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ORIGINAL ARTICLE

Variability of cut-off values for the detection of lupus anticoagulants: results of an international multicenter multiplatform study

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Essentials

- Between-lab variations of cut-off values in lupus anticoagulant detection are unknown.
- Cut-off values were calculated in 11 labs each testing plasma from 120 donors with 3 platforms.
- Major variation was observed even within the same platform.
- Cut-off values determined in different labs are not interchangeable.

Summary. *Background:* Cut-off values for interpretation of lupus anticoagulant (LA) detection are poorly investigated. *Aims:* (i) To assess whether results from healthy donors were normally distributed and (ii) the between-laboratories differences in cut-off values for screening, mixing and LA confirmation when calculated as 99th or 95th centiles, and (iii) to assess their impact on the detection rate for LA. *Methods:* Each of 11 laboratories using one of the three widely used commercial platforms for LA detection was asked to collect plasmas from 120 healthy donors and to perform screening, mixing and LA confirmation with two methods (activated partial thromboplastin time [APTT] and dilute Russell viper venom [dRVV]). A common set of LA-positive or LA-negative freeze-dried plasmas was used to assess the LA detection rate. Results were centralized (Milano) for statistical analysis. *Results and conclusions:* (i) Clotting times or ratios for healthy subjects were not *normally* distributed in the majority of cases. The take-home message is that cut-off values should be determined preferably by the non-parametric method based on centiles. (ii) There were relatively large inter-laboratory cut-off variations even within the same platform and the variability was marginally attenuated when results were expressed as ratios (test-to-normal pooled plasma). The take-home message is that cut-off values should be determined locally. (iii) There were differences between cut-off values calculated as 99th or 95th centiles that translate into a different LA detection rate (the lower the centile the greater the detection rate). The take-home message is that cut-off values determined as the 95th centile allow a better LA detection rate.

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Introduction

Lupus anticoagulants (LAs) are a heterogeneous family of immunoglobulins that upon binding to complexes of proteins and negatively charged phospholipids prolong phospholipid-dependent coagulation tests *in vitro*, but are responsible for venous/arterial thromboembolism and pregnancy complications [1]. Positivity for LA is considered to be one of the laboratory criteria to define the antiphospholipid syndrome [2] and its persistent positivity in combination with previous thrombotic events qualifies the patient for long-term anticoagulation [1]. Hence, the laboratory detection of LA is of paramount importance. Unfortunately, no specific test for LA exists and its detection rests on phospholipid-dependent coagulation tests combined with a set of diagnostic criteria developed and issued by the Scientific and Standardization Committee (SSC), Subcommittee for Lupus Anticoagulant/Phospholipid-Dependent Antibodies of the International Society on Thrombosis and Haemostasis (ISTH). They include the following: (a) evidence that one (or more) phospholipid-dependent coagulation test is prolonged beyond the upper limit of the normal range; (b) evidence that the prolongation does not revert to normal upon mixing equal portions of patient and normal plasma; and (c) evidence that the circulating anticoagulant is directed against negatively charged phospholipids, complexed with proteins, and not directed against specific coagulation factors. Criteria a, b and c are called screening, mixing and confirmation, respectively [3]. The LA ISTH guidelines recommend two types of tests for LA detection, including an activated partial thromboplastin time (APTT)-derived and a dilute Russell viper venom (dRVV)-derived test. Analyses with these two tests include screening, mixing and confirmation procedures. Results obtained with the above procedures should be interpreted by comparing the patients' values with specific cut-off values determined for each test and procedure.

There are no studies evaluating the variation in the LA cut-off values as determined in different laboratories with different platforms or the effect that the statistical analysis might have on interpretation of results. All the above issues prompted us to organize and carry out an international multicenter multiplatform study with the aim of assessing the variations in cut-off values specific for combinations of tests and coagulometers (here called platforms), run in different laboratories, when testing relatively large numbers of plasmas from healthy donors. Results have been compared in order to assess between-laboratory differences while using the same platform but different sets of plasmas from healthy donors. Other aims of the study were to assess whether results from healthy donors are *'normally'* distributed and the extent of differences in the cut-off values determined by different methods of calculation. A common set of LA-positive plasmas has been used to assess the impact that different cut-off values can have on the LA detection rate.

Material and methods

Design of the study

Manufacturers of three widely used platforms for LA detection were asked to support the study. Instrumentation Laboratory, Diagnostica Stago (Asnieres, France) and Siemens Healthcare Diagnostics (Marburg, Germany) were contacted and agreed to participate. With their help, we selected five (or more) laboratories within each platform and they were asked to join the study by collecting 120 plasma samples from individual healthy donors. Participants were provided with a protocol detailing how blood donors should be selected, handling of blood, plasma preparation and storage, and testing (see below). The reagents needed to run the tests in each laboratory were provided by the relevant manufacturers. At the end of the study, the results were entered into a (provided) data collection form and sent to the organizing laboratory (Milano) for centralized statistical analysis.

Selection of healthy donors

Participants were asked to select 120 male and female (60 : 60) healthy donors who ranged in age from 18 to 70 years, were in good health and were free from diseases or drugs known to affect coagulation. ISTH guidelines for LA detection suggest selecting donors who are less than 50 years of age [3]. We elected to expand the age range in order to increase the likelihood of individual centers accessing the requisite number of donors. Blood donors were acceptable provided that blood samples were collected by a separate venipuncture before or a long time after blood donation. Participants were free to collect the blood samples needed to complete the study on different days according to local availability.

