Clin. Lab. 2026;72:XXX-XXX

ORIGINAL ARTICLE

Diluted Russell Viper Venom Test for Lupus Anticoagulant Detection in Pregnancy Population

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SUMMARY

Background: **The association between lupus anticoagulants and pregnancy-related adverse outcomes is highly significant. Widely used in risk assessment, dilute Russell's viper venom time (dRVVT) is a common test for lupus anticoagulants during pregnancy. However, pregnancy induces complex changes in the coagulation system, leading to inconsistent trends in coagulation indicators compared to healthy individuals. This study aimed to investigate the impact of the pregnancy process on dRVVT detection and clinical applications.**
Methods: **From July 2021 to February 2022, data from 2,709 pregnant women's dRVVT tests were analyzed, with 71 healthy non-pregnant individuals as a control. Screening tests (LA1), confirmation tests (LA2), and the LA1/LA2 ratio were compared between early, mid, late pregnancy, and the control group. Using early pregnancy as the observational target, the correlation between the mentioned indicators and pregnancy outcomes were analyzed. Variance analysis and rank-sum tests were employed to assess the consistency, and logistic regression analysis was conducted for data analysis related to outcomes, with $p < 0.05$ considered statistically significant.**
Results: **There was no significant difference in LA1 between groups ($p > 0.05$). LA2 decreased and LA1/LA2 increased with advancing pregnancy ($p < 0.05$), consistently different from control group ($p < 0.05$). LA1 and LA1/LA2 in early pregnancy were correlated with pregnancy outcomes and served as independent prognostic factors for adverse pregnancy outcomes ($p < 0.01$), with LA1/LA2 being the optimal outcome prediction indicator.**
Conclusions: **As pregnancy progresses, LA2 decreases significantly, while LA1 remains unchanged. Existing dRVVT standards for adults may falsely elevate lupus anticoagulant detection during pregnancy. Establishing pregnancy-specific criteria for dRVVT is essential.**
(Clin. Lab. 2026;72:xx-xx. DOI: 10.7754/Clin.Lab.2025.250603)

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Manuscript accepted July 20, 2025

KEYWORDS

lupus anticoagulant, dRVVT, pregnancy

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune multisystem disorder characterized by the presence of antiphospholipid antibodies (aPL) and the occurrence of thrombotic events in arteries, veins, or microvasculature, as well as pathological pregnancies. Due to the nonspecific clinical manifestations of APS, the key to diagnosing APS is the laboratory confirmation of persistent aPL presence [1]. Among the three primary aPL

tests, lupus anticoagulant (LA) has been established as the most closely related indicator to pregnancy-related hypercoagulation and adverse pregnancy outcomes such as recurrent miscarriages [2,3]. It is currently widely employed for risk assessment and preventive treatment in pregnancy-related populations [4]. Given the heterogeneity of LA antibodies, current testing methods predominantly rely on *in vitro* coagulation assays. Typically, two testing systems with different analytical principles are chosen to maximize detection rates, often including dRVVT (dilute Russell's viper venom time) and APTT (activated partial thromboplastin time) assays, with the former being the most sensitive and used screening and diagnostic test for LA in pregnant individuals [5-7].

However, the pregnancy period is a unique physiological phase, and to support the processes of pregnancy and childbirth, the coagulation system undergoes a series of complex changes during pregnancy. These physiological changes result in a series of coagulation indicators showing distinct pregnancy-related trends compared to other healthy populations, such as fibrinogen concentration, D-dimer, and so on [8,9]. So, do these alterations in the coagulation system during pregnancy affect the detection and interpretation of LA results? In our previous research, we have already identified distinct characteristics in dRVVT assays in pregnant populations, while the mean data of LA screening tests (LA1) in the entire pregnancy-related population are close to those of non-pregnant populations, in the LA confirmatory tests with high phospholipid concentrations, the clotting times in pregnant populations are significantly lower than in non-pregnant populations [10]. In other words, pregnant individuals exhibit a particular response to high phospholipid concentrations, which will result in an elevation of one of the criteria for dRVVT assays, the LA1/LA2 ratio, in pregnant populations. Currently, the normalized LA1/LA2 ratio is recommended as the LA confirmation standard by numerous manufacturers and is supported by literature data [11,12]. However, considering the unique phenomenon we have observed, using ratios to confirm LA may lead to a significant increase in the LA positivity rate in pregnant populations, potentially resulting in incorrect diagnoses and excessive treatments. To confirm this phenomenon and understand the variations in LA1, LA2, and LA1/LA2 ratios between different gestational weeks, we have designed this study. Simultaneously, we have explored LA diagnostic criteria for pregnant populations.

