Significant differences in the analytic concordance between anti-dsDNA IgG antibody assays for the diagnosis of systemic lupus erythematosus—Implications for inter-laboratory testing

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ABSTRACT
Background: Anti-double stranded DNA (anti-dsDNA) autoantibodies are considered hallmark of systemic lupus erythematosus (SLE).
Methods: To determine concordance between assays for the detection of this marker, we analyzed 10 antinuclear antibody (ANA) positive sera with a homogeneous pattern and titers ≥1:160 by indirect immunofluorescence assay (IFA) on HEp-2 cells, 100 consecutive anti-dsDNA IgG ELISA-negative as well as 100 healthy control samples using six commercial ELISAs and the Centrifuge liquid immunofluorescence test (CLIFT).
Results: The positivity rates for the ELISAs in the ANA positive group ranged from 55.0 to 88.0% with specificities from 84.0 to 94.0%. The CLIFT had a positivity rate of 92.6% and specificity of 84.0%. In the previously screened anti-dsDNA IgG-negative group, the positivity rates ranged from 1 to 19.0%. The overall concordance between the ELISAs ranged from 73.0 to 89.5% and varied from 70.0 to 80.0% among specific ELISAs and CLIFT.
Conclusions: Our data show variable degree of concordance between anti-dsDNA IgG ELISAs which may significantly impact inter-laboratory testing as well as the diagnosis and management of SLE patients. Although some of the ELISAs show comparable performance to the CLIFT, the degree of concordance between these assays at high antibody levels suggests that CLIFT is still a relevant confirmatory tool.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease associated with the production of several antibodies. Of these, anti-double-stranded DNA (dsDNA) antibodies are the most prevalent and are part of the diagnostic criteria of the American College of Rheumatology (ACR) [1,2]. The significance of these antibodies in the diagnosis of SLE as well as in monitoring disease activity has led to an increase in demand for this test as evidenced by the number of commercially available kits [3-11]. With the increasing number of anti-dsDNA IgG assays, the potential for variability in the diagnostic accuracies is enormous as different antigens, assay principles and cutoff determinations are employed. Current recommendations require testing for anti-dsDNA antibodies only in individuals who test positive for antinuclear antibody (ANA) by indirect immunofluorescence assay (IFA) [12-14]. Due to the increased sensitivity but low specificities of the anti-dsDNA IgG detected by ELISAs it has also been proposed that the presence of these antibodies be confirmed by alternative assays that are more specific and highly predictive of disease [5,6,12]. Thus samples which test positive using anti-dsDNA IgG ELISAs are usually confirmed by assays such as the Centrifuge liquid immunofluorescence test (CLIFT) or the Farr radiolmmunoassay both of which are time-consuming. Of particular importance is the Farr assay that employs the use of radioactive element which limits its routine use in the clinical diagnostic laboratory [15]. Although the CLIFT is considered specific, subjective in reading and interpreting IFA is a major limitation due to intra- and inter-reader variability. As such, the CLIFT may not be a reliable tool in monitoring disease activity in SLE patients.

At ARUP Laboratories anti-dsDNA IgG antibodies are first screened by ELISA followed by confirmation of all positive results by CLIFT. As a reference laboratory, previously screened samples from other laboratories are sent for confirmation by the CLIFT. Although variation in the performance characteristics anti-dsDNA IgG antibodies has been reported [5,9,10], the effect of these correlations is not well established based on current testing practice. This is particularly important in the US where clinicians can no longer ascertain the laboratories in which their patients' specimens are evaluated. Although a few recent investigations have shown that some anti-dsDNA ELISA kits have diagnostic efficiencies that are similar to that o
the Farr assay and/or CLIFT [7,8,16], others have had conflicting outcomes [9,10,14]. This study was designed to determine the analytic agreement between anti-dsDNA IgG assays for the diagnosis of systemic lupus erythematosus and how this performance may affect inter-laboratory testing.

