Int J Clin Exp Pathol 2011;4(8):748-754
www.ijcep.com /IJCEP1109010

Original Article

Evaluation of a high avidity anti-dsDNA IgG enzyme-linked immunosorbent assay for the diagnosis of systemic lupus erythematosus

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Received September 27, 2011; accepted October 29, 2011; Epub October 30, 2011; Published November 31, 2011

Abstract: The high avidity (HA) anti-dsDNA IgG ELISA is considered highly specific for the diagnosis of systemic lupus erythematosus (SLE). The main objective of this study was to determine the performance of this test with existing assays for detecting anti-dsDNA IgG antibodies as well as assess its analytical characteristics. For method comparison studies, we investigated the correlation between the HA ELISA with 8 other assays for the detection of dsDNA IgG antibodies namely; six anti-dsDNA IgG ELISA, the Crithidia luciliae immunofluorescence test (CLIFT) and an in-house developed Farr radioimmunoassay (RIA). Overall, 125 patient (100 ANA-positive, 25 CLIFT-tested) and 100 healthy control samples were tested. The assay was also evaluated for imprecision, lot-to-lot consistency and the effect of interfering substances using commercial quality control materials based on the manufacturer's claims unless otherwise stated. Of the 100 ANA positive samples, 18 were positive in the HA ELISA with significant levels of antibodies in the six ELISAs and CLIFT. The HA ELISA had a specificity of 100% with an overall agreement of 84% with the RIA. Intra - and inter-assay imprecision ranged from 13.9-16.5% and the reproducibility between lots based on qualitative interpretation was 100%. Hemoglobin, bilirubin and lipemia showed variable interference with assay performance based on the manufacturer's claims and our in-house protocol. Our data suggest that the HA ELISA although less sensitive than the other dsDNA IgG assays evaluated, is specific and predicts high levels of anti-dsDNA IgG antibodies.

Keywords: Performance, agreement, imprecision, anti-dsDNA, antibodies

Introduction

The presence of anti-dsDNA IgG antibodies is considered diagnostic of systemic lupus erythematosus (SLE), an autoimmune disorder that is characterized by chronic inflammation and production of several autoantibodies [1-3]. Anti-dsDNA antibodies can be detected by a variety of test systems the most common of which include, enzyme-linked immunosorbent assay (ELISA), Crithidia luciliae immunofluorescence test (CLIFT) and the Farr radioimmunoassay (RIA) that is based on the ammonium sulfate precipitation of immune complexes [2-10]. These antibodies are heterogeneous with respect to avidity, class, cross-reactivity and clinical relevance. It has long been established that the analytical principle of the anti-dsDNA IgG antibody assay determines both its diagnostic and predictive capabilities in SLE [2, 4, 6-10]. High avidity anti-dsDNA antibodies as detected by CLIFT and/or Farr assays have been reported to have good positive predictive values for SLE while ELISAs have largely been reserved as screening tools [2, 5-6].

There are several evidences that point to SLE as an immune-complex disease in which inflammatory processes are initiated by local deposition of DNA or anti-dsDNA complexes. In this regard, some reports indicate that changes in the level of anti-dsDNA in an individual patient may provide clues to a patient's disease status in relationship to active disease or remission. Indeed, it has been reported that levels of anti-dsDNA antibodies in serum tend to reflect disease activity but not in all patients [11]. In patients who have both elevated levels of anti-dsDNA autoan-

tibodies and clinically quiescent disease, 80% have disease that becomes clinically active within 5 years after the detection of elevated levels of these antibodies [12]. In addition, high avidity anti-dsDNA antibodies are more closely associated with renal involvement and/or disease activity than intermediate or low-affinity anti-dsDNA antibodies [11, 13, 15-19].

A high avidity (HA) anti-dsDNA IgG ELISA (INOVA Diagnostics, San Diego, USA) formerly referred to as the FARRYZME high avidity anti-dsDNA IgG assay (The Binding Site, Birmingham UK) is designed to detect high avidity anti-dsDNA IgG antibodies [14-16]. Based on the ammonium sulphate precipitation of the dsDNA antigen, the principle of the HA ELISA is thought to be similar to that of the Farr radioimmunoassay with the advantage that no radioactive substance is employed. This study was designed to evaluate the analytical concordance of this HA ELISA with six commercially available ELISAs, the CLIFT and in-house developed Farr RIA for detecting anti-dsDNA IgG antibodies. The assay was also investigated for imprecision as well as the effect of interfering substances on test performance.

