

Application of different lupus anticoagulant diagnostic algorithms to the same assay data leads to interpretive discrepancies in some samples

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Abstract

Background: Gold standard lupus anticoagulant (LA) assays and reference plasmas do not exist and detection is based on inference in a medley of coagulation assays, creating potential for interpretive discrepancies when applying different algorithms.

Objectives: To investigate discrepancies from applying different algorithms to a common data set.

Methods: Diagnostic data on 311 non-anticoagulated patients LA-positive by dilute Russell's viper venom time (dRVVT) and/or dilute activated partial thromboplastin time (dAPTT) assays were employed to compare algorithms. Routine testing applied interpretive criteria from guidelines endorsing classification as LA-positive despite negative mixing tests, after exclusion of other clotting abnormalities. Integrated testing without mixing tests, and the classical algorithm where negative mixing tests preclude confirm tests, were then retrospectively applied to those data.

Results: Initial testing showed 92/311 (29.6%) were LA-positive by dRVVT only, 156/311 (50.1%) by dAPTT only, and 63/311 (20.3%) by both assays. All dAPTT-positive plasmas remained positive with integrated testing but eight dRVVT-positives became negative. Other data suggested they were false-negatives. The classical algorithm altered 52/155 (33.5%) dRVVT and 111/219 (50.7%) dAPTT interpretations to LA-negative because of normal mixing tests, most of which were apparently weak LA in undiluted plasma.

Conclusions: The classical algorithm improves diagnostic specificity and confidence but risks missing some genuine LA due to false-negative mixing tests. Integrated testing can be diagnostically accurate and logistically efficient but oversimplifies complex cases. Performing mix and confirm in response to an elevated screen with their interpretation based on clinical data, coagulation screens and the LA-assay design offers a potentially valuable option.

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KEYWORDS

activated partial thromboplastin time, antiphospholipid syndrome, diagnostic algorithms, dilute Russell's viper venom time, lupus anticoagulant

Essentials

- Lupus anticoagulant detection is based on inference from their effects on various clotting tests.
- Three different interpretive algorithms were applied to LA-positive data to compare outcomes.
- False-negative mixing tests were the main cause of interpretive discrepancies.
- Assaying confirm tests even if mixing tests are normal permits detection of weaker antibodies.

1 | INTRODUCTION

Antiphospholipid syndrome (APS) is diagnosed in patients with vascular thrombosis or pregnancy morbidity whose laboratory assays demonstrate persistent antiphospholipid antibodies (aPL).¹ Since thrombosis and pregnancy morbidity are non-specific for APS, the diagnosis is reliant on accurate and robust aPL detection. Two of the criteria antibodies, anticardiolipin antibodies (aCL) and anti- β_2 -glycoprotein I antibodies (β_2 GPI), are detected in semi-quantitative solid-phase assays, whilst lupus anticoagulants (LA) are detected by inference based on antibody behaviour in a medley of phospholipid-dependent coagulation assays.²

No single coagulation test is sensitive for all LA and two test systems of differing analytical principles are needed to maximise detection rates.²⁻⁵ All current guidelines recommend that dilute Russell's viper venom time (dRVVT) must be one of these tests²⁻⁵, mainly for specificity in detecting clinically significant LA, normally partnered with LA-responsive activated partial thromboplastin time (APTT). Classically, the medley for each test type comprises an initial screening test on undiluted plasma, which if elevated is followed by a mixing test to evidence inhibition, which if also elevated, is followed by a confirmatory test on undiluted plasma to demonstrate phospholipid dependence.²⁻⁵

Debate has ensued in recent years concerning the place of mixing tests in LA detection.⁶⁻¹⁵ Some contend that assaying screen and confirm tests and immediately assessing for phospholipid dependence, so-called integrated testing, will detect LA in most cases, even where other causes of elevated clotting times are present.⁸⁻¹¹ Others indicate that situations such as possible or known presence of other causes of elevated clotting times, potent antibodies and the "lupus cofactor effect," mean that mixing tests are crucial to accurate data interpretation and improved specificity.^{6,7,12,13} Either way, it is widely acknowledged that the dilution factor can make weaker LAs appear negative despite clear positivity from screen and confirm results in undiluted plasma.²⁻¹⁵