Blood collection and plasma preparation

Participants were asked to collect venous blood into vacuum tubes containing 1/10 trisodium citrate 109 mM and to prepare plasma from double centrifugation as recommended by the ISTH guidelines to obtain platelet-poor plasma [3]. Plasmas were to be stored frozen at −70 °C until testing. Samples collected before planning this study were permitted if their preparation fulfilled the recommendations in the ISTH guidelines [3] and they had been stored at −70 °C for no longer than 12 months.

Pooled normal plasma

Participants were advised to prepare the pooled normal plasma (PNP) locally by collecting blood samples from 30 healthy donors (15 : 15; male : female) into vacuum tubes containing 1/10 volume trisodium citrate 109 mM. Blood was centrifuged as for samples from blood donors used for the study. Equal amounts of the individual plasmas had to be pooled in a plastic container and after mixing the bulk of PNP had to be divided into small aliquots (~1.0 mL) in plastic capped tubes that had to be stored frozen at −70 °C and tested in parallel with the samples from blood donors. The same PNP had to be used to perform the mixing study. In case of unavailability of local PNP a lyophilized commercial preparation that fulfilled the SSC requirements for LA testing could be used.

Freeze-dried plasmas

Suitable numbers of vials of the 1st International Reference Panel for Lupus Anticoagulant, 13/172, consisting of freeze-dried plasma that was negative (plasma A) or positive for LA (B, weak; C, strong) were provided by NIBSC (Potters Bar, Hertfordshire, UK) and were sent to each participating laboratory as coded unknown plasmas in order to assess them for LA detection rate with different methods of calculating cut-off values. Information on how to reconstitute the plasmas was provided. Participants were asked to test plasmas A, B, C and B diluted 1 : 2 with the local PNP, on three test occasions.

Testing

Participants were asked to complete the study by testing the 120 samples from healthy donors and the NIBSC unknown freeze-dried plasmas using an APTT-based test and a dRVV-based test. Participants were free to split all the measurements on different days according to the laboratory workload. Single or duplicate measurements had to be taken according to the local practice. Participants were asked to run the following procedures**.**

Screening Testing was performed for the platelet-poor plasma with the phospholipid-dependent screening (APTT- and dRVV-based) tests according to the manufacturer's specification.

Mixing Testing was performed upon mixtures of equal portions of platelet-poor plasma from each donor and local PNP. The mixture was tested without pre-incubation.

Confirmation Testing was performed for the (undiluted) platelet-poor plasma with the confirmation tests of the same reagent for screening tests with higher concentrations of phospholipids as specified by the manufacturer.

Results (average values if duplicates) for the screening, mixing and confirmatory procedures had to be entered into the data collection form as clotting times (seconds), together with the clotting time of the PNP run in parallel. It should be noted that the methods provided by the three manufacturers and used in this study, although sharing the same principles (APTT-based or dRVV-based), are substantially different in terms of reagent composition and hence performance.

Data analysis

Results for the APTT-derived and dRVV-derived tests obtained with screening, mixing and confirmation have been analyzed to calculate cut-off values from data obtained by each participant. Raw data used for the analysis were clotting times for screening and mixing and percentage correction or Delta, calculated from results obtained with confirmation procedures performed at low and high phospholipid concentrations. Mixing has been assessed according to the widely used method of Rosner [4] by means of the index of circulating anticoagulants (ICA) as

$$\%\mathrm{ICA} = [(\mathrm{CT}_{\mathrm{mixture}} - \mathrm{CT}_{\mathrm{PNP}})/\mathrm{CT}_{\mathrm{patient}}] \times 100$$

where CT stands for the clotting time. Confirmation has been assessed as percentage correction (%correction) according to [5]

$$\%\mathrm{correction} = [(\mathrm{CT}_{\mathrm{lowPL}} - \mathrm{CT}_{\mathrm{highPL}})/\mathrm{CT}_{\mathrm{lowPL}}] \times 100$$

or Delta according to

$$\mathrm{Delta} = \mathrm{CT}_{\mathrm{lowPL}} - \mathrm{CT}_{\mathrm{highPL}}$$

where PL stands for the phospholipid concentrations. Cut-off values for the three procedures were also calculated by using ratios (patient-to-PNP) for clotting times.

Data were tested to assess for deviation from the *normal* (Gaussian) distribution by means of the Kolmogorov–Smirnov test with $P < 0.05$. Cut-off values have been determined by different methods of calculation for each participant, method and procedure without exclusion of outliers. Centiles were calculated from the distribution of the data according to the weighted average method. Results obtained for the freeze-dried NIBSC LA plasmas were used to calculate the LA detection rate achieved by the different methods of calculating cut-off values. For the calculations of detection rates, plasmas were considered LA positive when the result for each procedure and method was greater than the cut-off value determined in this study according to each of the investigated methods of calculation. Analyses were performed with the IBM SPSS Statistics version 23 Software (New York, NY, USA).

Results

Three groups, each composed of five participants, were identified among those laboratories that used routinely

one of the three commercial platforms (here called IL, Siemens and Stago). All of them agreed to participate and received the protocol. At the end of the study, results were received from four, two and five participants in the IL, Siemens and Stago groups, respectively. Eight participants were from Europe and there was one each from India, North America and South America. The LA detection platforms used in the study are shown in Table 1. One laboratory did not provide PNP results for the confirmation procedure; two reported duplicate rather than triplicate measurements for the freeze-dried LA plasmas and one did not perform confirmatory assays.

Deviation from the normal *(Gaussian) distribution*

The preliminary analysis showed that in many instances (> 50%) there was a significant deviation ($P < 0.05$) from the *normal* distribution (Table 2). In particular, deviations were more often observed when results were expressed as clotting times (average 60%) than as ratios (average 49%). Finally, deviations were more often observed for the APTT-derived methods (average, 57%) than for dRVV-derived (average, 51%) methods. Because of the above deviations, it was decided to calculate cut-off values by means of non-parametric statistics and the 99th and 95th centiles were chosen for further analysis.

