Myth and Reality: Practical Test System for the Measurement of Anti-DNA Antibodies in the Diagnosis of Systemic Lupus Erythematosus (SLE)

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The myth persists that only the labor intensive Farr radioimmunoassay and *Crithidia luciliae* immunofluorescence (CL-IFA) are systemic lupus erythematosus (SLE)-specific tests. We compared them to ELISA with bacteriophage λ DNA (EL-dsDNA) and denatured calf thymus DNA (EL-ssDNA). By percentile ranking, the specificity cut-off level was set both out of clinical context (SOCC) on 100 blood bank donors, and in clinical context (SICC) on 100 patients with either rheumatoid arthritis or scleroderma (50/50). Clinical sensitivity was calculated on 100 random SLE patients. At 95% SICC, the sensitivity of Farr, CL-IFA, EL-dsDNA, and EL-ssDNA was similar (95%CI): 76% (66–84), 76% (66–84), 63% (53–72), and 75% (65–83), respectively; 87% of the patients were positive by at least one method and 55%

by all methods. At 99% SICC, the sensitivity was also similar (95% CI): 57% (47–67), 47% (37–57), 58% (47–67), and 43% (33–53), respectively. The areas under ROC curve were similar (95% CI) when patients were used as controls for specificity. At 99% SOCC, EL-ssDNA identified 89% positive, 2 negative but positive by another method at 95% SICC, and 9 negative (i.e. 89/2/9), followed by CL-IFA (80/6/14), Farr (76/12/12), and EL-dsDNA (64/23/13). Thus, at relatively low cost and easy automation, under the same conditions of specificity, the two ELISA tests combined were at least as good, if not superior, to CL-IFA or Farr: they showed similar clinical sensitivity and also identified more patients with anti-DNA antibodies. J. Clin. Lab. Anal. 24:77–84, 2010.

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INTRODUCTION

The presence of anti-dsDNA antibodies is considered a very strong indicator of SLE, and inappropriate reporting of a result as either false positive or false negative has a significant impact on the diagnostic process. Three main methods are currently employed. First, *Crithidia luciliae* immunofluorescence (CL-IFA

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detects antibodies binding to the dsDNA of the kinetoplast of the protozoan *C. luciliae* (1). It is labor intensive and is not amenable to automation. Second, the Farr radioimmunoassay is often the standard by which all other methods of anti-dsDNA antibodies detection in SLE are judged (2). Farr is quantitative, with results that can be expressed in IU/ml, measures high avidity antibodies, and is considered specific for SLE when high quality, radiolabeled, procariotic DNA is used (2–4). It has its drawbacks (5): cannot be automated and is the most labor intensive. Finally, ELISA is the most widely used and is the most practical, since it is performed either manually or on automated instruments; it can be highly sensitive, isotype specific (6), and quantitative. ELISA usually detects the entire spectrum of avidity and affinity of IgG anti-dsDNA antibodies; however, high avidity antibodies are thought to be mainly associated with kidney disease in SLE. There is also the suspicion, mainly for ELISA tests constructed with S1 nuclease digested calf thymus DNA, that anti-ssDNA re-emerges and is also measured along with anti-dsDNA, and therefore erodes the SLE specificity of the test (7,8). In addition, this type of DNA, although it is most widely used in commercial products, appears to be inappropriate for predicting lupus nephritis (4). For reasons yet unknown, phage DNA, used in some commercial kits, maintains its double-stranded character and SLE specificity (9,10) and has been shown in separate studies to be as useful a predictor of kidney disease as the Farr assay (10–13).

In addition to anti-dsDNA, anti-ssDNA ELISA has also been proposed as being a valuable tool in the diagnosis and follow-up of SLE patients (10,14,15). However, the value of testing for anti-ssDNA in the diagnosis and management of SLE has been largely questioned in principle (8,16), without basis in experimental evidence. The antigen is mostly heat-denatured calf thymus DNA (9,10); therefore, the term ssDNA for this type of DNA is a misnomer, since all DNA epitopes are represented in this antigenic preparation. Anti-total DNA would be clearly more appropriate: the anti-dsDNA antibody is probably directed against shared determinants on the deoxyribose-phosphate backbone and the anti-ssDNA antibody against base-dependent structures not exposed in dsDNA (17,18).

