChB (UoN)
Registrar, Department of Human Pathology
University of Nairobi,
P. O. Box 19676 – 00202,
NAIROBI
Email: annebarasa@gmail.com
Student’s declaration
I, Dr. Anne Kasyoka, declare that this is my original work, and that as far as I am aware, it has not been presented to any other university or forum

Dr. Anne Barasa

Signed…………………………… Date……………………………………
Supervisors’ declaration
This dissertation has been submitted with our approval as supervisors:

1. Professor Walter O. Mwanda, MBChB, MD

   Associate Professor of Haematology

   Chairman – Department of Human Pathology

   University of Nairobi

   Signed…………………………… Date…………………………

2. Dr. Grace W. Kitonyi, MBChB, PGD-RM, FRCPath

   Senior Lecturer, Haematology & Blood Transfusion Unit

   Department of Human Pathology

   University of Nairobi

   Signed…………………………… Date…………………………
3. Dr. (Rtd. Major) Chris S. Gontier, MBChB, BSc (Anat), MMed (Path)

   Lecturer, Immunology Unit

   Department of Human Pathology

   University of Nairobi

   Signed………………………...                            Date ……………………
DEDICATION
To the loving memory of my aunt, the late Dr. Ketra Auma Muhombe, who encouraged me as I pursued my studies, but unfortunately departed her life after a brave battle with Lupus.
ACKNOWLEDGEMENTS
I would like to acknowledge the encouragement, guidance and support of Prof Walter Mwanda, Dr. Grace Kitonyi and Dr. Chris Gontier. I deeply appreciate the support and assistance given to me by Dr. Alexander Duncan from Emory University, who provided some of the reagents used to run the tests.

I wish to thank my two research assistants, Dr. Walter Akelo and Daniel Mkungo; and laboratory personnel Lilian, Ireri, Kamau, Gitonga and Bosco for their assistance with running the tests. I am thankful to my fellow students, who contributed ideas, feedback and advice, and to Dr. Peter Ukiru who helped me to source for some of the reagents.

A special thank you goes to my family for their patience and for believing in me.
## Table of Contents

Student’s declaration ........................................................................................................... ii
Supervisors’ declaration ....................................................................................................... iii
DEDICATION ........................................................................................................................... v
ACKNOWLEDGEMENTS ...................................................................................................... vi
LIST OF ABBREVIATIONS .................................................................................................... ix
LIST OF TABLES AND FIGURES ........................................................................................ xi
ABSTRACT .............................................................................................................................. xii
INTRODUCTION .................................................................................................................... 1

### LITERATURE REVIEW

- Antiphospholipid Antibodies ............................................................................................ 4
  - Historical Perspectives .................................................................................................... 5
- Epidemiology of Antiphospholipid Antibodies ................................................................. 6
- Clinical Manifestations of Antiphospholipid Antibodies .................................................. 7
- Laboratory Features of Antiphospholipid Antibodies ....................................................... 8

**RATIONALE** ..................................................................................................................... 10

### RESEARCH QUESTIONS
................................................................................................................................. 11

### STUDY OBJECTIVES
................................................................................................................................. 11
  - Broad objective ............................................................................................................ 11
  - Specific objectives ....................................................................................................... 11

### MATERIALS AND METHODS
................................................................................................................................. 12
  - Study Design .............................................................................................................. 12
  - Study Area ............................................................................................................... 12
  - Study Population ..................................................................................................... 12
  - Selection Criteria ..................................................................................................... 12
  - Data collection procedures ..................................................................................... 13
  - Data Handling .......................................................................................................... 17
  - Ethical Considerations .............................................................................................. 18

### RESULTS
........................................................................................................................................ 19

### DISCUSSION
...................................................................................................................................... 38

### CONCLUSION
.................................................................................................................................... 41

### RECOMMENDATIONS
.......................................................................................................................... 41

### STUDY LIMITATIONS
.......................................................................................................................... 42
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>A &amp; E</td>
<td>Accident and Emergency</td>
</tr>
<tr>
<td>ACL</td>
<td>Anticardiolipin antibodies</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>APA</td>
<td>Antiphospholipid antibodies</td>
</tr>
<tr>
<td>APS</td>
<td>Antiphospholipid Syndrome</td>
</tr>
<tr>
<td>APTT</td>
<td>Activated Partial Thromboplastin Time</td>
</tr>
<tr>
<td>β₂GP1</td>
<td>β₂ Glycoprotein 1</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep Venous Thrombosis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte Sedimentation Rate</td>
</tr>
<tr>
<td>KCT</td>
<td>Kaolin Clotting Time</td>
</tr>
<tr>
<td>Ksh</td>
<td>Kenya shillings</td>
</tr>
<tr>
<td>LA</td>
<td>Lupus anticoagulant</td>
</tr>
<tr>
<td>ml</td>
<td>millilitres</td>
</tr>
<tr>
<td>PBF</td>
<td>Peripheral blood film</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>TPHA</td>
<td>Treponema Pallidum Haemagglutination Assay</td>
</tr>
<tr>
<td>U/E/Cr</td>
<td>Urea, Electrolytes and Creatinine</td>
</tr>
<tr>
<td>VDRL</td>
<td>Venereal Disease Research Laboratory</td>
</tr>
</tbody>
</table>
VTE  Venous Thromboembolism
LIST OF TABLES AND FIGURES

Table 1: Demographic characteristics ................................................................. 19
Table 2: Social-economic characteristics ............................................................ 20
Table 3: Presenting symptoms ........................................................................... 21
Table 4: Clinical findings .................................................................................. 21
Table 5: Obstetric & Gynaecological characteristics ........................................... 23
Table 6: General examination findings ............................................................... 25
Table 7: Physical findings .................................................................................. 26
Table 8: Haemogram results .............................................................................. 27
Table 9: Haematology test results .................................................................... 28
Table 10: APTT results ..................................................................................... 30
Table 11: Lupus anticoagulant detection tests ..................................................... 31
Table 12: Immunology and Biochemistry test results ......................................... 32
Table 13: Summary of characteristics of patients demonstrating antiphospholipid antibodies ................................................................. 35
Table 14: Summary of characteristics of patients with thrombocytopenia ......... 36
Table 15: Bivariate analysis by gender ............................................................... 36

Figure 1: Gender of patients ............................................................................... 19
Figure 2: Occupation of patients ....................................................................... 20
Figure 3: Family history of DVT ......................................................................... 22
Figure 4: Obstetric and Gynaecological characteristics .................................... 24
Figure 5: Blood pressure categories .................................................................. 25
Figure 6: Red blood cell counts and Haemoglobin box plots ............................ 28
Figure 7: Platelet counts ................................................................................... 29
Figure 8: ESR results ......................................................................................... 30
Figure 9: VDRL results ..................................................................................... 33
Figure 10: Anticardiolipin IgG results ................................................................. 33
Figure 11: ACL IgG and anti-β2GP1 IgG box plots ........................................ 34
Figure 12: Creatinine results ............................................................................ 34
Figure 13: Correlation between ACL and APTT ............................................... 37
Figure 14: Correlation between Haemoglobin and Platelet count ..................... 37
ABSTRACT

Background: Antiphospholipid antibodies are autoimmune immunoglobulins directed against protein phospholipid complexes. They have been associated with, among other manifestations, an increased risk of thrombosis. The identification of patients with antiphospholipid antibody-associated thrombosis has an important implication on patient management, as these are patients who should benefit from prolonged anticoagulant therapy, to prevent recurrent thrombosis.

Objective: To determine the presence of antiphospholipid antibodies and to identify their clinical and laboratory associations in patients with venous thrombosis at Kenyatta National Hospital

Study design: A cross-sectional descriptive study

Setting: This study was conducted at Kenyatta National Hospital (KNH), a major referral and teaching hospital in Nairobi, Kenya, between January and November 2011. The study areas included the Accident and Emergency department, medical wards and outpatient clinics, Obstetrics and Gynaecology wards and outpatient clinics, and Biochemistry, Haematology and Immunology laboratories.

Participants: Male and female adult patients diagnosed with venous thrombosis confirmed by Doppler ultrasound or MRI.

Main outcome measures: Age, gender, clinical data (pain, pallor, jaundice, skin manifestations, site of thrombosis), associations (cigarette smoking, hypertension, previous thrombosis, recent surgery, pregnancy, puerperium, oral hormone contraceptive use), and laboratory results (presence or absence of lupus anticoagulant, titres of anticardiolipin (ACL)and anti-β2-GP1 antibodies (anti-β2-GP1), TBC, ESR, PBF, reticulocyte count, coagulation tests, serum creatinine and ALT,VDRL and TPHA).

Material and Methods: Demographic and clinical information was collected by direct interview of patients. Every patient was examined for clinical manifestations of APA and blood drawn for laboratory tests. A proforma questionnaire was used to collect all the information. The data collected was pooled, screened and entered into SPSS v.19 software for analysis.

Results: A total of 60 patients were studied. Majority of the patients, 52 (86.7%), were females, while males were 8 (13.3%). The mean age was 38.3 years (± 13.7), with a median (IQR) of
52 years (38.8, 58) for males vs. 32.5 years (24.8, 43.5) for females, p value <0.05). Fifty-five patients (91.7%) presented with pain and swelling of the lower limb. Twelve patients (20%) had previous history of DVT and 9 (15%) were hypertensive. Of all the patients, 55 (91.7%) were non-smokers. 43 (82.7%) of the female patients were not pregnant at presentation. Two patients (3.8%) were in puerperium. Fifteen patients (28.9%) were using oral hormonal contraceptives at the time of thrombosis. Thirteen patients (21.7%) had pallor, and 3 (5%) presented with jaundice. All 59 patients (98.3%) had no skin manifestations, except for a single case of a malar rash. Fifty-seven patients (95%) had lower limb thrombosis. The mean red cell count was 4.1 (SD ± 0.8), haemoglobin level 11.4 g/dL (SD ± 2), platelet count 275 x 10^9/L (SD ± 145.6), and absolute reticulocyte count 54.2 x 10^9/L (SD ± 29.8). The mean APTT value was 38.4 seconds (± 15.1) with 20 patients (33.3%) having prolonged values. Two patients (10%) had a prolonged KCT (RI >0.16; positive for LA), and all 20 (100%) who had a prolonged APTT had a negative DRVVT (NR <1.30; negative for LA). Fifty-five patients (91.7%) tested negative for VDRL. Of the 5 patients who tested positive for VDRL, 4 (80%) were also positive for TPHA. The mean anticardiolipin IgG titre was 107.4 U/mL (SD ± 62.4); 55 patients (91.7%) had a positive ACL result. The media anti-beta-2-glycoprotein IgG titre was 5 G units (IQR= 4.5, 6.5); 55 patients (91.7%) had a negative result. The mean serum creatinine and ALT concentrations were 98.2 µmol/L (SD ± 57.7) and 16.4 IU/L (SD ± 19.5) respectively. A significant positive correlation existed between APTT and ACL (r=0.39) and between ACL and β2GP1 (r=0.30), and a significant negative correlation (r= -0.29) between platelet count and haemoglobin levels.

**Conclusions:** Antiphospholipid antibodies (LA and anti-β2GP1 IgG antibodies) are present in a very small proportion of patients seen at KNH with venous thrombosis. ACL IgG antibodies may be induced by numerous factors and may not be related to thrombosis. DVT in our setting is more common in females of reproductive age, while affected males tend to be older. The lower limb is the most common site of DVT. Pathological antiphospholipid antibodies and their associated clinical and laboratory manifestations are uncommon in patients seen at KNH with VTE.

**Recommendations:** Screening for antiphospholipid antibodies in patients with venous thrombosis at KNH should be limited to those of a relatively young age, with unprovoked thrombosis or recurrent thrombosis. An LA assay in combination with a β2GP1 antibody assay would be more useful than ACL testing, unless local reference ranges are established.
INTRODUCTION
The antiphospholipid antibodies (APA) are a heterogeneous family of immunoglobulins directed against anionic phospholipids or protein phospholipid complexes. Lupus anticoagulant (LA) and anticardiolipin antibodies (ACL) are the two best clinically characterized antiphospholipid antibodies (1). Others are anti-β2-glycoprotein 1, antiprothrombin, antiphosphatidylserine, and antiphosphatidylethanolamine and antiphosphatidylinositol antibodies.

The frequency of APA in the normal population is approximately 3.6%, with most of these antibodies being induced by either infections or drugs. A high prevalence is seen in conditions such as peripheral vascular disease, recurrent foetal loss in women of reproductive age, acquired thrombotic episodes, autoimmune disorders, malignancies and cardiovascular disease, among others (1).

The antiphospholipid syndrome (APS) is a prothrombotic condition characterized by the presence of APA in patients with thromboembolic complications and/or recurrent pregnancy morbidity, as defined by Sydney Criteria of 2006(2). Evidence shows that the presence of antiphospholipid antibodies is associated with the development of vascular thrombosis (3)(4). Generally, APS is clinically classified into a primary form, with no associated systemic disease, and a secondary form, in which systemic lupus erythematosus (SLE) or a related connective tissue disorder is present. Primary APS has a similar prevalence in males and females. The secondary form is more frequent in females. Age distribution shows that most patients with APS lie between ages 25 and 40 years, however, there are reports of APS occurring in paediatric patients, as well as in patients above 50 years of age (1).

The key clinical consequence of APS is an increased risk for thrombosis, both venous and arterial. The most frequent site for venous thrombosis is the lower limb (3). Other sites that may be involved include retinal, renal and hepatic veins. The most frequent manifestation of arterial thrombosis is ischaemic stroke or transient ischaemic attack. Others features of APS include recurrent foetal loss and thrombocytopenia (3).

The tests for detection of APA are currently based on coagulation assays for lupus anticoagulant and enzyme-linked immunosorbent assays for anticardiolipin and anti-β2-glycoprotein 1 antibodies. Testing for LA should be limited to patients who have a significant probability of
having the APS, or have an unexplained prolonged APTT in the course of routine laboratory testing. Numerous variables can affect LA detection assays, giving a high variability in regards to sensitivity and specificity of the tests. The rates of false-negative and false-positive detections are high. There are recommended guidelines for optimal laboratory detection of LA, which describe the specimen collection and handling, the choice of tests, and the expression and transmission of results (5).

The ELISA tests aim to identify various isotypes (IgG, IgM and IgA) of antibodies directed towards phospholipids, or antibodies directed to phospholipids in complex with various cofactors (β2 glycoprotein 1, prothrombin). The assays directed towards phospholipids in association with cofactors (e.g. anti-β2-GP1) are more specific, as these antibodies are more strongly associated with thrombosis than ACL (6). However, these assays are less sensitive than ACL assays, which detect a wide range of additional antibodies.

In terms of antibody isotypes, IgG antibodies are more strongly associated with thrombosis than either IgA or IgM antibodies (6). This could be due to the fact that IgG response reflects a sustained immunity event, unlike the IgM response which reflects an initial immunity event.

From the literature, LA positivity has been found to be more strongly associated with a thrombotic risk than either ACL or anti-β2-GP1 positivity. For the solid-phase assays, anti-β-GP1 testing is more specific but less sensitive than ACL testing, and may lead to under-diagnosis, while ACL testing alone may lead to over-diagnosis of APS (6). Clinico-pathological correlation is recommended for diagnosis of the APS. There needs to be appropriate clinical features, plus two confirmatory laboratory findings.

The antiphospholipid syndrome is characterized by a significant risk of recurrent thromboembolism, especially on discontinuation of anticoagulants. Patients with APS and venous thrombosis therefore require prolonged or lifelong anticoagulant therapy (7).

This study was designed to determine the presence of antiphospholipid antibodies in, and to describe the clinical and laboratory characteristics of patients with venous thrombosis at Kenyatta National Hospital. This information provides some measure of the presence of APA in patients with DVT in our setting, as these are patients who would benefit from prolonged anticoagulant prophylaxis. It also gives some indication of the profile of the patient with DVT.
who may have APA, thus increasing the index of suspicion of clinicians to be vigilant for the other manifestations of APS in those patients found to have the antibodies. Venous thrombosis is a commonly encountered condition at KNH, but published literature on its local prevalence is not readily available.
LITERATURE REVIEW

Antiphospholipid Antibodies
Antiphospholipid antibodies represent a heterogenous group of autoantibodies that recognize various phospholipids, phospholipid-binding proteins, and phospholipid-protein complexes. The involved proteins, or antigenic targets, which are coagulation-regulating proteins, include β2 glycoprotein 1, prothrombin, activated protein C, and annexin V among others. Beta2-GP1 and prothrombin account for more than 90% of all the antibody binding activity (8). The autoantibodies involved include the lupus anticoagulant and anticardiolipin antibodies.

Lupus Anticoagulants
The lupus anticoagulant is an autoantibody that acts at the level of the prothrombin converter complex of the clotting cascade; a reaction which is catalyzed by phospholipids, to inhibit the conversion of prothrombin to thrombin (9). It therefore behaves as an acquired inhibitor of coagulation, by prolonging phospholipid-dependent coagulation. The LA is directed against β2-glycoprotein I (10) or prothrombin attached to negatively charged phospholipids (11). The LA is a misnomer since most patients with LA do not have lupus, and in addition, *in vivo* LA is a procoagulant rather than an anticoagulant (3). According to Lechner K *et al*, the presence of LA is reckoned to confer a 30% lifetime risk of a thrombotic event (12).

Anticardiolipin antibodies
Cardiolipin is the phospholipid antigen conventionally used in testing for APS. This phospholipid is mainly intracellular within the mitochondrial membrane, and antibodies to it are responsible for the false positive VDRL test for syphilis that is often seen in patients with APS (3). Pathogenic ACL antibodies are directed against β2-glycoprotein bound to cardiolipin (13).

ACL antibody assays may detect both antibodies to β2-GP1 as well as antibodies that directly bind to cardiolipin. The former are associated with APS, whereas the latter occur in various infections such as syphilis, leprosy, malaria, HIV and hepatitis C, and sometimes in normal individuals, and do not appear to be associated with APS (14).
β₂ glycoprotein 1 is the most important antigenic target of antiphospholipid antibodies. It is a 50-kDa plasma phospholipid-binding glycoprotein that can be expressed on cell membranes of endothelial and trophoblast cells. β₂-GP1 is mainly involved in anticoagulation (15,16). As well as anti-β₂-GP1 antibody activity, the sera of patients with APS may also contain antibodies directed against other proteins which interact with negatively charged phospholipids, such as prothrombin (17) and annexin V (18).

Anti-β₂-GP1 antibodies activate endothelial cells by producing a proinflammatory and procoagulant phenotype, sustained by the up-regulation and expression of adhesion molecules E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1, and resulting in the secretion of cytokines, chemokines, endothelin-1, and tissue factor, all contributing to the thrombophilic diathesis encountered in the APS (15,19).

Annexin V is a calcium-dependent phospholipid membrane-binding protein. It acts in vitro as an anticoagulant by competing for sites on phospholipid membranes where coagulation factors normally assemble into active complexes, and so mimics the lupus anticoagulant effect (20).

