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Validation of a commercially available kit to detect anti-phosphatidylserine/prothrombin antibodies in a cohort of systemic lupus erythematosus patients

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Abstract

Background

Antiprothrombin antibodies detection comprises two different ELISAs: prothrombin coated on irradiated plates (aPT) or phosphatidylserine/prothrombin (aPS/PT) as the antigen. While several commercial kits are available for the detection of aPT, aPS/PT are usually detected by in-house assays. Recently, a new commercially available kit was launched and, therefore, we decided to test its efficiency by comparing it to our in-house assay.

Methods

aPS/PT were tested by our in-house assay (aPS/PT^{ih}) in 75 SLE patients, using Immulon 1 plates coated with phosphatidylserine, purified human prothrombin and 1%BSA-TBS-CaCl as blocking and diluents. Data from this assay were compared to those obtained by the QUANTA Lite™ aPS/PT screen, IgG and IgM Elisa (INOVA Diagnostics, Inc, San Diego, USA) commercial kits (aPS/PT^{c}).

Results

aPS/PT were found in 41.3% and 46.7% of SLE patients by the aPS/PT^{ih} and the aPS/PT^{c}, respectively. There was a positive correlation between IgG aPS/PT^{ih} and aPS/PT^{c} assays ($R^2 = 0.861$ by Spearman test, $p = 0.0027$). Sensitivity and specificity for APS were 62.2% and 97.4% (AUC 0.780) for the aPS/PT^{ih} assay and 70.3% and 84.2% (AUC 0.858) for the aPS/PT^{c}. Shorter running times were also seen when comparing the aPS/PT^{ih} vs. aPS/PT^{c} (7 hours vs. 3 hours, respectively).

Conclusion

The aPS/PT^{c} is a reproducible and accurate test for the detection of aPS/PT, bringing also the advantage of shorter running times.

Keywords: prothrombin; phosphatidylserine-prothrombin complex; antiphospholipid syndrome; QUANTA Lite™ aPS/PT

Introduction

In clinical practice, anticardiolipin antibody (aCL), antiβ2glycoprotein I (aβ2GPI), detected by enzyme-linked immunosorbent assay (ELISA) and the lupus anticoagulant (LA) detected by clotting assays have been the tests of choice for the diagnosis of the antiphospholipid syndrome (APS). However, antiphospholipid antibodies (aPL) are a heterogeneous family of immunoglobulins that do not bind to phospholipids but to plasma proteins with affinity for anionic surfaces (i.e. phospholipids). Evidence gathered through years of work suggests that aPL are directed against specific phospholipid binding plasma proteins such as β2 glycoprotein I and prothrombin, among others [1] and [2]. Antibodies to prothrombin are frequently found in patients with systemic lupus erythematosus (SLE) and have been associated with thrombosis and recurrent pregnancy loss [3] and [4].

Antiprothrombin antibodies are commonly detected by ELISA, using irradiated plates (aPT) [3] and [5] or in complex with phosphatidylserine (aPS/PT) [3], [6] and [7]. The mode of presentation of prothrombin in solid phase seems to influence its recognition by antiprothrombin antibodies. In fact, antiprothrombin antibodies cannot bind when prothrombin is immobilised onto non-irradiated plates [5] and [6], but do so if prothrombin is immobilised on a suitable anionic surface, adsorbed on gamma irradiated plates or exposed to immobilised anionic phospholipids. While several commercial kits are available for the detection of aPT, aPS/PT are usually detected by in-house assays.

Recently, a new aPS/PT commercially available kit was launched and, therefore, we decided to test its efficiency and analyse its clinical performance by comparing the results to our in-house assay in a cohort of patients with SLE.

Patients and Methods

This study included 75 consecutive patients, all fulfilling the 1982 criteria for SLE [8]. Of these, 37 patients fulfilled criteria for definite APS [9] and [10]. Patients group comprised 70 female and 5 male with a mean age of 43.7 ± 11.9 years and mean disease duration of 13.1 ± 9.1 years. Overall, 46 patients had a history of thrombosis, including 18 arterial, 15 venous and 13 both arterial and venous thrombosis. Out of 49 women who had ever been pregnant, 16 had a history of miscarriages and 20 a history of fetal death. Demographics are summarised in Table 1.

Ethical approval was obtained from the Guy's and St Thomas' Ethics committee and all patients involved in this study gave their written consent.

