CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 1997, p. 185–188 Vol. 4, No. 2
1071-412X/97/$04.00+0

Comparison of Antinuclear Antibody Testing Methods: Immunofluorescence Assay versus Enzyme Immunoassay

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Received 15 August 1996/Returned for modification 14 October 1996/Accepted 25 November 1996

Performances of anti-nuclear antibody testing by immunofluorescence assay (ANA-IFA) and enzyme immunoassay (ANA-EIA) were compared in relation to patient diagnosis. A total of 467 patient serum samples were tested by ANA-IFA (Kallestad; Sanofi) and ANA-EIA (RADIAS; Bio-Rad), and their age, sex, diagnosis, disease status, and medications were obtained through chart review. Reference ranges were established by testing 98 healthy blood donor samples. Eighty-six samples came from patients with diffuse connective tissue diseases, including systemic lupus erythematosus, discoid lupus erythematosus, or drug-induced lupus (n = 71); systemic sclerosis, CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal motility abnormalities, sclerodactyly, and telangiectasia), or Raynaud's syndrome (n = 8); Sjögren's syndrome (n = 5); mixed connective tissue disease (n = 5); and polymyositis or dermatomyositis (n = 3). The sensitivity, specificity, positive predictive value, and negative predictive value for ANA-IFA were 87.2, 48.0, 29.1, and 93.9%, respectively, for the reference range of <1:160. For ANA-EIA, they were 90.7, 60.2, 35.8, and 96.4%, respectively, for the reference range of <0.9. ANA-EIA offers equivalent sensitivity and higher specificity compared to ANA-IFA.

Anti-nuclear antibody (ANA) testing is widely used as a screening test in connective tissue diseases (CTD) such as systemic lupus erythematosus (SLE), scleroderma, CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal motility abnormalities, sclerodactyly, and telangiectasia), Sjögren's syndrome, mixed connective tissue disease (MCTD), polymyositis, and dermatomyositis. However, positive ANA results are seen in a significant proportion of the elderly population (6, 17, 18, 20) and sensitivity of ANA testing varies widely from one clinical disease to another. For example, ANA testing has been reported to be positive in >95% of patients with SLE but in only 10 to 50% of patients with dermatomyositis and polymyositis (20).

The first description of ANA was made by Hargraves and colleagues in 1948 when they observed LE (lupus erythematosus) cells in the bone marrow of patients with SLE (4). Currently, the most commonly used method for ANA testing is ANA-immunofluorescence assay (ANA-IFA) in which slides prepared from human epithelioid cells (HEp-2 cells) as a substrate are incubated with diluted serum. The presence of autoantibodies is detected by fluorescent antiimmunoglobulin antibody, and characteristic morphologic patterns of fluorescent staining are observed. Certain ANA-IFA patterns are associated with the presence of autoantibodies to certain nuclear antigens which in turn are associated with certain clinical states (7, 13, 17, 20). For example, a diffuse or homogenous pattern is associated with such clinical states as SLE, rheumatoid arthritis, scleroderma, Sjögren's syndrome, and drug-induced lupus. The ANA-IFA is a subjective assay requiring skilled personnel and is a manual assay with a significant amount of hands-on time. Therefore, an ANA-enzyme immunoassay (ANA-EIA) is an attractive alternative to ANA-IFA, especially when the operation is automated. ANA-EIA should be able to reduce training time and hands-on time as well as eliminate the subjectivity in interpreting results. Studies on concordance of ANA-IFA and ANA-EIA results in which serum samples were tested by both methods have been reported previously (8, 11). However, the correlations between ANA results and the presence of CTD were not described in these studies. In this study, we compared the performance of ANA-IFA and ANA-EIA based on patient diagnosis. We conclude that ANA-EIA offers equivalent sensitivity and increased specificity compared to ANA-IFA.

MATERIALS AND METHODS

Subjects and chart review. Patient serum samples for which ANA-IFA was ordered as a part of routine medical care were entered into this study. They were from various locations within the University of California at San Francisco (UCSF) Medical Center including the emergency rooms, primary care clinics, specialty clinics, and inpatient services. The age, sex, diagnosis, disease status (active versus inactive), and medications of the patients were obtained by performing chart reviews. A total of 467 patient serum samples were collected from 13 April 1994 to 14 March 1995, of which 29 patient samples were eliminated because the charts could not be located before the completion of the study or the progress reports from pertinent visits were missing. Of the 438 samples entered into this study, 316 had an ANA-IFA titer of ≥1:80. During the entire study period, 1 patient had four serum samples and 13 patients had two serum samples sent for ANA-IFA.

