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Comparison of four methods of detection of anti-double-stranded DNA in SLE patients

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Abstract

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease. Anti-double stranded DNA (anti-dsDNA) detection is essential for diagnosis and assessment of disease severity and lupus nephritis. Variable laboratory tests for Anti-dsDNA detection have different qualities affecting the results and the disease diagnosis. This study aimed to compare the performance of four different methods of detection of anti-dsDNA among SLE patients in Sohag Governorate. This Case -control study was done in Sohag University Hospital during the period from March 2021 to June 2022 and included 81 cases diagnosed with SLE according to the ACR/EULAR 2019 classification criteria for SLE. We compared serum anti-dsDNA antibody levels by different commercially available kits including *Crithidia luciliae* indirect immunofluorescence assay (CLIFT), chemiluminescence immunoassay (CLIA), enzyme linked immunosorbent assay (ELISA) and dot immunoassay results. ELISA showed the highest positivity (75.3%), followed by CLIA (61.7%), dot immunoassay (49.4%) and CLIFT (48.1%), respectively. Combining the four methods of detection, 45.7% of the cases showed positive by all of the four detection methods. Most of the other cases were at least positive in two or three tests. Only 17.3% of the cases were negative by all of the four detection methods. None of the subjects in the control group were positive by any test. In conclusion for the detection of anti-dsDNA antibodies, ELISA showed the highest sensitivity. However, the combination of more than one method revealed higher sensitivity.

Keywords: Anti-double stranded DNA, Dot Immunoassay, and Indirect immunofluorescence assay.

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Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with multi-organ affection and formation of autoantibodies against nuclear and cytoplasmic antigens, among them the antinuclear antibodies (ANA) are the most common antibodies.[1, 2] A positive

ANA test by immunofluorescence remains a hallmark of SLE due to its high sensitivity ranging from 95% to 97%. The ANA test was included as an entry criterion in the American College of Rheumatology (ACR) / European League Against Rheumatism (EULAR) criteria of 2019 for SLE diagnosis.[3, 4]

Anti-double stranded DNA (anti-dsDNA) antibodies are one of the most distinguished ANA types, characterized by a high specificity (96%) for SLE.[5] Also, it was set as the highest weight element in the immunologic domain of the 2019 EULAR/ACR classification.[3] Positive anti-dsDNA antibodies were significantly linked to lupus nephritis.[5] These antibodies were found deposited in glomeruli, basement membrane in SLE patients with nephritis[2]. However, their diagnostic sensitivity is low (52% to 70%) as their presence in patient serum is transient and related to disease activity.[1, 5.]

Many laboratory testing methods are available to detect anti-dsDNA antibodies. These detection methods showed variable specificity which, consequently affect SLE diagnosis.[1] The oldest test, the Farr radioimmunoassay (Farr-RIA) which was considered as a gold standard test due to its high sensitivity but the most drawbacks of this test were the use of unsafe radioisotopes for precipitation of the dsDNA.[6] The test was widely replaced by the use of *Crithidia luciliae* indirect immunofluorescence assay (CLIFT) which is a fluorescence based method using the kinetoplast (compacted dsDNA in the mitochondrion) of the *Crithidia* cell, This method is time consuming and depend on personal skills with variable results between laboratories.[7]

With the development of the enzyme immunoassays (EIAs) as enzyme-linked immunosorbent assays (ELISAs) method which depend on antigen bound to the solid surface of microtiter plate with the advantage of quantification of bound antibodies. The method can be performed in automated or semi-automated system overcoming subjective skills and allow processing of many samples in the same time.[6] Also, relatively new technologies as chemiluminescent immunoassay (CIA),[8] fluorescence enzyme immunoassay (FEIA) based on fluorescence or luminescence as readout have been used. Recently, multiplex immunoassays (MIA) detecting more than one anti-antibody in the same test have been developed to help diagnosis of autoimmune diseases.[6, 9]

However, there is still difference in the specificity and sensitivity of these different tests among laboratories worldwide which affect the diagnosis of SLE.[5, 10, 11] This study aimed to compare the performance of four different commercial kits for detection of anti-dsDNA among SLE patients in Sohag University Hospital. They included; CLIFT, chemiluminescent immunoassay (CLIA), ELISA and dot immunoassay.