Cut-off values

Cut-off values calculated for the three procedures (screening, mixing and confirmation) for all the laboratories, platforms, tests (APTT-derived and dRVV-derived) and results are shown in Figs 1 and 2. Table 3 shows the median and range (minimum–maximum) of cut-off values for each platform/test and method of calculation. As expected, cut-off values were shorter when calculated as the 95th vs. 99th centile. Overall, there was a relatively large between-laboratory variability (even within the same platform) with no major improvement when results were expressed as a ratio (patient-to-PNP) for the APTT-derived or dRVV-derived methods.

LA detection rate

NIBSC freeze-dried plasmas have been used to assess the LA detection rate obtained for each laboratory with each platform for screening, mixing and confirming. Results are shown in Tables 4 and 5.

APTT derived Overall, detection rates for screening, mixing or confirming were excellent for plasma A (negative) regardless of the method of calculating cut-off values, good for plasma C (strongly positive), acceptable for B (weakly positive), but low for plasma B diluted 1 : 2 in PNP (Table 4). For plasma B (undiluted or diluted), the detection rate was better when cut-offs were calculated as

Table 1 Commercial platforms used throughout the study

		APTT-based		dRVV- based		
		Screen and mix	Confirm	Screen and mix	Confirm	
Platform	Laboratory	Low PL	High PL	Low PL	High PL	Coagulometer
IL	5	HemosIL SCT	HemosIL SCT	HemosIL dRVVT Screen	HemosIL dRVVT Confirm	ACL TOP 500
	6	HemosIL SCT	HemosIL SCT	HemosIL dRVVT Screen	HemosIL dRVVT Confirm	ACL TOP 700
	7	HemosIL SCT	HemosIL SCT	HemosIL dRVVT Screen	HemosIL dRVVT Confirm - IL	ACL TOP 700
	8	HemosIL SCT	HemosIL SCT	HemosIL dRVVT Screen	HemosIL dRVVT Confirm	ACL-TOP 500
Siemens	4	ACTIN FSL	Pathromtin SL	LA1 (Siemens)	LA2 (Siemens)	BCS
	9	ACTIN FSL	ACTIN FS	LA1 (Siemens)	LA2 (Siemens)	BCS
Stago	1	PTT-LA	STACLOT LA -/+ HX PL	STACLOT DRVV SCREEN	STACLOT DRVV CONFIRM	STAR EVOLUTION 3002
	2	PTT-LA	Not done	STACLOT DRVV SCREEN	STACLOT DRVV CONFIRM	STAR EVOLUTION 3002
	3	PTT-LA	STACLOT LA -/+ HX PL	STACLOT DRVV SCREEN	STACLOT DRVV CONFIRM	STAR EVOLUTION 3002
	10	PTT-LA	STACLOT LA -/+ HX PL	STACLOT DRVV SCREEN	STACLOT DRVV CONFIRM	STA compact
	11	PTT-LA	STACLOT LA -/+ HX PL	STACLOT DRVV SCREEN	STACLOT DRVV CONFIRM	STAR EVOLUTION 3002

IL, Instrumentation Laboratory; APTT, activated partial thromboplastin time; dRVV, dilute Russel viper venom.

Table 2 Details of the analysis to assess whether results from individual laboratories were *'normally'* distributed. Number (%) represents the proportion of deviation from *'normality'* ($P < 0.05$) for each method and expression of results

	Kolmogorov-Smirnov test							
	Clotting time (seconds)				Ratio (patient plasma to PNP)			
Test	IL	Siemens	Stago	All	IL	Siemens	Stago	All
APTT - screening	2/4	1/2	3/5	6/11	1/4	1/2	3/5	5/11
APTT - mixing (ICA%)	2/4	1/2	3/5	6/11	2/4	1/2	3/5	6/11
APTT - confirm (% correction)	2/4	1/2	4/4	7/10	1/3	0/2	3/4	4/9
APTT - confirm (Delta)	2/4	2/2	4/4	8/10	1/3	1/2	3/4	5/9
dRVV - screening	2/4	1/2	5/5	8/11	2/4	2/2	4/5	8/11
dRVV - mixing (ICA%)	0/4	0/2	3/5	3/11	0/4	0/2	0/5	0/11
dRVV - confirm (% correction)	1/4	1/2	3/4	5/10	0/3	1/2	3/4	4/9
dRVV - confirm (Delta)	2/4	1/2	4/4	7/10	2/3	1/2	4/4	7/9
Overall	13/32 (40)	8/16 (50)	29/36 (81)	50/84 (60)	9/28 (32)	7/16 (44)	23/36 (64)	39/80 (49)

APTT, activated partial thromboplastin time; ICA, index of circulating anticoagulant; IL, Instrumentation Laboratory; dRVV, dilute Russell viper venom test; PNP, pooled normal plasma.

