A new-generation dilute Russell's viper venom time assay system for lupus anticoagulants: evaluation of detection utilising frozen reagents and controls

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ABSTRACT

The dilute Russell's viper venom time (DRVVT) is one of the most widely used assays to detect lupus anticoagulants (LAs). Variation in diagnostic performance exists between DRVVT reagents from various manufacturers due to a variety of factors such as antibody heterogeneity, reagent phospholipid composition, venom heterogeneity, assay methodology and analytical technique. Recently, a new-generation DRVVT assay system has become available that utilises frozen reagents and controls that offer potential benefits to the diagnostic laboratory in terms of reagent quality and convenience of use. This study evaluates the diagnostic and analytical performance of these CryoCheck reagents and controls on a commonly employed automated coagulation analyser, the Sysmex CA 1500. Sensitivity is assessed by analysis of 60 samples shown to contain LAs by combinations of an alternative DRVVT, LA-sensitive dilute activated partial thromboplastin time and activated seven lupus anticoagulant assay. Specificity is assessed using 30 samples negative for LA, eight plasmas from non-LA orally anticoagulated patients and also immunodepleted factor-deficient plasmas. The CryoCheck reagents generated comparable diagnostic performance data to that previously reported for other reagents. There was a marked improvement in sensitivity when the BCSH recommended percent correction of ratio calculation for assessment of phospholipid dependence was employed in place of the manufacturer suggested test/confirm ratio. Slightly better diagnostic performance was achieved when using a frozen pooled normal control in place of a lyophilised normal control to generate sample/control ratios, giving sensitivity, specificity, positive predictive and negative predictive values of 88.2%, 86.8%, 90% and 85.2%, respectively. The combination of CryoCheck reagents and the Sysmex CA 1500 analyser provides a sensitive and specific LA detection technique comparable to those currently available.

KEY WORDS: Dilute Russell's viper venom time.
Lupus anticoagulants.
Frozen reagents.

Introduction

Lupus anticoagulants (LAs) comprise part of the heterogeneous spectrum of acquired autoantibodies termed antiphospholipid antibodies (APA).[1] The occurrence and persistence of APAs are associated with a wide range of clinical signs and symptoms, the most common of which are arterial and venous thrombosis, recurrent fetal loss and thrombocytopenia.[2]

Lupus anticoagulants are identified by their interference with one or more phospholipid-dependent coagulation assays[3] commonly involving sensitive screening tests that incorporate dilute phospholipid to accentuate their *in vitro* anticoagulant effect. Confirmation that an abnormal result is due to an LA is usually achieved by using a high phospholipid concentration in the same assay system that swamps, bypasses or neutralises the LA sufficiently to demonstrate a significant reduction of the screening test result. Mixing tests with normal plasma can demonstrate the inhibitory nature of LAs.

The most commonly used assay for LA detection in the UK is the dilute Russell's viper venom time (DRVVT),[4] which is widely available by automated analysis. However, the diagnostic performance of commercial DRVVT kits varies between reagents;[5] a situation that is compounded by local variations in data assessment.[6]

The present study assesses the diagnostic and analytical performance of a recently introduced frozen DRVVT reagent system, CryoCheck LA Check and LA Sure, on a Sysmex CA1500 analyser against an established DRVVT reagent, an LA-sensitive dilute activated partial thromboplastin time (DAPTT) and an activated seven lupus anticoagulant (ASLA) assay.

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Table 1. Result interpretations with clinical samples.

	Known LAs (*n*=60)		LA negative (*n*=30)		LA negative (OA) (*n*=8)		Factor-deficient plasmas (*n*=8)	
Interpretation	LA pos	LA neg	LA pos	LA neg	LA pos	LA neg	LA pos	LA neg
% correction (lyophilised NP)	48	12	0	30	6	2	1	7
% correction (frozen NP)	52	8	1	29	6	2	0	8
LA Check/LA Sure	35	25	0	30	6	2	0	8

OA: orally anticoagulated; NP: normal plasma.

