Anal Bioanal Chem (2007) 388:613–618
DOI 10.1007/s00216-007-1243-x

ORIGINAL PAPER

Analytical performance of the AtheNA MultiLyte® ANA II assay in sera from lupus patients with multiple positive ANAs

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Received: 16 January 2007 / Revised: 28 February 2007 / Accepted: 6 March 2007 / Published online: 3 April 2007

Abstract The purpose of this study was to evaluate the precision and accuracy of a commercial multiplexed kit for the measurement of 9 anti-nuclear antibodies (ANAs; anti-SS/A, anti-SS/B, anti-Sm, anti-RNP, anti-Jo-1, anti-Scl-70, anti-dsDNA, anti-Centromere B, and anti-Histone), and to compare these results to a subset of ANAs measured by enzyme-linked immunosorbent assays (ELISA) and immunodiffusion (ID). Sera were obtained from 22 systemic lupus erythematosus (SLE) patients, twelve controls and five others (commercial source) with various autoimmune diseases. ANA results from the AtheNA MultiLyte® ANA II Assay (AtheNA) were compared to ELISA results (controls) and patients (ID). The AtheNA interassay coefficients of variation (CVs, N=39, performed in duplicate; replicated 3×) ranged from 6.2% to 16.7% (mean = 9.8%), while the intra-assay CVs ranged from 5.8% to 14.3% (mean=10.8%). Compared to results for SLE cases and controls, the sensitivity of AtheNA ranged from 85.7% to 100% (mean=97.1%), while diagnostic specificity ranged from 16.7% to 100% (mean=71.6%). There was significant agreement (P values ranging from 0.0001 to 0.03) when analytes coanalyzed by AtheNA and ELISA/ID were evaluated using Cohen's kappa (κ values ranging from 0.376 to 1.000). No false positive ANA results were observed for either the control or commercial source autoimmune disease sera. These results indicate that the AtheNA assay is a precise and accurate alternative for performing multiple ELISAs or IDs in the diagnosis of autoimmune diseases, especially when the number of sera to be tested is large, such as in clinical screening or epidemiologic studies. It also appears that the AtheNA assay identifies positive ANA specificities which are missed by ID techniques, suggesting that it may have greater analytical sensitivity for some ANAs.

Keywords AtheNA MultiLyte® ANA system · Antinuclear antibodies · Autoimmune diseases · Systemic lupus erythematosus · ELISA

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Autoimmune diseases are a family of more than 80 chronic, and often disabling, illnesses that develop when underlying defects in the immune system lead the body to attack its own organs, tissues, and cells. While many of these diseases are rare, collectively they affect 14.7–23.5 million people in the US, and—for reasons unknown—their prevalence is rising. Since cures are not yet available for most autoimmune diseases, patients face a lifetime of illness and treatment. They often endure debilitating symptoms, loss of organ function, reduced productivity at work, and high medical expenses. Because most of these diseases disproportionately afflict women, and are among the leading causes of death for young and middle-aged

women, they impose a heavy burden on patients' families and on society [1].

The diagnosis of autoimmune disease is based upon clinical history, physical examination, and laboratory detection of anti-nuclear antibodies (ANAs). The detection of ANAs and the identification of their specificity have become well-established tools for the laboratory diagnosis of several autoimmune diseases, having both diagnostic and prognostic significance [2]. Testing for ANAs has been performed using immunodiffusion (ID), indirect immunofluorescence (IIF), counterimmunoelectrophoresis (CIE), enzyme-linked immunosorbent assays (ELISAs), as well as other methods [2–6]. Most autoimmune diseases involve polyclonal responses to self-antigens [7], yielding positive results for numerous ANA specificities simultaneously [8]. This necessitates the performance of numerous assays to determine individual ANA repertoires. ELISA techniques have lessened the burden involved when measuring numerous ANAs, since they have speed and increased throughput benefits compared to ID, IIF, and CIE. However, ELISA technology is designed to measure one analyte (or closely related analytes) per assay. When the number of samples and analytes to be measured becomes large, ELISA technology can also become quite cumbersome [9, 10]. An alternative technology is the use of microsphere-based liquid suspension arrays. The application of lasers and microspheres internally dyed with proprietary red- and infrared-emitting fluorochromes for spectral differentiation and a green reporter molecule allows for the measurement of numerous analytes simultaneously (multiplexing), in what is the equivalent of numerous ANA ELISAs. Furthermore, these methods provide a simple platform to obtain antibody patterns associated with autoimmune diseases, a process which has been shown to enhance the identification of individuals who would benefit from early referral to specialists [11].

