

Utility of phospholipid-sensitive and insensitive APTT pairs together with dRVVT in the diagnosis of lupus anticoagulant

Utilidad de pares de APTT sensible e insensible a fosfolípidos junto al dRVVT en el diagnóstico de anticoagulante lúpico

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Abstract

Introduction and objective: Laboratory diagnosis of antiphospholipid syndrome (APS) requires the determination of lupus anticoagulant (LA) activity by coagulation assays and antiphospholipid antibodies by solid-phase assays. Despite the years that have passed, the diagnosis of LA is still complex and requires well-established coagulation methods, properly obtained cut-off points and professional judgment for the interpretation of the results. **Method:** Two types of specific assays for LA based on different principles: dRVVT (dilute Russell's viper venom time) and sensitive APTT are recommended for the detection of LA. This is complemented by mixing assays with normal plasma and confirmatory assays. One of the interpretations is based on the calculation of the detection assay/confirmatory assay ratio. In an attempt to resemble the dRVVT detection/confirmatory assay, various combinations have recently been proposed using APTT with low or high sensitivity to phospholipids. In two centres (one with optical coagulometer and another with mechanical detection) we used PTT-LA as sensitive APTT and Pathromtin-SL and CK-Prest as insensitive APTTs. **Results:** Based on locally established cut-off points, in centre A 50 patients were positive for AL out of 173 evaluated. In centre B there were 36 positives out of 130 patients. In both centres the combination of paired APTTs (sensitive/insensitive) PTT-LA/Pathromtin or PTT-LA/CK-Prest demonstrated high sensitivity to detect AL. **Conclusions:** This strategy proved to be useful and economical for the diagnosis of AL according to current international standards.

Keywords: Lupus anticoagulant. APTT. Antiphospholipid syndrome.

Resumen

Introducción y objetivo: El diagnóstico de laboratorio del síndrome antifosfolípido (SAF) requiere la determinación de la actividad de anticoagulante lúpico (AL) por ensayos de coagulación y de anticuerpos antifosfolípidos por ensayos en fase sólida. A pesar de los años transcurridos el diagnóstico del AL siempre resulta complejo y requiere métodos de coagulación bien establecidos, puntos de corte adecuadamente obtenidos y criterio profesional para la interpretación de los resultados. **Método:** Dos tipos de ensayos específicos para AL basados en principios diferentes: dRVVT (tiempo de veneno de víbora Russell diluido) y APTT sensible son los recomendados para la detección del AL. Se complementa con ensayos de mezcla con plasma normal y con ensayos confirmatorios. Una de las interpretaciones se basa en el cálculo de la razón del ensayo de detección/ensayo de confirmación. Intentando semejarse al ensayo dRVVT detección/confirmatorio se han propuesto

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recientemente diversas combinaciones usando APTT con baja o alta sensibilidad a los fosfolípidos. En dos centros (uno con coagulómetro óptico y el otro con detección mecánica) utilizamos PTT-LA como APTT sensible y como APTT insensible al Pathromtin-SL y CK-Prest. **Resultados:** Basados en puntos de corte establecidos localmente, en el centro A se hallaron 50 pacientes positivos para AL de 173 evaluados. En el centro B hubo 36 positivos de 130 pacientes. En ambos centros la combinación de APTT apareados (sensible/insensible) PTT-LA/Pathromtin o PTT-LA/CK-Prest demostraron alta sensibilidad para detectar AL. **Conclusiones:** Esta estrategia resultó ser útil y económica para el diagnóstico de AL de acuerdo con las normas internacionales vigentes.

Palabras clave: Anticoagulante lúpico. APTT. Síndrome antifosfolípido.

Introduction

The laboratory diagnosis of antiphospholipid syndrome (APS) requires the determination of lupus anticoagulant (LA) activity through coagulation assays, and anticardiolipin and anti- β 2-glycoprotein I antibodies via solid-phase assays¹. LA detection is based on phospholipid (PL)-dependent coagulation assays. Despite advancements over the years, diagnosis remains complex, requiring well-established coagulation methods, accurately derived cut-off values, and professional expertise in result interpretation. Additional challenges arise in evaluating patients on anticoagulant therapy with heparin, vitamin K antagonists, and direct oral anticoagulants (DOACs) targeting factor Xa and antithrombin.

