High-Quality, Cost-Effective Strategy for Detection of Autoantibodies to Extractable Nuclear Antigens

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We evaluated methods for the detection of autoantibodies to extractable nuclear antigens (ENAs) to determine the strategy that yielded the most cost effective and clinically meaningful result. We prospectively compared counterimmunoelectrophoresis (CIEP) with and without serum prediffusion (SPD) and found that SPD significantly improved the quality of precipitation lines. This resulted in a decreased requirement for repeat testing and, consequently, was associated with a significant decrease in reagent costs and specimen turnaround time. We also retrospectively compared reactivity by CIEP, CIEP plus SPD, enzyme-linked immunosorbent assay (ELISA), and line immunoassay (LIA) of 52 serum samples that were previously determined to be positive for ENAs, and we correlated the results with clinical diagnoses. There was significant agreement among CIEP, CIEP plus SPD, ELISA, and LIA for the detection of anti-SS-A, anti-SS-B and anti-RNP. In general, CIEP, CIEP plus SPD, and LIA correlated better with the clinical diagnoses than ELISA, even though ELISA detected anti-ENAs more often than the other methods. CIEP plus SPD is therefore the most cost effective method for the identification of clinically meaningful ENAs. Based on our experience, we now screen for ENAs by CIEP, and positive samples are then typed by CIEP plus SPD. Samples that are difficult to interpret are then further assessed by an alternative method.

The detection of autoantibodies to extractable nuclear antigens (ENA) is useful in the diagnosis and assessment of prognosis of autoimmune connective tissue diseases (CTD) (5). These autoantibodies were originally described using the gel diffusion techniques double immunodiffusion (DID) and counterimmunoelectrophoresis (CIEP). Clinical correlates are, in general, based on test results produced by these methods. Both DID and CIEP are labor intensive, and in practice the specimen turnaround time is often slow, although CIEP is more rapid and more sensitive than DID. However, the precipitation lines (PLs) in CIEP are often absent or unclear, making interpretation of the gel difficult and necessitating repeat testing, further delaying the reporting of results.

Recently, more rapid and sensitive methods such as enzyme-linked immunosorbent assay (ELISA) and line immunoassay (LIA) have been introduced for use in the detection of ENA. These methods often use recombinant antigens that are costly and, in addition, lack the clinical specificity of the original gel diffusion techniques. Laboratories have tried to resolve these issues by developing different strategies for anti-ENA detection that utilize a combination of two or more methods, a strategy also recommended by a European consensus statement (6). We sought to evaluate the available methods for ENA detection in order to identify a strategy that would yield accurate, clinically useful, and cost-effective results by comparing the performances of CIEP with serum prediffusion (SPD), CIEP without SPD, a commercial ELISA, and LIA.

MATERIALS AND METHODS

Serum samples. Sera from 205 patients referred to our diagnostic laboratory for anti-ENA testing were screened by CIEP, and positive samples were typed by CIEP alone and CIEP plus SPD in parallel. In addition, 52 ENA-positive samples that had been stored at −70°C were analyzed by CIEP plus SPD and by ELISA, and the results were compared to those from the original CIEP and LIA. Clinical details were obtained from review of the patients’ medical records and from diagnoses made independently of the ENA test results.

Antigen extracts. Antigen extracts were prepared from lyophilized powder and stored at −70°C before use. Guinea pig kidney extract (Sigma, St. Louis, Mo.) was used to detect anti-SS-A, and rabbit thymus extract (Pel-Freez Biologicals, Rogers, Ark.) was used to detect anti-SS-B, anti-RNP, anti-Sm, anti-Jo-1, and anti-Scl-70.

Antibody controls. Purified antibodies to SS-A, SS-B, RNP, Sm, Jo-1, and Scl-70 (INOVA Diagnostics Inc., San Diego, Calif.) were used to determine lines of identity on the gels.

CIEP. CIEP was performed as described by Bunn and Kveder (2). Briefly, 1% agarose gels were freshly prepared, loaded with sera and antigen extract, and placed in an electrophoresis tank containing barbital buffer at pH 8.4. Electrophoresis was carried out at 180 V for 30 min. The gels were then washed in phosphate-buffered saline (PBS), soaked overnight in 5% sodium citrate, and stained with Paragon Blue (Beckman Instruments Inc., Fullerton, Calif.). CIEP plus SPD was performed as previously described (7). Briefly, 1% agarose gels were loaded with serum samples and antibody controls and allowed to diffuse in a moist chamber for 2 h before the antigen extracts were loaded and conventional CIEP was performed.

