Determination of cut-off values in healthy donors for lupus anticoagulant detection protocol in the Laboratorio Médico Echavarría, according to the international guidelines

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ABSTRACT

Background: The main types of antiphospholipid antibodies are: lupus anticoagulant, anticardiolipin antibodies and anti-β2 glycoprotein 1. Lupus anticoagulant has been associated with clinical findings such as thrombosis, consecutive fetal losses, and thrombocytopenia. Variables can affect the interpretation of the results. **Objective:** To determine the cut-off values in healthy donors for the group of tests required in detecting lupus anticoagulant, in order to improve the specificity of the test. **Methods:** Thirty-six healthy donors, from whom the cut-off values were calculated. The parameters measured in the study were: baseline activated partial thromboplastin time, aPTT 1:1 mix with normal plasma pool, and screen and confirmatory dilute Rusell viper venom time (dRVVTs/ dRVVTc). **Results:** The cut-off values obtained from percentage correction, Index of Circulating Anticoagulant, dRVVTs ratio, dRVVTc ratio, and final ratio for Laboratorio Médico Echavarría were: 27.6%, 12.18, 1.19, 1.11 and 1.24, respectively. **Conclusions:** Regarding the means of dRVVTs and dRVVTc ratios, the results were very similar to those obtained in other studies. The cut-off value obtained for the ICA and the correction rate were 12.2 and 27.6%, respectively, while the cut-off value reported in the literature is 15 and 10% for both tests, respectively.

RESUMEN

Antecedentes: *Los principales tipos de anticuerpos antifosfolípidos son anticoagulante lúpico, anticuerpos anticardiolipina y anti-β2 glucoproteína 1. El anticoagulante lúpico ha sido asociado con hallazgos clínicos como trombosis, pérdidas fetales consecutivas y trombocitopenia. Algunas variables pueden afectar la interpretación de los resultados.* ***Objetivo:*** *Determinar los valores de corte de donantes sanos para el grupo de pruebas necesarias para detectar anticoagulante lúpico con el fin de mejorar la especificidad de la prueba.* ***Métodos:*** *Treinta y seis donantes sanos, de quienes se calcularon los valores de corte. Los parámetros medidos en el estudio fueron el tiempo de tromboplastina parcial activada inicial, mezcla de aPTT 1:1 con plasma normal y el tiempo del veneno de víbora diluido de Rusell (dRVVTs/dRVVTc) presuntivo y confirmatorio.* ***Resultados:*** *Los valores de corte obtenidos de la corrección de porcentajes, índice de circulación de anticoagulante, relación dRVVTs, relación dRVVTc y relación final del Laboratorio Médico Echavarría fueron: 27.6, 12.18, 1.19, 1.11 y 1.24%, respectivamente.* ***Conclusiones:*** *En cuanto a los promedios de la relación dRVVTs y relación dRVVTc, los resultados fueron muy similares a los obtenidos en otros estudios de lupus anticoagulante. Los valores de corte obtenidos para la ACI y la tasa de corrección fueron 12.2 y 27.6%, respectivamente, mientras que el valor de corte descrito en la literatura es de 15 y 10% para ambas pruebas, respectivamente.*

INTRODUCTION

Antiphospholipid antibodies are a heterogeneous family of auto- and alloantibodies. Detection is performed through coagulation or immunological tests. Their presence is not always pathological. Associated with thrombotic events, they define a specific clinical entity: the Antiphospholipid Syndrome (APS), which may be primary or secondary to an autoimmune disease.[1] Its existence has been known since the work of Moore in 1952, which showed the occurrence of false-positive tests for syphilis serology. Indeed, false-positive occurred in the VDRL using phospholipids, while specific treponemal gave negative reactions (TPHA, Nelson).[2] The main types of antiphospholipid antibodies are lupus anticoagulant (LA), anti-

cardiolipin antibodies (aCA), and anti-β2 glycoprotein I (anti-β2GP1). LA is a heterogeneous group of antibodies directed against negatively charged phospholipids or against complexes formed between phospholipids and proteins (β2 GP1 or coagulation factors such as prothrombin). These anticoagulants interfere with coagulation tests in which phospholipids participate, such as the activated partial thromboplastin time (aPTT) and dilute Russell viper venom time (dRVVT), among others.[3]

LA has been documented in various medical articles, in association with clinical findings of thrombosis, recurrent fetal losses, and thrombocytopenia.[4]

There are variables that can affect the interpretation of the results for the diagnosis of LA, including the incorrect selection of patients according to clinical features, the partial ordering of laboratory tests without prior suspension of oral anticoagulation therapy, the high variability regarding the sensitivity and specificity of tests, such as the content and type of phospholipid reagents, activators, preparation of Normal Plasma Pool (NPP), expression of the results and the cut-off values.[5]

The ideal procedures for LA assay are those sensitive enough to detect weak LA and those specific enough to avoid incorrect conclusions.[6]

The aim of this study was to determine the cut-off values in a population of healthy donors for the group of tests required to detect LA in Laboratorio Médico Echavarría (LME), in order to improve the specificity of the test[5] and provide a detailed protocol that serves as a reference to those laboratories wishing to implement their own cut-off values for this test, as recommended by international guidelines.

