

Comparison of Three Multiplex Immunoassays for Detection of Antibodies to Extractable Nuclear Antibodies Using Clinically Defined Sera

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ABSTRACT: We set out to determine the agreement of three multiplex immunoassays for the detection of autoantibodies involved in connective tissue disease using clinically defined sera. Usefulness of the immunoassays will be defined by correlation to disease state. Using the immunoassays from Inova Diagnostics, Biomedical Diagnostics (BMD), and AtheNA, reactivity, to Smith (Sm), ribonucleic protein (RNP), SSA (Ro), SSB (La), Scl-70, and dsDNA or chromatin, we tested 273 clinically defined sera consisting of 57 systemic lupus erythematosus (SLE) sera, 69 rheumatoid arthritis (RA) sera, 47 sera defined as various other connective tissue diseases, and 100 normal donor sera. Samples were also tested for anti-nuclear antibody (ANA) on HEp-2 cells by IFA for analysis of discrepant results. Inova, BMD, and AtheNA assays demonstrated 57%, 89%, and 80% concordance, respectively, in the 47 connective tissue disease sera. The BMD assay was the most sensitive in detecting Scl-70. The immunoassays did not correlate well in the 57 SLE-defined sera; however, each had a variety of antibodies positive for each serum. The AtheNA assay demonstrated the highest degree of nonspecificity. Inova, BMD, AtheNA, and the Inova ANA HEp-2 IFA demonstrated 97%, 98%, 97%, and 99% specificity, respectively, using normal sera. Thus, all three assays showed a 97% or better negative predictive value. Positive correlation varied from 83% to 98%. Antibodies in SLE sera did not compare well among the three immunoassays. Significant variation in specificity and sensitivity due to individual characteristics of each assay was demonstrated, with the BMD assay showing the highest correlation with clinical diagnosis.

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INTRODUCTION

Connective tissue disease (CTD) is a group of autoimmune diseases, which include systemic lupus erythematosus (SLE), Sjögren's syndrome, scleroderma, polymyositis, and others. SLE, a predominant disease, is a protean autoimmune disease resulting in multiple antibodies to nuclear antigens.¹ Detecting some of these antibodies can be useful in diagnosing and/or monitoring the disease.^{1,2} SLE affects approximately 1 per 1000 of the population, predominantly women of child-bearing age, although it may occur at any age. As with all CTD, symptoms come and go and are often vague mimicking other illnesses, and many overlap with a variety of other diseases.^{1,3,4} Symptoms include fatigue, weight loss, fever, rash, poor sleep patterns, and chronic joint pain, among many others. Diagnosis of SLE should be determined by the clinician, based on patient symptoms, history, and a set of 11 criteria used for diagnosing SLE as published by the American College of Rheumatology (ACR).^{1,4-6} These 11 criteria are malar and discoid rash, photosensitivity, oral ulcers, arthritis (nonerosive, involving two or more peripheral joints), serositis, renal involvement, neurologic disorders, hematologic disorders, positive tests for antinuclear antibody (ANA) in the absence of drugs known to induce SLE, and a positive anti-double-stranded DNA test (dsDNA). A patient must present with four or more of these criteria to diagnose SLE.^{1,4-7} A negative ANA test virtually rules out SLE; however, a positive ANA is not specific for SLE or other CTD.^{1-3,7,8} Healthy people can demonstrate ANA immunofluorescent (IFA) titers as high as 1:320 with no sign of clinical disease.⁹⁻¹¹ Scleroderma, including the CREST syndrome, Sjögren's syndrome, polymyositis, and mixed CTD, among others, produce a variety of ANA titers and patterns based on the antibody they are producing. Because symptoms overlap, when a patient presents with vague complaints it has become routine for the physician to order an ANA to rule out the possibility of SLE and other CTD. Indirect immunofluorescence has been the gold standard for testing for ANA in the clinical laboratory. But ANA IFA is labor intensive and is dependent on the skills of a medical technologist who must manually manipulate the patient serum and HEp-2 substrate, then use his/her experience and knowledge interpreting titers and patterns under the fluorescent microscope.^{1,7-9} Using the ANA test as a screen to rule out CTD has diminished its usefulness. Therefore, it has been well established that a specimen positive for ANA should be followed up with testing for extractable nuclear antigen antibodies (ENA) to differentiate and substantiate the presence of disease.^{3,5,7,8,12,13} There are several enzyme-linked immunosorbent assays (ELISA) available for testing for ENAs.^{7,8} During the past few years a multiplex fluorescent microsphere immunoassay technology

has become available. This technology combines the use of flow cytometry with uniquely labeled fluorescent microspheres enabling the detection of multiple antibodies simultaneously with a small amount of patient sample in one well. Studies have demonstrated this technology to be sensitive and specific for the detection and semiquantitation of ENA antibodies with excellent correlation to well-established ELISAs.^{9,14-19}

The aim of this study was to evaluate the diagnostic performance and usefulness of three multiplex ENA assays for diagnosing SLE and other CTDs.

