

How to Achieve Standardization? Diluted Russell Viper Venom Test for Lupus Anticoagulant Detection in a Chinese Female Population

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Abstract

On an international scale, guidelines and proposals for lupus anticoagulant detection have been published over the last 20 years, but until now, standardization has not been completely realized. The aim of this study was to evaluate the different ways of interpreting the results of lupus anticoagulant detection for standardization. A retrospective review of 15 447 instances of lupus anticoagulant detection by the diluted Russell Viper Venom test for female patients presenting with problems relating to the areas of reproduction, gynecology and obstetrics was performed. Lupus anticoagulant data were compared between different departments, months, reagent lots and cutoffs. Significant differences were found in patient data between different reagent lots, especially between lots of screening reagents (monthly average: highest 37.96 s vs lowest 33.88 s) and in the positive rates of lupus anticoagulant by different detection cutoffs (47.58% by using LA1/LA2 > 1.20 without normalization as a cutoff in Lot 1 vs 1.52% by using LA1 > 44 s as a cutoff in Lot 3). Compared with the cutoff using the value above the 99th percentile of LA1 for the healthy donors per lot, the cutoff using integrated tests with normalization had the smaller deviation of positive rate between different reagent lots. Pregnant women had higher LA1/LA2 levels than nonpregnant women. Based on the results, normalization is needed because there are significant lot-to-lot variations. Integrated tests with normalization might be a better standard by which to confirm lupus anticoagulant. Pregnant women should have population-specific cutoffs because they have higher LA1/LA2 levels.

Keywords

coagulation, lupus anticoagulant, reproduction, obstetrics, gynecology

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Background

Lupus anticoagulants (LAs), which are detectable in laboratory tests, are linked to a phenomenon in which clotting times in vitro are prolonged under interference. Screening for LA can reflect prolonged clotting times in a phospholipid-poor environment and be confirmed by the recovery of clotting times in a phospholipid-rich environment.¹⁻³ Accurate detection of LA is essential for the diagnosis of antiphospholipid syndrome (APS), as the persistent presence of these antibodies correlates better with thrombosis and pregnancy morbidity than other APS-related antibodies, such as anticardiolipin antibodies.⁴⁻⁷

Numerous variables can affect assays used for LA detection. Among them, phospholipids in the reagent mixture, the

activator and, above all, the expression of results and cutoff values greatly influence the results. To standardize LA detection, guidelines for LA analysis were published as early as 1995.¹ In 2009, the Scientific Standardization Subcommittee

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(SSC) on LAC/aPL of the International Society of Thrombosis and Hemostasis (ISTH) provided more detailed guidelines on how to perform LA testing.⁵ This guideline recommended a diluted Russell Viper Venom assay (dRVVT) and recognized the existence of integrated test systems that consist of testing the plasma under investigation twice by means of the dRVVT or activated partial thromboplastin time (APTT) performed in parallel at low (screening) and high (confirmation) phospholipid concentrations.^{8,9} In 2014, the Clinical Laboratory Standards Institute (CLSI) issued the first edition of LA detection guideline H60. Although these guidelines have not yielded complete agreement about the testing flow and cutoff values, normalization of the results of screening and confirmation assays by means of calculating the cutoff value per lot or analyzing normal pooled plasma (NPP) with each batch is consistently recommended. Except for homemade or commercial NPP, the mean of a reference interval (RI) determined per lot of reagents can also be used for normalization.¹⁰⁻¹²

Until now, studies have noted poor compliance by laboratories in following the recommended guidelines for LA detection, eg, large numbers of labs (89%) not following the recommendation of in-house cutoff values calculated by the 99th percentile, as required by ISTH.^{13,14} In China's Sichuan Province, multiple detections are not incorporated into the uniform pricing followed by local governments, and normalization of the results by NPP per run is not an option whose cost is covered. Therefore, we decided to evaluate the different ways of interpreting the results of LA detection. During the course of LA detection, pregnancy is most concerning, because the coagulation systems of pregnant women have their own physiological characteristics.

Objective

To evaluate the different ways of interpreting the results of LA detection for standardization.