Subjects and Methods

This case-control study was done in Sohag University Hospital during the period from March 2021 to June 2022. A total of 81 newly diagnosed cases with SLE according to the 2019 ACR/EULAR classification criteria for SLE [2] were recruited from the patients visited the rheumatology clinic at Sohag University Hospital. Another 20 age and sex matched normal subjects were included as a control group.

Method

A venous blood sample (10 ml) was withdrawn from each subject under aseptic conditions and immediately delivered into vacutainer tubes. The serum was separated using the standard protocol of the hospital laboratory. We compared the Anti-dsDNA antibody levels as determined by CLIFT with, CLIA, ELISA and the dot immunoassay.

Detection of anti-dsDNA antibody by the Crithidia luciliae indirect immunofluorescence test (CLIFT)

Anti-dsDNA antibodies were detected by an indirect immunofluorescence (IF) method using commercial kits (ALPHADIA SA/NV, BELGIUM). *Crithidia lucilliae* was used as the substrate and approximately 20 – 30 µl of controls and 20 – 30 µl of patient sera (diluted 1:10) were added to each well. The slide was incubated for 30 minutes in a moist chamber at room

temperature. Slides were rinsed carefully with phosphate buffer saline (PBS), then the slide was placed into a Coplin jar filled with PBS for about 5 minutes and this was repeated using fresh PBS. Then, the slide was removed from the wash buffer and the excess PBS was drained and blotted from around the wells using a blotting strip. One drop (20-30 µl) of fluorescein isothiocyanate (FITC) IgG Conjugate was delivered per antigen well and incubated for an additional 30 minutes in a moist chamber at room temperature.

After washing with PBS as before, 4 – 5 drops of mounting media were applied to the slide and then a coverslip was placed gently over the slide, and the slide was read using a fluorescence microscope (Carl Zeiss Micro Imaging, GmbH 37081 Gottingen, Germany) at 40X power. A positive test was considered at a titer of 1:10 or above.

Assessment of anti-dsDNA Antibody by the chemiluminescent immunoassay (CLIA) Method

Anti-dsDNA IgG antibodies in serum specimens were detected using commercial CLIA-dsDNA IgG kits (Catalog #:C89015G, supplied by YHLO BIOTECH CO., P.R. China) on a chemiluminescent immunoassay analyzer (iFlash 3000 CLIA analyzer, China).according to the manufacturer's instructions. The antibody levels were expressed as IU/ml and a negative value was considered as < 24.0 IU/ml. A value of 24.0 to < 36.0 IU/ml was considered borderline and ≥ 36.0 IU/ml was considered positive.

Assessment of anti-dsDNA antibody by the enzyme-linked immunosorbent assay (ELISA) method

Anti-dsDNA antibodies were detected in serum samples using enzyme-linked immunosorbent assay (ELISA) commercial kits (Catalog #: ORG 604, ORGENTEC Diagnostika, Germany), according to the manufacturer's instructions.

The absorbance optical density (OD) of the final ELISA products was measured at 450 nm using a microplate reader (Thermo Fisher Scientific Multiskan EX Microplate Reader, Thermo Fisher Scientific, Finland). A negative value was considered at less than 20 IU/ml. A value of ≥ 20 IU/ml was considered positive.

Assessment of antinuclear antibodies (ANA) by the Dot Immunoassay method

Serum levels of ANA were assessed using commercially available dot immunoassay kits (Code: ANA19Q-24, BlueDiver Quantrix ANA19 IgG kit, D-Tek, Belgium) using immunoblot automated instrument (BlueDiver Instrument (BDI), D-Tek, Belgium), according to the manufacturer's instructions.

Dried strips were visually inspected for staining and imaged using a scanner (Bluescan scanner1, Eurpean Economic Community). The evaluation of the results was performed via the Dr DOT Software. The manufacturer's recommended a cut-off value of 6 U/ml for all antigens. Positive results for a specific antibody were considered when the result >12 U/ml. Low positive results (results comprised between 6 to 12 U/ml), although valid, were considered equivocal.

Statistical Methods

Data were analyzed using STATA version 14.2 (STATA Statistical Software: Release 14.2 College Station, TX: Stata Corp LP.). Quantitative data are represented as mean, standard deviation, median and range. Qualitative data are presented as number and percentage and compared using Chi square test. A p value of < 0.05 was considered significant.