2. Materials and methods

2.1. Study cohorts

We analyzed serum samples from 300 individuals based on 3 groups. The first group consisted of 100 ANA positive samples with a homogeneous pattern and titers greater ±1:160. The presence of ANAs in these samples was determined using the NOVA Line™ Hg 2 ANA assay (Inova Diagnostics, San Diego, CA) using an IgG-specific fluorescent isothiocyanate conjugate (FITC). This group consisted of 89 females and 11 males with a mean age of 47.3 years (± SD 20.5 years). The second group comprised of 100 (52 females, 48 males and 12 of unknown sex) previously tested samples in our reference laboratory for anti-dsDNA IgG antibodies using the Bio-Rad dsDNA IgG ELISA (Bio-Rad Diagnostics, Hercules, CA). The mean age of patients in this group was 55.7 years (± 30.4 years). To determine assay specificities of the different assays, sera from 100 healthy blood donors (57 females and 43 males with a mean age of 39.5 ± 12.2 years) were also tested. Samples were tested between December 2009 and March 2010 and were stored at –20 °C in aliquots until analyzed. All sera were de-identified prior to testing as per an approved study protocol by the University of Utah Institutional Review Board (IRB #7275).

2.2. Laboratory investigations for the detection of anti-dsDNA IgG antibodies

Five manufacturers of anti-dsDNA IgG diagnostic kits were invited to contribute in this study and all agreed to participate. The 6 ELISAs were obtained from Azeko Diagnostics (Wendelstein, Germany), The Binding Site (Birmingham, UK) provided by inviva Diagnostics, Bio-Rad Diagnostics, Euroimmun Ag (Lubeck, Germany), Dr. Fokke Laboratories (Neuss, Germany) and Inviva. The Binding Site anti-dsDNA ELISA used in this study is different from the more specific highly anti-dsDNA test (also referred to as Farr assay). As a confirmatory test, the CLIFT (Inova) was performed on all samples. The CLIFT assay was performed using C. lucilies as substrate. Titers greater than or equal to 1:10 were reported as positive. All 300 samples were tested manually within 1 month. All tests were performed by experienced laboratory personnel using procedures recommended by the manufacturers.

2.3. Statistical analysis

For binary analysis of the data, cut-offs as suggested by the manufacturers were applied to create positive and negative results from the continuous original observations. Agreement between assays was then quantified using Cohen’s kappa statistic. K coefficients > 0.75 signify substantial agreement [17]. We considered results statistically significant at p < 0.05. Positivity rates, specificities and Spearman correlation coefficient between assays were calculated as indicated using SAS® software, ver 9.2 of the SAS system for Windows, Cary, NC.

3. Results

3.1. Characteristics of anti-dsDNA antibody assays

Kits for this study were chosen based on differences in substrates used, their availability from manufacturers as well as published literature. Based on the kit-specific product inserts, all assays have been calibrated using the WHO standard [W8/80] [18]. However, there are variations in the types of antigen used, calibration set-up and cut-off values employed (Table 1). Except for the TBS ELISA which employs a γ-chain specific IgG conjugate, all assays refer to secondary antibody as anti-human IgG. Of the 6 anti-dsDNA ELISAs, none was identical in design. Therefore, although all 6 ELISA claim to measure the same analyte, some differences in the assay specific performance were anticipated.

<table>
<thead>
<tr>
<th>Table 1: Characteristics of anti-dsDNA IgG antibody assays.</th>
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<tr>
<td>Assay</td>
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<tr>
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<tr>
<td>AESEU</td>
</tr>
<tr>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Euroimmun</td>
</tr>
<tr>
<td>Dr. Fokke</td>
</tr>
<tr>
<td>Inviva</td>
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<tr>
<td>CLIFT</td>
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* The dsDNA IgG ELISA was provided by Inviva Diagnostics.
* IU denotes relative units.
* CLIFT is an indirect immunofluorescence assay (IFA) that employs CRV/1 cells as substrate.

To evaluate the prevalence of ELISA-positives in previous identified anti-dsDNA IgG negative samples tested by Bio-Rad ELI 100 samples were tested on all other ELISAs and by CLIFT. For these ELISAs, the positivity rate ranged from 1.0 to 19.0% (Fig. 1). Of the 300 sera tested, none were positive by all assays. The 25 were positive by one or more ELISAs and 3 by CLIFT. Of the discrepancy samples, 18 were positive by a single assay and remaining sera by 2 or 3 ELISAs. Of the three CLIFT positive samples has a positive result in at least one.