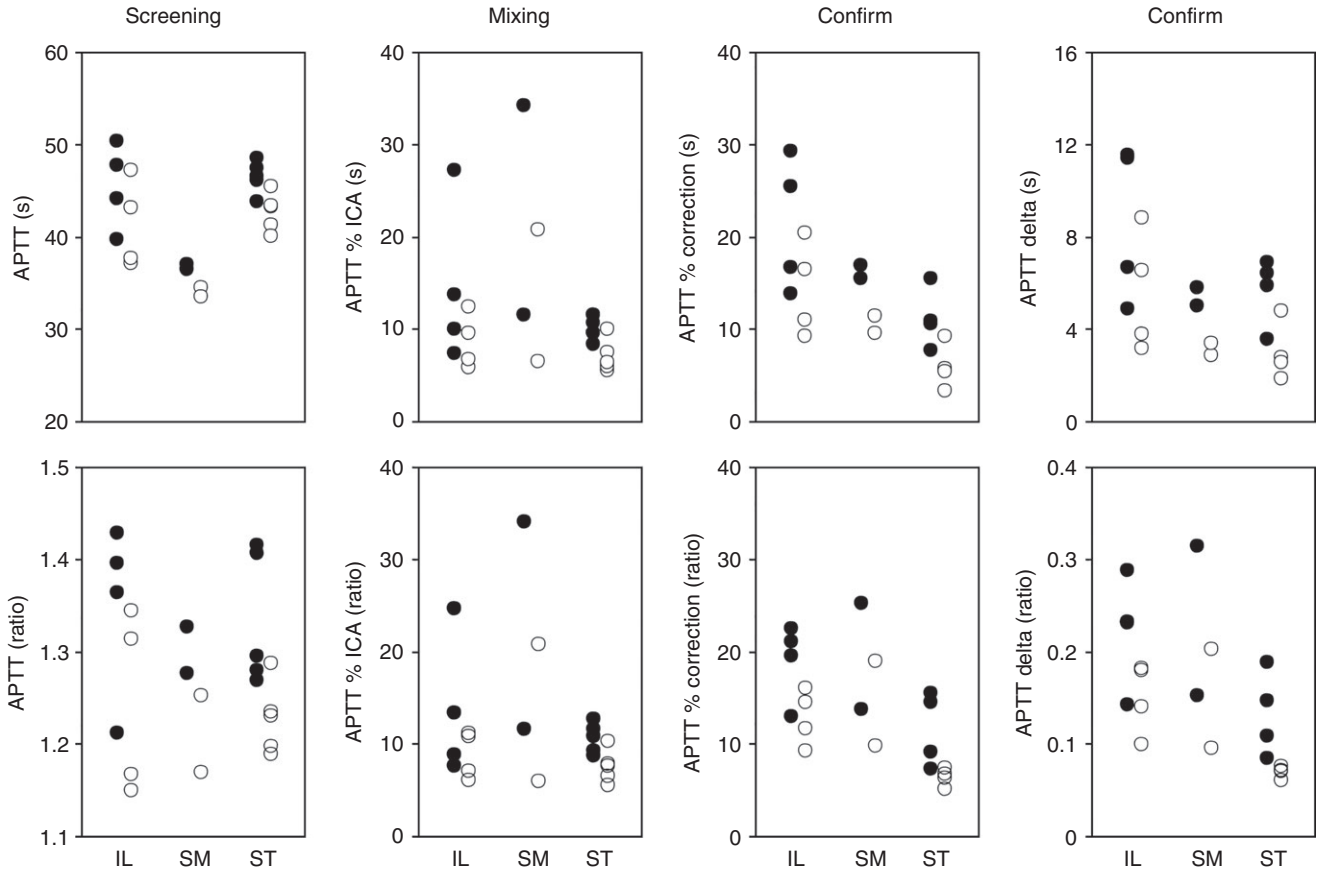

Fig. 1. APTT-derived cut-off values determined in different laboratories using three commercial platforms for LA detection for screening, mixing and confirmation procedures. Cut-off values have been determined according to clotting times (upper panels) or ratio of patient plasma to PNP (lower panels) as the 99th (closed symbols) or 95th (open symbols) centiles of distribution of results for healthy donors. APTT, activated partial thromboplastin time; ICA, index of circulating anticoagulant; LA, lupus anticoagulant; IL, Instrumentation Laboratory; PNP, pooled normal plasma; ST, Stago; SM, Siemens; ICA, index of circulating anticoagulant calculated as ICA = $[(CT_{mixture} - CT_{PNP})/CT_{patient}] \times 100$; % correction calculated as % correction = $[(CT_{lowPL} - CT_{highPL})/CT_{lowPL}] \times 100$. Delta calculated as Delta = $CT_{lowPL} - CT_{highPL}$.

95th centiles rather than as 99th centiles. Overall, detection rates for confirming were similar when cut-off values were calculated as Delta or as % correction. There were no major differences between the detection rates calculated from clotting times as compared with those calculated from ratios.

