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Original paper

FORMULATION, STANDARDIZATION AND VALIDATION OF AN ELISA TEST FOR DETERMINATION OF ANTICARDIOLIPIN ANTIBODIES

Slavica Marjanović, Snežana Golubović, Ljiljana Vićovac

Institute for the Application of Nuclear Energy – INEP, Zemun

Summary: Availability of a sensitive and a reliable test for anticardiolipin autoantibodies (aCL) is important in diagnosis and follow-up of the patients. This study presents the data on the assay protocol, analytical validation (detection limit, precision, linearity) and reference ranges for the ELISA aCL IgG and IgM assays developped at INEP. The determined characteristics of the assays, as well as the reference ranges, being 0–5.9 MPL U/mL for aCL IgM and 0–10.5 GPL U/mL for aCL IgG, are comparable to the assay from Axis-Shield Diagnostics Limited used as accepted procedure in the absence of the reference one. The tests described here can be included in to the standard laboratory panel for autoimmune conditions.

Key words: APA, anticardiolipin antibody (aCL), ELISA, IgM, IgG

Introduction

Antiphospholipid antibodies (APA) comprise a heterogenous group of antibodies of IgG, IgM and IgA classes, directed against anionic phospholipids and phospholipid-protein complexes (1, 2). Anticardiolipin antibodies (aCL) are most frequently determined APA (3) and they are found in patients with systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), rheumatoid arthritis and in some infectious diseases. In most cases, their presence is accompanied with arterial and/or venous thrombosis and recurrent fetal loss (4). It was originally believed that APA bound only to phospholipid antigens against which they were directed. However, further investigations demonstrated that aCL were also directed against cardiolipin antigen complexed with a plasma protein 50 kDa cofactor, known as β_2-glycoprotein I (β_2-GPI) (5).

Within normal population, the frequency of APA, i.e. of aCL, ranges between 1% in normal pregnancies (6) and 5.6%, in blood donors (7). Increased levels of aCL were found in acute infections (up to 32%), rheumatoid arthritis (between 4 and 25%), in medication-induced lupus (47%) and also in elder people without any characteristic symptoms (51%). Many of APL positive individuals are not affected by thrombocytic events (8).

The first standardized test for APA determination was an aCL ELISA test, which involves direct binding of antibodies present in the sample or standards to the immobilized cardiolipin (9). The test enables detection and/or quantitation of autoantibodies of different classes (IgG, IgM, IgA).

Availability of a sensitive and a reliable test for aCL is important for diagnosing and follow-up of patients. In the present study the characteristis of the ELISA aCL IgM and ELISA aCL IgG tests developed in our laboratory at INEP, inculding analytical validation (detection limit, precision, linearity) as well as the reference range for normal population, are presented.

Material and Methods

Cardiolipin, bovine serum albumin (BSA), and bovine serum were purchased from Sigma-Aldrich, USA. Horseraddish peroxidase (HRPO)-conjugated anti-hIgG and anti-hIgM antibodies were produced at INEP, Zemun. Characterized positive sera with high concentrations of either IgM or IgG were obtained from SLR Research, CA, USA.

Address for correspondence:

Dr Ljiljana Vićovac
Institute for the Application of Nuclear Energy – INEP
Banatska 31b
11080 Zemun
e-mail: vicovac@inep.co.yu

Commercial anticardiolipin test for IgM and IgG used for method comparison was purchased from Axis-Shield Diagnostics Limited, Scotland UK.

Sera of healthy individuals (n = 102) were from the Department for Blood Transfusion, Clinical Center Zemun, Serbia and Montenegro.

Preparation of components for INEP ELISA aCL

Coated microtiter plates. Cardiolipin was bound to microtitration plates by physical adsorption, immobilizing CL in a monomolecular layer. Briefly, microtiter wells were coated by the addition of 100 μL cardiolipin dissolved in etanol at 50 μg/mL. Ethanol was evaporated overnight. Plates were washed twice with phosphate buffered saline (0.05 mol/L PBS) and blocked for 1 h by adition of 150 μL per well of 1% bovine serum albumin in PBS (1% BSA-PBS). Plates were then washed twice with PBS, dried over night in a vacum oven and kept dessicated at 4 °C.

