International Council for Standardization in Haematology Field Study Evaluating Optimal Interpretation Methods for Activated Partial Thromboplastin Time and Prothrombin Time Mixing Studies

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• ***Context.***—**The prothrombin time (PT) and activated partial thromboplastin time (APTT) are screening tests used to detect congenital or acquired bleeding disorders. An unexpected PT and/or APTT prolongation is often evaluated using a mixing test with normal plasma. Failure to correct ("noncorrection") prolongation upon mixing is attributed to an inhibitor, whereas "correction" points to factor deficiency(ies).**

Objective.—**To define an optimal method for determining correction or noncorrection of plasma mixing tests through an international, multisite study that used multiple PT and APTT reagents and well-characterized plasma samples.**

Design.—**Each testing site was provided 22 abnormal and 25 normal donor plasma samples, and mixing studies were performed using local PT and APTT reagents. Mixing study results were evaluated using 11 different calculation methods to assess the optimal method based on the expected interpretation for factor deficiencies (correction) and noncorrection (inhibitor effect). Misprediction, which represents the failure of a mixing study interpretation method, was assessed.**

Results.—**Percentage correction was the most suitable calculation method for interpreting PT mixing test results for nearly all reagents evaluated. Incubated PT mixing tests should not be performed. For APTT mixing tests, percentage correction should be performed, and if the result indicates a factor deficiency, this should be confirmed with the subtraction III calculation where the normal pooled plasma result (run concurrently) is subtracted from the mixing test result with correction indicated by a result of 0 or less. In general, other calculation methods evaluated that performed well in the identification of factor deficiency tended to have high misprediction rates for inhibitors and vice versa.**

Conclusions.—**No single method of mixing test result calculation was consistently successful in accurately distinguishing factor deficiencies from inhibitors, with between-reagent and between-site variability also identified.**

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Prothrombin time (PT) and activated partial thromboplastin time (APTT) are screening tests used worldwide for the evaluation of patients suspected of congenital or acquired bleeding disorders; they are also used for monitoring purposes (eg, heparin or oral vitamin K antagonists [OVKAs] or replacement therapies) and in the identification of a lupus anticoagulant (LAC).[1,2] An unexpected prolongation of the PT and/or APTT can be attributed to a number of causes, including isolated or multiple factor deficiencies or the presence of an inhibitor.[3,4] A potentially useful screening mechanism to identify the cause of a prolonged PT or APTT is the mixing test, which is simply repeating the prolonged screening test after mixing the sample with normal pooled plasma (NPP). Failure to correct a prolonged PT or APTT upon mixing is often attributed to an inhibitor, whereas conversely, correction of the prolongation is attributed to single or multiple factor deficiencies.[5–7] The ability to differentiate between a factor deficiency and inhibitor will likely direct further evaluation and treatment of the patient.[8]

The traditional mixing test is performed by adding patient plasma to NPP at equal volumes/ratio.[7] Deviations from this have been described, including using 4 parts patient plasma to 1 part NPP or the addition of an incubation step at 37°C. Although the mixing test has been used for decades, standardization of mixing test performance, as well as methods of calculating results, is lacking.

Plasma mixing tests should be considered in the investigation of select patients with unexplained prolongation of PT and/or APTT. Guidance has been provided for mixing test use

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Table 1. Abnormal Sample Characteristics and Expected Mixing Study Results

	Salient Feature	Expected Baseline PT	Expected Baseline APTT	Expected PT Mix Study	Expected APTT Mix Study
FII DP	FII <1%	P	P	C	C
FV DP	FV <1%	P	P	C	C
FVII DP	FVII <1%	P	NP	C	—
FVIII DP	FVIII <1%	NP	P	—	C
FIX DP	FIX <1%	NP	P	—	C
FX DP	FX <1%	P	P	C	C
FXI DP	FXI <1%	NP	P	—	C
FXII DP	FXII <1%	NP	P	—	C
ABN 1	PT ~25 s; APTT ~60 s	P	P	C	C
ABN 2	PT ~44 s; APTT ~73 s	P	P	C	C
OVKA S1	INR 2.32	P	P	C	C
OVKA S2	INR 4.66	P	P	C	C
UFH	0.49 IU/mL anti-Xa	NP	P	—	NC
LMWH	0.68 IU/mL anti-Xa	NP	P	—	NC
Rivaroxaban[a]	266 ng/mL	P	P	NC	NC
Apixaban[a]	201 ng/mL	P	NP	NC	—
Edoxaban[a]	163 ng/mL	P	P	NC	NC
Dabigatran[a]	~200 ng/mL	P	P	NC	NC
FVIII inhibitor 1	0.6 BU	NP	P	—	NC
FVIII inhibitor 2	70 BU	NP	P	—	NC
LAC 1[b]	DRVVT ratio 2.07	NP	P	—	NC
LAC 2[b]	DRVVT ratio 1.53	NP	P	—	NC

Abbreviations: ABN, abnormal; APTT, activated partial thromboplastin time; BU, Bethesda unit; C, corrected; DP, deficient plasma; DRVVT, dilute Russell viper venom time; FII, FV, etc, factor II, factor V, etc; INR, international normalized ratio; LAC, lupus anticoagulant; LMWH, low-molecular-weight heparin; NC, not corrected; NP, not prolonged; OVKA, oral vitamin K antagonist; P, prolonged; PT, prothrombin time; UFH, unfractionated heparin.

[a] PT and APTT sensitivity to direct oral anticoagulants is highly variable.

