Evaluation of Multiplexed Fluorescent Microsphere Immunoassay for Detection of Autoantibodies to Nuclear Antigens

Thomas B. Martins, 1* Rufus Burlingame, 2 Carlos A. von Mühlen, 3 Troy D. Jaskowski, 1 Christine M. Litwin, 1, 4 and Harry R. Hill 1, 4

Associated Regional and University Pathologists Institute for Clinical and Experimental Pathology 1 and Department of Pathology, Pediatrics and Medicine, University of Utah School of Medicine, 4 Salt Lake City, Utah; INOVA Diagnostics Inc., San Diego, California 2; and Pontifical Catholic University School of Medicine, Porto Alegre, Brazil 3

Received 4 June 2004/Returned for modification 27 July 2004/Accepted 7 August 2004

Antibodies to extractable nuclear antigens (ENA) are found in a variety of collagen vascular diseases. Determining the individual specificities of these antibodies is extremely useful in establishing the disease diagnosis and in some cases the prognosis. With a multiplexed fluorescent microsphere immunoassay, reactivity to five of the most diagnostically useful ENA was measured in 249 serum samples, including samples from 56 patients previously documented to have systemic lupus erythematosus (SLE). Results of the multiplexed assay were compared to results from established ENA enzyme-linked immunosorbent assays (ELISAs), and the agreement, sensitivity, and specificity, respectively, for the five ENA evaluated were as follows: SSA, 99.1, 100.0, and 98.8%; SSB, 98.6, 88.9, and 99.5%; Sm, 97.6, 95.8, and 97.9%; RNP, 97.2, 92.7, and 98.8%; Scl-70, 93.6, 50.0, and 99.0%. In the 56 confirmed SLE patients, the frequency of significant concentrations of autoantibodies with the multiplexed assay was 21.4% for SSA, 7.1% for SSB, 10.7% for Sm, 32.1% for RNP, and 0% for Scl-70. The new flow cytometric bead-based multiplexed assay showed excellent correlation with the well-established single-analyte ELISA methods for four of five the ENA markers investigated in this study. The most notable discrepancies between the two assays were for the Scl-70 antigen, which was most often resolved in favor of the multiplexed assay. Our studies show that the multiplexed microsphere-based immunoassay is a sensitive and specific method for the detection and semiquantitation of ENA antibodies in human sera.
multiple carboxyl groups that function as sites for the covalent attachment of the antigens. The second layer excites a third fluorophore coupled to a reporter molecule, which allows quantitation of the interaction that has occurred on the microsphere surface.

In this study, we evaluated a Luminex-based assay for the simultaneous detection of five autoantibodies reacting with SSA, SSB, Sm, RNP, and Scl-70. Results of this multiplexed assay were compared to single-antigen ELISA results for these same five antigens.

MATERIALS AND METHODS

Clinical samples. This study included 193 serum samples submitted to our reference laboratory for autoimmune testing from patients with suspected collagen vascular diseases and 56 serum samples, from well-documented SLE patients who all meet Tan’s 1982 American College of Rheumatology criteria for the disease (30), that were provided by Carlos A. von Mühlen, a rheumatologist from Porto Alegre, Brazil. The samples were obtained from 56 consecutive patients who consented to having their blood drawn for a study on lupus erythematosus cell formation. All of the patient samples included in this study that were submitted to Associated Regional and University Pathologists laboratories were deidentified in accordance with the University of Utah Institutional Review Board-approved protocol (no. 7275) to meet the Health Information Portability and Accountability Act patient confidentiality guidelines.

ENA assays. Single-antigen ELISAs for SSA, SSB, Sm, RNP, and Scl-70 and the multiplexed ENA 5 Luminex-based assay used in this study were purchased from INOVA Diagnostics (San Diego, Calif.). All of the assays used in this study have received Food and Drug Administration clearance and are labeled for in vitro diagnostic use. The multiplexed ENA 5 assay consisted of native SSB, Sm, RNP, and Scl-70 antigens purified from calf thymus bound to microspheres from four different regions. Combined native calf spleen-derived and recombinant SSA antigens are bound to a microsphere from a fifth region. In addition to five antigen-coupled microspheres, a sixth microsphere coupled with human immunoglobulin G (IgG) was included. This microsphere acts as an internal control to ensure that the patient sample and the fluorescently labeled reporter antibody have been added to each individual reaction mixture. The five antigen-coupled and control beads are mixed together and used to coat the surface of a 96-well microtiter plate. Since no washing steps are involved in the assay, the SSA antigens are bound to a microsphere from a fifth region. In addition to the four different regions. Combined native calf spleen-derived and recombinant

