Practical Evaluation of Methods for Detection and Specificity of Autoantibodies to Extractable Nuclear Antigens

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Detection and specificity of autoantibodies against extractable nuclear antigens (ENA) play a critical role in the diagnosis and management of autoimmune disease. Historically, the detection of these antibodies has employed double immunodiffusion (DID). Autoantibody specificity was correlated with diagnoses by this technique. Enzyme immunoassays have been developed by many manufacturers to detect and identify the specificity ENA autoantibodies. To address the relationship of ENA detection by DID and enzyme immunoassay, the performances of five immunoassays were compared. These included two DID and three enzyme-linked immunoassays (ELISA) (both screening and individual antigen profile kits). The sample set included 83 ENA-positive, antinuclear-antibody (ANA)-positive specimens, 77 ENA-negative, ANA-positive specimens, and 20 ENA- and ANA-negative specimens. Sensitivity and specificity were calculated by two methods: first, by using the in-house DID result as the reference standard, and second, by using latent class analysis, which evaluates each kit result independently. Overall, the results showed that the ELISA methods were more sensitive for detection of ENA autoantibodies than DID techniques, but presence and/or specific type of ENA autoantibody did not always correlate with the patient’s clinical presentation. Regardless of the testing strategy an individual laboratory uses, clear communication with the clinical staff regarding the significance of a positive result is imperative. The laboratory and the clinician must both be aware of the sensitivity and specificity of each testing method in use in the clinical laboratory.

A diagnosis of autoimmune disease in patients is based upon clinical history, physical examination, and laboratory detection of antinuclear antibodies (ANAs). A particular class of ANAs specific for extractable nuclear antigens (ENA) was initially described in 1959 (3). Since that time, many different anti-ENA antibodies have been described. The detection of these autoantibodies and identification of their specificity have become well-established tools for the laboratory diagnosis of several autoimmune diseases. Studies of patients with ENA antibodies have shown that detection of these autoantibodies may have both diagnostic and prognostic significance, and the detection of anti-ENA antibodies has assumed an important role in the management of these patients (5, 16, 22). In most cases, ENA testing is ordered after an initial ANA screen. The indications for use are to establish a diagnosis in patients with suggestive clinical symptoms, to exclude a diagnosis of autoimmune disease in patients with few or uncertain clinical signs, to subclassify patients with a known diagnosis, and to monitor disease activity.

Testing for anti-ENA antibodies has historically relied on gel-based immunoprecipitation techniques such as double immunodiffusion (DID) and counterimmunoelectrophoresis (2, 14). The associations of specific types of ENA autoantibodies with rheumatological diseases were established by using these gel-based immunoassay techniques (15). In the last decade, enzyme-linked immunoassay (ELISA) systems have been developed to detect and determine the specificity of anti-ENA antibodies. ELISA systems permit more rapid processing of more specimens with a faster turnaround time than gel-based assays. ELISA-based methods may also have increased sensitivity for detection of ENA antibodies. However, the increased specificity of these ELISAs may influence the clinical relevance of their detection because diagnostic specificity may be reduced (10, 12, 17, 24). As yet, a set of reference standards with known antibody specificities against defined antigen preparations is not available for evaluation of various methods or kits. Serum reference panels are available from the Association of Medical Laboratory Immunologists (4), but the specificities of these sera were determined by consensus results from multiple laboratories. The purpose of this study was to address the relationship between DID and ELISA methods for the detection and identification of anti-ENA antibodies by evaluating and comparing two DID kits and three ELISA kits. We evaluated both screening ELISAs and monospecific antigen ELISAs to determine anti-ENA specificity.

MATERIALS AND METHODS

This study was approved by the Human Investigational Review Board of the University of North Carolina, Chapel Hill.

Kits. The immunoassay kits chosen for this study were based upon their representation in the listing of immunoassays utilized by participants in the College of American Pathologists proficiency surveys, as well as the manufacturer’s willingness to participate in this study by providing immunoassay kits. Three manufacturers of screening and individual antigen ELISA systems are as follows: Immuno Concepts (kit 2) (Sacramento, Calif.), INOVA Diagnostics, Inc. (kit 3) (San Diego, Calif.), and Diamedix (kit 4) (Miami, Fla.). Two DID kits

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RESULTS

Comparison of screening ELISAs for detection of ENA autoantibodies. Concordance, sensitivity, and specificity were calculated for each of the three screening ELISAs by using the in-house DID (kit 5) as the reference standard. The overall concordances between the screening immunounassays and the kit 5 DID for combined detection of Sm, RNP, SSA, and/or SSB were 95, 91, and 93%, respectively, for kits 2, 3, and 4 (Table 1). The sensitivities of the screening ELISA (kits 2, 3, and 4) were 91, 85, and 89%, respectively, with specificities of 100, 98, and 98%. The concordance between kit 1 (DID) and the reference test (kit 5) was 98%, with a sensitivity of 96% and specificity of 100%.

Using latent class analysis, the sensitivities of the screening assays were 99 to 100% for the three ELISA kits and 90 to 92% for the two DID kits (Table 2). Conversely, the two DID kits had specificities of 99 and 100%, while specificities for detecting anti-ENA antibodies were 98, 92, and 96% for ELISA kits 2, 3, and 4, respectively (Table 2).