[b] LAC 1 and 2 were the CRYOcheck LAC positive controls, lupus positive control and lupus weak positive control, from Precision BioLogic, respectively. For the purposes of this study, baseline results for each direct oral anticoagulant are indicated as prolonged, with the expected mixing study results as noncorrected given the inhibitory nature of these drugs. DRVVT ratios were derived from screen normalized ratio/confirmed normalized ratio.

in patients suspected of having a LAC as part of the diagnostic algorithm.[9–11] Where available, patient history, including recent drug exposure, must be reviewed prior to initiating testing, as this information could be useful for result interpretation. Mixing tests should not be performed on samples with normal baseline PT and/or APTT results, nor should they be performed in patients with known exposure to anticoagulant agents.

A recent international study of 990 laboratories revealed large variations in mixing test methodologies, with only 49% of responding participants providing accurate interpretation of the mixing test results.[12] This high percentage of inaccurate result reporting likely reflects the lack of standardization for both determining what constitutes a corrected mixing test result and the method for determining correction. As such, in 2019 the International Council for Standardization in Haematology (ICSH) agreed to provide funding for an international, multisite study to evaluate PT and APTT mixing tests using well-characterized plasma samples with the purpose of determining whether there is an optimal method for determining correction or noncorrection. In this field study, 11 different, previously described mixing test calculation methods to ascertain correction or noncorrection using a variety of PT and APTT reagent platforms were compared.

MATERIALS AND METHODS

This study was funded and supported by ICSH such that the test samples and NPP were provided at no cost to participating clinical laboratory sites. Buffered citrated plasma samples used in this study were prepared, stored, and shipped by a single-source commercial vendor (Precision BioLogic, Halifax, Nova Scotia, Canada). The ICSH mixing test kit consisted of frozen plasma sets including 22 abnormal samples, 25 normal donor samples, and NPP. Kits were shipped on dry ice for overnight delivery to each selected laboratory site. The characteristics of each abnormal sample are listed in Table 1. Normal donors consisted of 12 women (median age, 37 years; range, 22–50 years) and 13 men (median age, 40 years; range, 23–64 years). The NPP was collected from a minimum of 20 ostensibly healthy individuals with no evidence of hemostatic disease, then buffered with hydroxyethylpiperazine ethane sulfonic acid buffer. Single-factor–deficient plasmas were prepared by immunoadsorption, with each preparation quality tested to confirm normal and decreased factor activity levels as appropriate (Supplemental Table 1; see supplemental digital content containing 3 tables at https://meridian.allenpress.com/aplm in the August 2024 table of contents). Combined-factor–deficient plasma samples were prepared using either adsorbed plasma or plasma from patients on OVKA. Individual inhibitor-type anticoagulants were added to pooled normal plasma to achieve concentrations consistent with therapeutic or "on-therapy" concentrations.[13,14] Factor VIII inhibitor and LAC plasma samples were from well-characterized human donor sources (Supplemental Table 2).

All prepared plasma samples were quality tested for accuracy of sample characterization, aliquoted, rapidly frozen, and stored at −40°C to −80°C. Confirmation of kit receipt and sample status (residual dry ice present, samples maintained in frozen state) was made to the shipper and ICSH study investigators.

Twenty clinical laboratories that used the most common instrument/reagent platforms based on external quality assurance programs were sought for participation. The number of clinical laboratories using the

Table 2. Calculation Methods Used

Method ID	Formula	Explanation	Target Correction
Subtraction I	Test Result − URI	URI used for patient reporting subtracted from test result	Within local RI
Subtraction II	Test Result − Donor Mix URI	Donor mix URI (derived from donor plasma samples after mixing with NPP) subtracted from the test result	<Donor mix URI
Subtraction III	Test Result − NPP	NPP result (run concurrently) subtracted from test result	≤NPP
Ratio I	Test Result/Local Mean RI	Test result divided by the site-provided mean used for patient reporting	≤1.1 and ≤1.2
Ratio II	Test Result/Local Normal Donor + NPP RI	Test result divided by the locally determined mean from NPP + provided donor samples	≤1.1 and ≤1.2
Ratio III	Test Result/NPP	NPP value from run	≤1.1 and ≤1.2
% Correction	[Patient Baseline Result − 1:1 Mixture Result] ÷ [Patient Baseline Result − NPP Result]	See calculation	≥70%
Rosner index	[(1:1 Mixture Result − NPP Result) ÷ Patient Baseline Result] × 100	See calculation	≤15%

Abbreviations: NPP, normal pooled plasma; RI, reference interval; URI, upper reference interval.

same PT or APTT reagent platform was limited to 6. Testing sites had the option to use more than one reagent platform; however, a limiting factor was the provided test sample volume (1.0 mL). Laboratories using in-house or laboratory-developed PT or APTT methods were excluded from participation. In vitro diagnostic companies that provide PT and APTT reagents were separately invited to participate, although their participation required the ICSH mixing study kits to be purchased.

Each testing site was provided specific instructions for thawing the samples and performing PT and APTT mixing tests and was encouraged to perform such studies during a series of days using 2 or more technicians. For each mixing study run, laboratories were instructed to test the NPP as a stand-alone sample, as this was necessary for select calculations (Table 2). A clotting time that exceeded the upper limit of the reference interval (RI) provided by the testing site was considered an abnormal baseline result. After completion of baseline values, each sample (abnormal samples and normal donor samples) was mixed with NPP at a 1:1 ratio (immediate phase) and retested for PT and APTT. Mixing tests were performed on all samples, including those from normal donors, regardless of initial (baseline) values.

After completion of the immediate phase testing, capped mixed samples were incubated in a 37°C water bath for 1 hour (incubated phase) and screening PT and APTT were performed.