Method

Study Design

A retrospective review of LA data for dRVVT from patients older than 17 years of age presenting with problems relating to areas of reproduction, gynecology and obstetrics from July 2018 to January 2021 was performed. LA data were compared using means across months, reagent lots and patient classifications by different cutoffs.

Data Collection

All patient LA data were included except for those from certain lots of reagents that had been used for less than two months. Data from pregnant women, patients belonging to the gynecological department and patients diagnosed with recurrent spontaneous abortion (RSA) are summarized separately.

Blood Sampling

Samples were taken by 2.7 ml BD 3.2% buffered sodium citrate vacutainers. All pre-analytical management were following the requirements from Hematology and Coagulation Checklist of CAP (COLLEGE of AMERICAN PATHOLOGISTS).

Tests and Equipment Employed

Sysmex CS-5100 coagulation analyzers with original reagents, Siemens LA1 screening reagents and LA2 confirmation reagents were used to determine the LA1 and LA2 values. All quality control and management followed ISO15189/CAP requirements.

Cutoffs

Reference intervals were built from the data of at least 40 health donors from the Health Examination Center aged less than 50 years once LA1 or LA2 was performed by a new lot. The 99th percentiles of LA1 and LA1/LA2 of these healthy donor data were used as cutoff values for LA1 and the integrated test. The RI from these health donors was used to modify the formula of integrated tests to normalize the LA1 and LA2 results instead of NPP per run. LA1/LA2 > 1.20 was chosen as another cutoff for the integrated test.¹⁵

Five different cutoffs were used to designate positive results:

- LA1 > 44 s and LA1/LA2 > 1.20 (from the manufacturer's recommendation);
- LA1/LA2 > 1.20;
- LA1 > 99th percentile and LA1/LA2 > 1.20;
- LA1/RI ÷ LA2/RI > 1.20; and
- LA1/RI ÷ LA2/RI > 99th percentile.

Data Analysis

Comparisons were performed using the chi-squared test or Fisher's exact test for categorical variables and Student's *t* test for continuous variables. A *P* value ≤ .05 was considered statistically significant.

Table 1. Data on LA1 and LA2 for included patient.

Reagent	Durations	Data Number	Mean(s)	SD(s)
LA1	2018.09 to 2019.02	2255	37.49	4.07
	2019.04 to 2020.01	5080	34.59	4.07
	2020.02 to 2020.06	3225	34.29	3.56
	2020.08 to 2021.01	4887	35.71	3.76
LA2	2018.09 to 2019.02*	2255	31.04	2.23
	2019.04 to 2019.11	4130	30.08	2.19
	2019.12 to 2020.06	4175	29.77	1.84
	2020.08 to 2020.09*	1645	30.95	2.14
	2020.10 to 2021.01	3242	29.9	2.07

*: between Lot 1 and Lot 4 of LA2, *P* = .112.

