Guidelines on testing for the lupus anticoagulant

Lupus Anticoagulant Working Party on behalf of the BCSH Haemostasis and Thrombosis Task Force

In 1988 the results of a questionnaire from the Lupus Anticoagulation Working Party for the Haemostasis and Thrombosis Task Force showed that there was considerable pre-analytical and analytical variability among United Kingdom laboratories which perform tests for the lupus anticoagulant. In a subsequent quality control exercise these variables influenced the success of the various tests in identifying the presence of such inhibitors. One hundred British laboratories participated in a further exercise, using standardised methodology for two tests—namely, the dilute Russell's viper venom time (DRVVT) and the kaolin clotting time (KCT). This improved the rate of correct detection of lupus anticoagulant compared with the earlier study. As a result of these observations, methodological guidelines for laboratories wishing to test for the presence of lupus anticoagulatant were formulated.

The detection and positive identification of the lupus-like anticoagulant has become an important procedure for routine coagulation laboratories. Lupus anticoagulant is associated with arterial and venous thromboembolism and neurological disease. It has also been implicated in recurrent spontaneous abortion.[1 2] These inhibitors usually prolong the activated partial thromboplastin time (APTT),[3] and investigation for lupus anticoagulant is often prompted by an unexplained prolonged APTT result.

The activity of the inhibitor seems to be directed towards coagulation active phospholipid complexes in the coagulation cascade. Several phospholipid dependent coagulation tests have been advocated as being more sensitive and specific than the APTT, but there is no consensus on the most appropriate laboratory method. Anticardiolipin antibodies (ACA) have also been shown to be associated with lupus anticoagulant.[1 2]

The unrelated behaviour of lupus anticoagulant and ACA in the course of disease and in individual patients indicates that both assays are required when the antiphospholipid syndrome is suspected. Standardisation of methods for ACA assays has been recommended and has recently been reviewed.[4] A Working Party of the International Society on Thrombosis and Haemostasis on acquired inhibitors of coagulation made recommendations regarding definition and test procedures in 1983[5] but recent evidence suggests that a significant number of patients will be misdiagnosed using these criteria. Any definition of the lupus anticoagulant must include the phospholipid dependency of the inhibitory activity in clotting testing and the relative correction by lysed platelets or increased phopholipid concentration (table 1). The definition is now being reviewed by the Lupus Anticoagulant Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis and is soon to be published.

In view of the undoubted clinical importance of lupus anticoagulants and the lack of standardisation in their detection[6] a detailed national United Kingdom survey and quality control exercises in lupus anticoagulant testing have been undertaken.[7]

Factors which influence the performance of these tests have been identified and standardised methodology evaluated. As a result of these studies recommendations for standardised procedures for testing for lupus anticoagulants have been formulated and these are set out below.

Background

Lupus anticoagulant is frequently requested in routine coagulation laboratories. The methods of sample collection and handling before testing strongly influence lupus anticoagulant results. Inadequate removal of platelets in the test plasma adversely affects test results[7-10] and, furthermore, tests are frequently performed on frozen samples which inevitably leads to the presence of platelet fragments and lupus anticoagulant bypassing activity if the original plasma is not platelet free. Filtering or double centrifugation seems desirable.[7-9]

Various methods have been proposed but the APTT is the most frequently used screening test for lupus anticoagulant.[7] Studies have shown that sensitivity to the lupus anticoagulant defect varies considerably with different APTT reagents.[3 11] Reagents with low phospholipid content are the most sensitive.[3 11 12] Control and patient mixtures are often performed but a weak lupus anticoagulant defect may be corrected by a 50/50 mixture. These findings were confirmed in the recent United Kingdom survey.[7] The Austen and Rhymes modification of the APTT[13] using aluminium hydroxide absorption and heat stability has not proved a reliable test.[7] The

Table 1 Criteria for lupus anticoagulants

1	Prolongation of a phopholipid dependent clotting test
2	Clotting time of a mixture of test and normal plasma should be longer than the clotting time of normal plasma
3	There should be a relative correction of the defect by the addition of lysed platelets or phospholipids

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Dilute Thromboplastin Time Test[14] (tissue thromboplastin inhibition test) is prolonged by factor deficiences as well as the lupus anticoagulation defect[15] and sensitivity depends on thromboplastin dilution.[7] Some IgM lupus anticoagulants do not prolong this test, although they do prolong others.[16] In a recent review[17] the lack of specificity of the Dilute Thromboplastin Time Test was noted. The Kaolin clotting test (KCT)[18] and Dilute Russell's Viper Venom Test (DRVVT)[11] are particularly sensitive to lupus anticoagulants.[20 21] The mixture of normal and test plasma in the KCT offers some degree of specificity. The platelet correction procedure with the DRVVT using freeze-thawed or lyophilised platelets offers a good degree of specificity. This platelet correction procedure can also be used with the APTT[15 22] but experience with this test is limited.

