ORIGINAL ARTICLE

rpth
research & practice
in thrombosis & haemostasis

Ruling out lupus anticoagulants with mixing test–specific cutoff assessment and the index of circulating anticoagulant

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Abstract

Background: Lupus anticoagulant (LA) is classified in the antibody family that is recognized in antiphospholipid syndrome. Mixing tests are recommended for LA detection, and either a mixing test–specific cutoff (MTC) or index of circulating anticoagulant (ICA) is used for the interpretation. Although we previously showed MTC had higher sensitivity for LA than ICA, there are few studies investigating specificity.

Objectives: To investigate specificity of multiple activated partial thromboplastin time (APTT) and diluted Russell's viper venom time (dRVVT) reagents for inhibitors using plasmas with non-LA causes of prolonged clotting times, interpreted with MTC and ICA.

Methods: Seventy-six factor-deficient samples (either artificially prepared, hereditary deficiency, or warfarin), and 12 inhibitors (either coagulation factor inhibitors, rivaroxaban, or apixaban) were used. Samples were tested with 4 APTTs, 1 dilute APTT (dAPTT), and 2 dRVVT reagents, and all elevated screen ratios were followed up with mixing tests. Frequencies of corrected and not-corrected results were calculated.

Results: The frequency of MTC and ICA corrected results, suggesting factor deficiency, were 5% to 43% and 79% to 100%, respectively, except for dAPTT, where MTC and ICA performed similarly. Frequencies of MTC and ICA not-corrected results, suggesting inhibition, were 29% to 100% and 25% to 67%, respectively.

Conclusions: The data indicate that MTC has a tendency to generate not-corrected mixing tests in factor-deficient, warfarin, and other inhibitor samples, while ICA exhibited higher specificity. When we perform the mixing test and interpret the data, it is important to understand the characteristics of the indexes for maximizing the diagnostic potential of mixing test.

KEYWORDS
activated partial thromboplastin time, antiphospholipid antibodies, antiphospholipid syndrome, diluted Russell's viper venom time, lupus anticoagulant

Essentials

- Several indexes are available for mixing test interpretation in lupus anticoagulant detection.
- Mixing test–specific cutoff (MTC) and index of circulating anticoagulant (ICA) were used.
- ICA exhibited higher specificity than MTC in nonlupus anticoagulant samples with prolonged clotting times.
- It is important to understand the characteristics of indexes for mixing test interpretation.

1 | INTRODUCTION

The main symptoms of antiphospholipid syndrome (APS) are vascular thrombosis or pregnancy morbidity, and APS is diagnosed when laboratory assays demonstrate the presence of persistent antiphospholipid (aPL) antibodies in patients presenting with these symptoms.[1,2] Once APS is diagnosed, long-term anticoagulant therapy is considered because the risk of recurrent thrombosis is high.[3] Because thrombosis and pregnancy are nonspecific for APS, accurate detection of aPL antibodies in clinical laboratories is critical in securing a diagnosis of APS. Three types of aPL are defined as criteria antibodies in International Society on Thrombosis and Haemostasis (ISTH) guidance.[4] The antibodies detected in solid phase assays are anticardiolipin antibodies and anti-β2-glycoprotein I antibodies and are reported quantitatively. On the other hand, lupus anticoagulants (LAs) are detected by prolonged clotting times in uncalibrated coagulation assays.[3] A medley of phospholipid-dependent coagulation assays are employed for LA detection; screening tests to detect clotting time prolongation, mixing tests to evidence inhibition, and confirmatory tests to "bypass" the LA and shorten clotting times. Inherent difficulties and interferences with clotting assays complicate LA detection, and guidelines with broad but not complete agreement are available to lead best practice.[5–7]

All guidelines acknowledge that no single assay system will detect all LAs, and 2 different-principle assays are recommended for LA detection. The first test considered is diluted Russell's viper venom time (dRVVT), which is considered specific for LA detection in high-thrombosis-risk patients,[8] and the second test should be an LA-sensitive activated partial thromboplastin time (APTT). Testing order has proven controversial, and while ISTH guidelines recommend the traditional screen, then mix to detect inhibition and confirm only if the mix is positive,[5,6,9] other expert panels recommend alternative approaches. Concerns about false-negative mixing tests due to the dilution effect resulted in the Clinical and Laboratory Standards Institute guideline recommending initial measurement of screening and confirmation tests to evidence the phospholipid dependence of LA and performance of mixing tests when screening/confirmation test results are not clear-cut.[7,9] The British Society for Haematology guidelines suggest performing the full medley but indicate that apparently normal mixing tests can be disregarded in certain circumstances. In all guidelines, the mixing test is recommended, and it is useful and important for demonstrating the presence of LA and differentiating the inhibitor from a factor deficiency.