**Historical Perspectives**

In 1906, Wasserman identified sera from patients with syphilis that reacted with extracts of syphilitic tissues (21,22). The Wasserman reagin test was originally attributed to antibody reactivity against antigens derived from *Treponema pallidum*, until use of normal human and animal tissue was found to give similar results (21,23). In 1941, Pangborn isolated cardiolipin from bovine heart, identifying it as the antigenic component of the reagin test (24). The combination of cardiolipin, lecithin and cholesterol formed the basis of the flocculation test for syphilis referred to as Venereal Disease Research Laboratory test (21,23).

With development of more specific tests for syphilis, it became clear that infections other than syphilis could produce a positive Wasserman reagin or VDRL test (21). In 1952, Moore and Mohr identified two circumstances in which a biologic false positive serologic test result for syphilis could occur (25). Transient reactions followed acute viral infections and vaccination, whereas persistent (more than 6 months) reactions were associated with autoimmune disorders such as systemic lupus erythematosus (21).
The first description of lupus anticoagulant in 1952 associated this ‘circulating anticoagulant’ with haemorrhage (26) but subsequent reports failed to identify an increased risk of haemorrhage. It was detected by prolongation of a phospholipid-dependent in-vitro coagulation test. Subsequent work confirmed that the lupus anticoagulant was attributable to the biologic false positive serologic test result for syphilis (27,28) and, paradoxically, was associated with in-vivo thrombosis (29) rather than bleeding diathesis.

In 1990, it was demonstrated that pathological ACL antibodies were in fact anti-β2-GP1 antibodies. A year later, it was shown that majority of the antibodies that induce LA in plasma are also anti-β2-GP1 antibodies. The discovery that β2-GP1 is the real epitope for APA has raised questions on whether the ACL ELISA is appropriate for detection of a thrombotic risk due to APA (30). Anti-β2-GP1 assay has been found to be more specific in APA determination as compared to the ACL assay (31).

**Epidemiology of Antiphospholipid Antibodies**

Antiphospholipid antibodies have been detected in all races and geographical regions of the world (1). These antibodies can be identified in 4 – 21% of patients presenting with venous thromboembolism, a significantly higher prevalence than that of 1 – 5% observed in healthy individuals (32,33). These could be due to infections or could be drug-induced. Higher prevalence is seen in disease conditions such as peripheral vascular disease, recurrent foetal loss in women of reproductive age and acquired thrombotic episodes. Other disease conditions which show higher prevalence include pulmonary hypertension, heparin-induced thrombocytopenia, cerebral vascular disease in young patients, autoimmune disorders, lymphoproliferative diseases, drug reactions, infections, cardiovascular disease and malignancies(1). These antiphospholipid antibodies are usually of IgM isotype, are present at low levels, and are not usually associated with thrombotic events (34).

The proportion of subjects presenting with thrombosis who have persisting APA, and who fulfill the criteria for diagnosis of APS, is variable (35). One study quotes the prevalence of APA in unselected patients with DVT to be about 5% (36). In SLE, the prevalence of APA is much higher, with studies quoting between 15-30% of patients as having demonstrable LA, and the prevalence of ACL is even higher, at upto 86%(35).
Clinical Manifestations of Antiphospholipid Antibodies
Primary antiphospholipid antibodies are found equally in both males and females, while the secondary antibodies are more frequent in females. Age distribution shows that most patients with APA fall between ages 25 and 40 years, but the antibodies may also be present in paediatric and elderly patients (1).

Antiphospholipid antibodies are associated with the antiphospholipid syndrome, a non-inflammatory autoimmune disease defined by presence of APA in the plasma of patients with venous and/or arterial thrombosis and/or recurrent pregnancy complications (37,38). It is the most common acquired thrombophilia (39). The detection of APA in the blood of a patient with thrombosis or complications of pregnancy is the essential step in diagnosis of the syndrome, since these clinical manifestations occur relatively often in the general population (40).

APS has a wide diversity of clinical manifestations, which may affect virtually any organ in the body, including the lungs, skin, brain, liver, kidneys, adrenal glands, heart, and eyes (41). The recognised “classical” clinical presentations include peripheral deep venous thrombosis, pulmonary embolism, and/or arterial thrombotic events (42). Obstetric manifestations include recurrent miscarriages, and early-onset and severe preeclampsia. Deep venous thromboses are the most frequently reported association of the APA (15). Other clinical manifestations include thrombocytopaenia, thrombotic microangiopathic haemolytic anaemia, nephropathy, valvular heart lesions, livedo reticularis and neurological abnormalities (7). APS nephropathy is characterized by hypertension, acute or chronic renal failure, and a low-grade proteinuria (43).

Results from a case-control study conducted by de Groot et al between 1988 and 1993 demonstrated that presence of antiphospholipid antibodies is a risk factor for DVT in the general population (44). Venous thrombosis, particularly of the lower limb, occurs at some point in upto 55% of patients with antiphospholipid syndrome (45,47). Cross-sectional studies suggest about a two-fold increase in the risk of VTE with an anticardiolipin antibody and a 5- to 10-fold increase with a lupus anticoagulant in patients with, and without SLE (47,48).

Several studies have shown that additional non-APA prothrombotic risk factors may be associated with antiphospholipid antibody-related thrombosis (49-51). These additional risk factors include pregnancy, surgical procedures, oral hormonal contraceptive use, and cigarette...
smoking, among others. Local data on the magnitude of venous thrombosis and its associated aetiological risk factors is not readily available. The mechanisms underlying the generation of pathogenic antiphospholipid antibodies remain obscure (35).

There are data suggesting that antiphospholipid antibodies exert procoagulant effects through any one or more of several mechanisms (7,8). The first one involves interference by the antiphospholipid antibody with endogenous anticoagulant mechanisms {disruption of the annexin A5 anticoagulant shield (52), inhibition of protein C pathway (53,54), and inhibition of antithrombin (55)}. The second mechanism involves binding and activation of platelets by the antiphospholipid antibody (56,57). The third pathogenic mechanism involves interaction with endothelial cells and induction of expression of adhesion molecules and tissue factor (58,59). The fourth pathogenic mechanism of antiphospholipid antibodies involves activation of the complement cascade, generating split products that may lead to inflammation and thrombosis (60).

**Laboratory Features of Antiphospholipid Antibodies**

Testing for LA should be limited to patients with a high likelihood of having the antiphospholipid syndrome, or who have unexplained prolonged APTT in the course of routine laboratory testing. Appropriateness to search for lupus anticoagulants can be graded according to clinical characteristics into low, moderate and high. *Low*: venous or arterial thromboembolism in elderly patients; *Moderate*: accidentally found prolonged APTT in asymptomatic subjects, recurrent spontaneous early pregnancy loss, provoked venous thromboembolism in young patients; and *High*: unprovoked venous thromboembolism and unexplained arterial thrombosis in young patients (<50 years of age), thrombosis at unusual sites, late pregnancy loss, any thrombosis or pregnancy morbidity in patients with autoimmune diseases (5).

The presence of APA in plasma of patients should be detected by either a prolongation of phospholipid-dependent coagulation tests (for LA), or by solid-phase immunoassays, for the ant Cardiolipin and anti-β2GP1 antibody ELISA. Lupus anticoagulant is the assay of choice to detect the antiphospholipid syndrome (38).

In a meta-analysis of 25 studies, Galli et al (38) showed that lupus anticoagulant correlates much better than ant Cardiolipin antibodies with thromboembolic complications. An explanation for the
superiority of lupus anticoagulant assay over anticardiolipin assay is that lupus anticoagulant assay measures an “activity” of antiphospholipid antibodies, whereas in an ELISA set-up, both antibodies, one, that influence a functional activity and two, antibodies that do not influence an activity are measured. Thus, an ELISA measures the whole heterogenous population of antibodies whereas in a functional assay, only a specific subpopulation is measured (38).

Diagnosis of the APS is based on clinical criteria of vascular thrombosis or pregnancy morbidity, and laboratory findings of medium or high titre APA that are present on 2 or more occasions at least 12 weeks apart (2).
RATIONALE
Information on the prevalence and the role of APA and its associated disorders is not readily available locally, despite the fact that the role of antiphospholipid antibodies in development of thrombosis has been well defined. APA should be tested for when evaluating a patient with a hypercoagulable state (61,62). Conditions for antiphospholipid antibody testing include the presence of systemic lupus erythematosus, obstetric history that meets criteria for obstetric antiphospholipid syndrome, arterial or venous thrombosis before the age of 45 years, recurrent thrombosis, thrombosis at an unusual site, association of both venous and arterial events, and apparently unprovoked venous thromboembolism (35,63).

The clinical characteristics of patients with APA have also not been well established. Patients with APA-mediated thrombosis need to be identified so that they can be treated appropriately, as they are at a high risk for recurrence of thrombosis and therefore require long-term anticoagulant treatment. Obtaining more recent data on syndromic conditions like the APS will also increase the index of suspicion for our clinicians to enable them to look out for it in patients who present with some of the associated clinical manifestations. KNH is the major referral and teaching hospital in Kenya, with a capacity to diagnose, treat and follow-up clinical conditions associated with APA. Determination of the presence, type and titres of APA in patients with venous thrombosis at KNH will be beneficial not only to patients as they will be treated appropriately, but this information will also be used as teaching material and may also form a basis for further local research on APA.
**RESEARCH QUESTIONS**

Are there antiphospholipid antibodies in patients with venous thrombosis?

What are the clinical characteristics of patients with antiphospholipid antibodies and venous thrombosis?

What are the laboratory characteristics of patients with antiphospholipid antibodies and venous thrombosis?

**STUDY OBJECTIVES**

**Broad objective**
To determine the presence of antiphospholipid antibodies and to identify their clinical and laboratory associations in patients with venous thrombosis at KNH

**Specific objectives**
1. To determine the presence of antiphospholipid antibodies in patients with thrombosis at KNH

2. To determine the types of antiphospholipid antibodies (LA, anticardiolipin, anti-β2-glycoprotein 1) in patients with venous thrombosis at KNH

3. To identify associations between antiphospholipid antibodies and clinical characteristics of patients with venous thrombosis at KNH

4. To identify associations between antiphospholipid antibodies and laboratory characteristics of patients with venous thrombosis at KNH
MATERIALS AND METHODS

Study Design
This was a prospective cross-sectional descriptive study conducted between January and November 2011.

Study Area
The study was conducted at Kenyatta National Hospital, the largest referral and teaching hospital in Kenya, whose patients are drawn from throughout the Republic. The University of Nairobi Medical School is located within the KNH complex. As a referral and teaching institution, KNH has specialized medical personnel that are able to diagnose and effectively manage cases of thrombosis associated with antiphospholipid antibodies, with the help of qualified technicians from the various specialized laboratories. Being the largest referral institution in Kenya, the patients treated at KNH are a good representation of the country’s population. The study sites were the Accident and Emergency Department, Medical, and Obstetrics and Gynaecology wards and outpatient clinics, and the Haematology, Immunology and Biochemistry laboratories.

Study Population
All male and female adult patients seen at Kenyatta National Hospital with DVT confirmed by Doppler ultrasound or MRI, and who fulfilled the inclusion criteria.

Selection Criteria
Inclusion Criteria
Male and female adult patients with venous thrombosis confirmed by Doppler ultrasound or MRI; gave informed consent to participate in the study, and who were not on anticoagulant medication.

Exclusion Criteria
Patients documented as having a bleeding disorder, patients with documented co-morbidities (Sickle cell disease, polycythaemia)
Recruitment

Patients were recruited from the A&E department, the Haematology outpatient clinic, and the Medical, Surgical, Obstetric and Gynaecological inpatient wards and outpatient clinics. Medical personnel at these sites were notified about the study via oral and written communication to facilitate the recruitment of patients. The principal investigator’s contact was availed at these sites to facilitate easy notification once a potential patient was identified. Research assistants aided in the identification and recruitment of patients. Every patient who presented to these sites with a clinical suspicion of venous thrombosis was sent for Doppler ultrasound or MRI scanning. If the scan was confirmatory for venous thrombosis, the patient was screened (Appendix 2: Flow diagram) for inclusion and exclusion criteria through direct interview, followed by physical examination and review of the patient’s file. Those who fulfilled the inclusion criteria were informed about the study and written informed consent was obtained from those who agreed to participate in the study. Consecutive patients with DVT were recruited until the desired sample size was achieved.

Data collection procedures

Clinical procedures

After obtaining informed consent, the patient’s clinical details were obtained both by direct interview and review of the patient’s file. Assessment was conducted in the normal fashion: clinical history, followed by physical examination with a view to elicit pallor, jaundice, skin lesions and other associated clinical features. Blood was then collected for the laboratory tests, by the Principal Investigator or research assistant.

The data collected from each patient was captured in a pre-designed structured questionnaire and data extraction sheet (Appendix 4).

Specimen handling

The skin on the antecubital fossa was cleaned with 90% methylated spirit and allowed to dry spontaneously before being punctured. Where a tourniquet was used, it was applied just above the venepuncture site and released as soon as the blood began to flow into the syringe. The piston
of the syringe was withdrawn slowly. Fourteen millilitres of blood was drawn from an antecubital vein. After obtaining blood the needle was removed from the patient’s arm and a sterile swab strapped over the puncture. Pressure was applied on the swab for a few minutes to ensure that there was no more oozing. The puncture site was then covered with a small adhesive dressing.

The 14 ml of blood was then divided into (i) 4 ml into an EDTA vacutainer, (ii) 3.5 ml into a trisodium citrate vacutainer, (iii) 4 ml into a plain vacutainer, and (iv) 2 ml into another plain vacutainer. After collection, the containers were firmly capped. Anticoagulated specimens were mixed by inverting the containers several times.

Blood collected into the vacutainers containing EDTA anticoagulant were immediately transported to the haematology laboratory for haemogram, peripheral blood film, ESR and reticulocyte count. Blood collected into trisodium citrate was immediately transported to the haematology laboratory where platelet poor plasma was prepared by centrifugation at 3000 rpm for 10 minutes. The plasma samples were aliquoted and stored at -80°C, awaiting the coagulation tests (PT, APTT, KCT and LA test), which were done after batching of the samples.

The blood collected into plain vacutainers (4 ml) was transported to the Immunology laboratory, and then allowed to clot undisturbed for 1 hour at room temperature. The serum was separated from the clotted blood by centrifugation at 3000 rpm, then aliquoted and stored at -80°C. Analysis for VDRL, TPHA, ACL and anti-β2-GP1 antibodies was done after batching of the samples. The other 2 mls was immediately transported to the biochemistry laboratory for serum creatinine and alanine transaminase analysis.

Frozen specimens were thawed on the bench or in a water bath at room temperature, and then inverted several times to ensure homogeneity before use for a test.

**Laboratory procedures**

The laboratory tests were performed by the Principal Investigator, with the aid of qualified laboratory personnel at the Haematology, Immunology and Biochemistry laboratories.
**Haematological tests**

The haemograms were performed at the haematology laboratory by an automated cell counter (Cell-Dyn 3200). A peripheral blood film was prepared for each sample by placing a small drop of blood on a clean glass slide and spreading the blood, air-drying and then staining with May-Grünwald stain. Slides were also prepared for reticulocyte counts by adding 2 volumes of blood to 2 volumes of methylene blue dye solution in a plastic tube, incubating for 15 minutes at 37°C, mixing the solution and then films were prepared on glass slides. ESR was performed manually via the Wintrobe method. EDTA anticoagulated blood was drawn into a 100 mm long tube and the rate of fall of red blood cells measured in millilitres after one hour.

**Coagulation tests**

The PT and APTT were performed at the haematology laboratory using an automated coagulation analyser (ACL200). Hemosil™ PT-Fibrinogen HS PLUS and APTT Lyophilized Silica kits were used. Samples with prolonged APTT were subjected to LA detection procedures (**Appendices 5 and 6**). Two different testing methods were used, as recommended by the International Society of Thrombosis and Haemostasis guidelines (64), KCT (65,66) (LupoTek KCT kits), and DRVVT (67) (LupoTek DetecTin VL and LupoTek CorrecTin VL kits). LA prolongs phospholipid-dependent coagulation tests (APTT, DRVVT). The venom in these lupus anticoagulant testing kits directly activates Factor X without requiring Factor VII. The activated Factor X in conjunction with Factors V, II, calcium ions and phospholipid will generate thrombin which converts fibrinogen to fibrin, producing a clot.

LupoTek DetecTin VL, the low phospholipid reagent, is designed as the screening reagent to detect a prolongation of the clotting time. LupoTek CorrecTin VL is the high phospholipid reagent that neutralizes the LA and corrects the clotting time to normal, confirming the presence of a Lupus Anticoagulant. (**Appendix 5**)

The results of the KCT are expressed as a Rosners Index, with a cut-off of >1.30 being positive. The LA DetecTin and CorrecTin tests were performed simultaneously as recommended by the manufacturer. The results were expressed as a Normalized Ratio, with a cut-off value of >0.16 considered as positive.
**VDRL test**

This was performed using the Syphilis RPR Test kit (68), at the Immunology laboratory. The Syphilis RPR is a non-treponemal flocculation test used to detect and quantify reagin antibodies associated with syphilis. The RPR-antigen used in the kit is a modification of VDRL antigen which contains microparticulate charcoal to enhance the visual difference between a positive and negative result. For sera containing anti-reagin antibodies flocculation of antigen-charcoal particles is observed. In case of a non-reactive specimen, the specimen-antigen-suspension remains homogenous. (Appendices 5 and 6)

**TPHA**

This was performed at the Immunology laboratory on the VDRL positive samples. The Syphilis TPHA liquid kit was used. This is an indirect haemagglutination test for the detection of specific antibodies against *Treponema pallidum*. Avian erythrocytes are coated with *T. pallidum* antigen. In the presence of syphilitic antibodies the sensitized cells will agglutinate to form characteristic patterns in microtitration plates. (Appendices 5 and 6)

**Anticardiolipin and anti-β2-glycoprotein 1 antibody detection**

These were performed at the Immunology laboratory using ELISA kits. IMTEC-Cardiolipin-Antibodies IgG kit is a solid-phase enzyme immunoassay for quantitative measurement of IgG class autoantibodies against cardiolipin/β2-GP1 in human serum and plasma.

REAADS® IgG Anti-Beta 2 Glycoprotein 1 Semi-quantitative Test Kit is a solid-phase immunoassay for the semi-quantitative determination of IgG anti-β2-GP1 antibodies in human serum or plasma. (Appendices 5 and 6)

**Serum creatinine and ALT**

These were performed at the KNH biochemistry laboratory using an automated chemistry analyser (Olympus AU 640). Serum creatinine was measured via the Jaffe reaction and ALT activity by a transamination reaction.
Quality Assurance

There was strict adherence to protocol (see Specimen Handling) during sample collection, storage and processing. All tests were performed in accordance with manufacturers’ recommendations. Internal quality control materials were included during analysis; these were provided by the manufacturer. Validation of the ELISA tests was done according to the manufacturers’ recommendations (Appendix 6).

