Point

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Mixing studies for lupus anticoagulant: mostly yes, sometimes no

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Abstract: Lupus anticoagulants (LAs) represent one manifestation of the clinical condition called antiphospholipid syndrome (APS) and are associated with many adverse clinical outcomes, but primarily with thrombosis and/or pregnancy morbidity. LAs are identified by laboratory testing, principally using clot-based assays based on Russell viper venom time (RVVT) and activated partial thromboplastin time (APTT) test methods. All three of the most recent guidance documents for LA testing recommend using these tests, although they vary in regard to inclusion/exclusion of other test processes. Mixing studies form part of the process of LA identification/exclusion, since *in vitro* LAs act like coagulation inhibitors. Mixing studies are also supported by all three LA guidance documents, but recommendations vary in regard to relative importance and placement in the LA identification/exclusion algorithm. This Point article takes the position that mixing tests are usually indicated for appropriate identification/exclusion of LAs, but can occasionally be omitted.

Keywords: anticoagulants; lupus anticoagulant; mixing studies.

Introduction

Lupus anticoagulants (LAs) represent one manifestation of the clinical condition called antiphospholipid syndrome (APS) [1]. LAs are associated with many adverse clinical outcomes, but primarily with thrombosis and/or pregnancy morbidity. LAs are identified by laboratory testing, predominantly using clot-based assays based on Russell viper venom time (RVVT) and activated partial thromboplastin time (APTT) test methods [1–5]. There are three guidance documents for LA testing [2–4]. These suggest that both test types can be utilised for LA identification/exclusion, but guidelines vary in regard to their specific utilisation, including inclusion/exclusion of other test processes (see Table 1).

Mixing studies also form part of the process of LA identification/exclusion, since LAs act *in vitro* like coagulation inhibitors. Use of mixing studies for LA identification/exclusion are also supported by all three guidance documents, but these vary in regard to relative importance and placement of mixing studies in the LA identification/exclusion algorithm (Table 1). This Point article takes the position that mixing tests are usually indicated for appropriate identification/exclusion of LAs. However, mixing studies can occasionally be omitted.

A summary of the LA guidelines

The most recent guidance document from the International Society on Thrombosis and Haemostasis (ISTH) promotes both the RVVT and APTT in identification/exclusion of LA, and also strongly supports mixing studies as part of the diagnostic algorithm, but recommends no other test methods be employed to reduce the risk of false-positive LA identification (Table 1). The guidance document from the British Committee for Standards in Haematology (BCSH) also promotes both RVVT and APTT, and also mixing studies, as part of LA identification/exclusion, but instead identifies potential replacement of the APTT with, and/or inclusion of, other tests in order to provide a more comprehensive diagnostic test panel. Finally, the most recent guidance document, deriving from a working group of the Clinical and Laboratory Standards Institute (CLSI), also promotes RVVT and APTT (and other tests) as well as mixing studies. However, the guidance documents differ in respect to where and how mixing studies may be utilised (Table 1).

In theory, mixing studies are not required to identify LAs, since LAs can be defined without their performance. Thus, LAs can be defined should testing with screening

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Table 1: Summary of ISTH, BCSH and CLSI guidelines for LA detection related to tests and mixing studies.

Area of recommendation	ISTH2009 [2]	BCSH2012 [3]	CLSI2014 [4]
Assays to use	(d) RVVT & APTT	(d) RVVT & (APTT or others)	(d) RVVT & APTT (and/or others)
Mixing test	Perform on 1:1 mixture with NPP; Interpret with ICA or mixing test-specific cut-off	Perform on 1:1 mixture with NPP	Perform on 1:1 mixture with NPP; Interpret with ICA or mixing test-specific cut-off
Testing order	Screen – Mix – Confirm	Screen – Mix – Confirm	Screen – Confirm – Mix
Testing patients on VKAs	Undiluted plasma if INR <1.5; Mix with NPP if INR >1.5 < 3.0	Screen & confirm on 1:1 mixture with NPP; TSVT + ET or PNP	Screen & confirm on 1:1 mixture with NPP; TSVT + ET or PNP
Testing patients on UFH	Interpret with caution	Can detect LA in some cases where heparin neutraliser is effective	Not recommended
Testing patients on DOACs	Not covered	Not covered	Covered but not recommended