Each testing site provided its PT and APTT reagent source, manufacturer, local RIs, and local mechanism for interpreting mixing test results (Supplemental Table 3). Each testing site was provided a spreadsheet for result recording, and a single investigator performed the mixing study calculations (Table 2). Each testing site was blinded to other sites' participation and data.

Although for the purposes of this study all abnormal and normal samples underwent mixing studies, only baseline samples that were outside the upper end of the local RI were analyzed for interpretations. The RIs used in determining correction and noncorrection were based on 2 different determinations: (1) locally provided RI and (2) RI calculated using the mean + 2 SDs derived from the provided 25 normal donor plasmas mixed with the NPP. Three different subtraction methods were used. Subtraction I evaluated the mixing study result to the local upper RI, where mixing results that fell below were considered correction and above the upper RI noncorrection. Subtraction II compared mixing study results with upper RI, but the upper RI was determined using the study donor set and NPP result (mean + 2 SDs). Subtraction III compared the mixing study result with the NPP result from the run. Three mixing ratio determinations were also calculated: mixing study result divided by local RI mean, mixing study result divided by study donors + NPP mean, and mixing study result divided by NPP value obtained during the run. Samples that demonstrated any obvious operational or instrument errors were excluded from analysis. Samples that exceeded the upper limit for clotting time detection (eg, >90 seconds) were included in analysis and given a 1-second addition to threshold (eg, 91 seconds for >90 seconds reported value).

Test samples consisting of multiple or single factor deficiencies or OVKA-induced factor deficiencies were expected to demonstrate correction following NPP mix (Table 1). The expected mixing test result interpretation was determined to be noncorrected for those samples with baseline prolongation due to inhibitory anticoagulants and specific or nonspecific factor inhibitors.

To determine whether a reagent performed as expected, the concept of misprediction was used to analyze the pooled data set. Misprediction occurs when the reported mixing test interpretation or baseline PT and/or APTT result does not match the expected baseline result or mixing test interpretation. In addition, analysis was grouped by specific reagent if 3 or more participants used the same reagent(s). Misprediction was calculated for all baseline, immediate, and incubated mixing results. Multiple rationales for determining misprediction according to the parameters listed in Table 1 were used. First was that isolated or combined factor deficiencies are expected to correct after mixing with NPP. A second rationale for unfractionated heparin and low-molecular-weight heparin therapies is that the PT is expected to be normal, as commercial reagents contain heparin neutralizers. The APTT is expected to be prolonged in a sample containing therapeutic levels of unfractionated heparin, but with LWMH the APTT may be normal or elevated, depending on the reagent's sensitivity to low-molecular-weight heparin. A prolonged baseline APTT due to heparin effect is not expected to correct when performing mixing tests, but correction may occur in samples containing lower drug concentrations. A third rationale is that although it is well recognized that PT and/or APTT reagent sensitivity to direct oral anticoagulants (DOACs) (rivaroxaban, apixaban, edoxaban, and dabigatran) varies widely between platforms,[13,15,16] the desired or expected effect on the baseline PT and APTT for "on-therapy" drug concentrations would be a prolonged clotting time that does not correct with mixing studies.[13,14] A fourth rationale used is that factor VIII inhibitor samples will have a prolonged baseline APTT (and normal PT), which may or may not correct in immediate mixing test results but will prolong with incubated mix given that these antibodies tend to demonstrate time and temperature dependence.[17] Finally, as with DOACs and heparins, there is known between-APTT reagent variation in sensitivity to the presence of LAC.[18,19] For this reason, it has been suggested that LAC-insensitive reagents should be used for general (ie, not LAC) screening purposes.[18,19] The LAC interpretation data were assessed only on those samples with a reported baseline APTT prolongation, and the diagnostic criterion for LAC determination is noncorrection of a mixing study indicating an inhibitor.[19,20] The PT is rarely prolonged in patients with LAC, so a normal PT is expected in these samples.[11] As only prolonged baseline results were evaluated, mixing study

Table 3. Prothrombin Time (PT) Baseline and Immediate Phase Mixing Study Results: Misprediction Values for Abnormal Samples[a]

			Subtraction, %			Ratio, %							
						I		II		III			
Sample	Baseline, %	Mix N	I	II	III	>1.1	>1.2	>1.1	>1.2	>1.1	>1.2	% Correction	Rosner, %
FII-deficient plasma	0	28	0	46	100	0	0	14	0	0	0	0	0
FV-deficient plasma	0	28	21	100	100	39	0	75	0	71	0	0	0
FVII-deficient plasma	0	28	7	96	100	25	0	50	0	39	0	0	0
FX-deficient plasma	0	28	36	93	96	36	4	75	4	71	7	0	0
FXI-deficient plasma	ND	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FXII-deficient plasma	ND	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Abnormal control 1	0	28	21	100	100	50	7	71	7	68	7	4	4
Abnormal control 2	0	27	67	100	100	77	22	100	27	96	30	0	0
OVKA sample 1	0	28	50	100	100	70	11	93	19	93	29	0	4
OVKA sample 2	0	28	32	100	100	64	4	86	11	89	11	0	0
Unfractionated heparin	ND	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
LMWH	ND[b]	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Rivaroxaban	0	28	18	0	0	18	43	0	32	0	32	0	36
Apixaban	57	12	67	0	8	36	100	33	92	33	92	8	100
Edoxaban	4	27	48	0	0	32	78	19	59	19	48	4	74
Dabigatran	4	27	59	0	0	37	85	22	81	19	81	0	85
FVIII inhibitor sample 1	32	9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FVIII inhibitor sample 2	ND	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lupus positive control	39	11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Weak lupus positive control	ND[b]	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Abbreviations: FII, FV, etc, factor II, factor V, etc; LMWH, low-molecular-weight heparin; ND, not done because of normal baseline results; OVKA, oral vitamin K antagonist.

