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Models for releasing the lupus anticoagulant test

Modelos de liberação do teste anticoagulante lúpico

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ABSTRACT

Introduction: Thrombophilia is a thrombosis susceptibility of genetic, acquired or mixed nature. Among acquired causes, the antiphospholipid syndrome (APS) stands out as an autoimmune disease characterized by antiphospholipid antibodies, thrombotic events or recurrent gestational loss. Laboratory diagnosis is based on the detection of lupus anticoagulant (LAC), anti-β2-glycoprotein 1 and anticardiolipin; however the determination of LAC still demands uniformity. The last guideline published by the Clinical and Laboratory Standards Institute (CLSI) prioritizes the screening and confirmatory steps, to the detriment of the mixing phase. **Objectives:** To compare the forms of releasing the LAC and to adopt an investigation protocol in agreement with the international guidelines. **Methods:** Thirty-six samples with prolonged results in the screening step by the dilute Russell viper venom time (dRVVT) or activated partial thromboplastin time (APTT) were subjected to the mixing steps (1:1) and to the confirmatory steps with high concentrations of phospholipids. **Results:** For APTT, values whose indexes of circulating anticoagulant (ICA) were greater than 15% were considered positive. For dRVVT, the ratio between screening and confirmation was also used. Of the 36 tested samples, 14 showed correction in the mixing step, but only one resulted negative. **Conclusion:** ICA aided in identifying the weak antibodies that were probably diluted in the mixing step. There is no gold standard test for the diagnosis of APS, and LAC detection still requires standardization of technique and interpretation.

Key words: venous thrombosis; habitual abortion; antiphospholipid antibodies; lupus coagulation inhibitor.

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune disease, characterized by the presence of antiphospholipid antibodies (APLAs) associated with thrombotic events or recurrent gestational loss. According to international classification criteria, detection of autoantibodies is necessary, among them, lupus anticoagulant (LAC), anticardiolipin antibodies (ACA), and anti-β2-glycoprotein 1 (anti-β2GP1), associated with a clinical criterion, in which are included one or more reports of confirmed arterial or venous thrombosis in small vessels, tissues or organs and/or gestational complications, characterized with at least one unexplained fetal death of a proved normal fetus, occurring from the 10th gestational week; at least one birth of a morphologically normal newborn before the 34th week due to eclampsia, pre-eclampsia or placental insufficiency; or at least three consecutive spontaneous abortions, with no established cause before the 10th gestational week, excluding maternal anatomical, hormonal changes or parental chromosomal alterations[1, 2].

Laboratory investigation should be carried out preferably before the beginning or at the moment of deciding about suspension of anticoagulant treatment, and the tests currently available are classified into coagulation studies: LAC and immunoenzymatic assays – ACA and β2GP1. APLAs encompass a heterogeneous group of antibodies, therefore their investigation must contemplate the three tests as a group: LAC, ACA, and β2GP1, whenever possible[3, 4]. The concomitance of positivity in the three tests (triple positive) is associated with a greater clinical severity and higher thrombotic risk[5]; however, there is still not a golden standard for APS diagnosis and no standardization among laboratories. LAC investigation has limited specificity because its method is indirect and prolonged clotting times can be resulting from other non-specific inhibitors or the deficiency of factors induced by anticoagulation[2, 6]. The most commonly used tests are activated partial thromboplastin time (APTT) and dilute Russel viper venom time (dRVVT), since international guidelines recommend the use of two tests able to activate coagulation in distinct stages, simultaneously[7]. The reagents available for APTT test vary in relation to activator, composition and concentration of

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phospholipids. Those differences are responsible for heterogeneity of results. Besides, concentrations of factors of intrinsic and common pathways are capable of interfering in this research. dRVVT starts clotting by activating factor X, thus, it proves more specific for detecting LAC than APTT.

According to the guidelines by the International Society on Thrombosis and Haemostasis (ISTH) published in 2009[8], the mixing test should be done immediately after the detection of an altered result in the screening test, thus it remains implicit that the confirmatory test must only be carried out if the mixture is suggestive of the presence of an inhibitor. The British Committee for Standards in Haematology (BCSH), in its publication of 2012[9], indicates that the mixing test improves specificity in LAC investigation, but introduces the concept that the dilution could mask the presence of weak antibodies and generate false-negative results. Therefore, samples that present prolonged times in screening and confirmatory steps, even with normal mixing times, must be considered positive. Later, in 2014, the Clinical and Laboratory Standards Institute (CLSI) publishes its recommendations for LAC investigation, prioritizing screening and confirmatory phases in detriment to mixing phase, restricting the latter for cases in which the previous ones were inconclusive[10]. Antibody heterogeneity, variability of reagents and analyzers, the different ways of interpreting results, and the absence of a gold standard make APS laboratory diagnosis difficult.

OBJECTIVES

Verify the best way of releasing LAC results, comparing the current model (screening-mixing-confirmatory) with the new model proposed by the CLSI (screening-confirmatory-mixing, if necessary), correlating it with ACA and anti-β2GP1 tests and with the clinical picture of patients; evaluate interferents able to generate false-positive and false-negative results in the LAC investigation, as well as adopt a protocol of LAC investigation able to meet international norms and that is economically and technically viable for our institution.