Recommended methods

Conditions where testing for the lupus anticoagulant may be required to assist in diagnosis and management are listed in table 2.

SAMPLE COLLECTION AND HANDLING

Careful blood collection using a 19 gauge needle and minimal stasis is advised to avoid platelet activation. Blood should be processed as soon as possible and ideally within one hour of collection. It is important to obtain plasma with a platelet count of less than 10×10^9/l and to achieve this it is suggested that either double centrifugation or filtration is used.

Double centrifugation

(1) Platelet poor plasma is prepared by centrifuging citrated blood at $2000 \times g$ for 10 minutes, then removing the plasma avoiding the plasma buffy coat interface, and transferring to a plastic tube.

(2) The plasma is then recentrifuged at $2000 \times g$ for 10 minutes (or ideally in a microcentrifuge at $10\,000 \times g$ for five minutes) and the plasma again removed avoiding the interface.

Table 2 Situations in which lupus anticoagulant screening may be indicated

Venous thromboembolic disease, especially:
Spontaneous venous thrombosis at age younger than 40 years
Recurrent venous thrombosis
Unusual venous thrombosis, such as Budd-Chiari syndrome
Thromboembolic pulmonary hypertension
Arterial thrombotic disease, particularly:
Unexplained arterial occlusion at younger than 30 years
Unusual cerebrovascular events
Other conditions:
Recurrent unexplained fetal loss and early severe pre-eclampsia
Systemic lupus erythematosus and some other collagen vascular disorders
Immune thrombocytopenic purpura
Livedo reticularis
And
False positive serological tests for syphilis
Unexplained prolonged APTT; undue sensitivity of APTT to heparin

Filtration

Slow filtration of PPP through a 0·22 μm cellulose acetate syringe filter is adequate (Minisart R, Sartorius Ltd, GB-Belmont, Surrey, or Anotec, Banbury, Oxford). Where possible tests should be performed on fresh plasma. When frozen plasma is used rigorous care in preparation of the fresh platelet free plasma is advised.

Normal control plasma must be carefully prepared in a similar way to the test plasma. Commercial normal plasmas might not be free of platelet fragments and may therefore be unsuitable. Advice and specifications should be obtained from the manufacturer.

TEST PROCEDURES

These inhibitors are heterogeneous in their behaviour in phospholipid dependent coagulation tests and no single test for their identification is sufficient. At least two tests are advisable, one of which could be the screening test, the APTT. A flow diagram for the laboratory investigation for lupus anticoagulant is given in the figure.

A coagulation screen including prothrombin time, APTT, with thrombin time or fibrinogen estimation, is required before proceeding to lupus anticoagulant testing to exclude abnormalities unrelated to lupus anticoagulant.

SCREENING WITH APTT

The APTT should be performed on freshly prepared patient PPP, on pooled normal PPP, and on a mixture of four parts patient PPP to one part normal PPP (80%:20% mixture). Even the most sensitive APTT method will not detect all inhibitors and so an additional specific test should be performed in suspected cases of lupus anticoagulant, even if the APTT is normal.

CONFIRMATORY TESTS

These must confirm that the inhibitor activity is due to lupus anticoagulant, directed against procoagulant phospholipids, and not to an inhibitor to a single clotting factor. Though some degree of specificity can be achieved using mixtures of patient and control plasmas, better specificity is provided by use of a platelet correction procedure.

For two tests, the KCT and the DRVVT, standardised methodology has been shown to improve performance (table 3) and is therefore recommended (see below). For other tests, firm data evaluating their performance as regards specificity are still awaited and so these tests have not been included in the present recommendations.

Kaolin clotting time[18]

(A) REAGENTS AND MATERIALS

Plastic or glass coagulation tubes—for example, 75 × 10 mm polystyrene.

$CaCl_2$ (0·025 M).