[a] Refer to Table 2 for explanation of calculation methods.

[b] LMWH had 1 sample that generated a prolonged PT result; weak lupus control, 2 samples with prolonged PT results.

misprediction in LAC samples would be for those methods that indicated correction.

RESULTS

There were 20 clinical laboratories and 4 reagent manufacturers (Diagnostica Stago, Roche Diagnostics, Siemens Healthcare Diagnostics, and TCoag) that agreed to participate. Eight clinical sites were in the United States, 4 in Australia, 3 in the United Kingdom, 2 in Spain, and 1 each in Ireland, Switzerland, and Malta. Because of logistical issues with shipping, the clinical site in Malta was not able to complete the sample testing. One Australian clinical laboratory, because of staffing limitations secondary to the SARS-CoV-2 pandemic, was unable to perform sample testing.

Two clinical testing sites provided more than one reagent for APTT, and one provided more than one reagent for PT, whereas all industry participants provided more than one reagent for APTT and 2 of 4 industry participants provided more than 1 PT reagent for testing. Combining both clinical laboratory and industry data, there were 28 PT reagent results sets and 34 APTT reagent result sets used for evaluation. Different reagent lots were used between testing sites.

Of the clinical laboratory participants providing local guidance for mixing test correction, 7 of 18 (39%) indicated a mixing study correction if results returned to the RI, 2 of 18 (11%) if results returned to within 2 to 5 seconds of the upper limit of the RI, 2 of 18 (11%) using index of correction (Rosner index),[20] 2 of 18 (11%) using a percentage correction (or Chang score),[21] and 5 of 18 (28%) using other strategies, including ratios.[22]

PT Baseline and Mixing Test Results

For PT testing, baseline misprediction was based on an unexpected prolongation of the clotting time and occurred in 9 of 28 factor VIII–deficient and low-titer factor VIII inhibitor samples (32%) and 11 of 28 for the LAC samples (39%) (Table 3). For DOACs, baseline misprediction was based on a normal PT for 16 of 28 apixaban samples (57%), 1 of 27 edoxaban samples (4%), and 1 of 27 dabigatran samples (4%).

For immediate or incubated phase mixing test results, there was no interpretation method that correctly classified all samples (Tables 3 and 4). However, for percentage correction in the immediate PT mixing interpretation, there were single-laboratory mispredictions for one combined deficiency sample, the apixaban and edoxaban sample (Table 3). Subtraction method I, the most commonly performed method of interpretation by study participants, performed poorly for both deficient and inhibitor samples (Tables 3 and 4). The other 2 subtraction methods had decreased misprediction rates for inhibitors, but yielded nearly 100% misprediction for single or multiple factor deficiencies. The 3 ratio methods performed similarly to one another. In general, using a ratio greater than 1.2 improved the performance for factor deficiency interpretations but caused performance in the interpretation of factor inhibitors to worsen (Tables 3 and 4). The percentage correction interpretation demonstrated the most optimal method for interpreting immediate-phase PT mixing tests results. This was also apparent when 5 different PT reagents were evaluated individually, as there were no mispredictions in immediate-phase mixing results for both deficient and inhibitor samples, except for one reagent in the presence of edoxaban (Table 5).

Table 4. Prothrombin Time Incubated Phase Mixing Study Results: Misprediction Values for Abnormal Samples[a]

	Subtraction, %			Ratio, %							
				I		II		III			
Sample	I	II	III	>1.1	>1.2	>1.1	>1.2	>1.1	>1.2	% Correction	Rosner, %
FII-deficient plasma	7	88	100	18	0	18	0	36	0	0	0
FV-deficient plasma	43	100	100	61	14	82	7	93	21	0	0
FVII-deficient plasma	11	100	100	39	0	54	0	64	7	0	0
FX-deficient plasma	43	100	100	68	4	86	4	93	18	0	0
Abnormal control 1	29	100	100	46	4	50	7	68	11	4	4
Abnormal control 2	52	100	100	67	15	85	19	89	48	0	0
OVKA sample 1	71	100	100	75	14	100	15	96	46	0	0
OVKA sample 2	68	100	100	71	26	89	32	89	48	0	0
Rivaroxaban	7	4	4	11	29	0	18	0	18	82	19
Apixaban	58	8	1	36	100	17	100	17	92	92	93
Edoxaban	37	4	4	27	60	22	67	7	52	81	56
Dabigatran	30	4	4	26	63	11	59	11	44	78	44

Abbreviations: FII, FV, etc, factor II, factor V, etc; OVKA, oral vitamin K antagonist.

[a] Refer to Table 2 for explanation of calculation methods.

The Rosner index performed well in the correct interpretation of factor deficiencies, but there were frequent misinterpretations in the DOAC inhibitor samples. This was also apparent when 5 different PT reagents were evaluated individually, with the exception of one OVKA sample and one reagent where the Rosner index had frequent mispredictions in the DOAC samples. Misprediction rates markedly increased for all inhibitor sample results following incubation (Tables 4 through 6).

APTT Baseline and Mixing Test Results

For APTT results, baseline misprediction was based on an unexpected prolongation of the clotting time and occurred in 7 of 34 factor VII–deficient plasma samples (20%) and 17 of 34 apixaban samples (50%) (Table 7). For samples with expected prolongation of the baseline APTT, there were normal clotting times for 5 of 34 edoxaban samples (15%) and 4 of 34 (12%) and 9 of 34 (26%) strong and weak LAC samples, respectively.