Materials and methods

For this study, we used 100 anti-nuclear antibody (ANA) positive sera with a homogeneous pattern and titers ≥1:160 by indirect immunofluorescence assay (IFA) on HEp-2 cells and 100 adult "healthy" control (HC) samples as previously described [20]. To determine correlation between the Farr radioimmunoassay (RIA) and HA ELISA, 10 negative (<1:10) and 15 positive (≥1:10) previously tested specimens by CLIFT were evaluated. For the method comparison studies, all 100 ANA positive and 100 healthy control samples were screened for the presence of anti-dsDNA IgG antibodies using six commercial ELISAs from AESKU Diagnostics, The Binding Site (TBS), Bio-Rad Laboratories, Euroimmun, Dr. Fooke Laboratories, and INOVA Diagnostics, CLIFT (INOVA) and HA ELISA (INOVA) according to manufacturers' guidelines. To determine the correlation between the HA ELISA and Farr RIA, 25 previously tested specimens by CLIFT were identified and referred to Quest Diagnostics (Valencia, CA) for testing using their in-house developed assay.

To evaluate the analytical performance of the HA ELISA; lot-to-lot variation, imprecision, and interference studies were performed. For the determination of lot-to-to variation, 25 previously tested samples by CLIFT were tested with two distinct lots of the HA ELISA kits. Imprecision studies were performed with three samples; one negative, one low positive and one high positive which showed consistent results between the two lots. All three samples were stored at 4 degrees Celsius (4°C) until used. Samples were tested twice a day in triplicate, with a minimum of 2 hours separating each run for three consecutive days. The resulting data was used to calculate between (inter) and within -run (intra) CVs. For the interference studies, we employed previously tested specimens which were either negative, low positive or high positive in both lots of the HA ELISA. These samples were supplemented with various concentrations of hemoglobin (486 mg/dL), bilirubin (20.3 mg/dL) or lipemia (2000 and 1000 mg/dL) according to manufacturer's recommendation or our in -house protocol. Spiked pools were tested in triplicate by the HA ELISA and a deviation of more than 10% from the target concentration was considered to be a significant interference.

For statistical analyses, manufacturers' suggested cut-offs were applied to create positive and negative values from the continuous original observations. Positivity rates, specificities and Spearman correlation coefficient between assays were calculated as indicated using SAS® software, Version 9.2 of the SAS system for Windows. Copyright© 2002-2008 SAS institute Inc., NC, USA. The percent agreements between the assays investigated and HA ELISA were calculated with HA as reference test, unless where indicated. All other calculations were performed using Excel.

Results

To evaluate the performance characteristics of the newly developed high avidity anti-dsDNA IgG ELISA, we first compared its agreement with 7 other tests for the detection of this analyte in 100 ANA IFA positive sera which had a homogeneous pattern and titers greater than or equal to 1:160. For this analysis, we also included a cohort of 100 healthy donors. In the ANA positive group, the positivity rates ranged from 55.0 to 88.0% for the 6 anti-dsDNA IgG ELISAs and CLIFT while that of the HA ELISA was only 18%. Compared to all other assays which had specificities ranging from 84.0 to 92.0%, the HA

Table 1. Analytic concordance between the High Avidity ELISA and 7 other assays for detecting anti-dsDNA IgG antibodies

Assay	% Positive Agreement	% Negative Agreement	% Overall Agreement	Spearman's rho[z]
AESKU	100	52.8	57.0	0.71
TBS*	100	68.7	71.5	0.86
Bio-Rad	100	75.8	78.0	0.83
Euroimmun	100	77.5	79.5	0.68
Dr. Fooke	100	68.1	71.0	0.82
INOVA	100	76.9	79.0	0.76
CLIFT	100	21.4	67.0	0.72

TBS*: Total Binding Site (Kit was provided by INOVA Diagnostics). Spearman's rho[z]: Spearman's rank correlation coefficients were significant at $p<0.0001$ for the indicated anti-dsDNA IgG assays and HA ELISA in the ANA positive group only.