We evaluated the combined use of the broadly sensitive anti-ssDNA ELISA with the highly SLE-specific phage λ anti-dsDNA ELISA (9,10) as effective substitutes for Farr and/or CL-IFA. In addition, we investigated whether the generally accepted high SLE sensitivity and specificity of Farr and CL-IFA has any basis in reality, or is it only the consequence of differences in the establishment of cut-off values. Thus, the clinical sensitivity of all four tests was evaluated

under the same conditions of specificity (95 and 99%) out of clinical context (SOCC), using random blood bank donors as controls and in clinical context (SICC), using patients with similar collagen vascular diseases as controls (19). To minimize bias, the ELISA tests, Farr, and CL-IFA were performed on the same specimens at three separate institutions.

PATIENTS AND METHODS

All patient specimens used in this study were stored frozen after phlebotomy. To determine the quality of the storage we compared the levels of anti-dsDNA using the same λ phage-based anti-dsDNA ELISA test that was used when the specimens were fresh. Specimen quality after frozen storage showed that all negative specimens were still negative and only 4% of the low positive specimens became negative; the levels had a highly significant linear correlation of 0.959 ($P < 0.001$). The 100 SLE patients tested were at various stages of their disease and were used in a previous study (10); they fulfilled the ACR criteria for the SLE diagnosis (20,21). As controls we used 100 un-assayed blood bank donors and 100 disease controls, represented by 50 patients with RA and 50 with scleroderma, who also fulfilled the ACR criteria for their diagnosis. All RA patient specimens were collected before the introduction of anti-TNF inhibitors and all patients were diagnosed by attending rheumatologists. The study was approved by the participants' respective Institutional Review Boards.

Two ELISA tests for both anti-ssDNA and anti-dsDNA were performed at TheraTest Labs with the help of commercial kits. The anti-ssDNA was constructed with denatured calf thymus DNA and the anti-dsDNA with bacteriophage λ DNA (TheraTest Laboratories Inc., Lombard, IL). The Farr RIA (Diagnostic Products Corporation, Los Angeles, CA) was performed at Thomas Jefferson University and the *Crithidia luciliae* immunofluorescent assay (CL-IFA; INOVA Diagnostics, Inc., San Diego, CA) was performed at ARUP Laboratories (Salt Lake City, UT). All tests were performed according to the manufacturer's instructions. Each site was blinded to the results obtained by the other sites. Most specimens were tested to the assay's endpoint.

Statistics

Correlations were calculated with the help of Statistica (StatSoft, Tulsa, OK) software. Confidence intervals were calculated by Excel software (Microsoft, Seattle, WA). All specimens were considered in the nonparametric statistics (positive vs. negative), but only those with endpoint levels were used for correlations. Specificity cut-off values were calculated using percentile ranking

for non-Gaussian distribution of an analyte (22–24). ROC curves were calculated with the help of SPSS software (SPSS, Chicago, IL).

RESULTS

Specificity in and out of Clinical Context

Two specificity levels used in clinical practice were determined based on percentile ranking of 95 or 99%. Among the blood bank donors, none of the specimens showed a characteristic shift for an abnormal value identifying a clear outlier, i.e. 99 and 100% were essentially the same (Table 1). For the patient control group, however, there was a clear outlier for the highest recorded value, suggesting that it was likely to be a true positive (Table 1); it was a patient with scleroderma.

The first level was “specificity out of clinical context” (SOCC), based upon results on samples from 100 blood bank donors (Table 1). The second level, the “specificity in clinical context” (SICC), was based on testing specimens from 100 disease control patients (50 RA and 50 PSS) (Table 1). Overall, the cut-off values were higher for SICC than for SOCC. For example, the 99% SICC cut-off values were significantly higher: ssDNA 796 U/ml; dsDNA 74 IU/ml; Farr 13.8 IU/ml;

and CL-IFA 1/80, than the 99% SOCC: 117 U/ml, 18 IU/ml, 3.9 IU/ml, and 1/10, respectively. These specificity levels were used to compare the sensitivity of the four anti-DNA assays in 100 SLE patients. For all tests the values identified were close to those expected or suggested by the manufacturer’s instructions.