For immediate- or incubated-phase mixing tests there was no interpretation method that classified all samples correctly. Results for immediate APTT mixing mirror PT mixing results. Subtraction method I, the most commonly performed method of interpretation by study participants, performed poorly for both deficient and inhibitor samples (Tables 7 and 8). The other 2 subtraction methods had decreased misprediction rates for inhibitors but yielded nearly 100% misprediction for single or multiple factor deficiencies. The 3 ratio methods performed similarly. In general, using a ratio greater than 1.2 improved performance for factor deficiency interpretations but caused performance in the interpretation of factor inhibitors to worsen. All methods except subtraction II and III failed to detect a weak factor VIII inhibitor in the immediate mix, with some improvement following incubation (Tables 7 and 8). The percentage correction and Rosner index had nearly 100% misprediction for the weak FVIII inhibitor in both immediate

Table 5. Interpretation Misprediction Values for Respective Prothrombin Time Reagents Using Percentage Correction Method

	Recombiplastin 2G, % (n = 6)		Innovin, % (n = 6)		Thromborel S, % (n = 3)		Neoplastin CI Plus, % (n = 4)		NeoPTimal, % (n = 4)	
Sample	Immed	Incub	Immed	Incub	Immed	Incub	Immed	Incub	Immed	Incub
FII-deficient plasma	0	0	0	0	0	0	0	0	0	0
FV-deficient plasma	0	0	0	0	0	0	0	0	0	0
FVII-deficient plasma	0	0	0	0	0	0	0	0	0	0
FX-deficient plasma	0	0	0	0	0	0	0	0	0	0
Abnormal control 1	0	0	0	0	0	0	0	0	0	0
Abnormal control 2	0	0	0	0	0	0	0	0	0	0
OVKA sample 1	0	0	0	0	0	0	0	0	0	0
OVKA sample 2	0	0	0	0	0	0	0	0	0	0
Rivaroxaban	0	100	0	50	0	33	0	100	0	100
Apixaban	0	100	ND	ND	0	50	ND	ND	0	50
Edoxaban	0	80	17	67	0	33	0	100	0	100
Dabigatran	0	80	0	67	0	33	0	100	0	100

Abbreviations: FII, FV, etc, factor II, factor V, etc; Immed, immediate mix result interpretation; Incub, incubated mix result interpretation; ND, not done because of normal baseline results; OVKA, oral vitamin K antagonist.

Table 6. Interpretation Misprediction Values for Respective Prothrombin Time Reagents Using Rosner Index Method

	Recombiplastin 2G, % (n = 6)		Innovin, % (n = 6)		Thromborel S, % (n = 3)		Neoplastin CI Plus, % (n = 4)		NeoPTimal, % (n = 4)	
Sample	Immed	Incub	Immed	Incub	Immed	Incub	Immed	Incub	Immed	Incub
FII-deficient plasma	0	0	0	0	0	0	0	0	0	0
FV-deficient plasma	0	0	0	0	0	0	0	0	0	0
FVII-deficient plasma	0	0	0	0	0	0	0	0	0	0
FX-deficient plasma	0	0	0	0	0	0	0	0	0	0
Abnormal control 1	0	0	0	0	0	0	0	0	0	0
Abnormal control 2	0	0	0	0	0	0	0	0	0	0
OVKA sample 1	0	0	17	0	0	0	0	0	0	0
OVKA sample 2	0	0	0	0	0	0	0	0	0	0
Rivaroxaban	0	0	100	83	100	0	0	0	0	0
Apixaban	100	80	ND	ND	100	100	ND	ND	100	100
Edoxaban	80	80	100	100	100	0	75	25	0	25
Dabigatran	80	80	100	100	100	0	75	0	100	0

Abbreviations: FII, FV, etc, factor II, factor V, etc; Immed, immediate mix result interpretation; Incub, incubated mix result interpretation; ND, not done because of normal baseline results; OVKA, oral vitamin K antagonist.

and incubated mix results. Although the percentage correction and Rosner index performed well in correctly identifying factor deficient plasmas, there were frequent mispredictions in the inhibitor samples. Only subtraction method III correctly classified all inhibitor test samples, but it had nearly 100% misprediction for factor deficiency samples. Heparinized LAC and DOAC samples yielded misprediction interpretations for mixing study results for various APTT reagents using both percentage correction and the Rosner index. This is not surprising given the varied reagent responsiveness to heparin, DOACs, and LACs (Tables 9 and 10), but it should be noted that mispredictions for percentage correction on the incubated phase, with the exception of the weak factor VIII inhibitor, were mostly confined to a single laboratory (Table 9) for any given reagent.