MATERIALS AND METHODS

Clinical Samples

This study included 273 patient samples gathered from three independent sites and grouped into four categories. Category A consisted of 62 well defined SLE patients from two physicians diagnosed in accordance with the ACR criteria for SLE.^{1,4-6} Category B consisted of 42 sera representing a mix of CTDs and defined by extensive laboratory testing along with clinical diagnosis. Category C consisted of sera from 100 healthy donors considered disease free and representing a cross-section of the United States in regards to race, age, sex, and site of origin. Category D consisted of 69 sera from patients clinically diagnosed with rheumatoid arthritis (RA) by one rheumatologist using ACR 1987 criteria for classification of RA.¹⁸ Categories C and D were considered negative controls with categories A and B being the test patients. All patient samples in this study 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.

Assays

The three multiplex assays we compared used fluorescent antibody detection. This multiplex technology uses 5.6- μ m polystyrene microspheres that are internally labeled with different ratios of two fluorophores. When the microsphere passes through the flow cell it is interrogated by two lasers. The first laser, 635-nm, excites the fluorophores, causing them to emit at different wavelengths: 658 and 712 nm. By varying the intensities of emissions of the two wavelengths, 100 unique identifiable fluorescent microspheres are created. The Luminex 100 (Luminex Corp., Austin, TX) and FIDIS analyzer (Biomedical Diagnostics (BMD), Marne la Vallee, France) use digital signal processors to identify each microsphere. A reaction mixture is then formed

by coupling specific antigens to unique microspheres containing different dye ratios. Coupling of the antigen to the microsphere occurs by covalent bonding of antigen to carboxyl groups located on the microsphere. During the course of the test, a secondary anti-human antibody bound to a third fluorophore is added. This fluorescent-linked reporter molecule, when present, is excited by the second laser, which allows quantitation of the amount of analyte, in this case human antibody, bound to each antigen.

All three assays evaluated in this study have received Food and Drug Administration clearance and are labeled for *in vitro* diagnostic use. Each of the three assays, FIDIS connective 10 available through BMD, Inova QUANTA Plex™ ENA 8 (Inova Diagnostics, San Diego, CA), and AtheNA Multi-Lyte® ANA-II test system (Inverness Medical Professional Diagnostics, Princeton, NJ), present a slightly different test menu. For this study we compared the antibodies all three had in common, with one exception: the FIDIS and AtheNA contain dsDNA antigen while the ENA 8 uses chromatin. Along with dsDNA/chromatin analysis, we tested for Smith (Sm), ribonucleic protein (RNP), SSA (52kDa and 60kDa), SSB, Scleroderma 70 (Scl-70), and Jo-1. All sera were also tested by indirect immunofluorescence using HEP-2 substrate with anti-human IgG γ conjugate. All dsDNA and chromatin discrepant were resolved by dsDNA IFA using *Crithidia luciliae*. The assays differed in utilization of native antigens and/or recombinant antigens. There can be problems with either. A purified antigen may still have contaminants and might not have the full complement of native proteins. A recombinant antigen may have bacterial contamination or an altered structure, or may not contain all the necessary epitopes. All assays used human antigens. Two of the assays used a wash step, while the third did not. The number of microspheres counted per analyte varied from 50 to 200 among the assays. Each assay was performed according to the package insert using good laboratory practices. Due to the limited quantity of serum, assays were performed only once, with one exception, which will be addressed in the discussion.

RESULTS

Category A consisted of 64 clinically defined SLE sera. The results of the three assays in this category are shown in FIGURE 1. All three assays demonstrated overall agreement with the SSA. SSA 52 kDa antibody was detected in two sera by the BMD and Inova assays but not by the AtheNA assay. SSB, SM RNP, and dsDNA/chromatin antibodies were detected by all three assays with the AtheNA assay detecting positives in the highest number of sera in each category. Scl-70, an antibody not found in SLE, was positive in 7 SLE sera by the AtheNA assay. The Inova assay demonstrated a much lower detection of chromatin antibody compared to the dsDNA antibody detected by the other two assays. Jo-1, an antibody found in the cytoplasm that is associated with polymyositis, was not detected by any of the three assays.