RESULTS

Comparison of multiplexed ENA 5 to ELISA. The initial comparison included combined results from the 193 serum samples from patients with suspected collagen vascular diseases submitted to our laboratory for ENA antibody testing and the 56 clinical and laboratory-confirmed SLE patients. The multiplexed ENA 5 bead-based assay compared well to the standard single-antigen ELISA methods in these combined patient groups. When the SSA multiplexed-assay results were compared to the single-antigen ELISA results, there was an agreement of 99.1%, a sensitivity of 100%, and a specificity of 98.8% (Table 1). There were two discrepant samples that had positive results by the multiplexed assay and negative results by ELISA. There were 14 samples with equivocal results by ELISA and 9 samples with equivocal multiplexed-assay results for SSA. Linear regression analysis yielded a $y$ value of 1.67x - 4.58 and an $R^2$ value of 0.633 (Fig. 1a). The multiplexed SSB assay had an agreement of 98.6%, a sensitivity of 88.9%, and a specificity of 99.5% compared to the ELISA. There were three discrepant results, one sample that had a positive result by the multiplexed assay and was negative by the ELISA and two samples with positive ELISA results and negative multiplexed-assay results. Of the 239 samples tested for SSB, there were 19 equivocal results by the multiplexed assay and 4 by ELISA (Table 1). When linear regression was calculated for the two

<table>
<thead>
<tr>
<th>SSA ELISA (n = 239)</th>
<th>SSB ELISA (n = 239)</th>
<th>Sm ELISA (n = 239)</th>
<th>RNP ELISA (n = 239)</th>
<th>Scl-70 ELISA (n = 249)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ 52 2 16 1 23</td>
<td>+ 4 5 51 2 13</td>
<td>+ 2 2 6 6 6</td>
<td>+ 5 5 5 5 5</td>
<td>+ 2 2 2 2 2</td>
</tr>
<tr>
<td>− 168 8 2 199 1</td>
<td>− 1 1 183 5 4</td>
<td>− 5 5 5 5 5</td>
<td>− 2 2 2 2 2</td>
<td>− 2 2 2 2 2</td>
</tr>
<tr>
<td>equivocal 1 1 1 1 1</td>
<td>equivocal 1 1 1 1 1</td>
<td>equivocal 1 1 1 1 1</td>
<td>equivocal 1 1 1 1 1</td>
<td>equivocal 1 1 1 1 1</td>
</tr>
</tbody>
</table>

The INOVA ELISAs we used consisted of the five purified native ENA bound to the surfaces of separate polystyrene microtiter plates. Diluted patient samples and controls are added to the appropriate wells of each microtiter plate. After incubation at 20 to 26°C for 30 min, each plate is washed three times and horseradish peroxidase-conjugated anti-human IgG is added to each well. Following another 30-min incubation and a wash, a tetra-methylbenzidine substrate is added to each well. After another 30-min incubation, a stop solution is added to each well. The assay is then evaluated spectrophotometrically by measuring the optical density (OD) of the developed color. The OD of the patient sample is then divided by the OD of the cutoff calibrator control, and the result is then multiplied by a predefined factor to determine a semiquantitative result.

Data analysis. Reference ranges for determining semiquantitative patient results based on the amount of autoantibody measured were taken from the assay literature and were slightly different for the two assays. Ranges for the ELISAs were as follows: <20 U, negative; 20 to 39 U, weakly positive; 40 to 80 U, moderately positive; >80 U, strongly positive. Ranges for the multiplexed ENA 5 assay were <20 U, negative; 20 to 49 U, weakly positive; 50 to 100 U, moderately positive; >100 U, strongly positive. To determine agreement, sensitivity, and specificity between the multiplexed ENA 5 assay and the individual ELISAs, results were analyzed with two-by-two contingency tables (26). To simplify the analysis, samples with results of <20 U were considered negative. Samples with results that fell within the weakly positive range were considered equivocal, while samples having results of 40 U or greater for ELISA and 50 U or greater for the multiplexed assay were classified as positive. Samples with equivocal results were not included in the calculations of clinical agreement, sensitivity, and specificity. Linear regression analysis was also performed on the assays with the semiquantitative result.