Table 7. Activated Partial Thromboplastin Time Baseline and Immediate Phase Mixing Study Results: Misprediction Values for Abnormal Samples[a]

						Ratio, %							
			Subtraction, %			I		II		III			
Sample	Baseline	Mix N	I	II	III	>1.1	>1.2	>1.1	>1.2	>1.1	>1.2	% Correction	Rosner, %
FII-deficient plasma	0	34	0	0	38	0	0	0	0	3	0	0	0
FV-deficient plasma	0	33	6	52	100	27	0	9	0	15	0	3	0
FVII-deficient plasma	21	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FVIII-deficient plasma	0	34	32	82	100	53	21	56	3	79	0	0	0
FIX-deficient plasma	0	32	6	41	100	21	0	15	0	3	0	0	0
FX-deficient plasma	0	33	3	45	100	15	0	9	0	21	0	0	0
FXI-deficient plasma	0	34	28	79	100	59	12	65	3	65	3	0	0
FXII-deficient plasma	0	34	21	62	100	35	15	29	3	50	0	0	0
Abnormal control 1	0	32	18	69	82	26	6	38	6	58	3	3	3
Abnormal control 2	0	33	16	79	88	45	3	48	3	67	6	3	0
OVKA sample 1	18	28	4	0	74	7	0	0	0	4	0	0	0
OVKA sample 2	0	33	6	21	85	9	3	9	0	12	0	0	0
Unfractionated heparin	0	34	0	0	0	0	0	0	0	0	0	32	0
LMWH	0	34	6	0	0	0	15	0	6	0	0	3	0
Rivaroxaban	0	34	32	0	0	15	38	3	18	0	6	0	9
Apixaban	50	17	24	0	0	29	53	24	88	18	94	0	88
Edoxaban	15	29	41	0	0	26	62	21	59	15	48	0	52
Dabigatran	0	34	0	0	0	0	0	0	0	0	0	0	0
FVIII inhibitor sample 1	0	34	64	0	0	24	88	6	88	6	68	97	97
FVIII inhibitor sample 2	0	32	0	0	0	0	0	0	0	0	0	9	0
Lupus-positive control	12	30	27	3	0	20	40	10	30	3	23	7	37
Weak lupus-positive control	29	25	17	0	0	20	32	16	40	4	36	0	36

Abbreviations: FII, FV, etc, factor II, factor V, etc; LMWH, low-molecular-weight heparin; ND, not done because of normal baseline or expected normal baseline results; OVKA, oral vitamin K antagonist.

[a] Refer to Table 2 for explanation of calculation methods.

Table 8. Activated Partial Thromboplastin Time Incubated Phase Mixing Study Results: Misprediction Values for Abnormal Samples[a]

	Subtraction, %			Ratio, %							
				I		II		III			
Sample	I	II	III	>1.1	>1.2	>1.1	>1.2	>1.1	>1.2	% Correction	Rosner, %
FII-deficient plasma	3	0	62	6	0	0	0	12	0	0	0
FV-deficient plasma	19	41	100	44	3	15	0	53	6	3	0
FVIII-deficient plasma	44	64	100	62	24	44	0	88	12	0	0
FIX-deficient plasma	9	24	100	27	0	0	0	21	0	0	0
FX-deficient plasma	26	33	100	38	6	6	0	50	6	0	0
FXI-deficient plasma	45	52	100	65	21	38	0	79	15	3	0
FXII-deficient plasma	36	62	100	53	18	29	0	74	3	0	0
Abnormal control 1	24	30	82	35	3	9	3	59	9	3	3
Abnormal control 2	27	59	88	53	3	24	0	73	24	3	0
OVKA sample 1	7	4	93	14	0	0	0	21	4	25	0
OVKA sample 2	13	24	100	32	6	18	0	38	9	0	0
Unfractionated heparin	0	0	0	0	0	0	0	0	0	9	0
LMWH	3	3	0	0	0	3	6	3	3	3	6
Rivaroxaban	12	0	0	0	24	0	18	0	3	0	3
Apixaban	18	12	0	12	47	29	65	0	59	0	44
Edoxaban	14	3	0	0	39	26	55	15	24	3	24
Dabigatran	0	0	0	0	0	0	0	0	0	3	0
FVIII inhibitor sample 1	9	0	0	0	12	0	9	0	3	94	85
FVIII inhibitor sample 2	0	0	0	3	3	0	3	0	3	3	0
Lupus-positive control	13	3	0	7	27	7	43	0	13	0	17
Weak lupus-positive control	13	4	0	4	36	20	48	0	28	0	28

Abbreviations: FII, FV, etc, factor II, factor V, etc; LMWH, low-molecular-weight heparin; OVKA, oral vitamin K antagonist.

[a] Refer to Table 2 for explanation of calculation methods.

DISCUSSION

Although it is generally appreciated that PT and APTT reagents vary in their sensitivities to factor levels, heparins, DOACs, and LACs, it requires emphasis that in this study, misprediction was based on the inability for a mixing study interpretation to correctly identify a deficiency or inhibitor in a sample that demonstrated a prolonged baseline clotting time. Samples that did not demonstrate a baseline prolonged clotting time were not subject to mixing study evaluations. Factors that contributed to mixing study misprediction included reagent sensitivity, local RI determination, and inappropriate reagent use (ie, using a LAC-sensitive reagent for screening rather than a LAC-insensitive reagent, which would be more appropriate), further emphasizing the need for appropriate RI determination and local laboratory validation of its reagent sensitivity and mixing study interpretive methods.

This study revealed that no single mixing test interpretation method, regardless of reagent used, demonstrated zero misprediction in all samples. Calculation methods tended to perform well in the correct identification of factor deficiency or inhibitor, but not both. This suggests that a staggered approach in performing calculations may be optimal, especially for the more common APTT mixing study. Although the percentage correction method demonstrated the most accurate interpretation for PT mixing tests, for APTT mixing tests this interpretation method would be optimized using the subtraction III method when the percentage correction interpretation suggests a factor deficiency. This algorithm rules out a factor inhibitor. The calculation method reportedly used by the majority of laboratories participating in this study, specifically correction of the mix into the RI, performed poorly in correctly categorizing both PT and APTT factor-deficient and inhibitor samples (Table 7).