Profile ELISAs to detect antigen-specific ENA antibodies. Concordance, sensitivity, and specificity in relation to the in-house DID (kit 5) were calculated for the three antigen-specific ELISAs and DID kit 1 for the four major antigens, Sm, RNP, SSA, and SSB, regardless of the group that the specimen was in (i.e., ANA and ENA positive, ANA positive and ENA negative, or ANA and ENA negative [Table 3]). Compared to kit 5, concordance and specificity were ≥98% for detection of the four major antigens using kit 1. The sensitivities of kit 1 were 73% for detection of Sm antibodies, 100% for RNP antibodies, and 92 and 78% for SSA and SSB antibodies, respectively. The three ELISA profile kits performed similarly as a group. Kit 3, overall, had more discordant results than the other two ELISAs (kits 2 and 4) for the four major antigens (Table 3). The average sensitivity of detection of the Sm antigen by the kits tested compared to kit 5 was the lowest for the four antigens tested (75% ± 9%). Average concordance between the other kits and kit 5 for detection of Sm was also lower, on average, than for the other three antigens. Average concordance for detection of Sm antigen was 91% ± 12%, compared to 94% ± 7% for RNP, 95% ± 2% for SSA, and 94% ± 3% for SSB. The specificity of detection for all four antigens was similar regardless of the kit used. Limited testing was conducted for Jo-1 and Scl-70 antigens; however, in all cases where the in-house DID (kit 5) was positive for either antigen, kit 1 DID and all three ELISA profile kits for either Jo-1 or Scl-70 were positive.

Using latent-class analysis, the ELISA kits had greater sensitivity for detection of antibodies against Sm, RNP, and SSA than the DID kits (Table 4). The sensitivity of detecting SSB antibodies with DID was very low (64% and 69% for kits 1 and

| Kit | % Sensitivity (95% CI) | % Specificity (95% CI) |
|-----|------------------------|------------------------|
| 1   | 90 (84–96)             | 100                    |
| 2   | 100                   | 98 (95–100)            |
| 3   | 99 (97–100)           | 92 (86–98)            |
| 4   | 99 (97–100)           | 96 (93–100)            |
| 5   | 92 (87–98)            | 99 (97–100)            |

a Sensitivity and specificity were calculated based on analysis of 180 serum specimens to 95% confidence levels. CI, confidence interval.
5) compared to ELISA kits 2 and 3, both at 100%. However, ELISA kit 4 also had low sensitivity for detection of SSB at 55%. The specificity of the DID kits was ≥98% for detection of the four major antigens tested. The ELISA kits also showed similar results, except for kit 3, which had low specificity for Sm (73%) and RNP (86%).

**Evaluation of specimens with inconsistent results.** Fifteen specimens had varying results with screening and/or antigen specific immunoassays. Fourteen of the fifteen specimens with inconsistent results were from patients who were classified as ANA positive and ENA negative (DID kit 5). Chart review revealed that 11 of the 15 samples (67%) had either a previous diagnosis of autoimmune disease or symptoms consistent with a diagnosis of autoimmune disease (Table 5). ELISA kits detected ENA antibodies in 10 of those samples, while DID detected antibodies in only two of those samples. In both of those samples (no. 10 and 11), ENA reactivity by both DID and ELISA matched the predicted specificity. In four of the eight (50%) samples that were ELISA positive and DID negative (no. 2, 3, 9, and 12), ELISA specificity in one or more of the kits matched predicted ENA specificity.

There were three specimens (no. 6, 8, and 14) that were more difficult to interpret due to symptoms not necessarily consistent with autoimmune disease. However, for two of the samples (no. 6 and 8), all the ELISAs showed reactivity with SSA, and the samples were negative by DID. Sample 14, also negative by DID, showed Jo-1 reactivity by two of the ELISA kits, which was not predicted.

**DISCUSSION**

In recent years, ELISA methods have been developed to detect the presence and specificity of anti-ENA autoantibodies. This study was performed to compare the performance of several ELISA kits with conventional gel-based assays currently in use in a tertiary-care hospital.

The determination of the test characteristics of these assays is limited because no “perfect” reference standard is available. In using kit 5 (DID) as the reference standard, we assumed it had perfect characteristics. If a test is more sensitive, such as many of the ELISAs, some true-positive specimens may be misclassified as false positives, resulting in underestimation of test sensitivity. To address this issue we used latent-class analysis, which does not require the designation of a reference standard. Although this procedure assumes independence between the assays, the estimates are relatively robust to violation of this assumption (18). Results of this study indicate that the initial antibody screening ELISA had good correlation with the DID for detecting anti-ENA antibodies. One can reasonably assume that, based upon these results, the ELISA kits commercially available will detect specimens that contain anti-ENA antibodies. Both DID methods showed high levels of agreement for detection of specific ENA autoantibodies. By latent class analysis, the ELISA kits were more sensitive for antibody detection than the DID kits (Table 2), which was not unexpected, because other studies have drawn similar conclusions (1, 9). Lock et al. found that some patients who do not have systemic lupus erythematosus had levels of anti-Sm antibodies that were detectable by ELISA (8). This type of non-specificity may have important consequences, since the presence of anti-Sm is one of the diagnostic criteria described by the American Rheumatism Association for a diagnosis of systemic lupus erythematosus (15). The antigen source, native or recombinant, and method of purification are critical to sensitivity and specificity in relation to the Sm/RNP complex (7) and to the SSA multichain antigen (13, 21). Conformational changes, as a result of coating the plastic microwell, may result in loss of conformational epitopes recognized by ENA antibodies (6, 11). However, the DID technique may be more specific for detecting clinically significant anti-ENA antibodies.

