Measurement of Antinuclear Antibodies by Multiplex Immunoassay: A Prospective, Multicenter Clinical Evaluation

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ABSTRACT. ***Objective.*** We conducted a prospective, multicenter evaluation of autoantibody testing by multiplex immunoassay in patients with known or suspected connective tissue diseases (CTD). We evaluated agreement between multiplex immunoassay and enzyme immunoassay (EIA) and assessed the diagnostic utility of autoantibody profiles.

Methods. Samples from 908 patients with suspected CTD seen in rheumatology clinics were collected prospectively at 3 tertiary care centers. Diagnoses were established according to recognized classification criteria. Tests for autoantibodies were obtained by multiplex immunoassay and by EIA. The results of the multiplex immunoassay were analyzed using a previously validated interpretative algorithm, MDSS (Medical Decision Support Software), that suggests possible disease associations based on the pattern of results for the autoantibodies.

Results. The median patient age was 49.7 years; 83% were female. The most common diagnoses were rheumatoid arthritis in 352 patients and systemic lupus erythematosus (SLE) in 332 patients. Agreement between multiplex and EIA testing ranged from a high of 99% (95% CI 98% to 100%) for Jo-1 to a low of 79% (95% CI 76% to 82%) for antinuclear antibodies. The MDSS algorithm suggested an appropriate disease association in 75% to 100% of patients with SLE. The results varied depending on the disease and the autoantibodies present.

Conclusion. These results suggest that patterns of autoantibodies detected by multiplex immunoassay testing, when analyzed by an interpretative algorithm, are useful in the evaluation of patients with CTD in situations of high disease prevalence. Further testing is necessary to determine its utility in settings of low disease prevalence. (First Release April 1 2007; J Rheumatol 2007;34:978–86)

Key Indexing Terms:
CONNECTIVE TISSUE DISEASE SYSTEMIC LUPUS ERYTHEMATOSUS
AUTOANTIBODIES ANTINUCLEAR ANTIBODIES IMMUNOASSAY

The antinuclear antibodies (ANA) test performed by indirect immunofluorescence or enzyme immunoassay (EIA) is well accepted as clinically useful in the initial evaluation of patients suspected of having a connective tissue disease (CTD)[1,2]. Nevertheless, it is also widely recognized that many positive ANA results are seen in patients without a CTD. Identification of ANA-positive patients who have a CTD usually requires further testing for specific autoantibodies. Clinicians are thus presented with the problem of deciding which ANA-positive patients can best benefit from further testing, a particularly problematic situation when the prevalence of disease is low and the "false-positive" rate of the testing is high. Recent studies in the USA and Canada have shown that patients with signs or symptoms compatible with CTD are often difficult to characterize in the primary care setting[3,4]. New technologies that yield multiple test results from a single specimen may be useful to evaluate the significance of positive results and for interpreting complex patterns of autoantibodies. Patterns of test results may also be useful in patients with definite CTD in the specialty setting for recognizing phenotypes that are strongly associated with particular diseases.

Multiplex immunoassay test methods have recently been developed for use in the clinical laboratory. They utilize indi-

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vidually identifiable, fluorescent microspheres (beads), each coupled with a different antigen or antigen mixture to test for multiple autoantibodies simultaneously in the same tube. Patient serum is incubated with the bead mixture and antihuman IgG antibody conjugated to a second fluorophore is added to detect autoantibodies bound to the antigen-coated beads (Figure 1)[5-9]. With current immunofluorescence or EIA technology it is necessary to perform multiple tests to obtain the same profile of results available from a single multiplex test.

We present the results of a multicenter, prospective clinical evaluation of a recently developed multiplex immunoassay system for autoantibody testing[10] performed at 3 tertiary care rheumatology centers. The multiplex testing platform we evaluated includes software that analyzes patterns of autoantibody test results and compares the results to a database of previously characterized sera[11,12].

Our primary objective was to evaluate the agreement between multiplex and EIA testing in a population of patients with a high prevalence of CTD. We also sought to evaluate the agreement between physician diagnosis and the suggested diagnosis generated by the diagnostic software algorithm.

MATERIALS AND METHODS

Sera were collected from 908 patients at 3 rheumatology clinics (Mayo Clinic, Rochester, MN; the University of Washington Medical Center, Seattle, WA; and Cedars-Sinai Medical Center, Los Angeles, CA).