MATERIALS AND METHODS

Study design

A retrospective review of LA data for dRVVT from obstetric patients who attended West China Second Hospital from July 2021 to February 2022 was performed. LA data was compared across different trimesters of pregnancy. Then, by combining clinical information, the correlation between LA data in early pregnancy population and pregnancy outcomes were also analyzed. In addition, lupus anticoagulant screening cutoffs suitable for early pregnancy population were established.

Data collection

Observation of the differences in LA during different trimesters

From July 2021 to February 2022, a total of 2,709 obstetric patients who visited hospital were included in the study. After excluding those with thrombosis or active bleeding manifestations, concurrent autoimmune diseases, and the use of anticoagulant drugs, mainly low molecular weight heparin, and LA1 values exceeding the existing positive criteria (greater than the 97.5th percentile of healthy adults tested with this batch of reagents, 38.1 seconds), a final total of 2,279 cases were included. These cases were divided into the following groups: 866 cases in the early pregnancy group (1 - 12 weeks), 851 cases in the mid-pregnancy group (13 - 27 weeks), and 562 cases in the late pregnancy group (28 weeks and above), 71 normal people who came to hospital for pre-pregnancy physical examination during the same period were selected as control group.

The correlation between LA data in early pregnancy population and pregnancy outcomes

From July 2021 to February 2022, a total of 1,089 early pregnancy obstetric patients were included in the study. After excluding those with thrombosis or active bleeding manifestations, concurrent autoimmune diseases, and those lost to follow-up or transferred during the period, a final total of 1,055 cases were included. The correlation between dRVVT results and pregnancy outcomes was observed, with adverse pregnancy outcomes defined as miscarriage, preterm birth, stillbirth, fetal abnormalities, postpartum hemorrhage, etc.

Establishing LA screening cutoffs for the early pregnancy

Among the 1,055 cases of early pregnancy population included, after excluding those with a history or current adverse pregnancy outcomes in this study, a final total of 344 cases were included for calculating the cutoff threshold (97.5th percentile) for dRVVT.

Blood sampling

Samples (2.7 mL) were taken using BD 3.2% buffered sodium citrate vacutainers. All pre-analytical management was followed 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.

Statistical analysis

SPSS 10.0 (SPSS Statistics for Windows, Version 10.0.; SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad, USA) software were used for statistical analysis. The comparison of LAC values of the pregnant women and healthy controls was performed using the Mann-Whitney U test. For the association of clinical features of pregnant women with LAC values, chi-squared test was used to compare the intergroup differences. In multivariate analyses, Cox regression model was used to assess the prognostic factors. The p-value was established at 0.05 to be considered as statistically significant.

RESULTS

Detection of LA during different pregnant trimesters

According our previous study, normalization by 97.5th percentiles and RI from healthy non-pregnant donors was adopted as cutoffs. According to this cutoff value (LA1 > 38.1 seconds and LA1/LA2 > 1.17, the positive rate of dRVVT in pregnant women was 3.69% in first trimesters, 4.42% in second trimesters, and 9.27% in third trimesters.

In the LA data of pregnant individuals who meet the negative dRVVT standards for non-pregnant adults, it can be clearly seen that the screening experiment's LA1 levels during pregnancy are similar to those of non-pregnant adults ($p > 0.05$) and remain stable at various stages of pregnancy. However, unlike LA1, the diagnostic experiment LA2 during pregnancy is significantly lower than that of non-pregnant controls ($p < 0.001$) and decreases further with the progress of pregnancy ($p < 0.001$. Additionally, the asymmetric changes between LA1 and LA2 result in a higher LA1/LA2 ratio compared to controls, which significantly increases during pregnancy, as shown in Figure 1 and Table 1. This also affects the positivity rate of dRVVT during pregnancy when using the LA1/LA2 ratio to diagnose positive and negative result.

The detection of LAC in the first trimester is associated with the outcome of pregnancy

Due to the lower probability of using anticoagulants drugs and complications during the early stages of pregnancy, we established the cutoff values of LA1 and LA1/LA2 based on 97.5% of the normal population in the first trimester.

At the same time, the results of 97.5% quantile of the normal population were combined to calculate the univariate analysis of pregnancy outcomes.

Adverse pregnancy outcomes include spontaneous abortion, premature birth, overdue birth, stillbirth, low weight infants, macrosomia infants, neonatal asphyxia, birth defects, etc.

The chi-squared test results indicated that no matter which cutoff value is selected, as long as LAC is detected in early pregnancy, it is associated with adverse pregnancy outcomes. However, age and taking anticoagulants were not associated with pregnancy outcomes (Table 2).

Analysis of the RI/cutoff value affecting pregnancy outcome

Based on the data collected in the early stage of pregnancy and in combination with their clinical outcomes, we carried out ROC analysis. It was discovered that the results of LA1 and LA1/LA2 had better predictive value for prognosis. Meanwhile, it was found that when the ratio of LA1/LA2 was 1.17, it was the value with the best sensitivity and specificity.