Sensitivity as a Function of Specificity in and out of Clinical Context

At the highest cut-off for 99% SICC, which is most reliable clinically, the sensitivity of EL-dsDNA was 58% and did not differ significantly (95% CI) from that of 57% Farr, 47% CL-IFA, or 43% EL-ssDNA (Table 2). At 95% SICC, all four tests also appeared to be similar (95% CI); 76, 76, 63, and 75% respectively (Table 2). ROC curves, routinely used for comparative test evaluation of sensitivity vs. specificity, were also calculated separately for SLE with either patients (Fig. 2) or HBD (Fig. 3) as specificity controls. The area under the curves was not significantly different from each other (95% CI) for the four tests when patients were used as controls: 0.938 ssDNA (0.899–0.978), 0.869 dsDNA (0.811–0.926), 0.950 Farr (0.918–0.981), and 0.923 CL-IFA (0.880–0.965). Also, when HBD were used as controls, Farr, CL-IFA, and

TABLE 1. Ranking of the 10 Highest Values Recorded on 100 Blood Bank Donors (SOCC) and 100 Control Patients (SICC)

SOCC				SICC			
ssDNA (U/ml)	DsDNA (IU/ml)	Farr (IU/ml)	CL-IFA (titer)	ssDNA (U/ml)	dsDNA (IU/ml)	Farr (IU/ml)	CL-IFA (titer)
134	19	4	10	1525	186	23.3	160
117*	18*	3.9*	10*	796*	74*	13.8*	80*
96	13	3.9	<10	610	68	10.2	80
85	10	3.6	<10	464	53	5.7	80
75	10	3.4	<10	382	42	4.4	40
63**	9**	3.1**	<10	334**	39**	3.9**	40**
62	7	2.8	<10	269	37	3.1	40
62	7	2.8	<10	252	32	3.1	40
56	6	2.7	<10	237	24	3.1	<10
53	6	2.7	<10	208	22	3.1	<10

*99% and **95% cut-off values.

TABLE 2. Clinical Sensitivity for SLE Patients Based on Two Levels of SOCC and SICC (Ranges for 95% Confidence Interval)

Test considered	SOCC		SICC	
	95%	99%	95%	99%
1. Farr	80 (71–87)	76 (66–84)	76 (66–84)	57 (47–67)
2. CL-IFA	82 (73–89)	82 (73–89)	76 (66–84)	47 (37–57)
3. EL-dsDNA	80 (73–89)	76 (66–84)	63 (53–72)	58 (47–67)
4. At least one of the above (1–4)	92 (85–96)	91 (84–96)	87 (79–93)	59 (49–69)
5. EL-ssDNA	90 (82–95)	89 (81–94)	75 (65–83)	43 (33–53)
6. All of the above	72 (62–81)	68 (61–80)	55 (45–65)	48 (38–58)

Fig. 1. Results at 99% SOCC by anti-ssDNA ELISA (bottom row) and identification as positive at 95% SICC of 100 random SLE specimens by EL-ssDNA, EL-dsDNA-EL, Farr and CL-IFA. Each column is one patient: positive specimen = dark cell; negative specimen = clear cell. Note that some specimens are positive by all methods and some only by one or two methods. See details in Table 2.

Fig. 2. ROC curves for SLE patients compared to healthy blood bank donors as controls. The areas under the curves and the 95% CI ranges were as follows: ssDNA (—): 0.938 (0.899–0.978), dsDNA (.....): 0.869 (0.811–0.926), Farr (— — — —): 0.950 (0.918–0.981) and CL-IFA (- - - -): 0.923 (0.880–0.965).

EL-ssDNA were not significantly different from each other, whereas the area for EL-dsDNA was significantly lower than that of Farr, i.e. 0.813 (0.747–0.880) vs. 0.923 (0.885–0.962). Thus, according to this test evaluation method, the anti-ssDNA and Farr were very similar with both HBD and patients as controls.