The gels were read by at least two scientists blinded to the method used (CIEP or CIEP plus SPD) and a consensus was reached; if there was disagreement, the test was either repeated or further analyzed by LIA, or both. Samples were reported as positive if the PLs identified with monospecific control antibody, as equivocal if there was partial identity or unclear PLs, and as negative if the PLs did not identify with control antibody. In general, samples that gave unclear or absent PLs were retested and those that partially identified with control antibody were further analyzed by LIA.
ELISA. An ENA RELISA Kit (Immuno Concepts Inc., Sacramento, Calif.) was used according to the manufacturer’s instructions. The absorbance was read with a Dynatech MR5000 dual wavelength spectrophotometer at 450 nm, with the reference filter set at 630 nm. ENA units were calculated for each specimen and interpreted in accordance with the manufacturer’s recommendations as follows: <20 ENA units, negative; 20 to 30 ENA units, borderline; and >30 ENA units, positive.

LIA. An INNO-LIA ANA Kit (Innogenetics N.V., Ghent, Belgium) was used and interpreted according to the manufacturer’s instructions: the patient’s serum was considered positive if it reacted more strongly with the antigen band than the control serum provided with the kit. A serum sample was considered positive if it reacted with at least two of three RNP antigens (RNP-A, RNP-B, and RNP-C), and it was considered positive for anti-Sm only if it reacted with the SmD antigen.

Cost analysis. Costs were calculated based on the costs of reagents in Australian dollars (A$) at standard commercial prices, excluding labor. Although CIEP was more labor intensive, we have previously found that the time actually spent “hands-on” performing the assay is similar to that for, ELISA and LIA, and therefore labor costs were considered to be equivalent for each of the assays.

Turnaround time. Our laboratory routinely performs CIEP daily, and the average turnaround time for a positive test is 5 days (2 days to screen, 2 days to type, and 1 day for specimen processing). The laboratory batches the LIA samples and performs the test weekly. The average turnaround time for a sample requiring this test is 7 days.

Statistics. Comparison between proportions was performed by calculating the chi square with Yates’ correction, and agreement between tests was measured by calculating the kappa coefficient (1). Kappa was interpreted as follows: <0.20, poor agreement; 0.20 to 0.40, fair agreement; 0.41 to 0.60, moderate agreement; 0.61 to 0.80, good agreement; >0.80, very good agreement. The positive and negative predictive values of each method were calculated by correlating the results of the tests for anti-ENA with the clinical diagnoses: SS-A and SS-B for Sjögren’s syndrome and systemic lupus erythematosus (SLE); RNP for mixed connective tissue disease (MCTD) and/or SLE; Sm for SLE; Jo-1 for polymyositis/dermatomyositis (PM/DM); and Scl-70 for scleroderma.

RESULTS

CIEP plus SPD improves the quality of PLs and decreases the frequency of equivocal results. Twenty-eight, of 205 samples (14%) were positive for anti-ENA by CIEP. Analysis of sera from these 28 patients showed that SPD improved the quality of PLs and the ease of interpretation of the CIEP gels (Fig. 1). SPD reduced the number of equivocal results from 20 of 28 (71%) for conventional CIEP to 11 of 28 (39%) for CIEP plus SPD, a reduction of 32% (95% confidence interval [CI], 7 to 57%; P = 0.03). Repeat testing because of absent or unclear PLs was reduced (12 of 28 samples for CIEP, and 9 of 28 for CIEP plus SPD), and fewer LIAs were performed to characterize PLs that were difficult to interpret (8 of 28 for CIEP and 2 of 28 for CIEP plus SPD).

CIEP plus SPD decreases cost and turnaround time. CIEP costs A$8.00 per type, and LIA costs A$35.00 per type (see below). Over the 3-week study period, the cost of typing 28 specimens was A$600.00 by CIEP (28 gels x A$8.00 + 12 repeat gels x A$8.00 + 8 LIAs x A$35.00) and A$366.00 by CIEP plus SPD (28 gels x A$8.00 + 9 repeat gels x A$8.00 + 2 LIAs x A$35.00). This is a saving of A$234.00 (39%), or A$8.00 per specimen typed.

The turnaround time for characterizing 28 specimens by CIEP is 220 days (28 gels x 5 days + 12 repeat gels x 2 days + 8 LIAs x 7 days), or an average of 8 days. The turnaround time for characterizing 28 specimens by CIEP plus SPD is 172 days (28 gels x 5 days + 9 repeat gels x 2 days + 2 LIAs x 7 days), or an average of 6 days.