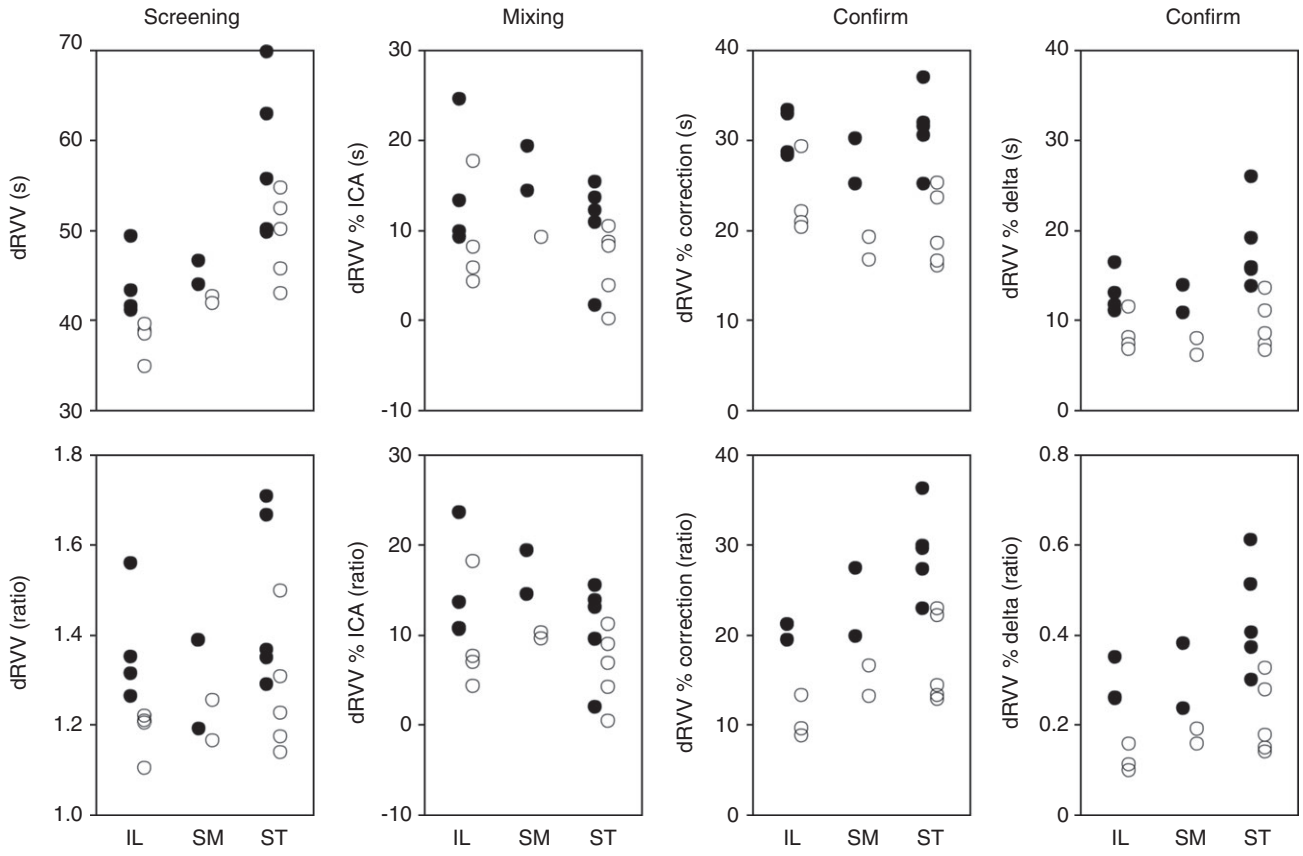

Fig. 2. dRVV-derived cut-off values determined in different laboratories using three commercial platforms for LA detection for screening, mixing and confirmation procedures. dRVV, dilute Russel viper venom. (see legend to Fig. 1 for other details).

dRVV derived Overall, detection rates for screening, mixing or confirming were excellent for plasma A (negative) and plasma C (strongly positive) regardless of the method of calculating cut-off values, acceptable for plasma B (weakly positive), but very low for plasma B diluted 1 : 2 in PNP (Table 5). For plasma B (undiluted or diluted), the detection rate was better when cut-off values were calculated as 95th centiles rather than as 99th centiles. Overall, detection rates for confirming were similar when cut-off values were calculated as % correction or as Delta. There were no major differences between the detection rates calculated from clotting times as compared with those calculated from ratios.

Discussion

Surveys performed over the years [6–13] have shown the variable performance of clinical laboratories with respect to sensitivity and specificity of LA tests. The rates of false-positive and false-negative detections remain relatively high. The former are of particular concern because they qualify the patients for long-term and unnecessary oral anticoagulant treatment [1]. Although some of the reasons for the poor performances have been elucidated [14], some others, such as the effect of cut-off values, although investigated in a multicenter study with a single platform [15], are still poorly understood. The ISTH guidelines for LA detection recommend testing plasmas from 40 or more healthy donors and taking the cut-off as the value corresponding to the 99th centile of their distribution for the screening and mixing procedures and as the mean percentage correction (low vs. high phospholipid concentration) for the confirmation procedure [3]. However, for most laboratories it is difficult to follow these recommendations, as plasmas from healthy donors are not readily available. Commercial frozen normal plasmas, if available, are difficult to access because of their cost. Furthermore, taking the 99th centile of the distribution of results from healthy donors as the cut-off value may give rise to bias when plasmas are less than 100. Finally, most laboratory operators are much more familiar with the cut-off values determined as the interval defined by the parametric methods (i.e. mean + SD), which was, however, not recommended in the ISTH guidelines as it was felt that the data were not *normally* distributed. Last but not least, most manufacturers

Table 3 Median (minimum and maximum) of cut-off values obtained by participants according to different platforms and models of calculation (99th or 95th centile, seconds or ratio [patient-to-PNP])