ELISA had an excellent specificity of 100%. Our agreement analyses between the HA ELISA and anti-dsDNA IgG ELISAs or CLIFT in the ANA-positive showed excellent positive agreement. However, the negative and overall agreements with specific assays were highly variable with implications for diagnostic use (**Table 1**). Since the principle of the HA ELISA is meant to be similar to that of the Farr RIA by utilizing conditions that preclude low avidity antibodies, we used a set of 25 sera that had previously been tested by CLIFT (15 positive and 10 negative samples) to evaluate the correlation between both assays. The overall agreement between the HA and the Farr (as reference test) was 84.0%, with positive and negative agreements of 85.7% and 81.8% respectively. Overall, both assays showed similar correlations with the results from the CLIFT analyses (data not shown).

We also investigated the association between HA assay and the different anti-dsDNA IgG tests based on antibody levels. Our data indicate significant correlation between the HA assay and elevated levels of anti-dsDNA IgG antibodies detected by the six different ELISAs and the CLIFT (**Table 2**). Of the HA- and CLIFT-positive samples, only 1 (5.6%) specimen had a CLIFT titer ≤1:20. Similarly, of the HA- and ELISA-positive samples, between 89.0 and 100% had levels greater than three times the cut-off of the respective assay (**Table 2**). The Spearman's rank correlation coefficients of the different assays ranged from 0.68 to 0.86 with The Binding Site assay showing the best correlation based on increasing levels of antibodies (**Table 1**).

Our observation that the positivity by the HA ELISA was associated with significantly elevated levels of anti-dsDNA IgG antibodies as well as the manufacturer's claims that this assay could possibly be used to monitor response to treatment and/or predict disease flares, prompted us to determine consistency in test performance between lots. To investigate these two factors, we selected 25 previously tested sera by CLIFT and tested all samples with two distinct lots of the HA ELISA. Based on our initial observations, we expected all CLIFT-negative samples to be negative and for some of the CLIFT-positive samples (based on the antibody titers) to be positive in both lots evaluated. For this reason, we simply examined the concordance between the expected and observed results as well as reproducibility of test results between the different lots. For both lots, we observed positivity and negativity rates of 56% and 46% respectively. The reproducibility between lots was 100%. Next, we determined imprecision using samples which tested negative (<30 IU/mL), low positive (>30.0 IU/mL but less than 100.0 IU/ml) or high positive (>100.0 IU/mL). A summary of results from the imprecision study is provided in **Table 3**. For the negative sample with a mean antibody concentration of 10.5 IU/mL, the within run (intra-assay) % CV was estimated to be 9.7% with an inter-assay CV of 16.5%. The low positive sample had a within run % CV of 2.0% and the inter-assay CV was estimated at 13.9%. The high positive sample had the least within run CV and an inter-assay CV of 14.2%. Overall, the HA assay demonstrated good intra-assay (CVs < 10%) with marginal inter-assay precision (CVs ranging from 13.9 to 16.5%).

Conditions of hemolysis, icterus and lipemia were simulated in an interference study and a deviation of more than 10% from the target con-

Table 2. Anti -dsDNA IgG antibody levels in the 18 HA ELISA-positive subjects

ID	ANA ≥1:160	AES ≥15.0 IU/mL	TBS ≥30.0 IU/mL	BR ≥25.0 IU/mL	EI ≥1.0 IU/mL	DF ≥1.0 RU*	IN >201.0 IU/mL	CLIFT >1:10	HA ≥30.0 IU/mL
1	320	50	363	609	1	3.5	744	>40	37
2	640	145	556	734	6.5	3.8	839	1320	38
3	1280	211	3947	752	7.7	7.3	1570	5120	355
4	160	171	222	544	3.7	3.4	504	80	66
5	320	78	358	489	4.8	3	511	320	133
6	5120	209	4304	761	7.8	5.8	1451	>40	311
7	160	116	406	676	3.6	2.8	637	160	43
8	640	191	1890	789	5.8	6.2	1335	1280	168
9	320	210	3706	778	5.7	7	1591	12560	291
10	10240	205	4104	784	8.6	7.1	1537	12560	286
11	1280	199	7550	756	7	5.9	1655	12560	417
12	640	140	391	521	6.6	4	889	80	41
13	320	198	1323	741	4.6	5.7	1131	1280	185
14	160	209	2707	755	5.3	6.8	1517	1280	93
15	1280	150	842	758	4.3	3.8	1259	640	101
16	160	202	965	756	4.1	5.9	1152	20	77
17	160	152	354	530	2.3	3.9	432	160	64
18	160	109	622	611	3	4.5	1055	160	30