The latest guidelines, published in 2020 by the Scientific Standardization Committee (SSC) of the International Society on Thrombosis and Hemostasis (ISTH)², outline a series of recommendations for laboratories conducting LA testing. These guidelines recommend a systematic profile of baseline assays (prothrombin time, standard APTT, and thrombin time) to assess the patient's baseline status. Additionally, two specific types of LA assays based on different principles are recommended: dRVVT (dilute Russell's viper venom time) and a sensitive APTT with low PL concentration. APTT should preferably use silica as the activator since, while ellagic acid shows some sensitivity, it is less sensitive than silica. Detection assays are considered prolonged (one or both) when the result exceeds the locally established cut-off in each laboratory after assessing normal plasma samples. The guidelines recommend reporting the patient/normal ratio. If the detection assay is prolonged, mixing studies with normal plasma and confirmatory assays are performed for the affected test². One interpretation approach is based on calculating the (normalized or unnormalized) ratio of the detection assay/confirmation assay. This result is considered positive if the ratio exceeds the 99th percentile of the normal distribution.

Cut-off points should be established by evaluating normal plasmas over several days, with a recommendation

of using 100–120 plasmas from apparently normal individuals³. Reference ranges for normality should be established between the 2.5th and 97.5th percentiles, while the cut-off for positivity is suggested to be the 99th percentile (P_{99}) of the distribution; thus, a test is positive when it exceeds the locally established cut-off. This applies to individual assays as well as to the ratios used (e.g., screen/confirm, patient+normal/normal, etc.). Outliers (values at the extremes of the normal distribution) should be excluded before establishing normal cut-offs². The Reed method is recommended for this purpose, primarily involving the exclusion of the longest result (in the case of LA assays).

CLSI (Clinical and Laboratory Standards Institute) guidelines H60 recommend conducting mixing and confirmatory assays simultaneously, and in the absence of another coagulopathy, if the confirmatory test is above the cut-off, LA is considered positive even if the sample corrects with a normal pool⁴. In this guideline, assay cut-offs are set at the 97.5th percentile ($P_{97.5}$).

The recommended methods for LA detection or screening are limited to two types: dRVVT and APTT with low PL concentration (Table 1). This combination provides a high probability of detecting LA if present. Another widely used commercial assay globally is the silica clotting time (SCT), which is essentially an APTT with silica as the activator. A recently validated assay for LA assessment utilizes two snake venoms that directly activate prothrombin⁵: the Taipan venom time, which is PL-sensitive and unaffected in patients on vitamin K antagonists or anti-FXa DOACs, and the Ecarin time, which is PL-insensitive and serves as the confirmatory test (Table 1).

In certain regions where SCT has not been approved by regulatory agencies for laboratory use, various combinations involving APTT with both high and low PL sensitivity have been proposed recently^{6–9}. In recent years, paired APTTs (sensitive/insensitive) have become increasingly popular for LA diagnosis due to their low cost.

Method

Population

Consecutive patients admitted to services for AL evaluation and who signed informed consent. In some cases, this was within the context of thrombophilia studies due to a history of venous and/or arterial thrombosis (center A: $n = 65$, center B: $n = 71$); in other cases, it was due to the presence of autoimmune diseases (center A: $n = 49$, center B: $n = 20$), a history of obstetric morbidity (center A: $n = 41$, center B: $n = 20$), or simply because they had prolonged APTT results in routine tests (center A: $n = 18$, center B: $n = 19$). Patients diagnosed with acquired hemophilia and those with factor deficiencies:

- Center A: 100 normal plasmas (blood donors) collected on different days were used to establish the reference range for each APTT reagent used, and the cutoff was set at the 99th percentile for the APTT sensitive/APTT insensitive ratios. The APTT reagents tested were PTT-LA (Diagnostica Stago) as APTT sensitive, and Pathromtin-SL (Siemens) and CK-Prest (Diagnostica Stago) as APTT insensitive. The PTT-LA activator is silica, the Pathromtin activator is silicon dioxide, and the CK-Prest activator is kaolin. Additionally, the cutoff (P_{99}) was established for the dRVVT screen (LA1, Siemens), dRVVT confirm (LA2, Siemens), and the dRVVT screen/confirm ratio.

These assays were evaluated simultaneously (APTT and dRVVT) in 173 patients referred for AL studies, using the dRVVT screen and confirm assay, and PTT-LA for initial diagnosis. All tests were performed on an automated BCSXP coagulation analyzer (Siemens) using an optical detection method.