ISTH, International Society on Thrombosis and Haemostasis; BCSH, British Committee for Standards in Haematology; CLSI, Clinical and Laboratory Standards Institute; DOAC, direct oral anticoagulants; (d) RVVT, (dilute) Russell viper venom time; APTT, activated partial thromboplastin time; NPP, normal pooled plasma; RI, reference interval; LA, lupus anticoagulant; ICA, index of circulating anticoagulant; VKA, vitamin K antagonist; INR, international normalised ratio; TSVT, Taipan snake venom time; ET, Ecarin time; PNP, platelet neutralisation procedure; UFH, unfractionated heparin. Summarised from Refs. [1–5].

assays [e.g. using RVVT and/or APTT with a low level of phospholipids, thereby defining dilute (d) RVVT for RVVT tests] identify a prolongation, and then subsequent testing with confirmation assays (e.g. RVVT and/or APTT, this time with a high level of phospholipids) be relatively shortened of 'corrected' compared to the screening assay. For example, a ratio of (d) RVVT screen/RVVT confirm ≥1.2 would be sufficient evidence for the identification of LAs, and thus mixing tests could theoretically be omitted.

Nevertheless, this Point article takes the view that mixing studies would be useful in a greater number of LA investigations than not. The reason for taking this position resides primarily in the type of samples received by contemporary laboratories, and how the information gathered from the results of LA testing, including mixing, informs on both the possibility of identification or exclusion of LA, but also the prevention of false positives and false negatives.

Anticoagulant therapy

Anticoagulant therapy is recommended for various reasons, but especially for the treatment and prevention of thrombosis [6–8]. The direct oral anticoagulants (DOACs), which act against thrombin (dabigatran) or activated factor X (e.g. rivaroxaban), are being increasingly prescribed, with usage exceeding that of vitamin K antagonists (VKAs; e.g. warfarin) in some locations [8, 9]. Of relevance to the current Point perspective, all anticoagulants, be they DOACs, VKAs or unfractionated heparin, may affect LA tests, leading to both false positives and false negatives [9–12]. Indeed, anticoagulant therapy can also affect the results of congenital thrombophilia investigations [13, 14], and a host of other hemostasis assays [10, 11, 15]. The different LA guidance documents vary in advice regarding testing of LA whilst patients are on anticoagulant therapy ([1–5]; Table 1). In part, the differences reflect historical variances. For example, the ISTH guidelines were published in 2009, before the formal release of most of the modern DOACs, and thus only covered testing in the presence of heparin and VKAs [2]. In the case of VKAs, the recommendation was to perform mixing studies, in part to normalise test results against the effects of VKAs, since these anticoagulants would otherwise prolong both the LA screen and confirm tests and complicate the generation of LA ratios. Mixing of VKA-affected patient plasma with normal plasma would essentially correct any effect of VKAs on both the LA screen and confirm assays, and thus lead to improved accuracy in LA identification/exclusion. Essentially, the same recommendation to perform mixing studies in the case of VKA therapy continued to be supported in later guidance documents ([3, 4]; Table 1). With regard to heparin therapy, most RVVT reagents contain a heparin neutraliser to permit therapeutic levels of unfractionated heparin to be neutralised; also, low molecular weight heparin does not greatly affect LA assays. Nevertheless, even in cases of heparin therapy, mixing studies

with RVVT reagents would also have some value in diluting out the heparin level to reduce its influence on assay clotting times. In contrast, as APTT tests are used in part for monitoring of unfractionated heparin [16], these assays tend to be quite sensitive to the presence of the drug. Nevertheless, the LA test guidance documents have varied recommendations around testing for LA whilst patients are on unfractionated heparin therapy ([1–5]; Table 1).

