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A quantitative enzyme-linked immunosorbent assay (ELISA) for the detection of anti-double-stranded DNA IgG antibodies

Reação imunoenzimática (ELISA) quantitativa para detecção de anticorpos IgG anti-DNA de dupla hélice

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ABSTRACT

Introduction: The detection of anti-double-stranded (ds) deoxyribonucleic acid (DNA) antibodies is one of the classification criteria for diagnosing systemic lupus erythematosus (SLE). **Objective:** To describe a quantitative enzyme-linked immunosorbent assay (ELISA) for detecting anti-dsDNA immunoglobulin class G (IgG) antibodies. **Methods:** The performance of ELISA was evaluated using the *Crithidia luciliae* indirect immunofluorescence test (CLIFT) as a reference. Anti-dsDNA IgG antibodies were screened by ELISA and CLIFT in serum samples from 127 patients with SLE, 56 patients with other diseases and 37 healthy persons. The Cochran Q test was used to compare the sensitivity and specificity of the reactions, with differences among the results being considered significant when $p \leq 0.05$. **Results:** ELISA had a sensitivity of 92.9% and a specificity of 94.6%, whereas the sensitivity and specificity of CLIFT were 85.8% and 100%, respectively. ELISA was significantly more sensitive than CLIFT ($p = 0.0027$), whereas CLIFT was significantly more specific than ELISA ($p = 0.0253$). **Conclusion:** ELISA showed excellent results in terms of sensitivity and specificity, with a potential use in research and routine diagnostics.

Key words: systemic lupus erythematosus; antinuclear antibodies; enzyme-linked immunosorbent assay; indirect fluorescent antibody technique.

RESUMO

Introdução: A detecção de anticorpos contra o ácido desoxirribonucleico (DNA) nativo (ds) é um dos critérios de classificação para o diagnóstico do lúpus eritematoso sistêmico (LES). **Objetivo:** Descrever uma técnica imunoenzimática enzyme-linked immunosorbent assay (ELISA) quantitativa para a detecção de anticorpos imunoglobulina da classe G (IgG) anti-DNAs. **Métodos:** O desempenho da técnica ELISA foi avaliado utilizando o teste de imunofluorescência indireta com *Crithidia luciliae* (CLIFT) como referência. Anticorpos IgG anti-DNAs foram pesquisados por ELISA e CLIFT em amostras de soros de 127 pacientes com LES, 56 pacientes com outras doenças e 37 indivíduos saudáveis. O teste Q de Cochran foi utilizado para comparar as sensibilidades e as especificidades das reações, considerando diferenças significantes entre os testes quando $p \leq 0,05$. **Resultados:** A técnica ELISA apresentou sensibilidade de 92,9% e especificidade de 94,6%, enquanto a sensibilidade e a especificidade da técnica CLIFT foram de 85,8% e 100%, respectivamente. A técnica ELISA apresentou sensibilidade significativamente maior do que a obtida com a técnica CLIFT ($p = 0,0027$); esta apresentou especificidade significativamente maior do que a obtida com a técnica ELISA ($p = 0,0253$). **Conclusão:** A técnica ELISA apresentou excelentes resultados em termos de sensibilidade e especificidade, podendo ser útil em pesquisa e rotina diagnóstica.

Unitermos: lúpus eritematoso sistêmico; anticorpos antinucleares; ensaio de imunoadsorção enzimática; técnica indireta de fluorescência para anticorpo.

RESUMEN

Introducción: La detección de anticuerpos contra el ácido desoxirribonucleico (ADN) de doble cadena (dc) es uno de los criterios de clasificación para el diagnóstico de lupus eritematoso sistémico (LES). **Objetivo:** Describir una técnica inmunoenzimática (ELISA) cuantitativa para detección de anticuerpos de inmunoglobulina de clase G (IgG) anti-ADNdc. **Métodos:** Se evaluó el desempeño de la técnica ELISA mediante el test inmunofluorescencia indirecta con *Crithidia luciliae* (IFI-CL) como referencia. Anticuerpos IgG anti-ADNdc fueron analizados por ELISA y IFI-CL en muestras de sueros de 127 pacientes con LES, 56 pacientes con otras enfermedades y 37 personas sanas. La prueba Q de Cochran fue utilizada para comparar la sensibilidad y la especificidad de las reacciones considerando diferencias significantes entre los tests cuando $p \leq 0,05$. **Resultados:** La técnica ELISA mostró sensibilidad del 92,9% y especificidad del 94,6%, mientras la sensibilidad y la especificidad de la técnica IFI-CL fueron del 85,8% y 100%, respectivamente. La técnica ELISA mostró sensibilidad significativamente mayor que la obtenida con IFI-CL ($p = 0,0027$); esta mostró especificidad significativamente mayor que la obtenida con ELISA ($p = 0,0253$). **Conclusión:** La técnica ELISA presentó resultados excelentes de sensibilidad y especificidad, con el potencial de ser utilizada en investigación y rutina diagnóstica.