- Center B: a total of 120 normal plasmas were used to establish the reference range for each APTT reagent, and the cutoff was evaluated at the 97.5th and 99th percentiles for the APTT sensitive/APTT insensitive ratios. The APTT reagents tested were PTT-LA as APTT sensitive and Pathromtin-SL and CK-Prest as APTT insensitive.

A total of 130 patients were referred for AL evaluation, and for the initial diagnosis, the dRVVT screen and confirm assay (Diagnostica Stago) and the SCT screen and confirm (Werfen) were used. In cases in which the SCT tested positive ($n = 36$), all 3 APTT assays were tested on the same day or within 4 hours of sample collection. All coagulation assays were performed on an automated STA-Compact Max 3

Table 1. Tests and combinations used in lupus anticoagulant diagnosis

	LA-sensitive test	LA-non-sensitive test
dRVVT (paired)	dRVVT screen (Werfen-Stago-Siemens-TCoag)	dRVVT confirm
APTT (paired)	SCT screen (Werfen)	SCT confirm
	PTT-LA (Stago)	Staclot LA (kit)
	PTT-LA (Stago)	CK-Prest
	PTT-LA (Stago)	Pathromtin-SL (Siemens)
	Cephen LS (Hyphen)	Cephen
	Actin FSL (Siemens)	Actin FS (Siemens)
T/E	Taipan test	Ecarin test

APTT: activated partial thromboplastin time; dRVVT: dilute Russell's viper venom time; LA: lupus anticoagulant; PTT-LA: commercial name for APTT; SCT: silica clotting time; T/E: Taipan/Ecarin.

(Diagnostica Stago) using a mechanical detection method.

None of the patients in either center were on anti-vitamin K, DOAC, or heparin therapy.

Both centers also evaluated the results of different APTT ratios in samples from patients with acquired hemophilia and in in vitro factor-deficient plasmas (factors VIII, IX, and XI).

Factor activity was determined using a 1-stage clotting method at three dilutions with factor-deficient Stago plasmas.

Factor-deficient samples were prepared by mixing different volumes of factor-deficient plasma with a normal plasma pool. The activity of each factor was determined by the clotting method mentioned above.

Statistics

Percentiles of the normal distribution were obtained using traditional statistical methods, and concordance between assays was assessed using the kappa coefficient.

Results

Center A

The normal cutoff (P_{99}) for the PTT-LA/CK-Prest ratio was 1.30, and for the PTT-LA/Pathromtin ratio, 1.31.

For the dRVVT screen/confirm ratio, the cutoff was set at 1.35 on the BCS-XP device.

A total of 50 (28.9%) out of the 173 patients studied tested AL positive. There were 21 cases (42%) with both ratios (dRVVT and APTT) positive, 16 (32%) with only the dRVVT ratio positive, and 13 (26%) with only the APTT PTT-LA/Pathromtin ratio positive (Table 2). Using the PTT-LA/CK-Prest combination, results were similar: 22 cases (44%) positive with both assays, 17 (34%) positive with only dRVVT, and 11 (22%) with only the PTT-LA/CK-Prest ratio positive.

The concordance level between the established method (dRVVT and PTT-LA) and the APTT sensitive/insensitive ratio, as measured by kappa correlation coefficients, was PTT-LA/Pathromtin 0.74 (95% confidence interval [CI], 0.96-0.49) and PTT-LA/CK-Prest 0.71 (95%CI, 0.93-0.46).

Overall, the dRVVT screen/confirm ratio tested positive in 74-78%, the PTT-LA/Pathromtin ratio in 68%, and the PTT-LA/CK-Prest ratio in 66% of AL-positive patients. Among AL-positive samples, the PTT-LA/Pathromtin ratio ranged from 1.47-4.05, the PTT-LA/CK-Prest ratio from 1.41-3.14, and the dRVVT screen/confirm ratio from 1.41-2.89.

Center B

The normal cutoff ($P_{97.5}$ and P_{99}) for the PTT-LA/Pathromtin ratio was 1.39 and 1.59, respectively. For the PTT-LA/CK-Prest ratio, the values were 1.50 and 1.62. For the dRVVT screen/confirm ratio, the cutoff was set at 1.20 ($P_{97.5}$) on the Compact Max device.