The DOAC and LA testing 'story' is even more complex. Each DOAC affects LA assays, but affects them differently [9–12]. The RVVT assay in particular is sensitive to the presence of all the current DOACs, with such presence potentially leading to false-positive LAs (especially with rivaroxaban; [11, 12]), or even false-negative LAs (with apixaban; [11]). As noted previously, due to publication vs. DOAC release timeframes, neither the ISTH guidance [2] nor the BCSH document [3] cover LA testing whilst on DOAC therapy. Although the CLSI document [4] does cover such testing, the basic recommendation is to not perform LA testing on patients under DOAC therapy.

However, irrespective of any guideline recommendation, it is inevitable that clinicians will request thrombophilia-related tests, including LA, on DOAC-treated patients [9, 13]. Indeed, local data suggest that around a third of samples received on clinical requests for thrombophilia testing, including LA, may arise from patients on concurrent anticoagulant therapy (where this is identified). Thus, in one investigation into LA testing covering a recent 2.5-year (2016–2018) audit, 1425 samples were identified to derive from patients on anticoagulant therapy compared to 2942 samples identified to come from non-anticoagulant treated patients (1425/4367; 32.6%) [9]. In a similar investigation into congenital thrombophilia testing covering the same 2.5-year period, 1232 samples were identified to derive from patients on anticoagulant therapy compared to 2374 samples identified to come from non-anticoagulant treated patients (1232/3606; 34.1%) [13].

One reason that clinicians may be testing patients whilst on anticoagulant therapy is that they do not understand that such therapy can lead to both false-positive and false-negative events. Another explanation is that anticoagulant therapy is prescribed for the very reasons (e.g. thrombosis) that patients are being investigated for 'thrombophilia'. Thus, the event timeline starts with a patient having suffered a thrombosis, that patient then being immediately started on anticoagulant therapy, and only later is the investigation into why the patient had the event initiated. Alternatively, the need for repeated LA testing for confirmation of positivity, some 12 weeks post the initial event [1–5], is likely to arise after the patient has initiated anticoagulant therapy.

In cases where anticoagulant therapy has commenced, mixing tests will help to dilute out any anticoagulant effect, and indeed such a dilution is often sufficient to abrogate the risk of false positives/negatives in LA due to anticoagulant effects.

Naturally, there is also a risk that LA, should it be present, may become diluted upon mixing, and this thus potentially lead to a false LA-negative event. Thus, any decision to undertake mixing studies has to balance the two competing elements of risk (i.e. anticoagulant-induced false-positive/false-negative LA vs. false LA-negative event due to LA dilution). Nevertheless, in our experience, anticoagulant therapy seems to be far more common in our patient cohort than true LA positivity, so reducing the risk of false positives and negatives induced by anticoagulant therapy via performance of mixing tests would strategically reflect a superior option than the potential for false-negative LA due to LA dilution. As mentioned above, up to one third of our test cohort may be on anticoagulant therapy [9, 13]. As a comparison, in the same identified LA audit [9], of 2376 LA tests performed in the non-anticoagulant-treated patients, only a minority of 226 (9.5%) were LA positive by the RVVT method. For rivaroxaban-treated patients (n = 121), 93 (76.9%) would have been identified as LA positive based on a raised RVVT LA ratio (≥1.2). As the prevalence of LA would not *per se* be expected to be higher in rivaroxaban-treated patients than in non-anticoagulant-treated patients, the vast majority of these 'LA positive' events (almost 4/5ths of the tested samples) are likely to be false positives. We have also identified a high rate of false LA-positive identification in a rivaroxaban-treated sample in a recent external quality assessment (EQA) exercise [12].