Samples collected by Dr. Moder at the Mayo Clinic were tested on the BioPlex ANA Screen at the Mayo Clinic Immunology Laboratory. Samples collected by Dr. Wener at the University of Washington were tested on the BioPlex ANA Screen at the University of Washington Medical Center Immunology Laboratory. Samples collected by Dr. Weisman at Cedars-Sinai Medical Center were tested on the BioPlex ANA Screen at Rheumatology Diagnostics Laboratory (RDL) in Los Angeles.

Consecutive patients with highly suspected or previously diagnosed CTD were included; patients solely diagnosed with osteoarthritis, fibromyalgia, and antiphospholipid syndrome were excluded. Patients with these conditions and healthy controls were the subject of a previous report[12].

All patients signed an informed consent to participate in the study. The study was approved by the institutional review board at each institution.

Patients were assigned to one or more disease states according to American College of Rheumatology (ACR) classification criteria. Other recognized classification criteria were used if no specific ACR criteria were available[13-19]. Patients with dermatomyositis were classified according to the Bohan and Peter criteria[14].

Tests for autoantibodies were performed by multiplex immunoassay using the BioPlex 2200® ANA screen kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). This ANA screen tests for the following autoantibodies simultaneously: dsDNA, chromatin, ribosomal P, SSA, SSB, Sm, SmRNP, RNP, Scl-70, Jo-1, and centromere B. All samples were also tested for the same autoantibodies using commercial, US Food and Drug Administration (FDA)-approved EIA kits from Bio-Rad Laboratories, from Inova Diagnostics (San Diego, CA, USA), and from Pharmacia (Freiburg, Germany; Table 1). More information regarding these antigens has been reported[12].

The serum was separated from the cells within 8 h of collection. The serum was then aliquoted into separate tubes for the BioPlex testing and the predicate testing (a separate aliquot for testing with the BioPlex and a sepa-