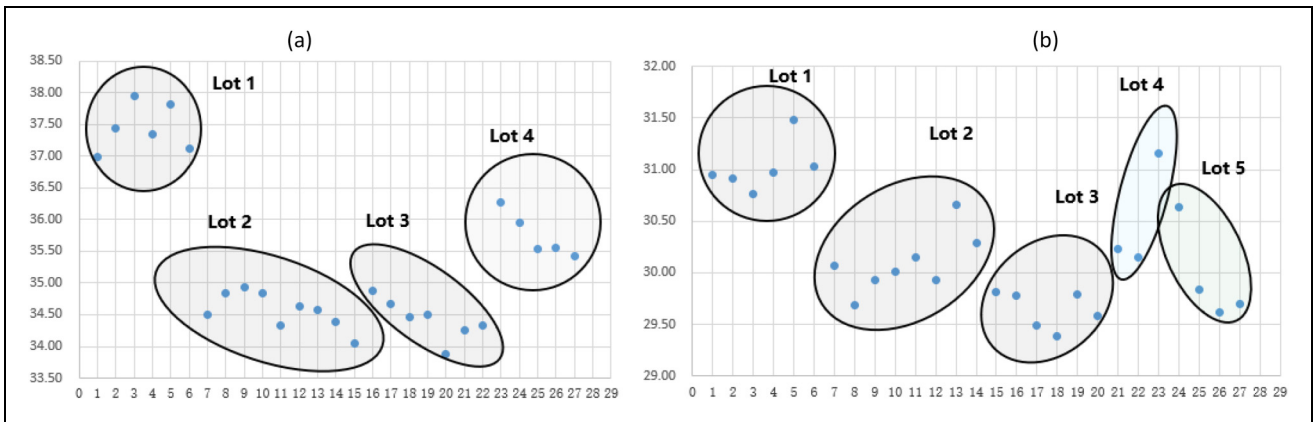


Figure 1. Monthly LA1 and LA2 patient data. Y-axis: average clotting time of patients a. LA1 and b. LA2 data, X-axis: month. Monthly data belonging to one reagent lot were in same circle. There were data from 29 months belonging to four lots of LA1 and five lots of LA2.

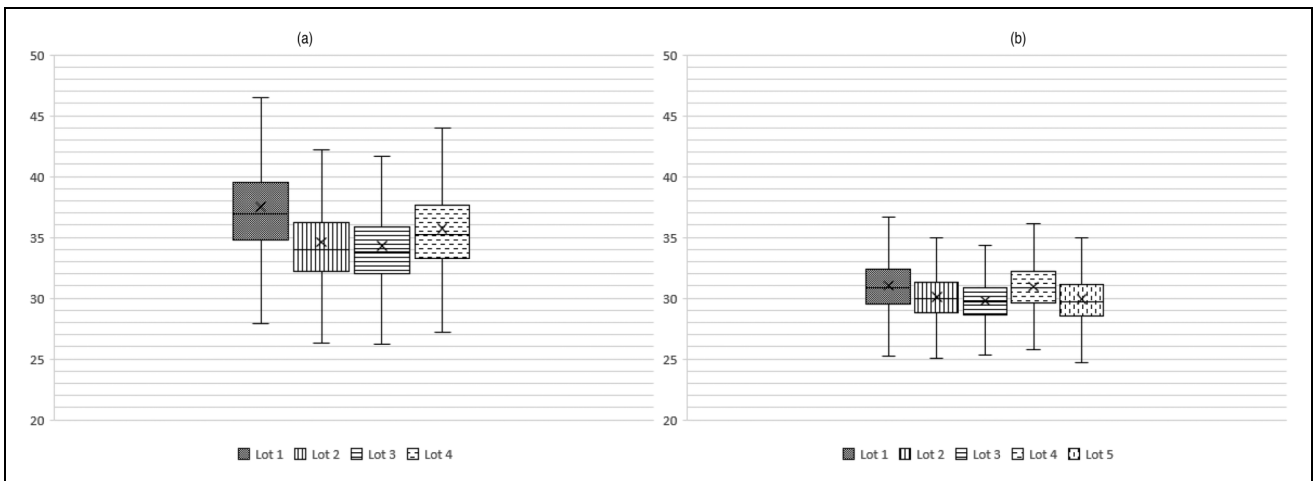


Figure 2. LA1 and LA2 patient data by lot. a. LA1 and b. LA2 patient data for each lot are shown as box plots. There were data from four lots of LA1 and five lots of LA2.

Results

Data Across Different Lots

Ultimately, 15 447 LA data points from four lots of LA1 and five lots of LA2 were included. Detailed information is listed in Table 1. According to these data, except between Lot1 and Lot4 of LA2 ($P=.112$), significant differences were found between each lot of LA1 or LA2 ($P<.05$). On average, there was a larger deviation in the patient data among lots of LA1 (3.2 s between Lot 1 and Lot 3) than among lots of LA2 (1.3 s between Lot1 and Lot3). The data distribution is shown by lot and month in Figures 1 and 2.

Positive Rates Using Different Cutoffs

In total, 746 data points from healthy donors aged below 50 years from the Health Examination Center were used to calculate

Table 2. Positive rates of LA by different cutoffs.