At the lowest cut-off of 95% SOCC, the sensitivity of all three tests designed for the specific measurement of anti-dsDNA antibodies, EL-dsDNA, Farr, and CL-IFA, was very similar, near 80% (Table 2). Over 90% of the specimens were identified as having abnormally high levels of anti-dsDNA by at least one dsDNA-specific test or by anti-ssDNA alone. At 95% SICC all specimens positive for EL-dsDNA were also positive for EL-ssDNA at 99% SOCC (Fig. 1), i.e., when the two ELISA tests are performed simultaneously, the anti-ssDNA test functions as a built-in quality control for all

Fig. 3. ROC curves for SLE patients compared to healthy blood bank donors as controls. The areas under the curves and the 95% CI ranges were as follows: ssDNA (—): 0.902 (0.854–0.949), dsDNA (.....): 0.813 (0.747–0.880), Farr 0.923 (— — — —): (0.885–0.962) and CL-IFA (- - - -): 0.923 (0.889–0.946).

positive anti-dsDNA tests. The same was seen for 99% SICC (data not shown). At all levels of specificity, each specimen was identified as positive by only one, two, three, or all four methods (Fig. 1 and Table 2). Only about half the SLE patients were identified as positive by all methods in clinical context (SICC) (Table 2).

EL-ssDNA Test as a Companion to EL-dsDNA

At both 95 and 99% SICC, some specimens were identified as positive by only one, two, or all three anti-dsDNA-specific methods (Fig. 1). If only one method would have been used, as is typical in clinical laboratories, the number of positive specimens remaining unidentified would have been represented by the difference between the number identified by at least one

of the methods (Table 2, line 4) and those identified by each method separately (Table 2, lines 1, 2, 3 and Fig. 1).

To minimize the number of SLE specimens missed for having any anti-DNA antibodies, we examined further the value of using the combination of EL-ssDNA and EL-dsDNA. Each specimen at 95% SICC was plotted as positive (dark cell) or negative (clear cell) for each of the four methods on an Excel workbook (Fig. 1). In addition, the EL-ssDNA results were also plotted at 99% SOCC (Fig. 1, bottom). The 100 SLE specimens could be split into three categories: positive, negative but positive by another method, and negative by all methods. At 99% SOCC, EL-ssDNA identified 89% as positive, two as negative but positive by another method, and nine as negative (i.e. 89/2/9); it was followed by CL-IFA (80/6/14), Farr (76/12/12), and dsDNA (64/23/13) (Fig. 1). Thus, ELISA for EL-ssDNA was the closest to the ideal method for identifying the presence of the largest spectrum of

anti-DNA antibodies, with only two specimens missed, one positive by Farr and one by CL-IFA.

Thus, when we combined the results at 95% SICC for the two ELISA methods, 78% of the specimens could be considered as having high probability for SLE, 11 were identified as probable to be positive by another method, and 11 were negative (Fig. 1). At the 99% SICC, these proportions were respectively 61, 13, and 26; the same two samples were missed (Table 3, bottom range) by ELISA methods.

Correlations Between Levels Measured by the Methods

Correlations were determined only on specimens that had pair-wise endpoint measurements. The specimens that lacked pair-wise endpoint data (e.g. Table 3, lines 1–10 for anti-ssDNA) could not be used in correlations, although all data were used in the nonparametric

TABLE 3. Ranking of 100 Anti-DNA Values Based on Anti-ssDNA Levels: Top, Middle, and Bottom Ranks Shown to Illustrate the Relationships Between the Levels of Anti-DNA Measured by Different Methods