ID: Sample identification, ANA: Antinuclear antibody by IFA; AES: AESKU; TBS: Total Binding Site (provided by INOVA Diagnostics); BR: Bio-Rad; EI: Euroimmun; DR: Dr. Fooke; IN: INOVA; HA: High Avidity ELISA. RU* denotes relative unit

Table 3. Summary of Imprecision studies

Sample	CV%		
	Mean Concentration (IU/mL)	Intra-assay	Inter-assay
Negative	10.5	9.7	16.5
Low positive	37.8	2.0	13.9
High positive	749.5	0.9	14.2

centration was considered to be a significant interference. Hemoglobin and bilirubin showed significant interference (>10%) with the negative but not the positive serum samples, at the concentrations reported by the manufacturer with no significant changes in the qualitative results. Based on our in-house protocol, lipemia showed significant interference with low-positive HA ELISA results at a concentration of 2000 mg/dL. Since we were unable to evaluate the effect of interference at the manufacturer's lipemic concentration of 1460 units, we further evaluated the effect of a lower level of lipemia (50% of the in-house lipemic concentration). At this concentration, no interference was observed (**Table 4**).

Discussion

The presence of anti-dsDNA is one of the diagnostic criteria for SLE by the American College of Rheumatology (ACR). In routine practice, clinical diagnostic laboratories use the ELISA, Farr RIA or CLIFT to detect and quantify these antibodies to diagnose and/or rule out SLE, monitor disease activity or predict flares. However, it has since been recognized that the assay principle of an anti-dsDNA IgG test determines its clinical relevance. In this study, we investigated the performance characteristics of the newly developed high avidity anti-dsDNA IgG antibody ELISA. Two main goals were accomplished in this investigation. First, we evaluated the analytical concordance of this relatively new assay with six commercially available ELISAs, the CLIFT and an in-house developed Farr RIA for detecting anti-dsDNA IgG antibodies. Secondly, we assessed the performance characteristics based on lot-to-lot variation, imprecision and the effect of common interfering substances that may affect its performance.

Based on the data from the ANA positive cohort, only 18 of the 100 samples tested positive by the HA ELISA. All 18 HA ELISA-positive specimens were also positive in the 7 other assays investigated with levels equal to or greater than

Table 4. Effect of interfering substances on the performance of the HA ELISA

Interfering substance & conc. tested	Specimen type	Conc. Unspiked (IU/mL)	Conc. Spiked (IU/mL)	Deviation observed (%)
Hemoglobin (486 mg/dL)	Negative	8.8	12.0	36.4
	Low positive	61.6	60.1	2.4
	High positive	719.6	760.0	5.6
Bilirubin (20.3 mg/dL)	Negative	8.8	5.9	33.0
	Low positive	61.6	48.5	21.3
	High positive	719.6	653.3	9.2
Lipemia*				
2000 mg/dL	Negative	8.8	7.9	10.2
	Low positive	61.6	49.4	19.8
	High positive	719.6	703.2	2.3
1000 mg/dL	Low positive	61.6	62.3	1.1
	High positive	719.6	710.1	1.3

*Evaluation of lipemia on the analytic performance of the HA ELISA was based on our in-house protocol as the exact conversion from 1460 Units suggested by the manufacturer to mg/dL could not be ascertained.

3 fold of their respective reference ranges. Since the HA assay showed good specificity but had a low positivity rate in the ANA positive cohort, we examined its correlation with the other assays to determine if it may have some clinical significances in the evaluation of SLE. The positive agreement data revealed considerable association between the HA ELISA and all assays with significant variability in the % negative agreements. The correlation between the HA ELISA and Farr RIA considered gold standard test for the detection of anti-dsDNA antibodies also revealed good concordance at elevated antibody levels. Both assays also displayed similar association with the CLIFT which is routinely used as a confirmatory test for anti-dsDNA IgG antibodies.