In both tests, 69 patients tested negative; 61 patients tested positive for AL: 27 positive in both SCT and dRVVT, 25 positive only in dRVVT, and 9 positive only in SCT.

The kappa correlation coefficient for concordance between the established method (SCT) and the APTT sensitive/insensitive ratio was: PTT-LA/Pathromtin 0.60 (95%CI, 0.81-0.38) and PTT-LA/CK-Prest 0.63 (95%CI, 0.85-0.42) at P_{99} , and PTT-LA/Pathromtin 0.77 (95%CI, 1.00-0.54) and PTT-LA/CK-Prest 0.75 (95%CI, 0.98-0.57) at $P_{97.5}$.

Using the combination of dRVVT and APTT ratios (including both tested combinations) with $P_{97.5}$ (for better kappa values), there were 27 cases (75%) with both ratios (dRVVT and APTT) positive and 8 (22%) with only the APTT ratio positive (Table 2).

Among AL-positive samples, the PTT-LA/Pathromtin ratio ranged from 1.42-2.92, the PTT-LA/CK-Prest ratio

Table 2. Distribution of results in the two evaluated centers. The table shows the number of patients with positive and negative results for the dRVVT ratio and/or APTT ratio. In center A, PTT-LA/Pathromtin was used, and in center B, two combinations of PTT-LA and P97.5 were applied

Center A	dRVVT ratio (+)	dRVVT ratio (-)	
APTT ratio (+)	21	13	34
APTT ratio (-)	16	123	139
Total	37	136	173
Center B	dRVVT ratio (+)	dRVVT ratio (-)	
APTT ratio (+)	27	8	35
APTT ratio (-)	25	70	95
Total	52	78	130

APTT: activated partial thromboplastin time; dRVVT: dilute Russell's viper venom time.

from 1.52-2.43, and the dRVVT screen/confirm ratio from 1.24-2.63.

In both centers, the APTT sensitive/insensitive ratios were negative in samples from patients with acquired hemophilia, congenital factor deficiencies, or in vitro factor-deficient samples with any APTT reagent combination tested (Tables 3 and 4).

Discussion

A crucial aspect of laboratory diagnosis for LA is the use of locally established cutoff points with no fewer than 100 normal plasma samples. These cutoffs can be defined at the 97.5th or 99th percentiles according to suggestions from international guidelines (CLSI or ISTH)^{2,4}. In this study, the cutoff points obtained in both centers were significantly different despite using the same reagent combinations (PTT-LA/Pathromtin: center A, 1.31 vs. center B, 1.59; PTT-LA/CK-Prest center A, 1.30 vs. center B, 1.62). Cutoff points cannot be exchanged across different laboratories or across different coagulometers within the same laboratory because the coagulometer detection system (optical or mechanical) significantly influences these definitions.

One of the earliest publications recommending a simple detection and confirmation assay is based on a 1997 report⁶. They used a rabbit brain cephalin and kaolin activator reagent as an APTT sensitive to LA, and Actin FS, containing plant-origin phospholipids (FL), as an LA-insensitive APTT. The ratio of the sensitive to insensitive APTT proved useful for detecting LA,

Table 3. Results of APTT sensitive/insensitive ratios in patients with inhibitors or congenital deficiencies (center A)

	PTT-LA/ Pathromtin ratio	PTT-LA/CK-Prest ratio
Anti-FVIII inhibitor 1	1.11	1.08
Anti-FVIII inhibitor 2	0.96	1.02
Anti-FVIII inhibitor 3	1.20	1.19
Anti-FIX inhibitor	0.96	1.05
FVIII 21%	1.03	1.08
FVIII 5%	1.10	1.15
FIX 29%	1.09	1.11
FIX 10%	1.12	1.16
FXI 11%	0.99	0.98
FXI 1%	1.10	1.12
FXII 25%	0.97	1.02
FXII 1%	1.09	1.05

Table 4. Results of APTT sensitive/insensitive ratios in patients with acquired deficiencies or deficiencies prepared *in vitro* (center B)

	PTT-LA/Pathromtin ratio	PTT-LA/CK-Prest ratio
Acquired HA 1	0.95	1.11
Acquired HA 2	0.78	1.17
Acquired HA 3	0.94	1.14
Acquired HA 4	0.87	1.16
FVIII 42%	0.99	0.99
FVIII 24%	0.96	0.96
FVIII 13%	0.96	0.96
FIX 37%	1.04	1.03
FIX 19%	1.14	1.11
FIX 7%	1.18	1.15
FXI 42%	1.00	0.98
FXI 24%	0.93	0.96
FXI 15%	0.95	1.02
FXII 40%	0.78	0.86
FXII 20%	0.83	0.91
FXII 10%	0.81	0.99

