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Comparative analysis of different enzyme immunoassays for assessment of phosphatidylserine-dependent antiprothrombin antibodies

Olga Amengual ¹, Tetsuya Horita ¹, Walter Binder ², Gary L Norman², Zakera Shums², Masaru Kato ¹, Kotaro Otomo ¹, Yuichiro Fujieda ¹, Kenji Oku¹, Toshiyuki Bohgaki¹, Shinsuke Yasuda ¹ and Tatsuya Atsumi ¹

¹ Division of Rheumatology, Endocrinology and Nephrology, Hokkaido University Graduate School of Medicine, Sapporo, Japan, ² INOVA Diagnostics, Inc, San Diego, USA

Corresponding author:

Olga Amengual, MD, PhD

Division of Rheumatology, Endocrinology and Nephrology
Hokkaido University Graduate School of Medicine,
N15 W7, Kita-ku, Sapporo 060-8638, Japan

Telephone: + 81 - 11 - 706 - 5915

Fax: + 81 - 11 - 706 - 7710

E-mail address: olgaam@med.hokudai.ac.jp

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Conflict of interest: Gary L Norman, and Zakera Shums are employees of INOVA Diagnostics, Inc., San Diego USA. Walter Binder was an employee of INOVA Diagnostics at the time of the study. The other authors declare that they have no

conflict of interest.

Abstract

Phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT) were strongly correlated with the presence of lupus anticoagulant showing a high specificity for the diagnosis of antiphospholipid syndrome. However, the main criticism for the clinical applicability of aPS/PT testing is the lack of reproducibility of the results among laboratories. In this study, we measured IgG and IgM aPS/PT using our original in-house enzyme-linked immunosorbent assays (ELISA) and commercial ELISA kits to assess the assay performance and to evaluate the accuracy of aPS/PT results.

The study included 111 plasma samples collected from patients and stored at our laboratory for aPS/PT assessment. Sixty-one samples were tested for IgG aPS/PT using two assays: 1) aPS/PT ELISA in-house ELISA and 2) QUANTA Lite™ aPS/PT IgG ELISA kit (INOVA Diagnostics, Inc., USA). Fifty samples were evaluated for IgM aPS/PT using two assays: 1) aPS/PT ELISA in-house ELISA and 2) QUANTA Lite™ aPS/PT IgM ELISA kit (INOVA Diagnostics).

Ninety-eight percent yielded concordant results for IgG aPS/PT and 82% for IgM aPS/PT. There was an excellent agreement between the IgG aPS/PT assays (Cohen κ = 0.962) and moderate agreement between the IgM aPS/PT assays (κ = 0.597). Statistically significant correlations in the aPS/PT results were obtained from both IgG and IgM aPS/PT assays (r = 0.749, r =0.622, p < 0.001, respectively).

In conclusion, IgG and IgM detection by ELISA is accurate. The performance of aPS/PT is reliable and concordant results can be obtained using different ELISA methods.

Introduction

Antiphospholipid antibodies (aPL) are a heterogeneous group of antibodies detected in patients with antiphospholipid syndrome (APS). Lupus anticoagulant (LA), detected by clotting assays, anticardiolipin antibodies (aCL) and anti β 2glycoprotein I (β 2GPI) antibodies, detected by enzyme linked immunosorbent assay (ELISA) are the laboratory tests included in the current classification criteria for definite APS [1]. However, a number of issues regarding the laboratory criteria for the diagnosis of APS are still under debate.

Antibodies against prothrombin, one of the major antigen target for aPL, are frequently found in patients with APS. The antiprothrombin antibody family comprises two types of antibodies: those detected by ELISA using prothrombin alone as the target antigen (aPT-A) and those directed against phosphatidylserine-prothrombin complexes, the so-called phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT) [2]. Numerous studies have investigated the implications of aPT-A in the clinical manifestation of APS with controversial results [3-6]. On the other hand, several groups have reported that the presence of aPS/PT strongly correlates with that of LA and that aPS/PT were highly specific for the diagnosis of APS [7-11]. However, the clinical applicability and diagnostic utility of aPS/PT testing have not yet been fully defined mainly as a result of the lack of a standardized procedure to test aPS/PT and the low reproducibility of the results between laboratories.