Blood Collection

Blood was collected by venopuncture from the antecubital vein into pre-cooled tubes containing 0.105 M sodium citrate and in non-anticoagulated tubes (Hemogard® 9NC and Hemogard® Z, respectively, Becton Dickinson, Rutherford, USA). Platelet free plasma was obtained by centrifugation at 2500 g for 20 minutes and filtration using a 0.2 μm surfactant free cellulose acetate membrane (Nalgene, Rochester, NY, USA). Plasma was stored at − 80 °C until used. For sera

preparation, blood was allowed to clot at room temperature and then centrifuged at 2000 g for 20 minutes. Sera were subsequently aliquoted and stored frozen at − 80 °C until use.

Antibodies to Phosphatidylserine-Prothrombin

Antibodies were tested by an in-house assay (aPS/PT^{ih}) and by a commercially available kit (aPS/PT^{c}).

aPS/PT^{ih} were tested as previously reported [3]. Briefly, Microtitre ELISA plates (Immulon 1, Dynatech, Virginia, USA) were coated with 30 µl of 50 µg/ml phosphatidylserine (Sigma) in methanol/chloroform (4:1) overnight at 4 °C. After blocking with 1% bovine serum albumine in Tris buffer saline and 5 mM CaCl2 (1%BSA-TBS-CaCl2), plates were washed twice with TBS-5 mM CaCl2–0.005% Tween and 10 µg/ml purified human prothrombin (Enzyme Research Laboratories Inc, Swansea, UK) were added and incubated at 37 °C. After one hour, serum samples diluted 1:100 in 1%BSA-TBS-CaCl2 were added in duplicate. Plates were incubated for one hour, followed by alkaline phosphatase-conjugated goat anti-human IgG or IgM (Sigma) and substrate. aPS-PT^{ih} titre of each samples was derived from a curve constructed by serial dilutions of a high binding sera used as standard. The cut off value was established at 2U for aPS-PT^{ih} IgG and 11U for aPS-PT^{ih} IgM, determined by the mean plus 3 standard deviations of 100 healthy controls. To avoid interference of anti-phosphatidylserine antibodies, all samples were tested in parallel in wells containing only phosphatidylserine.

aPS/PT^{c} were tested by using the QUANTA Lite™ aPS/PT screen, IgG and IgM Elisa (INOVA Diagnostics, Inc, San Diego, USA), according to manufacturer's recommendations.

Other aPL

The aCL and anti-β2GPI ELISAs were performed according to the current recommendations [2] and [11]. Anti-β2GPI were detected by ELISA as described previously [12], using irradiated microtitre plates (Nunc Maxisorp, Denmark).

Plasma samples were tested for the presence of LA according to the recommended criteria from the ISTH Subcommittee on Lupus Anticoagulant-Phospholipid-dependent antibodies [13] and [14]. All samples were tested by the activated partial thromboplastin time (aPTT - IL test™ APTT-SP, Instrumentation Laboratory, Italy) and the dilute Russell viper venom time (dRVVT- Diagen Russell's viper venom, Diagnostic Reagents Ltd, Oxon, UK) coagulation test. Both screen and confirm steps were performed.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 program (Microsoft software). Comparisons between patients and controls and patients groups were expressed as odds ratio with its 95% confidence interval (OR [95%CI]), where a lower limit > 1.0 was considered significant. The degree of linear association between aPS/PT^{ih} and aPS/PT^{c}was quantified by the Spearman correlation method. Differences between means were analysed by the Mann–Whitney test. All p

values were determined by Fisher's exact test. A p value of < 0.05 was considered statistically significant.

Results

Overall, aPS/PT were found in 41.3% and 46.7% of SLE patients by the aPS/PT^{ih} and aPS/PT^{c}, respectively. Total running time for the aPS/PT^{ih} was 7 hours vs. 3 hours for the aPS/PT^{c}. Data on prevalence of aPL, including aPS/PT are depicted in Table 2.

Overall results were in accord in 65/75 samples (86.6%) for IgG and/or IgM, with 63/75 (84%) for IgG and 61/75 (81.3%) for IgM, respectively. All data are summarised in Table 3. IgG and/or IgM aPS/PTih and aPS/PTc were found simultaneously in 28/38 (73.7%) patients found to be positive with any aPS/PT test. IgG were concomitantly found in 23/35 patients who were positive in any IgG aPS/PT test (65.7%), IgM aPS/PT in 14/28 (50%). All data are shown in Fig. 1.