DISCUSSION

The special changes of LA2 during pregnancy were the main reason for conducting this study. In the study, we confirmed that not only were the levels of LA2 significantly lower than those of the normal population throughout the entire pregnancy, but LA2 further decreased with the progression of pregnancy, while the levels of LA1 did not undergo significant changes. In other words, the ratio of LA1 to LA2 in the normal pregnant population showed a gradual increase during pregnancy stages. According to existing guidelines for LA testing, it is necessary to standardize the testing data of the normal population, by using the 97.5% or 99% percentile as the cutoff for positive judgment [11,13]. However, due to the particularity of the population, the standardization scheme based on the normal non-pregnant population may not be applicable to the pregnant population. For pregnant women, it is necessary to establish a standardization scheme and judgment criteria for lupus anticoagulant that match the gestational weeks.

Why LA1 levels remain stable in the normal pregnant population, while LA2 levels gradually decrease with the progression of pregnancy, is worth exploring. Compared to LA1 reagent, the characteristic of LA2 reagent is the excessive addition of phospholipids. In comparison to the normal population, plasma of pregnancy seems to exhibit a special high reactivity to this increased phospholipid component *in vitro*, and this reactivity is likely to intensify as the pregnancy progresses. We all know that during the development of pregnancy, the activity of coagulation factors such as FX gradually increases [14]. This increase seems not sufficient to affect the levels of activated partial thromboplastin time (APTT) or LA1. However, with the increase in phospholipid concentration, which is an important basement for the activation of coagulation factors, the increased phospholipids may amplify the effect of elevated coagulation factor levels, ultimately leading to a significant decrease in LA2. This could be a possible explanation for the special phospholipid hyperreactivity during

Table 1. Comparison of three groups of 97.5 percent quantiles.

Detection value	First trimester	Second trimester	Third trimester
97.5th of LA1			
Pregnancy	37.46	37.30	37.70
Control	37.58	37.58	37.58
p	0.8214	0.1118	0.4736
97.5th of LA2			
Pregnancy	33.30	33.10	32.80
Control	34.60	34.60	34.60
p	< 0.001	< 0.001	< 0.001
LA1/LA2			
Pregnancy	1.23	1.27	1.30
Control	1.17	1.17	1.17
p	< 0.001	< 0.001	< 0.001

Table 2. Association of LAC detection with pregnancy outcomes.

Variable	No. of patients	Outcome		p-value	Sensitivity	Specificity	Youden's index
		normal	abnormal				
LA1							
> 38.1	53 (5.9%)	43 (4.7%)	10 (1.1%)	0.000	0.21	0.95	0.16
≤ 38.1	851 (94.1%)	814 (90.0%)	37 (4.0%)				
LA1/LA2							
> 1.23	51 (5.6%)	36 (4.0%)	15 (1.7%)	0.000	0.32	0.96	0.28
≤ 1.23	853 (94.4%)	821 (90.8%)	32 (3.5%)				
LA1 > 38.1 and LA1/LA2 > 1.23							
Detected	30 (3.3%)	22 (2.4%)	8 (0.9%)	0.000	0.17	0.97	0.14
Not detected	874 (96.7%)	835 (92.4%)	39 (4.3%)				
LA1/LA2							
> 1.20	85 (9.4%)	61 (6.7%)	24 (2.7%)	0.000	0.5	0.93	0.43
≤ 1.20	819 (90.6%)	796 (88.1%)	23 (2.6%)				
LA1 > 38.1 and LA1/LA2 > 1.20							
Detected	35 (3.9%)	26 (2.9%)	9 (1.0%)	0.000	0.19	0.97	0.16
Not detected	869 (96.1%)	831 (91.9%)	38 (4.2%)				
LA1/LA2							
> 1.17	150 (16.6%)	122 (13.5%)	28 (3.0%)	0.000	0.60	0.86	0.46
≤ 1.17	754 (83.4%)	735 (81.3%)	19 (2.1%)				
LA1 > 38.1 and LA1/LA2 > 1.17							
Detected	42 (4.6%)	33 (3.7%)	9 (1.0%)	0.000	0.19	0.96	0.15
Not detected	862 (95.4%)	824 (91.2%)	38 (4.2%)				

pregnancy. Of course, it is also possible that other phospholipid-related mechanisms lead to changes in LA2. However, the existence of this phenomenon of high reactivity to phospholipids in the pregnant population may provide us with new perspectives on pregnancy-related hypercoagulability. Due to the existence of this