Results

The study included 101 subjects (81 SLE cases and 20 controls). The mean age of the study subjects was around 30 years, with a female predominance of over 85%.

Regarding the different methods of detection of anti-dsDNA, we found that ELISA showed the highest positivity (75.3%), followed by CLIA (61.7% positive; 6.2% equivocal), then Dot Immunoassay (49.4% positive; 28.4% equivocal) and lastly CLIFT (48.1% positive; 3.7% equivocal). Considering the equivocal subjects as positives, the dot immunoassay showed the highest positivity (78.8%), followed by ELISA (75.3%), then CLIA (63.9%) and lastly CLIFT (49.9%). None of the control subjects showed positive anti-dsDNA by any of the 4 methods (Table 1).

Table 1. Comparison between study Cases and Controls.

Item		Cases	Controls	*p* value
Age	Mean ±SD	30.20±10.03	30.75±8.12	NS
Sex	Male	10 (12.3%)	3 (15%)	NS
	Female	71 (87.7%)	17 (85%)	
Dot immunoassay	Titer (Mean±SD)	23.07±25.13	0.60±0.53	<0.001
	Negative	18 (22.2%)	20 (100%)	<0.001
	Equivocal	23 (28.4%)	0	
	Positive	40 (49.4%)	0	
CLIA	Titer (Mean±SD)	126.27±121.36	2.08±0.08	<0.001
	Negative	26 (32.1%)	20 (100%)	
	Equivocal	5(6.2%)	0	
	Positive	50(61.7%)	0	
ELISA	Titer (Mean±SD)	131.98±141.31	6.98±1.43	<0.001
	Negative	20 (24.7%)	20 (100%)	
	Positive	61 (75.3%)	0	
CLIFT	<1/10	39 (48.1%)	20 (100%)	0.003
	1/10	4 (4.9%)	0	
	1/20	5 (6.2%)	0	
	1/40	8 (9.9%)	0	
	1/80	13 (16%)	0	
	1/160	12 (14.8%)	0	
	Negative	39 (48.1%)	20 (100%)	<0.001
	Equivocal	3 (3.7%)	0	
	Positive	39 (48.1%)	0	

$p > 0.05$ is not significant (NS).

Combining the four methods of detection, we found that around 45.7% of the cases showed positive results (including equivocal cases) in all of the four detection methods. Most of the other cases were positives with at least two or three tests. Subjects singly positive with CLIA accounted for 4.9% and 2.5% singly positive with the dot immunoassay. On the other hand, 17.3% of the cases were negative by all of the four detection methods (Table 2).

Table 2. Single and combined methods for anti-dsDNA detection among studied subjects.

Test		Cases	Controls
Individual positive test	Negative all	14	17.3%
	Positive Dot immunoassay only	2	2.5%
	Positive CLIA only	4	4.9%
	Positive Dot + ELISA	5	6.2%
	Positive Dot + CLIA + ELISA	14	17.3%
	Positive Dot + ELISA + CLIFT	5	6.2%
	Positive All	37	45.7%
Number of positive tests	None	14	17.3%
	1	6	7.4%
	2	5	6.2%
	3	19	23.5%
	All	37	45.7%

Discussion

Anti-dsDNA antibody testing is essential for the diagnosis and monitoring of SLE disease activity. However, different laboratory methods yield considerably different results, which often lead to clinical misinterpretation. In this study, we evaluated four different methods for diagnosis and assessment of SLE disease activity based on detection of anti-dsDNA, and included CLIFT, CLIA, ELISA and Dot Immunoassay.

The current study included 81 newly diagnosed SLE cases and 20 controls. The mean age of the study groups was around 30 years, with more than 85% of both groups being females.

This female predominance is well recognized in SLE and was reported by many studies.[7, 12, 13] where 80 to 90% of their cases were females. However, the mean age of our study group is lower than reported by a recent study by Chang et al., 2021, where their patients mean age were 47 years.[8] Also, Dalgiç et al., 2020, reported mean age of 41 years of their study group.[14] In this study we focused on newly diagnosed patients to evaluate different tests detecting anti-dsDNA to diagnose SLE disease.