In this study, we aimed to assess the performance of two different ELISA methods, our original in-house assay [7] and commercial kits, to detect aPS/PT and to evaluate the reproducibility of the aPS/PT assay.

Material and Methods

The study comprised 111 patients' plasma samples submitted to our Department of Rheumatology for aPL assessment. Clinical/demographic data and laboratory findings were retrospectively extracted from their medical records.

The study was performed in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice. Approval was obtained from the Local Ethics Committee.

Plasma samples

Venous blood was collected into tubes containing a one-tenth volume of 0.105 M sodium citrate and was centrifuged immediately at 4°C. Plasma samples were depleted of platelets by filtration then stored at –80°C.

Phosphatidylserine-dependent antiprothrombin antibody assays:

1. In-house aPS/PT ELISA

For the detection of aPS/PT antibodies, an in-house ELISA was performed as previously described [7]. Briefly, non-irradiated microtiter plates (Sumilon type S, Sumitomo Bakelite, Tokyo, Japan) were coated with 30 µl of 50 µg/ml phosphatidylserine (Sigma Chemical Co., St. Louis, USA) and dried overnight at 4°C. To avoid nonspecific binding of proteins, wells were blocked with 150 µl of Tris-buffered saline (TBS) containing 1% fatty-acid free bovine serum albumin (BSA, A-6003, Sigma) and 5 mM CaCl₂ (BSA-Ca). After 3 washes in TBS containing 0.05% Tween 20 (Sigma) and 5 mM CaCl₂ (TBS-Tween-Ca), 50 µl of 10 µg/ml

human prothrombin (Diagnostica Stago, Asnieres, France) in BSA-Ca were added to half of the wells in the plates and the same volume of BSA-Ca alone (as sample blank) to the other half. After 1 hour incubation at 37°C, plates were washed and 50 µl of serum diluted in BSA-Ca in 1:100 were added in duplicate. Plates were incubated for 1 hour at room temperature, followed by alkaline phosphatase (ALP)-conjugated goat anti-human IgG or IgM. After one hour incubation at room temperature and four washes, 100 µl/well of 1 mg/ml 4-nitrophenylphosphate disodium (Sigma) in 1M diethanolamine buffer (pH) 9.8 were added. In all the assays, the samples were run in parallel on the phosphatidylserine/prothrombin-coated wells and in wells coated only with phosphatidylserine. Results were expressed as final optical density (OD) corresponding to OD detected in phosphatidylserine/prothrombin-coated wells minus OD detected in phosphatidylserine alone-coated wells. The aPS/PT titer of each sample was derived from the standard curve according to dilutions of the positive control. Normal ranges of IgG (>2.0 Units) and IgM (>9.6 Units) aPS/PT were previously established using non-pregnant 132 healthy controls of 99th percentile cut-off values.

2. aPS/PT commercial ELISAs

Samples were tested using two commercial ELISA kits from INOVA Diagnostics, Inc., San Diego, CA, USA (INOVA kits). QUANTA Lite™ aPS/ PT IgG ELISA was used for the detection of IgG aPS/PT and QUANTA Lite™ aPS/ PT IgM ELISA for IgM aPS/PT detection. aPS/PT testing was performed according to the manufacturer's instructions. Positive cut-off values for the IgG and IgM ELISA kits were set up by the manufacturer's as > 30 Units.

All the aPS/PT ELISAs were performed by the same person in our laboratory.

Statistical analysis

Statistical evaluation was carried out by Fisher's exact test or chi-squared test, as appropriate. Cohen's kappa test was applied to compare the results obtained using different tests in the same sample. The diagnostic accuracy of the assays was assessed by receiver operating characteristic (ROC) curve analysis. P values less than 0.05 were considered significant. All statistical analyses were performed using SPSS (Chicago, Illinois, USA).

