Thrombosis Research 139 (2016) 98–101

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Mixing test specific cut-off is more sensitive at detecting lupus anticoagulants than index of circulating anticoagulant

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ARTICLE INFO

Article history:
Received 13 November 2015
Received in revised form 5 January 2016
Accepted 26 January 2016
Available online 27 January 2016

Keywords:
APTT
Antiphospholipid antibodies
dRVVT
Index of circulating anticoagulant
Mixing tests

ABSTRACT

Introduction: Recent guidelines for lupus anticoagulant (LA) detection recommend mixing test interpretation with either a mixing test-specific cut-off (MTC) or index of circulating anticoagulant (ICA). Few studies directly compare efficacy of these approaches. We retrospectively applied MTC and ICA assessment to raw data of 350 LA-positive plasmas from non-anticoagulated patients to compare detection rates of inhibition.
Materials and methods: Screen and confirm dRVVT and dilute APTT assays were performed on undiluted plasma and 1:1 mixtures with normal pooled plasma. Samples were considered LA-positive if one or both screening test ratios were elevated and corrected by ≥10% with the confirmatory test. Mixing tests were assessed against locally derived cut-offs for MTC (dRVVT >1.13, dAPTT >1.15) and ICA (dRVVT >11.9%, dAPTT >13.2%).
Results: 105 of 350 (30%) were positive in dRVVT and dAPTT, 109/350 (31.1%) were dRVVT positive only and 136/350 were dAPTT positive only (38.9%), from undiluted plasma results. Of the 214 dRVVT positive plasmas, 53 (24.8%) were negative for inhibition by MTC and 65 (30.4%) negative by ICA. Of the 241 dAPTT positive plasmas, 48 (19.2%) were negative by MTC and 97 (40.2%) negative by ICA.
Conclusion: Whilst integrated testing often detects LA without mixing tests they are diagnostically useful in certain circumstances. Thus, it is valuable to maximise mixing test interpretation as the dilution can lead to false-negative results. These data on a large cohort of LA-positive plasmas reveal that, with the reagents and equipment employed, MTC is superior to ICA in detecting the in vitro inhibition of LA.

1. Introduction

Diagnosis of antiphospholipid syndrome (APS) is achieved when laboratory assays demonstrate the presence of persistent antiphospholipid antibodies (aPL) in patients presenting with thrombosis or pregnancy morbidity [1]. Since thrombosis and pregnancy morbidity are by no means specific to APS, the diagnosis is highly reliant on accurate and timely detection of aPL. Two of the criteria antibodies, anticardiolipin antibodies (aCL) and anti-β2-glycoprotein I antibodies (aβ2GPI), are detected in solid phase assays, whilst lupus anticoagulants (LA) are detected in coagulation assays. Issues such as antibody heterogeneity, reagent variability and differing interpretation strategies, among others, mean that standardisation issues persist and generation of gold standard assays and reference plasmas remains elusive [2, 3]. Whilst aCL and aβ2GPI assays do at least include calibrators, the presence of LA is inferred based on antibody behaviour in a medley of phospholipid-dependent coagulation assays. This adds a further layer of complexity to LA identification and several guidelines with broad but not complete concordance are available to guide best practices [4–6].

No single test is sensitive for all LA and two test systems of differing analytical principles should be employed to maximise detection rates [4–6]. Classically, the medley for each test type comprises (a) a screening test with low phospholipid content to accentuate the effect of LA, (b) performance of the screening test in a 1:1 mixture of index and normal pooled plasma (NPP) to evidence inhibition, and (c) recapitulation of the screening test but with concentrated phospholipid to demonstrate phospholipid dependence. Recent years have witnessed a debate on the efficacy and place of mixing tests in LA detection [7–9]. Some contend that assessment for screen and confirm discordance in undiluted plasma, so-called integrated testing, is sufficient to detect LA in most cases, even in situations of co-existing factor deficiency [10–12]. Others indicate that in certain situations, such as potent antibodies, possible presence of other causes of elevated clotting times and presence of the 'lupus cofactor effect', that mixing studies are sometimes crucial to

Abbreviations: aβ2GPI, anti-β2-glycoprotein I antibodies; aCL, anticardiolipin antibodies; aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; APTT, activated partial thromboplastin time; dAPTT, dilute activated partial thromboplastin time; DOAC, direct oral anticoagulant; dRVVT, dilute Russell's viper venom time; ICA, index of circulating anticoagulant; LA, lupus anticoagulant(s); MTC, mixing test-specific cut-off; NPP, normal pooled plasma; PPP, platelet poor plasma; PT, prothrombin time; RI, reference interval; SD, standard deviation.

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http://dx.doi.org/10.1016/j.thromres.2016.01.019
0049-3848/

accurate diagnosis [7, 8, 13, 14] and increase specificity [5]. What is widely acknowledged is that mixing index plasma with NPP introduces a dilution factor that can make weaker LA samples appear negative despite clear positivity in the screen and confirm results on undiluted plasma [4–11, 15, 16].

Accepting that mixing tests have a place in the LA diagnostic armoury, at least in some types of samples, warrants adoption of procedures to maximise diagnostic performance in light of test design limitations. Using a suitable NPP sufficiently platelet poor for LA testing in a 1:1 mixture with index plasma is accepted best practice and recommended in all current guidelines [4–6]. The current guidelines from the Scientific Sub-Committee of the International Society on Thrombosis and Haemostasis [4] were the first to recommend how mixing tests should be interpreted, with either a mixing test-specific cut-off (MTC) in place of the cut-off employed for undiluted plasma [16], or the index of circulating anticoagulant (ICA) [17], but studies comparing the two interpretation procedures are lacking. The present study assesses comparative performance of MTC and ICA in 350 plasmas positive for LA in the locally employed dilute Russell's viper venom time (dRVVT) and/or activated partial thromboplastin time (APTT) from non-anticoagulated patients to compare detection rates of inhibition in otherwise uncompromised samples.