**TABLE 3. Evaluation of profile kitsa using in-house DID (kit 5) as the reference standard**

| Antigen | Kit | Concordance (%) | Sensitivity (%) | Specificity (%) |
|---------|-----|-----------------|----------------|----------------|
| Sm      | 1   | 98              | 73             | 99             |
|         | 2   | 99              | 82             | 100            |
|         | 3   | 73              | 82             | 72             |
|         | 4   | 98              | 64             | 100            |
| RNP     | 1   | 99              | 100            | 99             |
|         | 2   | 94              | 100            | 94             |
|         | 3   | 84              | 100            | 84             |
|         | 4   | 95              | 100            | 95             |
| SSA     | 1   | 98              | 92             | 100            |
|         | 2   | 94              | 98             | 93             |
|         | 3   | 93              | 94             | 93             |
|         | 4   | 93              | 94             | 93             |
| SSB     | 1   | 98              | 78             | 100            |
|         | 2   | 94              | 83             | 95             |
|         | 3   | 91              | 83             | 92             |
|         | 4   | 91              | 56             | 95             |

*a One DID kit (kit 1) and three ELISA kits (kits 2, 3, and 4).

**TABLE 4. Latent-class analysis of profile kitsa**

| Antigen | Kit | % Sensitivity (95% CI) | % Specificity (95% CI) |
|---------|-----|------------------------|------------------------|
| Sm      | 1   | 89 (68–100)            | 99 (98–100)            |
|         | 2   | 100                    | 100                    |
|         | 3   | 100                    | 73 (67–80)             |
|         | 4   | 78 (51–100)            | 100                    |
|         | 5   | 100                    | 99 (97–100)            |
| RNP     | 1   | 90 (82–100)            | 99 (98–100)            |
|         | 2   | 100                    | 97 (94–100)            |
|         | 3   | 100                    | 86 (81–92)             |
|         | 4   | 100                    | 98 (95–100)            |
|         | 5   | 88 (78–98)             | 100                    |
| SSA     | 1   | 86 (76–95)             | 100                    |
|         | 2   | 98 (94–100)            | 97 (94–100)            |
|         | 3   | 98 (93–100)            | 97 (94–100)            |
|         | 4   | 98 (94–100)            | 97 (94–100)            |
|         | 5   | 90 (81–98)             | 100                    |
| SSB     | 1   | 64 (44–84)             | 100                    |
|         | 2   | 100                    | 99 (98–100)            |
|         | 3   | 100                    | 97 (95–100)            |
|         | 4   | 55 (34–76)             | 96 (93–99)             |
|         | 5   | 69 (49–88)             | 98 (96–100)            |

*a Sensitivity and specificity data were generated using latent-class analysis for ENA profile testing of five immunoassay methods. CI, confidence interval.
ELISA techniques are more likely to detect low affinity antibodies than gel immunodiffusion methods (8, 19). The variation in results of the ELISA kits may reflect differences in antigen preparations, possibly resulting in different antigen-binding epitopes and/or the presence of contaminating antigens; differences in antigen coating concentrations; different buffers affecting binding of antibodies, and different cutoff values for determination of positive and negative. Other studies have also shown inconsistent results between various ELISA kits (17). Additional studies are needed to determine the reasons for these inconsistencies, but this will be difficult to do until a standardized set of control antigens is available.

A majority of the specimens with inconsistent results that were noted in this study (67%) were from specimens with lower ANA titers, which has been noted in other studies as well (4). The lower ANA titers may reflect antibodies that have lower binding avidities. Among specimens with inconsistent results, review of the patient’s chart at the time of specimen collection showed that most of the patients had either a diagnosis or symptoms of autoimmune disease. The major discrepancy was due to the fact that the DID test was negative while the ELISAs were positive. Overall, the agreement between the kits for ENA specificity was good. Predicted ENA specificity matched measured specificity in 50% of the specimens.

There is still no one ideal test that is both highly sensitive and highly specific. So what is the strategy for anti-ENA testing? The European Consensus Workshops recommends that ENA testing be performed by two or more methods (20). Given the trade-off between sensitivity and specificity between the ELISA and DID methods, one might consider using the ELISAs to screen sera for anti-ENA antibodies, followed by DID testing of positive sera for identification of antibody specificity. Regardless of the testing strategy an individual laboratory selects, clear communication with the clinical staff regarding the significance of a positive result is imperative. Clinicians must be aware of the sensitivity and specificity of each testing method used.

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