	Clotting time (seconds)						Ratio (patient plasma to PNP)					
	IL		SM		ST		IL		SM		ST	
	99th	95th	99th	95th	99th	95th	99th	95th	99th	95th	99th	95th
APTTscreen												
Median	45.9	40.4	36.8	34.0	46.6	43.3	1.38	1.24	1.30	1.21	1.30	1.23
Min	39.8	37.2	36.5	33.5	43.8	40.1	1.21	1.15	1.28	1.17	1.27	1.19
Max	50.3	47.2	37.1	34.5	48.5	45.5	1.43	1.35	1.33	1.25	1.42	1.29
APTTmix*												
Median	11.9	8.2	22.9	13.7	10.7	6.4	11.17	8.93	22.89	13.42	10.85	7.66
Min	7.5	5.9	11.6	6.6	8.5	5.6	7.67	6.12	11.61	5.98	8.75	5.58
Max	27.2	12.4	34.2	20.8	11.6	10.0	24.73	11.17	34.16	20.86	12.71	10.29
APTTcorr†												
Median	21.1	13.7	16.3	10.6	10.8	5.6	21.2	11.8	19.7	14.5	12.0	6.6
Min	13.9	9.3	15.6	9.7	7.8	3.4	13.1	9.4	13.9	10.0	7.4	5.3
Max	29.3	20.4	17.0	11.5	15.5	9.3	22.6	14.6	25.4	19.1	15.6	7.6
APTTdelta‡												
Median	9.0	5.2	5.4	3.2	6.2	2.7	0.23	0.14	0.23	0.15	0.13	0.07
Min	4.9	3.2	5.0	2.9	3.6	1.9	0.14	0.10	0.15	0.10	0.09	0.06
Max	11.5	8.8	5.8	3.4	6.9	4.8	0.29	0.18	0.31	0.20	0.19	0.08
dRVVscreen												
Median	42.4	38.7	45.3	42.3	55.7	50.1	1.33	1.21	1.29	1.21	1.37	1.23
Min	41.1	34.9	44.0	41.9	49.8	43.0	1.27	1.10	1.19	1.17	1.29	1.14
Max	49.4	39.7	46.6	42.7	69.8	54.7	1.56	1.22	1.39	1.26	1.71	1.50
dRVVmix*												
Median	11.6	7.0	16.8	9.3	12.1	8.3	12.1	7.3	17.0	10.0	13.1	6.8
Min	9.3	4.3	14.4	9.3	1.7	0.2	10.6	4.3	14.5	9.6	2.0	0.4
Max	24.5	17.7	19.3	9.3	15.4	10.4	23.6	18.2	19.4	10.3	15.5	11.2
dRVVcorr†												
Median	30.8	21.5	27.6	18.0	31.5	18.6	21.2	9.6	23.7	14.9	29.6	14.4
Min	28.3	20.3	25.1	16.7	25.1	16.1	19.4	8.9	19.9	13.2	23.0	12.9
Max	33.3	29.3	30.1	19.2	36.9	25.2	21.2	13.3	27.4	16.6	36.3	23.0
dRVVdelta‡												
Median	12.5	7.8	12.5	7.2	16.0	8.6	0.26	0.11	0.31	0.17	0.40	0.18
Min	11.2	6.9	10.9	6.3	13.9	6.8	0.26	0.10	0.24	0.16	0.30	0.14
Max	16.5	11.6	14.0	8.1	26.0	13.7	0.33	0.16	0.38	0.19	0.61	0.33

APTT, activated partial thromboplastin time; dRVV, dilute Russell viper venom tests; ICA, index of circulating anticoagulant; IL, Instrumentation Laboratory; SM, Siemens; ST, Stago; CT, clotting time; PNP, pooled normal plasma; PL, phospholipids. *Calculated as % ICA = $[(CT_{mixture} - CT_{PNP})/CT_{patient}] \times 100$. †% correction = $[(CT_{lowPL} - CT_{highPL})/CT_{lowPL}] \times 100$. ‡Delta = $CT_{lowPL} - CT_{highPL}$.

provide in the package insert for their products, indications on cut-off values to be used in each laboratory to interpret results obtained with the same combination of reagent and coagulometer (platform). The validity of this procedure has, however, not been thoroughly investigated to see whether small variations in the application of testing in different laboratories may introduce local bias in the determination of cut-off as well as patient values and hence in the interpretation of results.

The present study aimed to answer some of these questions. We identified three widely used commercial platforms for LA detection and selected five clinical laboratories across the world using each of the three platforms. Each laboratory was asked to include in the study at least 120 healthy donors and a common set of freeze-dried plasmas negative or positive for LA that were to be tested along with the donors' plasmas. The collaborative study gave the opportunity to collect and analyze huge numbers of data (i.e. > 20 000) that are not completely analyzed here and will form the basis of additional reports.

Interestingly, there were differences in dealing with the LA detection policy between different platforms. All included in their protocol APTT-derived and dRVV-derived tests as recommended by the ISTH guidelines [3]. However, three platforms used integrated tests (i.e. dual tests at low and high phospholipid concentrations) for the dRVV-derived tests. For the APTT-derived test, the approach was different. Laboratories with the Siemens platform used a brand of APTT for screening/mixing and a different brand of APTT for confirmation. Laboratories with the Instrumentation Laboratory (IL) platform used an integrated test and laboratories with the Stago platform used a brand of APTT for screening/mixing and a

Table 4 APTT-derived LA detection rate according to different cut-off values. Numbers represent the percentage of positive detection obtained by participants [(n. of positive LA/n. of observations) × 100] while testing the common LA-negative (plasma A), LA-strongly-positive (plasma C), LA-weakly-positive (plasma B) and plasma B diluted 1:2 in PNP

	Clotting time (seconds)								Ratio (patient plasma to PNP)							
	IL		SM		ST		TOT		IL		SM		ST		TOT	
Plasmas	99th	95th	99th	95th	99th	95th	99th	95th	99th	95th	99th	95th	99th	95th	99th	95th
Screening APTT-derived																
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	100	100	0	50	100	100	81	91	100	100	0	33	100	100	81	88
C	100	100	50	50	100	100	91	91	100	100	50	50	100	100	91	91
B diluted1:2	57	86	0	0	43	100	37	74	71	100	0	0	43	93	41	74
Mixing APTT-derived*																
A	0	0	0	0	0	14	0	6	0	0	0	0	0	0	0	0
B	83	100	0	60	100	100	77	94	100	100	0	40	100	100	84	90
C	100	100	60	60	100	100	94	94	100	100	40	60	100	100	90	94
B diluted1:2	71	100	0	0	79	100	62	81	71	100	0	0	71	100	58	81
Confirm APTT-derived as % correction†																
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	92	100	0	60	60	90	63	89	100	100	0	40	60	80	65	81
C	92	100	60	60	78	100	81	92	100	100	60	60	78	89	84	88
B diluted1:2	57	100	0	20	67	89	48	76	50	100	0	20	67	89	45	75
Confirm APTT as Delta‡																
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	100	100	0	60	80	90	74	89	100	100	20	60	80	90	77	89
C	100	100	60	60	89	100	88	92	100	100	60	60	78	89	84	88
B diluted1:2	57	100	0	40	67	89	48	81	50	100	0	20	67	89	45	75