In the present report, we have evaluated the analytical performance (intra- and interassay coefficients of variation and comparison with ELISA and ID coanalyzed analytes) of the AtheNA MultiLyte® ANA II Assay (AtheNA).

Methods and materials

Human sera Human sera were obtained from subjects who were part of the Carolina Lupus Study (N=34), a population-based case-control study of recently diagnosed systemic lupus erythematosus (SLE) patients designed to examine hormonal, environmental, and genetic risk factors for the disease [8]. Based on case definitions (using the 1997 revised American College of Rheumatology classification criteria for SLE) [12], sera were dichotomized as case (N=22; selected as having multiple positive ANAs) or control (N=12). Five human well-characterized, disease-state sera were also obtained from individuals diagnosed with scleroderma (two sera), mixed connective tissue disease, Sjogren's syndrome or SLE (Zeus Scientific, Raritan, NJ, USA). Sera were stored frozen at −60 °C and only thawed once. Where appropriate, the use of human sera was approved by the National Institute of Environmental Health Sciences Human Subjects Review Board.

Measurement of ANAs Sera (supplied masked to the laboratory) were tested using the heterogenous AtheNA MultiLyte® ANA-II Test System (Product No. A21001; Inverness Medical Professional Diagnostics, Princeton, NJ, USA [AtheNA]), in duplicate on three separate days, using calibrators, standards, controls and reagents obtained from the manufacturer. Briefly, the AtheNA system is a multiplexed, microsphere-based, FDA-cleared (section 510[k] of the Federal Food, Drug, and Cosmetic Act [21CFR866.5750]) assay for the qualitative measurement of eight ANAs and the quantitative measurement of anti-dsDNA in serum. The multiplexed suspension contains a mixture of spectrally distinguishable 5.6-μM-diameter microspheres (composed of polystyrene, divinylbenzene, and methacrylic acid) internally dyed with proprietary red- and infrared-emitting fluorochromes. By adjusting the concentrations of each fluorochrome, spectrally addressable microsphere sets are obtained. A 635-nm classification laser excites and classifies the microspheres based on embedded red and infrared fluorochromes. A 532-nm reporter laser excites the green fluorescent molecules bound to the surface of the microspheres. Using this method, concentrations of numerous analytes can be measured simultaneously by isolating detector responses (green fluorescence) from numerous spectrally addressable microspheres simultaneously (multiplexing). In the AtheNA assay, different addressable microsphere sets are conjugated separately with different ANA antigens: SS/A, SS/B, Sm, RNP, Jo-1, Scl-70, dsDNA, Centromere B, Histone, and an extract of HeP-2 cells (HeP-2NA). These conjugated microspheres are mixed with additional spectrally addressable microspheres (one microsphere set designed to detect nonspecific antibodies in the sample [if present] and four separate bead sets used for assay calibration [Intra-well CalibrationTechnology]) are included in the assay. Essentially, a multipoint standard curve corrected for nonspecific binding is run within each sample. To perform an assay, 50 μl of the AtheNA microspheres are added to the wells of a microtiter plate followed by 10 μl of diluted (1:21) sera or control and these are allowed to incubate at room temperature for 30 min, during which the ANAs bind to their respective microsphere-bound antigens. After incubation with diluted sera and washing of the micropheres, phycoerythrin (PE)-conjugated goat anti-human IgG is added, which binds to

the microphere-bound ANA and produces a fluorescent signal. The resultant microsphere-(conjugated-ANA-antigen)-ANA-goat-anti-human IgG PE complex was measured in a Bio-Plex suspension array instrument (Bio-Rad Laboratories, Hercules, CA, USA) fitted with a microplate autosampler. Luminex^{100} software (ver. 2.3, Luminex, Inc., Austin, TX, USA) was used to acquire data (the Luminex software operates in the background), which are analyzed using AtheNA software. The AtheNA software uses lot-specific regression analyses of the internal standards (on a CD supplied with the kits) to calculate IU/ml for dsDNA and AU/ml for the eight ANAs. Positive anti-HeP-2NA results are not reported unless the results for all nine other analytes are negative, indicating that the specific ANA is not directed against one of the nine specific analytes contained in the multiplex microsphere suspension. Results were considered positive at >120 AtheNA Units (AU)/ml or IU/ml, as per the manufacturer's recommendation.