Results

The 111 plasma specimens belonged to 87 patients, 72 woman and 15 men with a mean age of 46 years (range 23-70 years). Forty seven patients (54%) were diagnosed as having APS according to the classification criteria for definite APS [1], twenty patients had primary APS and in 27 patients systemic lupus erythematosus (SLE) was diagnosed in association with APS. Seventeen patients (20%) had SLE, 20 patients (23%) other autoimmune diseases and 3 patients (3%) had positive aPL in the absence of any diseases.

Twenty four patients (28%) had history of arterial thrombotic events, 19 venous thrombosis (40%) and 10 females had history of pregnancy complications (14%). IgG/ IgM aCL, IgG /IgM anti β 2GPI antibodies and LA were found in 36 (41%), 34 (39%) and 61 (70%) patients, respectively.

Sixty one samples from 58 patients were tested for the IgG aPS/PT and 50 samples from 48 patients were assayed for the IgM aPS/PT. Samples were selected to be tested in the IgG or in the IgM aPS/PT ELISA based on previous data obtained with our in-house aPS/PT ELISA. We analyzed the results for each sample at the first determination in the in-house and commercial assays. Ninety-eight percent of samples yielded concordant results for IgG aPS/PT, while 82% samples displayed concordant IgM aPS/PT results (Table 1). One sample displayed discrepant results for IgG aPT/PT and 9 samples were discrepant for IgM aPS/PT results (Table 2). There was an excellent agreement between the IgG aPS/PT assays ($\kappa = 0.962$) and a moderate agreement in the IgM aPS/PT assays ($\kappa = 0.597$).

There was a statistical significant correlation in the OD values and Units of IgG aPS/PT, as well as, IgM aPS/PT obtained with homemade and commercial

ELISAs (Pearson correlation coefficients: $r = 0.835$, $r = 0.749$ and $r = 0.719$, $r = 0.622$, $p < 0.001$, for IgG aPS/PT and IgM aPS/PT respectively)(Figure 1).

The distribution of aPS/PT and classical aPL in 58 patients tested for IgG aPS/PT and in 48 patients tested for IgM aPS/PT is shown in Table 3.

We performed ROC analysis and evaluated the sensitivity, specificity, likelihood ratio positive and likelihood ratio negative of the aPS/PT assays for the diagnosis of APS. The area under the curve (AUC) values were 0.799 and 0.808 for in-house and INOVA IgG aPS/PT and 0.791 and 0.705 for in-house and INOVA IgM aPS/PT, respectively (Figure 2).

The sensitivity, specificity, likelihood ratio positive and likelihood ratio for APS diagnosis were 93.6%, 59.3%, 2.30, 0.11, 93.6%, 63.0%, 2.53, 0.10, 75.0%, 84.6%, 2.33, 0.24, 88.5%, 50.0%, 1.77, 0.23 for IgG aPS/PT in-house ELISA, IgG INOVA ELISA, IgM aPS/PT in-house ELISA and IgG INOVA ELISA, respectively.

Discussion

In this manuscript, we assessed the performance of two ELISA methods to determine aPS/PT showing that the detection of aPS/PT is accurate.

Antiprothrombin antibodies family are commonly detected by ELISA-based methods. ELISAs using gamma-irradiated plates coated with prothrombin reveal aPT-A [12] and ELISAs in which prothrombin is exposed to immobilized phosphatidylserine identified aPS/PT [7]. The good correlation between aPS/PT ELISA and LA supports the use of aPS/PT as one of the “screening” or “confirming” assays for APS-associated LA [11,13]. Moreover, aPS/PT are associated with the clinical manifestations of APS [11, 14, 15] and the determination of aPS/PT would potentially contribute to a better recognition of APS, especially in cases of suspected APS, but without evidence of aCL, antiβ2GPI antibodies or LA [16].