APTT, activated partial thromboplastin time; LA, lupus anticoagulant; dRVV, dilute Russell viper venom test; IL, Instrumentation Laboratory; SM, Siemens; ST, Stago; TOT, total; ICA, index of circulating anticoagulant; CT, clotting time; PNP, pooled normal plasma; PL, phospholipids. *Calculated as $ICA = [(CT_{mixture} - CT_{PNP})/CT_{patient}] \times 100$. †% correction $= [(CT_{lowPL} - CT_{highPL})/CT_{lowPL}] \times 100$. ‡Delta $= CT_{lowPL} - CT_{highPL}$.

different brand of APTT with dual tests without and with hexagonal phospholipids for confirmation. From the scrutiny of results, the following conclusions on cut-off values and LA detection rates can be drawn.

Cut-off values

As expected, there were differences in the cut-off values for the different platforms when results were expressed as clotting times and these differences were not attenuated when results were expressed as a ratio (see Figs 1 and 2, Table 3). It is of more interest to note that in general there were differences in the cut-off values determined from results stemming from participants using the same platform. This is (apparently) an unexpected finding, but the most likely explanation rests on the between-laboratory differences of the selected population of donors combined with small differences brought about by the different lots of reagents employed in different laboratories, together with small between-laboratory differences in the application of the same platform. In contrast to a previous paper that reported cut-off values determined in five laboratories and for a single platform using the same lot of reagents [15], in the present study the between-laboratory differences were only marginally attenuated when results were expressed as a ratio. The reason probably rests with the variability of PNP prepared in different laboratories. Perhaps, a common international standard PNP is needed to harmonize results between laboratories. However, this notwithstanding, clinical laboratories should be encouraged to express their results as ratios, as this minimizes the day-to-day variability. Another reason for the between-laboratory variation in cut-off observed in the study when using the same platform may be the different lots of reagents or blood collecting systems used in different laboratories with the same platform. Indeed, two laboratories that used the same platform and shared the same lot of reagents obtained similar cut-off values (not shown). Overall, the relatively high inter-laboratory variability in the cut-off values even within the same platform observed in this study should be indicative of the notion [16], until now not directly documented in a large collaborative study involving many laboratories and three widely used commercial platforms, that cut-off values should be determined locally and that the values reported in the reagent package insert must be taken only as preliminary information.

The study also investigated whether results from healthy donors are or are not *normally* distributed. On average, more than half of the datasets were not *normally* distributed and there were differences between methods and platforms. Hence, the practice of using the

Table 5 dRVVT-derived LA detection rate according to different cut-off values. Numbers represent the percentage positive detection obtained by participants [(n. of positive LA/n. of observations) × 100] while testing the common LA-negative (plasma A), LA-strongly positive (plasma C), LA-weakly positive (plasma B) and plasma B diluted 1:2 in a PNP (see also legend to Table 4)

	Clotting time (seconds)								Ratio (patient plasma to PNP)							
	IL		SM		ST		TOT		IL		SM		ST		TOT	
Plasmas	99th	95th	99th	95th	99th	95th	99th	95th	99th	95th	99th	95th	99th	95th	99th	95th
Screening dRVV-derived																
A	0	0	0	17	0	0	0	3	0	0	0	17	0	0	0	3
B	67	100	100	100	57	100	69	100	67	100	100	100	57	93	69	97
C	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
B diluted1:2	0	29	0	33	0	31	0	31	0	14	17	17	0	23	4	19
Mixing dRVV-derived*																
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	67	75	0	100	100	100	69	91	67	75	0	83	79	100	59	88
C	75	100	100	100	100	100	91	100	92	100	100	100	100	100	97	100
B diluted1:2	0	14	0	0	0	33	0	20	0	0	0	0	8	17	4	8
Confirm dRVV-derived as % correction†																
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	58	92	100	100	29	100	53	97	46	100	100	100	0	79	36	90
C	92	100	100	100	100	100	97	100	100	100	100	100	79	79	90	90
B diluted1:2	0	43	0	100	0	31	0	50	0	43	17	83	0	23	4	44
Confirm dRVV as Delta‡																
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	75	92	100	100	43	100	66	97	55	100	100	100	14	79	45	90
C	92	100	100	100	100	100	97	100	100	100	100	100	79	79	90	90
B diluted1:2	0	43	0	100	0	46	0	58	0	43	17	83	0	23	4	44

LA, lupus anticoagulant; dRVV, dilute Russell viper venom test; ICA, index of circulating anticoagulant; IL, Instrumentation Laboratory; SM, Siemens; ST, Stago; TOT, total; CT, clotting time; PNP, pooled normal plasma; PL, phospholipids. *Calculated as ICA = $[(CT_{mixture} - CT_{PNP})/CT_{patient}] \times 100$. †% correction = $[(CT_{lowPL} - CT_{highPL})/CT_{lowPL}] \times 100$. ‡Delta = $CT_{lowPL} - CT_{highPL}$.

parametric method for calculating cut-off, based on the mean + SD, is questionable and the more robust non-parametric calculation based on centiles should be implemented. As expected, the cut-off values were smaller when calculated as 95th rather than 99th centiles. It should, however, be pointed out that the choice between the two models of calculation should be based on the LA detection rate determined with truly positive or negative plasma rather than on statistical considerations. This is especially important if one considers that persistently positive LA combined with previous thrombosis qualifies patients for long-term anticoagulation [1].