METHODS

A prospective study was conducted, determining the cut-off values for LA test in a population of healthy donors, according to the Scientific Standardization Committee (SSC) for this test.[5]

Exclusion criteria: Clinical and demographic factors that could affect the results in the measurement of the parameters for determining the cut-off values, according to the C28 -A2 CLSI guide,[7] such as: living outside the metropolitan area, subjects older than 50 years of age, being under special medical treatment; having viral, bacterial, parasitic, malignant and/or hematologic diseases; being under oral anticoagulation therapy; having personal or family history of autoimmune or thrombotic diseases, thrombocytopenia; personal history of venous and arterial thrombosis, having skin ulcers on the legs, consuming any actual medications, and –in the case of women– past

medical history of obstetric complications, early and/or late fetal losses.

Selection of reference population

The study included healthy male and female donors, which did not submit the exclusion criteria specified by a survey of risk; those who met the inclusion criteria were required to fill out an informed consent form, which unveiled all about the study.

Procedure for sampling

1. **Sample collection:** Sample taking was performed in one of the LME service points in the morning. Samples were collected mostly without tourniquet use to prevent microscopic hemolysis, release of tissue factor and platelet aggregation. For healthy donors, two tubes of 4 mL sodium citrate anticoagulant to 3.2% were taken in a 9:1 ratio, through the vacuum tube system in the ulnar region of the arm. Following the protocol for obtaining blood samples from LME, which is adjusted according to the CLSI recommendations for this type of test.[8]

2. **Sample analysis:** Each sample obtained was centrifuged at 2,000 g for 15 minutes at room temperature; the plasma was removed with a plastic pipette and centrifuged again at 2,500 g for 10 minutes. After separating plasma, we proceeded to make the NPP from samples of healthy donors. Platelet count, aPTT and percentage of activity of factor VIII were determined in NPP.

   The analyses required for LA testing were performed with ACL TOP® 300, equipment of Instrumentation Laboratory®, with reagents: **HemosIL® dRVVT Screen (dRVVTs)** -0020301500/**dRVVT Confirm (dRVVTc)** - 0020301600, **aPTT SP** - 0020006300, and **Factor VIII deficient Plasma** - 0020011800.

   For quality control, material of the same trading house was used in normal, low and high concentrations (Lots: N1021609, N0228997, N1121957, respectively) for aPTT test. For Factor VIII, normal control was used alone (Lot N1021609). For dRVVTs and dRVVTc tests, they were controlled with positive (0020012500) and **negative** (0020012600) controls for LA (Lots N0329326 and N0329327, respectively).

3. **Measured parameters:** Baseline **aPTT**, **aPTT 1:1 mix with NPP**, **dRVVTs and dRVVTc**

   For each dRVVTs and dRVVTc reagent, a new normal range was determined according to CLSI C28- A2 document. The reference range was expressed as

normal mean ± 2 SD. The mean was used as constant denominator in the calculation of ratios.

3.1 dRVVTs

   3.1.1 For each healthy donor, the result in seconds was obtained and then this was divided by the average of dRVVTs of all donors.

3.2 dRVVTc

   3.2.1 For each healthy donor, the result in seconds was obtained and then this was divided by the average of dRVVTc of all donors.

3.3 Normalized dRVVT ratio: DRVVTs ratio was divided by dRVVTc ratio.

3.4 Other calculations: Rosner Index (RI) or Index of Circulating Anticoagulant (ICA) and the percentage of correction were calculated from the following formulas:

ACI or RI = [CT (aPTT) of mix 1:1 - CT (aPTT) NPP/CT (aPTT) patient]* 100

Percentage of correction: (dRVVt screen – dRVVt confirm/ dRVVT screen)* 100

CT = coagulation time.

4. Interpretation: The final result was expressed as normalized dRVVT ratio. The percentage of correction for dRVVT was applied, which was previously suggested for Kaolin clotting time.[9] This percentage takes into account the degree of relative correction on normal initial values. This calculation method is recommended by the British Society for Haematology (BCSH) and the SSC LA Guidelines.[5,10]

An Excel 2010 matrix was constructed for recording and calculating the results of the mean, standard deviation, normal range ± 2 SD and cut-off values. The cut-off values were obtained from the 99th percentile (99% Confidence Interval) for screening, study of mixtures, and confirmatory tests, following the SSC recommendations for LA detection protocol.

RESULTS

Voluntarily, 46 adults signed up; six of them were excluded (medication use, family history of thrombosis, autoimmune diseases and fetal losses). Four healthy volunteers had altered results on tests, which did not allow their participation in the study.