HPRO-conjugated anti- hIgM and -hIgG. Polyclonal antibodies from the serum of sheep immunized with purified H chain of human IgG or IgM, were isolated by rivanol and ammonium sulphate precipitation. Affinity purified antibodies were labeled with horseradish peroxidase, by periodate method as described by Wilson and Nakane (10). The prepared conjugate was stored in 50% (by volume) glycerol at –20 °C. Working dilution of the conjugate was prepared immediately before the assay.

Assay procedure

Anticardiolipin test for IgM and IgG (aCL IgM and aCL IgG) Axis-Shield. According to the manufacturer's instructions 100 μL of standard, control or diluted human serum (1/100) are incubated in microtitre plates. After 60 min of incubation at room temperature, plates are washed four times with 200 μL of wash buffer. This is followed by incubation with 100 μL of corresponding conjugate (anti-IgG or anti-IgM) for 30 min. The same washing procedure as above is applied, and 100 μL of substrate is subsequently added and incubated for 30 min. The reaction is stopped with 100 mL of stop solution. Absorbance is measured at 540 nm. Levels of anticardiolipin IgG and IgM antibodies are expressed as GPL or MPL units, with 1 unit being the cardiolipin activity of 1 g/L of affinity-purified IgG or IgM anticardiolipin antibodies from a standard serum sample (11).

aCL IgM and aCL IgG (Anticardiolipin test IgM and IgG) – INEP. Assays developed at INEP are performed according to an established procedure (12). The unknown samples (diluted 1/100 in 1% BSA-PBS) and the IgM- and IgG-standards are added to the previously coated ELISA microtiter plates and incubated for 1 hour at 37 °C. Plates are then washed four times with PBS and incubated for 30 minutes with HRPO-conjugated anti-hIgG or anti-hIgM. After another wash cycle, an enzyme reaction is performed by incubation of 50 μL of substrate (H_2O_2) and 50 μL of chromogene (TMB) for 30 minutes at 37 °C. The reaction is stopped with 100 μL stop solution (2 mol/L H_2SO_4). Plates are then read at 450 nm using ELISA reader (LKB).

Statistical analysis

All statistical calculations (mean values, standard deviations, SD and coefficients of variation, CV) for each group of data were performed with the Statistical Software Program version 5.0 (Primer of Biostatistics, Mc Graw-Hill Companies, Inc., New York, NY).

Results and Discussion

Standard curves

The optimal conditions for immunological reactions were studied in detail (12) during method developpment phase. The standard procedure for INEP anticardiolipin ELISA test was established after examination of the effects of variations of volume, reactant concentrations, time, and temperature on the reaction kinetics and assay performances compared to the chosen commercial assay. The final test procedure (described in *Material and Methods*), as well as an assay range, was shown comparable to the procedure and the results by the commercial test (Axis-Shield). Typical standard curves obtained by the two ELISA tests are represented in *Figure 1*, ELISA tests for aCL IgM and in *Figure 2*, ELISA tests for aCL IgG. Each point on the curve represents the mean value obtained by measurement of two replicates.

Analytical validation

Analytical validation of the two developped ELISA aCL IgM and IgG tests was done according to an established procedure for immunoassay analysis (13, 14). Detection limit, within-test and between-test precision and linearity, as well as reference range were determined and analysed (results below).