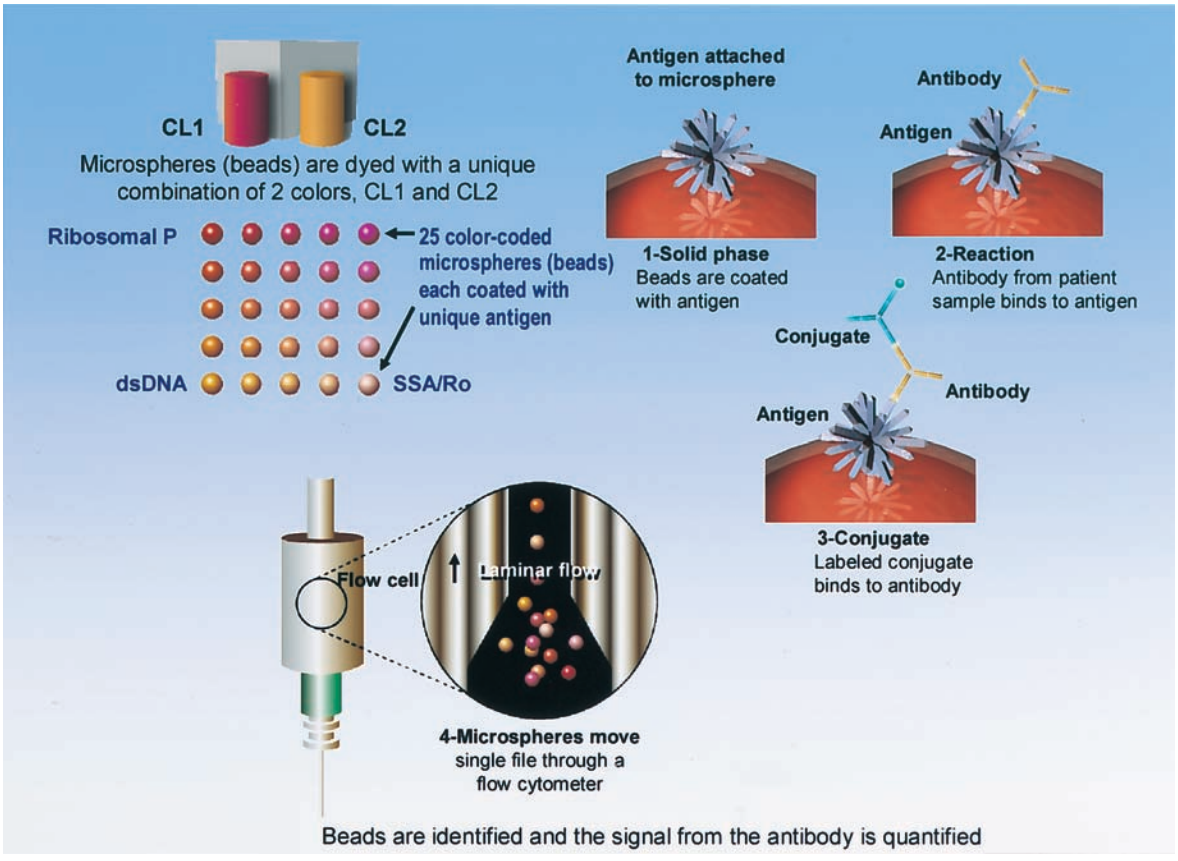

Figure 1.

rate aliquot for testing with the predicates). All sample aliquots were stored at Mayo Clinic at –20°C; at University of Washington at –70°C; and at RDL at –20°C. Sample aliquots were thawed just prior to testing with either the BioPlex or the predicate.

Positive test results obtained on the BioPlex 2200 were analyzed using a software module based on a k-nearest neighbor (kNN) pattern recognition method to identify antibody patterns associated with CTD. The software module, called MDSS (Medical Decision Support Software), compares the results for a test patient to a stored library of results from patients with clinically characterized diseases, as well as non-disease patients[11,12]. For example, the detection of elevated levels of both anti-SSA and anti-SSB is a typical finding in primary Sjögren's syndrome, but is also a common observation in systemic lupus erythematosus (SLE), with or without secondary Sjögren's syndrome[20]. When seen in combination with anti-dsDNA and/or anti-chromatin, the pattern recognition algorithm will propose SLE; but in the absence of an elevation of an SLE-specific antibody, it will generally propose "SLE or Sjögren's syndrome."

In our statistical analysis we estimated the agreement between EIA results for individual autoantibodies, and we calculated the diagnostic accuracy of the BioPlex MDSS as compared to clinical diagnosis. To estimate agreement, we calculated for each autoantibody the proportion of individuals with a positive result, the percentage agreement between EIA, and also Cohen's kappa

Table 1. Performance characteristics of BioPlex 2200 and comparison methods.

Bioplex 2200 Method	Bioplex 2200 Calibration	Bioplex 2200 Reportable Range	Comparison Method	Comp. Method Calibration	Comp. Method Reportable Range
ANA Screen	Qualitative response based on following analytes	Positive if any of the following 11 analytes exceeds positive cutoff	Bio-Rad (Helix) Autoimmune EIA ANA Screening Test	Single point; calibrator=25U	0.1-12.0 U; ≥1.0 Positive
dsDNA	6 point curve	1-300 IU; 5-9 Indeterm.; ≥10 Positive	Pharmacia Varelisa® dsDNA Antibodies	6 point curve	1-200 IU; 35-55 Equivocal; >55 Positive
SS-A (SS-A 52 & SS-A 60)	4 point curve	0.2-8.0 AI; ≥1.0 Positive	INOVA Diagnostics, Inc.QUANTA Lite™ SS-A (52 + 60)	Single point; calibrator=25U	1-500 U; >20 Positive
SS-B	4 point curve	0.2-8.0 AI; ≥1.0 Positive	Bio-Rad (Helix) Autoimmune Anti-SS-B/LA Test	Single point; calibrator=25U	1-500 U; >25 Positive
Sm	4 point curve	0.2-8.0 AI; ≥1.0 Positive	INOVA Diagnostics, Inc.QUANTA Lite™ Sm	Single point; calibrator=25U	1-500 U; >20 Positive
SmRNP	4 point curve	0.2-8.0 AI; ≥1.0 Positive	INOVA Diagnostics, Inc.QUANTA Lite™ RNP	Single point; calibrator=25U	1-500 U; >20 Positive
RNP (RNP 68 & RNP A)	4 point curve	0.2-8.0 AI; ≥1.0 Positive	Pharmacia Varelisa® RNP Antibodies (68 + A+C)	Single point; calibrator=1.0U	0.1-10.0 U; >1.4 Positive
Scl-70	4 point curve	0.2-8.0 AI; ≥1.0 Positive	Bio-Rad (Helix) Autoimmune Anti-Scl-70 Test	Single point; calibrator=25U	1-500 U; >25 Positive
Jo-1	4 point curve	0.2-8.0 AI; ≥1.0 Positive	Bio-Rad (Helix) Autoimmune Anti-Jo-1 Test	Single point; calibrator=25U	1-500 U; >25 Positive
Centromere	4 point curve	0.2-8.0 AI; ≥1.0 Positive	Bio-Rad (Helix) Autoimmune Anti-Centromere Test	Single point; calibrator=25U	1-500 U; >25 Positive
Chromatin	4 point curve	0.2-8.0 AI; ≥1.0 Positive	INOVA Diagnostics, Inc.QUANTA Lite™ Chromatin	Single point; calibrator=25U	1-500 U; >20 Positive
Ribosomal Protein	4 point curve	0.2-8.0 AI; ≥1.0 Positive	INOVA Diagnostics, Inc.QUANTA Lite™ Ribosome P	Single point; calibrator=25U	1-500 U; >20 Positive