Rank	EL-ssDNA (U/ml)	EL-dsDNA-EL (IU/ml)	Farr (IU/ml)	CL-IFA (1/n)
<i>Top 10 rank</i>				
1	> 1803	275	21	80
2	> 1793	293	9.1	160
3	> 1777	861	20.1	1280
4	> 1631	507	39.6	2560
5	> 1597	490	34.8	1280
6	> 1568	257	43.4	2560
7	> 1559	611	48.3	1280
8	> 1536	235	42.2	80
9	> 1498	90	2.5	< 10
10	> 1450	465	41.6	1280
<i>Middle 10 rank</i>				
45	703	302	35.4	320
46	698	27	50	160
47	683	0	7.3	10
48	638	98	45.7	160
49	630	90	33.2	160
50	607	98	33.6	40
51	564	89	6.2	40
52	563	108	7.5	40
53	549	28	7.9	40
54	546	61	2.6	320
<i>Bottom 10 rank</i>				
89	129	22	33	160
90	70	24	29.6	80
91	27	0	3.1	< 10
92	18	15	2.7	< 10
93	7	0	1	< 10
94	7	28	3.4	10
95	0	0	2.6	160
96	0	0	1	10
97	0	0	1	10
98	0	0	3.1	< 10
99	0	0	3.1	< 10
100	0	0	2.6	< 10

TABLE 4. Linear and Spearman Rank Correlations (*R*) Between the Four Types of Measurements of Anti-DNA Antibodies (Significance; *P*)

Test considered	Correlate			
	EL-ssDNA	EL-dsDNA	Farr	CL-IFA
<i>Linear correlation (significance)</i>				
EL-ssDNA		0.59 (<0.001)	0.46 (<0.001)	N/A
EL-dsDNA	<i>N</i> = 63		0.51 (<0.001)	N/A
Farr	<i>N</i> = 59	<i>N</i> = 92		N/A
CL-IFA	<i>N</i> = 63	<i>N</i> = 63	<i>N</i> = 93	
<i>Spearman rank (P < 0.05)</i>				
EL-ssDNA		0.70	0.50	0.50
EL-dsDNA			0.62	0.54
Farr				0.74

analyses (Table 2 and Fig. 1); this resulted in a variable number of specimens shown for each correlation (Table 4).

The linear correlations between measurements of anti-DNA antibodies were modest but highly significant. Spearman ranked correlation was best between Farr and CL-IFA ($R = 0.62$) and between EL-ssDNA and EL-dsDNA ($R = 0.70$). Although the overall ranking of values showed clear agreement for some individual specimens, there was still a very large difference in the ranking obtained by different methods (Table 3). For example, for specimen #3, the value for Farr was in the low range and for CL-IFA in the high range; for specimen #8, the values for ELISA and Farr were in the high range and for CL-IFA in the low range.

DISCUSSION

Contrary to the common myth, under the same conditions of specificity, the methods used for the measurement of anti-dsDNA antibodies in the diagnosis of SLE—Farr, CL-IFA, ELISA with phage dsDNA—did not differ significantly (95% CI) from each other in sensitivity. Moreover, they did not differ significantly either in or out of clinical context with EL-ssDNA (the method generally considered as useless for the diagnosis of SLE). Clinically, the most useful results, and at the same time the easiest to perform on automated instruments, were obtained when the specimens were tested simultaneously by ELISA with phage DNA (EL-dsDNA) and denatured calf thymus DNA (EL-ssDNA). This is the first report on the diagnostic value of combining the two ELISA tests.

Our cohorts of SLE and control patients appeared similar to those used in other studies. One study on 440 mixed SLE patients reported the sensitivity of the Farr assay at 95 and 99% SICC to be 72 and 56%,

respectively (19), which is similar to our 76 and 57% and to the results from other previous studies (3). By combining the results for specimens identified as positive by the EL-ssDNA and EL-dsDNA at 99% SOCC with results from EL-dsDNA at the 95% SICC, three categories of specimens emerged: those very likely to have SLE (78%), likely to have SLE, but in need of confirmation by either Farr or CL-IFA (11%), and normal by any method (11%; Fig. 1). At 99% SOCC EL-ssDNA missed 2 specimens, CL-IFA 6, and Farr 12. The two specimens missed by EL-ssDNA could be considered as false negative, since they were identified as positive by Farr or CL-IFA (Table 3). Or they may have been false positive by those two methods. Possible explanations for false positive results include identification of IgM anti-DNA by the Farr assay (25) or presence of acute phase reactants in infection (5). The CL-IFA might have detected antibody to unique antigenic determinants present only in *C. luciliae* kinetoplast DNA (26).