Data Handling

Sample size

\[ n = \frac{Z^2 \times P \times (1-P)}{d^2} \]

\[ n = \text{sample size (n = 60)} \]

\[ Z = \text{confidence level at 95% (Z = 1.96)} \]

\[ P = \text{prevalence of APA at 4% (32,33) (P=0.04)} \]

\[ d = \text{margin of error at 5% (d = 0.05)} \]

Variables

- Independent variables
  - Demographic characteristics – age, gender
  - Medical history - cigarette smoking, uncontrolled hypertension, SLE, previous history of thrombosis, recent abdominal or orthopaedic surgery, pregnancy, prior foetal loss, hormonal contraceptive use, site of thrombosis, pregnancy, hypertension, skin manifestations (malar rash, livedo reticularis)
Dependent variables

- Laboratory characteristics – TBC, ESR, PBF, reticulocyte count, PT, APTT, KCT, Viper venom test time, VDRL, TPHA, IgG ACL, IgG β2-GP1, serum creatinine and ALT

The data collected using a structured questionnaire (Appendix 4) and pre-designed extraction sheets was entered into Ms Excel computer database, cleaned and verified, then imported into SPSS (v.19) statistical software for analysis.

Descriptive statistics on socio-demographic characteristics was presented using percentages and frequencies for categorical or nominal data. Continuous variables were presented using means (standard deviations) if normally distributed and medians (inter-quartile range) for non-normally distributed variables. Tables and appropriate charts were used to display the results.

T-test or ranksum tests were used as appropriate to compare difference in means for two groups of continuous variables. Chi-square or Fisher’s tests for independence were used to assess association between two nominal or categorical variables. The level of significance was set at 5% with p-values of ≤0.05 being considered significant.

Correlation analysis to assess for any linear association was done using Pearson correlation coefficient for the continuous variables, and considered significant at 5% level.

Ethical Considerations
Approval for the study was obtained from Kenyatta National Hospital Ethical and Research Committee prior to commencement of the study (P272/08/2010, Appendix 7). In addition, informed consent was obtained from each study participant. Samples were collected according to standard procedure. Patient details including results were kept confidential, and participants in whom disease was diagnosed had their physicians notified.
RESULTS

A total of 62 eligible participants were enrolled into the study. Two were excluded due to mislabeling of the specimens. The remaining 60 met the inclusion criteria and were evaluated for the requirements of the study.

Baseline Characteristics

**Gender and Age:**

Table 1: Demographic characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years, mean (SD)</td>
<td>38.3 (±13.7)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8 (13.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>52 (86.7%)</td>
</tr>
</tbody>
</table>

Figure 1: Gender of patients

Majority of the patients were females, 52 (86.7%), (Table 1, Figure 1). The mean age was 38.3 years (± 13.7), with a median (IQR) of 32.5 years (24.8, 43.5) for females and 52 years (38.8, 58) for males.
**Social-economic characteristics:**

Table 2: Social-economic characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occupation</td>
<td></td>
</tr>
<tr>
<td>Housewife</td>
<td>12 (20%)</td>
</tr>
<tr>
<td>Farmer</td>
<td>5 (8.3%)</td>
</tr>
<tr>
<td>Artisan</td>
<td>6 (10%)</td>
</tr>
<tr>
<td>Professional*</td>
<td>18 (30%)</td>
</tr>
<tr>
<td>Unemployed</td>
<td>19 (31.7%)</td>
</tr>
</tbody>
</table>

*Professions - businessmen and business ladies, hairdressers, security officers, teachers, community health workers

Figure 2: Occupation of patients

Sizable proportions of patients were mostly either unemployed 19(31.7%) or professionals (businessmen and business ladies, hairdressers, security officers, teachers, community health workers) 18(30%), with a good number being housewives 12(20%) as indicated in (Table 2, Figure 2).
Clinical Characteristics

Table 3: Presenting symptoms

Presenting symptoms (n = 60)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Presenting symptom</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presenting symptom</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limb pain</td>
<td></td>
<td>1 (1.7%)</td>
</tr>
<tr>
<td>Limb swelling</td>
<td></td>
<td>2 (3.3%)</td>
</tr>
<tr>
<td>Others*</td>
<td></td>
<td>2 (3.3%)</td>
</tr>
<tr>
<td>Limb pain and swelling</td>
<td></td>
<td>55 (91.7%)</td>
</tr>
</tbody>
</table>

*Other symptoms – shortness of breath, abdominal pain, one-sided weakness

Table 4: Clinical findings

Clinical findings (n=60)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Past Medical History</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DVT recurrence</td>
<td></td>
<td>12 (20%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td>9 (15%)</td>
</tr>
<tr>
<td>Recent surgery</td>
<td></td>
<td>2 (3.3%)</td>
</tr>
<tr>
<td>Not significant</td>
<td></td>
<td>37 (61.7%)</td>
</tr>
</tbody>
</table>

| Family History of DVT               |                      |       |
| Positive history                    |                      | 3 (5%) |
| Negative history                    |                      | 57 (95%) |

| Cigarette Smoking                   |                      |       |
| Smokers                             |                      | 5 (8.3%) |
| None smokers                        |                      | 55 (91.7%) |
Fifty-five patients (91.7%) presented with limb pain and swelling, (*Table 3*). As shown in *Table 4*, 12 (20%) patients had previous history of DVT, 9 (15%) had hypertension and 3 (5%) had a family history of DVT, (*Figure 3*). Most of the patients, 55(91.7%), were non-smokers.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant at presentation</td>
<td></td>
</tr>
<tr>
<td>Pregnant</td>
<td>9 (17.3%)</td>
</tr>
<tr>
<td>Not pregnant</td>
<td>43 (82.7%)</td>
</tr>
<tr>
<td>History of foetal loss</td>
<td></td>
</tr>
<tr>
<td>Positive history</td>
<td>8 (15.4%)</td>
</tr>
<tr>
<td>Negative history</td>
<td>44 (84.6%)</td>
</tr>
<tr>
<td>History of pre-eclampsia or eclampsia</td>
<td></td>
</tr>
<tr>
<td>Positive history</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Negative history</td>
<td>51 (98%)</td>
</tr>
<tr>
<td>Recent Delivery</td>
<td></td>
</tr>
<tr>
<td>In puerperium</td>
<td>2 (3.8%)</td>
</tr>
<tr>
<td>Not in puerperium</td>
<td>50 (96.2%)</td>
</tr>
<tr>
<td>Oral Hormonal Contraceptive use</td>
<td></td>
</tr>
<tr>
<td>OCP use</td>
<td>15 (28.9%)</td>
</tr>
<tr>
<td>No OCP use</td>
<td>37 (71.1%)</td>
</tr>
</tbody>
</table>
As shown in Table 5 and Figure 4, a majority 43(82.7%) of the female patients were not pregnant. Only a small proportion of the women, 8(15.4%), had any history of foetal loss. Virtually all patients 51(98%) had no history of pre-eclampsia or eclampsia. Two patients (3.8%) were in puerperium. Fifteen patients (28.9%) were using oral hormonal contraceptives at the time of thrombosis.
**Examination findings:**

Table 6: General examination findings

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure n (%)</td>
<td></td>
</tr>
<tr>
<td>Normal BP</td>
<td>53(88.3%)</td>
</tr>
<tr>
<td>Elevated BP</td>
<td>7(11.7%)</td>
</tr>
<tr>
<td>Pallor of mucus membranes n (%)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>13 (21.7%)</td>
</tr>
<tr>
<td>Absent</td>
<td>47 (78.3%)</td>
</tr>
<tr>
<td>Jaundice n (%)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>3 (5%)</td>
</tr>
<tr>
<td>Absent</td>
<td>57 (95%)</td>
</tr>
</tbody>
</table>

As shown in Table 6 and Figure 5, 53 patients (88.3%) were normotensive, with blood pressures of 90/60 - 120/80 mmHg; 13 (21.7%) had pallor, and only 3 (5%) presented with jaundice.
Table 7: Physical findings

<table>
<thead>
<tr>
<th>Physical findings (n=60)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Characteristic</strong></td>
</tr>
<tr>
<td>Skin manifestations</td>
</tr>
<tr>
<td>n (%)</td>
</tr>
<tr>
<td>Malar rash</td>
</tr>
<tr>
<td>None</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anatomic site of thrombosis</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower limb</td>
<td>57 (95%)</td>
</tr>
<tr>
<td>Upper limb</td>
<td>1 (1.7%)</td>
</tr>
<tr>
<td>Dural venous sinus</td>
<td>1 (1.7%)</td>
</tr>
<tr>
<td>Jugular vein</td>
<td>1 (1.7%)</td>
</tr>
</tbody>
</table>

Virtually all 59(98.3%) patients had no skin manifestations, except for a single case of a malar rash; (Table 7). Concerning the anatomic site of thrombosis the majority, 57(95%), had lower limb involvement.
### Laboratory Characteristics

#### Table 8: Haemogram results

<table>
<thead>
<tr>
<th>Haemogram results (n=60)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Characteristic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red cell count (x10^{12}/L), mean (SD)</td>
<td>4.1 (± 0.8)</td>
<td></td>
</tr>
<tr>
<td>Red cell count categories</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytopaenia</td>
<td>19 (31.7%)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>35 (58.3%)</td>
<td></td>
</tr>
<tr>
<td>Erythrocytosis</td>
<td>6 (10%)</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dL), mean (SD)</td>
<td>11.4 (± 2)</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin categories</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaemia</td>
<td>37 (61.7%)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>21 (35%)</td>
<td></td>
</tr>
<tr>
<td>Polycythaemia</td>
<td>2 (3.3%)</td>
<td></td>
</tr>
<tr>
<td>White cell count (x10^9/L), mean (SD)</td>
<td>7.0 (± 3.0)</td>
<td></td>
</tr>
<tr>
<td>White cell count categories</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucopaenia</td>
<td>5 (8.3%)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>49 (81.7%)</td>
<td></td>
</tr>
<tr>
<td>Leucocytosis</td>
<td>6 (10%)</td>
<td></td>
</tr>
<tr>
<td>Platelet count (x10^9/L), mean (SD)</td>
<td>275.5 (± 145.6)</td>
<td></td>
</tr>
<tr>
<td>Platelet Count categories n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombocytopaenia</td>
<td>3 (5%)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>47 (78.3%)</td>
<td></td>
</tr>
<tr>
<td>Thrombocytosis</td>
<td>10 (16.7%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 9: Haematology test results

**Haematology test results (n=60)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulocyte count (x10⁹/L), mean (SD)</td>
<td>54.2 (± 29.8)</td>
</tr>
</tbody>
</table>

**Reticulocyte Count categories**

- Reticulocytopaenia: 26 (43.3%)
- Normal: 30 (50%)
- Reticulocytosis: 4 (6.7%)

**Peripheral blood film n (%)**

- Normal: 37 (61.7%)
- Normocytic normochromic anaemia: 11 (18.3%)
- Neutrophilia: 1 (1.7%)
- Macrocytosis: 3 (5%)
- Microcytic hypochromic anaemia: 6 (10%)
- Lymphocytosis: 1 (1.7%)
- Thrombocytopaenia: 1 (1.7%)

**ESR (mm in the 1st hr), mean (SD)**

- Elevated: 46 (76.7%)
- Normal: 14 (23.3%)

Table 9: Haematology test results 1

Figure 6: Red blood cell counts and Haemoglobin box plots
As shown in Table 8, the mean red cell count was 4.1 x 10^{12}/L (SD ± 0.8). Majority 35(58.3%) of the patients had a normal red blood cell count (3.8 - 4.8 x 10^{12}/L in females and 4.5 - 5.5 x 10^{12}/L in males). The mean haemoglobin level was 11.4 g/dL (SD ± 2; Range 5.28 – 17.2 g/dL), (Figure 6). Majority 37 (61.7%) of the patients were anaemic, with only 21 (35%) having a normal haemoglobin level (12-15 g/dL in females and 13-17 g/dL in males). The mean white cell count was 7.0 x 10^{9}/L (SD ± 3.0); with 5 (8.3%) patients having leucopaenia and 6 (10%) having leucocytosis. The mean platelet count was 275 x 10^{9}/L (SD ± 145.6; Range 66.8–788 x 10^{9}/L), with a majority of patients 47 (78.3%) having a normal count (150-400 x 10^{9}/L), Figure 7. Three patients (5%) had thrombocytopaenia (<100 x 10^{9}/L).

The mean absolute reticulocyte count was 54.2 x 10^{9}/L (SD ± 29.8), (Table 9) with most patients, 30(50%) having either a normal count (50-100 x 10^{9}/L) or reticulocytopaenia, 26 (43.3%). A large proportion 37(61.7%) of the patients had a normal peripheral blood film, with a sizable number 11(18.3%) exhibiting normocytic normochromic anaemia.
The mean ESR was 35.6 (SD ± 20.3) with a majority 46(76.7%) having elevated ESR values, (Figure 8).

Table 10: APTT results

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTT (seconds), mean (SD)</td>
<td>38.4 (± 15.1)</td>
</tr>
<tr>
<td>APTT categories n (%)</td>
<td></td>
</tr>
<tr>
<td>Shortened (&lt;28.3 s)</td>
<td>9 (15%)</td>
</tr>
<tr>
<td>Normal (28.3 – 38.3 s)</td>
<td>31 (51.7%)</td>
</tr>
<tr>
<td>Prolonged (&gt;38.3 s)</td>
<td>20 (33.3%)</td>
</tr>
</tbody>
</table>

As shown in Table 10, the mean APTT value was 38.4 seconds (± 15.1), with a majority of the patients having either normal 31(51.7%) or prolonged values 20 (33.3%). (Normal control 28.3 – 38.3 seconds)

KCT was performed on the 20 patients with prolonged APTT, and the results expressed as Rosners Index (RI). The mean RI was 0 (± 0.3), with majority of the patients 18 (90%) having a negative value and 2 (10%) having a positive value.
Table 11: Lupus anticoagulant tests

<table>
<thead>
<tr>
<th>Tests for LA (n=20)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td>Results</td>
</tr>
<tr>
<td>Rosner's Index (KCT), mean (SD)</td>
<td>0 (± 0.3)</td>
</tr>
<tr>
<td>Rosner's Index KCT categories n (%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>18 (90%)</td>
</tr>
<tr>
<td>Positive</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Normalized Ratio (LA), mean (SD)</td>
<td>1.2 (± 0.1)</td>
</tr>
<tr>
<td>Normalized ratio Viper Venom Test categories n (%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>20 (100%)</td>
</tr>
</tbody>
</table>

The Lupus anticoagulant DetecTin and CorrecTin tests were also performed on samples with prolonged APTT, and the results expressed as a Normalized Ratio (NR). The mean NR was 1.2(± 0.1) with all the samples 20 (100%) testing negative for LA, as shown in Table 11.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDRL n (%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>55 (91.7%)</td>
</tr>
<tr>
<td>Positive</td>
<td>5 (8.3%)</td>
</tr>
<tr>
<td>TPHA n (%) (n = 5)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Positive</td>
<td>4 (80%)</td>
</tr>
<tr>
<td>Anticardiolipin IgG (U/mL), mean (SD)</td>
<td>107.4 (± 62.4)</td>
</tr>
<tr>
<td>ACL categories n (%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>5 (8.3%)</td>
</tr>
<tr>
<td>Positive</td>
<td>55 (91.7%)</td>
</tr>
<tr>
<td>Antibeta2glycoprotein IgG (G units), median (IQR)</td>
<td>5 (4.5, 6.5)</td>
</tr>
<tr>
<td>Antibeta2glycoprotein IgG categories n (%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>55 (91.7%)</td>
</tr>
<tr>
<td>Positive</td>
<td>5 (8.3%)</td>
</tr>
<tr>
<td>Creatinine (µmol/L), mean (SD)</td>
<td>98.2 (± 57.7)</td>
</tr>
<tr>
<td>Creatinine categories n (%)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>5 (8.3%)</td>
</tr>
<tr>
<td>Normal</td>
<td>35 (58.3%)</td>
</tr>
<tr>
<td>High</td>
<td>20 (33.3%)</td>
</tr>
<tr>
<td>ALT (IU/L), mean (SD)</td>
<td>16.4 (± 19.5)</td>
</tr>
<tr>
<td>ALT categories n (%)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2 (3.3%)</td>
</tr>
<tr>
<td>Normal</td>
<td>58 (96.7%)</td>
</tr>
</tbody>
</table>
Figure 9: VDRL results

Figure 10: Anticardiolipin IgG results
Figure 11: ACL IgG and anti-β2GP1 IgG box plots

Figure 12: Creatinine results
Fifty-five patients (91.7%) tested negative for VDRL, (Table 12 and Figure 9). Out of the 5 who tested positive, 4 (80%) also tested positive for TPHA. The mean anticardiolipin IgG titre was 107.4 U/mL (SD ± 62.4) with almost all 55 (91.7%) having a positive ACL result, (Figure 10). With regard to anti-beta-2-glycoprotein IgG, the median was 5 G units (IQR= 4.5, 6.5) with almost all 55 (91.7%) the patients exhibiting a negative result, (Figure 11).

The mean serum creatinine concentration was 98.2 µmol/L (SD ± 57.7), with a majority of patients having either normal results 35(58.3%) or elevated creatinine levels 20(33.3%); (Figure 12). The mean serum ALT concentration was 16.4 IU/L (SD ± 19.5), with almost all patients 58(96.7%) having a normal ALT levels, as indicated in Table 12.