Additional considerations

A second category of patients exists in which mixing studies are critical for the accurate identification of LA. These are patients with very strong LAs, where false negatives may arise if mixing studies are not performed [17, 18]. Although potentially rare, these cases provide sobering reflection on the importance of mixing studies for some patients. Also relevant here is the original intent of ISTH LA guidelines [19], aiming to identify the 'inhibitory' nature of LA, something that is imbedded into the initial LA criteria. Thus, *in vitro*, LA are 'inhibitors' of coagulation tests, and 'inhibitors' can be identified by lack of correction in mixing studies [20].

Table 2: Anticipated patterns of routine test results for different anticoagulants.

Coagulation test	Effect of					
	Anti-IIa DOAC (dabigatran)	Anti-Xa DOAC (rivaroxaban)	Anti-Xa DOAC (apixaban)	Vitamin K antagonists	Unfractionated heparin	Low molecular weight heparin
Prothrombin time/International normalized ratio (PT/INR)	↑	↑↑	(↑)	↑↑	–/(↑)	–
Activated partial thromboplastin Time	↑↑	↑	(↑)	↑	↑↑	–/(↑)
Thrombin time	↑↑↑	–	–	–	↑↑↑	(↑)/–
Anti-Xa assay	–	↑↑↑	↑↑↑	–	↑↑	↑↑
Russell viper venom time screen	↑↑↑	↑↑↑	↑↑	↑↑	–/↑	–
Russell viper venom time confirm	↑↑↑	↑↑	↑↑↑	↑↑	–/↑	–
Russell viper venom time screen/confirm ratio	–	↑	↓	↑/↓	–/↑/↓	–

Unfractionated heparin at therapeutic levels will not affect PT or RVVT when these reagents contain a heparin neutraliser; however, excess unfractionated heparin will affect these assays when neutraliser limits are exceeded. –, not affected; ↑, slightly affected (prolonged or raised); ↑↑, affected (prolonged); ↑↑↑, greatly affected (very prolonged); ↓, affected (reduced); multiple entries, such as –/(↑) or (↑)/– or –/↑ or ↑/↓ or –/↑/↓, indicate possibility for variable affects (may or may not be prolonged/raised/reduced dependent on reagent and level of anticoagulant, and/or heparin-neutralising ability of reagent (etc.)).

Using 'coagulation testing' patterns to help distinguish true LA positives/negatives from false positives/negatives

In a wholistic sense, in the current landscape of test requests, inclusive of many patients on anticoagulant therapy, the accurate identification or exclusion of LA requires a comprehensive approach. Looking at the results of multiple tests, not just LA based (e.g. RVVT screen and confirm), but also routine coagulation tests, helps to identify test patterns that can distinguish true LA from false-positive LA (e.g. due to DOACs) [15]. Mixing studies can assist in this process ([20]; Table 2). However, such an approach would be very time consuming and expensive given the high proportion of anticoagulated patients being tested for 'thrombophilia' (including LA). Thus, incorporating mixing studies early in testing can mitigate the occurrence of false-positive LAs due to anticoagulant interference, and avoid having to trouble-shoot a falsely prolonged LA ratio, especially as it is not always clear if the prolongation reflects a false-positive event.

Conclusions

This Point perspective takes the position that in the year 2020, mixing studies for appropriate LA identification/exclusion has become even more important than simply identifying an 'inhibitory' element of LA, as imbedded into the original LA criteria [19]. In particular, mixing studies act to effectively neutralise the effect of VKA therapy [1–5], and also 'dilute out' the potential effects of other anticoagulants such as unfractionated heparin and DOACs. In our experience, anticoagulant therapy can be identified in patients under investigation of LA at a rate at least three times that of true LA, and thus the risk of false-positive LA (in particular with rivaroxaban), exceeds the risk of false-negative LA due to LA dilution. Thus, on balance, mixing studies in LA testing, at least for (d) RVVT testing, should be the default position for LA investigation, and then additional LA testing by RVVT can be reflexed to use neat patient plasma on the rare occasion that a weak LA may be anticipated.

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