There was a positive correlation between IgG aPS/PTih and aPS/PTc by Spearman test ($R2 = 0.861$, $p = 0.0027$) (Fig. 2A). Although less strong than that found for the IgG isotype, a positive correlation was also seen between IgM aPS/PTih and aPS/PTc ($R2 = 0.54$, $p = 0.003$) (Fig. 2.B).

Sensitivity and specificity for APS were 62.2% and 97.4% (AUC 0.780) for the aPS/PTih and 70.3% and 84.2% (AUC 0.858) for the aPS/PTc, respectively. The presence of aPS/PT by both, aPS/PTih and aPS/PTc was associated with thrombosis. All data are summarised in Table 4.

Discussion

In this study we aimed to test the efficiency and analyse the clinical performance of a new commercially available kit for aPS/PT testing by comparing the results to our in-house assay.

aPS/PT have been previously reported to be associated with thrombosis and recurrent pregnancy loss [3], [6] and [7]. Recent data from our group [15] confirms that their inclusion in the panel of antibodies tested for APS can result in a higher diagnostic power and in helping to stratify the thrombotic risk in patients with SLE.

To the best our knowledge, no commercial ELISA test has been previously evaluated and this is the first study testing the clinical accuracy of a commercial kit for aPS/PT. As these antibodies are not part of the routinely tested panel of aPL and no consensual standardized method exists for their detection, the validation of an available commercial kit is largely desirable.

In this study, the comparison of the overall diagnostic performance by AUCs, a conventional method [16], confirmed that both the assays, aPS/PTih and aPS/PTc, reached a good level of accuracy (AUC 0.780 and 0.858, respectively), and were able to identify patients with APS, both in terms of thrombosis and/or pregnancy loss.

Although sensitivity and specificity were similar between the two assays, the aPS/PTcassays showed a slightly higher number of positive results, with the consequential higher sensitivity to the expense of the specificity.

We report that the aPS/PTc ELISA test is also a rapid and simple method. The mean-time of the analysis is reduced with the commercial method which includes fewer time-consuming procedures (data not shown). This results in the reduction of potential analytical variables thanks to a simpler

protocol and a shorter testing time. In addition, it offers a valid diagnostic tool for APS diagnosis mainly for those centres where in-house assays are not available or affordable. In addition to these technical advantages, adopting a commercial kit may help in guaranteeing the comparability of results between laboratories, although a closer collaboration with kit companies is necessary for the success of this sort of initiative.

While the need for routine testing for aPS/PT is still under debate, there is overwhelming clinical evidence that these antibodies are associated with thrombosis and pregnancy loss in patients with APS and that their presence increases the risk of developing such an event [17], [18] and [19]. Therefore, the technical assessment of available methods for the detection is timely.

In conclusion, the results of this study demonstrated that the analysed commercially available ELISA kit is a satisfactory and time-effective method for the testing of aPS/PT, at least when compared to our in-house assay.

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Table 1. Demographic characteristics.

	N = 75 (%)
Female (%)	70 (93.3)
Mean age ± SD	43.7 ± 11.9
Mean disease duration ± SD	13.1 ± 9.1
APS*	37 (49.3)
Thrombosis	46 (61.3)
-Arterial thrombosis †	31 (41.3)
-Venous thrombosis †	28 (37.3)
Pregnancy loss ‡	22 (44.9)
- Miscarriages (≥ 1)	16 (32.7)
- Miscarriages (≥ 3)	6 (12.2)
- Fetal death	20 (40.8)

SLE: systemic lupus erythematosus, APS: antiphospholipid syndrome, *some patients fulfil criteria for both SLE and APS, † 13 patients from each group have both arterial and venous thrombosis, ‡ pregnancy loss defined by criteria [9] and [10]. All pregnancy data percentages calculated over the total number of female who had ever been pregnant (n = 49).