TABLE 1. Frequency of ENA identified

| ENA  | CIEP (n = 52) | CIEP+SPD (n = 52) | LIA (n = 51) | ELISA (n = 46) |
|------|--------------|------------------|------------|--------------|
| SS-A | 22 (42)      | 19 (37)          | 27 (53)    | 29 (62)      |
| SS-B | 6 (12)       | 5 (10)           | 13 (25)    | 12 (25)      |
| RNP  | 10 (19)      | 13 (25)          | 8 (16)     | 25 (53)      |
| Sm   | 5 (10)       | 4 (8)            | 4 (8)      | 3 (6)        |
| Jo-1 | 1 (2)        | 0                | 1 (2)      | 2 (4)        |
| Scl-70 | 3 (6)    | 1 (2)            | 3 (6)      | 5 (11)       |
There is significant agreement among CIEP, CIEP plus SPD, ELISA, and LIA for SS-A, SS-B, and RNP. Fifty-two samples, previously typed by CIEP and LIA, were reanalyzed by CIEP plus SPD, and 47 of these were analyzed by ELISA. Anti-ENA identified by the four methods are shown in Table 1. Overall, anti-SS-A was detected most frequently (37 to 42%), followed by anti-RNP (16 to 53%) and anti-SS-B (10 to 25%). Antibodies to Sm (6 to 10%), Scl-70 (2 to 11%), and Jo-1 (0 to 4%) were infrequent, and agreement among tests was not calculated for these three anti-ENA. There is moderate to very good agreement among CIEP, CIEP plus SPD, ELISA, and LIA for anti-SS-A and anti-SS-B (kappa, 0.44 to 0.90) and fair to good agreement for anti-RNP (kappa, 0.30 to 0.78) (Table 2). SPD did not adversely affect the frequency of anti-ENA detection, and there was good agreement between CIEP and CIEP plus SPD for anti-SS-A (kappa = 0.72) and anti-RNP (kappa = 0.78) and very good agreement for anti-SS-B (kappa = 0.90). Agreement between CIEP with or without SPD and LIA was better than that between CIEP with or without SPD and ELISA.

CIEP and CIEP plus SPD correlate better with clinical diagnoses than LIA and ELISA. Clinical details were obtained for 45 of the 52 patients (Table 3). The commonest diagnoses were SLE (15 of 52, or 29%), Sjögren’s syndrome (11 of 52, or 21%) and MCTD (6 of 52, or 12%). Clinical correlations of anti-ENA detected by the different methods are shown in Tables 4 and 5. In general, the positive predictive values of CIEP, CIEP plus SPD, and LIA were greater than that of ELISA, particularly for anti-RNP, anti-Sm, and anti-Scl-70; however, this difference was not statistically significant. Similarly, the negative predictive values of CIEP, CIEP plus SPD, LIA, and ELISA were not statistically different.

CIEP and CIEP plus SPD are the most cost effective methods of ENA detection. Screening by CIEP costs $A0.70 in reagents per sample. Typing by CIEP costs $A8.00 per sample, and the use of commercial monospecific antibody controls contributes the majority (98%) of the cost. SPD did not increase the cost of CIEP, as no additional materials were required. Typing by ELISA costs $A29.00 per sample, and typing by LIA costs $A35.00 per sample.

**DISCUSSION**

The detection of autoantibodies that are highly specific for autoimmune CTD can be pivotal in the diagnosis of these conditions. In the general population, the prevalence of autoimmune connective diseases is low, and positive tests for anti-nuclear antibodies (ANAs) and anti-ENA have low positive predictive value as screening tests (4). It is therefore not surprising that a survey of 104 clinicians determined that they considered that the results of testing for ANAs and ENA had no clinical value in 66% of cases (3). The use of highly specific methods for anti-ENA detection can, however, increase the positive predictive value and therefore the clinical utility of the test.

The use of CIEP to screen for ENA is highly specific and cost-effective. We sought to determine the optimum strategy for subsequent identification of the autoantibodies to ENA. We confirmed that SPD improves the quality of PLs and the ease of CIEP gel interpretation, as reported by Walravens et al. (7), and can be easily integrated into a diagnostic laboratory’s work flow. Indeed, the use of CIEP plus SPD was associated with a significant improvement in the quality of the results compared to those with conventional CIEP. This resulted in decreased repeat testing and less need for testing by an alternate, more expensive method. Overall, CIEP plus SPD