Each method used for detecting anti-dsDNA identifies only part of the spectrum of anti-dsDNA antibodies produced by a patient (3). This phenomenon reflects the presence of antigenic determinants in DNA that are unique to the DNA source and its unique interaction with the individual's antibody (26). Indeed, at 95% SICC, 87% (79–93; 95% CI) of the specimens in our SLE cohort were identified as having anti-dsDNA by at least one method (Table 2), but only 55% were identified (45–65; 95% CI) by all methods (Table 2 and Fig. 1) and some by only one or two methods.

EL-dsDNA was the most useful companion for the highly sensitive EL-ssDNA. It is plausible to interpret the highest sensitivity of the EL-ssDNA data with the detection of the majority of the spectrum of anti-DNA antibodies because practically all dsDNA-specific IgG antibodies in ELISA also bind with comparable avidity to denatured DNA (ssDNA). Additionally, a significant proportion of specimens react only with ssDNA and not with dsDNA (27), as we have also seen here. This characteristic could make the EL-ssDNA test the best screening method for laboratories that can practice all methods. In addition, considering that only 60–70% of the patients have anti-dsDNA by any one method, it is clearly an advantage to know that some anti-DNA antibody is present, even if it is only anti-ssDNA; it is an abnormality that may be useful in the context of other clinical symptoms suggestive of SLE and could be followed longitudinally (10,14,28). Obviously, the combined use of EL-ssDNA and EL-dsDNA provides an additional dimension of reliability or “built-in quality control” by having the requirement that all specimens positive for anti-dsDNA to be also positive for anti-ssDNA at 99% SOCC.

Surprisingly, the area under the curve for EL-ssDNA, which is considered as lacking any clinical value (8,16,29), was very close to that of the Farr assay (Figs. 2 and 3), which is considered the "gold standard" for anti-dsDNA testing in SLE diagnosis or CL-IFA. Thus, any of these tests can be SLE-specific depending on the cut-off values considered to identify "positive" specimens; the high SLE specificity of the Farr and CL-IFA is obviously a myth.

A large proportion (91%) of this mixed SLE cohort, at various stages in their disease, had anti-dsDNA at higher levels than those seen in 99% of healthy blood bank donors by at least one method (Table 2). Considering that levels vary with disease activity (10,13,14,28,30,31), it is likely that most, if not all, SLE patients have abnormal and detectable levels of circulating anti-dsDNA at sometime during their disease course.

The antigen used in solid phase assays for the measurement of anti-dsDNA antibodies varies widely: bacteriophage λ DNA (9,10), plasmid DNA (19,27,32), or calf thymus DNA cut with S1 nuclease to remove ssDNA strands (33). Unfortunately, the latter type is the most widely used in clinical laboratories and yet has the lowest clinical sensitivity (4,7). We reproduced the comparatively low clinical sensitivity results seen by others with a commercial test designed with S1 nuclease cut DNA (data not shown).

The main reason cited for the superiority of the Farr assay for SLE is the good statistical correlation of levels with disease activity and kidney involvement (34). It is also part of the myth regarding Farr assay. In this study the disease activity was not taken into consideration and was not a purpose of this investigation. However, phage-based ELISA for anti-dsDNA as well as anti-ssDNA have also been shown in a previous report to correlate well with current or future changes in disease activity on the same cohort used here (10) and in other cohorts (13,14,31,34). However, clinical laboratory correlations are statistical concepts, valuable for our understanding of pathogenesis (35), but difficult to apply to any individual patient for active disease management (36). The second reason for the superiority myth is the purportedly high sensitivity and specificity of the Farr assay and CL-IFA (8). But for the same sensitivity requirements there was no significant difference in SLE specificity between Farr, CL-IFA, EL-dsDNA (Table 2 and Fig. 1), or even EL-ssDNA.

In many clinical settings, the physician seeks to obtain diagnostic information by ordering a number of autoantibody tests to complement clinical findings and a positive ANA screening test: anti-chromatin, anti-Sm, RNP, SSA, and anti-cardiolipin, to name a few. In this context, it is worth noting that the simultaneous use of

ELISA with denatured calf thymus DNA and phage λ DNA provide, on their own, a large part of the answers.

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