Owren's Buffer

5·825 g sodium diethylbarbiturate

7·335 g sodium chloride

Dissolve in 750 ml distilled water

Add 0·1M hydrochloric acid to give pH 7·35
Adjust volume to 1000 ml with distilled water
Kaolin (20 mg/ml in Owren's buffer, pH 7·35)
Normal control plasma

(B) TEST PROCEDURE
Perform tests in duplicate on normal plasma, test plasma, and on a 1:4 mixture of test and normal plasma. A full curve is not essential, but the ratios of test to normal and 1 to 4 parts mixture to normal are calculated, as shown below:

$$\text{test ratio} = \frac{\text{test (seconds)}}{\text{normal (seconds)}}$$

$$\text{mixture ratio} = \frac{\text{1:4 mixture (seconds)}}{\text{normal (seconds)}}$$

1 Place 0·2 ml plasma in the plastic tube at 37°C.
2 Add 0·1 ml kaolin and tilt three times. Incubate for three minutes at 37°C.
3 Add 0·2 ml $CaCl_2$, start stopwatch, and tilt three times.
4 At 60 seconds slowly tilt and record time of end point.

(C) INTERPRETATION
A test ratio of more than 1·2 indicates an abnormal result; a mixture ratio of > 1·2 should be considered a positive result for lupus anticoagulant; and a ratio between 1·1-1·2 equivocal.
NB A control time of less than 60 seconds suggests contamination of the normal control plasma with platelet fragments, and invalidates the results.

Dilute Russell's Viper Venom Test (DRVVT) and Platelet Correction Procedure (PCP)[19]

(A) REAGENTS AND MATERIALS
Glass tubes (75 × 10 mm rimless)
$CaCl_2$ (0·025M)
Imidazole buffer (0·05M, pH 7·3)
3·4 g imidazole (Glyoxaline)
5·85 g NaCl
Dissolve in 900 ml distilled water
Adjust pH with HCl
Make volume to 1000 ml with distilled water
100 mM EDTA in buffer
3·74 g Na_2 EDTA diluted in 100ml of buffer to achieve this concentration
Imidazole buffer with albumin:
Dissolve 0·1 g bovine serum albumin (fraction V, 99% pure—for example, Sigma Chemical Co Ltd, Dorset, Poole) in 10 ml imidazole buffer (pH7·3). Store at −20°C and then thaw for use, or use during the working day.
Calcium free Tyrode's buffer (pH 6·5):
8·0 g NaCl
0·2 g KCl
0·065 g $NaH_2PO4_4 . 2H_2O$
0·415 g $MgCl_2 . 6H_2O$
1·0 g $NaHCO_3$
Dissolve in 900 ml distilled water adjust pH bring to one litre 1μg Iloprost (Schering, UK) or Epoprostenol (Wellcome, Dartford, Essex) diluted one in 100 in buffer
(that is, 10 ng/ml)
TRIS buffered saline:
Stock solution
60·5 g TRIS per litre water with HCl to pH 7·6
Working buffer
Dilute stock solution 1 in 10 in 0·15 M saline
10 mM EDTA in buffer
0·37 g Na_2 EDTA in 100 ml buffer
Russell's Viper Venom (Diagnostic Reagents,