For purposes of this table: IU = International Units, AI = Antibody Index, U = Arbitrary Units.

statistic, which accounts for agreement by chance. We calculated 95% confidence intervals (CI) for percentage agreement and for kappa[21]. To calculate the diagnostic accuracy of BioPlex, we assumed clinical diagnosis as the gold standard and calculated the sensitivity, specificity, and positive and negative likelihood ratios of the BioPlex MDSS for the CTD prevalent in our study population. We calculated 95% CI for the measures of accuracy[22] and for the likelihood ratios[23]. All analyses were performed using the R statistical programming software (R Development Core Team 2005).

Those samples with discordant results were not retested. Retesting was done if a known technical error occurred, or if there was an instrument malfunction or an invalid assay. The BioPlex instrument gives error messages and depending on the message, the sample may have been retested. The predicate assay results were monitored to assure that all assays were valid and all calibrators and controls met acceptance criteria.

RESULTS

The study group of patients, their demographic characteristics, and clinical diagnoses are summarized in Table 2. Rheumatoid arthritis (RA, 39%), SLE (37%), and scleroderma (5%) accounted for more than 80% of disease diagnoses in these patients and 83% were female. The agreements between all tests for autoantibodies performed by the multiplex immunoassay and individual EIA are summarized in Table 3. Taken together, the overall agreement for all tests was 79.2%. Including the ANA screen test, observed agreement was greater for negative results than for positive results (Table 3). Stated differently, the multiplex immunoassay generated few false-positive results for any of the autoantibodies tested compared to the EIA methods. Across the entire study group, the agreement for positive test results varied from more than 90% for SSA and centromere B autoantibodies to less than 60% for Scl-70 and Jo-1 autoantibodies. Agreement of positive test results for the ANA screen test and dsDNA antibodies test was 70% and 77%, respectively (Table 3).

The intraassay reproducibility for the 13 antibodies, measured at 2× to 3× cutoff, was 1.9%–6.6%, and for the interassay reproducibility it was 5.8%–12.8% at the principal author's site.

Table 2. Study group of patients: demographics and clinical diagnoses.

Median age	49 yrs (range 18–92)	
Sex	750/908 female (83%)	
Ethnicity, n (%)	744 (83) Caucasian	
	62 (7) Hispanic	
	45 (5) Asian	
	39 (4) African American	
	18 (2) Unknown or other	
Diseases, n (%)	Rheumatoid arthritis	352 (39)
	Systemic lupus erythematosus	332 (37)
	Scleroderma	41 (5)
	Mixed connective tissue disease	16 (2)
	Sjögren's syndrome	16 (2)
	Dermatomyositis	15 (2)
	Polymyositis	12 (1)
	CREST	6 (< 1)
	No CTD	77 (8)
	Raynaud's	10 (1)
	Other CTD	21 (2)

CREST: Calcinosis, Raynaud's phenomenon, esophageal dysfunction, sclerodactyly, telangiectasia; CTD: connective tissue disease.

The test results in patients with defined CTD (excluding RA) are summarized by disease in Table 4. Among patients with SLE, the multiplex ANA screen result was less often positive than the ANA screen by EIA. This is because the multiplex assay considered only those sera that were positive for a specific antibody to have a positive ANA; the ANA by EIA recognizes other antigens not in the multiplex assay[24].