Two mixing test interpretation methods, mixing test–specific cutoff (MTC) and the index of circulating anticoagulant (ICA) were described in the guidelines. MTC is derived from the upper limit of population distribution data for screening test ratios performed on 1:1 mixtures with a common normal pooled plasma. Ratios are calculated as: 1:1 mix sample (seconds)/1:1 mix reference interval mean (seconds). The formula for ICA is ([1:1 Mix Sample (seconds) − Normal Pooled Plasma (seconds)]/Screen Patient (seconds)) × 100. Because weaker LA can generate negative mixing tests despite clear screen and confirm positivity in undiluted plasma,[6,7,10,11] it is crucial to perform mixing tests with high sensitivity reagents and an appropriate index. Aside from low-potency antibodies, false-negative mixing tests can be induced by a less sensitive reagent, higher cutoff values, and some clinical conditions. We previously reported higher sensitivity to LA with MTC than ICA with multiple APTT and dRVVT reagents.[12] It is important to investigate with multiple reagents because sensitivity and specificity for LA, especially with APTT, varies due to differences in phospholipid composition and concentration.[13–15] However, that study focused on only sensitivity to known LAs, and specificity was not investigated. There are few data comparing MTC and ICA in the context of LA specificity, and the present study aims to complement the previous study and compare performance of MTC and ICA with multiple reagents in samples with non-LA causes of prolonged clotting times.

2 | MATERIALS AND METHODS

2.1 | Plasma samples

The methods of blood collection and sample preparation were as previously described.[16] Briefly, blood samples were collected into Vacuette tubes (Greiner Bio-One Ltd, Tokyo, Japan), which include a one-tenth volume of 0.105 mol/L (3.2%) trisodium citrate. These samples were double-centrifuged and stored at −80°C until use. The plasma samples were derived from 5 patients with hemophilia A (factor VIII [FVIII], 2.2-10.7 IU/dL), 4 with hemophilia B (factor IX [FIX], <1-23.8 IU/dL), one with factor XII (FXII) deficiency (FXII, 39.9 IU/dL), and 25 non-APS warfarinized patients with a prolonged APTT using an LA-insensitive reagent (see below). In addition, 6 samples with FVIII inhibitors were included (1.02-69.76 NBU/mL). Plasmas from 6 patients anticoagulated with direct factor Xa (FXa) inhibitors (5 on rivaroxaban, 1 on apixaban) for reasons other than APS were also tested. The drug concentrations were 15 to 54 and 295 ng/mL, respectively. For further analysis, artificial factor-deficiency samples were prepared to assess specificity at different degrees of single-factor reductions. Lyophilized plasmas, immunodepleted of single coagulation factors II, V, VIII, IX, X, XI, or XII (Siemens Healthineers, Erlanger, Germany) were mixed with standard human plasma (Siemens Healthineers) to produce 6 samples for each factor at concentrations of <1%, 1%, 2%, 5%, 10%, and 20%. The levels of the nondepleted factors have previously been shown to be normal in these lyophilized plasmas.[17] The <1% factor II plasma was excluded from data analysis because of a no-coagulation error.

TABLE 1 Cutoff ratios for each index and reagent

	APTT				dAPTT	dRVVT	
	SLA	FSL	SP	Cephen	PTT	dRVVT A	dRVVT B
Screen ratio	1.13	1.12	1.15	1.09	1.20	1.17	1.12
Mix ratio	1.07	1.07	1.08	1.04	1.15	1.07	1.06
ICA	12.4	10.4	13.6	12.0	13.2	11.9	12.0

APTT, activated partial thromboplastin time; Cephen, Cephen 2.5 LS; dAPTT, dilute activated partial thromboplastin time; dRVVT, diluted Russell's viper venom time; FSL, Actin FSL; ICA, index of circulating anticoagulant; PTT, PTT-LA; SLA, Thrombocheck APTT-SLA; SP, APTT-SP.

2.2 | Coagulation screening tests

Prothrombin time (PT), APTT, fibrinogen by the Clauss method, and thrombin time were performed as coagulation screening tests. The reagents were Dade Innovin, Actin FS, Thrombin-Reagent, and Thromboclotin (Siemens Healthineers), respectively. All tests were performed in a Sysmex CS-2100i (Sysmex UK, Milton Keynes, UK).