Blood donor serum samples (n = 98) from the UCSF Blood Donor Center were also entered into this study. All units were from volunteer random or designated donors. None of the units came from autologous donors. An approval from the institution's Committee on Human Research was obtained for the use of these patient and blood donor serum samples for the purpose of this study. For the blood donors, age and sex were obtained.

Assays. ANA-IFA (Kallestad QUANTAFLUOR; Sanofi Diagnostics Pasteur, Inc., Chaska, Minn.) was performed according to the manufacturer's instructions. The serum samples were kept frozen (−20°C) until they were ready to be tested by ANA-EIA by using the automated analyzer RADIAS (Bio-Rad Laboratories, Hercules, Calif.). The ANA-EIA plates for the RADIAS are coated with a HEp-2 cell extract containing ANA antigens which include double-stranded DNA (dsDNA), Sjögren's syndrome antigens A and B (SS-A and SS-B) Sm, ribonucleoprotein (RNP), Jo-1, and Scl-70.

For selected samples, an autoantibody testing panel for dsDNA, Sm/RNP, and SS-A/SS-B was also performed. Autoantibody to dsDNA was tested by using an EIA method (Kallestad Anti-dsDNA Microplate EIA; Sanofi Diagnostics Pas-

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TABLE 1. Patient samples with diagnosis of CTD

Diagnosis	No. of samples	
	Overall period	Common period
SLE, DLE, drug induced	71	13
Scleroderma, CREST, Raynaud's syndrome	8	2
Sjögren's syndrome	5	0
MCTD, overlap syndromes	5	1
Polymyositis/dermatomyositis	3	2
Total	86[a]	15[a]

[a] A number of patients had multiple diagnoses of CTD.

teur, Inc.). Immunodiffusion (Ouchterlony) was performed for Sm/RNP (NOVA Gel "T" Sm/RNP; INOVA Diagnostics, Inc., San Diego, Calif.) and SS-A/SS-B (NOVA Gel "T" SS-A/SS-B, INOVA Diagnostics, Inc.).

Statistical analysis. For statistical analysis, SLE, discoid lupus erythematosus (DLE), drug-induced lupus, scleroderma, CREST syndrome, Raynaud's syndrome, Sjögren's syndrome, MCTD, overlap syndromes, polymyositis, and dermatomyositis were considered disease positive whereas rheumatoid arthritis, polyarteritis nodosa, and polymyalgia rheumatica were considered disease negative. The rationale was to define certain diagnoses of CTD (14) for which ANA is commonly positive as disease positive. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated by using standard formulae (20).

The analysis was done in two parts; the first part of the analysis included all 438 samples collected between 13 April 1994 and 14 March 1995, and this is referred to as the overall period. The analysis from the overall period would not reflect the prevalence of CTD patient samples encountered at UCSF because it includes samples from a time period when only ANA-IFA-positive (titer ≥ 1:80) samples were collected and ANA-IFA-negative samples were excluded. Because the UCSF Clinical Immunology Laboratory routinely kept only ANA-IFA-positive (≥1:80) samples, sera with ANA-IFA results of ≤1:40 were not available when this study was initiated. Therefore, a second analysis was performed between 5 January and 23 February 1995, referred to as the common period, to truly reflect the patient population at UCSF. During this period, 46 of 166 patient samples had ANA-IFA titers of ≥1:80.

Data were analyzed by statistical methods that account for the paired results from the ANA-IFA and ANA-EIA diagnostic tests within patients. To test the null hypothesis that two receiver-operating characteristic (ROC) curves (19) arose from the same binormal curve, a CLABROC algorithm was used, which is a version of a CORROC algorithm (10) that has been modified to analyze continuously distributed data (9). In addition, for specific cutoff values (1:40 and 1:160 for ANA-IFA and 0.9 for ANA-EIA), the exact McNemar's statistic (1) (StatXact; Cytel Software Corporation, Cambridge, Mass.) was used to compare sensitivities and specificities, and a z statistic (i.e., normally distributed) was used to compare positive and negative predictive values from the ANA-IFA and ANA-EIA assays. The latter statistic accounts for some subjects' responses being statistically independent (e.g., positive by one assay but not the other) and some subjects' responses being dependent (e.g., positive by both assays).