Materials and methods

Blood collection, manipulation and storage

Blood was collected by venepuncture into evacuated siliconised glass tubes (Becton-Dickinson Vacutainer System, New Jersey, USA) where nine parts blood was mixed with one part 0.105 mol/L sodium citrate. Plasma for LA screening was prepared by double centrifugation to obtain plasma with a platelet count $<10 \times 10^9$/L, as previously described.[7] The platelet-poor plasma for LA testing was stored at –70°C until use.

Coagulation screening tests

Coagulation screen comprising prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and Clauss fibrinogen were performed on a Sysmex CA 1500 (Sysmex UK, Milton Keynes, UK) using Innovin recombinant thromboplastin, Actin FS, Thromboclotin and Thrombin Reagent (Dade-Behring, Marburg, Germany). Coagulation screens were performed to assess ongoing anticoagulant therapy and to help exclude coagulopathies that could mask, mimic or co-exist with LAs.

Lupus anticoagulant assays

Initial LA screening was undertaken on the Sysmex CA1500 analyser using two assays. Dilute Russell's viper venom time was performed with Gradipore LA Screen and LA Confirm reagents (BioMérieux UK, Basingstoke, UK). Dilute activated partial thromboplastin time was performed using PTT-LA (Diagnostica Stago, Asniéres, France) in the screen with a platelet neutralisation procedure employing Biodata Platelet Extract Reagent (Alpha Laboratories, Hampshire, UK) in the confirmatory test.

Samples negative by these assays were subsequently analysed using the ASLA assay,[8] which was performed using rFVIIa (NovoSeven, Novo Nordisk A/S, Bagsvaerd, Denmark) reconstituted to 0.55 mg/mL and then diluted 1 in 8 in 0.05 mol/L imidazole buffer (pH 7.3) using Bell and Alton platelet substitute (Diagnostic Reagents, Thame, UK) diluted 1 in 4 in imidazole buffer for the screen, and the Platelet Extract Reagent for the confirmatory test.

Samples positive by DRVVT and/or DAPTT with sufficient surplus plasma also received ASLA testing. All elevated screens received the confirmatory test, plus a screen and confirmatory test on equal-volume mixing studies with normal plasma to detect inhibition.[9,10] Lyophilised platelet-poor plasma (Technoclone, Dorking, UK) was used as the normal plasma throughout, as it is manufactured specifically to be sufficiently platelet-free for use in LA assays.

Results were assessed for the presence of LAs according to published guidelines and previous reports by converting clotting times to ratios in relation to the normal plasma. A ≥ 10% correction of an elevated screen ratio by the confirmatory test ratio was regarded as indicative of the presence of an LA after exclusion of other causes of prolonged clotting times.[5–10]

The CryoCheck LA Check and LA Sure (Precision Biologic, Dartmouth, Canada) screen and confirm tests were performed on the Sysmex CA1500 by adding 75μL reagent to an equal volume of plasma at 37°C and recording the clotting time. Mixing studies were not performed due to insufficient quantities of test plasmas. CryoCheck frozen pooled normal plasma, lupus-positive control and weak lupus-positive control were used with the LA Check and LA Sure reagents.

Reference ranges

Reference ranges in ratio format for the established assays had been derived locally.[6] The CryoCheck manufacturer recommends a reference range for LA Check using clotting times, and if the clotting time is more than two standard deviations (2SD) above the locally derived mean normal, the confirmatory test should be performed. Assessment for the presence of an LA is achieved by calculating the LA Check/LA Sure ratio (LA Check clotting time/LA Sure clotting time). If this ratio is greater than the upper limit of a locally derived 2SD reference range, the test is positive for LA.

Reference ranges for clotting times, LA Check/LA Sure ratio, and ratios against normal plasma using either the lyophilised platelet-poor plasma or the frozen CryoCheck pooled normal plasma were generated from 37 donor plasmas with normal coagulation screens and no evidence of haemostatic disease.