2.3 | Reagents for mixing tests

Four APTT, 1 dilute APTT (dAPTT), and 2 dRVVT screening reagents having high LA sensitivity were employed for the mixing tests in this study. The APTT reagents were Thrombocheck APTT-SLA (SLA) (Sysmex Corporation, Kobe, Japan), Actin FSL (FSL) (Siemens Healthineers), APTT-SP (SP) (Instrumentation Laboratory Company, Bedford, MA), and Cephen 2.5 LS (Hyphen BioMed, Neuville-sur-Oise, France), and the dAPTT reagent was PTT-LA (PTT) (Diagnostica Stago UK, Theale, UK). The two dRVVT screening reagents were STACLOT DRVV Screen (dRVVT A) (Diagnostica Stago) and LA1 Screening reagent

(dRVVT B) (Siemens Healthineers). These tests were performed in a CS-2400 (Sysmex Corporation). The compositional characteristics of each reagent were previously described.[12] All clotting time results were determined once, as imprecision is low with automated coagulometers.

2.4 | Mixing tests and the cutoff values

The plasma samples used in this study were mixed with normal pooled plasma (NPP) in a ratio of 1:1, and mixing tests were performed without incubation. CRYOcheck frozen pooled normal plasma (Precision BioLogics Inc., Dallas, TX) was used as the NPP. Samples with elevated APTT or dRVVT screen ratios in undiluted plasma subsequently received mixing tests, which were interpreted with both MTC and ICA. The screen ratio, mix ratio, and ICA formulas were as follows: Screen Ratio = Screen Patient (seconds)/Reference Interval Mean (seconds), Mix Ratio = 1:1 Mix Sample (seconds)/1:1 Mix Reference Interval Mean (seconds), ICA = ([1:1 Mix Sample (seconds) – Normal Pooled Plasma (seconds)]/Screen Patient (seconds)) × 100. All the mixing tests were performed on the
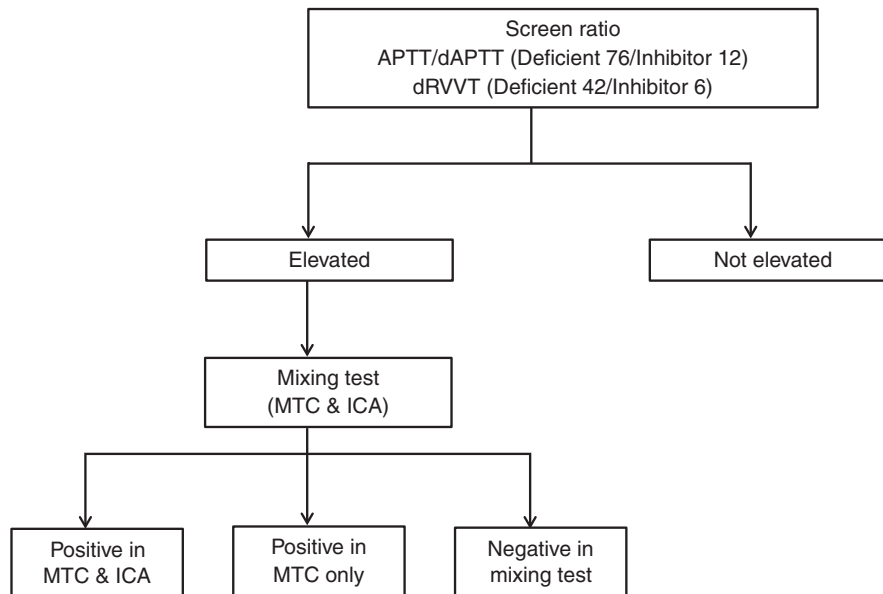

FIGURE 1 Mixing tests algorithm for factor deficient and inhibitor samples. Screen ratios from undiluted plasma were calculated for all samples in APTT/dAPTT and dRVVT. When the initial screen ratio was elevated, the mixing test was performed and MTC and ICA were calculated. The samples with elevated screen ratios were divided into 3 groups: (1) not-corrected in MTC and ICA, (2) not-corrected in MTC only, and (3) corrected in mixing test. There were no samples that were not-corrected in only ICA. Abbreviations: APTT, activated partial thromboplastin time; dAPTT, dilute activated partial thromboplastin time; dRVVT, diluted Russell's viper venom time; ICA, index of circulating anticoagulant; MTC, mixing test–specific cutoff

TABLE 2 Mixing test results of MTC and ICA in the samples with elevated screen ratio

