Clinical Value of Multiplexed Bead-Based Immunoassays for Detection of Autoantibodies to Nuclear Antigens

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The advent of multiplexed bead assays in recent years has introduced a new dimension of testing for complex diseases such as lupus, which can involve multiple autoantibodies. The ability to rapidly identify multiple autoantibodies, with high sensitivity and specificity in an automated fashion, is highly attractive. The aim of this study was to assess the performance and clinical value of multiplexed bead-based (AtheNA Multi-Lyte ANA-II test system) immunoassays both by comparing the results with those achieved by indirect fluorescent-antibody assay (IFA) or conventional enzyme immunoassays (EIAs) and by independent identification of autoantibodies in well-characterized samples. To achieve this goal, 984 samples were tested for seven analytes (SS/A, SS/B, Sm, RNP, Scl-70, double-stranded DNA [dsDNA], and centromere B) in both traditional and bead-based assays. The average concordance for the different analytes was 91%, ranging from 81% (dsDNA) to 97% (centromere B). The average relative specificity and sensitivity for the analytes were also high, 92% and 81%, respectively. An examination of 93 “normal controls” demonstrated a 7% false-positive rate, which was comparable to IFA. Percentages of different autoantibodies found in patients with a variety of disease conditions (34 with calcinosis, Raynaud’s phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia; 41 with mixed connective tissue disease; 24 with scleroderma; and 35 with Sjögren’s syndrome) were well within the range expected from each group. A scrutiny of results from AtheNA and IFA and Farr results for 185 systemic lupus erythematosus samples revealed comparable results by both methods, with the exception of SS/A and dsDNA, where AtheNA had a higher percentage of SS/A-positive results compared to IFA (51% versus 29%) and a lower percentage of dsDNA-positive results (18% versus 28% at a cutoff of 5 IU/ml).

Detection of antinuclear antibodies (ANAs) has a significant role in diagnosis and prognosis for clinically indicated patients with a variety of autoimmune vascular diseases. Traditionally, the “gold standard” test for detection of ANAs has been the indirect fluorescent-antibody assay (IFA). The advent of using the human HEp-2 cell line for detection of ANAs in the past 20 years has provided sensitivity and brought more standardization and therefore acceptance of this test globally (11). It has provided superior resolution for detection of different staining patterns that was not available before. The increased sensitivity also brought forth a more reliable use of titer cutoffs for determining positive results. Though reliable, ANA testing by IFA has had its share of problems and criticism over the years. The test has been deemed “subjective” and highly dependent on the competence of the technician reading the slides (10). IFA testing is also an issue for high-volume laboratories performing ANA screens routinely. To circumvent these problems, researchers have evaluated ANA screening methods using enzyme immunoassays (EIAs) that are usually prepared from HEp-2 cells. A few studies have shown comparable sensitivity and specificity with IFA testing (6, 7). The advocates of EIA-ANA testing herald the objectivity of the results and the ability to automate and run multiple samples reliably (1). Most critics, though, cite issues with sensitivity (either too sensitive, resulting in a high number of false positives, or the opposite, resulting in false negatives) (2), the range of specificity (number of different extractable nuclear antigens [ENAs] detected), and the lack of the ability to detect different patterns available by IFA. There have been suggestions that these shortcomings can be overcome by testing each sample with EIAs specific for testing single ENAs. This idea, although theoretically sound, defeats the purpose of ANA testing by IFA, since it considerably increases cost and time to result, not to mention erroneous diagnosis based on a single test result.

Advances in technology have recently provided a new methodology for ANA testing (8, 12, 14). Fluorescent bead-based flow cytometry, pioneered by Luminex Inc., has allowed different manufacturers to produce kits capable of detecting multiple autoantibodies to ENAs simultaneously. Recent papers have evaluated these kits against IFA and EIA and shown excellent concordance, sensitivity, and specificity (12, 14). We compared the results of 984 clinical samples that were originally tested by IFA for ANA screen by EIA (Inova) for anti-Ro (SS-A), anti-La (SS-B), anti-Sm, anti-RNP, anticentromere, anti-Jo1, anti-Scl70, and antihistone and by the Farr method for dsDNA with AtehNA Multi-Lyte, a multiplexed microparticle immunoassay for antibodies to ANAs. Our goals were to determine if these systems could reliably be used as a screen to replace ANA testing by IFA. Furthermore, we sought to determine if the test was specific and sensitive enough to be used for monitoring flares for patients that are monitored by rheumatologists.