Detection limit

Detection limit is defined as the smallest concentration of an analyte that can be detected with reasonable certainty for a given analytical procedure (15). It is recorded as double standard deviation of the determined zero standard concentration (14). Twenty replicates of zero standard were assayed for aCL IgG and IgM using INEP tests. Standard devia-

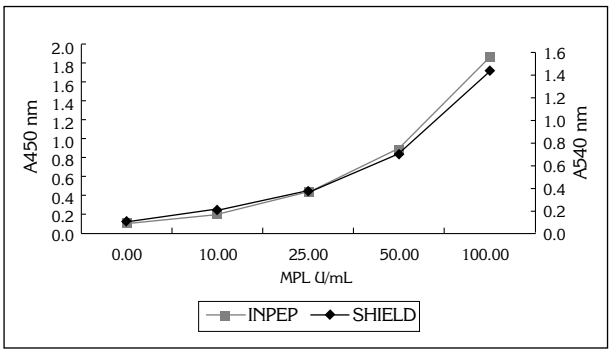

Figure 1 Typical standard curves obtained using the two ELISA tests for aCL IgM. Each point on the curve represents the mean value obtained by measurement of two replicates.

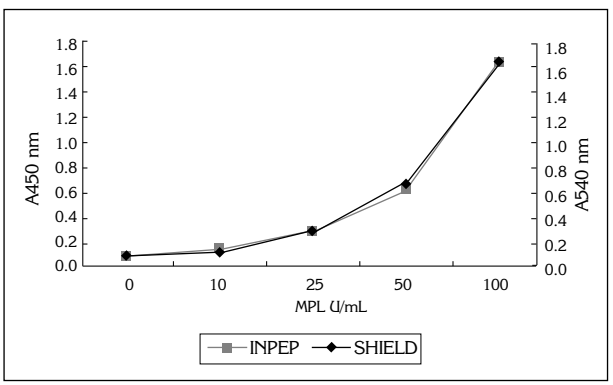

Figure 2 Typical standard curves obtained using the two ELISA tests for aCL IgG. Each point on the curve represents the mean value obtained by measurement of two replicates.

tion for the measured values was calculated and the sum of its double value and the mean value of the zero standard, was plotted on a standard curve. The detection limit determined in a such way for the developed assays is: 5.0 MPL U/mL for IgM aCL, and 2.6 GPL U/mL for IgG.

Within-test precision and between-test precision

Test precision was assessed as reproducibility of measurement (13). Within-test precision was determined by measuring aCL concentration in 10 replicates of the same sample in one test. Precision of the measurement is then calculated as a coefficient of variation (CV) as represented in *Table I*.

Precision between the tests was determined by measuring aCL concentration of the same sample (in duplicate) in 10 independent runs of both IgG and IgM tests. The calculated data for a coefficient of variation (CV) are presented in *Table II*.

Table I ELISA aCL IgM test and ELISA aCL IgG test – within test precision

Samples	Mean value	SD	CV (%)
	MPL, U/mL	MPL, U/mL	
1.	23.3	2.39	10.3
2.	42.0	3.22	7.7
3.	103.8	7.47	7.2
	GPL, U/mL	GPL, U/mL	
4.	22.8	3.98	17.5
5.	47.1	6.69	14.2
6.	88.6	7.41	8.4

Table II ELISA aCL IgM test and ELISA aCL IgG test – between test precision

Samples	Mean value	SD	CV (%)
	MPL, U/mL	MPL, U/mL	
1.	25.6	2.19	8.5
2.	48.3	1.21	2.5
3.	104.8	4.06	3.9
	GPL, U/mL	GPL, U/mL	
4.	24.7	2.03	8.2
5.	45.7	5.16	11.3
6.	106.3	6.80	6.4

Linearity

The linearity of an analytical procedure is its ability to produce results that are directly proportional to the concentration of analytes in the samples. The range of the procedure is an expression of the lowest and highest levels of analyte that can be determined with acceptable precision, accuracy and linearity.

The linearity of both established ELISA aCL tests was evaluated by preparing dilutions of sera with high aCL concentration. Comparisson of measured with the calculated values is presented in *Table III*.