LA1 Lot	Data Number	Positive Rate (%)				
		Cutoff a	Cutoff b	Cutoff c	Cutoff d	Cutoff e
1	2255	5.94	47.58	5.81	5.59	9.18
2	5080	2.11	52.82	2.42	4.26	8.58
3	3225	1.52	33.57	5.18	4.24	8.93
4	4887	2.86	34.83	5.38	4.21	9.86

Cutoff a: LA1 > 44 s and LA1/LA2 > 1.20; Cutoff b: LA1/LA2 > 1.20; Cutoff c: LA1 > 99th percentile and LA1/LA2 > 1.20; Cutoff d: LA1/RI+LA2/RI > 1.20; Cutoff e: LA1/RI+LA2/RI > 99th percentile.

cutoffs for each lot. The LA1 cutoff value was 44.16 s for lot 1 ($n=95$, age = 41.1 ± 6.0 years), 43.11 s for lot 2 ($n=431$, age = 37.5 ± 7.1 years), 40.24 s for lot 3 ($n=124$, age = 41.5 ± 7.5 years) and 42.2 s for lot 4 ($n=96$, age = 39.0 ± 6.7 years).

The cutoff values for normalized LA1/LA2 were 1.16 for lot 1, 1.15 for lot 2, 1.16 for lot 3 and 1.15 for lot 4. The positive rate by different cutoffs is shown in Table 2. According to the data, the cutoff LA1/LA2 > 1.20 led to the highest positive rate, while the cutoffs LA1 > 44 s and LA1/LA2 > 1.20 led to the lowest positive rates, and there were obvious unstable positive rates among lots according to both of these standards. There were also differences found between positive rates for the remaining three cutoffs, even though these cutoffs were all normalized relative to the data from the healthy donors. Between different reagent lots, the positive rates established by the LA1/RI ÷ LA2/RI > 99th percentile and the LA1/RI ÷ LA2/RI > 1.20 cutoffs were more consistent than those established by the LA1 > 99th percentile or LA1 > 44 s cutoffs.

Positive Rates by Different Classifications of Patients

All testing data were divided into three groups according to the patient classification:

Group A: data from pregnant women;

Group B: data from patients with problems relating to the gynecological domain; and

Group C: data from nonpregnant women having been diagnosed with RSA.

The positive rates of these different groups for the LA1 > 99th percentile and LA1/LA2 > 1.20 cutoff and the normalized LA1/LA2 > 1.20 cutoff are shown in Figure 3. According to the data, there was a significant difference between the two different cutoffs for the positive rates of group A. When the cutoff was LA1 > 99th percentile and

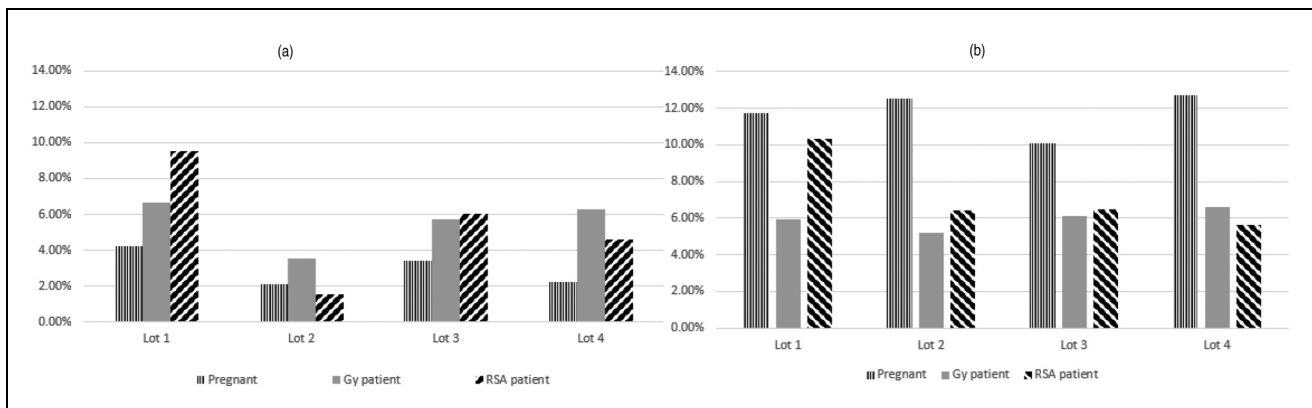


Figure 3. Positive rates of LA for the different groups.