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 M (3.2%) trisodium citrate and double centrifuged to obtain platelet poor plasma (PPP) with a platelet count of $<10 \times 10^9$/L [4–6]. The PPP for LA testing was stored at −80 °C until use.

2.2. Lupus anticoagulant assays

Dilute Russell's viper venom time was performed with Life Diagnostics LA Screen and LA Confirm reagents (Diagnostica Stago UK, Theale, UK). Dilute APTT employed Stago PTT-LA (Diagnostica Stago) in the screen and addition of Bio/Data Corporation LA Confirmation Reagent (Alpha Labs, Eastleigh, UK) for the confirmatory test. All elevated screens received the confirmatory test plus screen and confirmatory test on 1:1 mixing studies with normal plasma. CRYOcheck™ Normal Reference Plasma (Alpha Labs) was used for 1:1 mixing tests. All LA assays were performed on a Sysmex CS2000i analyser (Sysmex UK, Milton Keynes, UK). Screen and confirm clotting times were each converted to normalised ratios via the reference interval (RI) mean clotting times [6, 10, 11, 18]. Results were defined as consistent with the presence of a LA if the screening test ratio was greater than the upper limit of the RI with ≥10% correction by the confirmatory test ratio [1, 4–6, 16, 18]. Mixing tests with both screen and confirm assays were performed to increase specificity [5, 6], particularly in situations where confirmatory test ratios were themselves elevated [6, 7, 13, 14]. In view of potential differences between NPP and RI mean clotting times [18], mixing test ratios were derived from using NPP results as denominator in order that the ratios were a reflection of the effect of index plasmas on the NPP in which they were mixed. Mixing tests were considered positive if the mixing test screen ratio was above the MTC. Samples with clear LA-positivity in undiluted plasma and no evidence of other causes of elevated clotting times were regarded as LA-positive even if mixing tests were normal [4–6, 9, 10, 16, 19]. The RIs, and thus cut-offs, for all assays had previously been locally derived from 43 normal donor plasmas [4–6, 20]. All had Gaussian distributions and were calculated as ±2 standard deviations of the mean [5, 6, 20].

Cut-offs for ICA in dRVVT and APTT were derived from 73 normal donor plasmas. Both had Gaussian distributions and RIs were calculated as ±2 standard deviations (SD) of the mean [5, 6, 20, 22, 23]. Index of circulating anticoagulant was calculated as follows:

$$\text{ICA}\,(\%) : \left(\frac{\text{screen 1:1 mix (s)} - \text{screen NPP (s)}}{\text{screen undiluted test plasma (s)}}\right) \times 100.$$

2.3. Coagulation screening tests

Coagulation screening was performed to exclude factor deficiencies and undisclosed anticoagulation. Prothrombin time (PT), 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 [6, 9, 21].

2.4. Patients

The mixing test results from diagnostic testing of 350 LA-positive non-anticoagulated patients were retrospectively assessed with ICA to compare frequencies of detection of inhibition with MTC in a dRVVT and APTT reagent pairing. All patients were either being investigated for APS in response to appropriate clinical signs and symptoms [4–6] or were known to have APS and/or systemic lupus erythematosus.

2.5. Statistics

Reference intervals were generated and Spearman's correlation performed using Analyse-it for Excel, version 2.11 (Analyse-it Software Ltd, Leeds, UK). Unpaired *t*-test was performed using GraphPad QuickCalcs (GraphPad Software Inc., La Jolla, USA). Data were considered statistically significant at $P < 0.05$.

3. Results

One hundred and five of 350 (30%) samples were positive for LA in both dRVVT and dAPTT, 109/350 (31.1%) were positive only in dRVVT and 136/350 (38.9%) were positive only in dAPTT. Cut-offs for ICA by dRVVT and dAPTT were calculated as 11.9% and 13.2% respectively. Table 1 shows the range, mean and median values of dRVVT screen ratio, mix ratio, ICA and confirm ratio for samples that evidenced inhibition by both MTC and ICA, MTC only or were negative by both MTC and

Table 1
dRVVT results on 214 LA positive samples.

dRVVT	Cut-off	Positive in mixing test by MTC & ICA ($n = 149$)	Positive in mixing test by MTC only ($n = 12$)	Negative mixing test by MTC and ICA ($n = 53$)
Screen ratio	>1.17			
Range		1.18–3.26	1.18–1.51	1.18–1.42
Mean		1.60	1.30	1.25
Median		1.45	1.25	1.24
Mix ratio	>1.13			
Range		1.16–2.63	1.14–1.18	0.82–1.13
Mean		1.43	1.15	1.10
Median		1.32	1.15	1.11
ICA (%)	>11.9			
Range		12.0–55.6	9.1–11.8	−12.5–10.3
Mean		23.4	10.9	7.3
Median		20.8	10.9	8.2
Confirm ratio	>1.10			
Range		0.94–1.86	1.01–1.23	0.90–1.19
Mean		1.13	1.10	1.05
Median		1.10	1.11	1.05

dRVVT, dilute Russell's viper venom time; LA, lupus anticoagulant; MTC, mixing test-specific cut-off; ICA, index of circulating anticoagulant.