MATERIALS AND METHODS

Clinical samples. A total of 984 clinical samples were obtained from Rheumatology Diagnostics Laboratory (Los Angeles, CA). These samples were in
three distinct groups. One group consisted of 452 samples from patients who had
documented adverse drug reactions (systemic lupus erythematosus [SLE]; Sjögren’s
syndrome; scleroderma; calcinosis, Raynaud’s phenomenon, esophageal dysmo-
tility, sclerodactyly, and telangiectasia [CREST]; Raynaud’s phenomenon; drug-
induced lupus, and mixed connective tissue disease [MCTD]) and test results by
different methodologies. Another group consisted of 438 samples which had only
test results by different methodologies. The third group consisted of 94 patient
samples that were deemed to be “normal controls” or at the time of their visit
with the physician did not demonstrate any symptoms or clinical signs of illness.
All samples obtained were only identified by a number to meet the Health
Information Portability and Accountability Act patient confidentiality guidelines.

**ANA screen and centromere ENA detection by IFA.** The RhiGene Titer-Fluor
ANA test system was used as the ANA screening methodology for the obtained
samples. The RhiGene Titer-Fluor ANA test system is an indirect fluorescent
antibody assay utilizing HEp-2 tissue culture cells as a substrate for the qualita-
tive and/or semiquantitative determination of antinuclear antibodies in human
serum. A titer calibrator was utilized by preparing eight serial dilutions to
incorporate a range of 1:2,560 to <1:40. After processing the HEp-2 slides
according to the manufacturer’s suggested protocol, the Rhigene ImageTiter
workstation was used to determine the appropriate titer for each sample. Sam-
ples with titers of 1:40 or greater were considered positives. The same method-
ology was used for detection of centromere patterns. Similarly, samples with
titers of 1:40 and a centromeric pattern were determined to be positive for
centromere autoantibodies.

**ENA assays.** Inova’s QUANTA Lite enzyme-linked immunosorbent assays
(ELISAs) were used for the semiquantitative detection of Sm, RNP, SS-A (60
kDa and 52 kDa), SS-B, and Scl-70 antibodies in the serum. Purified Sm-RNP, SS-
A, SS-B, and Scl-70 antigens were bound to the wells of a polystyrene micro-
well plate under conditions that preserve the antigen in its native state. The
manufacturer’s suggested protocol was used for all of the assays. The following
formula was used for calculating the sample results: sample value = (sample
optical density/test low positive) × test low positive, where test low positive refers
to the low value of the positive control provided with the kit. Samples with values
of 20 or above by using the above formula were considered positives.

**dsDNA antibodies by the Farr technique.** DPC’s anti-DNA radioimmunoassay
was used for detection of antibodies against double-stranded or native DNA. The
assay is based on principles of the Farr technique (4). The assay utilizes 125I-
labeled recombinant DNA for detection of antibodies against double-stranded
DNA. The concentration of the DNA-bound antibodies in the serum is deter-
mined by readings against a calibration curve ranging from 0 to 50 IU/ml. A 25-μl
volume of undiluted serum was used for this assay. The manufacturer’s suggested
protocol was followed for performing this assay. All samples with results exceed-
ing 5 IU/ml were considered positive samples.

**ANA detection by multiplexed bead-based immunoassay.** We chose the
AtheNA Multi-Lyte ANA-II test system for testing our 984 samples. AtheNA Multi-
Lyte utilizes a suspension that contains separate distinguishable 5.6-μm polystyrene
beads that are conjugated with the following autoantigens: SS/A, SS/B, Sm, mRNP B/B’, U1 snRNP 60, U1 snRNP A, U1 snRNP C, Scl-70, Jo-1, centromere B, dsDNA, histone H, and histone HLY. Samples were diluted 1:21
and incubated with the bead mixture for 30 min. Samples were then washed to
remove all unbound serum components. Phycoerythrin-conjugated goat anti-
human immunoglobulin G (γ chain specific) was added to the samples, the
samples were incubated for another 30 min, and the samples were read on a
Luminex 100 system. The manufacturer’s suggested protocol was followed for
performing this assay. All samples with results exceeding 120 arbitrary units
(AU)/ml were considered positive samples. Samples below 100 AU/ml were
classified as negative. All samples with results between 100 and 120 AU/ml were
classified equivocal and were repeated for resolution. Samples that stayed in
the equivocal range after repeat testing were excluded from our study.