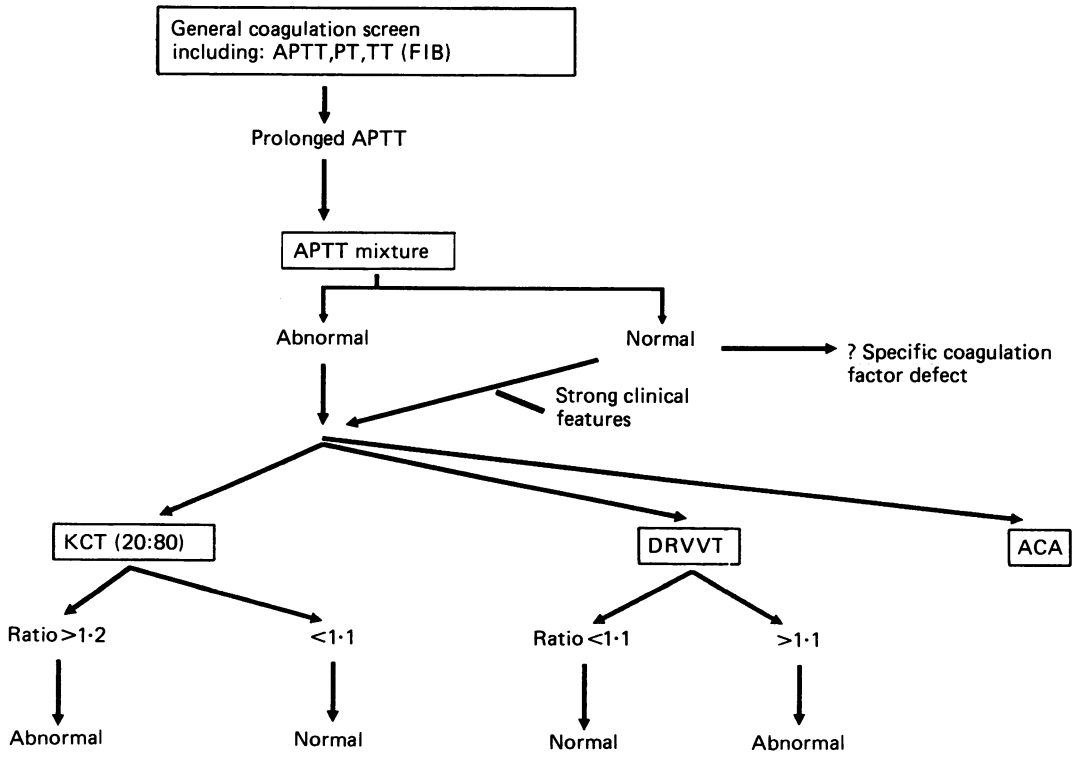

Figure 1 Flow diagram for laboratory investigation when lupus anticoagulant is suspected. To confirm the presence of lupus anticoagulant two positive tests are advisable, one of which could be the screening APTT test.

Table 3 DRVVT interpretations in United Kingdom lupus anticoagulant surveys

Plasma	01	02	03	04	05	06
Defect	Weak	Strong	Absent	Absent	Moderate	Moderate
Methodology	Non-standardised			Standardised		
Proportion correct	42%	61%	55%	93%	87%	66%
KCT interpretations in United Kingdom lupus anticoagulant surveys						
Plasma	01	02	03	04	05	06
Defect	Weak	Strong	Absent	Absent	Moderate	Moderate
Methodology	Non-standardised			Standardised		
Proportion correct	43%	83%	67%	98%	81%	85%

Plasmas 01, 02, and 03 were included in the first United Kingdom Lupus quality control survey.[6] KCT and DRVVT results from this survey are tabulated.

In a second exercise three further lyophilised plasmas 04, 05, 06 were included which were, respectively, normal and two moderate intensity lupus positive samples. Participants were requested to test by KCT or DRVVT with standardised methodology using method sheets included in the survey. These results are tabulated for comparison with the results of the first survey. The standardised methods form the basis for the recommendations in these guidelines for KCT and DRVVT.

Thame, Oxford; Wellcome Diagnostics, or Sigma Chemical Co. Ltd. All are suitable). Reconstitute to give a stock solution containing 1 mg/ml. Store in 20 μl aliquots at -20°C or below.

Phospholipid—use cephalin, such as Diagen "Bell and Alton", Phospholipid Reagent, Diagnostic Reagents, Ltd, UK, which does not contain activator, from a sensitive APTT method. Reconstitute according to the manufacturer's instructions for use in the APTT.

(B) REAGENT PREPARATION

1 *Dilute RVV*

The stock solution is thawed and 10 μl is added to 5 ml imidazole buffer with albumin. The venom concentration is further adjusted to give a RVV clotting time of between 30-35 seconds in a mixture of 0·1ml of RVV, 0·1 ml normal control PPP, 0·1 ml of undiluted phospholipid and 0·1 ml $CaCl_2$ at 37°C. Store the RVV solution on ice and use within four hours.

2 *Dilute phospholipid*

The RVV test is repeated using normal control PPP, dilute RVV, and phospholipid diluted in imidazole buffer 1 in 2, 1 in 4, 1 in 8 and 1 in 16. From these results, a dilution of phospholipid is selected which gives a DRVV time of between 35-40 seconds (two to five seconds greater than the time with undiluted phospholipid). This is subsequently used in testing normal control and patients plasmas.