ELISA and ID ANA methods Antinuclear antibodies were determined by immunofluorescence using HEp-2 cells (The Binding Site Inc., San Diego, CA, USA) as substrate. Positive sera at 1:40 titration underwent further dilution (1:160 and 1:640) for semi-quantitation. Control sera samples positive at titer levels of ≥1:160 were further assessed for the presence of IgG antibodies against Sm, RNP, dsDNA, SS/A, and SS/B by commercial ELISA kits (Bio-Rad Helix EIAs, Bio-Rad Laboratories, Hercules, CA, USA). These kits were applied following the manufacturer's directions, using supplied reagents and standards. The reference range for anti-dsDNA, SS/A and SS/B was 0–25 and 0–20 for anti-Sm and anti-RNP antibodies. Case sera samples were evaluated for ANAs by ID with direct visualization using methods reported previously [8].

Statistical analysis Intra-assay and interassay coefficients of variation (CVs) for the sera evaluated in the AtheNA system (N=39) were calculated as described previously [13]. Results of duplicate determinations were dichotomized on positive (>120 A(I)U/ml) or negative (≤120 A[I] U/ml) status, transformed to binary outcomes, and concordance calculated. For the three replicates, the dichotomous duplicate outcomes were evaluated for concordance. Sensitivity and specificity of the AtheNA assay was calculated using the following definitions (compared to ELISA or ID results): TP: true-positive diagnostic test result; TN: true-negative diagnostic test result; FN: false-negative diagnostic test result; and FP: false-positive diagnostic test result. Sensitivity (TP/[TP + FN] × 100) was computed as the percentage of positive test responses in subjects with a positive ELISA or ID response for a specific ANA. Specificity (TN/[FP + TN] × 100) was computed as the percentage of negative tests in subjects with a negative ELISA or ID response for a specific ANA. Cohen's kappa (κ) was used to evaluate agreement for coanalyzed measurands (SPSS Inc. Chicago, IL, USA). A type 1 error value of 0.05 was considered statistically significant.

Results

The inter- and intra-assay coefficients of variation (CVs) for the AtheNA system are shown in Table 1. The interassay CVs ranged from 6.2% to 16.7% (mean=9.8%), while the intra-assay CVs ranged from 5.8% to 14.3% (mean= 10.8%). Using 120 A(I)U/ml as a positive cutoff there was 100% concordance between duplicates (39 samples, positive/negative) for anti-SS/A, anti-SS/B, anti-Sm, anti-RNP, anti-Jo-1, anti-Scl-70, anti-Centromere B, and anti-Histone antibodies. Discordant duplicates were seen for 2/39 (5%) anti-dsDNA samples. For the three replicates, one serum had discordant replicate results for anti-SS/B, anti-Histone, and anti-Scl-70, yielding 97% replicate concordance for these analytes, while anti-SS/A, anti-Sm, anti-RNP, anti-Jo-1, and anti-Centromere B showed 100% concordance between replicates. The replicate concordance for anti-dsDNA was 97%. There were no sera which gave isolated positive anti-HeP-2NA results.

When the five well-characterized, disease-state commercial sera were compared to results obtained by the kit's manufacturer using the AtheNA system, 100% concordance for all five sera were observed for the positive/negative status of anti-SS/A, anti-SS/B, anti-Sm, anti-RNP, anti-Jo-1, anti-Scl-70, anti-dsDNA, anti-Centromere B, and anti-Histone. The mixed connective tissue disease sera had positive results for anti-Sm and anti-RNP; one scleroderma serum had positive results for anti-SS/A and anti-Scl-70, while the other had a positive result for anti-Scl-70 only; the Sjogren's disease and SLE sera were positive for anti-SS/A and anti-SS/B.

Table 1 Coefficient of variation (CV) of AtheNA MultiLyte® II test system (N=39 sera, tested three separate times in duplicate)

	Coefficients of variation (%)	
Analyte	Interassay	Intra-assay
anti-SS/A	12.0	10.5
anti-SS/B	12.6	14.0
anti-Sm	7.3	9.6
anti-RNP	5.7	6.2
anti-Scl-70	7.8	12.0
anti-Jo-1	16.7	14.3
anti-dsDNA	8.6	11.1
anti-Centromere B	11.7	14.1
anti-Histone	6.2	5.8

Table 2 Kappa statistic for specific autoantibodies coanalyzed by AtheNA and ELISA/ID (N=34)