**RESULTS**

**Concordance, relative specificity, and relative sensitivity of bead-based assay to ELISA and Farr.** A total of 984 serum samples were compared to determine the concordance of the AtheNA Multi-Lyte assay with the traditional individual ELISAs for individual ENAs and dsDNA by Farr. All of the 984 samples were tested by the AtheNA system, but a fraction of these samples had appropriate test results for each individual
antibody. The number of samples with test results for Jo-1

<table>
<thead>
<tr>
<th></th>
<th>No. of samples</th>
<th>Concordance (%)</th>
<th>Relative specificity (%)</th>
<th>Relative sensitivity (%)</th>
</tr>
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<tbody>
<tr>
<td>SS/A</td>
<td>Positive</td>
<td>187</td>
<td>51</td>
<td>86.1</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>45</td>
<td>408</td>
<td></td>
</tr>
<tr>
<td>SS/B</td>
<td>Positive</td>
<td>98</td>
<td>17</td>
<td>93.2</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>30</td>
<td>546</td>
<td></td>
</tr>
<tr>
<td>Sm</td>
<td>Positive</td>
<td>92</td>
<td>29</td>
<td>92.8</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>32</td>
<td>688</td>
<td></td>
</tr>
<tr>
<td>RNP</td>
<td>Positive</td>
<td>160</td>
<td>78</td>
<td>87.5</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>25</td>
<td>563</td>
<td></td>
</tr>
<tr>
<td>Scl-70</td>
<td>Positive</td>
<td>63</td>
<td>21</td>
<td>92.8</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>27</td>
<td>559</td>
<td></td>
</tr>
<tr>
<td>dsDNA</td>
<td>Positive</td>
<td>215</td>
<td>153</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>21</td>
<td>527</td>
<td></td>
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<table>
<thead>
<tr>
<th></th>
<th>No. of samples</th>
<th>Concordance (%)</th>
<th>Relative specificity (%)</th>
<th>Relative sensitivity (%)</th>
</tr>
</thead>
</table>

* dsDNA was analyzed by Farr assay with a cutoff of 5 IU/ml.

**Determination of false positive rate.** Ninety-four “normal control” samples were screened by both AtheNA and IFA (Table 2). Seven samples (7.4%) were positive by both IFA and AtheNA. The other 87 samples were negative with both AtheNA and IFA. Four of the seven positive samples were just barely positive by IFA, with titers of 1:40. Two samples had high titers of 1:640. Further investigation of these high-titer samples showed high SS/A results by AtheNA. All seven samples had only one antibody present (either SS/A or RNP) when tested by both AtheNA and conventional methods. The frequency of these positive results in “normal control” specimens

(n = 6) and histone (n = 47) were far below the number of samples with results for other assays and therefore they were eliminated from this study. The results of the concordance between the other seven assays and their relative specificities and sensitivities based on the agreement numbers are detailed in Table 1. The results obtained by the AtheNA system com-
pared well with the results from the individual ELISA tests and the Farr assay. SS/B, Sm, centromere B, and Scl-70 had a greater than 92% concordance and specificities in excess of 95%. SS/A and RNP had lower levels of concordance (86% and 87%, respectively) but still demonstrated greater than 88% relative specificity. dsDNA was least concordant (81%). A cutoff value of 5.0 IU/ml was chosen when the samples were originally run by the Farr assay. The concordance increases to
83% if the cutoff of 10 IU/ml is used.

**Table 1. Concordance and relative sensitivity and specificity of Wampole's AtheNA MultiLyte compared to Inova’s Quanta Lite ELISA and DPC’s anti-DNA radioimmunoassay**

Wampole's AtheNA MultiLyte compared to Inova's Quanta Lite ELISA and DPC's anti-DNA radioimmunoassay was used for detection of antibodies against double-stranded or native DNA. The assay is based on principles of the Farr technique (4). The assay utilizes labeled recombinant DNA for detection of antibodies against double-stranded DNA. The concentration of the DNA-bound antibodies in the serum is determined by readings against a calibration curve ranging from 0 to 50 IU/ml. A 25-μl volume of undiluted serum was used for this assay. The manufacturer’s suggested protocol was followed for performing this assay. All samples with results exceeding 5 IU/ml were considered positive samples. Samples below 100 AU/ml were considered negative. All samples with results between 100 and 120 AU/ml were considered equivocal and were repeated for resolution. Samples that stayed in the equivocal range after repeat testing were excluded from our study.
was well within the range previously reported in the literature (11, 15).