In contrast to the ANA results, similar numbers of sera tested positive for antibodies to dsDNA and chromatin using both methods. However, individual sera from SLE patients with positive results for dsDNA, chromatin, Sm, or ribosome P antibodies by either method showed agreement between the 2 methods only about half the time (48% to 68%; Table 4). The high rates (81% to 95%) of overall agreement of test results between the 2 methods for these antibodies were due to the fact that most specimens gave negative results with both methods.

Among patients with the other CTD, including Sjögren's syndrome, scleroderma, CREST (calcinosis, Raynaud's phenomenon, esophageal dysfunction, sclerodactyly, telangiectasia), mixed connective tissue disease, and polymyositis, there were high rates of agreement for the various disease-specific autoantibodies overall and among positive sera. The only exception to this generalization was the tendency to lower rates of positivity for the ANA screen by the multiplex method compared to the EIA in scleroderma patients (Table 4).

The relationships between autoantibodies detected by the multiplex method and diagnoses suggested by the interpretative algorithm are shown in Table 5 for 4 different autoantibodies often found in patients with SLE. The relationships are shown for the individual autoantibodies and for 6 combinations of 2 autoantibodies. In each comparison, the interpretative algorithm suggested the expected diagnosis of SLE in at least 84% of cases; and for several comparisons, the algorithm suggested a diagnosis of SLE in 100% of cases. Conversely, some patients with individual autoantibodies had a diagnosis other than SLE, indicating that the presence of a single antibody was not absolutely disease-specific. In many such instances, the interpretative algorithm correctly suggested the possibility of a disease other than SLE. Analogous comparisons for the other autoantibodies and CTD are shown in Table 6. Once again, the interpretative algorithm suggested the correct clinical diagnosis in most instances in which one or more results were positive.

The accuracy and positive and negative likelihood ratios for each CTD based upon results generated by the multiplex assay and interpretative algorithm are shown in Table 7. For these 5 CTD viewed collectively, the negative likelihood ratios of the ANA screen test by the multiplex immunoassay and EIA were 0.34 and 0.29, respectively.

DISCUSSION

Our objectives were to evaluate a new analytical method for

Table 3. Agreement between Multiplex and EIA assays for screen and 11 autoantibodies.

Autoantibody	n	Positive Result		Multiplex Agreement with EIA			
		Multiplex	EIA	Positive	Negative	Total (95% CI)	Kappa (95% CI)
ANA screen	908	0.43	0.55	0.70	0.90	0.79 (0.76, 0.82)	0.59 (0.54, 0.64)
dsDNA	832	0.13	0.13	0.77	0.97	0.94 (0.92, 0.95)	0.73 (0.66, 0.80)
Chromatin	908	0.19	0.17	0.62	0.91	0.86 (0.83, 0.88)	0.51 (0.44, 0.59)
Ribosomal P	908	0.04	0.02	0.86	0.98	0.98 (0.96, 0.98)	0.61 (0.46, 0.76)
SSA	908	0.19	0.19	0.92	0.98	0.97 (0.95, 0.98)	0.89 (0.85, 0.93)
SSB	908	0.08	0.09	0.83	0.98	0.96 (0.95, 0.97)	0.75 (0.67, 0.83)
Sm	908	0.07	0.04	0.88	0.97	0.97 (0.95, 0.98)	0.68 (0.58, 0.79)
Sm-RNP	908	0.11	0.12	0.80	0.98	0.96 (0.94, 0.97)	0.81 (0.75, 0.87)
RNP	879	0.12	0.11	0.83	0.97	0.95 (0.94, 0.97)	0.77 (0.70, 0.84)
Scl-70	908	0.03	0.02	0.53	0.98	0.97 (0.96, 0.98)	0.40 (0.22, 0.59)
Jo-1	908	0.01	0.01	0.55	1.00	0.99 (0.98, 1.00)	0.66 (0.41, 0.92)
Centromere	908	0.04	0.04	0.97	0.99	0.99 (0.98, 1.00)	0.88 (0.80, 0.96)

NB: Indeterminate and equivocal samples were excluded; EIA: enzyme immunoassay.

Table 4. Performance of the Multiplex and EIA methods for ANA and 11 specific antibodies (sorted by disease state).