RESULTS

Establishing reference ranges. Of the 98 blood donors, 68% (67 of 98) had ANA-IFA results of <1:40. Of the 31 blood donors who had ANA-IFA titers of ≥1:40, 71% (22 of 31) had a titer of 1:40. The remaining 29% (9 of 31) had an ANA-IFA titer of ≥1:80. However, these nine samples had an ANA-EIA result of ≤0.9. By defining 95% of the blood donors as normal, a reference range of <1:160 for ANA-IFA was established for this study.

Ninety-seven percent (n = 95) of the 98 blood donors had ANA-EIA results of <0.9. The ANA-EIA results of three remaining donors were 0.9, 1.5, and 1.7. The manufacturer of ANA-EIA (RADIAS) defines the result of <0.9 as negative, 0.9 to 1.1 as indeterminate, and >1.1 as positive. The result of 1.0 is set at 2.5 standard deviations above the mean of "normals."

The ages of the blood donors ranged from 15 to 75 years, with a median of 34. Four blood donors were over 60 years of age. All four elderly donors had an ANA-IFA titer of 1:40, with a speckled pattern and an ANA-EIA result of ≤0.5. There were slightly more male donors (n = 57) than female donors (n = 41). The reference ranges calculated separately for male and female donors were identical (both <0.9) for ANA-EIA.

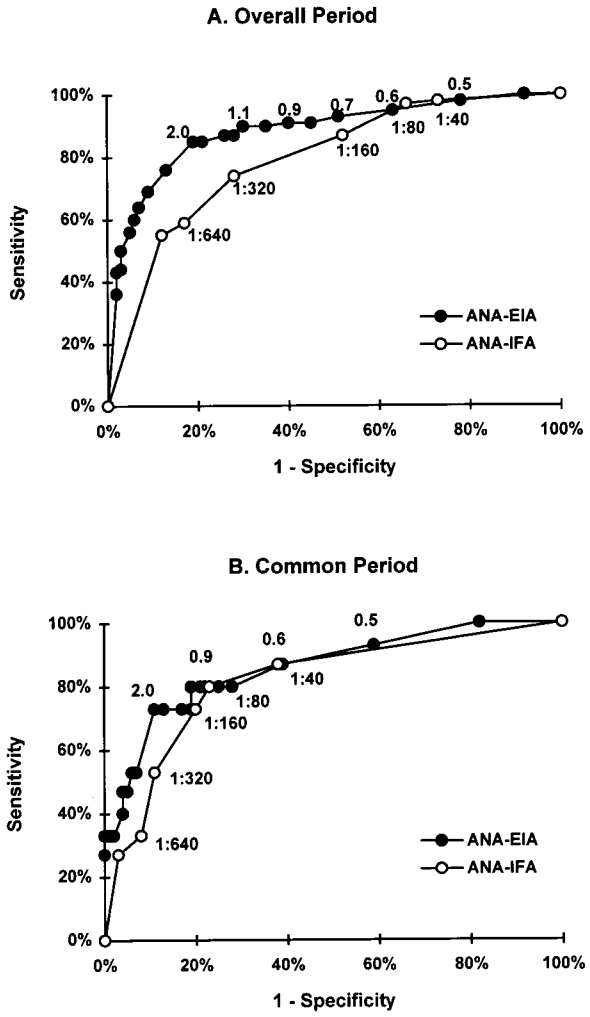

FIG. 1. (A) ROC curves for ANA-IFA (●) and ANA-EIA (○) for the overall period (P = 0.0004). (B) ROC curves for ANA-IFA (●) and ANA-EIA (○) for the common period (P = not significant).

Comparison of ANA-IFA and ANA-EIA. Of the 438 patient serum samples collected during the overall period, 20% (n = 86) had a diagnosis of CTD other than rheumatoid arthritis, polyarteritis nodosa, and polymyalgia rheumatica (Table 1). The majority of these samples came from patients with some form of lupus (71 of 86), but systemic sclerosis, CREST syndrome, Raynaud's syndrome, Sjögren's syndrome, MCTD, overlap syndromes, polymyositis, and dermatomyositis were also identified. A number of patients had multiple diagnoses of CTD.