Current guidelines from the International Society on Thrombosis and Haemostasis (ISTH)³ and British Society for Haematology (BSH)⁴ endorse the classical algorithm. However, the BSH guidelines state that in the absence of other causes of prolonged clotting times,

samples with negative mixing tests but positive screen and confirm results in undiluted plasma can be classified as LA positive. This implies that all three tests are performed and then interpreted in light of assay design instead of using mixing test results as a diagnostic decision point. The Clinical and Laboratory Standards Institute (CLSI) guideline reprioritizes test order to screen-confirm-mix, recommending that the mixing test is initiated only when screen and confirm analysis is not clear-cut and/or when other causes of prolonged clotting times are known or suspected.⁵ The present study retrospectively assesses diagnostic data from 311 plasma samples from non-anticoagulated patients deemed LA-positive according to the BSH guideline, and by extension the CLSI guideline, by subsequently applying interpretations based on integrated testing, and the classical algorithm.

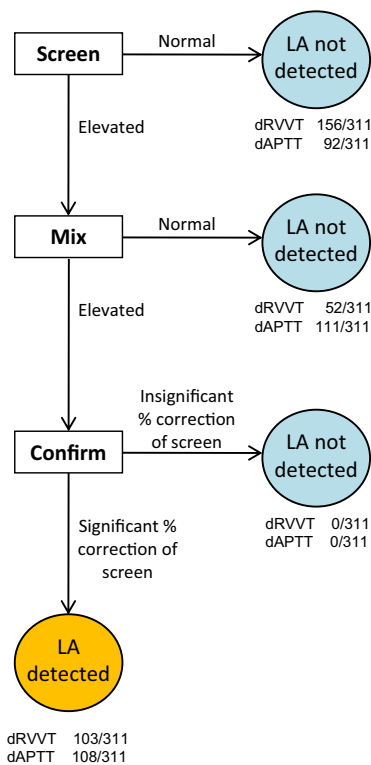
2 | MATERIALS AND METHODS**2.1 | Blood collection, manipulation, and storage**

Blood was collected into Vacuette tubes (Greiner Bio-One Ltd, Stonehouse, UK) containing a one-tenth volume of 0.105 mol L^{-1} (3.2%) tri-sodium citrate and double centrifuged to obtain platelet poor plasma (PPP)³⁻⁵ which was stored at -80°C until use.

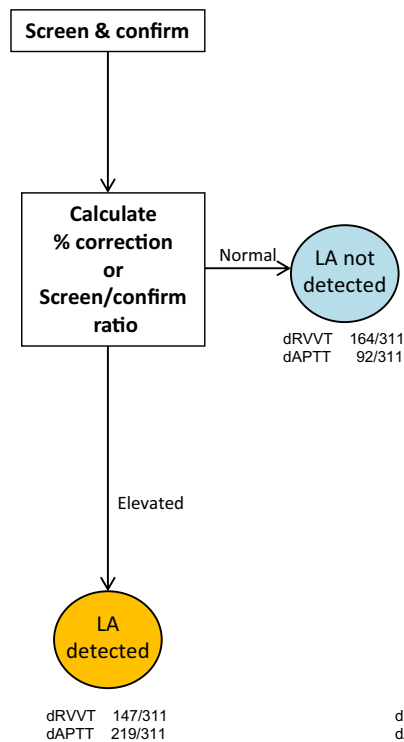
2.2 | Lupus anticoagulant assays

Routine dRVVT was performed with Life Diagnostics (LD) LA Screen and LA Confirm reagents (Diagnostica Stago UK, Theale, UK). Dilute APTT (dAPTT) employed Stago PTT-LA (Diagnostica Stago) in the screen and addition of Bio/Data Corporation LA Confirmation Reagent (Alpha Labs, Eastleigh, UK) for the confirm. All elevated screens received the confirm test plus screen and confirm on 1:1 mixing studies with normal plasma. CRYOcheck™ Normal Reference Plasma (Alpha Labs) was used for 1:1 mixing tests. The LA assays were performed on a Sysmex CS2000i analyser (Sysmex UK, Milton Keynes, UK). Screen and confirm clotting times were converted to normalized ratios via their reference interval (RI) mean clotting times.^{5,10,16,17} Results were defined as consistent with the presence of a LA if the screen ratio was greater than the RI upper limit with $\geq 10\%$ correction by the confirm ratio^{2-5,15,16,18-20}, which had been previously locally validated.^{15,16,20} Mixing tests with screen and confirm assays were performed for