LA detection rate

Although defining truly positive or negative LA plasmas is difficult because of the lack of specific tests, we attempted to investigate the LA detection rate using a common set of previously validated LA plasmas that had been tested by the participants along with the plasmas from donors. From scrutiny of the results, the following considerations can be derived.

LA-negative plasma It is reassuring that no false-positive LAs were found by participants when testing plasma A (LA-negative), both for the APTT-derived and dRVV-derived tests (screening, mixing and confirming), whatever the methods for calculating cut-off or expressing results, except for the screening dRVV Siemens platform when cut-off was calculated as the 95th centile with results expressed as clotting times (see Tables 4 and 5). The inherent limitation of the above conclusions rests on the fact that plasma A was negative for LA but had normal clotting time. A negative-LA plasma with abnormal clotting time would have been needed to draw definite conclusions.

LA-strongly-positive plasma Overall, the LA detection rate obtained by participants when using the APTT-derived test was high for plasma C (strongly positive) (range of values, 81–94%) (see Table 4). The detection rate was slightly better when using more stringent cut-off values (95th centiles) regardless of the expression of results. For the dRVV-derived tests, the detection rates were higher than those observed for the APTT-derived tests (range of values, 90–100%), with slightly better detection rates when using more stringent cut-off values (95th centiles) regardless of the expression of results (see Table 5).

LA-weakly-positive plasmas For plasma B (weakly positive), detection rates for the APTT-derived methods were (as expected) smaller than those observed for the strongly positive plasma (range of values, 63–94%), with a better

detection rate when using more stringent cut-off values (95th centiles) regardless of the expression of results. Finally, when plasma B (weakly positive) was diluted 1 : 2 in PNP, the LA potency for the APTT-derived test became very weak and the detection rate considerably lower (range of values, 37–81%), with a better detection rate when using more stringent cut-off values (95th centiles) regardless of the expression of results (see Table 4). For the dRVV-derived tests, detection rates for plasma B were similar to those observed for the APTT-derived tests (range of values, 53–100%). It is of interest that detection rates for the diluted plasma B were smaller for the dRVV-derived (range of value, 0–58%) than those observed for the APTT-derived tests (37–81%) (see Tables 4 and 5). However, no definitive conclusions can be drawn on the performance of the APTT-derived vs. the dRVV-derived tests in detecting LA, as the results pertain to the specific set of LA plasmas used in this study, which are not necessarily representative of the whole population of patients with LA. In addition, none of the plasmas in the panel had abnormal prolonged clotting time because of the presence of coagulation factor deficiencies or presence of an inhibitor other than LA. There were differences in the LA detection rate according to the method of calculating cut-off: those based on 99th centiles were smaller than those based on 95th centiles (see Table 3). These findings may be of considerable practical interest. Owing to the lack of standardization of LA reporting [17], the potency of LA in individual patients cannot be accurately determined. Therefore, although surmised [18], it is not yet known with certainty if weak LA positivity is as clinically relevant as strong LA positivity. It can be argued that if the goal of the laboratory is to pick up not only the strongly positive but also the weakly positive patients, more stringent cut-off values based on the 95th centiles should be selected. This choice would carry the risk of increasing the numbers of false-positive patients and should therefore be carefully considered.

Although we recognize that the presence of outliers could overstate the estimated variability, in this preliminary evaluation, we elected to analyze all the results stemming from the participants without detection (and rejection) of outliers. Some guidance on the methods that are useful for outlier detection when calculating cut-off values for laboratory parameters of clinical interest has been given previously [see 19 for more details]. However, no clear recommendations on the best method to use, nor evaluation of the impact that different methods may have on interpretation of result, have been established. Furthermore, owing to the complexity of the diagnostic procedures, the detection of outliers in the LA setting might require strategies that are different from those used for other parameters. Hence, we believe that further analyses of the data collected in this collaborative study are appropriate to investigate the impact of different models of outlier detection and these will form the basis of a specific report that is in preparation.

In conclusion, the collaborative study involving 11 laboratories collecting plasmas from hundreds of donors and using the three widely used commercial platforms for LA detection leads to the following considerations. (i) Clotting times or ratios for healthy subjects were not *normally* distributed in the majority of cases. The take-home message is that cut-off values should be determined preferably by the non-parametric method based on centiles. (ii) There are relatively large between-laboratory cut-off variations even within the same platform and the variability is marginally attenuated when results are expressed as ratios. The take-home message is that cut-off values should be determined locally. (iii) There are major differences between cut-off values calculated as 99th or 95th centiles that translate into a different LA detection rate (the lower the centile the greater the detection rate). The take-home message is that cut-off values determined as the 95th centile allow a better LA detection rate.

Addendum

A. Tripodi conceived the study, reviewed results, and wrote the manuscript. V. Chantarangkul collected data, carried out statistical analyses, and reviewed the results and the manuscript. M. Cini, K. Devreese, J. S. Dlott, R. Giacomello, C. Legnani, M. E. Martinuzzo, P. Pradella, A. Siegemund, S. Subramanian, P. Suchon, and S. Testa selected donors, collected plasmas, supervised measurements at their own laboratories, and reviewed and accepted the manuscript. E. Gray prepared and characterized the freeze-dried LA-positive or negative plasmas, and reviewed and accepted the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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