METHODS

The present study was carried out in the Central Laboratory of Hospital das Clínicas da Universidade de São Paulo (HCUSP). Thirty-six positive samples were randomly selected from routine in the screening step of LAC investigation. All eligible cases to this study were referred to LAC investigation by the assistant physician and no additional test was carried out besides those ordered.

Pre-analytical variables

Tubes containing 3.2% sodium citrate anticoagulant were used, with the ratio of nine parts of blood to one part of anticoagulant. Screening for possible interferents in sample turbidity (hemolysis, jaundice, and lipemia) was performed as laboratory routine, and samples in those conditions were excluded from the study. Double centrifugation was standardized to obtain platelet-poor plasma, since the membrane of these cells is source of phospholipids and their presence can cause false-positive results in the LAC assay.

Preparation of pooled normal samples

Twenty healthy adults were selected, with normal values of prothrombin time (PT) and APTT. Those younger than 18 years and older than 65 were excluded. Samples were subjected to double centrifugation at 4,000 rpm for 20 minutes for obtainment of platelet-poor plasma. Equal proportions of each plasma aliquot from these donors were transferred to a single tube, homogenized, and a pooled sample was obtained.

Sample storage

Samples that could not be analyzed in the same day were stored in aliquots and frozen at -80°C up to the moment of analysis. Thawing was done in a water bath at 37°C and after the test, they were discharged.

Steps of lupus anticoagulante testing

Before beginning the test, the following were conducted: platelet count after centrifugation, ensuring that plasma had a number of platelets below 10,000/mm^3; and PT and thrombin time (TT), so that samples contaminated with anticoagulants could be identified, and the interpretation of results took into consideration this possible interference. LAC detection followed the three steps recommended by the 2009 guideline: screening, mixture and confirmatory[8]. At screening, two tests able to activate coagulation, based on different principles, were used: dRVVT (LA 1 Screening Reagent® kit – Siemens) and silica-activated APTT (Pathromtin® SL reagent kit – Siemens).

Result interpretation

We verified repetition of positive tests after 12 weeks and the correlation with ACA tests [immunoglobulin class M and G (IgM

and IgG)] and anti-β2GP1 (IgM and IgG) whenever available. Samples with prolonged screening test results were further analyzed by the mixing test, which consisted of the addition of control plasma (pooled) to the patients' plasma, at 1:1 proportion. Next, samples underwent confirmatory final step, in which high phospholipid concentration reagents (Dade® Actin® FS Activated PTT Reagent – Siemens and LA 2 Confirmation Reagent® – Siemens), were used, able to prove the phospholipid dependence of antibodies already identified in earlier steps. ICA calculation helped in result interpretation, with values above 15%[11] being considered positive for LAC investigation.

RESULTS

Among the 36 patients included in this study, 28 (80.5%) were females and eight (19.4%) were males. The mean age was 43 years and there was predominance of white people (72%), followed by blacks (11%) and mixed-race people (9%); in 8% there was no race description. Among the diseases that induced LAC investigation, associated or not to thrombosis or gestational morbidity, collagen diseases corresponded to 67% of the cases; 5% of the patients presented chronic kidney failure (CKF); 5%, intestinal inflammatory disease; 3% neoplasms; and the remaining (17%) were characterized as other causes, such as neurologic, psychiatric and infectious diseases. The research was motivated by the exclusive presence of gestational morbidity in just 3% of the cases. Among the patients with thrombotic events and/or gestational morbidity, 19 had thrombosis in the venous territory; five, arterial thrombosis; and two, gestational morbidity. Among the 24 patients with collagen diseases, nine (37%) demonstrated association with venous thrombotic events; four (17%), with arterial thrombotic events; and just one (4%) was associated with gestational morbidity.

Among the patients with venous thrombosis with no association with collagen diseases, two presented CFK; two, Crohn's disease; one, pulmonary neoplasm; one was associated with chronic hepatitis B; three, associated with immobility (car accident trauma, medullary aplasia, and schizophrenia); and just one case of spontaneous cerebral venous thrombosis (CVT). The only patient that presented arterial thrombosis without association with collagen diseases presented immobility caused by multiple sclerosis as a risk factor.

Regarding LAC investigation, 24 patients demonstrated prolonged APTT and dRVVT; nine, just dRVVT; and three, just APTT. None of the samples prolonged in screening for dRVVT revealed correction in the mixing step, and ICA calculations and the relation between screening and confirmation proved equally positive for LAC investigation. Among the three samples with prolonged screening results by APTT, all demonstrated correction in the mixing step. However, when the confirmatory step and the ICA calculation were conducted, just one proved negative.

By the end of this study, out of the 35 patients that presented positive LAC, just 10 had repeated tests after 12 weeks, and there was confirmation of positivity in 100% of the cases.