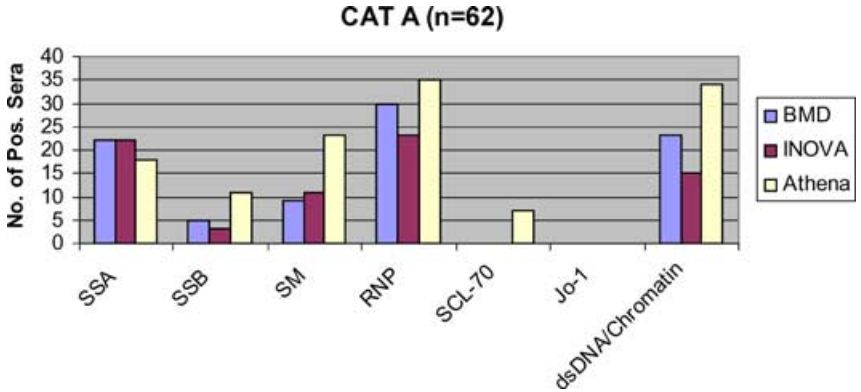


FIGURE 1. SLE clinical samples (ANA IFA = 97% positive).

The BMD, Inova, and AtheNA assays demonstrated overall agreement for SSA, Sm, RNP, and Jo-1 antibody detection in the 48 CTD sera shown in FIGURE 2. The Inova assay again had a low level of detection of chromatin antibodies compared to the other two assays, which detected a higher number of antibodies to dsDNA. The Inova assay also did not detect Scl-70 antibodies. As with the SLE sera, the AtheNA assay detected the highest number of antibodies overall in the CTD sera.

Category C, the 100 healthy donor sera (FIG. 3), was expected to show little or no ENA antibody detection. The BMD assay fulfilled this expectation. The Inova assay detected antibody in one sera each for Scl-70 and Jo-1 and detected antibodies in low concentration to chromatin antigen in seven of the sera. The AtheNA assay detected positives for all antigens except Sm, Jo-1, and dsDNA. All 100 healthy donor sera were tested by ANA IFA using the Inova HEp-2

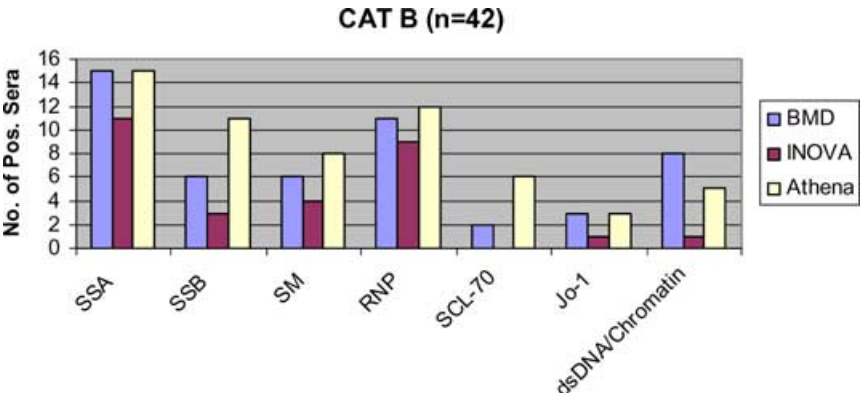


FIGURE 2. CTD clinical samples (ANA IFA = 96% positive).

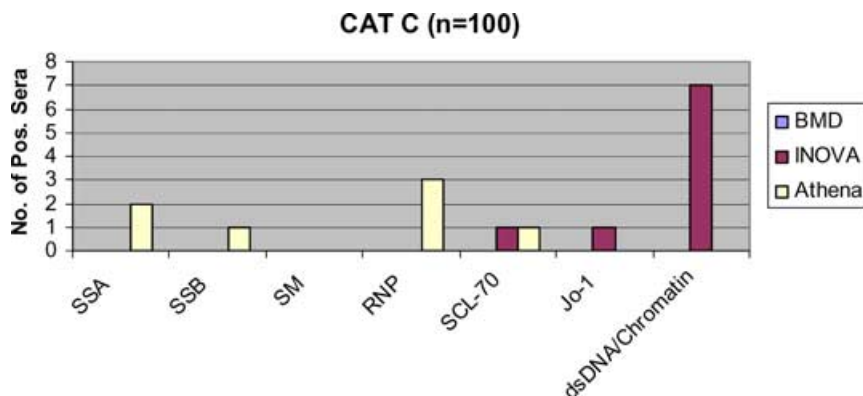


FIGURE 3. Healthy donors (ANA IFA 99% negative).

substrate. One sera tested positive with a 1:1,280 ANA titer with a centromere pattern. The other 99 sera were negative on HEp-2 cells.

The 69 RA sera that comprised Category D were used as a negative control for ENA testing. FIGURE 4 shows the results of the three multiplex assays for the detection of antibody in the sera. The AtheNA assay again demonstrated a high number of positive antibodies for all antigens except Jo-1, while the Inova and BMD assays demonstrated antibodies to SSA in two and three, respectively, and one SSB positive sera each. Only one of these sera was positive for ANA IFA using the Inova HEp-2 substrate.