Evaluation with clinical samples

CryoCheck DRVVT testing was performed on 60 plasma samples confirmed to contain LAs by either Gradipore DRVVT and/or DAPTT and/or ASLA to assess sensitivity. Specificity was evaluated using 30 plasma samples taken from routine thrombophilia investigations with normal coagulation screens and negative for LA by Gradipore DRVVT, DAPTT and ASLA; eight plasma samples from orally anticoagulated non-LA patients (international normalised ratio [INR] range: 1.81–7.03, mean: 3.15) and a series of lyophilised Dade-Behring immunodepleted plasmas separately deficient in factors II, V, VII, VIII, IX, X, XI and XII.

Table 2. Positive interpretations for each calculation in relation to the primary detection methods.

	DAPTT DRVVT ASLA (*n*=7)	DAPTT DRVVT (*n*=25)	DRVVT ASLA (*n*=1)	DAPTT only (*n*=2)	DRVVT only (*n*=17)	ASLA only (*n*=8)
% correction (lyophilised NP)	7	23	0	0	14	4
% correction (frozen NP)	7	24	1	1	15	4
LA Check/LA Sure	7	16	0	0	8	4

The 60 known LA-positive samples are divided into groups based on the detecting primary assay(s).
NP: normal plasma.

Table 3. Diagnostic performance data.

	% correction of ratio (lyophilised NP)	% correction of ratio (frozen NP)	LA Check/LA Sure
Sensitivity (%)	83.3	88.2	70.1
Specificity (%)	86.8	86.8	88.5
PPV (%)	90.0	90.0	90.1
NPV (%)	79.3	85.2	64.8

Diagnostic performance data is based on interpretation of screen and confirm results.
NP: normal plasma; PPV: positive predictive value; NPV: negative predictive value.

Results

Reference ranges

The reference range for clotting times(s) for LA Check was 34.9–48.1 (mean: 41.5), and for LA Sure was 30.7–35.8 (mean: 33.3). LA Check ratio reference range with the lyophilised plasma was 0.88–1.20, (mean: 1.04), and with the frozen plasma was 0.85–1.20 (mean: 1.03). LA Sure ratio reference range with the lyophilised plasma was 0.91–1.10 (mean: 1.01), and with the frozen plasma was 0.84– 1.00, (mean: 0.92). The LA Check:LA Sure ratio reference range was 1.07–1.40 (mean: 1.24). All reference ranges were calculated as ± 2SD of the mean.[10]

Testing Cryocheck DRVVT reagents with clinical samples

Numbers of positive and negative interpretations for the presence of LAs using either the lyophilised normal plasma or the frozen normal plasma to generate screen and confirm ratios, or the LA Check:LA Sure ratio for all the clinical samples, are presented in Table 1. Numbers of positive interpretations for each calculation in relation to the primary detection methods are shown in Table 2. Dilute Russell's viper venom time screening ratios for LA-positive results using the lyophilised normal plasma ranged from 1.23 to 2.86 (mean ratio: 1.6). The range when using the frozen normal plasma was 1.23–2.97 (mean ratio: 1.62). Samples positive for LA by the LA Check:LA Sure ratio gave a range of 1.41–2.66 (mean ratio: 1.78). Diagnostic performance data are given in Table 3.

LA Check and LA Sure assay performance characteristics

Intra- and inter-assay coefficients of variation (CV) of ratios for the lyophilised normal plasma, frozen normal plasma and frozen weak and strong lupus anticoagulant controls are presented in Table 4. Inter-batch variability was not assessed. The manufacturers claim that the reagents can be refrozen and thawed once. Testing fresh samples of the CryoCheck weak LA-positive and LA-positive plasmas on a set of LA Check and LA Sure reagents that had been frozen and thawed at time points of zero, 1, 2, 5, 6 and 7 h showed little deterioration in diagnostic performance (Table 5). Strict instructions for thawing the reagents are provided by the manufacturer and these were adhered to during the study to assess the reagents under these conditions. No cryoprecipitate formation was apparent on thawing the control plasmas.