Fig. 1. Prevalence of anti-dsDNA in the different cohorts based on assay employed. Histogram plots show the positivity rates of the different assays using manufacturers-suggested cut-off values.
Table 2

<table>
<thead>
<tr>
<th>Assay</th>
<th>% Overall agreement</th>
<th>Delta-BioRad</th>
<th>Euroimmun</th>
<th>Dr. Focke</th>
<th>Inova</th>
</tr>
</thead>
<tbody>
<tr>
<td>AESCU</td>
<td>76.3</td>
<td>76.0</td>
<td>75.5</td>
<td>80.0</td>
<td>72.0</td>
</tr>
<tr>
<td>TBS</td>
<td>87.5</td>
<td>83.0</td>
<td>87.5</td>
<td>87.5</td>
<td>85.0</td>
</tr>
<tr>
<td>BioRad</td>
<td>89.5</td>
<td>88.0</td>
<td>89.0</td>
<td>87.5</td>
<td>85.0</td>
</tr>
<tr>
<td>Euroimm</td>
<td>83.5</td>
<td>83.0</td>
<td>89.0</td>
<td>83.0</td>
<td>85.0</td>
</tr>
<tr>
<td>Dr. Focke</td>
<td>83.5</td>
<td>83.0</td>
<td>89.0</td>
<td>83.0</td>
<td>85.0</td>
</tr>
<tr>
<td>Inova</td>
<td>77.0</td>
<td>76.0</td>
<td>75.5</td>
<td>80.0</td>
<td>72.0</td>
</tr>
</tbody>
</table>

The overall percent agreements between paired assays for the ANA-positive and healthy control groups are shown. TBS represents total binding site, lots from this manufacturer were provided by Innova Diagnostics.

Table 4

<table>
<thead>
<tr>
<th>Assay</th>
<th>% ELISA positive &amp; CLIFT negative</th>
<th>% ELISA negative &amp; CLIFT positive (1:100)</th>
<th>% ELISA negative &amp; CLIFT positive (1:20)</th>
<th>Spearman correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>AESCU</td>
<td>12.0</td>
<td>11.0</td>
<td>3.5</td>
<td>0.05</td>
</tr>
<tr>
<td>TBS</td>
<td>10.5</td>
<td>12.0</td>
<td>4.0</td>
<td>0.70</td>
</tr>
<tr>
<td>BioRad</td>
<td>4.0</td>
<td>16.0</td>
<td>4.5</td>
<td>0.81</td>
</tr>
<tr>
<td>Euroimm</td>
<td>48.0</td>
<td>18.0</td>
<td>7.0</td>
<td>0.87</td>
</tr>
<tr>
<td>Dr. Focke</td>
<td>8.0</td>
<td>12.0</td>
<td>3.5</td>
<td>0.71</td>
</tr>
<tr>
<td>Inova</td>
<td>40.0</td>
<td>16.5</td>
<td>6.5</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Estimates for % positive and negative agreement are based on data from the healthy control group. Spearman correlation coefficient analyses significant at p < 0.0001 for the indicated ELISAs and CLIFT in the ANA positive group only.

3.3. Analytical correlation between anti-dsDNA antibody ELISAs and CLIFT

To determine if substantial differences exist between the performance characteristics of the anti-dsDNA IgG ELISAs, we compared the % agreement between specific combinations of these assays (Table 2). The % agreement between any 2 ELISAs ranged from 73.0 to 89.5% in the ANA-positive and healthy control groups with the best correlation observed between the Euroimmun and BioRad assays (89.5%). Overall, the Dr. Focke ELISA showed the best analytical concordance with all the ELISAs (80.0–88.0%). The agreement between the AESCU assay (which had the highest sensitivity and lowest specificity) and the other ELISAs ranged from 73.0 to 80.0%.

