Original Article

APhL antibody ELISA as an alternative to anticardiolipin test for the diagnosis of antiphospholipid syndrome

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Abstract: Background: Persistent levels of antiphospholipid (aPL) antibodies [lupus anticoagulant (LA), anticardiolipin (aCL), anti-beta 2 glycoprotein I (aβ2GPI) IgG and/or IgM] in association with clinical features of thrombosis and/or pregnancy associated morbidity are indicative of antiphospholipid syndrome (APS). Of the aPL antibodies, aCL is the most sensitive for APS, however, their lack of specificity constitute a laboratory and clinical challenge. IgG/IgM antibodies directed against APhL (a mixture of phospholipids) has been reported to predict APS more reliably than aCL tests. The main objective of this study was to evaluate the performance characteristics of the APhL IgG/IgM ELISA, relative to the aCL and aβ2GPI tests. Methods: Sixteen (16) clinically confirmed APS and 85 previously tested serum (PTS) samples for aCL and aβ2GPI IgG/IgM antibodies were evaluated with the APhL IgG/IgM ELISA. Clinical specificity was determined in 100 serum samples (50 healthy and 50 infectious disease controls [parvo- and syphilis-IgG/IgM positive]). Results: The IgG antibody prevalence for aCL and APhL in the APS and PTS groups was comparable with marginal differences in clinical specificities. In contrast to the aCL IgM ELISA, the APhL test showed improved clinical specificities (72% aCL vs 94% APhL in the healthy controls; 38% aCL vs 78% APhL in the infectious disease controls) with implications for increased reliability in the diagnosis of APS. The overall agreement of the APhL with the aCL or aβ2GPI for the IgG tests was 89% and 95% respectively, and that of the APhL IgM to the aCL or aβ2GPI IgM tests was 72% and 86% respectively. Conclusion: Routine use of the APhL IgG/IgM ELISA may substantially reduce the high number of false positives associated with the aCL test without loss in sensitivity for APS.

Keywords: Anticardiolipin, APhL, antiphospholipid antibodies, method comparison

Introduction

The anti-cardiolipin (aCL) and anti-beta 2 glycoprotein I (aβ2GPI) IgG and/or IgM immunosays together with the lupus anticoagulant (LA) test are considered ‘criteria’ laboratory markers for the diagnosis of definite antiphospholipid syndrome (APS), an autoimmune disorder characterized by pregnancy-related morbidity, arterial and/or venous thrombosis [1-2]. Based on the laboratory recommendations for APS, a confirmed positive result of one immunoassay, i.e. aCL or aβ2GPI IgG or IgM is sufficient for classifying patients with vascular thrombosis and/or pregnancy related morbidity as having APS [1]. Of the ‘criteria’ immunoassays for APS, aCL is the most sensitive while aβ2GPI antibodies are considered highly specific with low sensitivity for APS. Although the increased sensitivity of the aCL ELISA makes it a favorable test in the initial diagnostic work-up of APS patients, their lack of specificity with associated high degree of false positive results constitute both a laboratory and clinical challenge. Indeed, several clinical studies as well as systematic review of the literature indicate that IgG isotype of either aCL or aβ2GPI is more strongly associated with APS than that of IgM [3-8]. The inherent difficulty in the standardization of aCL and aβ2GPI IgM as well as their unreliability in the context of infectious diseases and interfering substances like IgM rheumatoid factor poses significant challenges in the determination of this antibody isotype in APS [3, 9-15].

The aCL IgM antibodies in particular have been shown to occur in infections such as chronic hepatitis C, leprosy, syphilis, Kala-azar, parvovirus B19 among others [10, 12-13, 16]. The presence of these antibodies in different infec-
tious diseases and the recognition that they do not usually correlate with thrombotic events and/or pregnancy-related morbidity in APS makes testing at 2 time points necessary for differentiation of APS-associated from infection-associated aPL antibodies [1]. Based on these observations, there have been suggestions to replace aCL and aβ2GPI measurements from routine laboratory determinations with more reliable tests for the diagnosis of APS [17, 18]. Indeed alternative tests to aCL IgG/IgM antibodies and other potential diagnostic markers for APS have been described [19-23]. Of these, the APhL IgG/IgM as determined by ELISA has been reported to have improved specificity with optimal sensitivity for the diagnosis of APS [19]. The main objective in this study was to evaluate the performance characteristics of the APhL IgG/IgM ELISA relative to the aCL and aβ2GPI IgG/IgM antibody tests. Recognizing the inherent challenge of comparing the APhL assays to the sensitive aCL ELISAs, we sought to investigate its performance in 4 distinct groups to reduce selection bias. These groups included: 16 confirmed APS patients, 85 previously tested samples for aCL and aβ2GPI IgG/IgM, 50 healthy and 50 infectious disease (syphilis or parvovirus B19 IgG/IgM positive) controls. Our data shows comparable performance of the APhL and aCL IgG assays with significant difference in the clinical specificities for the IgM iso-type. Use of the APhL IgG/IgM ELISA may be useful as an alternate assay to aCL IgG/IgM ELISA without loss of diagnostic accuracy for APS.