Disease (Total no. of samples)	Result	No. Antibody-Positive by BioPlex 2200	No. Antibody-Positive by EIA	Agreement Between Methods on Positive Samples (n) %	Overall Agreement Between Methods (n) %
SLE, N = 332	ANA screen	220	270	(213/277) 77	(270/332) 81
	dsDNA	84	87	(65/96) 68	(265/281) 93*
	Chromatin	122	113	(86/147) 59	(269/332) 81
	Sm	49	33	(30/52) 58	(310/332) 93
	RiboP	30	19	(16/33) 48	(315/332) 95
Primary Sjögren's syndrome, N = 16	ANA screen	15	15	(15/15) 100	(16/16) 100
	SSA	15	15	(15/15) 100	(16/16) 100
	SSB	14	13	(13/14) 93	(15/16) 94
Scleroderma, N = 41	ANA screen	26	35	(25/36) 69	(30/41) 73
	Scl-70	7	7	(6/8) 75	(39/41) 95
	Centromere	9	9	(9/9) 100	(41/41) 100
CREST, N = 6	ANA screen	6	6	(6/6) 100	(6/6) 100
	Centromere	4	4	(4/4) 100	(6/6) 100
MCTD, N = 16	ANA screen	16	16	(16/16) 100	(16/16) 100
	SmRNP	15	15	(15/15) 100	(16/16) 100
	RNP	15	14	(14/15) 93	(15/16) 94
Polymyositis, N = 12	ANA screen	6	9	(6/9) 67	(9/12) 75
	Jo-1	2	2	(2/2) 100	(12/12) 100

* Results that were in the equivocal/indeterminate range by either method were excluded; CREST: calcinosis, Raynaud's phenomenon, esophageal dysfunction, sclerodactyly, telangiectasia; MCTD: mixed connective tissue disease.

detecting autoantibodies associated with CTD in comparison to commonly used FDA-approved tests. We also sought to determine whether the detection of multiple autoantibodies simultaneously by multiplex technology would enable accurate classification of patients with known or suspected CTD when compared to consensus clinical criteria. The results of the multiplex immunoassay and individual comparative EIA generated similar results for most autoantibodies.

A unique advantage to the multiplex system is the inclusion of internal controls in each reaction for monitoring serum addition, nonspecific binding, and tube to tube variation. This is a powerful quality assurance measure not available in conventional immunoassays.

The most frequent discordant results between the multiplex method and the comparative EIA methods were obtained for the generic ANA test. ANA screen results reported by multiplex immunoassay were derived from 11 specific antibodies tested using individual antigen-coated beads. The EIA ANA may detect antigens not tested in the multiplex panel[24]. It is not surprising that some discordant results were obtained between ANA measured by EIA and by the multiplex immunoassay panel. In our study, the multiplex ANA displayed lower rates of positive results in patients with clinical diagnoses of SLE and scleroderma than the ANA by EIA. These data suggest that the lower sensitivity of the multiplex ANA might limit its usefulness for excluding the diagnosis of SLE or scleroderma in certain clinical settings as suggested in a recent evidence-based guideline[25].

Table 5. Pattern recognition results for antibody patterns with SLE (sorted according to physicians' diagnosis).

Antibody Finding(s)	N	Expected Diagnosis	No. of Patients with Expected Diagnosis	% of kNN Results Mentioning Expected Diagnosis	No. of Patients with Different Diagnoses	% of kNN Results Offering Different Diagnoses	Overall Ability of kNN to Offer an Appropriate Association, %
dsDNA positive	119	SLE	92	94.6	27	74.1	89.9
Chromatin positive	168	SLE	122	84.2	46	50.0	74.8
Sm positive	60	SLE	49	100.0	11	45.5	90.0
Ribosomal P positive	37	SLE	30	96.7	7	28.6	83.8
dsDNA and chromatin positive	77	SLE	74	100.0	3	33.3	97.4
dsDNA and Sm positive	36	SLE	34	100.0	2	100.0	100.0
dsDNA and ribosomal P positive	22	SLE	22	100.0	0	NA	100.0
Chromatin and Sm positive	52	SLE	45	100.0	7	42.9	92.3
Chromatin and ribosomal P positive	27	SLE	27	100.0	5	20.0	87.5
Sm and ribosomal P positive	18	SLE	16	100.0	2	0.0	88.9

kNN: k-nearest neighbor pattern recognition method.