Due to the low prevalence of HA anti-dsDNA antibodies in the ANA-positive cohort, our data suggest that this assay can be used as a confirmatory marker in individuals who test positive in either the anti-dsDNA IgG ELISAs and/or CLIFT. This observation is further supported by the good correlation between HA ELISA positivity and high level anti-dsDNA IgG antibodies in all 7 assays. Although the importance of this observation is limited by the absence of clinical data, one can infer that HA anti-dsDNA IgG positivity selects for patients with elevated levels of these antibodies compared to those who are negative but positive by the other ELISAs and/or the CLIFT. Indeed, it is widely recognized that high avidity anti-dsDNA IgG antibodies are related more frequently to the occurrence of nephritis, whereas low avidity anti-dsDNA antibodies are associated with milder form or early disease [2, 6-8, 10-13, 15-19]. Since the HA ELISA is designed to predict the presence of high avidity anti-dsDNA IgG antibodies, one would expect the assay to be less sensitive for the diagnosis of SLE as antibodies of low-to-medium avidity will be missed. Based on some published studies, the HA dsDNA ELISA has been reported to correlate significantly with disease activity [14, 16, 19]. A study by Heidenreich and colleagues reported a moderate sensitivity but good specificity of this assay in the differential diagnosis of lupus nephritis [15]. The later study used this marker only in the differential diagnosis and not to monitor disease activity following treatment or predict disease remission or flares.

Our study also showed the HA ELISA to have good intra-assay reproducibility (CVs <10%) with some variability in the inter-assay precision at the assigned cut-off for both low- and high-positive samples. However, this does not seem to affect qualitative interpretation of results as demonstrated in the evaluation of lot-to-lot performance. With respect to the interfering substances evaluated, hemoglobin and bilirubin showed significant interference with some of the samples at the manufacturer's claim of no

interference. Likewise, interference was observed in the presence of lipemia at in-house concentrations, but disappeared when lipemic concentrations were decreased by 50%.

One of the shortcomings of our study is the absence of clinical data for the 125 patients samples evaluated. Although we chose to determine the correlation between the HA ELISA and other assays for the detection of anti-dsDNA IgG antibodies with different principles in ANA positive samples, it is widely recognized that the ANA by IFA has low specificity for SLE [1-5]. To minimize the lack of clinical information in our disease cohort, we designed our study simulating laboratory recommendations for testing these antibodies [2-3]. Since anti-dsDNA IgG antibodies are highly recommended to be tested in individuals with positive ANA, we selected 100 ANA positive sera with homogeneous pattern and titers greater than or equal to 1:160 as previously described [20]. We decided on titers ≥1:160 as this offers the best predictive value for disease [3]. To rule out bias in pre-selecting samples that are ANA-positive, the specificities of all assays were assessed using sera from 100 healthy donors.

In conclusion, the HA ELISA is highly specific and predictive of the presence of highly elevated levels of anti-dsDNA antibodies. Due to its excellent concordance with elevated levels of anti-dsDNA IgG antibodies in the assays evaluated here, it may be suitable as a confirmatory tool for anti-dsDNA antibodies. In addition, this test may also be a useful adjunct to other biomarkers such complement factors 3 and 4 (C3 and C4) as well as the anti-C1q assays in assessing SLE activity especially in individuals with lupus nephritis. Our analytical data suggest that further improvement in the precision of the assay is necessary. Also, based on our interference studies, we recommend that hemolysed, lipemic, and icteric serum samples be avoided as their presence significantly affects the accuracy of results. Although high avidity anti-dsDNA antibodies may be used to predict outcome, our study has not addressed this aspect of laboratory testing.

Acknowledgements

All kits for this study were provided free-of-charge by AESKU, Dr. Fooke, Euroimmun, Bio-Rad and INOVA Diagnostics. The authors would like to thank Shanna Stromberg, Diana Knapp, Jeanette Enbody, Jacob Barnhill, Taylor Jackson, Susan Copple, Anndorie Sachs and Holly Hatch for excellent technical support. This work was supported by the ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories and Department of Pathology, University of Utah, Salt Lake City. This work was supported by the ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories and Department of Pathology, University of Utah, Salt Lake City.

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