Determination of false-negative rate. A total of 137 well-characterized SLE samples with negative AtheNA results were scrutinized for determination of the false-negative rate. Of these samples, 124 were also negative for all autoantibodies by ELISA and for dsDNA by Farr. Out of the remaining 13, 1 (0.7%) was positive for SSA, 4 (2.9%) were positive for RNP, and 8 (5.8%) were positive for dsDNA by Farr (cutoff, 10 IU/ml).

Determination of individual ENA frequencies in different disease states. Well-characterized samples from different vascular disease states (34 CREST, 41 MCTD, 24 scleroderma, and 35 Sjogren’s syndrome) were scrutinized for the frequency of different ENAs when tested by AtheNA. All samples with negative results were retested by ELISA and were confirmed to be negative for all ENAs. The results of these determinations are detailed in Table 3.

Bead assay sensitivity of well-characterized SLE patients. One hundred eighty-five clinically diagnosed and well-characterized SLE patients were examined to determine the bead assay sensitivity. The same samples were also tested by ELISA for ENAs and by Farr for dsDNA. Table 4 details the results of this analysis. dsDNA results by Farr were separated and analyzed with three different cutoffs, 5 IU/ml, 10 IU/ml, and 15 IU/ml.

DISCUSSION

Bead-based assays are fast becoming a mainstay in clinical laboratories. The advantage of multiplexing combined with labor and reagent cost savings makes these assays extremely attractive. The ability to share a single platform to perform multiple assays is an added bonus. Our goal for this study was to determine the clinical value of these assays as it pertains to screening and detection of autoantibodies and, furthermore, their ability to accurately follow autoantibody changes during flares. Traditionally, screening for autoantibodies has been performed by IFA on Hep-2 cells. The sensitivity of this assay and its reliability have made it the gold standard for screening purposes. On the other hand, a low throughput, subjectivity, and the need for further testing with single autoantibodies for identification of individual autoantibodies have been the shortcomings for this assay. Screening by ELISA has been suggested for overcoming throughput and technician subjectivity, but lack of specificity has held it back from replacing the IFA test. Bead-based assays now seem to fulfill this shortcoming by their ability to multiplex. Concordance values were in excess of 90% for four out of seven autoantibodies between traditional methods and our bead-based assay (AtheNA Multi-Lyte). The two autoantibodies with concordance levels below 90% were RNP and SSA. The low level of concordance with RNP can be explained by the fact that the ELISA kit used for detection of RNP detected the Sm/RNP combination, while the AtheNA kit, due to its recombinant nature, detected only RNP. This was more evident when we scrutinized 185 clinically diagnosed SLE patients, where the difference in percentage of RNP detected by the AtheNA and ELISA methods matched the percentage of Sm detected by both. Similarly, the low concordance in SSA can be attributed to the differences in antigens used in the kits. Further investigation from manufacturers allowed us to realize that the AtheNA SSA/Sm has a bias towards the 60-kDa fragment of SSA (Inova claims an equal concentration of both fragments), although it includes both the 60- and the 52-kDa fragments. This fact might also explain the discrepancy in the percentage of positive SSA samples in our characterized SLE patient group, since lupus samples have been shown to have a higher bias towards the 60-kDa fragment of SSA (13). This fact, though, did not diminish the sensitivity of the AtheNA towards the Sjogren’s samples, which have been shown to have a higher bias towards the 52-kDa fragment.

After establishing concordance with a proven methodology, we looked at false-positive and false-negative rates of the bead