3 *Freeze thawed washed platelets*

Platelet rich plasma (PRP) is prepared by centrifuging citrated whole blood at $170 \times g$ for 10 minutes. The supernatant PRP is carefully removed and placed in a plastic tube. The platelets are washed three times in either (i) calcium free Tyrode's buffer plus 10 ng/ml Iloprost, or epoprostenol and 10 mM EDTA; or (ii) imidazole buffer (pH7·3) plus 10mM EDTA; or (iii) TRIS buffered saline (TBS)(pH7·6) plus 10mM EDTA, by repeated suspension in buffer, followed by recentrifugation at $2000 \times g$ for 10 minutes. Finally they are resuspended at a concentration of $200\text{-}500 \times 10^9$ in the selected buffer without Iloprost, epoprostenol, or EDTA and stored in plastic phials at -20°C or below. It is advisable to dilute the PRP 1 in 2 with buffer (and inhibitors where appropiate) before the first centrifugation step. The platelets are rapidly thawed for use, mixed well, and used in place of the dilute phospholipid in the DRVVT as part of the DRVVT platelet correction procedure (PCP). Some commercial platelet preparations—for example, Biodata Platelet Extract Reagent, Lep Scientific, Milton Keynes—are available, which are designed for use in lupus anticoagulant tests.

(C) DRVVT AND PCP METHOD

Into clean glass tubes at 37°C, pipette:
1 0·1 ml diluted phospholipid
2 0·1 ml normal control plasma
Mix and incubate for 30 seconds
3 0·1 ml dilute RVV reagent
Incubate for exactly 30 seconds then add:
4 0·1 ml $CaCl_2$ and time clot formation
5 Repeat steps 1-4 with patient plasma
6 If the result with patient plasma is longer than that with normal control plasma repeat steps 1-4 for normal and patient plasmas, substituting washed freeze-thawed platelets for the dilute phospholipid reagent.
7 Calculate the ratio of patient clotting times to normal clotting times for both DRVVT and PCP procedures.

(D) INTERPRETATION

1 Normal ratio 0·9-1·09
2 DRVVT ratios of $>1{\cdot}1$ should be retested using the PCP, and a significant shortening (10%) of the DRVVT is suggestive of the presence of lupus anticoagulant.
3 A normal control plasma must be tested with each batch of patient plasmas, and should be repeated at regular intervals (at least every hour) to check for loss of activity of the RVV reagent.

Lupus anticoagulant testing in the presence of anticoagulation treatment

There are no reliable methods for testing for the presence of the lupus anticoagulant when the patient is receiving heparin or oral anticoagulants. If the patient is receiving heparin testing should be delayed until treatment with heparin has stopped. Mixtures of patient and normal plasma may correct the coumarin defect, without neutralising the lupus anticoagulant inhibitory activity. Consequently a 50% normal to 50% test mixture giving a ratio of more than 1·1 with the

DRVVT is suggestive of the presence of lupus anticoagulant. Similarly, a 80% normal to 20% test mixture giving a ratio of more than 1·2 with KCT suggests the presence of the lupus anticoagulant inhibitor. Firm conclusions cannot be made, however, unless testing is repeated after discontinuing oral anticoagulation.

Discussion

The clinical diversity of the primary antiphospholipid syndrome has recently become widely recognised[23] and the identification of the lupus anticoagulant is important in the diagnosis and management of this condition. Consequently lupus anticoagulant testing has become an essential routine procedure for haemostasis laboratories. Nevertheless, there is considerable controversy about the most appropriate methods for detecting lupus anticoagulant.[17] Further problems were highlighted in the first United Kingdom national survey by the Lupus Anticoagulant Working Party when three freeze dried samples were distributed to 183 laboratories.[7] These problems included preanalytical and analytical factors as well as choice of test type. Based on these results the methodology for two of the most widely used confirmatory tests were specified as described above.

The benefits of this approach for improving the identification and interpretation of the lupus anticoagulant test were shown when a second group of three freeze dried samples were distributed to routine laboratories. Standardisation of the two confirmatory tests led to considerable improvement in the correct identification of negative and moderately positive lupus anticoagulant samples. The relative ease with which this methodology can be introduced into a laboratory was shown by a successful wet workshop when over 60 participants performed the standardised assays satisfactorily.

The main aim of these guidelines and the Lupus Anticoagulant Working Party is to encourage standardised methodology for laboratories wishing to test for lupus anticoagulant. The current recommendations suggest that laboratories should perform at least one standardised confirmatory test in addition to the APPT screening test.

Correct reporting of a positive lupus anticoagulant test will generate increased confidence in the diagnosis of the antiphospholipid syndrome. This will allow multicentre clinical trials to determine the incidence and treatment response of the thrombotic episodes and recurrent fetal loss which are associated with this condition.

Addendum

The Scientific and Standardization Committee of the ISTH has recently published the following guidelines: Exner T, Triplett DA, Taberner D, Machin ST. Guidelines for testing and revised criteria for lupus anticoagulants. *Thromb Haemostas* 1991;**65**:320–2.

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