Table 6. Pattern recognition results for antibody patterns with other connective tissue diseases (sorted according to physicians' diagnosis).

Antibody Finding(s)	N	Expected Diagnosis	No. of Patients with Expected Diagnosis	% of kNN Results Mentioning Expected Diagnosis	No. of Patients with Different Diagnoses	% of kNN Results Offering Different Diagnoses	Overall Ability of kNN to Offer an Appropriate Association, %
Jo-1 positive	6	Polymyositis	3	100.0	3	100.0	100.0
Scl-70 positive	23	Scleroderma	7	71.4	16	56.3	60.9
SSA and SSB positive	60	Sjögren's syndrome	13	100.0			
		SLE	39	100.0			
		(neither of these)			8	0.0	86.7
RNP and Sm-RNP positive	73	MCTD	15	86.7			
		SLE	48	100.0			
		(neither of these)			10	0.0	86.3
Centromere positive	38	SLE	11	81.8			
		Scleroderma	9	100.0			
		CREST	3	100.0			
		(None of these)			15	6.7	57.9

kNN: k-nearest neighbor pattern recognition method.

Table 7. Accuracy and likelihood ratios for multiplex software as compared to clinical diagnosis for selected diseases.

Disease	Prevalence	Sensitivity	Specificity	LR Positive	LR Negative
SLE	0.37	0.56 (0.51, 0.62)	0.82 (0.79, 0.85)	3.1 (2.6, 3.8)	0.53 (0.47, 0.61)
MCTD	0.02	0.81 (0.54, 0.95)	0.98 (0.96, 0.98)	32.9 (20.5, 53.0)	0.19 (0.07, 0.53)
Polymyositis	0.01	0.17 (0.03, 0.49)	1.00 (0.99, 1.00)	149.3 (14.51, 537.8)	0.83 (0.65, 1.07)
Scleroderma	0.05	0.34 (0.21, 0.51)	0.97 (0.95, 0.98)	10.6 (6.0, 18.5)	0.68 (0.55, 0.85)
Sjögren's Syndrome	0.03	0.54 (0.34, 0.72)	0.97 (0.96, 0.98)	17.5 (10.5, 29.0)	0.48 (0.32, 0.71)

It is unlikely that the multiplex analytical system missed detecting clinically significant antibodies, in that the multiplex method had excellent analytical sensitivity (see below). Conversely, the lower frequency of positive results for ANA by the multiplex method may actually be advantageous, as it might lead to fewer weakly-positive results not associated with measurable disease-specific autoantibodies.

Discordant results were also noted with some frequency for dsDNA antibody testing. Although the 2 methods actually gave similar overall percentages of positive results in patients

with SLE, this is likely the result of inclusion of large numbers of SLE patients in the clinical study group. The 2 test methods may have different analytical sensitivities for dsDNA antibodies of lower versus higher affinities. This conclusion is supported by the differences in reference ranges and cutoffs for positive results for dsDNA testing, since both methods trace their calibration to the same reference preparation (Wo/80) and it is probable that differences can be accounted for by methodological variables. Because the positive cutoff for the multiplex assay is considerably lower than for the comparative EIA, it is possible that the multiplex assay may be less susceptible to positive results caused by lower affinity antibodies.

Specimens in our study were collected on consecutive patients. Blood draws were not necessarily triggered by suspicions of change in disease activity and may differ from blood draws performed as part of the standard of care. This is important to consider for the following reasons. Antibodies such as dsDNA antibody can correlate with disease activity. Rising dsDNA antibody levels may help predict SLE flares[26,27] and may decrease after the flare has resolved[28]. Antibodies to dsDNA are associated with active disease, especially active nephritis[29]. Testing of patients with a pretest likelihood of active SLE has been recommended as the optimal usage of dsDNA antibody testing, and many study samples collected do not fit that description[30].

Further, antibodies to dsDNA have been shown to be affected by treatment of SLE. Two studies of patients with SLE treated solely on the basis of increasing dsDNA antibody (regardless of disease activity) with the addition of prednisone 30 mg per day or mycophenolate mofetil 2000 mg per day to a conventional regimen resulted in significant decreases in dsDNA antibody[31,32]. Patients who were previously positive for dsDNA antibody with active nephritis treated with cytotoxic drugs, corticosteroids, and mycophenolate might subsequently have a negative dsDNA test result. This would affect results based on the MDSS. The pattern recognition reported in Table 4 would prove to be problematic if accurate SLE categorization relied on the presence of DNA positivity.