Reagent	Number of samples with elevated screen ratio (%)	Not-corrected in mixing test by MTC and ICA	Not-corrected in mixing test by MTC only	Corrected in mixing test
(A)				
SLA	75/76 (98.7)	0	48	27
FSL	76/76 (100)	16	38	22
SP	73/76 (96.1)	1	46	26
Cephen	74/76 (97.4)	1	41	32
PTT	68/76 (89.5)	1	3	64
dRVVT A	42/42 (100)	1	29	12
dRVVT B	42/42 (100)	1	39	2
(B)				
SLA	8/12 (66.7)	2	6	0
FSL	9/12 (75.0)	5	2	2
SP	6/12 (50)	2	4	0
Cephen	8/12 (66.7)	2	5	1
PTT	7/12 (58.3)	2	0	5
dRVVT A	3/6 (50)	2	0	1
dRVVT B	5/6 (83.3)	2	2	1

(A) The coagulation factor–deficient group includes artificially prepared plasmas deficient in single coagulation factor II, V, VIII, IX, X, XI or XII, hereditary deficiencies of factors VIII, IX, and XII, and warfarin. (B) The inhibitor group includes samples containing factor VIII inhibitors and the direct factor Xa inhibitors rivaroxaban and apixaban.

APTT, activated partial thromboplastin time; Cephen, Cephen 2.5 LS; dAPTT, dilute activated partial thromboplastin time; dRVVT, diluted Russell's viper venom time; FSL, Actin FSL; ICA, index of circulating anticoagulant; MTC, mixing test–specific cutoff; PTT, PTT-LA; SLA, Thrombocheck APTT-SLA; SP, APTT-SP.

CS-2400 (Sysmex Corporation) employing the automatic dilution function. The cutoff values established in our previous study were employed for screen ratio, mix ratio, and ICA for all reagents shown in Table 1,[12] and are comparable to those in other reports.[11,18]

2.5 | Mixing tests algorithm for deficient and inhibitor samples

The mixing test algorithm for factor-deficient and inhibitor samples is shown in Figure 1. All factor-deficient and inhibitor samples were analyzed with each reagent. Samples were divided into 3 groups: (1) not-corrected in MTC and ICA, (2) not-corrected in MTC only, and (3) corrected in mixing test. No samples were not-corrected in ICA only. As would be anticipated, the samples deficient in FVIII, FIX, and FXII and with inhibitors toward FVIII did not elevate dRVVT screen ratios and were excluded from data analysis.

3 | RESULTS

3.1 | Screen ratios, mix ratios, and ICA in each reagent

The numbers of samples with elevated screen ratios with each reagent and their mixing test results are shown in Table 2. In 76 factor-deficient and warfarin samples analyzed with APTT and dAPTT, 75, 76, 73, 74,

and 68 had elevated screen ratios in SLA, FSL, SP, Cephen, and PTT, respectively. The 42 samples containing common pathway deficiencies or from warfarinized patients all generated prolonged clotting times in both dRVVT A and dRVVT B. Of the 12 samples from patients with FVIII inhibitors or on direct FXa inhibitors, elevated screen ratios were generated in 8, 9, 6, 8, and 7 of the 4 APTT, and dAPTT reagents, respectively. The 6 samples from patients on direct FXa inhibitors generated 3 and 5 elevated screen ratios in dRVVT A and dRVVT B, respectively. More than half of the factor-deficient and warfarin samples were not-corrected by MTC in multiple APTT and dRVVT reagents. Only PTT reagent showed similar results between MTC and ICA, and 4 samples generated not-corrected results by MTC. The MTC was not-corrected for more than half of the FVIII inhibitor and direct FXa inhibitor samples. Only 2 of these samples generated an elevated ICA in APTT/dAPTT, both of which were plasmas containing FVIII inhibitors, whose titers were 22.9 and 69.76 NBU/mL. In the dRVVT A and dRVVT B reagents, 1 rivaroxaban and 1 apixaban sample were ICA not-corrected; the concentrations were 54 and 295 ng/mL, respectively. Overall, the frequency of samples with MTC elevation was higher than for ICA.