HA: hemophilia.

yielding normal results in patients on heparin and other hemostatic disorders that prolong the APTT. In 23 patients with LA, 22 had positive sensitive/insensitive APTT ratios (mean 2.08). They generally observed that results with the insensitive APTT were approximately half the time in seconds of those obtained with the sensitive APTT. In patients with factor deficiencies or anti-FVIII inhibitors, the ratios were close to 1.0 since prolongations were proportional across both reagents.

A different study compared the Cephen LS and Cephen APTT reagents⁷. The Cephen LS/Cephen ratio yielded positive results in 33 of 105 samples previously classified as LA-positive, with 31 also showing positive ratios using the dRVVT test (detection and confirmation). However, this reagent combination demonstrated lower sensitivity than previously obtained with the dRVVT ratio and PTT-LA as the sensitive APTT.

In 2016, a 4-year study in a pediatric center identified 161 patients with prolonged APTT, confirmed in a second sample⁸. They routinely used Platelin LS for its versatility in detecting both factor deficiencies and LA. As a confirmatory APTT for LA, they used Actin FS, an LA-insensitive reagent. The presence of LA was demonstrated in 64/88 (73%). Platelin LS (with silica) was prolonged in this patient group, and only 4 of the 64 patients showed prolongation with Actin FS. The established cutoff was 1.29, and the Platelin LS/Actin FS ratio demonstrated statistical significance with LA ($p < 0.05$). Using this pair of APTTs, sensitivity for LA detection increased to 82-86%.

In Switzerland, a study aimed to determine whether using an LA-sensitive/insensitive APTT ratio could be a useful tool in LA testing⁹. They included samples from patients with factor deficiencies, anti-FVIII or anti-FIX inhibitors, and those on heparin or vitamin K antagonists. The primary group evaluated LA in 1553 patients over a 3-year period. They used Pathromtin-SL and PTT-LA, calculating the PTT-LA/Pathromtin-SL ratio in addition to dRVVT testing. The sensitivity of Pathromtin-SL for LA was 59%, PTT-LA was 82.1%, and the PTT-LA/Pathromtin-SL ratio was 92.3%.

Pathromtin-SL contains silica as an activator and plant-origin FL, while PTT-LA contains silica and cephalin. LA was identified in 78 out of the 1553 patients (5%), with the PTT-LA/Pathromtin-SL ratio cutoff established at 1.40. They demonstrated that calculating the ratio between clotting times obtained with the sensitive (PTT-LA) and less sensitive (Pathromtin) APTTs improved the performance of the PTT-LA and represented a simple, more cost-effective, and sensitive strategy based on the APTT assay.

The choice of CK-Prest was considered because it is a kaolin-based reagent with a high concentration of phosphatidylethanolamine. Thus, center A chose to use CK-Prest as the confirmatory APTT reagent for PTT-LA, which was our sensitive reagent for LA. The CK-Prest reagent has the disadvantage of requiring constant agitation, which is not efficiently achieved on our Siemens automated equipment, so manual shaking of the reagent was required for each use.

In both centers, the PTT-LA/Pathromtin combination yielded highly significant results for detecting LA and is now the combination used in our routine testing, consistent with findings from a different study⁹.

Conclusions

The study and interpretation of LA still face certain challenges today due to the diversity of commercially available reagents with different sensitivities to LA, the lack of adherence by many laboratories to ISTH/CLSI guidelines, and the absence of local validation of cutoff points. These factors hinder the correct interpretation of LA presence or absence in test samples. Many laboratories only perform the dRVVT test, so the inclusion of paired APTTs (sensitive/insensitive) is very useful, as it complements the recommendations in international guidelines and offers a more economical option within the LA study panel.

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Conflicts of interest

None declared.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article. Furthermore, they have acknowledged and followed the recommendations as per the SAGER guidelines depending on the type and nature of the study.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Use of artificial intelligence for generating text. The authors declare that they have indeed used generative artificial intelligence, specifically ChatGPT-4, for the efficiency analysis of the 3 treatments, through the analysis of the results reported in the different studies.

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