Table 10: Summary of characteristics of patients demonstrating antiphospholipid antibodies

<table>
<thead>
<tr>
<th>Study No</th>
<th>21</th>
<th>37</th>
<th>39</th>
<th>45</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>39</td>
<td>28</td>
<td>28</td>
<td>42</td>
<td>55</td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td>Previous DVT</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Pregnant</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Abortion History</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>OCP use</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Platelet count</td>
<td>352</td>
<td>268</td>
<td>360</td>
<td>178</td>
<td>157</td>
</tr>
<tr>
<td>(150-400 x 10^9/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APTT (28.2 – 38.2 seconds)</td>
<td>34.5</td>
<td>42.5⁺</td>
<td>30</td>
<td>27.6</td>
<td>33.2</td>
</tr>
<tr>
<td>VDRL (Neg/Pos)</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>KCT (cut-off is 0.16)</td>
<td>-</td>
<td>1.0⁺</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TPHA (Neg/Pos)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Negative</td>
</tr>
<tr>
<td>ACL IgG titres</td>
<td>255.75</td>
<td>131.5</td>
<td>109.4</td>
<td>150.28</td>
<td>147.24</td>
</tr>
<tr>
<td>(cut-off is 48 U/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiβ2-GP1 IgG titres</td>
<td>29⁺</td>
<td>4.9</td>
<td>22⁺</td>
<td>31⁺</td>
<td>48⁺</td>
</tr>
<tr>
<td>(cut-off is 20 G units)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td>86</td>
<td>54</td>
<td>70</td>
<td>91</td>
<td>153</td>
</tr>
<tr>
<td>(40-110 µmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT (upto 40 IU/L)</td>
<td>3</td>
<td>5</td>
<td>25</td>
<td>8</td>
<td>20</td>
</tr>
</tbody>
</table>

* prolonged APTT
⁺ positive KCT
⁺⁺ positive for antiβ2GP1 antibodies
Table 11: Summary of characteristics of patients with thrombocytopenia

<table>
<thead>
<tr>
<th>Study No.</th>
<th>7</th>
<th>18</th>
<th>34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count (150-400 x 10^9/L)</td>
<td>97.3</td>
<td>66.8</td>
<td>95</td>
</tr>
<tr>
<td>APTT (28.3 – 38.3 sec)</td>
<td>17.3</td>
<td>24.6</td>
<td><strong>48.3</strong>*</td>
</tr>
<tr>
<td>VDRL (Neg/Pos)</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>KCT (cut-off is 0.16)</td>
<td>-</td>
<td>-</td>
<td><strong>0.14</strong></td>
</tr>
<tr>
<td>ACL IgG (cut-off is 48 U/mL)</td>
<td><strong>86.63†</strong></td>
<td>19.82</td>
<td><strong>78.6†</strong></td>
</tr>
<tr>
<td>Anti-β2GP1 (cut-off is 20 G units)</td>
<td>4.0</td>
<td>3.6</td>
<td>5.00</td>
</tr>
</tbody>
</table>

*prolonged APTT
†positive for ACL antibodies

Table 12: Bivariate analysis by gender

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male</th>
<th>Female</th>
<th>Test statistic</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>Ranksum test</td>
<td>0.013*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median(IQR)</td>
<td>52 (38.8,58)</td>
<td>32.5 (24.8,43.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cigarette Smoking</td>
<td>Fisher’s exact test</td>
<td>&lt; 0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>4 (50%)</td>
<td>1 (1.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None smokers</td>
<td>4 (50%)</td>
<td>51 (98.1%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant Result (p <0.05)
Non-significant results not shown.

As shown in Table 15, only two characteristics differed significantly among the males and females. The male patients were older than the females, median age (IQR) 52 (38.8, 58) vs. 32.5 (24.8, 43.5), p value <0.05; and there was a significant difference (p value <0.01) with respect to their smoking history, with half of the males being smokers. Majority of the female patients 51(98.1%), were non-smokers.
Correlations were done using Pearson correlations coefficients and statistical significance determined at 5% level. A significant positive correlation existed between APTT and ACL (r=0.39; p value <0.05) and between ACL and β2GP1 (r=0.30; p value <0.05), and a significant negative correlation between platelet count and haemoglobin level(r= -0.29; p value <0.05).
DISCUSSION
This study evaluated 60 patients, most of who were females (86.7%). The male participants were significantly older than the females, with a median age of 52 years and 32.5 years respectively. In a local study by Magada et al on patients with DVT, 94% of the study participants were females, and the mean age of the participants was 36.4 (± 10.0) years (69). DVT predominantly affects men and women older than 45 years (70). However, a higher incidence is seen in females of reproductive age, due to association with specific risk factors, mainly pregnancy, the postpartum period and use of oral contraceptive pills (70,71). These risk factors could account for the higher incidence of DVT among the females in evaluated in this study.

One of the objectives of this study was to describe the clinical characteristics of patients with DVT, some of which are independent risk factors for development of venous thrombosis. Cigarette smoking was established in 8.3% of the patients, while hypertension was identified in 15%. One-fifth of the patients had a previous history of DVT. Prior DVT increases the risk of another DVT by upto 30% (72). While antiphospholipid antibodies have been associated with recurrent venous thrombosis, a suboptimal duration of initial anticoagulant therapy after a first episode of DVT also predisposes to recurrence (73). A limitation of this study was that it did not determine the duration of previous anticoagulant therapy for those with a recurrent DVT.

Most of the patients evaluated in this study did not have a history of surgery within the three months prior to development of DVT. There is a significant association between surgery and DVT (74), but the reduced incidence of post-operative DVT seen in this study may be due to prophylactic measures to prevent occurrence of venous thrombosis after surgery in at-risk patients.

The most predominant anatomical site of thrombosis was the lower limb, with pain and/or swelling of the affected limb constituting the most frequent presenting symptoms. However, one patient had upper limb thrombosis, one had thrombosis of the dural venous sinus and another had thrombosis involving the jugular vein. Venous thrombosis can potentially involve any section of the venous system, with common manifestations being DVT of the lower limbs and pulmonary embolism. Uncommon manifestations involve thrombosis at atypical sites, like the cerebral sinus
and the upper limbs (75). These different manifestations are seen due to different inherited and acquired thrombophilic states, for example the presence of antiphospholipid antibodies.

Few female patients had past history of foetal loss, while one had a history of pre-eclampsia in a previous pregnancy. Foetal loss and pre-eclampsia are clinical consequences of APA (63), and venous thrombosis in a patient with a similar obstetric history should raise the clinical suspicion of presence of APA. Only one patient evaluated in this study had skin manifestations, in this case, a malar rash, which was suggestive of an autoimmune condition.

On general examination, some of the patients evaluated in this study had pallor of the mucus membranes, while only three were icteric. Pallor and jaundice are suggestive of haemolytic anaemia, another association of APA. On further evaluation of the patients with clinical jaundice, only one was anaemic, with an increased reticulocyte count. All the three patients had a normal liver function as evidenced by a normal ALT enzyme level. However, one limitation of this study is that serum bilirubin assays were not performed to confirm the presence of jaundice.

Slightly more than half of the patients recruited into this study were anaemic, with normocytic normochromic anaemia being the most predominant type, of anaemia, followed by microcytic hypochromic anaemia. Either a normal or reduced reticulocyte response was noted in most of the patients.

A few patients had thrombocytopaenia, although this may have been caused by other factors, as it was not related to the presence of antiphospholipid antibodies. The ESR was elevated in most of the patients, but is a non-specific marker of an ongoing inflammatory process.

The APTT was prolonged in a third of the patients evaluated in this study. The local laboratory reference range used for interpretation of APTT results is 28.3 – 38.3 seconds, and values exceeding the upper limit of this range were considered prolonged. The causes of the prolonged APTT in these patients’ samples may have been due to the presence of heparin, LA, or a coagulation factor deficiency. Thrombin test would have been useful to confirm for the presence of heparin in these samples, as it would cause prolongation of test. However, this was another limitation of this study, as the thrombin test was not performed. The viper venom test reagent used in this study contained a heparin neutralizer, but unfractionated heparin in excess of therapeutic levels may not be completely neutralized by these neutralizers, and this would
interfere with the LA results. Few patients had a shortened APTT, which may have been due to difficulties during sample collection, with partial clotting of the specimens before analysis.

LA was positive in two patients by the KCT test, while the viper venom test was negative for all. However, one of the patients with a positive KCT test had syphilis, which may have interfered with the test results to give a false positive LA. The incidence of LA positivity in this study is low (1.6%), compared to those from the West, and is similar to the incidence found in the general healthy population (1%) (76). Simioni et al, found five LA positive patients among 59 unselected patients with DVT (8.5%) (77), while Ginsberg et al, found nine LA positive patients out of 65 (14%) (33).

VDRL test was negative in most patients. Out of the five with a positive VDRL, only one was a false-positive result, as evidenced by a negative TPHA. The other 4 were confirmed to have syphilitic infection by the TPHA test. Almost all the patients recruited into this study were positive for IgG anticardiolipin antibodies. Several factors may explain this; firstly, the ACL antibodies were measured in the blood after the thrombosis. An assumption made by this study was that the APA measured after the thrombotic event reflects the antibody status before the event. Transiently elevated ACL antibody levels are found in many patients after a venous thrombosis, suggesting that the antibodies may be a result, rather than a cause of thrombosis in these patients (78).

Secondly, analytic issues in the ACL assays may also contribute to false-positive results. Even when a β2-dependent ACL assay is used, the recommended dilutions during testing enable other endogenous proteins in the serum, apart from β2-GP1, to be present in a sufficiently high concentration that allows binding in a non-β2-GP1-dependent fashion, thus reducing the specificity of the test. Thirdly, the cut-off values used in this study for interpretation of the ACL assay were those recommended by the manufacturer of the reagent used. It is recommended that local cut-off values be used whenever possible (6); however, local reference ranges for ACL have not been established for this population.

Five patients (8.3%) were positive for anti-β2-glycoprotein IgG antibodies. This finding is similar to that of a study by Zanon et al, who found a prevalence of 8.4% of anti-β2GP1 antibodies in patients with acute thromboembolic events (79).
The serum creatinine was elevated in one-third of the patients while most patients had normal serum ALT levels. There was no significant correlation between these biochemical markers and the presence of antiphospholipid antibodies; therefore the elevated serum creatinine may have been due to other co-morbidities.

CONCLUSION
Antiphospholipid antibodies (LA and anti-β2GP1 IgG antibodies) are present in a small proportion of patients seen at KNH with venous thrombosis. The positive tests for anti-β2GP1 were more common than for LA. ACL IgG antibodies may be induced by numerous factors and may not be related to thrombosis. As in other centers, DVT in our setting is more common in females of reproductive age, while affected males tend to be older, with the lower limb being the most common anatomical site involved. Pathological antiphospholipid antibodies and their associated clinical and laboratory manifestations are uncommon in patients seen at KNH with VTE. This study may be used as an algorithm for investigating patients with venous thrombosis, as the protocol incorporates both the clinical and laboratory aspects that are important to aid in identification of the cause or underlying risk factor for the thrombotic episode.

RECOMMENDATIONS
Screening for antiphospholipid antibodies in patients with venous thrombosis at KNH should be limited to those in whom there is a high index of suspicion for the presence of these antibodies. These include relatively young patients with unprovoked thrombosis or recurrent thrombosis, patients with a history of recurrent pregnancy loss, or those with the other clinical and/or laboratory features associated with the antiphospholipid syndrome. An LA assay together with an anti-β2-GP1 assay may be more useful than an ACL antibody assay, unless local cut-off values for ACL are established. Other haematological and biochemical laboratory investigations for the associated complications of the antiphospholipid antibodies should also be performed in these patients.
STUDY LIMITATIONS

- Presence of acute phase proteins has potential to mask the lupus anticoagulant, or to provide false positives

- Thrombin test was not performed as part of this study. This would have been useful to rule out the presence of heparin in samples that had a prolonged APTT.

- Diagnosis of the antiphospholipid syndrome cannot be established on the basis of a single test result. The current diagnostic criteria require that medium or high titre antiphospholipid antibodies be present on 2 or more occasions at least 12 weeks apart. Repeat evaluation of a positive result to confirm the presence of antiphospholipid antibodies was not performed in this study.
REFERENCES


APPENDICES

APPENDIX 1: TIMELINE AND BUDGET

Timeline

<table>
<thead>
<tr>
<th>MONTH</th>
<th>ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>JANUARY 2010 – JUNE 2010</td>
<td>Proposal development</td>
</tr>
<tr>
<td>JUNE 2010 - AUGUST 2010</td>
<td>Presentation to the department</td>
</tr>
<tr>
<td>AUGUST 2010 – OCTOBER 2010</td>
<td>ERC approval</td>
</tr>
<tr>
<td>JANUARY 2011 – NOVEMBER 2011</td>
<td>Data collection</td>
</tr>
<tr>
<td>JANUARY – FEBRUARY 2012</td>
<td>Data analysis</td>
</tr>
<tr>
<td>MAY 2012</td>
<td>Presentation of results</td>
</tr>
</tbody>
</table>

Budget

<table>
<thead>
<tr>
<th>ITEM</th>
<th>AMOUNT IN KSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERC</td>
<td>1, 000</td>
</tr>
<tr>
<td>STATIONERY, PRINTING AND BINDING</td>
<td>15, 000</td>
</tr>
<tr>
<td>STATISTICIAN</td>
<td>10, 000</td>
</tr>
<tr>
<td>VACUTAINERS, SYRINGES, NEEDLES, SWABS, CRYOVIALS</td>
<td>10, 000</td>
</tr>
<tr>
<td>COAGULATION KITS</td>
<td>150, 000</td>
</tr>
<tr>
<td>ACL &amp; Anti-β2 GP1 KITS</td>
<td>115, 000</td>
</tr>
<tr>
<td>LABORATORY TESTS</td>
<td>84, 000</td>
</tr>
<tr>
<td>VDRL KIT</td>
<td>2, 500</td>
</tr>
<tr>
<td>TPHA KIT</td>
<td>2, 500</td>
</tr>
<tr>
<td>RESEARCH ASSISTANT</td>
<td>10, 000</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>400, 000</strong></td>
</tr>
</tbody>
</table>
APPENDIX 2: FLOW DIAGRAM

Patient with confirmed thrombosis, at A&E Department, ward or clinic

Obtain informed consent
Administration of questionnaire
Physical examination

Specimen collection

Haematology laboratory for analysis
(Appendix 5)

Biochemistry laboratory for analysis
(Appendix 5)

Immunology laboratory for analysis
(Appendix 5)
APPENDIX 3: STUDY INFORMATION AND CONSENT FORM
INFORMATION SHEET

My name is Dr. Barasa, and I am a post-graduate student of the University of Nairobi. I am conducting a study to determine the presence of antiphospholipid antibodies in patients with venous thrombosis at Kenyatta National Hospital.

Antibodies are proteins in the blood that the body produces to fight off foreign agents. Sometimes the immune system does not function properly and makes antibodies against normal organs and tissues in the body. These are called autoantibodies. Antiphospholipid antibodies are autoantibodies that react with proteins in the blood that are bound to phospholipid, a type of fat that is present on blood cells and the lining of blood vessels. Why patients develop these autoantibodies is not yet completely understood. Antibodies to the phospholipids increase the risk of developing blood clots in the veins or arteries, and an increased risk of miscarriage or stillbirth among pregnant women. Clots forming in the veins most frequently affect the legs.

These antibodies were first discovered in people who have lupus, but it is not necessary to have lupus to have these antibodies. It is not yet understood why a person’s immune system begins to manufacture these antibodies. They are measured using blood tests.

If a person has had a thrombotic or clotting complication and has antiphospholipid antibodies, treatment consists of “thinning” the blood to prevent future clots. This is usually done with warfarin tablets. If a person has antiphospholipid antibodies but has never had a thrombotic complication, treatment is not currently recommended. However, it is good to reduce other risk factors for clotting, such as being overweight or smoking.

The interview of patients and physical examination is to allow for proper selection of study participants will be done. Selected patients will be required to sign a consent form and then fill a questionnaire regarding patient characteristics. Venous blood will then be collected and taken to the laboratory for analysis and measurement of the antiphospholipid antibodies.

Anticipated risks of the study are minor, and include a little pain at the injection site, and haematoma formation, which will subside within a few days.

The benefits of the study include detection of the antiphospholipid antibodies, which will improve management of patients with venous thrombosis. The laboratory results will be communicated to the attending doctor immediately for patient management.

Study approval has been obtained from the Kenyatta National Hospital Ethical and Research Committee. Test results will be sent back to you through your doctor.

Your decision to participate in the study is voluntary. No compensation will be offered to those who choose to participate. Your identity will be kept strictly confidential throughout the study as well as during the publication of the study findings. Your decision to participate or not to
participate in this study will not affect the quality of your care. You can also withdraw from the study at any time.

Kindly fill the consent form below:

CONSENT FORM:

I Mr./Mrs./Miss ……………………………………………………………………………………, agree to enroll myself into this study, being fully aware of its purpose and my rights as explained to me by Dr. Barasa. I consent to the investigations.

Signature:

a) Participant ……………………… Date …………………

b) Witness (P. I) Name……………………… Date ………………..

During this study, if you have any concerns, contact me, Dr. Barasa K. A. (Principal Investigator), on telephone number 0722-849024.

If you have further questions about the study you can contact Dr. Paul N. Mungai, Chairperson of the Ethics and Research Committee, on telephone number 02726300 (extension 44102).
APPENDIX 4: QUESTIONNAIRE

1.0 SOCIODEMOGRAPHIC DATA

1.1 Study No: 

1.2 Interviewer names:

1.3 Patient’s names:

1.4 Hospital number: 

1.5 Age (years): 

1.6 Sex:

1.6.1 Male: 

1.6.2 Female: 

1.7 Occupation:

1.7.1 Housewife: 

1.7.2 Farmer: 

1.7.3 Artisan: 

1.7.4 Professional (specify): 

1.7.5 Unemployed: 

2.0 MEDICAL HISTORY

2.1 Presenting complaint:

2.1.1 Pain: 

2.1.2 Swelling: 

2.1.3 Other (specify):
2.2 Past medical history:

2.2.1 DVT: [ ] 2.2.2 Hypertension: [ ]

2.2.3 Recent surgery (abdominal or orthopaedic): [ ]

2.2.4 Not significant: [ ]

2.3 Family history of DVT:

2.3.1 Yes: [ ] 2.3.2 No: [ ]

2.4 History of cigarette smoking:

2.4.1 Yes: [ ] 2.4.2 No: [ ]

2.5 Obstetric & Gynaecological history:

2.5.1 Date of last menstrual period:

2.5.2 Have you ever been pregnant?:

2.5.2.1 Yes: [ ] 2.5.2.2 No: [ ]

2.5.3 If yes, how many live births?: [ ]

2.5.4 How many still births?: [ ]

2.5.5 How many abortions?: [ ]

2.5.6 History of high blood pressure during pregnancy:

2.5.6.1 Yes: [ ] 2.5.6.2 No: [ ]
2.5.7 History of unusual or excessive body swelling during pregnancy:

2.5.7.1 Yes: [ ]  2.5.7.2 No: [ ]

2.5.8 When was your last delivery?:

2.5.9 Contraception:

2.5.9.1 Are you using any family planning method?:

2.5.9.1.1 Yes: [ ]  2.5.9.1.2 No: [ ]

2.5.9.2 If yes, specify:

2.5.9.2.1 Pills: [ ]  2.5.9.2.2 Injections: [ ]

2.5.9.2.3 Implants: [ ]  2.5.9.2.4 IUCDs: [ ]

2.5.9.2.5 Condoms: [ ]  2.5.9.2.6 Spermicides: [ ]

2.5.7.2.7 Bilateral tubal ligation: [ ]

2.5.9.3 If stopped, why?

3.0 SALIENT EXAMINATION FINDINGS

3.1 General examination:

3.1.1 Pulse rate: [ ]  3.1.2 Temperature: [ ]

3.1.3 Respiratory rate: [ ]

3.1.4 Blood pressure: Systolic: [ ]  Diastolic: [ ]

3.1.5 Pallor:

3.1.5.1 Present: [ ]  3.1.5.2 Absent: [ ]

3.1.6 Jaundice:

3.1.6.1 Present: [ ]  3.1.6.2 Absent: [ ]
3.1.7 Oedema:

3.1.7.1 Present: □  3.1.7.2 Absent: □

3.2 CNS:

3.3 Respiratory system:

3.4 CVS:

3.5 Gastrointestinal system:

3.6 Skin:

3.7 Site of venous thrombosis:

3.7.1 Lower limb: □  3.7.2 Upper limb: □

3.7.3 Other (specify): □

4.0 LABORATORY RESULTS

4.1 Rbc count: □  4.2 Wbc count: □  4.3 Platelet count: □

4.4 Haemoglobin: □  4.6 Reticulocyte count: □

4.7 Peripheral blood film:

<table>
<thead>
<tr>
<th>Film</th>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rbc</td>
<td></td>
</tr>
<tr>
<td>Wbc</td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td></td>
</tr>
<tr>
<td>Comment</td>
<td></td>
</tr>
</tbody>
</table>
4.8 Coagulation tests:

4.8.1 PT:  
4.8.2 APTT:  
4.8.5KCT:  
4.8.4 DRVVT:  

4.9 VDRL:  

4.10 TPHA:  

4.11 Anticardiolipin antibodies:

4.11.1 ACL IgG:  

4.12 Anti-β2-Glycoprotein 1 antibodies:

4.12.1 β2GP1 IgG:  

4.13 Creatinine:  
4.14 ALT:  

57
APPENDIX 5: TEST PRINCIPLES AND PROCEDURES

- Prothrombin Time
- Activated Partial Thromboplastin Time
- Direct Russell’s Viper Venom Test (LupoTek DetecTin/CorrecTin VL)
- Lupus anticoagulant plasma control
- Kaolin Clotting Time
- Rapid Plasma Reagin Test (VDRL)
- Treponema Pallidum Haemagglutination Assay
- Anticardiolipin ELISA
- Anti-beta2-glycoprotein 1 ELISA
- Validation of the ELISA assays
INTENDED USE

LupoTek DetecTin VL and CorrecTin VL test kits are qualitative tests intended for the detection of lupus anticoagulant in plasma by the dilute Russell’s viper venom method in professional clinical laboratories.