The major limitation of aPS/PT determination is the lack of standardization of the aPS/PT ELISA [17]. In this study, we aimed to evaluate the accuracy of the results using different aPS/PT ELISAs that are currently used to detect aPS/PT. We analyzed the results obtained for each samples at the first determination in the in-house and commercial ELISAs and observed a high agreement, indicating that aPS/PT results are precise.

For the IgG aPS/PT assays, we found discrepant results for only one sample. This sample was obtained from a patient with SLE and displayed low-positive IgG aPS/PT titer in the in-house ELISA but negative results in the INOVA test (21.5 units, cut off >30). The sample was re-tested and the low-positive results in the in-house assay was confirmed. The differences in the interpretation of this specimen is likely due to the cut-off selected for each assay. Although the INOVA result was

interpreted as negative it fell in the upper portion of the normal range.

For the IgM aPS/PT assays, 9 samples displayed discrepant results. Three samples, (S-2, S-9, S-10, Table 2) became negative after re-testing, implying false positive aPS/PT results at the first determination. Therefore, true discrepancy for the IgM aPS/PT assay was detected in 6 out of 50 samples leading to 88% concordance. The discrepant results in the IgM aPS/PT assays could be explained by the different methodologies used in each ELISA systems. In the commercial kits, plastic microtiter plate wells were coated with purified phosphatidylserine /prothrombin complexes and then stabilized. In the in-house assay, plastic microtiter plate wells were coated with phosphatidylserine and dried overnight at 4°C. Prothrombin was then added and samples were run in parallel on the phosphatidylserine/prothrombin-coated wells and in wells coated only with phosphatidylserine. In the commercial ELISA, the presence or absence of aPS/PT is determined by measuring and comparing the direct color intensity that develops in the sample wells with that of a calibration curve. However, in the in-house assay, the presence of aPS/PT is determined by measuring the color intensity that develops in the sample phosphatidylserine-coated wells and subtracted from the color intensity that developed in the sample phosphatidylserine/prothrombin-coated wells. Subtracted color intensity is compared with that of a calibration curve. Interestingly, samples with positive IgM aPS/PT results only in the commercial ELISA showed a high binding to phosphatidylserine alone-coated wells in the in-house assay, suggesting that the positive binding might be related to IgM binding to phosphatidylserine rather than true binding to phosphatidylserine/prothrombin complexes. The aPS/PT false positive pattern, ie. IgM binding positive to phosphatidylserine alone but negative to

aPS/PT could be found in some cases, but the clinical significance needs to be clarified. In addition, in general IgM ELISAs shows more variation compared with IgG assays presumably due to the low-affinity characteristics of the IgM isotype.

We observed high correlations between the IgG or IgM results from both assays, implying that accurate results can be obtained using IgG and IgM aPS/PT available ELISAs.

The APS-Score [18, 19] and GAPPSS [20] showed that multiple positivity for aPL will increase the thrombotic risk. Testing aPS/PT would be useful for the prediction of the thrombotic risk in patients with APS.

We believe that our findings add additional evidence suggesting that aPS/PT testing should be considered as a tool for the diagnosis of APS. Standardization of aPS/PT assay is a necessary step for the worldwide implementation of aPS/PT testing and will potentially lead to the inclusion of aPS/PT as one of the laboratory criteria for the APS classification and to a better identification of patients with APS.

In conclusion, our data demonstrated that the performance of the currently available aPS/PT assays is accurate and reliable.

Table 1 Detection of aPS/PT

	Tested samples	Concordant Positive results	Concordant Negative results	Total Concordance
IgG aPS/PT assays*	61	41 (67%)	19 (31%)	60 (98%)
IgM aPS/PT assays#	50	29 (58%)	12(24%)	41 (82%)

*In-house IgG aPS/PT ELISA, IgG aPS/PT INOVA ELISA kit, # In-house IgM aPS/PT ELISA, IgM aPS/PT INOVA ELISA kit.