To further investigate the impact of the variable performance between the ELISAs, we examined their individual and collective correlation with the CLIFT as confirmatory assay. The % positive agreement between the different ELISAs and CLIFT ranged from 57.1 to 76.2% (Table 3) however, the overall agreement between either of these assays and the CLIFT did not exceed 30.0% (for both the ANA-positive and healthy control groups). Cohen's kappas analyses for correlation did not demonstrate any significant association between any of the ELISAs and the CLIFT (Table 3). In the ANA positive cohort, 65/65 (52.2%) of the samples were positive in all 5 ELISAs and CLIFT while 13/32 (40.6%) were negative in all ELISAs and CLIFT (data not shown). Thus, in these 2 groups the overall agreement between the ELISAs and CLIFT was estimated to be 58% (data not shown). To determine the prevalence of discordant results between specific ELISAs and CLIFT, we analyzed the % of ELISA-positive CLIFT-negative as well as the % ELISA-negative CLIFT-positive in both the ANA-positive and healthy control groups. The percentage of samples that were positive in one ELISA and negative in the CLIFT or vice versa is shown in Table 4. With exception of the AESCU and TBS assays, all other ELISAs had a prevalence of 4.0–8.0 in the ELISA positive and CLIFT negative category. In the ANA positive group, 11/13 (84.6%) samples were positive one ELISA and negative by CLIFT (came from the AESCU assay). For the ELISA assay and CLIFT positive samples we also evaluated the role of low antibody titers (1:10 vs 1:20) on this association. As indicated in Table 2, the prevalence of ELISA negative CLIFT positive results ranged from 12.0 to 18.0% with a CLIFT titer of 1:10 compared 3.3–7.8% at cut-off. This difference may be attributable to 13.5% of the CLIFT positive samples which all had a 1:10 titer in the healthy group. Adjusting cut-off of the assay from 1:10 to 1:20, significantly improves specificity (100%) with a concordant drop in sensitivity to 54% (not shown). Based on our observations that correlations between ELISA and CLIFT results appeared to be tenuous at low levels, examined the overall relationship between these levels in the positive group. Our scattershot demonstrated good correlations between high levels of anti-dsDNA IgG independent of the CLIFT with increasing CLIFT antibody titers (Fig. 2). Spearman correlation coefficients between the different ELISAs and the CLIFT ranged from 0.65 to 0.81 (Table 4) and were significant (p < 0.0001).

4. Discussion

Our study was initiated to determine the correlation between ELISAs for detecting anti-dsDNA IgG antibodies and their association with CLIFT, an assay considered less sensitive but specific for SSA. From a reference laboratory standpoint, an appreciation of analytical concordance between these assays is important in assessing laboratory performance and impact on SLE diagnosis management. This is particularly important in the US where serum screening may be performed in one laboratory and sent to another for confirmation. Based on laboratory practices, some previouse screened samples may be subjected to further screening for confirmatory test is being performed leading to different outcomes and so that we may have conflicting results. In the absence of clinical specimens we designed our study simulating laboratory recommendations testing these antibodies [12–14]. Since anti-dsDNA IgG antibody 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. Titers of ≥ 1:160 were chosen as this offers the best predictive value for disease [12]. To our bias in pre-selecting samples that are ANA- positive and to further evaluate the prevalence of discordant ELISA screening results we analyzed 100 previously screened anti-dsDNA IgG antibody negative (BioRad) samples were also included in this investigation. Lastly, the specificity of all assays were assessed using sera from healthy donors.

The prevalence of anti-dsDNA IgG antibodies for the clilf assay in the cohorts utilized was highly variable with implications for patient care especially if testing is performed in multiple laboratories of note, the ELISAs with the highest sensitivities had the low specificities. It is widely recognized that the clinical relevance of dsDNA IgG is largely dependent on the assay principle as most of it assays either have high sensitivity or high specificity but not both...
SLE [4,5,18]. In this regard, it has been suggested that testing for these antibodies be reserved for individuals who are ANA IFA positive [11-13]. Furthermore, due to the high false positive rates of anti-dsDNA IgG ELISAs, some investigators have proposed that testing by ELISA should be reserved for screening with positive results confirmed by more specific tests such as the CLIFT and Farr radioimmunoassay. Anti-dsDNA antibodies of low avidity are generally not detected by the Farr assay due to the dissociating reaction conditions of the ammonium sulfate precipitation step employed whereas ELISAs have been reported to detect antibodies of low avidity [3]. The ELISA platform however, offers several potential advantages for the clinical immunology laboratory compared to either the CLIFT or Farr radioimmunoassay [15]. Unlike the Farr assay, the ELISA and the CLIFT avoid problems of radioactive contamination and disposal as well as the ability to distinguish between the different anti-dsDNA antibody isotypes. With respect to the CLIFT, the ELISA is less subjective, can easily be automated and requires less training to perform [13].