As shown in Table 2, compared to ANA-IFA with the reference range of <1:160, ANA-EIA had equivalent sensitivity (90.7% versus 87.2%; P = not significant), higher specificity (60.2% versus 48.0%; $P < 0.0001$), higher positive predictive value (35.8% versus 29.1%; $P < 0.0001$), and higher negative predictive value (96.4% versus 93.9%; $P = 0.04$). The same observations can be drawn from the ROC curves (Fig. 1A). At lower cutoffs, the ROC curves for ANA-IFA and ANA-EIA overlap. However at higher cutoffs, ANA-EIA has a lower false

positive rate at equivalent sensitivity. The comparison of the ROC curves indicates that the ANA-EIA is a better diagnostic test than is ANA-IFA ($P = 0.0004$).

At the reference range for ANA-IFA of <1:40 normally used at UCSF, ANA-IFA has higher sensitivity than ANA-EIA (97.7% versus 90.7%; $P = 0.03$) (Table 2). However, it is probably inappropriate to compare the sensitivity of ANA-IFA at <1:40 and that of ANA-EIA at <0.9 in this study since the data from the 98 healthy blood donors indicated that only 68% had ANA-IFA titers of <1:40. In addition, the ROC curves in Fig. 1A indicate that the cutoff for the ANA-EIA at <0.5 would be roughly equivalent to an ANA-IFA titer at <1:40.

Similar analyses were performed for patient serum samples ($n = 166$) collected during the common period. During this period, patient serum samples for which ANA was ordered as a part of routine medical care, regardless of ANA-IFA titer, were entered into the study. Nine percent (15 of 166) of the samples were from patients with diagnoses of CTD (Table 1). As expected, this rate was lower than the 20% observed during the overall period, in which more ANA-IFA-positive (titer of ≥1:80) patient serum samples were entered into the study. Some form of lupus was still the most common diagnosis. Comparisons of sensitivity, specificity, positive predictive values, and negative predictive values, using the reference range of <0.9 for ANA-EIA and that of <1:160 for ANA-IFA, did not reach statistical significance (Table 2). The ROC curves for the common period were also not statistically significantly different (Fig. 1B).

Sensitivity of ANA-EIA. Of the 86 serum samples from CTD patients collected during the overall period, 74 were positive by both ANA-IFA (≥1:160) and ANA-EIA (≥0.9) and 7 were negative by both methods. Four samples were positive for ANA-EIA (≥0.9) but negative for ANA-IFA (<1:160), and one sample was negative for ANA-EIA (<0.9) but positive for ANA-IFA (≥1:160). Therefore, the agreement between the two methods for samples from CTD patients was 94%.

Eight of 86 samples from CTD patients had negative ANA-EIA results and are therefore considered false negatives by this diagnostic test. As mentioned above, one of eight was positive by ANA-IFA (≥1:160) but negative by ANA-EIA (0.5). This patient had severe skin manifestations of lupus but was not treated with any medication. Five of eight patients had ANA-IFA results of 1:80, of whom three had SLE but were stable, one was diagnosed with Raynaud's syndrome, and the other had Sjögren's syndrome, which was stable, and a possible history of SLE which could not be verified. Two of eight false-negative samples had ANA-IFA results of <1:40. In addition, all six samples from CTD patients which were negative by ANA-EIA (<0.9) but which had a titer of ≥1:40 by ANA-IFA tested negative for autoantibodies to dsDNA, Sm/RNP, and SS-A/SS-B.

Pattern. Since ANA-EIA does not reveal patterns, clinically valuable information may be lost because immunofluorescence patterns have been associated with certain clinical states (7, 17, 20). For example, a centromere pattern is associated with CREST syndrome (7, 12, 13, 20) while a nucleolar pattern is associated with systemic sclerosis (7, 17, 20). In this study, there were 8 patient serum samples with a centromere pattern and 19 patient serum samples with a nucleolar pattern. One of the samples had both centromere and nucleolar patterns, and it came from a patient with a diagnosis of CREST syndrome/scleroderma. Two other patients with a diagnosis of CREST syndrome had a centromere pattern, whereas no other samples with a nucleolar pattern were from patients with scleroderma.