This study encompasses multiple reagent platforms using a wide selection of abnormal samples, in contrast to other publications that focused on mixing studies for LAC assessment,[11,23] only addressed APTT and not PT prolongations,[22] or used only contrived samples that may mimic coagulation abnormalities.[8] In a large-single site retrospective study of APTT mixing studies using a single platform, Liu and colleagues[24] evaluated modified thresholds for different interpretative methods, but it is unclear whether these same thresholds could be applicable to other platforms. In the present study, these same thresholds were evaluated using multiple platforms. Results from this study provide additional clinical and laboratory guidance as to the use of PT mixing studies, which are often neglected given the rarity of inhibitors to extrinsic and common pathway factors.[25–27] With increased use of anti–factor Xa DOACs, there may be increased demand for PT mixing studies in acute settings in patients with unknown drug exposure.[7,28]

A surprising number of mispredictions were reported for baseline sample results. Factor VIII–deficient and low-titer factor VIII inhibitor (0.6 Bethesda units) samples should yield a normal PT, yet 32% (9 of 28) reported a prolonged PT for those sample types. Similar to the expectation that NPPs used in mixing tests should contain more than 80 IU/dL of each coagulation factor,[6,8] manufacturer-provided information for the deficient plasmas used in this study indicated that all levels other than intended deficiencies were 50 IU/dL activity or more, and thus a deficiency or inhibitor of a factor

Table 9. Interpretation Misprediction Values for Respective Activated Partial Thromboplastin Time Reagents Using Percentage Correction Method[a]

	SynthasIL, % (n = 6)		Actin FS, % (n = 4)		Actin FSL, %(n = 4)		Pathromtin SL, % (n = 3)		APTT-A, % (n = 4)		Cephascreen, % (n = 3)	
Sample	Immed	Incub	Immed	Incub	Immed	Incub	Immed	Incub	Immed	Incub	Immed	Incub
FII-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
FV-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
FVIII-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
FIX-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
FX-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
FXI-deficient plasma	0	0	0	25	0	0	0	0	0	0	0	0
FXII-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
Abnormal control 1	0	0	0	0	0	0	0	0	0	0	33	33
Abnormal control 2	0	0	0	0	0	0	0	0	0	0	0	0
OVKA sample 1	0	20	0	50	0	50	0	0	0	0	0	0
OVKA sample 2	0	0	0	0	0	0	0	0	0	0	0	0
Unfractionated heparin	0	0	75	25	0	0	100	33	25	0	0	0
LMWH	0	0	25	0	0	25	0	0	0	0	0	0
Rivaroxaban	0	0	0	0	0	0	0	0	0	0	0	0
Apixaban	0	0	ND	ND	ND	ND	0	0	0	0	0	0
Edoxaban	0	0	0	25	0	0	0	0	0	0	0	0
Dabigatran	0	0	0	25	0	0	0	0	0	0	0	0
FVIII inhibitor sample 1	100	100	75	50	100	100	100	100	100	100	100	100
FVIII inhibitor sample 2	17	0	0	0	0	0	33	0	0	0	0	0
Lupus-positive control	0	0	ND	ND	0	0	0	0	0	0	0	0
Weak lupus-positive control	0	0	ND	ND	0	0	0	0	0	0	0	0

Abbreviations: FII, FV, etc, factor II, factor V, etc; Immed, immediate mix result interpretation; Incub, incubated mix result interpretation; ND, not done because of normal baseline results; OVKA, oral vitamin K antagonist.

[a] Refer to Table 2 for explanation of calculation methods.

other than factor VIII cannot explain this misprediction. The basis of this high misprediction rate requires further investigation. The high degree of baseline misprediction with DOAC was not unexpected, as it is well known that APTT and PT reagents vary in their responsiveness to these agents.[13,15,16,29] For APTT reagents, there were also varying degrees of baseline misprediction between reagent platforms, as some reagents were identified as LAC sensitive and others not. The misprediction in baseline results for weak and strong LAC of 12% (4 of 34) and 29% (9 of 34), respectively, emphasizes the need to use APTT reagents that are sensitive to LAC when screening for the presence of these antibodies or laboratories should seek other testing schemes.[9,10] An unexpected prolongation of the PT and APTT in these otherwise normal samples could be due to inappropriate methodologies used for local reference range generation, yet these seemingly abnormal results may lead to erroneous diagnosis, unnecessary laboratory investigation, unnecessary delays in treatment, or patient mismanagement.[30] Previous studies have also demonstrated such differences between reagent platforms and the varied factor sensitivities of both PT and APTT reagents.[31,32]

The best calculation method for PT mixing tests was the percentage correction followed by the Rosner index. The Rosner index (also known as the index of circulating anticoagulant), performed poorly in the identification of inhibitor samples, for which it was originally designed. Misprediction rates dramatically increased when incubated-phase calculations were used; possible contributors could be partial loss of labile factors and pH drift. These data suggest that only immediate-phase and not incubated PT mixing tests should be performed, as incubation tends to result in misprediction of inhibitor samples.[33–35] The authors are unaware of the existence of time- and temperature-dependent antibodies requiring incubated PT mixing studies.