Recently, the rationale for screening anti-dsDNA IgG antibodies by ELISA and confirming positive results by alternate platforms has been challenged due to reported comparable performance of some ELISAs with the CLIFT and/or Farr assays [7,8]. Some investigators also question the low sensitivity of CLIFT and suggest that anti-dsDNA as detected by ELISA would identify more SLE patients [7]. Although some of the ELISAs investigated in this study appear to have equivalent performance characteristics to CLIFT, there are substantial differences in their correlation with one another as well as with the CLIFT. For example, in the ANA positive group, 4 of the 6 ELISAs show poor percent positivity rates which are reflected in their specificities. However, the overall percent agreement between individual ELISAs ranged from 72.0 to 89.5% while the total concordance between the ELISAs and the CLIFT varied between 70.9 and 89.5%. In addition, our data also shows significant variability in the correlation between low level of antibodies as detected by the ELISA and CLIFT. Of importance, the correlation between the ELISA and CLIFT appears to be least relevant at high titters. This observation lends support for using CLIFT as the confirmatory assay or the approach of using at least two test systems with different assay principles [5].

For the solid-phase anti-dsDNA IgG assays, there is some indication that the performance of the test system may be influenced by the source of antigen [5,7,11,18]. For example, assays that employ recombinant dsDNA as antigen have been reported to be more sensitive than others using calf thymus extract (CTE), nucleosome purified dsDNA (sources other than CTE) [5,7,8]. Our observations also show any specific bias in concordance based on assay price (data not shown). However, the 2 ELISAs that employ recombinant antigens did demonstrate the highest sensitivities albeit in different specificities. Based on our data, the prevalence of discord between a screening ELISA and CLIFT is dependent on how specific ELISA is designed. It is therefore logical to speculate that a ELISA (irrespective of antigen utilized) may be detecting different kinds or subsets of anti-dsDNA antibodies based on its principle how cut-off levels have been established.

Our study has three shortcomings, the increased sensitivity and specificity of the CLIFT compared to published reports [5,7,8,10,24]. Lack of clinical information for the ANA positive and Bio-Rad Ig negative samples and, lastly, the absence of ANA IFA results for the 2 SLE samples. Nevertheless, the inclusion of Bio-Rad Ig negative group even though a highly sensitive specific assay has been described [20,21], the sensitivity of the CLIFT is estimated to range from 43.0 to 50.2% with specificities of 65.0 to 87.3% [5,7,8,10]. With the consistent performance of the CLIFT in all groups investigated, it is very likely that factors relating to the use of the substrates and/or secondary antibody rather than test reading and interpretation of the slides may be responsible for the high positivity and specificity observed in this study. In addition, lack of contradiction in the performance of the CLIFT as well as ELISAs in the different cohorts further validates our interpretation results despite the absence of clinical information. With respect to use of clinically defined specimens in our evaluation, we have been cautious in selecting ANA positive samples with titers greater than or equal to 1:160 with a corresponding homogenous pattern. Although the ANA IFA homogenous pattern is largely associated with the presence of dsDNA antibodies and there are other specific associated with this pattern, the clinical relevance of anti-dsDNA antibodies is the absence of ANA as determined by IFA is unknown. Furthermore, of the ANA positive SLE patients, a variable percent will test positive for anti-dsDNA antibodies [3,11,13]. Finally, Bio-Rad anti-dsDNA IgG negative group lacks results for ANA IFA which makes the interpretation of the data especially for the discrepant samples somewhat difficult. While the significance of this observation was not determined in this study, the finding that majority of
positive samples came from a single ELISA reaffirms our interpretation that these assays have variable performance characteristics.

Our study raises 2 key issues regarding anti-dsDNA antibody testing. The first being how best to standardize these assays given the substantial differences in the design of the anti-dsDNA antibody ELISAs that are currently available. The second issue has to be with discordant results between screening and confirmatory tests especially knowing that these assays lack standardization, may have different clinical uses (based on assay principle) as well as the fact these antibodies may precede disease onset by several years [3–8,10–15,19,22]. With respect to discordance between screening and confirmatory assays, there is some evidence that ELISA positive and CLEF negative results may have some clinical consequence [7]. On the other, false negative screening assays have also been reported [10]. While this study by no means directly addresses these issues, our data show significant variations in the performance of commonly used assays for the detection of anti-dsDNA IgG antibodies in the US. The actual impact of this in routine practice and how patient care may be affected remains unknown.

In conclusion, our data demonstrate significant differences in the analytical concordance between ELISAs for detecting anti-dsDNA IgG antibodies and the CLEF confirmatory with implications for inter-laboratory testing. An understanding of the strengths and limitations of these assays and how they may impact the diagnosis of SiE is important.

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