Classical algorithm



Integrated testing



BSH/CLSI algorithms

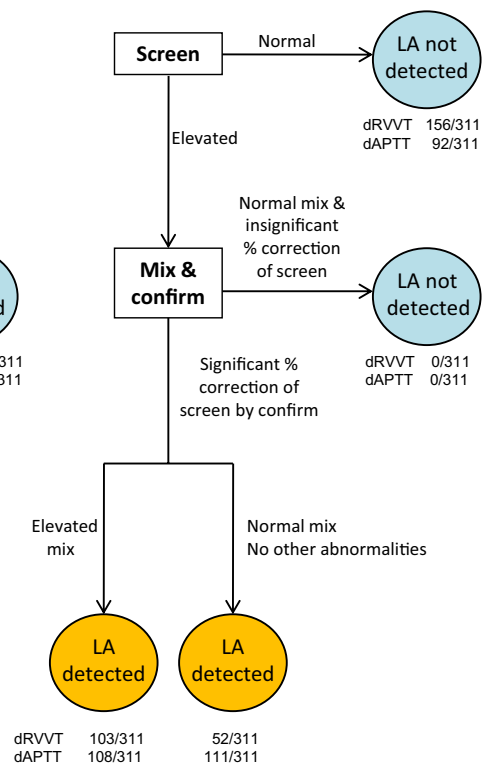


FIGURE 1 Lupus anticoagulant diagnostic algorithms. Numbers of the 311 samples classified as LA-positive or -negative by dRVVT and/or dAPTT are shown for the junctures of each algorithm.

dRVVT and dAPTT to increase specificity^{4-6,12,13}, particularly where confirm ratios were themselves elevated.^{5,6,12,13} In view of potential differences between normal pooled plasma (NPP) and RI mean clotting times,^{5,16} mixing test ratios were derived from NPP results as denominator so that the ratios reflected the effect of index plasmas on the NPP in which they were mixed.²⁰ Mixing test ratio specific cut-off was employed to determine presence of inhibition.^{3,5,20,21} The RIs, and thus cut-offs, had previously been locally derived from 43 normal donor plasmas.^{3-5,22} All had Gaussian distributions and were calculated as ± 2 standard deviations (SD) of the mean.^{2,4,5,22,23} The ISTH recommendation of 99th percentile cut-offs was not applied as it has proven controversial, and lower cut-offs have been shown to allow better detection rates.^{2,4,5,24}

2.3 | Coagulation screening tests

Coagulation screening was performed to exclude factor deficiencies and undisclosed anticoagulation. Prothrombin time, APTT, thrombin time, and Clauss fibrinogen were performed on a Sysmex CS2100i analyser (Sysmex UK) using Dade Innovin recombinant thromboplastin, Actin FS, Thromboclotin™ and Thrombin-Reagent (Siemens Healthcare, Marburg, Germany) respectively. Actin FS was employed as it is a LA-unresponsive routine APTT reagent and suited to exclusion of other causes of elevated clotting times.^{5,8,25}

2.4 | Patients

Diagnostic data from routine LA testing at Guy's and St. Thomas' Hospitals for 311 non-anticoagulated patients interpreted as LA-positive by following the BSH/CLSI algorithms were re-evaluated by applying integrated testing alone to screen and confirm results from undiluted plasma, and also the classical algorithm where an elevated screen but negative mixing test precludes performance of a confirm test and allows classification of the sample as being LA-negative. The ratio generated from integrated testing was derived from screen ratio/confirm ratio and not from raw clotting times because clotting times for the paired screen and confirm reagents with NPP differed for both dRVVT and dAPTT.^{2,18} Schematics of the algorithms are shown in Figure 1.

One hundred and six of the patients had antiphospholipid syndrome (APS), 35 had systemic lupus erythematosus (SLE) and persistent LA, 26 had SLE and first LA-positive testing, and 144 were clinically appropriate patients being investigated for APS. Seventy of the 106 (66%) APS patients also had elevated aCL and/or $\text{a}\beta 2\text{GPI}$.