Materials and methods

To evaluate the APhL IgG/IgM assays, we used two groups of samples that had previously been tested for APS. Sixteen (16) clinically confirmed APS patient sera obtained from the APLA 2010 wet workshop (Courtesy of Dr. Silvia Pierangeli, UTMB Galveston, TX) and 85 serum specimens obtained after completion of clinical testing for aCL and aβ2GPI IgG/IgM (INOVA Diagnostics, San Diego, CA) at ARUP laboratories (previously tested samples, PTS) were employed. The PTS group was made up of 16 males and 69 females, mean age 45.5 years (±19.0 years). To determine clinical specificity, two groups of control serum samples were investigated. The first group consisted of 50 serum samples from self-reported healthy individuals (HC), 10 males and 40 females with mean age 38.3 (±12.3 years). The second control group (n=50) was made up of 36 parvovirus IgG/IgM positive patients (mean age ± standard deviation: 30.3 ± 15.2 years) and 14 syphilis IgG/IgM positive cases (mean age ± standard deviation: 39.6 ± 21.1 years). IgG/IgM antibodies to parvovirus were determined using a commercial ELISA (Biotrin, Dublin, Ireland). Syphilis testing was performed with a Treponema pallidum antibody IgG/IgM ELISA (Trep-check, Bremancos Diagnostics Inc., Canada).

All 201 sera were tested with the APhL IgG/IgM ELISA (Louisville APL Diagnostics, Inc., Sea- brook, TX) as well as the aCL and aβ2GPI IgG/IgM commercial ELISAs (INOVA Diagnostics, San Diego, CA) by investigators who were blinded to the original aPL antibody results following manufacturers’ recommendations. Test results were interpreted in one of two ways. First, based on the manufacturers’ suggested cut-off values [aCL IgG/IgM (15 GPL and 13 MPL), aβ2GPI IgG/IgM (20 SGU and 20 SMU) and APhL IgG/IgM (15 GPL and 15 MPL)] and secondly adjusted to exclude the equivocal samples according to manufacturers’ recommendations [aCL IgG/IgM (20 GPL and 20 MPL), aβ2GPI IgG/IgM (20 SGU and 20 SMU) and APhL IgG/IgM (27 GPL and 38 MPL)].

To evaluate the analytical performance of the different assays, the prevalence, sensitivity or specificity of each aPL antibody was estimated in the different sample groups using the manufacturers’ suggested and adjusted cutoff values. The correlation between the different aPL assays was evaluated using EP evaluator release 8 (Data Innovations LLC, South Burlington, VT).

Results and discussion

In this study we assessed the performance characteristics of the APhL IgG/IgM ELISA relative to current ‘criteria’ immunoassays (aCL and aβ2GPI IgG/IgM) for the diagnosis of APS using four distinct groups of samples. The first group consisted of known APS samples that were evaluated in the Wet Workshop of the 13th International Congress on Antiphospholipid Antibodies (APLA 2010), in Galveston, Texas. In addition to these samples, we examined previously tested samples (PTS) for APS using aCL and aβ2GPI IgG/IgM assays (INOVA Diagnostics, San Diego, USA). As controls, two sets of samples from 50 reported healthy and 50 infectious
disease controls (36 parvo- and 16-syphilis-antibody positive sera). In each of these groups, we determined the prevalence of APhL, aCL and aβ2GPI IgG/IgM antibodies as recommended by the manufacturers. Figure 1A shows the antibody prevalence for the APhL, aCL and aβ2GPI IgG/IgM assays in the study cohort. At the manufacturers’ suggested cutoff values, the aPL antibody prevalence was highest in the aCL IgG/IgM assay in all groups evaluated. While the APhL IgG/IgM antibody prevalence in the APS and ‘suspected’ APS patient group was comparable to that of the aCL assays, its specificities in the healthy and infectious disease control cohorts were comparable to those of the aβ2GPI tests. The most significant difference in antibody prevalence between these assays was observed in the infectious disease group where the aCL IgG had a prevalence of 20% that was 5-fold greater than the APhL IgG assay and 10-fold more than the aβ2GPI IgG tests. With regards to the IgM antibody isotype, the prevalence of aCL was 62% compared to 24% and 12% for the APhL and aβ2GPI tests respectively. In addition, the unadjusted specificities of the IgG aPL assays were significantly higher than those of the IgM analytes in both control groups (Figure 1A). Thus, the aCL and APhL assays showed comparable sensitivities but distinct clinical specificities for APS (Table 1).

Since the prevalence of the APhL were comparable to those of the aCL for the IgG in the clini-

<table>
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<tr>
<th>Assay</th>
<th>Suggested Cut-off (%)</th>
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<th>Specificity (%)</th>
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<td>Healthy Controls</td>
<td>Infectious Disease Controls</td>
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<td></td>
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<td>aβ2GPI IgG/IgM</td>
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*Sensitivity for the different assays was determined only for confirmed APS group. Data for clinical specificity is presented based on the healthy and infectious disease control groups. No recommendation to adjust cut-off values for aβ2GPI assays is provided by the manufacturer, N/A represents not available.