Table 2. aPL distribution in SLE cohort.

aPL	n = 75 (%)
aCL IgG/IgM	31 (41.3)
- aCL IgG	30 (40)
- aCL IgM	15 (20)
Anti-β2GPI IgG/IgM	27 (36)
- Anti-β2GPI IgG	25 (33.3)
- Anti-β2GPI IgM	6 (8)
LA	25 (30.3)
aPS/PT^{ih} IgG/IgM	31 (41.3)
- aPS/PT^{ih} IgG	25 (33.4)
- aPS/PT^{ih} IgM	19 (25.3)
aPS/PT^{c} IgG/IgM	35 (46.7)
- aPS/PT^{c} IgG	33 (44)
- aPS/PT^{c} IgM	23 (30.7)

IgG/IgM = IgG and/or IgM for both aCL and anti-β2GPI.

Table 3. Level of agreement/disagreement between commercial and in-house assays.

aPS/PTih	aPS/PTc	N = 75	(%)	
IgG and/or IgM*			65	(86.6)
	+	+	28	(37.3)
	-	-	37	(49.3)
			10	(13.3)
	-	+	7	(9.3)
	+	-	3	(4)
IgG			63	(84)
	+	+	23	(30.6)
	-	-	40	(53.4)
			12	(16)
	-	+	10	(13.3)
	+	-	2	(2.7)
IgM			61	(81.3)
	+	+	14	(18.6)
	-	-	47	(62.7)
			14	(18.6)
	-	+	9	(12)
	+	-	5	(6.6)

aPS/PTih: in house assay; aPS/PTc: QUANTA Lite™ aPS/PT kit; + = positive; - = negative. *and/or = any isotype.

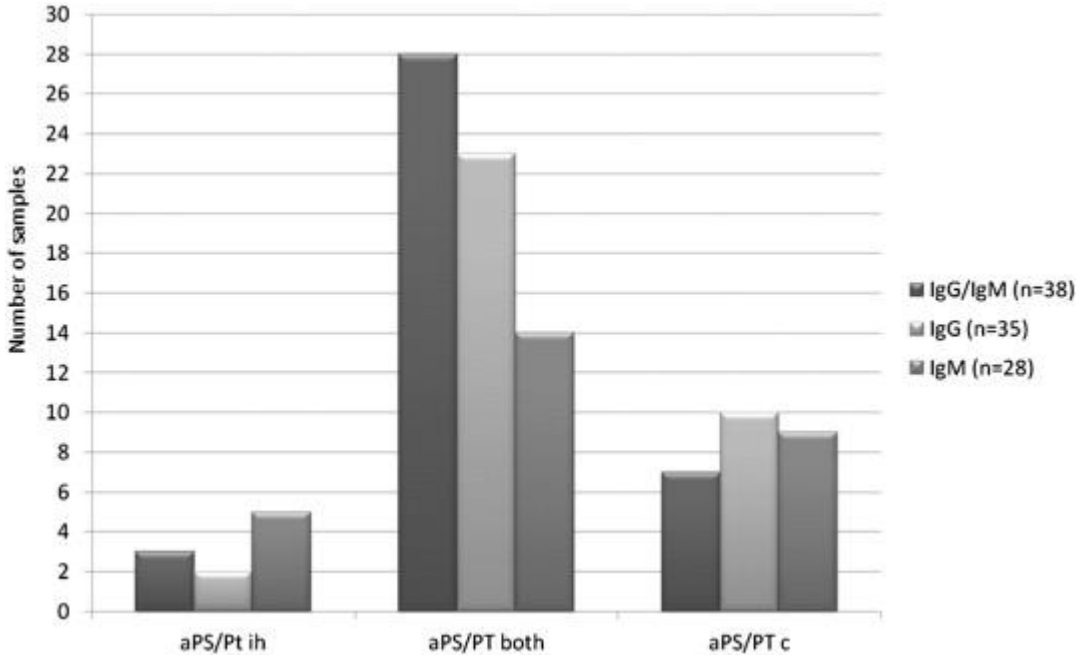

Fig. 1. Distribution of aPS/PT positivity between assays.