3 | RESULTS AND DISCUSSION

The lack of gold standard assays and reference plasmas continues to hamper standardisation of LA detection. Instead, diagnostic

practitioners aim to employ reagents recognised as LA-sensitive and make choices on suitable algorithms based on published guidelines, personal experience, manufacturer recommendations, and/or logistical and cost considerations.^{26,27} The classical algorithm has been questioned in recent years due to concerns on false-negative mixing tests arising from the dilution effect,^{2,4,5,7-10,15} but also to the increasing adoption of integrated testing that circumvents traditional testing order and allegedly detects LA without mixing tests.^{2,8-11,17} Recent reports investigating the effect of excluding mixing tests have shown interpretation discordance between the integrated and classical algorithms but these studies concentrated only on the use of dRVVT.^{6,13} We present a study on 311 samples interpreted as being LA-positive via a BSH/CLSI directed approach. These results are herewith reinterpreted with integrated testing and the classical algorithm applied to dRVVT and dAPTT.

From the routine testing and application of BSH/CLSI algorithms, 92/311 (29.6%) were LA-positive by dRVVT only, 156/311 (50.1%) by dAPTT only, and 63/311 (20.3%) in both dRVVT and dAPTT. Diagnostic data are summarised in Table 1. Because patients were not anticoagulated and routine APTT was LA-insensitive, these samples could be designated as LA-positive when mixing tests were negative.^{4,5} The ISTH guideline acknowledges that confirm tests in LA-positive samples do not always return into the reference range and the CLSI algorithm indicates that apparently LA-positive samples showing elevated confirm ratios should undergo mixing tests to increase specificity by excluding factor deficiencies and most anticoagulation.^{2,5,6,13} Twenty of 311 (6.4%) had mildly elevated routine APTT ratios (range 1.3-1.5, mean/median 1.34/1.30, cut-off 1.2). All were LA-positive by dAPTT testing (screen ratio range 1.22-3.19, mean/median 1.62/1.45), 12 of which were also dRVVT testing-positive. The mild elevations were considered to be due to the LA and not undisclosed anticoagulation. Two each had INR of 1.3 (cut-off 1.2) but were dRVVT testing-positive (screen ratios 2.26/1.66) and dAPTT testing-positive (screen ratios 1.88/1.35), and were also considered due to the LAs.

Thirty four of 155 (21.9%) dRVVT-positive samples had confirmatory test ratios above the cut-off of 1.10, (range 1.11-1.87, mean 1.21, median, 1.16), 4 of which had negative mixing tests. All 4 had normal routine APTT and one was also dAPTT-positive, and in the absence of evidence for other causes of elevated clotting times, were considered genuine LA-positive.^{2,4} Seventeen of 34 were also dAPTT-positive. Twenty of 219 (9.1%) dAPTT-positive samples had

confirmatory test ratios above the cut-off of 1.18, (range 1.19-1.54, mean 1.26, median, 1.22), 5 of which also had mildly elevated routine APTT ratios of 1.3-1.4, broadly concordant with their dAPTT confirmatory test ratios (range 1.21-1.54). Two of 20 had negative mixing tests. Their confirmatory test ratios were 1.26 and 1.21 and both had routine APTT ratios of 1.4 (cut-off 1.2), yet they achieved 21.7% and 16.6% correction and were consequently considered LA-positive.⁴ Twelve of 20 were also dRVVT-positive. Devreese⁶ and Devreese and De Laat¹³ reported a higher prevalence of dRVVT-positive samples with elevated confirm results yet the majority were in patients on vitamin K antagonists. Our study was specifically performed on non-anticoagulated patients to assess interpretation discordances in otherwise uncompromised plasma samples. Such patients could have a LA capable of overcoming some of the overwhelming effect of high concentration phospholipid confirm reagents,^{2,6,12} a co-existing factor deficiency,^{2,6} or in the case of APTT testing, a clinically insignificant contact factor deficiency.²⁸

Generating the integrated testing ratio from screen and confirm ratios in undiluted plasma and interpreting elevated integrated ratios as LA-positive irrespective of mixing test results altered 8/155 (5.2%) of dRVVT interpretations to LA-negative (Table 2). Four of those were dAPTT-positive, one of which also had elevated aCL and a β_2 GPI, two had elevated mixing tests, one of which had had elevated aCL and a β_2 GPI, and the other 2 had established, persistent LA. Their dRVVT screen ratios ranged from 1.18-1.29, mean 1.23, median 1.23. Thus, they were all likely false-negatives arising from differences in sensitivity between the percent correction and integrated ratio cut-offs. The 10% correction value has been advocated by BSH guidelines since 1991^{4,18,29} and had been locally validated for dRVVT and dAPTT from reference range data.³ All dAPTT interpretations remained LA-positive with integrated testing.