DISCUSSION

The multiplex immunoassay platform lends itself well to ENA testing; however, antibodies testing positive among the three different assays in each category varied. The BMD assay couples the SSA 52 kDa and SSA 60 kDa on separate microspheres, allowing us to see that two of the SSA positive sera detected by the BMD assay contained only antibody to SSA 52 kDa. These two sera were also positive by the Inova assay, but not by the AtheNA assay. The SSB antibody is not a commonly found in SLE, but was often detected by ENA testing in our studies. SSA and SSB are associated with a common RNA, making it difficult to completely separate the two.¹ This may be the reason each assay produced some reactivity to the SSB antigen. Scl-70 antibody is not associated with SLE; however, the AtheNA assay gave a positive result for antibodies to this antigen in seven of the sera. None of the three assays showed reactivity to Jo-1. This was to be expected since Jo-1 antibodies, which are found in the cytoplasm, are associated with polymyositis and not with SLE or other CTD.

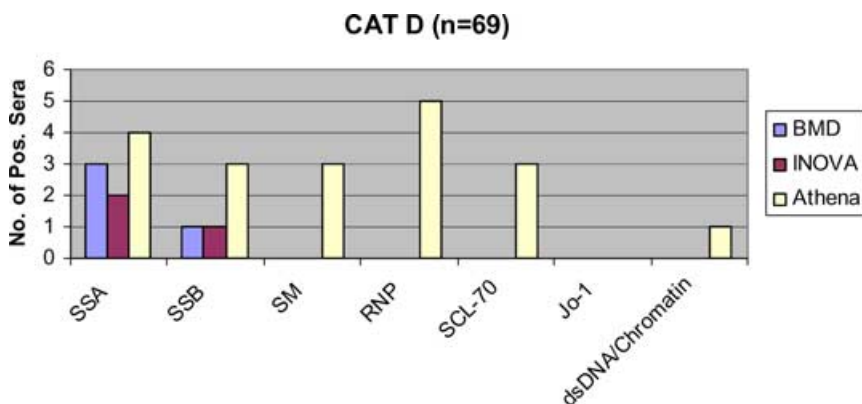


FIGURE 4. RA clinical samples (ANA IFA 99% negative).

During the past few years many studies have compared anti-chromatin (nucleosome) antibody assays to the traditional dsDNA antibody assays due to the emerging role of the nucleosome. It is believed that the nucleosome, a large protein–nucleic acid complex, is the autoantigen responsible for the production of numerous antinuclear antibodies. Studies have shown that antinucleosome antibodies are formed prior to dsDNA antibodies and are often detected in patients negative for anti-dsDNA.^{20–24} One would expect the Inova multiplex assay to produce a higher number of positive results for chromatin antibody than the assays that use dsDNA. Our test results did not show that. The low reactivity to chromatin by the sera that tested positive to dsDNA antigen may be due to better coupling of the dsDNA to the microsphere compared to that chromatin. The discrepant sera were tested on a second lot number of the Inova multiplex assay, and once again demonstrated negative results. When these samples were tested on Inova's chromatin ELISA assay, which contained the same chromatin antigen used on their microsphere, all sera were positive. These sera were further tested by IFA on *Crithidia luciliae*, considered the gold standard, and resulted in high titers for dsDNA. Clearly, the Inova microsphere assay for chromatin antibody need additional validation.

The BMD and Inova assays demonstrated excellent agreement with ANA IFA even though the BMD assay incorporates a wash step in their procedure and Inova does not.

The AtheNA assay, which also uses a wash step in their test, appeared to have a 33% false-positive ANA rate in the RA sera when compared to the gold standard ANA IFA HEp-2 substrate. There can be several reasons for the false positives, including altered structure of the antigen or contamination. The AtheNA assay's high rate of positivity in the RA sera suggests the possibility that the high number of positives in the other categories may also reflect at least some false positives.

CONCLUSION

The multiplex microsphere IFA assay is an effective tool for testing multiple ENAs associated with SLE and other CTDs. Overall, the assays correlated well with each other. The BMD assay correlated most closely with IFA results. The AtheNA assay appeared to have a high false-positive rate. Each assay demonstrated positivity in the CTD and SLE sera, but did not necessarily agree with each other on which antibodies were present. The use or lack of a wash step and the number of microspheres counted with each assay did not appear to give any advantage to a particular assay. The native or recombinant antigens used by the assays and the coupling of the antigen to the microsphere appear to be key factors in how the assay performs. The most important information for the clinician and the laboratorian to know is the limitations of the assay being used in the particular population being tested.^{1,3,7,8,15,25}

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