Pregnant: Lot 1 to Lot 4, $n = 809,1595989$ and 1908 ; Gy patient: Lot 1 to Lot 4, $n = 405,712245$ and 333 ; RSA patient: Lot 1 to Lot 4, $n = 252,717415$ and 587 .

Y-axis: positive rate by cutoff a. LA1 > 99th percentile and b. LA1/LA2 > 99th percentile of healthy donors, X-axis: lots 1 to 4 of LA1. Gy patients means patients with problems relating to gynecology.

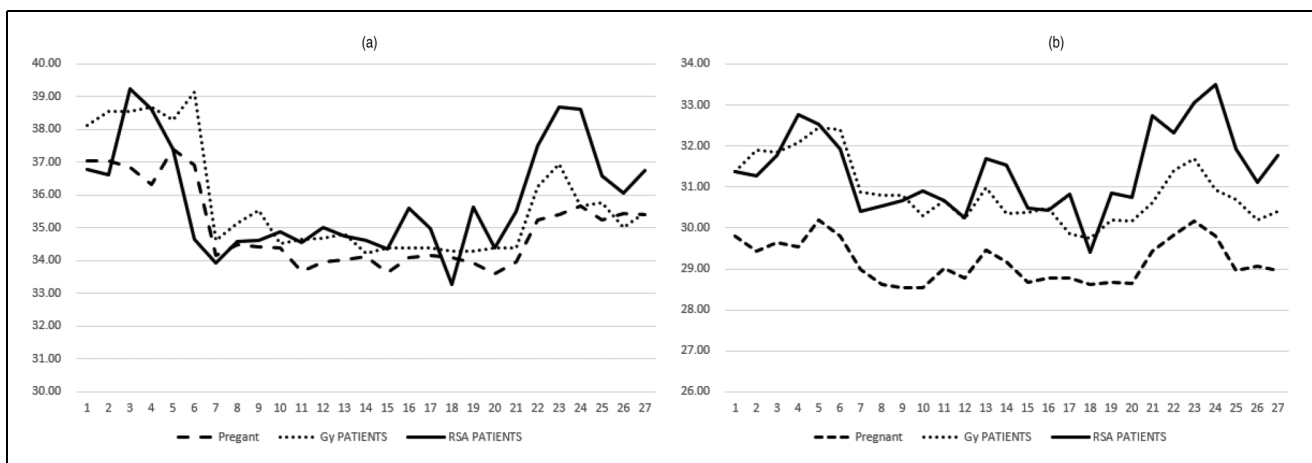


Figure 4. Monthly LA1 and LA2 patient data for the different groups.

Y-axis: average clotting time of patients a. LA1 and b. LA2 data, X-axis: month. Gy patients means patients with problems relating to gynecology.

LA1/LA2 > 1.20, the positive rate for pregnant women was lowest in almost every lot, except in lot 2, but when the cutoff was normalized to LA1/LA2 > 99th percentile, the positive rate for pregnant women became the highest among all the groups. This difference was caused by a phenomenon specific to pregnant women in that their LA2 values were much lower than those for nonpregnant women (means: 29.2 s vs 31.2 s, $P < .001$, deviation = 2.0 s), while there was a smaller difference between the pregnant and nonpregnant in LA1 (means: 34.9 s vs 35.8 s, $P < .001$, deviation = .9 s), making the former group's LA1/LA2 higher. The monthly LA1 and LA2 values for the different groups are shown in Figure 4.

Discussion

LA testing is essential for APS diagnosis and thrombophilia screening. However, similar to other coagulation tests, a lack of standardization has hindered its clinical use. According to previous studies, without standardization, the positive rate of LA would change from less than 10% to more than 40%.^{16,18} To ensure adequate clinical performance, LA testing at minimum needs to maintain a stable and appropriate positive rate in certain fixed people. In Sichuan Province and even across China, LA testing is affected by many factors, eg, break-even matter, and the fixed screening cutoff (LA1 > 44 s) suggested by the manufacturer is the most commonly used cutoff for LA. However, according to our study, notable lot-to-lot variations can be found between reagent lots, especially between lots of LA1, making it impossible to obtain stable LA1 data when testing with different lots for certain fixed people. This also means that the normalization of results is quite necessary.