This study demonstrates that for APTT-based mixing tests, mixing study subtraction calculation methods that correctly identified inhibitors had high misprediction rates in identifying factor deficiencies and vice versa. Subtraction method III correctly identified all inhibitor samples but performed poorly on samples with single or multiple factor deficiencies. Calculations using the percentage correction or Rosner index showed better performance in factor-deficient samples than any of the subtraction- or ratio-based calculations evaluated. Both percentage correction and Rosner index, however, did not perform correctly for all samples, and surprisingly, both methods performed poorly in identifying the weak FVIII inhibitor sample as an inhibitor in both the immediate and incubated mixing studies. When evaluating individual APTT reagents (Tables 9 and 10), the percentage correction method demonstrated fewer mispredictions than the Rosner index method. Further investigation is needed to determine if using locally derived cutoffs instead of standard 1.1 and 1.2 cutoffs for the ratio methods could improve assay performance, particularly if these cutoffs are derived from normal donors mixed with the same NPP in local use.[11]

In this study, multiple laboratories, regardless of the APTT reagent used, had difficulty identifying the weak factor VIII inhibitor, even though all sites demonstrated a prolonged baseline APTT in this sample. This could be because a 1-hour incubation may be insufficient for detecting weak factor VIII inhibitors. For other factor inhibitors studied, the incubated mixing test (versus the immediate) substantially improved

Table 10. Interpretation Misprediction Values for Respective Activated Partial Thromboplastin Time Reagents Using Rosner Index Method

	SynthasIL, % (n = 6)		Actin FS, % (n = 4)		Actin FSL, % (n = 4)		Pathromtin SL, % (n = 3)		APTT-A, % (n = 4)		Cephascreen, % (n = 3)	
Sample	Immed	Incub	Immed	Incub	Immed	Incub	Immed	Incub	Immed	Incub	Immed	Incub
FII-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
FV-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
FVIII-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
FIX-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
FX-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
FXI-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
FXII-deficient plasma	0	0	0	0	0	0	0	0	0	0	0	0
Abnormal control 1	0	0	0	0	0	0	0	0	0	0	33	33
Abnormal control 2	0	0	0	0	0	0	0	0	0	0	0	0
OVKA sample 1	0	0	0	0	0	0	0	0	0	0	0	0
OVKA sample 2	0	0	0	0	0	0	0	0	0	0	0	0
Unfractionated heparin	0	0	0	0	0	0	0	0	0	0	0	0
LMWH	0	0	0	25	0	25	0	0	0	0	0	0
Rivaroxaban	0	0	25	25	0	0	0	0	25	0	0	0
Apixaban	75	0	ND	ND	ND	ND	67	0	100	100	100	100
Edoxaban	33	20	75	50	100	0	0	0	75	50	67	33
Dabigatran	0	0	0	0	0	0	0	0	0	0	0	0
FVIII inhibitor sample 1	100	100	100	75	100	75	100	100	100	100	67	67
FVIII inhibitor sample 2	0	0	0	0	0	0	0	0	0	0	0	0
Lupus-positive control	0	0	ND	ND	50	0	67	67	0	0	67	0
Weak lupus-positive control	0	0	ND	ND	100	100	100	100	0	0	67	33

Abbreviations: FII, FV, etc, factor II, factor V, etc; Immed, immediate mix result interpretation; Incub, incubated mix result interpretation; ND, not done because of normal baseline results; OVKA, oral vitamin K antagonist.

inhibitor detection using the subtraction and ratio methods. In patients with suspected factor VIII inhibitors, because of its potential serious consequences, additional studies, such as performance of factor VIII activity levels in addition to the performance of an incubated mixing study, should be considered.

Given the variability in misprediction rates between calculation methods demonstrated in this study, validation, or, at a minimum, verification by individual laboratories of the accuracy of the mixing calculation used is paramount. This should be conducted using a variety of deficient and inhibitor sample types. Furthermore, in those situations where a complete patient history including medications is not available, more selective laboratory methods to screen for anticoagulant presence are recommended, as mixing studies should not be performed when inhibitor-type anticoagulants are present.

The strength of this study is the multinational clinical laboratory and industry participation, which represents multiple reagent testing platforms, variable RIs (even when using the same reagent/instrument system), and differing lots within the same reagent platforms. Study weaknesses include that some samples used were considered surrogate or contrived samples, which is a commonly used method for assessing reagent performance but may not have the same testing characteristics as patient samples. This study used a 1-hour incubation period, which may not be sufficient to detect weak factor VIII inhibitors, and further studies are warranted to elucidate the ideal incubation time for detecting these weak titer inhibitors. Additionally, only a single source and type (buffered) of NPP was used; it may differ from the NPP source and characteristics used in many laboratories, which may influence misprediction rates. Hence the importance of laboratories validating their mix test interpretations under local test conditions.

CONCLUSIONS

The APTT and to a lesser degree the PT mixing tests have long been a diagnostic staple for clinicians as a guide to suggest whether prolongation of the clotting time is due to factor deficiency versus an inhibitor. Standardization of mixing test performance as well as limited evidence for accurate interpretative methods is lacking. Although limited result variation between testing sites reportedly using the same reagent platforms and interpretative methods was evident, the following conclusions are notable. Testing of samples with normal baseline PT or APTT results and in patients with known inhibitory anticoagulants, such as DOACs or heparins, should be avoided. The PT mixing test is optimal for the immediate phase only, as incubated PT mixing studies dramatically increased misprediction. This study suggests that no single mixing study interpretation method, regardless of reagents used, demonstrates zero misprediction in all samples tested. For the PT and APTT mixing tests, the percentage correction was the most suitable interpretation method, but APTT studies may be further optimized when used in conjunction with a subtraction method to the NPP value used in the percentage correction calculation. As differences in misprediction between laboratories using the same PT and APTT reagents was evident, each laboratory should validate or at a minimum verify the performance of their mixing study interpretive method(s) using well-characterized samples expected to yield corrected and noncorrected interpretations, before the results of mixing

tests are reported clinically. Given that no single approach for mixing study interpretations had zero misprediction, clinical correlation with laboratory findings is required.

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