Palabras clave: lupus eritematoso sistémico; anticuerpos antinucleares; ensayo de inmovilización enzimática; técnica del anticuerpo fluorescente indirecta.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease in which the production of antibodies to a large variety of self-components may result in a wide spectrum of clinical manifestations and laboratory abnormalities⁽¹⁻⁶⁾. Although the etiology of SLE is still not fully understood, genetic interactions with environmental factors have been implicated in the development of the disease^(5,7-9). Epidemiological studies have shown that SLE can affect persons of all ages and ethnic groups and both sexes, but women of childbearing age and certain ethnic groups (African-Americans and Asians) have a greater predisposition to develop the disease^(1,6,10).

The diagnosis of SLE is based on clinical and laboratory findings. In most cases, diagnosis of the disease is challenging because of the polymorphism of the clinical symptoms. The detection of anti-double-stranded (ds) deoxyribonucleic acid (DNA) antibodies is part of the classification criteria for diagnosing SLE^(11,12). Anti-dsDNA antibodies have usually been detected by Farr radioimmunoassay (Farr-RIA), indirect immunofluorescence using *Crithidia luciliae* as substrate (CLIFT) and enzyme-linked immunosorbent assay (ELISA).

The Farr technique has important characteristics such as its high sensitivity and specificity and the possibility of expressing results in a quantitative form. However, the major disadvantage of the technique is the need to use radioactive materials. The CLIFT technique has a high specificity, but usually has a lower sensitivity than the Farr and ELISA techniques. Other disadvantages of this

technique include the difficulty of processing large numbers of samples, the expression of semi-quantitative results, its operator-dependent nature on their reading, which may lead to subjectivity in interpretation of the results. The ELISA technique permits the simultaneous processing of a large number of samples, allowing the expression of qualitative or quantitative results and shows relatively high sensitivity compared to CLIFT⁽¹³⁻¹⁶⁾. In this study, we describe a quantitative ELISA for detecting anti-dsDNA immunoglobulin class G (IgG) antibodies and compare its performance to that of CLIFT, used as a reference.

METHODS

Chemicals

All chemicals were reagent grade or better and, unless otherwise stated, were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Serum standards

A human serum pool positive for anti-dsDNA IgG antibodies (DNA_{PO}) was prepared by mixing equal parts of serum from 10 patients with SLE who had a positive CLIFT test $\geq 1:640$. The DNA_{PO} was arbitrarily designed as having 100 arbitrary antibody units (AU) per ml. Artificial serum standards containing different AU/ml were prepared by diluting the DNA_{PO} with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T). A human serum pool negative for anti-dsDNA IgG antibodies (DNA_{NE}) was prepared by mixing equal parts of serum from 10 healthy persons

who had a negative CLIFT test and no clinical evidence of SLE. The DNAPo and DNANe pools were used as positive and negative controls in the ELISA and CLIFT reactions.

Determination of optimal reagent concentrations

The ELISA was standardized using excess amounts of all reagents, except for that being tested. For antigen titration, increasing amounts of calf-thymus DNA (0.3 to 100 µg/ml) (Sigma-Aldrich) were used. For conjugate (peroxidase-labeled goat anti-human IgG, Sigma-Aldrich), dilutions from 1:500 to 1:64,000 were tested.

Linearity of substrate conversion

The linearity of substrate conversion was determined using a serum standard containing 50 AU/ml. The rate of substrate conversion was assessed at different intervals of time from 1 to 30 min of incubation at room temperature (RT) in the dark.