Eleven patients (30%) presented prolonged international normalized ratio (INR) due to the use of vitamin K antagonist oral anticoagulants (between 1.25 and 3), and no patient demonstrated prolonged TT.

ACA investigation was conducted in 19 of the 36 studied patients: five presented positivity for IgG, IgM fraction or both (values above 40 GPL units). Anti-β2GPI assay was conducted in 12 of the 36 patients. Just four of them demonstrated positivity of the IgM fraction (values above 20). It was possible to document two cases of triple positivity (LAC, ACA, and anti-β2GPI).

DISCUSSION

APS diagnosis is a challenge under clinical and laboratory points of view. Despite the limitations of this study, it was possible to observe that LAC positivity is more common in youngsters and people with collagen diseases, according to medical literature[2, 12, 13]. PT analysis (INR) permitted identify patients in use of oral anticoagulants, and depending on their levels, recommend investigation after therapy suspension. At our institution, APS investigation during anticoagulation therapy occurred in 30% of the patients in this study.

Heterogeneity of APLAs makes laboratory interpretation of LAC steps more difficult, once specific situations (transient antibodies in inflammatory or infectious processes, anticoagulant medications, or even undetermined causes) can prolong clotting times and cause false-positive results. Permanence of positive results after 12 weeks and the correlation with other laboratory tests (ACA and anti-β2GPI) contribute for the definite APS diagnosis[3, 4]. In the current study, ACA and anti-β2GPI were not performed in most patients, due to disruptions in reagent supply. Two cases stand out, however, of triple positivity (ACA, anti- β2GPI and LAC) with recurrent thrombotic events. It is possible that other patients have similar profiles, as 28% of the involved in the study demonstrated clinical criteria (thrombosis or gestational morbidity) associated with LAC sustained positivity.

Altered values in the screening step that are corrected in the mixture can be due to deficiency of coagulation factors or dilution of weak antibodies. Confirmatory tests, in these situations, decrease the chances of false-negative results. Two cases of prolonged APTT were verified in the screening step and correction with the mixture, although the results of the confirmatory test and ICA calculation have proved positive for LAC investigation. These cases alert to the importance of conduction of a confirmatory test to identify weak antibodies, according to CLSI publication, in 2014, which recommends screening and confirmation steps as priority in investigation, restricting the mixing step for specific cases in which investigation is inconclusive[10]. The calculation used for result interpretation (ICA) did not reveal discrepancies. On the contrary, it helped identify the possible weak antibodies that underwent dilution when in the 1:1 mixture step[14]. As expected, dRVVT proved the most sensitive and specific method for LAC detection, considering its action on coagulation factor X, activating it directly and with smaller dependency on other factors in the generation of fibrin[8].

CONCLUSION

The obtained results confirmed APS heterogeneity and the importance of laboratory interpretation along with clinical manifestations and in compliance with international guidelines. There was adequacy of LAC investigation protocol in our institution, which started to consider clinical criteria, PT and TT tests before LAC conduction, and confirmation test for all samples with prolonged screening step, regardless of the result obtained in the mixture step. ICA calculation became part of the algorithm of result interpretation.

RESUMO

Introdução: *Trombofilia é a suscetibilidade à trombose, de natureza genética, adquirida ou mista. Entre as causas adquiridas, destaca-se a síndrome do anticorpo antifosfolípide (SAF) – doença autoimune caracterizada por anticorpos antifosfolípides, eventos trombóticos ou perda gestacional recorrente. O diagnóstico laboratorial baseia-se na detecção do anticoagulante lúpico (ACL), do anti-β2-glicoproteína 1 e da anticardiolipina; entretanto a execução do ACL ainda demanda uniformização. A última diretriz publicada pelo Clinical and Laboratory Standards Institute (CLSI) prioriza as etapas de triagem e confirmatória, em detrimento da mistura.* ***Objetivos:*** *Comparar as formas de liberação do ACL e adotar um protocolo de investigação em anuência às normas internacionais.* ***Métodos:*** *Trinta e seis amostras com resultados prolongados na etapa de triagem pelo ensaio do tempo do veneno da víbora de Russel (dRVVT) ou tempo de tromboplastina parcial ativada (TTPA) foram submetidas às etapas de mistura (1:1) e confirmatórias com altas concentrações de fosfolipídios.* ***Resultados:*** *Para o TTPA, foram considerados positivos os valores cujo cálculo do índice de circulação de anticoagulante (ICA) resultasse superior a 15%. Para o dRVVT, utilizou-se também o valor da razão entre triagem e confirmatória. Das amostras testadas, 14 revelaram correção na etapa da mistura, mas somente uma resultou em pesquisa negativa.* ***Conclusão:*** *O cálculo do ICA auxiliou na identificação dos anticorpos fracos que possivelmente sofreram diluição na etapa da mistura. Não há um exame padrão-ouro para o diagnóstico da SAF, e a pesquisa do ACL ainda demanda uniformização da técnica e da interpretação.*

Unitermos: *trombose venosa; aborto; anticorpos antifosfolipídios; anticoagulante lúpico.*

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