DISCUSSION

The comparison of ANA-EIA and ANA-IFA based on patient diagnosis has shown that the performance of ANA-EIA is

TABLE 2. Performances of ANA-IFA and ANA-EIA

Method (reference range [titer])	Rate (no. of samples/total) [P] for CTD[a]									
	Overall period				Common period					
	Sensitivity	Specificity	PV+	PV−	Sensitivity	Specificity	PV+	PV−
ANA-IFA (<1:40)	0.977 (84/86) [0.03]	0.267 (94/352) [<0.0001]	0.246 (84/342) [<0.0001]	0.979 (94/96) [NS]	0.867 (13/15) [NS]	0.623 (94/151) [0.0001]	0.186 (13/70) [0.03]	0.979 (94/96) [NS]
ANA-IFA (<1:160)	0.872 (75/86) [NS]	0.480 (169/352) [<0.0001]	0.291 (75/258) [<0.0001]	0.939 (169/180) [0.04]	0.733 (11/15) [NS]	0.801 (121/151) [NS]	0.268 (11/41) [NS]	0.968 (121/125) [NS]
ANA-EIA (<0.9)	0.907 (78/86)	0.602 (212/352)	0.358 (78/218)	0.964 (212/220)	0.800 (12/15)	0.781 (118/151)	0.267 (12/45)	0.975 (118/121)

[a] SLE, DLE, drug-induced lupus, scleroderma, CREST syndrome, Raynaud's syndrome, Sjögren's syndrome, MCTD, overlap syndromes, polymyositis, and dermatomyositis were included. P was compared with that of ANA-EIA (<0.9). PV+, positive predictive value; PV−, negative predictive value; NS, not significant.

at least as good as that of ANA-IFA. Establishing reference ranges was important in this study in order to statistically compare the sensitivity, specificity, and positive and negative predictive values of these two methods. The reference range established for ANA-EIA, 0.9, was in agreement with that previously established by the manufacturer. The reference range established for the purpose of this study of <1:160 for ANA-IFA was higher than that previously established (<1:40). In this study, ANA-EIA with a reference range of 0.9 demonstrates equivalent sensitivity and somewhat higher specificity compared to ANA-IFA, with a reference range of <1:160. The comparison of the ROC curves from the overall period also indicates that ANA-EIA performance is superior to that of ANA-IFA ($P = 0.0004$). One must keep in mind, however, that data from the overall period should only be used to make a comparison between the two methods, since an unusually high number of ANA-IFA-positive samples were included in the analysis during this time period.

Critics of ANA-EIA have voiced concerns about its low sensitivity (2). However, in this study, of all the samples tested, only one sample was positive by ANA-IFA (reference range of <1:160) but not by ANA-EIA (reference range of <0.9). This patient had dermatologic manifestations of lupus, not SLE, and no detectable levels of autoantibodies to dsDNA, Sm/RNP, and SS-A/SS-B.

Data on patient samples with centromere and nucleolar patterns show that, while some clinically valuable information may be lost by using ANA-EIA, the number of such cases is small. Previously, the presence of anti-centromere antibody in 96% (26 of 27) and 88% (7 of 8) of patients with CREST syndrome has been reported (3, 12). However, only 38% (3 of 8) of patients with anti-centromere antibody had a diagnosis of CREST syndrome in our study. Similarly, although the presence of nucleolar antibodies in 54% (13 of 24) of patients with systemic sclerosis has been shown (13), only 5% (1 of 19) of patients with nucleolar antibody had a diagnosis of scleroderma in our study. While the likelihood of a patient with a certain disease having a particular autoantibody can be quite high, the opposite is not necessarily true. Associations between certain diseases and the presence of autoantibodies to nuclear antigens, which can be seen in patients with established diagnoses, may not be confirmed in unselected patients (16).

We acknowledge that the use of patient diagnoses, determined by retrospective chart reviews, to compare performances of diagnostic tests has its limitations. Since patient samples came from various clinical settings, the same standards for making diagnoses were unlikely to have been applied in all cases. In addition, physicians established diagnoses for some patients by using the ANA-IFA results reported in this study but not the ANA-EIA results. However, a definitive diagnostic test is not available and it is not possible to determine the extent or direction of bias in this case.

Since UCSF is primarily a tertiary-care center, the patient population enrolled in this study is not typical of many healthcare settings. This is reflected in the number of samples from patients with lupus and rheumatoid arthritis. During the overall period, 71 samples were obtained from patients with lupus while 11 samples were obtained from patients with rheumatoid arthritis. The estimated prevalence of SLE is between 4 and 250 per 100,000 people (15) while that of rheumatoid arthritis is 300 to 1,500 per 100,000 people (5). Therefore, analogous comparison of ANA-IFA and ANA-EIA may be indicated in a patient population from a predominantly primary-care setting.

ACKNOWLEDGMENTS

This study was financially supported by Bio-Rad Laboratories.

We thank Richard Edwards and Susan Breglio for technical guidance and Francis Kong and Jason Chang for clerical assistance.

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