ELISA procedure

The wells of U-bottomed ELISA plates (Nunc MaxiSorp®, Thermo Fisher Scientific, USA) were filled with 100 µl of 0.1 M carbonate-bicarbonate buffer, pH 9.5 containing poly-L-lysine [(PLL), 20 µg/ml] (Sigma-Aldrich). After incubating the plate for 2 h at RT and overnight at 4°C, the PLL solution was discarded and the wells were washed three times with 0.15 M PBS, pH 7.4. Calf-thymus DNA diluted to 20 µg/ml in 0.15 M Tris-HCl/NaCl buffer, pH 8, was then added to the PLL-coated wells (100 µl). After a 3.5-h incubation at RT the wells were washed once with PBS-T and the reactive sites in the polystyrene matrix were blocked with 100 µl of PBS-T containing 0.1% bovine serum albumin (PBS-T-BSA). After incubation for 10 min at RT, the wells were washed once with PBS-T and then filled with 100 µl of 50 mM sodium acetate/acetic acid buffer, pH 4.6, containing S1 nuclease (40 U/ml) and BSA (5 mg/ml). Following a 1.5-h incubation at RT, the wells were washed three times with PBS-T, and 100 µl of the serum samples diluted 1:200 in PBS-T-BSA was added to the wells. After a 1-h incubation at RT and washing three times with PBS-T, 100 µl of the conjugate diluted 1:4,000 in PBS-T was added to each well. After a further 45-min incubation at RT and washing three times with PBS-T, 100 µl of the substrate system (0.42 mM tetramethylbenzidine and 1.42 mM H₂O₂ in 0.1 M sodium acetate/acetic acid buffer, pH 5.5) was added to the wells and the plates incubated for 10 min at RT in the dark. After this period, the reactions were stopped by adding 50 µl of 2 N H₂SO₄ to each well, and the resulting absorbance was measured at 450 nm using an ELISA reader (Labsystems, Helsinki, Finland). Each serum sample was assayed in triplicate and the mean absorbance values were calculated. A serial two-fold dilution of positive standard controls (100, 50, 25, 12.5, 6.25, 3.12 and 1.56 AU/ml) was included

in each plate and used to convert the optical density of each serum sample into AU/ml. The cut-off value for the assay was determined using a receiver operating characteristic (ROC) curve.

CLIFT

Anti-dsDNA IgG antibodies were detected by CLIFT using Immuno Concepts (Immuno Concepts N.A. Ltd, Sacramento, CA, USA) or Scimedex (Scimedex Corporation, Dover, NJ, USA) kits, according to manufacturer's instructions. A CLIFT antibody titer $\geq 1:10$ was considered positive.

Patients and controls

Anti-dsDNA IgG antibodies were screened by ELISA and CLIFT in serum samples from 127 patients with SLE (122 females, 5 males, mean age = 34 years), 56 patients with other diseases (36 females, 20 males, mean age = 48 years) (rheumatoid arthritis – $n = 26$; Sjögren's syndrome – $n = 3$; systemic sclerosis – $n = 3$; multiple myeloma – $n = 9$; hepatitis A – $n = 4$; hepatitis B – $n = 6$; cytomegalovirus (CMV) infection – $n = 3$; and infectious mononucleosis – $n = 2$) and 37 healthy persons (23 females, 14 males, mean age = 41 years). All patients were treated at the university hospital of the Universidade Estadual de Campinas (Unicamp), Campinas, SP, Brazil. Control serum samples from healthy persons were obtained from students and laboratory personnel of Unicamp with no history of SLE. This study was approved by the Ethics Committee of the School of Medical Sciences (FCM), Unicamp, in accordance with the resolutions of the Brazilian National Ethics Committee (CAAE: 45707815.5.0000.5404).

Data analysis

The diagnostic accuracy of ELISA and CLIFT was evaluated by the kappa (κ) coefficient, using the SAS® System for Windows, version 9.2 (SAS Institute Inc., Cary, NC, USA). The intra- and inter-assay variations were studied on serum standard containing 50 AU/ml. For determination of intra-assay variation, the standard was tested 27 times on the same plate. The inter-assay variation was determined by assaying the serum standard on 14 alternate days. The Cochran Q test⁽¹⁷⁾ was used to compare the sensitivity and specificity of the reactions. Differences among results were considered significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Based on DNA and conjugate titrations, antigen and conjugate excess was achieved at concentrations > 10 µg/ml and dilutions $< 1:8,000$, respectively. Linearity studies showed that the substrate

conversion rate was linear for at least 12 min for a serum standard containing 50 AU/ml. The ELISA was standardized using a DNA concentration of 20 µg/ml and a conjugate dilution of 1:4,000, and the reaction was stopped 10 min after addition of the substrate system.