<table>
<thead>
<tr>
<th>Antigen</th>
<th>% Samples with autoantibodies detected by AtheNA (no. of samples tested)</th>
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<tbody>
<tr>
<td></td>
<td>CREST (34)</td>
</tr>
<tr>
<td>SSA</td>
<td>3</td>
</tr>
<tr>
<td>SSB</td>
<td>3</td>
</tr>
<tr>
<td>Sm</td>
<td>0</td>
</tr>
<tr>
<td>RNP</td>
<td>0</td>
</tr>
<tr>
<td>Scl-70</td>
<td>18.2</td>
</tr>
<tr>
<td>dsDNA</td>
<td>0</td>
</tr>
<tr>
<td>Centromere B</td>
<td>69.7</td>
</tr>
</tbody>
</table>
 assay, for its use as a screening assay, it was important to determine if AtheNa produced a false, higher percentage of positive results (unspecific binding) than IFA when a normal population was scrutinized. Conversely, it was important to know the limits of the AtheNa assay when it came to incorrectly missing positive samples. For false positives, 7 out of 94 (7.4%) normal controls tested positive by AtheNa. Repeat testing with IFA showed that four out these seven samples had a titer of 1:40 and three had a titer of >1:160. These values are well within the established prevalence of positive ANA results in normal persons (20% to 30% with titers of >1:40 and 5% with titers of >1:160) (11). The other 87 samples were negative with both AtheNa and IFA. For false negatives, we specifically looked at well-characterized SLE samples, all of which were positive by IFA. Since SS/A and RNP are not specific to lupus and their incidence is well below the occurrence in normal persons, we only considered the false-positive results from the dsDNA in this group to be of significance. Considering the sensitivity of the Farr assay and its utility in monitoring flares, AtheNa’s performance in missing only 6% of these samples was an adequate indicator of its sensitivity towards not missing positive samples. It is important to consider this result in the context of the samples tested. Almost all of the samples used for the study came from well-established SLE patients who have been through therapy for years. When we looked at our SLE group of patients positive by AtheNa, out of 185 samples we did not have a single sample that was positive for dsDNA alone. In fact, it is not very common to have new lupus patients with just elevated dsDNA antibodies, although this phenomenon is commonplace for patients who have been in therapy and are undergoing a flare. In this light and considering the high negative predictive value of the test, we believe that the test can be used as an effective screen for antinuclear antibodies.

To assess the utility of bead-based assays for reflex testing (conducting further tests to determine more detail) of positive ANA screens, we looked at previously diagnosed patients in order to have a better picture of the frequency of different autoantibodies detected by AtheNa. The results detailed in Table 3 show equal or better frequencies of autoantibodies associated with specific diseases. All of the negative samples by AtheNa were further confirmed by ELISA and were negative for all autoantibodies. In particular, we tested the positive samples from the SLE patients for a detailed comparison. The frequency percentages detailed in Table 4 show excellent similarity in all autoantibodies except SS/A, which we suspect is due to the differences in the percentages of antigens (52 kDa and 60 kDa) used by the different manufacturers. The other notable fact from these data was the difference in the percentages of dsDNA determined by Farr and AtheNa. It seems that the sensitivity of the AtheNa test is equivalent to 15 IU/ml by Farr.

It is important to note that the results from multiplex assays should always be interpreted within the clinical context, since the amount of information generated by such assays can act as a double-edged sword. As with any diagnostic test system, use of this information without proper clinical presentation, especially for screening tests, can lead to misdiagnosis and improper therapy, since positive ANA results are quite common in healthy individuals. It is also important to understand the fundamentals and limitations of these assays before using them. Patients with a positive ANA result by IFA will almost always have a positive result for ANA regardless of the fact that they are being treated or they are experiencing a flare (3, 5, 9). But, patients with a positive ANA result by AtheNa will not stay positive after they are treated. The difference, it is important to remember, comes from the fact that the AtheNa ANA test is composed of nine individual analytes towards specific autoantibodies which do subside while the patient is on therapy. On the other hand, the IFA test is comprised of all the components present in the HEp-2 cells. While specific autoantibodies subside due to therapy, there are still other components in the cell that do react with the patient’s serum (16, 17). This new and innovative difference in assay design could lead to serious consequences in the real world if not understood precisely. For example, many patients do change physicians, and with an autoimmune history, the physician expects to have a positive ANA result from a patient’s serum. But, if these patients were in treatment and the laboratory utilized a bead-based assay to determine the ANA status, there might be a negative ANA result, which could be very confusing to the physician. This phenomenon is very real and does cause problems and mistrust in a new test such as this one when misunderstood. The reality, on the other hand, is that there really is no need for an ANA screening test if a patient comes in with an extensive autoimmune history, since the physician already expects the test result for this patient to be positive. This is not to say that the bead-based assays are at a disadvantage for detecting ANA-positive patients who are being screened for the first time and that have not received any treatment. In such cases, the results of our study do show that the test is sensitive and specific enough to act as a screening test.

In conclusion, we found the multiplexed bead assay’s performance to be satisfactory for screening, for reflex testing a positive result to determine the presence of specific autoantibodies, and for monitoring patients throughout the course of their disease by detecting flares.

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REFERENCES