3.2 | The percentage of corrected and not-corrected samples in MTC and ICA

Theoretically, the factor-deficient and warfarin samples should achieve corrected mixing test results. The frequencies of corrected

TABLE 3 Percentage of corrected results calculated from MTC and ICA in samples with high screen ratio

	APTT				dAPTT	dRVVT	
	SLA	FSL	SP	Cephen	PTT	dRVVT A	dRVVT B
(A)							
Number of samples with an elevated initial screen ratio in undiluted plasma	75	76	73	74	68	42	42
Percentage of samples with an elevated initial screen ratio in undiluted plasma (%)	99	100	96	97	90	100	100
Percentage of samples with an MTC-corrected mixing test (%)	36	29	36	43	94	29	5
Percentage of samples with an ICA-corrected mixing test (%)	100	79	99	99	99	98	98
(B)							
Number of samples with an elevated initial screen ratio in undiluted plasma	8	9	6	8	7	3	5
Percentage of samples with an elevated initial screen ratio in undiluted plasma (%)	67	75	50	67	58	50	83
Percentage of samples with an MTC not-corrected mixing test (%)	100	78	100	88	29	67	80
Percentage of samples with an ICA not-corrected mixing test (%)	25	56	33	25	29	67	40

(A) The coagulation factor–deficient group includes artificially prepared plasmas deficient in single coagulation factor II, V, VIII, IX, X, XI or XII, hereditary deficiencies of factors VIII, IX, and XII, and warfarin. The numbers of samples with elevated screen ratios in undiluted samples are presented because mixing tests are performed only when the initial screen is elevated. The percentage of samples with elevated initial screen ratios in undiluted plasma was calculated in APTT/dAPTT and dRVVT. The total numbers with elevated initial screens were 76 and 42, respectively. The sample groups were the same as Table 2. (B) The inhibitor group includes samples containing factor VIII inhibitors and the direct factor Xa inhibitors rivaroxaban and apixaban. The total number of samples with elevated initial screen ratios were 12 and 6 for APTT and dRVVT, respectively. The sample groups were the same as Table 2.

APTT, activated partial thromboplastin time; Cephen, Cephen 2.5 LS; dAPTT, dilute activated partial thromboplastin time; dRVVT, diluted Russell's viper venom time; FSL, Actin FSL; ICA, index of circulating anticoagulant; MTC, mixing test–specific cutoff; PTT, PTT-LA; SLA, Thrombocheck APTT-SLA; SP, APTT-SP.

results in MTC and ICA were calculated in APTT/dAPTT and dRVVT for 76 and 42 samples, respectively (Table 3). The mixing tests were corrected more frequently with ICA than MTC except for the PTT reagent, which is the only one specifically formulated for LA testing. The 95th percentile confidence intervals for MTC and ICA for all the reagents were 13.8% to 63.9% and 89.0% to 103.0%, respectively. On the other hand, coagulation inhibitor, rivaroxaban, and apixaban samples would theoretically be expected to generate not-corrected mixing test results. Application of MTC revealed a higher frequency of detection of inhibition than ICA with all reagents except PTT and dRVVT A, and the 95th percentile confidence intervals were 54.8% to 100.1% and 24.2% to 54.4%, respectively. The consistent identification percentages were also calculated by both deficient and inhibitor samples to find how many samples were correctly identified as samples with deficiency or inhibitor in MTC and ICA. The consistent identification percentage was defined as (Number of Samples Correctly Identified in the Mixing Test/Total Number of Samples) × 100 (%), and was calculated using the results from all factor-deficient and inhibitor samples with each reagent. In all reagents, the consistent identification percentages for ICA were higher than those of MTC (Table 4).

4 | DISCUSSION

Although recommendations for test order vary among the 3 current LA guidelines, mixing tests are recommended in all 3 despite acknowledged limitations, so there is value in maximizing diagnostic performance.[5-7] The present study investigated performance of mixing studies on non-LA plasma samples with elevated screen ratios in APTT, dAPTT, and/or dRVVT prior to dilution in NPP to evaluate specificity of the 2 recommended mixing test interpretive parameters in LA detection, MTC, and ICA.

A higher frequency of corrected mixing tests in samples with reduced coagulation factor levels was encountered when applying ICA than MTC in all reagents except PTT, which exhibited a similar frequency of corrected values for both ICA and MTC. High specificity for ICA has been previously described for multiple-LA-sensitive APTT reagents when comparing samples with reduced coagulation factor levels against those known to contain LA.[19] However, recent studies have shown that ICA is less sensitive than MTC to the presence of LA in multiple reagents.[12,16,20] The most likely explanation for the apparent reduced specificity of MTC is that the broad principle of mixing tests expects that 50% of a given factor is sufficient to

TABLE 4 Percentages correctly identified as deficiency or inhibitor in MTC and ICA