Using the cut-off determined by the ROC curve (7.65), ELISA was positive in 118 (92.9%) of the 127 serum samples from SLE patients and negative in all 37 serum samples from healthy persons. Twenty-one of the 127 SLE patients had a positive test for anti-Smith (Sm) antibodies. Although the frequency of detection of anti-Sm antibodies in SLE is low, these antibodies are highly specific for the disease⁽¹⁸⁾. All SLE patients with anti-Sm antibodies also showed significant titers of anti-dsDNA IgG antibodies by ELISA. The ELISA was also positive in five (8.9%) of the 56 serum samples from patients with other diseases: two with rheumatoid arthritis [both patients had positive tests for rheumatoid factor and the third generation of anti-cyclic citrullinated peptide (CCP3) antibodies of IgG isotype], one with systemic sclerosis (the patient had a positive test for topoisomerase 1), one with hepatitis B infection [the patient had a positive test for hepatitis B virus surface antigen (HbsAg)] and one with infectious mononucleosis [the patient had positive immunoglobulin class M (IgM) and IgG anti-Epstein-Barr virus viral capsid antigen (EBV-VCA) tests, with an Epstein-Barr virus nuclear antigen (EBNA) negative assay]. None of these five patients had clinical evidence of SLE. Other studies also detected positive reactions to anti-dsDNA antibodies by ELISA in patients with rheumatoid arthritis⁽¹⁹⁻²¹⁾, systemic sclerosis^(15, 22), hepatitis B⁽²³⁾, and mononucleosis^(15, 20).

The CLIFT assay was positive (titers $\geq 1:10$) in 109 (89%) of 127 serum samples from SLE patients, and negative in all 56 serum samples from patients with other diseases and in the 37 serum samples from healthy persons. Nine patients with SLE had a positive anti-dsDNA antibody result with ELISA and negative result with CLIFT. Based on their clinical history, these nine patients had a confirmed diagnosis of SLE, according to the American College of Rheumatology (ACR) criteria. Nine patients with SLE had no anti-dsDNA antibodies detected by CLIFT and ELISA. All these nine patients were on immunosuppressive drugs at the time of serum sample collection.

ELISA and CLIFT showed high diagnostic accuracy, with k values of 0.87 and 0.84, respectively. The intra- and inter-assay coefficients of variation of ELISA were 3.1% and 9.6%, respectively.

The results obtained with ELISA and CLIFT for detecting anti-dsDNA IgG antibodies are shown in the **Table**. In summary, ELISA showed sensitivity of 92.9% and specificity of 94.6%, whereas CLIFT showed sensitivity and specificity of 85.8% and 100%, respectively. Based on the statistical analysis: 1. ELISA had significantly greater sensitivity than CLIFT ($p = 0.0027$); and 2. CLIFT had significantly greater specificity than the ELISA ($p = 0.0253$).

The ELISA described here was standardized by taking into account two requirements for a quantitative technique, namely, 1. all reagents except the one being tested (the antibodies, in this case), were present in excess; and 2. the enzymatic activity was measured during the initial linear portion of the reaction, when the rate of substrate conversion was directly proportional to the antibody concentration. In addition, the use of a standard curve to determine the concentration of anti-dsDNA antibodies and subsequent expression of the results in arbitrary units of antibodies markedly reduced the variability of the findings^(24, 25).

The literature shows great variation in the performance of ELISAs for anti-dsDNA IgG antibodies, with sensitivity varying from 19% to 98% and specificity from 73% to 99%^(15, 18, 19, 26-29). The ELISA described here showed excellent performance, with 92.9% sensitivity and 94.6% specificity. Variations in the performance of serological reactions used to detect anti-dsDNA antibodies are expected and are probably related to several factors, such as the heterogeneity of the patients included in the studies, the patients' immune status at the time of their participation in the study, the intrinsic properties of the techniques used for antibody screening, the antigen preparation, the diversity of antibodies produced by the patients and the cut-off calculation method.

CONCLUSION

The excellent performance of the ELISA described here makes this assay a potentially useful tool for detecting anti-dsDNA IgG antibodies in research and routine diagnosis.

TABLE – ELISA and CLIFT results in patients with SLE, patients with other diseases and healthy persons

	ELISA				CLIFT	
	Patients with SLE ($n = 127$)	Patients with others diseases ($n = 56$)	Healthy persons ($n = 37$)	Sen (%)	Spe (%)	Sen (%)
$\bar{x}$	44.3	4.35	4.4	92.9	94.6	85.8
V	(3.25-100)	(0.78-16.2)	(1.66-7.47)			100

ELISA: enzyme-linked immunosorbent assay; CLIFT: Crithidia luciliae indirect immunofluorescence test; SLE: systemic lupus erythematosus; Sen: sensitivity; Spe: specificity; $\bar{x}$: arithmetic mean (AU/ml); V: variation (minimum and maximum values, in AU/ml); AU: arbitrary units.

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