One omission from our study is integrated testing on samples deemed LA-negative from BSH/CLSI or classical algorithms because dRVVT and dAPTT screen ratios were within reference ranges. Whilst it can be reasonably argued that normal screen ratios do not fulfill one of the crucial diagnostic criteria, being elevation of a phospholipid-dependent screening test, a converse view exists suggesting that screen and confirm discordance will detect weaker LA that prolong screen clotting times above a patient-specific baseline but not beyond the cut-off of from a population distribution.^{2,8,30} A separate debate exists on the significance of apparently weaker LA but the purpose of

TABLE 1 Diagnostic data on 311 plasma samples positive for lupus anticoagulant by dRVVT and/or dAPTT analysis according to BSH and CLSI recommended algorithms

Assay	n	Screen ratio				Percent correction of screen ratio by confirm ratio			Positive in mixing test
		Cut-off	Range	mean	median	Range	mean	median	n (%)
dRVVT	155	>1.17	1.18-3.12	1.45	1.31	10.2-64.7	24.2	20.8	103 (66.5)
dAPTT	219	>1.20	1.21-4.49	1.45	1.33	10.2-72.9	26.1	24.1	108 (49.3)

BSH, British Society for Haematology; CLSI, Clinical and Laboratory Standards Institute; dAPTT, dilute activated partial thromboplastin time; dRVVT, dilute Russell's viper venom time.

TABLE 2 Diagnostic data on eight plasma samples negative for lupus anticoagulant by dRVVT integrated testing

Clinical details	dRVVT screening test ratio (CO >1.17)	dRVVT confirm test ratio (CO >1.10)	dRVVT integrated test ratio (CO >1.13)	dRVVT percent correction (CO >10%)	dRVVT mixing test ratio (CO >1.13)	dAPTT	aCL	ap ₂ GPI
APS	1.23	1.09	1.13	11.4	1.14	Positive	Elevated	Elevated
CTD, first LA testing	1.29	1.15	1.12	10.9	1.17	Negative	Elevated	Elevated
SLE with persistent LA	1.18	1.06	1.11	10.2	1.02	Positive	Normal	Not done
? APS, first LA testing	1.21	1.07	1.13	11.6	1.20	Negative	Not done	Not done
APS	1.19	1.05	1.13	11.8	1.12	Positive	Normal	Normal
SLE with persistent LA	1.22	1.08	1.13	11.5	1.11	Negative	Normal	Normal
APS	1.23	1.10	1.12	10.6	1.12	Negative	Normal	Normal
Thrombocytopenia, first LA testing	1.28	1.15	1.11	10.2	1.20	Positive	Normal	Not done

ap₂GPI, anti-β₂glycoprotein I antibodies; aCL, anticardiolipin antibodies; APS, antiphospholipid syndrome; CTD, connective tissue disease; CO, cut-off; dAPTT, dilute activated partial thromboplastin time; dRVVT, dilute Russell's viper venom time; LA, lupus anticoagulant.

TABLE 3 Interpretive outcomes from classical algorithm of 311 plasma samples positive for lupus anticoagulant by dRVVT and/or dAPTT analysis according to BSH and CLSI recommended algorithms

Assay and classical algorithm interpretation	n	Screen ratio		Percent correction		Integrated test ratio		Mixing test ratio	
		Range	Mean/median	Range	Mean/median	Range	Mean/median	Range	Mean/median
dRVVT									
positive	103	1.20-3.12	1.55/1.38	10.2-64.7	27.8/25.0	1.11-2.83	1.44/1.33	1.14-2.57	1.41/1.26
negative	52	1.18-1.44	1.26/1.25	10.2-28.7	17.2/16.0	1.11-1.40	1.21/1.19	0.91-1.13	1.10/1.11
dAPTT									
positive	108	1.21-4.49	1.58/1.40	10.2-72.9	27.1/23.9	1.11-3.69	1.44/1.31	1.16-3.33	1.48/1.28
negative	111	1.21-1.68	1.33/1.29	10.2-44.1	25.1/25.4	1.11-1.79	1.35/1.34	0.93-1.15	1.10/1.10