The normalization method suggested by existing guidelines is to establish a new cutoff per lot using at least 40 healthy donors aged less than 50 years as a reference and/or to use the results of NPP per run to modify the patient data of LA1 and LA2. Therefore, in our study, we used the data from healthy donors to establish the 99th percentiles of LA1 and LA1/LA2 as cutoffs for each lot. Considering the cost factor, we did not choose NPP testing per run to modify the patient data but used the reference intervals from the healthy donors instead. Due to the high quality of the existing closed detection system with Siemens reagent and matching Sysmex equipment, the monthly averages of the patient data and the daily internal quality control values were very stable within a specific lot, which indicates that the deviation within the run was not obvious. Therefore, we believe that using RI per lot to modify patient data could have an effect similar to that of using NPP per run, as other studies have also reported.¹² In our study, there were stable positive rates among different lots by several kinds of normalization methods, including using RI.

We believe that the integrated test system, which consists of testing the plasma under investigation twice by means of the dRVVT performed in parallel at low and high phospholipid

concentrations, was an ideal method to express the LA data because this system reflects the nature of LA, namely, that dRVVT is prolonged at low but recovered at high phospholipid concentrations. The most powerful evidence of LA is the gap between LA1 and LA2 rather than the amplitude of LA1 prolongation. However, using integrated test systems means that the lot-to-lot variations of not only LA1 but also LA2 will interfere with the screening of LA, causing a more complex situation. Therefore, in our study, we found that cutoff b, ie, LA1/LA2 > 1.20, was the worst choice because it could not obtain a stable positive rate and would lead to more than half of the data in some lots showing LA positivity, which is even higher than that in SLE patients.¹⁹ However, after normalization by RI, the integrated test systems showed an even more stable positive rate than using the 99th percentile of LA1 among different lots. Presumably, because RI uses the average of reference from a limited number of healthy donors, the average would be less affected by the distribution or outlier of reference data than the 99th percentile used by the cutoff of LA1.

To our surprise, although 99th percentiles from the same reference data were used in both cutoff c and e, there were still obvious differences in the positive rates. The positive rate established by the LA1/RI > LA2/RI > 99th percentile cutoff was much higher than that established by the LA1 > 99th percentile cutoff. When using the LA1/RI > 99th percentile cutoff, the positive rates in pregnant patients were even higher than those in RSA patients and gynecology patients, most of whom had tumors, which is not reasonable. In a subsequent study, we found that this "pseudohigher" positive rate was due to a special phenomenon in which LA1 and LA2 were shorter in pregnant women than in nonpregnant women, and most importantly, the amplitude of LA2 shortening was much more pronounced than that of LA1, making LA1/LA2 higher, which has not been mentioned in any previous study. This special phenomenon would not be influenced by RI or NPP because they come from healthy donors excluding healthy pregnant women. Therefore, even when modified by RI, using the LA1/RI + LA2/RI > 99th percentile cutoff leads to a much higher positive rate in pregnant women than using LA1 alone. This phenomenon may be caused by the physiological hypercoagulability of pregnant women, which may be further amplified by the high phospholipid concentrations, making LA2 much more shortened than LA1. None of the cutoffs used in our study could solve this problem, especially cutoffs from an integrated test system that used both LA1 and LA2 for screening. Consequent to this finding, establishing special population cutoffs for pregnant women is needed. Prior to such cutoffs being established, using normalized LA1 for screening is preferred for pregnant women.

Conclusion

Due to the existence of notable lot-to-lot variations, the normalization of LA testing results is quite necessary. Normalization by 99th percentiles and RI from healthy donors for each lot to

modify patient data could yield stable positive rates among different lots. The deviation of the positive rates between lots from the integrated test system was smaller than those from LA1 alone. For pregnant women, special population cutoffs are needed to account for their higher LA1/LA2 values.

Declaration of Conflicting Interests

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