The ELISA method has the advantages of quantitative results, availability in laboratories and ability to detect antibodies of both low and high avidity.[15] In our study the ELISA sensitivity was 75.3%. The high sensitivity of ELISA was reported by many studies. A study by Chang et al., 2021, reported sensitivity between 59% and 66.7% depending on disease activity.[8] The study by Cavalcante et al., 2019, reported 92.9% sensitivity.[11] The study by Zhao et al., 2019, reported sensitivity of 66, 8% in a study of Chinese patients.[16] Also, Dalgiç et al., 2020, observed that the sensitivity of ELISA was 83% which was higher compared to CLIFT sensitivity (72%).[14]

In this study CLIFT showed the lowest sensitivity (48.1%). The test results depend on manual processing steps and on the technician skills and tend to have variable results among laboratories. However, many studies reported higher sensitivity of the test as a study by Cavalcante et al., 2019, reported sensitivity of 85.8%.[11] In a systemic review of 13 studies the sensitivity of CLIFT ranged from 5.7 % to 55.8 %.

However, the test was reported to have the advantage of high specificity 96.5%–99%.[6] In our study all of the positive cases by CLIFT were positive by any other method ensuring high specificity.

In this study the sensitivity of CLIA was 61%. This is slightly higher than that reported by He et al., 2023, in a study included SLE patients, healthy controls and patients with other autoimmune diseases. They reported that the sensitivity of YHLO CLIA and CLIFT were 58.2% and 55.3%, respectively and their specificities were 95.1% and 99.3%, respectively17. In a recent review of literature by Cockx et al., 2021, included 6 studies which used CIA, the reported specificity and median (range) were 87.4% (70.7%–97.7%) and the reported sensitivity and median range 66.5% (20.0%-86.6%).[6] These studies used different kits than ours however, the results are similar. In a former study by van der Pol et al., 2018, reported that the sensitivity of CIA was 99% using fully automated immunoassay system.[9]

In this study we used automated enzyme immunoassay, the Dot Immunoassay (BlueDiver Quantrix ANA19 IgG), the test detected 19 autoantibodies. The sensitivity of anti-dsDNA detection was 49.4% which is comparable to CLIFT sensitivity (48.1%). In addition, it has the advantage of detecting other autoantibodies as anti-smith antibody which is one of the immunological criteria of ACR/EULAR classification.[3]

The use of multiple testing methods to detect Anti-dsDNA was greatly recommended by many studies to increase the sensitivity of detection.[5, 6, 8] In the current study, ELISA showed the highest positivity (75.3%), followed by CLIA (61.7% positive; 6.2% equivocal), then Dot Immunoassay (49.4% positive; 28.4% equivocal) and lastly CLIFT (48.1% positive; 3.7% equivocal). Only 6 cases were negative by ELISA and positive by other methods (4 were positive by CLIA and 2 were positive by dot immunoassay). So, the sensitivity of the combination of these methods would increase from 75.3% if we used ELISA alone to 83.7% if we used ELISA + CLIA + Dot Immunoassay. In this study, all of the four tests showed 100% specificity, which may be due to the limited number of controls, and due to the fact that we included apparently healthy controls.

Limitations of this study include low number of control subjects. Moreover, these controls were apparently healthy controls, which would be expected to test negative for anti-dsDNA whatever the test done, and this affects the specificity statistics calculations. It would be better to include enough number of other autoimmune disorder patients (such as rheumatoid arthritis, systemic sclerosis, polymyositis/dermatomyositis etc.) who may show positive anti-dsDNA.

In conclusion, for the detection of anti-dsDNA, ELISA showed the highest sensitivity, followed by CLIA then Dot Immunoassay and lastly CLIFT. The combination ELISA with Dot immunoassay and CLIA revealed higher sensitivity.

Author Contributions

AHA, reviewed the protocol, laboratory work, wrote and submitted the manuscript, the corresponding author. HMH and AMG, Reviewed the protocol, laboratory work, shared in writing and reviewing the manuscript**.** MAI, Reviewed the protocol, collecting data, clinical assessment of the patients, shared in writing and reviewing the manuscript**.** AK and HS, approved the protocol, clinical assessment of the patients, reviewed the manuscript. ARR, Approved the protocol, statistical analysis, reviewed the manuscript.

Declaration of Conflicting Interests

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Ethical approval

The study protocol was reviewed and approved by the Medical Ethical Committee of the Faculty of Medicine, Sohag University (IBR#: Soh-Med-21-03-13).

Informed consent

All of the study participants signed informed consents before inclusion in the study.

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