In patients with SLE, published data suggest that clustering of autoantibodies is predictive of clinical subsets and disease damage[33]. Having the full autoantibody pattern from multiplex testing in patients with SLE initially may influence diagnosis, treatment, and clinical outcomes. Data from this evaluation suggest that the detection of certain SLE-specific autoantibodies such as chromatin, Sm, and ribosomal P is enhanced using multiplex technology compared to EIA (Table 4).

A limitation of the current multiplex assay is that it does not include tests commonly ordered in patients with suspected RA, such as rheumatoid factor or cyclic citrullinated peptide antibodies. These tests are of documented value in the diagnosis and prognostic assessment of patients with RA[34]. RA was the most common CTD at each of the 3 participating centers.

A unique feature of this multiplex system is the MDSS algorithm for comparing results in individual patients to a database of patients previously studied and characterized. Overall the MDSS system performed well in suggesting the appropriate clinical diagnoses. The presence of dsDNA antibodies in addition to 3 other autoantibodies (Sm, ribosome P, and chromatin) each increased the likelihood that the pattern would be reported as being associated with SLE (Table 5). The MDSS did not make incorrect associations based solely on the presence of one autoantibody. For example, Jo-1 is an autoantibody associated with dermatomyositis and polymyositis[35]. The presence of Jo-1 antibody was associated by the MDSS with polymyositis only 50% of the time (3 of 6 samples), with the algorithm correctly assigning patterns involving Jo-1 antibody to other diseases in the other 3 cases. The presence of additional autoantibodies in the same specimens as anti-Jo-1 served as an indicator of a non-myositis pattern in these instances.

For the remaining antibodies listed in Table 6, the association of antibody patterns with disease was not as strong as observed for patterns associated with SLE (Table 5). Several specimens with weakly-reactive Scl-70 antibodies had other diagnoses. These results can be explained by noting that the positive results were close to the positive cutoffs in each case. The 3 other patterns listed in Table 5, antibodies to SSA and SSB, to RNP and SmRNP, and to centromere, also showed good associations with the expected diagnoses.

With the exception of SLE, the likelihood ratios reported in Table 7 compare favorably with the results of a recent meta-analysis[25]. The high positive likelihood ratios for MDSS results benefit from both the improved specificity of the antibody results and the ability of pattern recognition to identify nonspecific patterns. The positive likelihood ratio for SLE is slightly lower, because a possible SLE association must be proposed for many antibody patterns that do not contain any SLE-specific antibodies (Table 6). Summary data for all the autoantibodies can be found in Table 8.

It is important to consider that this testing was done at only one point in time. It is recognized that patterns of autoantibodies evolve over time in individual patients, similar to the evolution of their clinical manifestations. That these tests were performed cross-sectionally, in a group of subjects with different disease durations has to be considered a weakness in the interpretation and extrapolation of the results.

In summary, the multiplex immunoassay displayed excellent overall comparability to established enzyme immunoassays for several different autoantibodies. Results obtained with the multiplex method displayed excellent analytical sensitivity and negligible rates of false-positive test results, suggesting that the multiplex method should have very good clinical usefulness in appropriate clinical settings. The ability to associate results with appropriate clinical diagnoses is an added feature that may have considerable clinical value.

Table 8. Results of all autoantibodies.

Antibody/Antibody Group	dsDNA	Chromatin	Ribosomal-P	SSA	SSB	Sm	SmRNP	RNP	Scl-70	Jo-1	Centromere
N	832	908	908	908	908	908	908	879	908	908	908
BioPlex and EIA positive	83	98	18	156	57	35	90	79	9	6	31
BioPlex positive and EIA negative	25	70	19	17	19	25	13	25	14	0	7
BioPlex and EIA negative	699	680	868	721	820	843	782	759	877	897	869
BioPlex negative and EIA positive	25	60	3	14	12	5	23	16	8	5	1
% Overall agreement	94	86	98	97	97	97	96	95	98	99	99
95% Confidence interval	92–96%	83–88%	97–99%	95–98%	95–98%	96–98%	95–97%	94–97%	97–99%	99–100%	99–100%

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