	APTT				dAPTT	dRVVT	
	SLA	FSL	SP	Cephen	PTT	dRVVT A	dRVVT B
Number of samples with high screen ratio in undiluted plasmas	83	85	79	82	75	45	47
Percentage samples with high screen ratio in undiluted plasmas (%) Total: APTT/dAPTT, 88 samples; dRVVT, 48 samples	94	90	90	93	85	94	98
MTC consistent identification percentage in mixing test (%)	42	34	41	48	88	31	13
ICA consistent identification percentage in mixing test (%)	93	76	94	92	92	96	92

The number of samples with elevated initial screen ratios in undiluted plasmas for both deficient and inhibitor groups for APTT/dAPTT and dRVVT were 88 and 48, respectively. The consistent identification percentages were calculated from the total number of samples in both deficient and inhibitor groups. The sample groups were the same as Table 2.
APTT, activated partial thromboplastin time; Cephen, Cephen 2.5 LS; dAPTT, dilute activated partial thromboplastin time; dRVVT, diluted Russell's viper venom time; FSL, Actin FSL; ICA, index of circulating anticoagulant; MTC, mixing test–specific cutoff; PTT, PTT-LA; SLA, Thrombocheck APTT-SLA; SP, APTT-SP.

restore a clotting time to within the reference range, yet the nature of generating mixing test–specific ranges leads to lower cutoffs[11] such that a more marked deficiency may well return into the reference range for undiluted plasma but not the mixing test range.
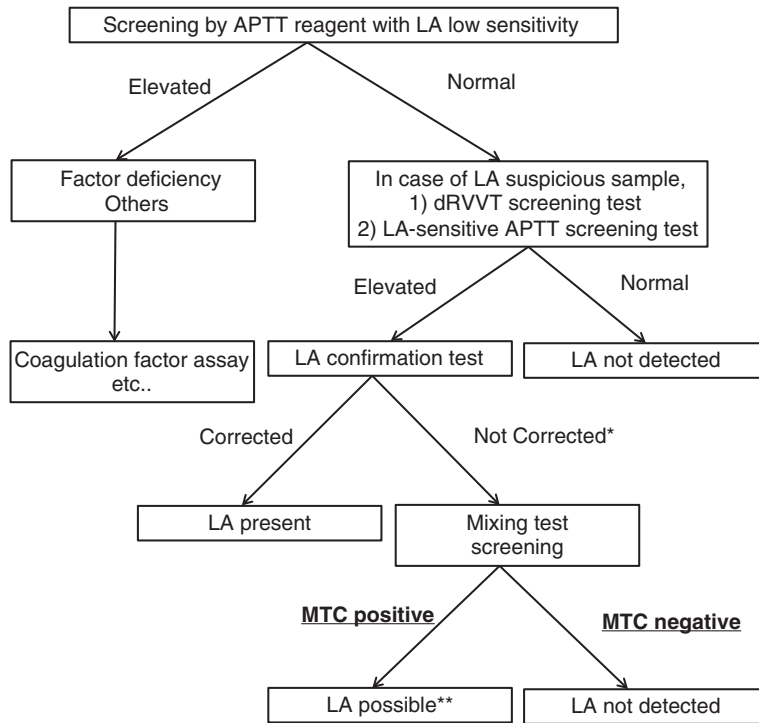
Mixing tests serve to differentiate between inhibitors and factor deficiencies,[21-23] yet they are not specific for LA-induced inhibition, which is the role of confirmatory tests, so samples containing FVIII inhibitors and direct FXa inhibitors were included in this study. Although FVIII inhibitors are progressive, sufficiently potent or avid antibodies can manifest in immediate APTT mixing tests,[22] which was encountered in some of the samples in this study. Similarly, direct FXa inhibitors have inevitably been shown to prolong APTT and dRVVT measurements in undiluted plasma and mixing tests.[24-27] The frequencies of elevated MTC and ICA in the multiple reagents were 29% to 100% and 25% to 67%, respectively, attesting to the lower sensitivity of ICA to the presence of inhibition compared to MTC. Manifestation of direct FXa inhibitors in a given assay and its mixing test is a function of drug concentration and reagent responsiveness. Rivaroxaban and apixaban are inhibitory by design and ex vivo samples with a range of drug concentrations were employed to assess