Reference range

Sera of blood donors used for this study were mainly the sera of healthy, young individuals (secondary school students, n = 102). In the group of analysed sera there were two, i. e. 2%, positive for IgM aCL and five sera, i.e. 5%, that were positive for IgG aCL. All of the positive sera were low positive, i.e. the aCL concentrations determined were between 10 and 15 GPL U/mL. All results obtained for each parameter were shown as percent without the lower 2.5% and upper 97.5% values. The mean value ($\bar{x}$) and the standard deviation (SD) for aCL IgM within the population

Table III Linearity of ELISA aCL IgM test and ELISA aCL IgG test

aCL concentration was measured in samples diluted in an assay dilution buffer. aCL concentration was determined both in ELISA aCL IgM and in ELISA aCL IgG. Mean values obtained by measurement of two replicates and percentage (%) of measured concentrations in relation to expected concentrations are presented in table.

Dilution	Expected concentration		Measured concentration		%	
	MPLU/mL	GPLU/mL	MPLU/mL	GPLU/mL	ELISA aCL IgM	ELISA aCL IgG
1:100		/	104	100	/	/
1:200	52	50	48.9	53	94	106
1:400	25	25	24.7	23	99	92
1:800	12.5	12.5	12.2	13	98	104
1:1600	6.25	6.25	6.4	7.7	102	123
1:3200	3.13	3.13	2.6	2.9	83	92

of healthy individuals were 1.41 MPL U/mL and 1.923 MPL U/mL, respectively. The mean value ($\bar{x}$) and the standard deviation (SD) for aCL IgG within the population of healthy individuals were 3.26 GPL U/mL and 2.729 GPL U/mL, respectively. According to the results obtained the reference ranges were determinated to be: 0–5.9 MPL for aCL IgM and 0–10.5 GPL for aCL IgG.

The procedure adopted for the tests developed is comparable to other procedures in commercial ELISA tests. The results presented here enable us to conclude that the established ELISA tests for aCL IgM and aCL IgG may be used for laboratory testing of patients. These tests can be useful in diagnostic procedures where involvement of the autoimmune component is suspected, and paticularly for identification of and folow-up in antiphospholipid syndrome. Using the aCL ELISA tests by INEP described here, several groups of patients that are know to have an increased incidence of aCL, such as SLE (16) and idiopatic infertility (17) have been analysed. In both groups of patients positive individuals were detected with the comparable incidences to those previously reported by other authors. These findings further confirm usefuleness and reliability of the tests introduced.

FORMULISANJE, STANDARDIZACIJA I VALIDACIJA ELISA TESTA ZA ODREĐIVANJE AUTOANTITELA NA KARDIOLIPIN

Slavica Marjanović, Snežana Golubović, Ljiljana Vićovac

Institut za primenu nuklearne energije – INEP, Zemun

Kratak sadržaj: ELISA testom moguće je detektovati prisustvo i/ili odrediti koncentraciju autoantitela na kardiolipin (aCL) određene klase. U ovom radu prikazane su analitičke karakteristike testova za detekciju autoantitela na kardiolipin (aCL) razvijenih u INEP-u. Prikazan je način izvođenja aCL ELISA testa, određen je detekcioni limit, preciznost i linearnost. Opseg normalnih vrednosti za aCL IgM je od 0 do 5,9 MPL U/mL, za aCL IgG od 0 do 10,5 GPL U/mL. Performanse prikazanih INEP aCL IgG i aCL IgM testova, kao i utvrđeni opsezi normalnih vrednosti su uporedivi sa komercijalnim testom Axis-Shield Diagnostics Limited, Scotland UK. Analiza aCL se može koristiti kao dopunski laboratorijski parametar za dijagnostiku i praćenje autoimunih bolesti, a posebno antifosfolipidnog sindroma.

Ključne reči: antikardiolipinska antitela (aCL), antifosfolipidni sindrom (APA), enzimski test na čvrstoj fazi, ELISA

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