SUMMARY

Lupus anticoagulants are anti phospholipid autoantibodies targeted against components of the clotting system. They are associated with autoimmune diseases (1), recurrent fetal loss (2) and unexplained thrombosis, both venous and arterial (3). Circulating anticoagulants are usually detected by the presence of a prolonged clotting time in whole blood coagulation tests (4) which does not correct on mixing patient plasma (1) with normal plasma. These tests are not specific and cannot distinguish between a factor deficiency, heparin contamination and a true anti phospholipid antibody without further studies.

The hallmark characteristics of lupus anticoagulants is their phospholipid dependency that is, the prolonged clotting time seen with low phospholipid reagents is corrected with high phospholipid reagents.

The Dilute Russell’s Viper Venom Time (DRVVT) is a simple one stage clotting test which can be used with carefully matched low and high phospholipid reagents to detect Lupus Anticoagulants with minimal interference from other types of circulating anticoagulants (5).

PRINCIPLE

LupoTek DetecTin VL and LupoTek CorrecTin VL use Vipera lebetina venom rather than Vipera russelli (Russell’s) Viper venom. Vipera lebetina venom, like Russell’s viper venom, will directly activate Factor X without requiring Factor V. The activated Factor X in turn, activates the inner coagulation cascade, resulting in procoagulant clot formation.

LupoTek DetecTin VL uses a low phospholipid reagent, designed as the screening reagent to detect a prolongation of the clotting time. LupoTek CorrecTin VL is the high phospholipid reagent that neutralises the LA and corrects the clotting time to normal, confirming the presence of a Lupus Anticoagulant.

REAGENTS

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY.

LupoTek DetecTin VL. Catalog number 85-202, 10 x 2 ml vials.

Ingredients: Vipera lebetina venom, low concentration of phospholipids, anti-heparin agents, calcium ions, buffers, stabilizers and a blue dye. Sodium azide (0.05%) is used as a preservative.

Preparation for Use: Reconstitute the vial with 2 ml distilled water. Mix well, do not shake and leave at room temperature for 10 minutes before use.

LupoTek CorrecTin VL. Catalog number 85-201, 10 x 1 ml vials.

Ingredients: Vipera lebetina venom, high concentration of phospholipids, anti-heparin agents, calcium ions, buffers, stabilizers and a blue dye. Sodium azide (0.05%) is used as a preservative.

Preparation for Use: Reconstitute the vial with 1 ml distilled water. Mix well, do not shake and leave at room temperature for 10 minutes before use.

Storage and Stability

The phospholipid reagents are stable until the expiration date printed on the vials. After reconstitution, the reagents are stable for 24 hours at 2-8°C or 8 hours at room temperature.

WARNING: SODIUM AZIDE. Both LupoTek DetecTin VL and LupoTek CorrecTin VL contain sodium azide, which can form highly explosive metal azides. Handle to load or dispose of in a flammable Waste. Any such material should be added into a sink with large volumes of water to minimize such a risk.

SPECIMEN HANDLING

Specimen Collection and Preparation

Plasma is obtained from whole blood anticoagulated with 1 part 3.8% sodium citrate to 9 parts whole blood. Process the collected whole blood and handle the plasma according to the CLSI guidelines HE1-A5 (11).

To achieve an optimum sample, the plasma should contain less than 10 x 10^9 platelets (6). Double centrifugation or filtration through a 0.22 micron filter type filter before testing is recommended (7). This is particularly important if plasma is to be frozen before testing.

Storage and Stability

Store plasma according to the CLSI guidelines HE1-A5 noted above. It is strongly recommended that plasma should be double centrifuged or filtered before freezing as outlined above. Any residual platelets will rupture on freezing and thawing and can neutralize a lupus anticoagulant by the removal of phospholipids from the damaged membranes. Thaw rapidly at 37°C before use.

Materials Required but not Provided

Pooled Normal Plasma

TEST PROCEDURE

Please contact 22 Diagnostics for validated applications for LupoTek DetecTin VL and LupoTek CorrecTin VL in individual models of coagulation analyzers.

Quality Control

Quality control of coagulation tests involves multiple components. Each laboratory should establish a quality control program that includes both normal and abnormal control plasma. 22 Diagnostics normal control plasma PlasmaCova N and LA positive control plasma PlasmaCova LA are suitable controls. Quality control testing with the LupoTek DetecTin VL and CorrecTin VL reagents should be carried out at the same time.

RESULTS

For optimal results, testing using LupoTek DetecTin VL and CorrecTin VL should be done at the same time. If the LupoTek DetecTin VL time is in the normal range no further testing is necessary. If the clotting time with the DetecTin VL reagent is above the upper limit of the normal release range further testing with the CorrecTin VL reagent is justified.

The clotting times obtained with the DetecTin VL and CorrecTin VL reagents are used to express results in a ratio format, as outlined below. The use of a normalized ratio (NR) in which the patient’s clotting time is divided by the clotting time of pooled normal plasma, minimizes any impact of differences in the normal ranges due to lot to lot reagent variability.

\[ \text{NR} = \frac{\text{DetecTin VL Time of Patient}}{\text{DetecTin VL Time of Pooled Normal Plasma}} \]

INTRODUCTION OF RESULTS

In an internal study of 122 patient samples, a ROC analysis suggested a cutoff of 1.30 for the Normalised Ratio. Samples above this level were considered as LA positive. The example cutoff value described above is not absolute and each laboratory is encouraged to establish its own specific ratio for the normal reference and patient population.

Patient plasma yielding below the reagent is should be repeated and correlated with the clinical findings if indicated. Patient plasma that have long clotting times with both LupoTek DetecTin VL and CorrecTin VL, irrespective of the ratio, may have some other defects such as a Factor deficiency or be on oral anticoagulant therapy.

Mixing Studies

Mixing studies are recommended to differentiate between factor deficiency states and diluting inhibitors (8). A failure to correct on mixing with normal plasma (11) is more indicative of an inhibitor, while correlation is more suggestive of a factor deficiency state. Mixing studies should be carried out under carefully controlled conditions using well characterized pooled normal plasma (8, 9).

63
REFERENCE VALUES

Ranges for normal plasma with LipoTec DetectIn VL are in the 0.01 to 0.44 second range when assessed on the STA Compact.

Reference ranges for normal plasma with LipoTec CorrectIn VL are in the 0.2 to 1.6 second range when assessed on the STA Compact.

These results are illustrative only. Each laboratory must establish its own normal reference ranges for the test procedures and instruments used in the laboratory.

LIMITATIONS

All testing for lupus anticoagulants requires plasma samples that are platelet-poor (<10^5/μL) or preferably platelet-free. Samples not double centrifuged or filtered should be tested before freezing. Both DetectIn VL and CorrectIn VL tests should be performed on either a fresh sample or a frozen sample.

Factor deficiencies, anticoagulants, and other antibody-type inhibitors can lengthen the clotting times of both LipoTec DetectIn VL and CorrectIn VL. Mixing studies are recommended to differentiate between these and clinical conditions (6, 8, 9).

DetectIn VL and CorrectIn VL incorporate a heparin neutralization agent that is effective to 0.6 U/mL of unfractionated heparin. Testing of samples with higher heparin levels is not recommended. The effects of low molecular weight heparins and direct thrombin inhibitors have not been determined.

The performance characteristics of DetectIn VL and CorrectIn VL have not been evaluated in pediatric populations.

PERFORMANCE CHARACTERISTICS

Precaution

CLSI EP-2-A2 (10) precision estimates of LipoTec DetectIn VL and CorrectIn VL on the STA Compact, as %CV of the clotting time, were:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Plasma</th>
<th>Repeatability</th>
<th>Total (Within Instrument) Imprecision</th>
</tr>
</thead>
<tbody>
<tr>
<td>DetectIn VL</td>
<td>Normal</td>
<td>3.8%</td>
<td>8.5%</td>
</tr>
<tr>
<td></td>
<td>Abnormal</td>
<td>4.4%</td>
<td>5.7%</td>
</tr>
<tr>
<td>CorrectIn VL</td>
<td>Normal</td>
<td>4.1%</td>
<td>5.7%</td>
</tr>
<tr>
<td></td>
<td>Abnormal</td>
<td>3.8%</td>
<td>2.6%</td>
</tr>
</tbody>
</table>

Interferences

Interference studies of LipoTec DetectIn VL and CorrectIn VL were determined on a standard STA Compact analyzer. Interference was splined into pooled normal plasma and the maximum concentration tolerated in the assay was defined as the highest concentration of interferent wherein no consistent drift relative to the recovered value of the base PHF clotting time was less than 15%. The maximum concentrations were:

<table>
<thead>
<tr>
<th>Interferent class</th>
<th>Maximum Concentration (PLS/μL)</th>
<th>Maximum Tolerated Concentration (PLS/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysate</td>
<td>300 mg/dL hemoglobin</td>
<td>300 mg/dL hemoglobin</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>30 mg/dL</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Lithium</td>
<td>2,000 mg/dL</td>
<td>2,000 mg/dL Lithium</td>
</tr>
<tr>
<td>Heparin</td>
<td>2.0 U/mL unfractionated heparin</td>
<td>0.5 U/mL unfractionated heparin</td>
</tr>
</tbody>
</table>

Method Comparison

One hundred twenty-two patient samples were analyzed on the STA Compact. In an internal study to determine the cutoff for the LipoTec DetectIn VL / CorrectIn VL normalized ratio, there were a total of another one-hundred fifty-five patient samples that were evaluated in three laboratories using LipoTec DetectIn VL and CorrectIn VL, and a Dacron SSA V200 Screen and Confirm reagents or STA Compact analyzers. All samples were a mix of known LA patients and other miscellaneous clinical conditions. Positive percent agreement and negative percent agreement were calculated using the normalized ratio of the test and predicate devices according to the FDA guidance document 1630, "Statistical Guidance on Reporting Results from Studies Evaluating Diagnostic Tests". The results were:

<table>
<thead>
<tr>
<th>Dacron SSA V200 SSA Screen/confirm</th>
<th>Positive Percent Agreement</th>
<th>Negative Percent Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>98%</td>
<td>98%</td>
</tr>
</tbody>
</table>

References:

PlasmaCon LA
ABNORMAL COAGULATION CONTROL PLASMA FOR LUPUS ANTICOAGULANTS

INTENDED USE

PlasmaCon LA is intended for use as an LA positive, abnormal-quality control plasma to monitor the performance of diagnostic assays, performed in professional clinical laboratories, for the presence of lupus anticoagulant in clotted plasma.

SUMMARY

Modern quality control practices require that test procedures be accompanied with test materials of known performance for, or concentration of, those constituents to be assayed. PlasmaCon LA is a lyophilized Lupus Anticoagulant (LA) positive plasma suitable for use as a quality control plasma for in vitro diagnostic assays in the clinical coagulation laboratory sensitive for the presence of LA.

PRINCIPLE OF THE PROCEDURE

PlasmaCon LA can be used in all tests in the same manner as any clotted plasma sample. PlasmaCon LA can be used to monitor testing variables in laboratory QC systems (e.g., instrumentation, reagents and techniques) for assays sensitive to the presence of LA.

REAGENT

FOR IN-VITRO DIAGNOSTIC USE ONLY
Catalog No: 150-200. 10 x 0.5 mL, vials
Catalog No: 150-201. 10 x 1.0 mL, vials

PlasmaCon LA is prepared from clotted plasma(s) from known LA donors and normal donors. The plasma control contains buffer and stabilizers and is lyophilized. PlasmaCon LA does not contain any preservatives.

WARNING: Potential Hazards: PlasmaCon LA has been found negative for Hepatitis B Antigen (HBsAg) and antibodies to HCV and HIV by FDA licensed tests. However, the control should be handled with the same precautions as those observed when handling potentially infectious patient plasma.

Preparation for Use: PlasmaCon LA should be reconstituted with the volume of deionized or distilled water indicated on the vial label. Stir gently, do not shake. Allow the plasma to stand for 15 minutes at room temperature before use.

Storage and Stability: Unopened PlasmaCon LA is stable until the expiration date shown on the label when stored at 2-8°C. Reconstituted plasma is stable (e.g., less than a 10% shift in the baseline values) for 8 hours when stored capped at 2-8°C or 4 hours at room temperature (23-25°C).

INSTRUMENTS

PlasmaCon LA may be used as a control when performing LA assays on any mechanical or photo-optical coagulation instrument in conjunction with suitable reagents.

PROCEDURE

PlasmaCon LA is treated in the same manner as the unknown specimen in accordance with the instructions outlined in the procedure used in the laboratory.

LIMITATIONS

PlasmaCon LA, like any control plasma, is subject to the limitations of the assay system. Quality control of coagulation assays involves multiple components, including reagents, pipets, distilled water, buffers and instruments. Each laboratory should establish a Quality Control program that includes both normal and abnormal control plasmas. If any of the controls are outside the reference range established by the laboratory, then the assay should be considered invalid, no patient results should be reported, and the assay and controls investigated to determine and correct the source of the problem(s).

EXPECTED VALUES

The results obtained with PlasmaCon LA depend on several factors including instrumentation, types of reagents and laboratory-to-laboratory variation. Results for PlasmaCon LA should fall above the normal reference ranges established by the laboratory for the LA sensitive tests of interest.

Laboratories should establish the mean values and expected control ranges for their particular laboratory's instrument-reagent system for each new lot of control, on an instrument service, or a change in test procedure.

PERFORMANCE CHARACTERISTICS

Precision: Precision estimates of PlasmaCon LA with multiple lots of 2 Diagnosticon®/Logitek Dextroan VL, and Logitek® Controls V/LA (LA confirmatory reagent) were determined in a two-run per day, twenty day study on a Diagostics STA Compact analyzer as described in the CLSI guidelines EP5-A3b. "Evaluation of Precision Performance of Quantitative Measurement Methods & Appraisals Guidelines, 2nd Edition, 2004. The mean clotting times and average precision results as %CV were:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Mean clot time, seconds</th>
<th>Repeatability</th>
<th>Total Imprecision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextroan VL</td>
<td>33.7</td>
<td>1.4%</td>
<td>3.5%</td>
</tr>
<tr>
<td>Controls V/LA</td>
<td>39.7</td>
<td>2.8%</td>
<td>9.8%</td>
</tr>
</tbody>
</table>

These performance results should be considered illustrative only. Each laboratory should establish their own control ranges and Quality Control program for their reagents and instruments according to the appropriate Clinical Laboratory Improvement Amendment (CLIA) regulations and Clinical Laboratory Standards Institute (CLSI) guidelines.
LupoTek KCT

For the detection of circulating anti-coagulants

FOR INVESTIGATIONAL USE ONLY

INTENDED USE
LupoTek KCT is intended for use in the detection of circulating anticoagulants.

SUMMARY
The Kaolin Clotting Time (KCT)1 was first used to study lupus anticoagulants (LA) in 19782. The KCT test has been reported to be the most sensitive test for the detection of circulating anticoagulants such as LA1. Platelet contamination can cause problems in kaolin-based APTT reagents and KCT tests3. The APTT and KCT tests detect all classes of inhibitors, including those directed against factor VIII and contact activation as well as heparin. However, in many clinical situations (studies of LA in pregnancy), it is often desirable to retain a broad screening method such as the LupoTek KCT for anticoagulants. Use of the Rosner Index (R1)4 to report KCT results increases the specificity of the test for LA. The LupoTek KCT is suitable for use both as a manual technique and in coagulation analyzers.

PRINCIPLE
The LupoTek KCT is a modified activated partial thromboplastin time (APTT) reagent without any added phospholipids. Phospholipids are believed to be a major target of anti-phospholipid antibodies that are often associated with lupus anticoagulants. When these antibodies are present, the test is prolonged.

REAGENTS
WARNING: FOR INVESTIGATIONAL USE ONLY.
1. KCT Reagent
   Ingredients: Each vial of LupoTek KCT contains 5 mL of a low turbidity, slow-settling kaolin specifically intended for automated KCT tests.
   Preparation for Use: LupoTek KCT is provided as a liquid suspension, which can settle upon storage. Ensure the reagent is completely re-suspended and thoroughly mixed before use.
   Storage and Stability: The reagent should be stored at 2-8°C and is stable until the date printed on the vial.

2. 0.025M Calcium Chloride Reagent
   Ingredients: Each vial contains 5 mL 0.025M Calcium Chloride reagent stabilized with 0.02% Sodium Azide.
   Preparation for Use: The reagent is packaged ready for use.
   Storage and Stability: The reagent should be stored at 2-8°C and is stable until the date printed on the label.