Data analysis. Reference ranges for determining semiquantitative patient results based on the amount of autoantibody measured were taken from the assay literature and were slightly different for the two assays. Ranges for the ELISAs were as follows: <20 U, negative; 20 to 39 U, weakly positive; 40 to 80 U, moderately positive; >80 U, strongly positive. Ranges for the multiplexed ENA 5 assay were <20 U, negative; 20 to 49 U, weakly positive; 50 to 100 U, moderately positive; >100 U, strongly positive. To determine agreement, sensitivity, and specificity between the multiplexed ENA 5 assay and the individual ELISAs, results were analyzed with two-by-two contingency tables (26). To simplify the analysis, samples with results of <20 U were considered negative. Samples with results that fell within the weakly positive range were considered equivocal, while samples having results of 40 U or greater for ELISA and 50 U or greater for the multiplexed assay were classified as positive. Samples with equivocal results were not included in the calculations of clinical agreement, sensitivity, and specificity. Linear regression analysis was also performed on the assays with the semiquantitative result.
FIG. 1. Linear regression analysis of the reported semiquantitative results of the multiplexed assay compared with ELISA results. Panels: a, SSA correlation; b, SSB correlation; c, Sm correlation; d, RNP correlation; e, Scl-70 correlation.

Panel a:
- $n = 239$
- $y = 1.67x - 4.58$
- $R^2 = 0.633$

Panel b:
- $n = 239$
- $y = 1.44x + 0.759$
- $R^2 = 0.752$

Panel c:
- $n = 237$
- $y = 1.69x + 0.353$
- $R^2 = 0.731$

Panel d:
- $n = 235$
- $y = 1.30x - 3.90$
- $R^2 = 0.795$

Panel e:
- $n = 249$
- $y = 0.655x + 3.04$
- $R^2 = 0.617$

FIG. 1. Linear regression analysis of the reported semiquantitative results of the multiplexed assay compared with ELISA results. Panels: a, SSA correlation; b, SSB correlation; c, Sm correlation; d, RNP correlation; e, Scl-70 correlation.
yielded linear regression results of by ELISA (Table 1). A total of 249 samples were tested for antibodies to the Sm antigen; four were positive by the multiplexed assay and negative by ELISA, and one was positive by ELISA and negative by the multiplexed assay (Table 1). These discrepant samples had positive results slightly above the cutoff for ELISA (47 and 46 U; cutoff = 40 U) and negative results by the multiplexed assay. The two other discrepant samples had positive results slightly above the cutoff for the multiplexed assay. ANA IFA assay results were unavailable for these samples, but one sample was from a diagnosed SLE patient. Antibodies to SSB are typically found in 5 to 15% of SLE patients.

A total of five results were discrepant between the multiplexed assay and the ELISA for detection of antibodies to Sm antigen; four were positive by the multiplexed assay and negative by ELISA, and one was positive by ELISA and negative by the multiplexed assay (Table 1). These discrepant samples are hard to resolve as none were from diagnosed SLE patients and all had reactivity to at least one of the other four ENA.

For antibodies to the RNP antigen, there were six discrepant samples, two positive by the multiplexed assay and negative by ELISA and four positive by ELISA but negative by the multiplexed assay. Of the 56 SLE patients, two positive by the multiplexed assay and negative by ELISA, and one sample was from a diagnosed SLE patient. Antibodies to SSB are typically found in 5 to 15% of SLE patients.

When the discrepant results of the combined patient groups for the SSA antigen were examined, there were two samples that had positive results by the multiplexed assay and negative results by the ELISA. One of these discrepant samples had a result of 54 U and was just above the positive cutoff of 50 for the multiplexed assay. The other SSA discrepant sample, which was highly reactive (262 U) by the multiplexed assay, may be explained by a difference in the antigens used in the multiplexed assay and the ELISA. The microsphere used in the multiplexed assay is coupled with a mixture of native 60-kDa SSA antigen and a 52-kDa recombinant SSA antigen, which have both been shown to be diagnostically significant (13). At the time of this study, the recombinant 52-kDa antigen was not included in the SLE ELISA. When further testing was performed on this sample with specific SSA 60-kDa and SSA 52-kDa ELISAs, the sample was highly reactive to the 52-kDa antigen but showed no reactivity to the 60-kDa antigen. A mix of both antigens has since been included in the INOVA SSA ELISA.