Analyte	Kappa	P value
anti-SS/A	1.00	0.0001
anti-SS/B	0.376	0.03
anti-Sm	0.821	0.0001
anti-RNP	0.825	0.0001
anti-dsDNA	0.626	0.001

The 22 case SLE sera had 86 positive ANAs for the nine analytes measured by the AtheNA assay. The 12 control sera were negative for these analytes. When the five ANAs coanalyzed by AtheNA and ID (anti-SS/A, anti-SS/B, anti-Sm, anti-RNP, and anti-dsDNA) were collated, the 22 case SLE sera had 68 positive analytes by the AtheNA and 57 positive ANAs by ID (no positive ANAs were observed for the control sera by ELISA). The concordance of the AtheNA assay for analytes coanalyzed by ID was 100% for SS/A, 85.3% for SS/B, 91.2% for Sm and RNP, and 82.4 for dsDNA, yielding an overall concordance of 90.0%. There was significant agreement (P values ranging from 0.0001 to 0.03) when analytes coanalyzed by AtheNA and ID were evaluated using Cohen's kappa (κ values ranging from 0.376 to 1.000, Table 2). The AtheNA system identified 19 case sera with between two and seven multiple positive results. By far, the pattern of multiple positive ANAs included anti-SS/A, anti-Sm, anti-RNP, anti-dsDNA and anti-Histone (Table 3). The AtheNA assay identified at least one positive ANA analyte in 19/22 (86.4%) case sera which had HEp-2 titers ≥1:160 measured by IIF. Samples positive by ID are described in Table 4.

The sensitivity of the AtheNA assay vs. ELISA/ID was 100% for anti-SS/A, anti-SS/B, anti-Sm, and anti-RNP, while it was 85.7% for anti-dsDNA (mean=97.1%). The specificity of the AtheNA assay vs. ELISA/ID was 100% for anti-SS/A, 16.7% for anti-SS/B, 81.3% for anti-Sm, 83.3% for anti-RNP, and 76.9% for anti-dsDNA (mean=71.6%).

Discussion

The benefits of multiplexed suspension array technology over other technologies for the measurement of multiple analytes simultaneously includes speed, the ability to multiplex, the ability to measure all antibody concentrations for all analytes with a single serum dilution, and more desirable reaction kinetics of the liquid phase in a suspension array [10]. A drawback of multiplexed suspension array technology is the initial acquisition of a flow cytometer or dedicated instrument needed to perform the assays, which can cost more than alternative methods. However, because the cost of buying one multiplexed kit for nine analytes is substantially less than buying nine individual ELISA kits,

Table 3 Distribution matrix of positive ANAs in sera from 22 SLE case patients measured by the AtheNA MultiLyte® II Test System (shaded cells are positive results)

	ANA Analyte									
Subject ID	SS/A	SS/B	Sm	RNP	Scl-70	Jo-1	dsDNA	Cent B	Histone	No. of Positive Analytes
1048										0
1058										0
1077										0
1230				■						1
1575	■	■								2
1821	■		■	■						3
1880	■			■					■	3
1899			■	■			■		■	4
1901			■	■			■		■	4
2466	■		■	■					■	4
2472			■	■			■		■	4
2800	■		■	■			■		■	5
2805	■		■	■			■		■	5
2807	■		■	■			■		■	5
3146	■		■	■			■		■	5
3152	■	■	■	■					■	5
3157	■		■	■			■		■	5
3163	■	■	■	■			■		■	6
3164	■		■	■	■		■		■	6
3170	■	■	■	■			■		■	6
3183	■	■	■	■			■		■	6
3200	■	■	■	■	■		■		■	7

Table 4 Distribution matrix of positive ANAs in sera from 22 SLE case patients measured by immunodiffusion (ID; shaded cells are positive results)

	ANA Analyte					
Subject ID	SS/A	SS/B	Sm	RNP	dsDNA	No. of Positive Analytes
1048			■	■	■	3
1058			■	■	■	3
1077	■		■		■	3
1230	■		■	■		3
1575	■			■	■	3
1821				■		1
1880	■		■	■	■	4
1899	■			■	■	3
1901	■		■		■	3
2466	■		■	■	■	4
2472	■	■			■	3
2800						0
2805	■			■	■	3
2807	■		■		■	3
3146	■			■	■	3
3152						0
3157	■		■	■		3
3163	■		■	■		3
3164						0
3170	■		■	■		3
3183			■	■	■	3
3200	■		■	■		3

depending on test volume, the initial cost of the multiplex instrumentation could be recouped rapidly.