Frozen positive control samples

The mean clotting times for the weak lupus-positive control with LA Check and LA Sure taken from seven separate runs were 65.9 sec (range: 64.7–67.3) and 38.6 sec (range: 38.1–38.9), respectively. Mean ratios using the lyophilised normal plasma were 1.69 (range: 1.66–1.72) and 1.14 (range: 1.13–1.15), respectively, and using the frozen normal plasma were 1.71 (range: 1.55–1.77) and 1.08 (range: 0.91–1.14), respectively. The mean LA Check:LA Sure ratio was 1.71 (range: 1.67–1.74). The mean clotting times for the lupus-positive control with LA Check and LA Sure taken from the same runs were 109.5 sec (range: 105.9–110.9) and 43.1 sec (range: 42.6–43.5), respectively. Mean ratios using the lyophilised normal plasma were 2.81 (range: 2.72–2.86) and 1.27 (range 1.25–1.32), respectively, and using the frozen normal plasma were 2.83 (range: 2.55–2.97) and 1.24 (range 1.17–1.29), respectively. The mean LA Check:LA Sure ratio was 2.54 (range: 2.49–2.59).

Table 4. Assay performance characteristics for LA Check and LA Sure on the Sysmex CA1500.

	Intra-assay values			Inter-assay values		
	n	LA Check CV (%)	LA Sure CV (%)	*n*	LA Check CV (%)	LA Sure CV (%)
Lyophilised normal plasma	32	1.4	1.6	7	1.2	0.9
Frozen normal plasma	31	3.6	2.7	7	6.1	3.4
Frozen weak LA control	31	1.7	1.4	7	4.8	7.6
Frozen strong LA control	11	3.5	1.9	7	5.6	3.5

LA: lupus anticoagulant; CV: coefficient of variation.

Table 5. Results for CryoCheck LA-positive control plasmas using repeatedly frozen and thawed LA Check and LA Sure reagents.

		Lyophilised normal control		Frozen normal control		
Time point (h)	Control type type	LA Check (ratio)	LA Sure (ratio)	LA Check (ratio)	LA Sure (ratio)	*LA Check* LA Sure
0	Weak LPC	1.70	1.13	1.77	1.13	1.71
	LPC	2.72	1.25	2.83	1.24	2.49
1	Weak LPC	1.72	1.15	1.79	1.12	1.73
	LPC	2.76	1.28	2.88	1.25	2.49
2	Weak LPC	1.66	1.12	1.75	1.11	1.67
	LPC	2.64	1.23	2.78	1.21	2.43
5	Weak LPC	1.72	1.13	1.80	1.13	1.68
	LPC	2.79	1.24	2.91	1.24	2.47
6	Weak LPC	1.70	1.15	1.66	1.08	1.62
	LPC	2.67	1.23	2.62	1.16	2.37
7	Weak LPC	1.74	1.15	1.70	1.08	1.63
	LPC	2.66	1.24	2.59	1.16	2.32

Weak LPC: CryoCheck weak lupus-positive control.
LPC: CryoCheck lupus-positive control.

Discussion

As with many other DRVVT reagent kits, the CryoCheck screening reagent contains a low phospholipid concentration, with the confirmatory reagent utilising a higher concentration of the same phospholipid preparation. Dilute Russell's viper venom time reagents from other manufacturers exhibit variations in diagnostic performance and the phospholipid concentration and composition are crucial determinants in the sensitivity of assays to LA.[11–14]

Other factors that contribute to performance variability are the type of analytical equipment used,[5] methodological differences,[14–16] venom heterogeneity,[17] reference range generation[6] and the calculation used to assess phospholipid dependence.[6] The diagnostic performance of the LA Check/Sure reagents on the Sysmex CA1500 analyser was evaluated by employing locally derived reference ranges that were specific to the methodology and analyser, and the use of calculations recommended by the reagent manufacturer and of published guidelines.

The clotting times and ratios in relation to normal pool controls for normal plasmas obtained with the CryoCheck reagents, and hence the reference ranges using these data, were similar to those reported previously for other DRVVT reagents.[5,6,10,17,18] Clotting times that are excessively long or short would achieve inadequate discrimination between normal and abnormal samples, and the CryoCheck reagents appear to have been titrated to operate at similar clotting times to currently available reagents and recommended values.[10] However, the upper limit of normal for the LA Check:LA Sure ratio of 1.4 was markedly higher than the ratio of 1.2 suggested by the manufacturer, although this was derived on a different analyser. This test:confirm ratio has been shown to vary widely[6,10] and is not recommended in expert guidelines as it does not take into account variations in the clotting time obtained with normal pooled plasma, operator performance or reagent stability/quality.[10]

As no gold standard exists for LA detection, the results for any such study are relative to the primary detection assays employed. The three primary detection assays used covered each of the intrinsic, extrinsic and common pathways, and the reagents used have proved sensitive to LAs in previous studies.[5,8,14,19,20] These assays were performed and interpreted according to national[10] and international[9] guidelines.