detection of direct FXa inhibitor–induced inhibition.[26] Samples from patients on edoxaban and dabigatran were not locally available but not-corrected mixing tests have been previously demonstrated.[28] As direct oral anticoagulant (DOAC) levels are infrequently performed in the clinical diagnostic setting, the number of DOAC-containing samples was limited in this study and further work is planned to achieve a wider assessment of DOAC interference in mixing tests with multiple reagents. In addition, an attempt to establish thresholds with best balances between sensitivity and specificity for factor deficiency and inhibitor in each mixing test index is planned.
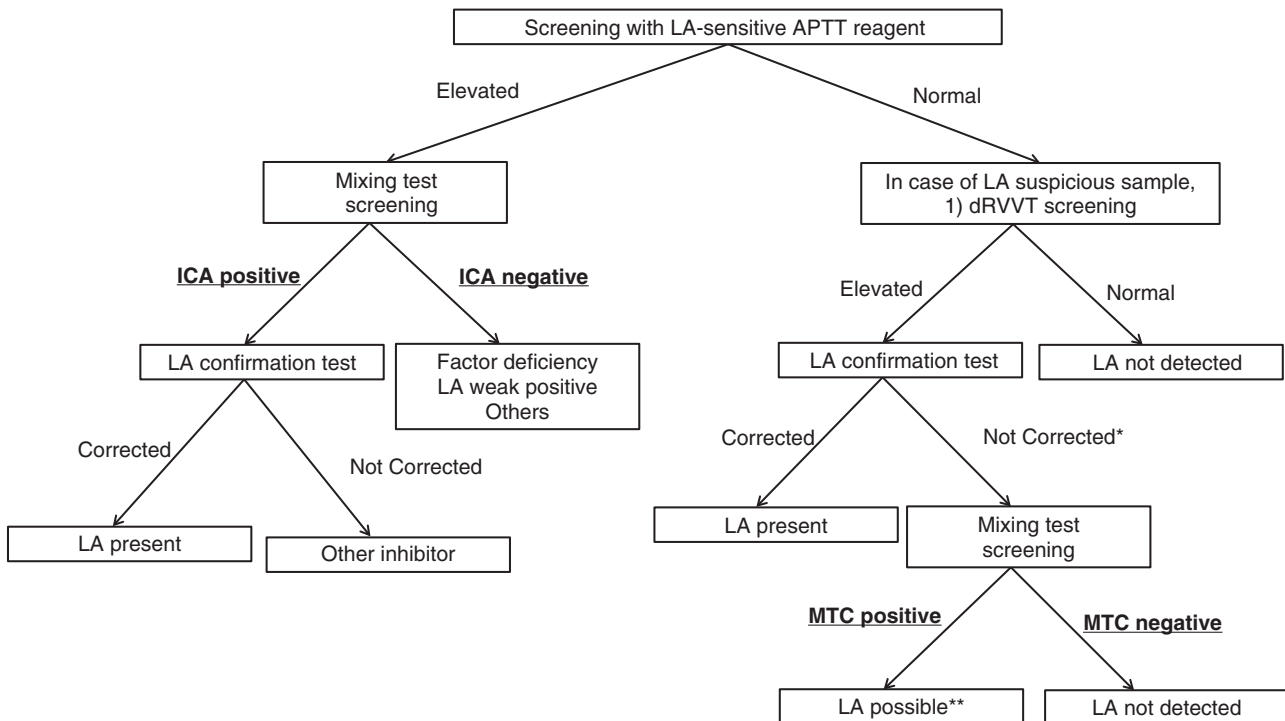
Based on the greater specificity for non-LA causes of prolonged clotting times with ICA but greater sensitivity to inhibition for MTC, we suggest LA diagnostic algorithms incorporating different approaches, depending on whether the APTT reagent in use has high or low LA sensitivity (Figure 2). In the routine coagulation screening tests for this study, Actin FS, recognized as an LA-insensitive APTT reagent, was used in our laboratory.[29] When the clotting time is prolonged with Actin FS in a nonanticoagulated patient, factor deficiency is considered first, as LA is largely excluded, with recognition that potent LA can elevate

FIGURE 2 Lupus anticoagulant (LA) diagnostic flow with mixing tests in LA-sensitive and LA-insensitive APTT screening reagents. A, When the routine coagulation screening is performed with an LA-insensitive APTT reagent, factor deficiency is the first consideration for prolonged results. Where investigation for LA is specifically being undertaken, (1) dRVVT screening and (2) APTT with high LA sensitivity should be performed. In this case, the sensitivity is important for the mixing test and MTC is the preferred option. B, When the routine coagulation screening is performed with an LA-sensitive APTT reagent, it is important to differentiate between inhibitor samples like LA and factor deficiency at this point. If ICA is not-corrected, the LA confirmatory test should be performed, and if ICA is corrected, factor deficiency is the first consideration. In addition to the LA-sensitive APTT reagent, dRVVT screening should be performed whether or not an LA has been demonstrated in APTT. Deficiencies of factors II, V, and X and fibrinogen could manifest in the dRVVT screening test, but application of MTC to the mixing test may not exhibit correction, whereupon the confirmatory test will provide crucial information on phospholipid dependence, and ICA may also be valuable. *If a confirmatory test corrects the screening test but does not itself normalize, a mixing test can be useful to identify whether there is a concomitant abnormality. **Some potent/avid LA can partially or wholly overcome the swamping effect of confirmatory reagents, and it can be informative to also perform a confirmatory test mix where the dilution effect can permit screen and confirm discordance to manifest. APTT, activated partial thromboplastin time; dRVVT, diluted Russell's viper venom time; ICA, index of circulating anticoagulant; MTC, mixing test–specific cutoff