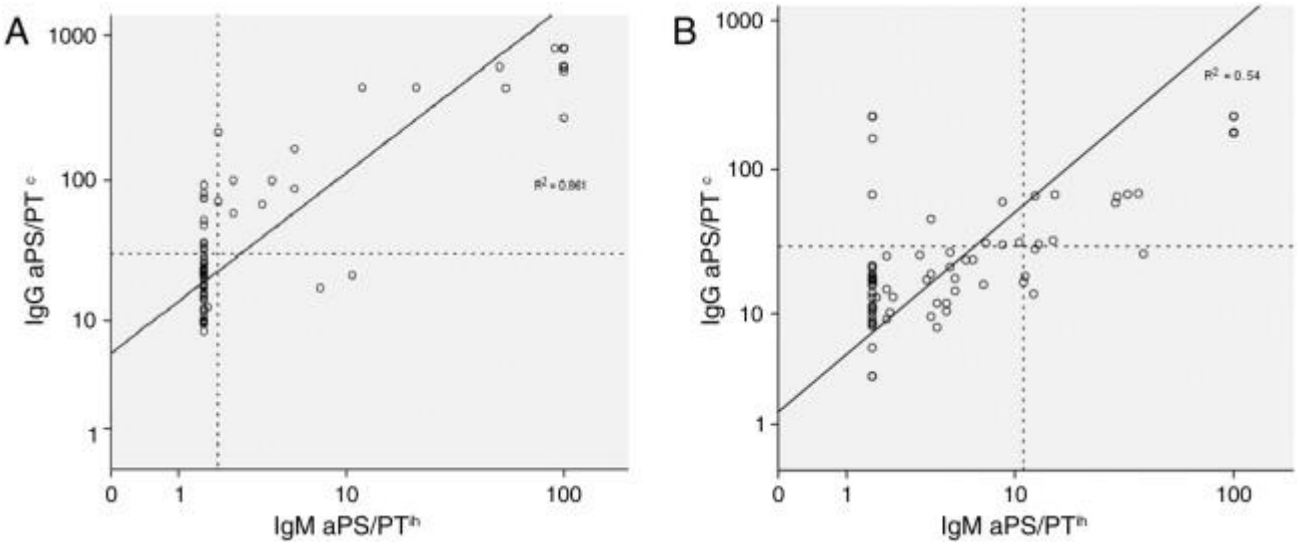

Fig. 2. Correlation between IgG (A) and IgM (B) aPS/PTih and aPS/PTc.

Table 4. aPS/PT in thrombosis and pregnancy loss (PL).

Antibody	Thrombosis						PL*					
	Any thrombosis		arterial		venous		Any PL		miscarriages		fetal Death	
	OR [95%ic]	p	OR [95%IC]	p	OR [95%IC]	p	OR [95%IC]	p	OR [95%IC]	p	OR [95%IC]	p
aPS/PT^{ih} IgG/IgM	23.03 [4.86-109.17]	0.0001	3.30 [1.26-8.65]	0.013	8.18 [2.83-23.67]	0.0001	5.33 [1.55-18.30]	0.006	2.10 [0.35-12.67]	NS	5.70 [1.60-20.28]	0.005
aPS/PT^{ih} IgG	30.55 [3.83-243.7]	0.0001	2.47 [0.96-6.60]	NS	14.43 [4.49-46.96]	0.0001	6.13 [1.74-21.51]	0.003	3.38 [0.55-20.55]	NS	5.84 [1.67-20.41]	0.0042
aPS/PT^{ih} IgM	7.91 [1.67-37.51]	0.036	3.34 [1.13-9.87]	0.0026	2.35 [0.81-6.78]	0.11	1.98 [0.59-6.63]	NS	1.04 [0.17-6.35]	NS	1.75 [0.52-5.87]	NS
aPS/PT^{c} IgG/IgM	19.81 [5.13-76.42]	0.0001	4.50 [1.68-12.05]	0.0001	7.07 [2.45-20.40]	0.0001	9.00 [2.34-34.61]	0.0007	1.74 [0.29-10.52]	NS	6.55 [1.73-24.70]	0.0036
aPS/PT^{c} IgG	7.47 [2.41-23.14]	0.002	2.08 [1.04-6.90]	0.0394	4.98 [1.81-13.66]	0.0013	5.09 [1.50-17.23]	0.0072	2.53 [0.49-15.30]	NS	3.53 [1.07-11.67]	0.035
aPS/PT^{c} IgM	25.67 [3.22-204.8]	0.001	4.22 [1.49-11.94]	0.0052	2.53 [1.06-8.06]	0.034	3.43 [1.03-11.41]	0.04	0.76 [0.13-4.65]	NS	3.21 [0.97-10.65]	NS

*PL: pregnancy loss. All pregnancy data are calculated over the total number of female who had ever been pregnant (n = 49).