Diagnostic performance data for the CryoCheck reagents were comparable to those reported for other commercially

available DRVVT reagent kits.[5,6,18] Sensitivity of the CryoCheck reagents for LA was significantly higher when using the percent correction of ratio calculation instead of the LA Check:LA Sure ratio, although specificity and positive predictive values were almost identical. Negative predictive value was significantly lower using the LA Check:LA Sure ratio. Use of the frozen normal plasma to generate ratios identified more LAs than did the lyophilised normal plasma, thus a slightly higher sensitivity value was generated.

Sensitivity of the CryoCheck reagents was assessed against plasmas positive for LAs in tests other than DRVVT, although only 10 of 60 were negative by the Gradipore DRVVT. The CryoCheck reagents detected LAs in five of these samples, which is a manifestation of the heterogeneity of the antibodies and differences between test systems. Both CryoCheck-positive controls generated consistently elevated screening test results, and markedly reduced results were seen in the confirmatory test, clearly indicating phospholipid dependence. However, the screening test ratios for the weak positive control were considerably higher than the reference range cut-off point and thus may not prove effective in checking assay performance for true weak antibodies with minimal screening test elevation.

Inter- and intra-assay CVs for each of the control plasmas indicated good analytical precision, although the values for the frozen normal plasma were conspicuously higher than those obtained with the lypohilised preparation. When using the ≥ 10% correction of ratio formula to demonstrate phospholipid dependence, it is crucial that intra-assay CVs for each of the screen and confirm reagents do not exceed 5%. Above this level, random errors could conspire to generate a screen ratio above the reference range and produce ≥ 10% correction by the confirmatory test ratio, leading to false-positive results. In this respect, the CryoCheck reagents and controls in conjunction with the Sysmex CA1500 analyser are suitable for use in this approach to the assessment of phospholipid dependence.

Frozen reagents and controls offer potential benefits (eg convenience and result quality) to a busy diagnostic laboratory. Reconstitution errors are eliminated and reagents/controls can be used almost immediately once thawed. The freeze-drying process can alter the conformation and functionality of certain proteins, so use of frozen control plasmas results in comparison of patient sample results with those of controls in the same state. Use of the frozen normal control to generate ratios detected more LAs than did the lyophilised control.

Lyophilised plasmas tend to show a degree of turbidity once reconstituted, but this is not present in frozen plasmas. Turbidity is known to affect performance of photo-optical clot detection analysers,[5] such as the Sysmex CA1500 used in this study, and this, in part, may explain the slightly improved sensitivity obtained when using frozen plasma.

Accurate detection of LAs is important because the clinical manifestations of persistent LAs are associated with increased morbidity and mortality. The DRVVT is one of the most widely used assays to detect LA antibodies, and new reagents need to demonstrate comparable diagnostic performance to existing products. This is of particular relevance to DRVVT, as it is often used in isolation to detect LAs,[21] despite the fact that expert guidelines indicate the need to use a minimum of two tests of different assay types in response to antibody heterogeneity.[9,10]

The CryoCheck reagents and controls, used with the Sysmex CA1500 analyser, represent a sensitive and specific DRVVT test system for LA detection, and enjoys the additional benefits associated with the use of frozen reagents. Furthermore, use of the percent correction of ratio, in place of the manufacturer's recommended test:confirm ratio, increases sensitivity. As demonstrated in the results from this and other studies,[4–6,8,12,17,18] no single test, or test type, will identify all LAs and the CryoCheck reagents should be used in conjunction with at least one other LA assay type in order to maximise detection rates.

CryoCheck reagents and controls were provided by Alpha Laboratories, Eastleigh, Hampshire, UK.

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