SPECIMEN COLLECTION
Specimen Collection and Handling
NOTE: After initial whole blood collection, during testing all test tubes, syringes and pipettes should be plastic.
Specimen: Plasma obtained from whole blood anti-coagulated with 0.1 M sodium citrate.
Specimen Preparation: Centrifuge the whole blood samples at 1500 x g for 15 minutes (NCCLS H21-A2,1991). Immediately separate the plasma from the red blood cells if necessary using a plastic pipette and place it in a plastic test tube at 2-8°C until assayed. Perform the test within 4 hours.

Storage and Stability: Before and during testing, the plasma sample should be maintained in the plastic tubes at 2-8°C to ensure stability of the factors. If testing is delayed for more than 2 hours, the plasma may be stored at -20°C for two weeks or at -70°C for up to one month. Frozen samples should be thawed rapidly at 37°C before testing.

Materials Provided: Materials needed for LupoTek KCT assays are provided in the following packaging configuration.
LupoTek KCT
   KCT Reagent, 5 x 5 mL vials
   0.025M Calcium Chloride Reagent, 5 x 5 mL vials

Materials and Equipment Required but not Provided:
Coagulation Instrument or 37°C water bath and timer
Reaction Cups or plastic test tubes
Pipettes to deliver 0.5 and 0.1 mL
Concetration Distilled or deionized water
Control Plasmas: PlasmaCon N
PlasmaCon L-2

PROCEDURE
1. CLOTTING TEST:
A simplified LupoTek KCT technique identical to that for an Activated Partial Thromboplastin Time (APTT) is now recommended for automation. Any instrument protocol for performing an APTT can be used for determining the Kaolin Clotting Time by substituting LupoTek KCT for the APTT reagent.
This method is to be used as a guideline and should be adapted to suit individual instruments.
Technical assistance can be obtained from R2 Diagnostics.
   a) Pipette 100 µL of test plasma into a plastic test tube.
   b) Add 100 µL LupoTek KCT reagent and incubate for 3-5 minutes at 37°C.
   c) Add 100 µL 0.025M calcium chloride.
   d) Time and record clotting endpoint (in seconds).

2. MIXING TESTS:
Mixing tests are recommended for use with the LupoTek KCT reagent in order to reduce the effect of non-LA related clotting abnormalities. To make LupoTek KCT more specific for circulating inhibitors the test should be carried out on mixtures of patient and normal plasma2. Both 1:1 and 1:4 (patient: normal) proportions have been recommended for screening purposes2. R2 recommends the 1:1 mix for most testing purposes.
Normal plasma pools used for the mixing studies should be a carefully prepared pool of normal donors. The donor plasma should be prepared in a similar fashion to the patient samples. All donor plasma should be filtered to remove platelet fragments prior to being added to the pool. The normal pool should be well
characterized to ensure at least 75% activity of all coagulation factors. The normal pool may be aliquoted and stored at < - 50°C to provide some measure of standardization.

To prepare samples, mix 50 µL patient plasma with 50 µL normal plasma for a 50:50 (1:1) or 20 µL patient plasma with 80 µL normal plasma for a 20:80 (1:4) mix and carry out the KCT with the resulting 100 µL of test plasma exactly as described for the clotting test above. Larger volumes can be prepared for duplicate testing.

QUALITY CONTROL
The KCT of normal quality control plasmas is largely dependent on the level of platelet contamination. For adequate sensitivity to LA, baseline KCT results should be in the range 80-120 sec. Commercial QC normal plasmas yielding such results should be tested with each batch of product. Filtered normal plasmas should yield 100-150 sec.

Commercial QC normal and abnormal plasmas should be tested with each new lot of KCT. A known LA positive sample (if available) should also be tested.

EXPRESSION OF RESULTS
KCT results should be expressed as follows:

Rosser Index (RI) = \frac{[\text{KCT} \text{ (mix)} - \text{KCT} \text{ (normal pool)}]}{\text{KCT} \text{ (patient)}}

This expression reduces the effect of any coagulation factor deficiencies within the patient plasma.

Alternatively, the results may also be expressed as follows:

a) Raw results, preferably in a mix, relative to a normal reference range established by the laboratory.

b) As a ratio of delta KCT: the difference between the KCT in a 20:80 mix and the KCT of normal plasma, to the result on normal plasma alone.

INTERPRETATION OF RESULTS
A prolonged KCT is likely to be a result of LA when:

RI > 0.16

or if the delta KCT ratio is > 0.1.

LIMITATIONS:
Note that HEPARIN and DIRECT THROMBIN INHIBITORS interfere with the KCT. The KCT test is not suitable for patients undergoing hirudin or Direct Thrombin Inhibitor therapy.

Note that fresh plasma or filtered frozen plasma is recommended for testing. Platelet contamination may shorten the KCT.

REFERENCES:


SYPHILIS RPR TEST
Quicktest for the Qualitative and Quantitative Determination of Reagin Antibodies in Serum or Plasma

Package Sizes

<table>
<thead>
<tr>
<th>REF</th>
<th>50001</th>
<th>100 Tests Complete Test Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50002</td>
<td>500 Tests Complete Test Kit</td>
</tr>
<tr>
<td></td>
<td>50016</td>
<td>500 Tests Reduced accessories</td>
</tr>
</tbody>
</table>

IVD

Principle
The SYPHILIS RPR TEST is a non-Newtonian flocculation test used to detect and quantify reagin antibodies associated with syphilis. The RPR antigen is a modification of VDRL antigen which contains microparticulate charcoal to enhance the visual difference between a positive and negative result. For sera containing anti-Reagin antibodies, flocculation of antigen-charcoal particles is observed. In case of non-reactive specimen the specimen-antigen-suspension remains homogenous.

Contents, Reagent Composition

<table>
<thead>
<tr>
<th>AGS</th>
<th>1 or 5 x 1.6 ml RPR Antigen Suspension (white cap)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cardiolipin suspension, containing microparticulate charcoal</td>
</tr>
<tr>
<td></td>
<td>0.3 % Sodium azide</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PC</th>
<th>0.5 or 1 ml RPR control serum positive (red dropper)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stabilised liquid control, reactive with RPR antigen, human</td>
</tr>
<tr>
<td></td>
<td>Sodium azide 0.095 %</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NC</th>
<th>1 ml RPR control serum negative (green dropper)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stabilised liquid control, non-reactive with RPR antigen, human</td>
</tr>
<tr>
<td></td>
<td>Sodium azide 0.095 %</td>
</tr>
</tbody>
</table>

Material Provided
Disposable test cards with 10 cells
Dispensing needle for AGS (1.6 μl)
Dispensing bottle
Disposable dispensers for serum or plasma (50 μl) (Not supplied with REF 50016)

Stability
AGS, PC, and NC are stable up to the expiry date when stored at 2 - 8°C. Do not freeze AGS!

Specimen
Plasma, serum heated or unheated. The samples should be free from contamination and non-haemolysed.
Fresh serum samples may be stored for 5 days at 2 - 8°C or for 4 weeks at -20°C.

Procedure

A. Qualitative Test

Bring AGS, PC, NC and samples to room temperature.

| AGS | mix reagent thoroughly and suck up the suspension through the needle into the dispensing bottle. The suspension must be totally homogenous before use. |

Place in separate cells of the test card using the disposable serum dispensers or droppers and spread the fluid over the entire area of the test cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1 drop (50 μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC (red dropper)</td>
<td>1 drop</td>
</tr>
<tr>
<td>NC (green dropper)</td>
<td>1 drop</td>
</tr>
</tbody>
</table>

Hold the dispenser bottle vertical

Do not restir.

Tilt the test card and forth slowly for 8 minutes or place the card on an automated rotator and rotate at 100 r.p.m. for 8 minutes.

Interpretation of Results
Immediately after the 8 minutes rotation read the results macroscopically in direct light. A reactive result is indicated by large aggregates in the centre or the periphery of the test circle. Very weak reactivities are indicated by the presence of small aggregates around the edge of the test circle. A negative (non-reactive) result will display a smooth, even appearance with no aggregates visible. Samples with positive results should be retested in the semi-quantitative test.

B. Semi-quantitative Test

Prepare dilutions of the samples with physiological saline (0.9%) as indicated: 1:2, 1:4, 1:8, 1:16, 1:32. Proceed as in the qualitative test.

Interpretation of Results
The last dilution step that contains macroscopic aggregates indicates the titre of the sample. If the last dilution gives a positive result the dilution series should be extended to 1:64, 1:128, 1:256 and 1:512.

Quality Control
AGS and PC should be run with each series of samples and incorporated in reading the results:
NC - homogeneous suspension with no aggregates
PC - within 5 min. distinct macroscopic aggregates.

Notes
1. As well as reagent tests, the SYPHILIS RPR TEST may give false positive results. These results can be caused by diseases such as leprosy, lupus erythematosus, infectious mononucleosis, malaria, vaccine and viral pneumonia. All reactive test samples should undergo a further serological test (e.g. TPHA).
2. The final diagnosis should be based on a correlation of test results with other clinical findings.
3. Contaminated sera and a longer reaction time may cause false positive results.
4. After each day's testing, the needle should be removed from the dispensing bottle, rinsed with distilled water and air dried. Recap dispensing bottle for storage.
5. The long term stability of the antigen may be reduced if it is stored in the dispensing bottle. It is therefore preferable to return the antigen suspension to the original glass bottle after use. The dispensing bottle and needle must be thoroughly washed in distilled water and air dried after use.
6. PC has been tested for HbAg and antibodies to HIV and HCV and shown to be negative using FDA approved methods. However, the material should still be regarded and handled as potentially hazardous.
7. AGS, PC, and NC contain sodium azide. Do not swallow. Avoid contact with skin and mucous membranes.

Performance Characteristics
Typical performance data can be found in the Verification Report, accessible via www.human.de/products/db/vrlt/rpr.pdf or www.human.de/products/db/vrlt/rx-rpr.pdf

References
SYPHILIS TPHA liquid
Hemagglutination Test for the Qualitative and Quantitative Determination of Antibodies against Treponema pallidum

Package Size
REF 50/101 100 Tests Complete test kit

Method
The SYPHILIS TPHA liquid test is an indirect hemagglutination test for the detection of specific antibodies against Treponema pallidum. Avian erythrocytes are coated with T. pallidum antigen. In the presence of specific antibodies, the sensitized cells will agglutinate to form characteristic patterns in microtiter plates. Antibodies to nonpathogenic Treponema are absorbed by an extract of Reiter's Treponemas included in the cell suspensions.

Contents
- 2 x 4 ml TPHA Test Cells (white cap)
- 2 x 5 ml TPHA Control Cells (blue cap)
- 0.5 ml TPHA Control Serum Positive (red cap)
- 0.5 ml TPHA Control Serum Negative (green cap)
- 20 ml TPHA Diluent (rabbit serum)

Material Required but not Supplied
- Microtiter or microplug (25 µl, 75 µl, 100 µl)
- Rigid type (styrene) U-well microtiter plates (e.g. Dynex M24A from Labsystems)

Stability
Stable if unopened up to the expiry date when stored at 2–8°C. After opening the reagents are stable for 6 weeks at 2–8°C and 2 weeks at 10–25°C (avoid contamination).

Specimen
Serum (do not use plasma). Avoid contamination and hemolysis. Fresh serum samples may be stored for max. 24 h at 2–8°C, or for 4 weeks at -20°C.

Procedure
A Qualitative Test
1. Using a micropette place 100 µl [XX] in well 1 and 25 µl in well 2 and 3 each.
2. Add 25 µl of serum sample or [XX] to well 1. Mix the contents of well 1 with a 25 µl micropette or microplug and transfer 25 µl to well 2 (control well). Mix and transfer 25 µl to well 3 (test well). Mix and discard 25 µl from well 3.
3. Add 75 µl of the carefully resuspended [XX] to well 2 and 75 µl of the carefully resuspended [XX] to well 3.
4. Shake the plate gently to ensure the contents are thoroughly mixed.
5. Place the plate on a white level surface, away from vibration and direct sunlight. Leave for 45–60 minutes before reading the result. The plate may be left overnight.

Interpretation of Results
Negative result: defined button of non-agglutinated cells, with or without a very small hole in the centre.
Indeterminate result: button of cells with a small hole in the centre (appearance of a well defined, thin dense ring with a clear background). Such samples should be retested.
Positive result: partial or total agglutination of cells (appearance of smooth mat of agglutinated cells, possibly surrounded by a circle of cells). Weak positive samples show a ring with a frayed border surrounded by agglutinated cells. Positive samples should be retested in the quantitative test.

B Quantitative Test
1. Dispense [XX] as for the qualitative test but continue to place 25 µl each to well 4 to 10 in the same line.
2. Instead of discarding the last volume of dilution from well 3 continue to transfer 25 µl to well 4, mix, transfer to well 5 and so on. Discard 25 µl of well 10.
3. Dispense 75 µl [XX] to well 2 and 75 µl each of [XX] to the test wells 3–10.
4. Continue as in the qualitative test (A).

Importance of Results
A serum showing agglutination in the test wells should be reported as positive, provided that no agglutination is reported in the control wells. The titre is defined as the final dilution showing a positive reaction (well 3, 1, 0, 00, 0, 1, 550). Samples with titres ≥ 1:50 are considered reactive for T. pallidum antibodies.

Quality Control
With every series of samples both controls should be run. [XX] should give a titre of 1:250 ± 1 titre step. The degree of agglutination should decrease from well 3 to 9.

Important Notes
1. The SYPHILIS TPHA liquid test is a screening test. Positive samples should be retested with a confirmatory test. External evaluation of the SYPHILIS TPHA liquid test revealed an overall diagnostic sensitivity of 98.5% and an overall diagnostic specificity of 100%. Primary syphilitic samples, mostly containing specific IgM antibodies, may yield negative results. If syphilis infection is still suspected in such cases, the test should be repeated after about 2 weeks with a freshly drawn sample. Anamnestic information should always be carefully considered. Due to long-term persisting specific antibodies in the circulation in patients with treated syphilis, positive test results may be obtained in such cases. Although not supported by own results, the literature reported about false positive results with similar test systems in case of leprosy, infectious mononucleosis and connective tissue disorders. The final diagnosis therefore should consider further clinical and diagnostic findings.
2. Serum samples with invalid results have to be absorbed with 5 µl [XX] and incubate for 30 minutes. Centrifuge 5 minutes and pipette 25 µl of the absorbed supernatant to 75 µl of [XX] in one well. Continue and read as in the qualitative test (A).
3. The reagents and controls contain sodium azide as preservative (0.09%). Do not swallow. Avoid contact with skin and mucous membranes.
4. Each individual unit of human blood used in the manufacture of this test kit has been tested on the donor level for presence of HIV-1, antibodies to HCV and HIV and found negative using FDA approved methods. However, the material should still be regarded and handled as potentially hazardous.

Performance Characteristics
Typical performance data can be found in the Verification Report, accessible via www.human.de/datenlib/pdf/LX-TPHA.pdf or www.human.de/data/LX-TPHA.pdf

References
9. ISO 15223 Medical devices - Symbols to be used with medical device labels, labelling and information to be supplied

LX-TPHA
INF 50/101 01 GB 01.30.01.15
Human Gesellschaft für Biochemikum und Diagnostika mbH
Max-Planck-Ring 21 - D-65205 Wiesbaden - Germany
Tel.: +49 6122 9999-0 - Telefax: +49 6122 9999-100 - eMail: human@human.de

69
Test Instructions
IMTEC-Cardiolipin-Antibodies IgG

Enzyme Immunoassay for the quantitative Determination of IgG Antibodies against Cardiolipin

REF: TC 59071

Please read the instructions carefully before testing.
Procedural precautions:
▸ Do not use the reagents beyond the date of expiry.
▸ Never mix reagents from different test kits nor lots.
▸ Store reagents at 2-8°C.

1. Clinical Use
Over the past years, the determination of autoantibodies against phospholipids, detected e.g. as anti-cardiolipin antibodies (aCL) or as lupus anticoagulant (LAC), has become increasingly important due to its association with arterial and venous thrombosis, tendency to abortion, neurological symptoms and signs as migraine and chorea, pulmonary hypertonia as well as thrombocytopenia and hemolytic anemia.

2. Principle of the Test
The test is based on the immobilisation of cardiolipin in a biological active vesicle-like structure to a solid phase (polystyrene) and subsequent binding of the aCL. A better presentation of the antigenic epitop is achieved because of specially purified human β2-glycoprotein I (the anti-phospholipid cofactor) was added and the sample buffer also contains β2-glycoprotein I. The bound antibodies are detected with a peroxidase-labeled secondary antibody that is directed against human IgG. After addition of substrate solution, a color stain develops. Its intensity is proportional to the concentration and/or the avidity of the detected antibodies.
The IMTEC standards are calibrated against the internationally accepted Sapporo standard (acc. to Koike et al, monoclonal antibody HCAL).

3. Materials Provided
- [MTP]: Cardiolipin coated microtiter strips, (1x10), breakable
- [CAL]: standards, ready to use, 750 μL
  1. 31.25 U/mL = 6.3 mg/mL = 6.5 GPL/mL per vial
  2. 52.5 U/mL = 12.5 ng/mL = 13 GPL/mL 1 vial
  3. 125 U/mL = 25 ng/mL = 26 GPL/mL each
  4. 250 U/mL = 50 ng/mL = 52 GPL/mL
  5. 500 U/mL = 100 ng/mL = 104 GPL/mL
  (all standards contain sodium azide and are linked according to concentration)
- [CONTROL-]: negative control serum, ready to use, contains sodium azide 1 vial
- [CONTROL+]: positive control serum, ready to use, contains sodium azide 1 vial
- [WASH]: washing buffer concentrate (10x) 1 bottle
- [WASH]: sample buffer, ready to use 100 mL
- [CONJ]: rabbit IgG-HRP, HRP-Conjugate, anti-human IgG, ready to use 12 mL
- [SUBS]: TMB, TMB solution, HRP substrate, ready to use 12 mL
- [SOLN]: stop, ready to use, sulfury acid, handle with care, corrosive!

4. Preparation of Reagents
Attention! Allow the testkit and all its components to reach room temperature completely before executing it!
Please do not use any polystyrene vessels for handling of HRP conjugates.
If the test is performed automatically, we recommend the use of fresh conjugate each run and to discharge traces of old conjugate entirely. Remove washing buffer after washing steps completely.
4.1. Preparation of Washing Buffer
If any salt has been crystallized inside the bottle, it must be resolved before use. Dilute 1 part washing buffer concentrate [BUFF] WASH [10X] with 9 parts distilled water. The diluted buffer is stable for 6 weeks stored at 2 - 8 °C.

4.2. Stopping Solution, Sample buffer, Standards, Control Sera, HRP-Conjugate and TMB Solution
Stopping solution, sample buffer, standards, control sera, HRP-conjugate and TMB solution are ready to use. Used bottles should be closed carefully and stored at 4 - 8 °C. Store TMB solution also protected from light.