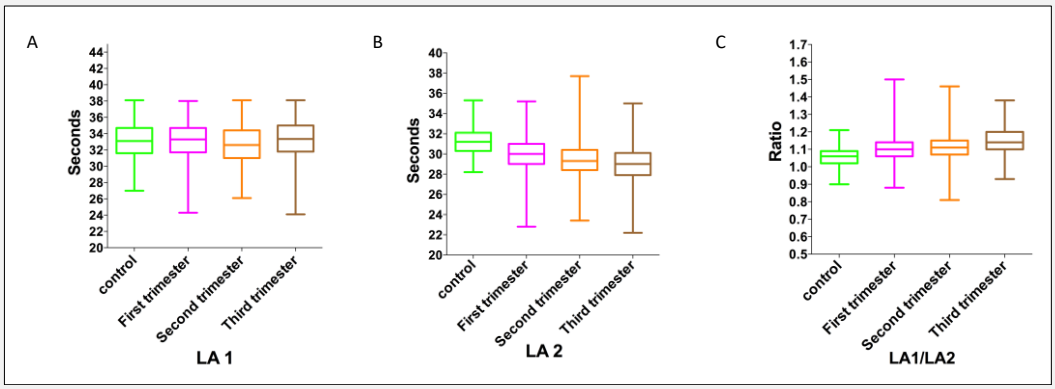

Figure 1. LA in each trimester.

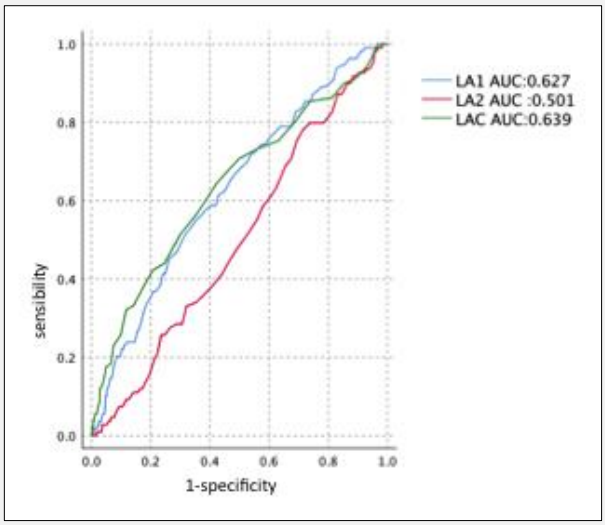

Figure 2. ROC analysis of LA data.

phenomenon, it will also compel us to design new solutions for lupus anticoagulant testing in the pregnant population. Apart from the aforementioned establishment of standardization schemes and judgment criteria matching gestational weeks, we may also plan to adopt approaches that bypass phospholipids to address the specificity of the pregnant population. For example, maintaining LA1 unchanged and using phospholipid-independent coagulation reactions as diagnostic experiments, such as Ecarin time (ECT) [15], could be effective solutions. These are also directions for our future research.

Given the impact of different gestational stages on LA testing, and also to minimize the influence of anticoagulant drugs such as low-molecular-weight heparin [16], and to obtain a sufficient number of research subjects, we separately divided the early pregnancy population for a study on the correlation between lupus anticoagulant and pregnancy outcomes. It was also demonstrated that the levels of LA testing during early pregnancy are related to pregnancy outcomes. In populations experiencing thrombotic events and adverse pregnancy outcomes, the levels of LA1 and LA1/LA2 are higher than those in the normal population, and LA1/LA2 may be the better indicator related to clinical outcomes, similar to findings in other non-pregnant studies [17,18]. Based

on this, the study also verified cutoff values for lupus anticoagulant testing LA1/LA2 for the early pregnancy population using statistical methods, and it seems that the LA1/LA2 > 1.17 still is a better choice with a higher Youden's index. However, considering the persistent changes in the values of LA1/LA2 during different stages of pregnancy and the influence of inter-batch differences in LA-related reagents on the thresholds, adjusting LA1/LA2 thresholds when changing batches is a problem that needs to be addressed. In situations where resources are limited and establishing a cutoff value for the local pregnant population is not feasible, prioritizing screening using the LA1 standard instead of the LA1/LA2 ratio for non-pregnant adults may be a more feasible approach. Further validation is needed for the population at each stage of pregnancy.

CONCLUSION

The significant physiological changes during pregnancy will markedly affect the results of dRVVT, rendering the standards for non-pregnant adults inappropriate for the pregnant population. Nevertheless, the levels of LA remain an independent prognostic factor for adverse pregnancy outcomes. Therefore, establishing a diagnostic system for LA suitable for the pregnant population is crucial.

Source of Funds:
2024YFFK0269 A Modified Laboratory Diagnostic Protocol for Lupus Anticoagulant Testing in Pregnant Populations - Based on the Ecarin Time (Ecarintime) Assay Using Saw-scaled Viper Venom.

Declaration of Interest:
The authors declared no potential conflicts of interest to the research, authorship, and/or publication of this paper.

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