There were three discrepant samples between the multiplexed assay and the SSB ELISA. One of these samples was positive by the multiplexed assay for SSB but negative for the other four ENA markers (SSA, Sm, RNP, and ScI-70). This sample was negative by the SSB ELISA, as well as the ANA IFA assay, and therefore this appears to be a false-positive SSB result by the multiplexed assay. The two other discrepant samples had positive results slightly above the cutoff for ELISA (47 and 46 U; cutoff = 40 U) and negative results by the multiplexed assay. ANA IFA assay results were unavailable for these samples, but one sample was from a diagnosed SLE patient. Antibodies to SSB are typically found in 5 to 15% of SLE patients.

When the reactivity to the Sm antigen was examined, there were three samples that had positive results by the multiplexed assay and negative results by ELISA, and one was positive by ELISA and negative by the multiplexed assay. The two other discrepant samples were found to have positive results slightly above the cutoff for ELISA (47 and 46 U; cutoff = 40 U) and negative results by the multiplexed assay. The discrepant samples are hard to resolve as none were from diagnosed SLE patients and all had reactivity to at least one of the other four ENA.

For antibodies to the RNP antigen, there were six discrepant samples, two positive by the multiplexed assay and negative by ELISA and four positive by ELISA but negative by the multiplexed assay. While the RNP antigen contains epitopes that are unique to RNP, they also contain epitopes that are immunologically identical to Sm antigens. The two discrepant samples that were positive by the multiplexed assay also had reactivity to the Sm antigen. Of the six discrepant samples, only one was from a diagnosed SLE patient. This patient was negative for all five of the ENA markers in the multiplexed assay and positive for only RNP in the ELISA. Antibodies to RNP are observed in up to 50% of patients with SLE. Thus, this may be a false-negative RNP result by the multiplexed assay.

Of the five ENA examined in this study, discrepant results between the multiplexed assay and the ELISA were found most often with the ScI-70 antigen. Of the 15 discrepant samples, 2 were positive by the multiplexed assay and negative by the ELISA. One of the discrepant results is likely a false positive by the multiplexed assay. It had a value of 52 U, just
over the cutoff value of 50 U, and was negative by the Scl-70 ELISA and the ANA IFA assay. The remaining 13 discrepant samples were positive by the ELISA and negative by the multiplexed assay. Three of these 13 samples were from the diagnosed SLE patients and are likely ELISA false positives. Scl-70 is a very specific marker for scleroderma but is very rarely found in SLE patients. Additionally, 9 (69%) of the 13 samples that were positive for Scl-70 antibodies by ELISA but negative by the multiplexed assay had positive results for Sm antigen. Antibodies to Sm antigen are highly specific for SLE but found in <1% of scleroderma patients. No matter what the autoimmune disease state, reactivity to Sm antigen and reactivity to Scl-70 antigen are very rarely linked. These samples, therefore, are also most likely false positives by the ELISA for Scl-70.

Further studies have shown good agreement between the ELISA and the multiplexed assay for Scl-70 in patients with scleroderma and a possible interaction of anti-DNA antibodies with the Scl-70 ELISA (unpublished data).

These results compared favorably with those of Rouquette et al. (21), who used the FIDIS Luminex-based ANA immunoassay in their study. They compared the results of 222 patient samples generated by the FIDIS system to those obtained by individual ELISAs from Biomedical Diagnostics (Marne la Vallée, France). For the five ENA compared in our study (SSA, SSB, Sm, RNP, and Scl-70), they reported concordances (agreements), sensitivities, and specificities of 100% for all five markers. In our study of 249 patient samples, results ranged from 93.6 to 99.1% agreement, 50 to 100% specificity, and 97.9 to 99.5% sensitivity for the same five ENA markers. The lowest results for agreement (93.6%) and sensitivity (50.0%) came from the numerous discrepant samples we found with the Scl-70 antigen. Rouquette et al. (21) found no discrepancies for the Scl-70 antigen in their study, in which 11 of their 222 patient samples were positive by both FIDIS and ELISA. Their sample population contained 10 scleroderma patients, but their results did not correlate the 11 FIDIS and ELISA Scl-70-positive results with the patients’ diagnoses.