4.3. Preparation of Serum or Plasma
Use serum or plasma freshly collected or freeze samples at -20 °C. Do not use samples that are repeatedly thawed and frozen. Do not use serum or plasma inactivated by heat treatment at 56 °C. Allow the samples to reach room temperature (30 min). Dilute samples 1:100 with sample buffer [DIL] SPE (10 μL sample to 1 mL buffer).

4.4. Microtiter Strips
The strips are ready to use. Unused strips should be sealed in the lockable original bag at 2-8°C.

5. Test Procedure
- Pipette 100 μL serum or plasma dilution or undiluted standards [CAL] into each well, for blanks use sample buffer [DIL] SPE instead of serum dilution, seal wells with adhesive foil.
- Incubate for 1 hour at room temperature (RT).
- Rinse the wells 3x using at least 200 μL washing buffer per well.
- Discard buffer and knock out residues on an absorbent paper or cloth.
- Pipette 100 μL HRP-conjugate [CONJ] [human IgG] [HRP] into each well, seal wells with adhesive foil.
- Incubate for 30 minutes at RT.
- Rinse the wells 3x using at least 200 μL washing buffer per well.
- Discard buffer and knock out residues on an absorbent paper or cloth.
- Pipette 100 μL TMB solution [SUBL] TMB into each well.
- Incubate for 10 min at RT in the dark. At room temperatures above 25 °C the substrate incubation should be shortened, but should never fall short of 5 minutes.
- Pipette 100 μL stopping solution [SOLN] STOP per well.
- Measure at 450 nm within the next 30 min after stopping.

6. Interpretation of Results
Calibrate measured absorbance against concentration/units of standards [CAL] (31.25 U/mL, 62.5 U/mL, 125 U/mL, 250 U/mL, 500 U/mL) in semi log. Determine the units of the examined sera or plasma samples from the standard curve directly.

The following histogram shows a determination of Anti-Cardiolipin IgG antibodies in 300 blood donors:

<table>
<thead>
<tr>
<th>Unit</th>
<th>Cut-off IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>U/mL</td>
<td>48 U/mL</td>
</tr>
<tr>
<td>Sapporo</td>
<td>9.6 ng/mL</td>
</tr>
<tr>
<td>Louisville</td>
<td>10 GPL/mL</td>
</tr>
</tbody>
</table>

It is also possible to calibrate the test in Sapporo units (ng/mL, acc. to Koike et al. - related to a sample dilution of 1:100) or Louisville units (GPL/mL, acc. to Harris et al.) respectively. Using these, results above the respective cutoff values listed in the following table, are considered positive.

Precautions
For in vitro diagnostic use only.

The human Control Sera and Standards in this kit have been prepared from blood donations which have been tested for Hepatitis B Surface Antigen, anti-HCV- and anti-HIV-1/2 antibodies and shown to be NEGATIVE.

However, as no known test can guarantee the absence of an infectious virus, all reagents and samples must be handled carefully and disposed of in accordance with local legislation.

IMTEC Immundiagnostika GmbH
Robert-Rössle-Straße 10
13125 Berlin
GERMANY
Tel.: +49 (30) 948936-00
Fax: +49 (30) 948936-15
www.imtec-immundiagnostika.de
imtec@mdc-berlin.de
An enzyme-linked immunosorbent assay (ELISA) for the semi-quantitative determination of IgG anti-Beta 2 Glycoprotein I (β2GPI) antibodies in human serum or citrated plasma (3.2% sodium citrate).

**INTENDED USE**

For the detection and semi-quantitation of IgG anti-β2GPI antibodies in individuals with systemic lupus erythematosus (SLE) and lupus-like disorders (anti-phospholipid syndrome).

**SUMMARY AND EXPLANATION OF THE ANTI-BETA 2 GLYCOPEPTIDE I TEST**

Anti-phospholipid antibodies are a heterogeneous group of immunoglobulins that bind to several anionic phospholipids, including cardiolipin and phosphatidylycerine.\(^1\) High serum levels of anti-phospholipid antibodies are frequently detected in patients with autoimmune (e.g., SLE) and non-autoimmune diseases, as well as in apparently healthy individuals.\(^3\) These antibodies have been associated with an increased risk for recurrent arterial and venous thrombotic events, thrombocytopenia, and fetal loss. These manifestations are the main features of the anti-phospholipid syndrome (APS).\(^5,10\)

Most autoimmune anti-phospholipid antibodies require a serum cofactor (β2GPI) for optimal binding.\(^7\) It has been shown that many anti-phospholipid antibodies may react to a neoepitope formed on the β2GPI molecule by the interaction between the phospholipid and β2GPI.\(^11\) Most assays for anti-phospholipid antibodies contain bovine serum as the source of cofactor. More recently, it has been shown that the binding of β2GPI to the microwell surface may produce a neoepitope similar to that when combined with a phospholipid, and the results with this system showed a good correlation with the anti-phospholipid syndrome.\(^12\) The serologic detection of anti-β2GPI antibodies provides enhanced clinical sensitivity for thrombosis. The REAADS Anti-β2GPI ELISA Test Kit uses the well known ELISA format to detect anti-β2GPI antibodies in human serum.

Patients with positive reactions to both anti-phospholipid and anti-β2GPI assays were more likely to have clinical complications than those positive for only one. Higher prevalence and mean serum levels of IgG anti-β2GPI antibodies have been reported in autoimmune patients. In addition, anti-β2GPI antibodies in SLE patients correlated with clinical manifestations of anti-phospholipid syndrome.\(^17\)

**PRINCIPLE OF THE TEST**

The test is performed as an indirect ELISA. Diluted serum or plasma samples, calibrator sera, and controls are incubated in microwells coated with purified human β2GPI. Incubation allows the anti-β2GPI antibodies present in the samples to react with the immobilized antigen. After the removal of unbound serum or plasma proteins by washing, antibodies specific for human IgG, labeled with horseradish peroxidase (HRP), are added forming complexes with the β2GPI bound antibodies. Following another washing step, the bound enzyme-antibody conjugate is assayed by the addition of a single solution containing tetramethylbenzidine (TMB) and hydrogen peroxide (H₂O₂) as the chromogenic substrate. Color develops in the wells at an intensity proportional to the serum concentration of anti-β2GPI antibodies.
Results are obtained by reading the O.D. (optical density or absorbance) of each well in a spectrophotometer. Calibrator sera are provided, with the IgG anti-β2GPI antibody concentrations expressed in G units. The user has the option of running either a single point calibrator or a four-point calibration curve. For single point calibration, dividing the concentration value of the calibrator sera by the O.D. value of the calibrator provides a conversion factor. The O.D. values of the other samples are multiplied by the conversion factor to obtain IgG anti-β2GPI antibody concentrations in G units. For multipoint calibration, perform a linear regression analysis with calibrator values against calibrator O.D.s. Controls and patient results are determined from the calibration curve. These units are traceable to available reference preparations.

REAGENTS

Store at 2 - 8 °C. Do Not Freeze.

Each REAADS IgG Anti-β2GPI 96-Microwell Test Kit contains the following reagents (volumes may vary depending on the kit size and configuration):

- 12 x 8 stabilized β2GPI (from human serum) coated microwells with frame
- 60 mL Sample Diluent IV (blue-green solution)
- 3 vials (0.250 mL) IgG β2GPI Calibrator Serum* (1-high, 2-moderate, 3-low)(human); see vial label for antibody concentration in G units. Calibrator 3 should be used when performing single point calibration
- 0.250 mL IgG β2GPI Positive Control Serum* (human); see vial label for expected G unit range
- 0.250 mL Normal Control Serum* (human); see vial label for expected G unit range
- 15 mL anti-human IgG (goat) HRP-Conjugated Antibody Solution (blue solution)
- 15 mL One Component Substrate Solution (TMB and H2O2); ready to use
- 15 mL Stopping Solution (0.36 N sulfuric acid)
- 2 bottles (30 mL) Wash Concentrate (33X PBS/Tween)

**CAUTION: Contains sodium azide**

WARNINGS AND PRECAUTIONS

**For In Vitro Diagnostic Use**

1. Human source material used to prepare the calibrators and controls included in this kit has been tested and shown to be negative to HBsAg, HCV, and HIV 1 & 2 by FDA required tests. However, all human blood derivatives, including patient samples, should be handled as potentially infectious material.

2. Do not pipette by mouth.

3. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.

4. Wear disposable gloves while handling kit reagents and wash hands thoroughly afterwards.

5. Certain components of this product contain sodium azide as a preservative. Sodium azide has been reported to form lead and copper azides when left in contact with these metals. These metal azides are explosive. Any solutions containing azide must be thoroughly flushed with copious amounts of water to prevent the build-up of explosive metal azides in the plumbing system.

6. One-Component Substrate Solution can cause irritation to the eyes and skin. Use gloves when handling substrate and wash thoroughly after handling. Keep reagent away from ignition sources. Avoid contact with oxidizing agents.

7. Certain components are labeled with the following:
   Irritating to eyes (R 36). Avoid contact with skin (S 24). Avoid contact with eyes (S 25). In case of contact with eyes, rinse immediately with plenty of water and seek medical advice (S 26). If swallowed, seek medical advice immediately and show this container or label (S 46).

Irritant 🍗, Biological Risk 🪤.
SPECIMEN COLLECTION AND PREPARATION

Serum or citrated plasma (3.2% sodium citrate) should be used as the sample matrix.

Blood should be collected by venipuncture and the serum separated from the cells by centrifugation after clot formation. If not tested immediately, the specimens should be stored at 2 to 8°C. If specimens are to be stored for more than 72 hours, they should be frozen at -20°C or below. Avoid repeated freezing and thawing. Do not use hemolyzed, icteric, or lipemic serum or plasma as these conditions may cause aberrant results.Specimens containing visible particulate matter should be clarified by centrifugation before testing.

If citrated plasma is to be used, blood should be collected by venipuncture and the plasma separated from the cells immediately by centrifugation at 1500g for 10 minutes. The supernatant must be carefully removed after centrifugation to avoid contamination with platelets. Repeating the centrifugation and separation steps may be advisable to minimize platelet contamination. Lysed or aged platelets can lead to aberrant results. If not tested immediately, plasma samples should be stored as described for serum.

INSTRUCTIONS FOR USE

Materials Provided

REAADS Anti-β2GPI Test Kit; see “Reagents” section for a complete list.

Materials Required but not Supplied

- Reagent grade water to prepare PBS wash solution and to zero or blank the plate reader during the final assay step
- Graduated cylinders
- Precision pipettors capable of delivering between 5 µL and 1000 µL, with appropriate tips
- Miscellaneous glassware appropriate for handling small volumes
- Flasks or bottles, 1 liter
- Wash bottles, preferably with the tip partially cut back to provide a wide stream, or an automated or semi-automated washing system
- Disposable gloves
- Plate-reading spectrophotometer capable of reading absorbance at 450 nm (650 nm reference if dual beam)
- Multichannel pipettors capable of delivering to 8 wells simultaneously
- Microdilution tubes and a 96-well microdilution tube holder for sample dilutions and rapid delivery to microwell plate

Procedural Notes

1. Bring serum or plasma samples and kit reagents to room temperature and mix well before using; avoid foaming. Return all unused samples and reagents to refrigerated storage as soon as possible.
2. All dilutions of calibrators, controls, and test sera or plasma must be made just prior to use in the assay.
3. A single water blank well can be included in each plate with each run. No sample or kit reagents are to be added to this well. Instead, add 200 µL of reagent grade water to the well immediately prior to reading the plate in the spectrophotometer. The plate reader should be programmed to “zero” or “blank” against air or a water well.
4. Good washing technique is critical for optimal performance of the assay. Adequate washing is best accomplished by directing a forceful stream of wash solution into the bottom of the microwells from a plastic squeeze bottle with a wide tip. Wash solution in the water blank well will not interfere with the procedure. An automated plate washing system can also be used.
5. IMPORTANT: Failure to adequately remove residual wash solution can cause inconsistent color development of the Substrate Solution.
6. Use a multichannel pipettor capable of delivering to 8 wells simultaneously when possible. This speeds the process and provides more uniform incubation and reaction times for all wells.
7. Carefully controlled timing of all steps is critical. All calibrators, controls, and samples must be added within a five minute period. Batch size of samples should not be larger than the amount that can be added within this time period.
8. For all incubations, the start of the incubation period begins with the completion of reagent or sample addition.
9. Addition of all samples and reagents should be performed at the same rate and in the same sequence.
10. Incubation temperatures above or below normal room temperature (18 - 26°C) may contribute to inaccurate results.
11. Avoid microbial and cross-contamination of reagents when opening and removing aliquots from the primary vials.
12. Do not use kit components beyond expiration date.
13. Do not use kit components from different kit lot numbers.

**Reagent Preparation**

**Wash Solution (PBS/Tween):** Measure 30 mL of Wash Concentrate and dilute to 1 liter with reagent grade water. The pH of the final solution should be 7.35 ± 0.1. Store unused wash solution in the refrigerator at 2 - 8°C. Discard if the solution shows signs of microbial or cross-contamination.

**Assay Procedure**

1. The assay can be performed with a single point calibration (Calibrator 3) or a four-point calibration curve (Calibrators 1, 2, and 3 plus sample diluent/reagent blank as Calibrator 4 equal to 0 G units). A reagent blank control should also be run with both the single point and multipoint calibration methods. Sample Diluent without serum or plasma is added to the well. This well will be treated the same as a control or patient sample in subsequent assay steps.
2. Remove any microwell strips that will not be used from the frame and store them in the bag provided.
3. Prepare a 1:50 dilution of the calibrators, controls, and patient samples in sample diluent (blue-green solution); e.g., 10 µL sample added to 490 µL Sample Diluent equals a 1:50 sample dilution.
4. Add 100 µL of diluted calibrators (including the reagent blank/Calibrator 4), controls, and patient samples to the appropriate microwells.
5. Incubate 15 minutes at room temperature. After the incubation is complete, carefully invert the microwells and empty the sample fluid. Do not allow samples to contaminate other microwells.
6. Wash 4 times with wash solution. Each well should be filled with wash solution per wash. Invert microwells between each wash to empty fluid. Use a snapping motion of the wrist to shake the liquid from the wells. To retain microwell modules during washing, the frame must be squeezed at the top and bottom of the longer sides. Blot on absorbent paper to remove residual wash fluid. Do not allow wells to dry out between steps.
7. Add 100 µL anti-human IgG HRP-Conjugated Antibody Solution (blue) to the wells.
8. Incubate for 15 minutes at room temperature. After the incubation is complete, carefully invert the microwells and empty the conjugate solution.
9. Wash 4 times with wash solution, as in step 6. Use a snapping motion to drain the liquid and blot on absorbent towels after the final wash. Do not allow the wells to dry out.
10. Add 100 µL One-Component Substrate to each well and incubate for 10 minutes at room temperature. Add substrate to the wells at a steady rate. Blue color will develop in wells with positive samples.
11. Add 100 µL Stopping Solution (0.36 N sulfuric acid) to each well to stop the enzyme reaction. Be sure to add the acid to the wells in the same order and at the same rate as the Substrate was added. Blue Substrate will turn yellow and colorless solution will remain colorless. Blank or zero the plate reader against an air or a water blank well. Read the O.D. of each well at 450 nm (and 650 nm reference if dual beam). The O.D. values should be measured within 5 minutes of the addition of the Stopping Solution.
Results

Single Point Calibration
1. Calculate the mean O.D. values if duplicates of Calibrator 3, Controls and patient samples were performed.
2. Divide the concentration value of Calibrator 3 (printed on vial label) by the O.D. or mean O.D. value of the calibrator serum to obtain the conversion factor.
3. Multiply the O.D. or mean O.D. values for each of the controls and patient samples by the conversion factor to obtain an anti-β₂GPI antibody concentration value expressed in G units.

<table>
<thead>
<tr>
<th>Conversion Factor =</th>
<th>( \frac{\text{Anti-β₂GPI Concentration of Calibrator 3}}{\text{Absorbance Value of the Calibrator 3 (O.D.)}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-β₂GPI Concentration of Sample =</td>
<td>Conversion Factor X Absorbance of the Sample (O.D.)</td>
</tr>
</tbody>
</table>

4. The Conversion Factor must be calculated for each assay run. Using a Conversion Factor from another assay will invalidate the results.

Multi-Point Curve Calibration
1. Calculate the mean O.D. values if duplicates of the calibrators, controls and patient samples were performed.
2. Perform linear regression analysis with the four calibrator values against the mean O.D.s for each calibrator. (See vial labels for G units; Calibrator 4 [sample diluent] is equal to 0 G units.)
3. The calibrator curve can be plotted either automatically using a validated software program or manually with graph paper. It is recommended to use a zero intercept when generating the regression line to avoid negative values. If this option is not available, any negative values should be reported as zero units. When generating the curve manually, draw a best fit line through the plotted points using a zero intercept.
4. Determine the control and patient sample values from the calibrator curve.
5. Example of a multi-point curve calibration.

![Multi-Point Calibration Curve](image)

Using the example calibration curve provided, a specimen O.D. of 0.860 at 450 nm would correspond to a calculated value of 60 units. The calibration curve provided is an example only and should not be used to calculate patient results. A new calibration curve should be performed with every test run.
Quality Control

1. The O.D. value of Calibrator 3 should be ≥0.400 to assure that the kit is functioning properly. Calibrator 3 O.D. readings of less than 0.400 may indicate that the kit is no longer suitable for use.
2. The O.D. of Calibrator 4 or reagent blank should be less than 0.050 when the spectrophotometer has been blanked against air or a water well. Readings greater than 0.050 may indicate possible reagent contamination or inadequate plate washing.
3. The anti-β2GPI values obtained for the control sera should be within the ranges indicated on the vial labels. Occasional small deviations outside these ranges are acceptable.
4. O.D. values for duplicates of the controls or patient samples should be within 20% of the mean O.D. value for samples with absorbance readings greater than 0.200.
5. Each laboratory should periodically determine its own normal cut-off values for the appropriate population of patients.
6. Samples with anti-β2GPI values greater than 200 G units may be reported as “greater than 200 G units.”
7. Assure that all quality control parameters have been met before reporting test results.

NORMAL RANGE

Serum samples from 120 healthy blood donors were tested for IgG anti-β2GPI antibodies. The following normal range was established:
- Less than 20 G units

PERFORMANCE CHARACTERISTICS

Clinical Specificity

Normal Samples:
Serum samples from 121 healthy blood donors were assayed for the presence of IgG anti-β2GPI antibodies. Using the established cutoff value of 20 G units, this normal population demonstrated 100% specificity (mean value = 2.1 G units).

Serum samples from 41 infectious disease (syphilis), 42 progressive systemic sclerosis (PSS), and 42 rheumatoid arthritis (RA) patients were assayed for the presence of IgG anti-β2GPI antibodies. These patient groups demonstrated similar results compared to the healthy blood donor population (mean values = 3.9, 2.1, and 2.0 G units respectively). Results of these groups along with the healthy blood donors are summarized in the table below.