Table 2 Discrepant results using in-house ELISAs and commercial ELISAs

ELISA isotype	Sample	In-house ELISA (cut off > 2 U)		INOVA ELISA (cut off >30 U)			
		<i>U</i>	<i>Results</i>	<i>U</i>	<i>Results</i>	Diagnosis	LA
IgG	S-1	2.9	(low +)	21.5	(-)	SLE	(-)
IgM		(cut off >9.6 U)		(cut off >30U)			
	S-2	12.5	(low +)	5	(-)	ITP	(-)
	S-3	15.5	(low +)	14.5	(-)	PAPS	(+)
	S-4	7.2	(-)	130	(high +)	PAPS	(+)
	S-5	3.5	(-)	125	(high +)	APS/SLE	(+)
	S-6	<1.5	(-)	140	(high +)	aPL (+) only	(+)
	S-7	2.7	(-)	49	(low +)	SLE	(-)
	S-8	8.8	(-)	37	(low +)	RA	(+)
	S-9	9	(-)	72	(low +)	APS/SLE	(+)
	S-10	<1.5	(-)	32	(low +)	RA	(-)

LA: lupus anticoagulant, SLE: systemic lupus erythematosus, ITP: idiopathic thrombocytopenic purpura, PAPS: primary antiphospholipid syndrome, aPL: antiphospholipid antibodies, RA: rheumatoid arthritis. S: sample number, U: Units, (+): positive results, (-) negative results,

Table 3 Profile of antiphospholipid antibodies in the population analyzed

	APS	Non-APS	X ²	p value
aPS/PT IgG				
Tested patients N=58	31 (53%)	27 (47%)		
aPS/PT IgG in-house	29 (50%)	11 (20%)	18.80	< 0.001
aPS/PT IgG INOVA	29 (50%)	10 (17%)	20.92	< 0.001
aCL (IgG/M)	14 (24%)	5 (9%)	4.65	n.s
antiβ2GPI (IgG/M)	12 (21%)	8 (14%)	0.53	n.s
LA	30 (52%)	7 (12%)	31.34	< 0.001
aPS/PT IgM				
Tested patients N=48	26 (54%)	22 (46%)		
aPS/PT IgM in-house	22 (46%)	8 (17%)	11.84	0.001
aPS/PT IgM INOVA	23 (48%)	11 (23%)	8.53	0.005
aCL (IgG/M)	18 (24%)	4 (8%)	12.51	0.001
antiβ2GPI antibodies (IgG/M)	14 (29%)	6 (13%)	3.46	n.s
LA	25 (52%)	8 (17%)	19.83	< 0.001

APS: antiphospholipid syndrome, aCL: anticardiolipin antibodies, β2GPI: β2Glycoprotein I, IgG/M: IgG and/or IgM positive
LA: lupus anticoagulant X²: Pearson's chi-squared test, n.s: no significant

Figure Legend

Fig. 1 Correlation of the optical density values of aPS/PT obtained with in-house ELISAs and commercial ELISAs

a) Correlation of the IgG aPS/PT optical density (OD) values between INOVA ELISA kit and in-house ELISA.

b) Correlation of the IgM aPS/PT OD values between INOVA ELISA kit and in-house ELISA.

There was a statistically significant correlation in the aPS/PT OD values obtained with both IgG and IgM ELISAs. aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies.

Fig. 2 Receiver operating characteristic (ROC) curves for the diagnosis of APS.

a) IgG aPS/PT assays

The area under the ROC curve were 0.799 and 0.808 (95% confidence interval [95% CI] 0.685-0.913 and 0.690-0.926) for in-house ELISA and INOVA ELISA, respectively.

b) IgM aPS/PT assays

The area under the ROC curve were 0.791 and 0.705 [95% CI] 0.666–0.917 and 0.545–0.866 for in-house ELISA and INOVA ELISA, respectively.