The positive and negative qualitative results were in close agreement between the ELISA and the multiplexed assays, as was the correlation of the semiquantitative values when analyzed by linear regression (Fig. 1). The $R^2$ values ranged from 0.617 to 0.795, indicating that most of the samples showed similar relative intensities in both assay systems. Overall, the multiplexed assay produced higher unit values than did the individual ELISAs for all of the ENA except Scl-70. The difference is most likely due to the increased dynamic range of the fluorescence-based detection of the Luminex system versus the spectrophotometrically measured OD used by the ELISA system. For this reason, it is best to use the semiquantitative values from only one system when monitoring patient results over time.

When the results of the 56 clinically confirmed SLE patients alone were examined, 23.2% had a positive SSA result by ELISA while 21.4% had a positive result by the multiplexed assay (Table 2). These percentages are only slightly lower than reports in the literature, which cite SSA positivities ranging from 25 to 35% in non-drug-induced SLE patients. The sensitivity for autoantibodies to SSB was 10.7% for ELISA and 7.1% for the multiplexed assay. This closely matches reports in previous studies in which antibodies to SSB range from 5 to 15% in SLE. Autoantibodies to Sm antigen are very specific for SLE but only occur in 25 to 30% of patients. In our study of 56 SLE patients, only 12.5% were positive for Sm by ELISA and 10.7% were positive by the multiplexed assay. Antibodies to the RNP antigen were detected in 33.9% of the patients by ELISA and in 32.1% of the patients by the multiplexed assay. These frequencies match closely those of 30 to 45% found in the literature. The incidence of Scl-70 reactivity is less than 1% in SLE but was found in 5.4% of our SLE patients by ELISA. In contrast, none of the 56 SLE patients had positive Scl-70 results by the multiplexed assay, suggesting that the ELISA may have given false-positive results.

Both the ELISA and the multiplexed assay contained positive and negative controls and a calibrator to ensure that the assays on the whole were functioning properly. A unique advantage of the Luminex multiplexed system is the ability to add true internal controls to each individual sample. In the INOVA multiplexed system, this is accomplished by the use of an additional microsphere coupled with human IgG. This additional microsphere functions as a control for the addition of a conjugate. The patient sample addition control works in a competitive fashion in which human IgG in the serum competes with the human IgG bound to the microsphere for the goat anti-human conjugate. If the patient sample is left out of the reaction mixture, there will be more conjugate available to bind to the human IgG on the microsphere, resulting in elevated fluorescence signals. If the patient sample is not added to the well, the ratio of the median fluorescence intensity (MFI) of the human IgG-coupled bead of the patient compared to the MFI of the human IgG bead of the high control will be greater than 0.9. Studies of different sample populations showed that 80 to 97% of patients, depending on their origin and serum IgG concentrations, will have ratios of less than 0.9. This same internal control also ensures that the fluorescently labeled reporter antibody, goat anti-human IgG, was added to the reaction mixture. If the conjugate was accidentally omitted, the MFI of the IgG bead for the patient will be below 300, while it will always be above 3,000 if the conjugate was added.

The new flow cytometric bead-based multiplexed assay showed excellent correlation with the well-established single-analyte ELISA methods for four of the five (SSA, SSB, Sm, and RNP) ENA investigated in this study. Agreements for these analytes were all 97% or greater. There was a poor correlation, however, between the ELISA and the multiplexed assay for detection of Scl-70 autoantibodies. When the discrepant results were further analyzed, the resolution was most often in agreement with the multiplexed assay, as the ELISA found Scl-70 reactivity in 3 of the SLE patients, as well as dual reactivity for both Sm and Scl-70 antigens in 10 patients. The multiplexed assay also has the advantage of reporting results for all five antigens from a single reaction, resulting in reagent, labor, sample volume, and storage space savings. When the Luminex-based system is used in conjunction with the automated Tecan liquid-handling system, a highly automated, cost-effective system for the detection of antibodies to the most common ENA was achieved.
REFERENCES