(A) LA-insensitive APTT screening reagent

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                    Screening by APTT reagent with LA low sensitivity

         Elevated                                          Normal

   Factor deficiency                  In case of LA suspicious sample,
       Others                           1) dRVVT screening test
                                        2) LA-sensitive APTT screening test

                                   Elevated                    Normal

  Coagulation factor assay      LA confirmation test        LA not detected
         etc..

                           Corrected            Not Corrected*

                       LA present              Mixing test
                                                screening

                            MTC positive                MTC negative

                              LA possible**          LA not detected
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(B) LA-sensitive APTT screening reagent

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                    Screening with LA-sensitive APTT reagent

            Elevated                                    Normal

         Mixing test                        In case of LA suspicious sample,
          screening                             1) dRVVT screening

    ICA positive      ICA negative          Elevated            Normal

  LA confirmation test   Factor deficiency  LA confirmation test   LA not detected
                         LA weak positive
                            Others

   Corrected      Not Corrected      Corrected          Not Corrected*

  LA present    Other inhibitor     LA present          Mixing test
                                                         screening

                                              MTC positive        MTC negative

                                                LA possible**    LA not detected
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clotting times and ratios even with this reagent. For patients who are specifically being investigated for APS, dRVVT and dAPTT testing are subsequently performed. In this diagnostic flow, a normal routine APTT via Actin FS permits interpretation of dRVVT and dAPTT testing unencumbered by the possibility of numerous interferences. Having excluded factor deficiencies via a different mechanism than mixing tests, adoption of MTC to interpret the LA assay mixing test will increase detection rates of inhibition. On the other hand, LA-sensitive APTT reagents are also used as a broader screening test in many laboratories.[30] In this case, they serve to

detect all abnormalities and circumvent the need for a separate APTT reagent when specifically investigating for LA. However, this introduces a requirement to effectively distinguish between LA and other causes of prolonged APTT at this stage in the diagnostic flow. At this step, we suggest employing a mixing test interpreted with ICA due to its superior specificity, as there are few false positives for inhibition when the initial elevated screening test is due to factor deficiency, as shown in Table 4. When ICA is positive at this juncture, the LA confirmatory test should be performed to complete the medley. In cases of an ICA corrected result, factor deficiency is the first consideration, but the possibility of a weak LA must be borne in mind because of the lower LA sensitivity of ICA compared to MTC. Even if the clotting time or ratio of an LA-sensitive APTT are not prolonged, reagent and antibody heterogeneity require that dRVVT screening also be performed when investigating for LA.[5–7] When dRVVT screening, confirmation, and mixing tests are examined, MTC should be adopted for mixing test interpretation, as it has superior LA sensitivity to ICA,[12,16,20] and common pathway factor deficiencies will have been largely excluded by the APTT investigative approach described above, in tandem with prothrombin time, with acknowledgment that sensitivity of APTT reagents to LA, coagulation deficiency, coagulation inhibitor, and drugs vary because of the compositional differences.[5]

There were some limitations in our study, such as the small numbers of samples containing hereditary factor deficiencies, FVIII inhibitors, and direct FXa inhibitors, which may not fully reflect the spread of mixing test results encountered in the routine diagnostic setting. Additionally, while the artificial factor-deficient samples gave an indication of assay behavior at different levels of isolated coagulation factor reductions, they cannot reproduce variability in clinical samples arising from different molecular variants.

In conclusion, ICA exhibited superior specificity to MTC in multiple reagents when investigating non-LA causes of prolonged clotting times. Alternative approaches to adoption of these indexes are proposed based on local approach to routine coagulation screening, with recognition that neither index provides diagnostic certainty.

ACKNOWLEDGMENTS

RELATIONSHIP DISCLOSURE

AUTHOR CONTRIBUTIONS

OK performed the measurement, analysis, and interpretation of the data and wrote the manuscript. GWM designed the research, interpreted data, and critically revised the manuscript.

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