Sequential and/or contemporaneous measurement of numerous analytes from a biological or clinical sample is wrought with numerous potential sources of error. Unless the biological media is stored as many independently frozen aliquots, or other contingencies are used to ensure sample integrity, sample degradation is likely. Errors in the estimates of the concentrations of individual analytes are likely, as the individual methods most probably have their own unique inter- and intra-assay coefficients of variation and recovery. Propagation of these errors in each individual analysis could lead to potentially large combined errors, especially for the relative ratios of concentrations of individual analytes [14].

In the present report, the AtheNA method showed 100% duplicate concordance (positive/negative) for anti-SS/A, anti-SS/B, anti-Sm, anti-RNP, anti-Jo-1, anti-Scl-70, anti-dsDNA, anti-Centromere B, and anti-Histone, and 95% duplicate concordance for anti-dsDNA. Replicate concordance (N=3) was also excellent, between 97% and 100% for all analytes. This information strongly suggests that the AtheNA method for measuring ANAs is extremely precise. The level of precision indicates that the method can be performed in singlicate (one multiplexed assay for nine analytes per serum), albeit with appropriate quality control monitoring/techniques, such as masked splits [13].

When ANA results from the AtheNA system were compared to results measured by ELISA and ID in sera (N= 34) for co-nalyzed analytes (anti-SS/A, anti-SS/B, anti-Sm, anti-RNP, and anti-dsDNA), sensitivities ranged from 85.7% (anti-dsDNA) to 100%. It is worth noting that our observed sensitivity of 85.7% for AtheNA-measured anti-dsDNA compared to ELISA and ID agrees well with the 86% sensitivity described by others using the AtheNA method [15]. Another investigator who compared ELISA vs. AtheNA values for anti-SS/A, anti-SS/B, anti-Sm, and anti-RNP in sera from 639 patients with physician-diagnosed autoimmune disease reported a mean combined kappa of 0.572 for these analytes [16]. Our mean combined kappa for these same analytes was 0.756 using 22 SLE case sera.

Reference ELISAs do not exist for the coanalyzed measures compared in this study [15], and alternative methods (such as ID) have subjective endpoints [17, 18], so there is really no “gold standard” for the measurement of ANAs. Specificities (compared to ELISA/ID) in the present study ranged from 16.7% (anti-SS/B) to 100% (anti-SS/A). Interestingly, all control sera samples and commercially available disease sera had 100% agreement for all analytes when measured by either ELISA or AtheNA. By far the most important reason for the decreased specificity in the AtheNA assay was due to the AtheNA system yielding positive discordant results compared to ID. This phenomena has been described by others, who speculated that

differing antigens or increased dilutions of sera in other assays were responsible [15, 16, 19, 20] and that the AtheNA results might be ID or ELISA false negatives. In the present work we did not have access to a sufficient number of multiple positive discordant ID/AtheNA positive sera to investigate this hypothesis. However, the lack of AtheNA "false positives" in the control and commercial sera argues for the concept that the AtheNA assay is more sensitive than ID.

Multiplex assays provide the opportunity for high-throughput screening at a lower cost in terms of time and reagents than individual ELISA assays. For example, performing a ANA ELISA for one analyte in a 96-well plate format requires about 4 h for loading samples, conjugate, substrate incubations, washing, and reading of plates. The AtheNA method in the present report takes about 3 h to obtain results on nine separate analytes. The AtheNA method may also be useful in large epidemiologic studies to efficiently screen for multiple autoantibodies in stored specimens of limited volume, as it requires only one dilution of the sample to measure nine analytes vs. numerous separate dilutions for ELISA or ID. There is no doubt that the AtheNA method has a throughput advantage over ELISA and ID, which would be magnified as the number of analytes or samples become large [10].

In conclusion, we have shown the AtheNA multiplexed ANA assay is precise and accurate (albeit with the consideration that no rigorously validated reference standard assays exist). The level of precision is such that the assay can be performed in singlicate, it has the potential for extremely high throughput, and it should be a beneficial addition for the diagnosis and study of autoimmune diseases.

Acknowledgements Mention of a product or company name does not constitute endorsement by NIOSH. This work was supported in part by an interagency agreement between NIOSH and NIEHS (Y1-ES-0001-Clinical Immunotoxicity). The content and conclusions of this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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