Cut-offs for dRVVT screen ratio, percent correction, integrated ratio and mixing test ratio respectively were 1.17, 10.0, 1.13, 1.13, and for dAPTT, 1.20, 10.0, 1.10, 1.15. Of the 52 dRVVT mixing test negative samples, 20 were from patients with APS, seven were from patients with SLE (three of which had persistent LA), and the majority of the remainder were being tested in response to thrombosis or recurrent miscarriage; 22 of 52 (42.3%) had elevated aCL and/or ap₂GPI. Of the 111 dAPTT mixing test negative samples, 25 were from patients with APS, 26 were from patients with SLE (six of which had persistent LA), four were in response to an elevated routine APTT, and the majority of the remainder were being tested in response to thrombosis or recurrent miscarriage; 40 of 111 (36.0%) had elevated aCL and/or ap₂GPI. ap₂GPI, anti-β₂GPI antibodies; aCL, anticardiolipin antibodies; APS, antiphospholipid syndrome; BSH, British Society for Haematology; CLSI, Clinical and Laboratory Standards Institute; dAPTT, dilute activated partial thromboplastin time; dRVVT, dilute Russell's viper venom time.

the present study was to evaluate differences in samples fulfilling the standard elevated screening test criterion.

ISTH and CLSI Guidelines recommend interpreting mixing tests with a mixing test-specific cut-off (MTC) or calculation of the index of circulating anticoagulant (ICA).^{2,3,5} Recent reports suggest that MTC achieves higher detection rates.^{20,21,31} Employing a locally derived MTC^{3,5} in this study maximised mixing test detection of LA. Yet, applying the classical algorithm altered 52/155 (33.5%) dRVVT and 111/219 (50.7%) dAPTT interpretations to LA-negative as mixing test ratios were below cut-offs. Similar frequencies of false-negative mixing tests have been previously reported^{6,9,13–15,20,21,31} and external quality assessment schemes have reported false-negative mixing test results as a common cause of misclassification of weaker LA.^{8,26} More specific assays have been reported to be less affected⁹, which is mirrored in the data for this study where dRVVT was less affected by false-negative mixing tests than dAPTT. Comparative data between LA-positive and LA-negative samples via this algorithm are given in Table 3. The dilution effect is an accepted phenomenon and therefore our routine LA detection strategy does not adopt mixing test values as a decision point but responds to all elevated screens with confirm and mixing tests and interprets accordingly. The data suggest that stronger antibodies, as implied by degree of screening test elevation, are more likely to elevate mixing test ratios than weaker antibodies (those with lower screen ratios) and prompt confirmatory test performance in the classical algorithm. However, there was some degree of cross-over with some samples whose screen ratios in undiluted plasma were close to cut-offs nonetheless elevating mixing tests, whilst some others with moderately elevated screen ratios (dRVVT up to 1.44, dAPTT up to 1.68) did not. Manifestation in mixing tests seems to be a function of more than just potency, other possible contributory factors include epitope specificity, antibody avidity, and reagent composition.²⁰

In summary, the number of samples classified as LA-positive differed depending on the method of interpretation, particularly in the case of the classical algorithm where a design limitation of mixing tests precluded completion of the assay medley that could otherwise reveal an LA. The discrepancies occurred mainly with samples whose screen ratios were slightly, and occasionally moderately, elevated, a common area of between-center discordance in external quality assurance challenges.⁸ The absence of reference plasmas and gold standard assays means that we cannot be certain which of these arguably ambiguous result sets reflect genuine LA and those with positive mixing tests can engender increased confidence in specificity. A major contributor to between-department discrepancies with lower titer antibodies irrespective of the algorithm employed is cut-off generation. It is widely accepted, and recommended in all guidelines, that cut-offs must be locally derived yet laboratories vary in their approach to this issue and some laboratories continue to adopt manufacturers' generic cut-offs. Conversely, no assay is perfect and the dilution effect is a genuine confounder, so performing mix and confirm tests in response to an elevated screen coupled with informed interpretation based on clinical data, routine coagulation screening tests and LA-assay design offers us a potentially valuable interpretive middle ground.

AUTHOR CONTRIBUTION

G.W. Moore initiated the study, re-evaluated the diagnostic data and wrote the manuscript. J.C. Maloney, N. de Jager, C.L. Dunsmore, D.K. Gorman and R.F. Polgrean performed and interpreted initial diagnostic testing and revised the manuscript. M.L. Bertolaccini critically revised the manuscript.

RELATIONSHIP DISCLOSURE

G.W. Moore is a Coagulation Advisory Board member for Roche Diagnostics International Ltd.

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