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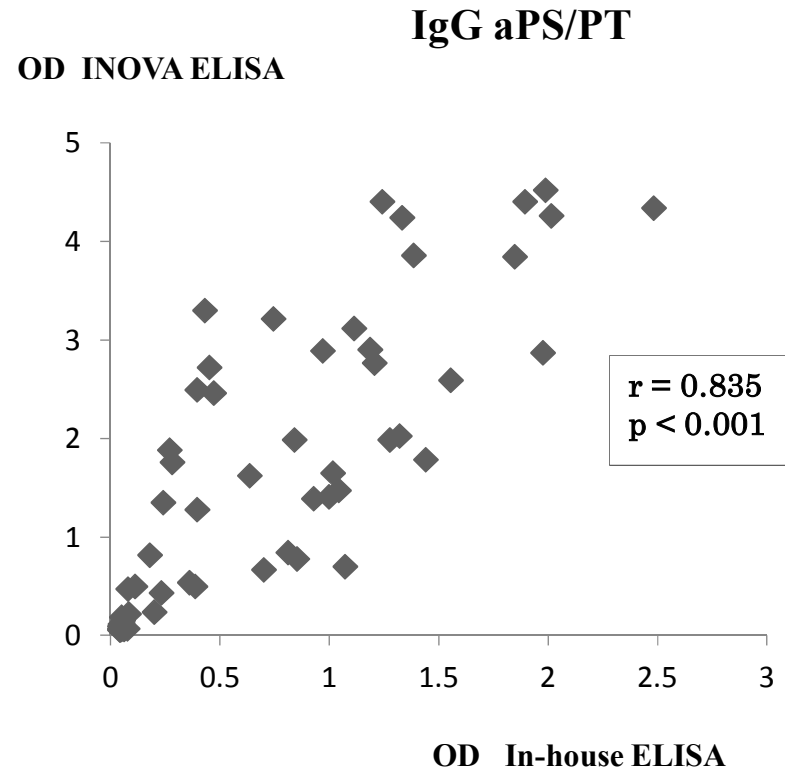
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(a)



(b)

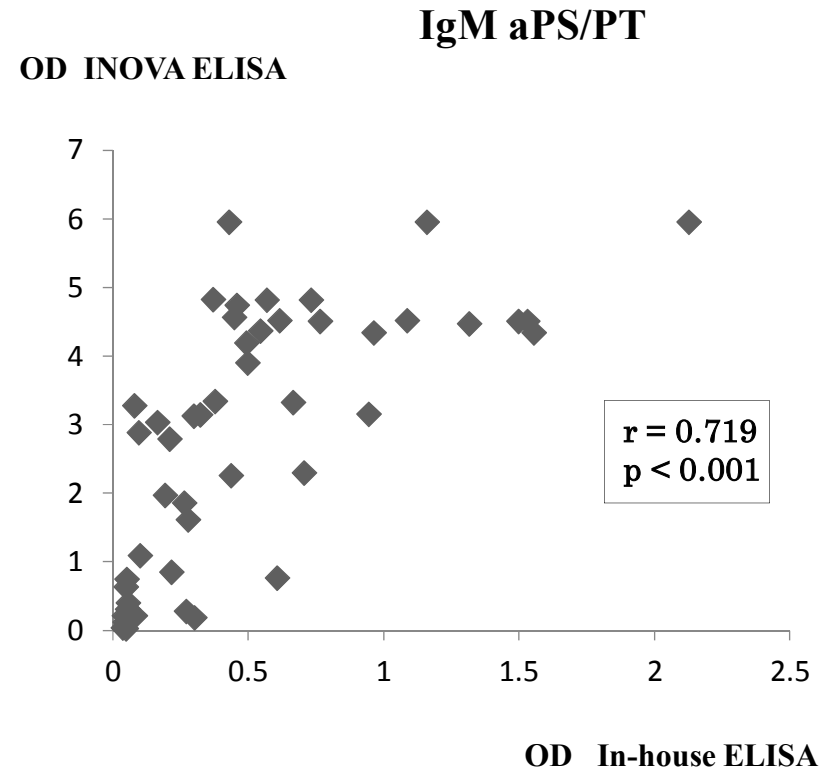


Figure 2