Figure 1. Prevalence of specific aPL antibodies is dependent of cut-off determination. The per cent aCL, APhL or aβ2GPI IgG/IgM antibody isotype is shown for the confirmed APS, previously tested and controls groups based the manufacturers’ (A) suggested and (B) adjusted cut-off values.

Since the prevalence of the APhL were comparable to those of the aCL for the IgG in the clini-
cut-off values based on manufacturers’ recommendations for all assays (except aβ2-GPI tests) and compared the outcomes (Figures 1B, 2A and 2B). Except in the APS confirmed group, the adjustment in cut-off values resulted in a general decrease in the αCL and APhL IgG/IgM antibody prevalence in all groups (Figure 1B). However, compared to the APhL IgM assay which showed significant improvement in clinical specificity (78% versus 98%), the change in specificity for the αCL IgM assay was also significant, but not as high (38% versus 64%). Overall, adjusting the cut-off values for the assays increased the specificities for the αCL IgG/IgM (38% to 62%) and APhL IgG/IgM (76% to 96%) for the infectious disease groups. Further examination of analyte-specific (αCL, APhL and aβ2-GPI) clinical performance based on specific infectious disease serologies showed the αCL assays to be the most affected (Table 1, Figure 2A and 2B). Although significant improvement in specificities was observed with the APhL assays and αCL IgG following adjustment of cut-off values, the effect on the αCL IgM isotype was minor. Our results indicate that the use of the APhL IgG/IgM assays may provide substantial improvement in clinical specificity for APS without a significant loss in sensitivity and supports a recent report that the choice of the cut-off value used can markedly influence the outcome of aPL antibody testing and hence APS diagnosis [23].

Assay selection bias in the evaluation of alternate tests for αCL is any inherent challenge for a study of this nature. It is well recognized that αCL is required for optimal sensitivity in the diagnosis of APS [24]. In addition, for the most confirmed APS cases, the use of cut-off values representing the 99th percentile or 40 GPL or MPL units [1] allow for a relatively homogenous patient population, i.e. those with high risk for disease. Thus, comparison of methods under unselected conditions which mimic routine laboratory practice represents an appealing approach to evaluate the APhL assay. Using specimens which have been tested for αCL and aβ2-GPI IgG/IgM at our reference laboratory, our concordance analyses show that the agreement between the αCL and APhL for IgG isotype antibody to exceed 80% with a positive agreement of 68% between the IgM assays (data not shown). Furthermore, the overall correlation between the αCL and APhL IgG assays was greater than 80% (Table 2). Due to the differential clinical specificities of the aPL IgM assays, the positive agreements for the αCL and APhL IgM tests differed significantly with comparable concordance between the APhL and aβ2-GPI analytes. Consequently, the APhL assay may probably have similar clinical sensitivity to the aCL ELISA but with better specificity particularly in the context of infectious disease.

Although, we have sought to reduce bias in our investigation by using a diverse number of study groups to evaluate the performance characteristics of the APhL IgG/IgM assays for APS, there are some limitations in our study design. The most obvious weakness of this investigation is the lack of clinical data for patients in our suspected APS disease group. This was unavoidable as our efforts to recruit patient samples without assay selection bias was unsuccessful since most of the patients in our database have...
been diagnosed using INOVA aCL and aβ2GPI IgG/IgM assays. Thus for this study, we elected to use a limited number of APS confirmed patients as well as an unselected population of samples that have previously been tested for aCL and aβ2GPI antibodies. A second potential limitation of our study is the use of parvovirus B19 antibody positive patient samples to evaluate the clinical specificities of the aPL assays given that this infection may be a potential trigger of APS and/or SLE [16]. While antibody responses to specific B19 viral proteins and estimation of parvovirus B19 DNA in the specimens have not been performed to rule out acute, persistent or past infectious, we included known parvovirus IgG and/or IgM-positive sera to determine interference on aPL antibody testing especially since both APhL and aCL assays are β2GPI-dependent. Of the parvo-antibody positive samples, those which were only IgG positive had the least number of aPL antibody prevalence (data not shown). Overall, the APhL and aβ2GPI IgG/IgM assays had similar positivity rates in the parvovirus antibody positive group (Figure 2A) while the aCL IgM had the highest prevalence at the manufacturer’s recommended cut-off.

Current laboratory criteria for the diagnosis of APS are dependent on the demonstration of the presence and persistence of 1 or more of LA, aCL, and aβ2GPI IgG and/or IgM aPL antibodies. Although these guidelines are meant for research purposes, they are the best available tool for the evaluation of APS in clinical practice. The aCL ELISA is most sensitive test for the diagnosis of APS but lacks specificity despite efforts for clinical improvement. With an array of alternative and emerging diagnostic assays for APS, it is highly anticipated that choosing tests with the best medical benefit would outweigh the use of established analytes with suboptimal clinical value. Our data demonstrate that routine use of alternate tests such as the the APhL IgG/IgM ELISA may substantially reduce the high number of false positives associated with the aCL IgG/IgM tests without loss in diagnostic sensitivity for APS.

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