<table>
<thead>
<tr>
<th>Healthy</th>
<th>Infectious (syphilis)</th>
<th>PSS</th>
<th>RA</th>
</tr>
</thead>
<tbody>
<tr>
<td># of Samples (n)</td>
<td>121</td>
<td>41</td>
<td>42</td>
</tr>
<tr>
<td>Mean (G units)</td>
<td>2.1</td>
<td>3.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.1</td>
<td>10.3</td>
<td>3.9</td>
</tr>
<tr>
<td>% Negative</td>
<td>100%</td>
<td>97.5%</td>
<td>97.8%</td>
</tr>
</tbody>
</table>

Clinical Sensitivity

Systemic Lupus Erythematosus (SLE):
Serum samples from 40 unselected (consecutive) patients with SLE were tested with the kit. Nine of the samples (sensitivity of 22.5%) were positive for IgG anti-β2GPI antibodies (mean value = 24.5 G units). A good correlation was found between IgG anti-β2GPI with both IgG anti-phosphatidyserine (r = 0.928) and IgG anti-cardiolipin (r = 0.884) antibody levels in this group.

Serum samples from 12 selected female patients with SLE who had a clinical history of thrombosis, thrombocytopenia, or recurrent fetal loss were evaluated for IgG anti-β2GPI antibodies. Seven of the samples (sensitivity of 58%) were positive in this population (mean value = 69 G units).
SLE Controls:
Serum samples from six selected female patients with SLE who had a history of thrombocytopenia (no thrombosis) were tested for IgG anti-β2GPI antibodies. Only one sample (17%) tested weak positive (36 G units), with a mean value of 12.7 G units for this group.

Serum samples from 10 selected female patients with SLE who were known not to have had thrombotic episodes, nor any other feature of the anti-phospholipid syndrome, were tested in the assay. Two of the samples (20%) were weak positive for IgG anti-β2GPI antibodies, with a mean value of 8.5 G units.

Primary Anti-phospholipid Syndrome (APS):
Serum samples from nine patients with the diagnosis of primary anti phospholipid syndrome (APS) were tested on the REAADS IgG anti-β2GPI assay. Most, if not all, specimens were expected to be positive (above 20 G units) in this population. Eight of the nine samples resulted positive (88.9% sensitivity) with a mean value of 111 G units. A summary of sensitivity testing is presented below.

<table>
<thead>
<tr>
<th></th>
<th>Unselected SLE</th>
<th>Selected SLE</th>
<th>Primary APS</th>
</tr>
</thead>
<tbody>
<tr>
<td># of Samples (n)</td>
<td>40</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Mean (G units)</td>
<td>24.5</td>
<td>10.1</td>
<td>69.0</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>49.6</td>
<td>12.7</td>
<td>66.2</td>
</tr>
<tr>
<td>% Positive</td>
<td>22.5%</td>
<td>16.8%</td>
<td>56.0%</td>
</tr>
</tbody>
</table>

Technical Performance Comparison
Two disease populations (unselected SLE and primary APS) were tested on REAADS IgG anti-β2GPI ELISA assay and a predicate device to study the correlation between positive and negative results. The results are summarized in the table presented below.

<table>
<thead>
<tr>
<th>Predicate Device IgG Anti-β2GPI</th>
<th>Negative</th>
<th>Positive</th>
<th>Relative Sensitivity</th>
<th>Relative Specificity</th>
<th>Agreement:</th>
</tr>
</thead>
<tbody>
<tr>
<td>REAADS IgG Anti-β2GPI</td>
<td></td>
<td></td>
<td>100%</td>
<td>80%</td>
<td>84%</td>
</tr>
<tr>
<td>Predicate Device IgG Anti-β2GPI</td>
<td>32</td>
<td>8</td>
<td>100%</td>
<td>80%</td>
<td>84%</td>
</tr>
</tbody>
</table>

Precision
Three samples with known G unit values (one low, one moderate, and one high) were assayed in 23 replicates on three different occasions. The mean intra-assay and inter-assay coefficients of variation (%CVs) are presented in the following table. The reported intra-assay coefficient of variation is the mean of the three separate intra-assay %CVs. Inter-assay %CV is the coefficient of variation obtained from three plates from one lot.

<table>
<thead>
<tr>
<th>Value Range</th>
<th>Mean Intra-assay %CV</th>
<th>Mean Inter-assay %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>30 - 50 G units</td>
<td>4.3%</td>
</tr>
<tr>
<td>Moderate</td>
<td>60 - 70 G units</td>
<td>4.7%</td>
</tr>
<tr>
<td>High</td>
<td>&gt;110 G units</td>
<td>3.4%</td>
</tr>
</tbody>
</table>
LIMITATIONS OF THE TEST

The anti-β₂-GPI antibody concentration values obtained from this assay are an aid to diagnosis only. Each physician must interpret these results in light of the patient's history, physical findings, and other diagnostic procedures. If clinical findings suggest the presence of anti-phospholipid antibodies and the patient is negative for anti-β₂-GPI antibodies, some investigators recommend testing for anti-cardiolipin antibodies, anti-phosphatidylserine antibodies, and the lupus anticoagulant to confirm the negative result. A patient may be considered positive for anti-phospholipid antibodies if one or all of the tests give positive results.

WARRANTY

This product is warranted to perform as described in this package insert. Corgenix, Inc. disclaims any implied warranty of merchantability or fitness for a particular use, and in no event shall Corgenix, Inc. be liable for consequential damage.

For Technical or Customer Service in the United States, phone 1-800-729-5661. Outside the United States, phone (303) 457-4345, fax (303) 457-4519, email: techsupport@corgenix.com or contact a Corgenix authorized distributor.
APPENDIX 6: STANDARD OPERATING PROCEDURES (SOPs) AND INTERPRETATION OF LABORATORY TESTS

Haematological tests

Haemogram

The haemograms were performed on EDTA anticoagulated blood using an automated cell counter (Cell-Dyn 3200).

1. Mix the EDTA anticoagulated blood sample thoroughly by gentle inversions
2. Hold the specimen under the machine probe and aspirate by pressing the touch plate and wait for the instrument to do the analysis
3. The results are recorded on the screen and on a print out
4. The results are interpreted according to the appropriate local reference ranges(Appendix 8)

Peripheral blood film

1. Place a small drop of EDTA anticoagulated blood on a clean glass slide
2. Spread the blood using a spreader glass slide
3. Air-dry
4. Stain with May-Grünwald Giemsa stain
5. Examine the film via light microscopy and record the findings together with the haemogram report

Reticulocyte count

1. Deliver 2 drops of new methylene solution into 975 x 10 mm glass or plastic tube by means of a Pasteur pipette
2. Add 2 drops of the patient’s EDTA anticoagulated blood to the solution of new methylene blue and mix
3. Incubate the solution at 37°C for 15 minutes
4. Resuspend the red blood cells by gentle mixing the solution and then prepare a film on a glass slide
5. Allow the film to dry and examine using x100 oil immersion objective
6. Count the cells in an area where they are not distorted and the staining is good
7. The percentage of reticulocytes is obtained by counting reticulocytes and red blood cells in the number of fields
Calculation of reticulocyte count:

Number of reticulocytes in n fields = x
Average number of red cells per field = y
Total number of red cells in n fields = n X y
Reticulocyte percentage = \[\frac{x}{n \times y}\] X 100%
Absolute reticulocyte count = % X red cell count

_Erythrocyte sedimentation rate_

ESR was performed manually via the Wintrobe method.

1. Using a 230 mm glass pipette, slowly fill the tube with EDTA anticoagulated blood, avoiding air bubbles
2. Adjust the meniscus of the sample to the “0” mark at the top of the tube
3. Place the tube in an upright position in a labelled rack
4. Set the time for one hour
5. After one hour, read the fall of erythrocytes in the tube, reading from the top downwards
6. The ESR is the reported as the fall of red blood cells in mm per hour

_Coagulation tests_

The coagulation tests were performed on sodium citrate anticoagulated blood using an automated coagulation analyser (ACL200).

_Prothrombin time and Activated Partial Thromboplastin Time_

1. Centrifuge blood at 3000 rpm for 10 minutes
2. Freeze the plasma immediately at -70°C
3. On the day of analysis, thaw the frozen plasma samples at 37°C before testing
4. Put 0.5 mls of test plasma in the sample cups, and 0.5 mls of pooled normal plasma in another sample cup.
5. Put the sample cups in the sample tray, starting with the first empty tray
6. Load a new rotor or select empty clean positions on the rotor
7. Select the required test (PT and APTT) from the options available on the screen and enter
8. Confirm that the reagents are adequate in the reagent tray and press the downward arrow to begin the analysis
9. After the analysis, the results are displayed on the screen and also printed on the printer paper
Results are expressed in seconds
Normal reference range for PT: 12.8 – 14.8 seconds
Normal reference range for APTT: 28.3 – 38.3 seconds

10. Samples with a prolonged APTT (>38.3 seconds) are subjected to LA detection procedures using the KCT and Viper venom tests for LA.

*Kaolin clotting time (KCT)*

1. For the clotting test, replace the APTT reagent in the machine with the KCT reagent, load the sample tray with the sample cups containing 0.5 mls of test plasma, and one containing 0.5 mls of pooled normal plasma
2. Select the required test from the screen to begin the analysis
3. For the mixing test, mix 0.25 ml patient plasma with 0.25 ml normal plasma for a 50:50 (1:1) mix and carry out the KCT test as above.

The mixing test with pooled normal plasma reduces the effect of non-LA related clotting abnormalities.

Results are expressed as Rosner index

RI= \[\frac{\text{KCT mix} - \text{KCT normal pool}}{\text{KCT patient}}\]

The manufacturer recommends a cut-off value of 0.16 for the RI, and a sample is considered positive for LA if the RI is >0.16

*Viper venom tests for Lupus anticoagulant*

Testing using LupoTek DetecTin VL and CorrecTin VL should be done at the same time.

1. Place the LupoTek DetecTin VL reagent in place of PT reagent and the CorrecTin VL reagent in place of the APTT reagent in the respective reagent trays.
2. Load the sample tray with the sample cups containing 0.5 mls of test plasma and select the required test from the screen to begin analysis.

The clotting times obtained with the DetecTin VL and Correctin VL reagents are expressed in a ratio format. The patient’s clotting time is divided by the clotting time of the pooled normal plasma to obtain a Normalized Ratio (NR).

\[\text{NR} = \frac{\text{DetecTin VL time of patients}}{\text{DetecTin VL time of pooled normal plasma}} \div \frac{\text{Correctin VL time of patient}}{\text{Correctin VL time of pooled normal plasma}}\]
The manufacturer’s cut off value for the Normalized Ratio is 1.30. Samples with a ratio above this level are considered as LA positive.

**Mixing tests**

Perform PT and APTT on control, patients and a 50:50 mixture of the control and platelet plasma

Interpretation – if the prolongation is the result of a deficiency of a clotting factor, the PT or APTT of the mixture should return to within a few seconds of normal

If the APTT is prolonged, and normal plasma fails to correct the APTT, an inhibitor should be suspected. An inhibitor screen and tests for a lupus anticoagulant should be performed.

**Immunological tests**

**VDRL**

1. Bring RPR antigen suspension, positive control, negative control and patient samples to room temperature.
2. Mix RPR antigen suspension thoroughly until totally homogenous, and suck up the suspension through the needle, into the dispensing bottle.
3. Place in separate cells of the test card using the disposable serum dispensers or droppers and spread the fluid over the entire area of the test cells.
   a. 1 drop (50μl) of sample
   b. 1 drop of positive control
   c. 1 drop of negative control
4. Place 1 drop of antigen suspension in each cell
5. Tilt the test card back and forth slowly for 8 minutes or place the card on an automated rotator and rotate at 100 rpm for 8 minutes.
6. Immediately after 8 minutes rotation read the results macroscopically in direct light. A reactive result is indicated by large aggregates in the centre or periphery of the test circle. A non-reactive result will display a smooth, even appearance with no aggregates visible.

**Treponema pallidum haemagglutination assay**

1. Place 100 μl TPHA diluent in well 1 and 25 μl in well 2 and 3 each
2. Add 25 μl of serum sample or positive control, negative control to well 1.
3. Mix the contents of well 1 with a 25 μl micropipette and transfer 25 μl to well 2 (control well)
4. Mix and transfer 25 μl to well 3 (test well)
5. Mix and discard 25 μl from well 3
6. Add 75 μl of the carefully resuspended TPHA control cells to well 2 and 75 μl of the carefully resuspended TPHA test cells to well 3
7. Shake the plate gently to ensure the contents are thoroughly mixed
8. Place the plate on a white level surface. Leave for 45-60 minutes before reading the result

Interpretation:
Negative result – defined button of non-agglutinated cells, with or without a very small hole in the center
Positive result – partial or total agglutination of cells

Anticardiolipin antibodies
1. Bring the test sera to room temperature
2. Pipette 100 μl test serum dilutions, standards, positive and negative controls into respective wells.
3. Seal wells with adhesive foil and incubate at room temperature for 1 hour
4. Rinse the wells three times using washing buffer
5. Discard buffer and knock out residues on an absorbent paper
6. Pipette 100 μl HRP-conjugate into each well
7. Seal wells with adhesive foil and incubate for 30 minutes at room temperature.
8. Rinse the wells three times using washing buffer
9. Discard buffer and knock out residues on an absorbent paper
10. Pipette 100 μl TMB solution into each well
11. Incubate for 10 minutes at room temperature in the dark.
12. Pipette 100 μl stopping solution per well
13. Measure at 450 nm within next 30 minutes
Interpretation:
A cut-off value of 48 U/ml was used, according to the manufacturer’s recommendations

Anti-β2-glycoprotein I antibodies

1. Prepare a 1:50 dilution of the calibrators, controls and patient samples in sample diluent
2. Add 100 μl of diluted calibrators, controls and patient samples to the appropriate microwells.
3. Incubate at room temperature for 15 minutes
4. Empty the microwells and wash four times with wash solution. Blot on absorbent paper to remove residual wash fluid.
5. Add 100 μl anti-human IgG HRP-Conjugated Antibody Solution to the wells
6. Incubate for 15 minutes at room temperature.
7. Wash 4 times with wash solution and drain on absorbent paper.
8. Add 100 μl One-Component Substrate to each well and incubate for 10 minutes at room temperature.
9. Add 100 μl Stopping Solution to each well to stop the enzyme reaction
10. Blank the plate reader and read the optical density of each well at 450 nm, within 5 minutes of addition of the Stopping solution

Interpretation:

Anti-β2GPI concentration of the sample = Conversion factor X Absorbance (O.D) of sample

A cut-off value of 20 G units was used, according to the manufacturer’s recommendations

Biochemical tests

Serum creatinine and ALT tests were performed using an automated chemistry analyser (Olympus AU 640). Mindray creatinine kit was used for the Serum creatinine assay, and Mindray ALT kit for the ALT assay.
Validation of the ELISA tests
This was done for the anticardiolipin and anti-beta_{2}-glycoprotein 1 ELISA assays.
Intra-run precision or reproducibility of results was ascertained using control materials, to ensure that the coefficient of variation was less than 10%. Precision of the assay was checked before testing the study participants’ samples. Accuracy or true value was established by running 2 levels of control materials.
Linearity, or ability of the test to obtain results that vary in a manner directly proportional to changes in the concentration of analyte in the sample was provided by the manufacturer, but was also validated to ensure that the test was linear at the upper and lower assay limits. A sample that was above the assay limit was diluted down to the lower assay limit.
APPENDIX 7: ETHICAL APPROVAL

KENYATTA NATIONAL HOSPITAL
Hospital Rd. along, Ngong Rd.
P.O. Box 20723, Nairobi.
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP*, Nairobi.
Email: KNHplan@Ken.Healthnet.org
23rd November 2010

Ref: KNH-ERC/ A/840

Dr. Anne Kasyoka Barasa
Dept.of Human Pathology
School of Medicine
University of Nairobi

Dear Dr. Barasa

RESEARCH PROPOSAL: “ANTIPHOSPHOLIPID ANTIBODIES IN PATIENTS WITH VENOUS THROMBOSIS AT KENYATTA NATIONAL HOSPITAL” (P272/08/2010)

This is to inform you that the KNH/UON-Ethics & Research Committee has reviewed and approved your above revised research proposal for the period 23rd November 2010 to 22nd November 2011.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimens must also be obtained from KNH/UON-Ethics & Research Committee for each batch.

On behalf of the Committee, I wish you a fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of the data base that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely,

[Signature]

PROF A N GUANTAI
SECRETARY, KNH/UON-ERC

cc. The Deputy Director CS, KNH
    The HOD, Records, KNH
    The Dean, School of Medicine, UON
    The Chairman, Dept.of Human Pathology, UON
    Supervisors: Prof. Walter O. Mwanda, Dept.of Human Pathology, UON
    Dr. Grace W. Kitonyi, Dept.of Human Pathology, UON
## APPENDIX 8: REFERENCE RANGES AND UNITS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rbc count</td>
<td>$x10^9/L$</td>
<td>Female: 4.3 ± 0.5; Male: 5.0 ± 0.5</td>
</tr>
<tr>
<td>Wbc count</td>
<td>$x10^9/L$</td>
<td>4.0 – 10.0</td>
</tr>
<tr>
<td>Platelet count</td>
<td>$x10^9/L$</td>
<td>150 - 400</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>g/dL</td>
<td>Female: 13.5 ± 1.5; Male: 15.0 ± 2.0</td>
</tr>
<tr>
<td>ESR</td>
<td>mm/hr</td>
<td>upto 15</td>
</tr>
<tr>
<td>Reticulocyte count</td>
<td>%</td>
<td>0.5 – 2.5</td>
</tr>
<tr>
<td>PT</td>
<td>Seconds</td>
<td>12.8 – 14.8</td>
</tr>
<tr>
<td>KCT</td>
<td>Rosner Index (RI)</td>
<td>&lt;0.16</td>
</tr>
<tr>
<td>APTT</td>
<td>Seconds</td>
<td>28.3-38.3</td>
</tr>
<tr>
<td>DRVVT</td>
<td>Normalized Ratio (NR)</td>
<td>&lt;1.3</td>
</tr>
<tr>
<td>VDRL</td>
<td>Negative or positive</td>
<td>_</td>
</tr>
<tr>
<td>TPHA</td>
<td>Negative or positive</td>
<td>_</td>
</tr>
<tr>
<td>ACL IgG</td>
<td>Units/mL</td>
<td>&gt;48: positive</td>
</tr>
<tr>
<td>Anti-β2-GP1 IgG</td>
<td>G units</td>
<td>&gt;20: positive</td>
</tr>
<tr>
<td>Creatinine</td>
<td>μmol/L</td>
<td>Female: 40-110; Male: 60-130</td>
</tr>
<tr>
<td>ALT</td>
<td>IU/L</td>
<td>Upto 40</td>
</tr>
</tbody>
</table>