In total, 36 healthy adults participated: 8 (22.2%) male and 28 (77.7%) female, with average ages of 30.3 and 32.8 years, respectively.

Platelet count, aPTT, dRVVTs, dRVVTc, and factor VIII tests were performed in NPP, with results of: 7,000 platelets/uL, 30.6 seconds, 31.8 seconds, 28.5 seconds and 85% activity, respectively.

The cut-off values obtained from the 99th percentile to the screen and confirmatory tests of LA detection protocol are described in *table I*.

The cut-off values suggested by the trading house and some references were compared with those obtained in the LME, which are described in *table II*.

The results of screening and confirmatory tests for LA obtained directly from the NPP and obtained as a statistical average of the healthy donors included in the study are described in *table III*.

Table I. Cut-off values for screen and confirmatory test for lupus anticoagulant.

Parameters	Age	aPTT seg	aPTT mix1:1 seg	dRVVt s seg	dRVVt s ratio	dRVVt c seg	dRVVt c ratio	Final ratio	CAI %	% correction /confirmatory test
Mean	32.3	29.1	30.0	32.2	1.00	28.8	1.00	1.00	-0.3	10.0
SD	8.4	2.5	1.8	3.0	0.09	1.5	0.05	0.09	6.0	7.5
Min	23.8	24.4	26.5	26.1	0.80	25.9	0.80	0.82	–	–
Max	40.6	34.2	33.5	38.8	1.20	31.9	1.10	1.18	–	–
2 SD Range	8.4	24.4 - 34.2	26.5 - 33.5	26.1- 38.8	0.80 - 1.20	25.9 - 31.9	0.80 - 1.10	0.80 - 1.20	12.0	27.0
99th Percentile	48.7	34.3	34.0	38.4	1.19	32.1	1.11	1.24	12.2	27.6

ICA= Index of Circulating Anticoagulant.

Table II. Diferences between manufacturer Cut-off values and Laboratorio Médico Echavarría.					
Reference values	% correction/confirmatory test	ICA	dRVVt s ratio	dRVVt c ratio	Final ratio
Ref. 1	ND	>15.0	ND	ND	ND
Manufacturer	ND	ND	1.20	1.20	1.20
Ref. 2	>10.0	ND	1.10	1.10	1.10
LME	27.6	12.2	1.19	1.11	1.24

ND = No data; Ref. 1:[8]; Trading House:[3]; Ref. 2:[10]; ICA = Index of Circulating Anticoagulant.

Table III. Diference in the 99 percentil (cut-off) using directly value of NPP or statistic average.				
Parameter	NPP	Statistic average	Bias (Un)	Bias (%)
Screen seg	31.8	32.23	0.43	1.35
Screen ratio	1.21	1.19	-0.02	-1.65
Confirm seg	28.5	28.83	0.33	1.16
Confirm ratio	1.12	1.11	-0.01	-0.89
Final ratio	1.24	1.24	0.00	0.00

DISCUSSION

According to the guidelines of the International Society of Thrombosis and Hemostasia,[4,11] we recommend using at least 40 healthy donors younger than 50 years old to determine the cut-off values. In our study, after applying the exclusion criteria in line with the C28 A 2 CLSI document, considering the biological and environmental factors that could affect the results of this test, a total population of 36 healthy donors were evaluated, with a mean age of 32 years in the female population and 30 in the male population; agreed to the inclusion of young adults to reference values of the same guideline cited above.

The results of analytical determinations performed on all donors were in line with the parameters described by the SSC and the recommendations of the manufacturer to test LA; nonetheless, only coagulometric Factor VIII was evaluated in the study of factors, considering its importance in the intrinsic coagulation pathway. The results of quality controls were also found within the expected values described by headquarters.

As for the means of the dRVVTs and dRVVTc ratios, the results were very similar to those obtained in a study of four diagnostic centers with three different kits;[12] in the case of the final ratio, LME mean was equal to that reported by headquarters.

The cut-off value obtained for the ACI index and the correction rate was 12.2 and 27.6%, respectively, while the cut-off reported in the literature is 15% and 10% for both tests.[12] Previous studies have shown that performance is influenced by the analyzer used,[5] which shows the need for each institution to calculate its own normal reference ranges.

The calculation of ratios depends on the value of normality used as the denominator in the formula; the difference was observed in the ratios obtained from the NPP and the statistical average obtained from 36 healthy donors.

Detection rates of false positive and false negative LA test remain relatively high, being the first of particular interest due to the prescription of long and unnecessary oral anticoagulation therapy.[4]

Finally, our interest is to facilitate the understanding of the protocol to fix the cut-off local values of this test, in order to avoid incurring in false-positive interpretations.

ACKNOWLEDGEMENTS

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Saldarriaga-Saldarriaga M et al. *Determination of cut-off values in healthy donors for lupus anticoagulant detection protocol*

149

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