**TABLE 2. Agreement between tests as measured by the Kappa coefficient**

| Tests compared | SS-A | SS-B | RNP | Sm | Jo-1 | Scl-70 |
|---------------|------|------|-----|----|------|--------|
| CIEP vs SPD   | 0.72 (0.53-0.91) | 0.90 (0.70-1.00) | 0.78 (0.58-0.99) | ND* | ND   | ND     |
| CIEP vs LIA   | 0.65 (0.44-0.86) | 0.56 (0.26-0.86) | 0.68 (0.41-0.95) | ND  | ND   | ND     |
| CIEP vs ELISA | 0.44 (0.19-0.70) | 0.50 (0.18-0.82) | 0.42 (0.16-0.67) | ND  | ND   | ND     |
| SPD vs LIA    | 0.61 (0.40-0.83) | 0.48 (0.15-0.81) | 0.58 (0.30-0.87) | ND  | ND   | ND     |
| SPD vs ELISA  | 0.48 (0.23-0.73) | 0.51 (0.18-0.85) | 0.33 (0.06-0.60) | ND  | ND   | ND     |
| LIA vs ELISA  | 0.43 (0.17-0.69) | 0.66 (0.41-0.91) | 0.30 (0.04-0.57) | ND  | ND   | ND     |

* ND, not done because numbers were too small.

**TABLE 3. Clinical diagnoses of the 52 patients tested**

| Diagnosis                              | No. (%) of patients |
|----------------------------------------|---------------------|
| SLE                                    | 15 (29)             |
| Sjögren’s syndrome                     | 11 (21)             |
| MCTD                                   | 6 (12)              |
| Scleroderma                            | 3 (6)               |
| PM/DM                                  | 2 (4)               |
| Rheumatoid arthritis                   | 2 (4)               |
| Primary Raynaud’s phenomenon           | 2 (4)               |
| Other                                   | 4 (8)               |
| Unknown                                 | 7 (13)              |

**TABLE 4. Positive predictive value of the anti-ENA for their associated clinical diagnoses in the selected population**

| ENA      | Disease            | No. positive by the indicated test/no. positive by clinical diagnosis (%) |
|----------|--------------------|-------------------------------------------------------------------------|
| SS-A     | SLE or SLE         | CIEP 17/19 (89) | CIEP plus SPD 15/17 (88) | LIA 18/22 (82) | ELISA 19/27 (70) |
| SS-B     | SS or SLE          | CIEP 4/4 (100)  | CIEP plus SPD 3/3 (100)  | LIA 10/11 (91) | ELISA 8/10 (80)  |
| RNP      | MCTD               | CIEP 6/10 (60)  | CIEP plus SPD 5/12 (42) | LIA 4/7 (57)  | ELISA 6/16 (38)  |
| MCTD or SLE |                  | CIEP 9/10 (90) | CIEP plus SPD 11/12 (92) | LIA 6/7 (86)  | ELISA 15/22 (68) |
| Sm       | SLE                | CIEP 2/3 (67)   | CIEP plus SPD 2/3 (67)  | LIA 0/4 (0)   | ELISA 1/3 (33)   |
| Jo-1     | PM/DM              | CIEP 1/1 (100)  | CIEP plus SPD 0/1 (0)   | LIA 1/1 (100) | ELISA 1/2 (50)   |
| Scl-70   | Scleroderma        | CIEP 2/2 (100)  | CIEP plus SPD 1/1 (100) | LIA 3/3 (100) | ELISA 2/2 (100)  |

* SS, Sjögren’s syndrome.
significantly decreased the cost and turnaround time for sample analysis.

There was significant agreement among CIEP, CIEP plus SPD, LIA, and ELISA for the detection of the common anti-ENA (anti-SS-A, anti-SS-B, and anti-RNP). Overall, anti-ENA detected by CIEP and CIEP plus SPD correlated better with their respective clinical diagnoses than anti-ENA detected by LIA and ELISA.

We conclude that SPD significantly improves the quality of CIEP at no additional cost and concomitantly, significantly decreases cost and turnaround time. Anti-ENA identified by CIEP, with or without SPD, correlate well with their associated clinical conditions and have the greatest clinical utility. Although LIA also correlates well, it is significantly more expensive to perform. Therefore, we recommend that anti-ENA be screened by CIEP and typed by CIEP plus SPD. The minority of samples that cannot be interpreted by these methods alone should be further assessed by an alternate method, preferably LIA, because it has a higher predictive value than ELISA. This strategy optimizes the positive predictive value and clinical utility of the ENA test and, at the same time, decreases cost. This study demonstrates that in an era of cost containment, it is still possible to improve the quality of a laboratory service and simultaneously to reduce the cost and provide results with greater clinical significance.

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